European Journal of Immunology

Peer review correspondence

Leptin deficiency impairs maturation of dendritic cells and enhances induction of regulatory T and Th17 cells

Pedro M.M. Moraes-Vieira; Rafael A. Larocca; Enio J. Bassi; Jean Pierre S. Peron, Vinícius Andrade-Oliveira; Frederick Wasinski; Ronaldo Araujo; Thomas Thornley; Francisco J. Quintana; Alexandre S. Basso; Terry B. Strom; Niels O.S. Câmara

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Handling Executive Committee member: Dr. Andrea Cooper

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision – 6 May 2013

Dear Dr. Camara,

Manuscript ID eji.201343592 entitled "LEPTIN DEFICIENCY IMPAIRS DENDRITIC CELL MATURATION AND ITS ABILITY TO INDUCE REGULATORY T AND TH17 CELLS" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. You will see that referee 3 has substantial concerns about both your data and the way it is presented and written in the manuscript, and should know that both the Executive Editor and the editorial office feel that these requests are necessary to enhance the impact of your paper.

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You should also pay close attention to the editorial comments included below. In particular, we strongly recommend that you have your manuscript proofread by a native English speaker before re-submission.

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by both the original and new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Karen Chu

On behalf of Dr. Andrea Cooper

Dr. Karen Chu
Editorial Office
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e-mail: ejied@wiley.com

****

Reviewer: 1
Comments to the Author
The paper describes a comprehensive series of experiments that extend the effects of leptin on the inflammatory process.

Reviewer: 2
Comments to the Author
The work by Moraes-Vieira et al. identified a unique phenotype of dendritic cells (DCs) derived from leptin-deficient ob/ob mice with potent function in enhancing Treg and Th17 cell generation in culture. This well performed study provides further insight in understanding the role of leptin in regulating T-cell response via modulating DC function.

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A few points of concerns are mentioned below:

Major points:

1. Figure 1: CD11c was used as an exclusive marker for DC. Moreover, the gating for CD11c+ cells was not adequately set. Thus, co-staining with MHC-II should be performed for DC phenotypic analysis. Moreover, both frequency and total cell number of DCs from the spleen of WT and Lepob/ob mice should be provided to better assess the effect of leptin deficiency on DC generation.
2. Figure 3: The total number of Teg cells generated in co-cultures with BM-derived DCs from WT and Lepob/ob mice should be provided for comparison. In contrast to the findings on a direct function of leptin in suppressing Treg generation (De Rosa V et al, Immunity, 2007), current experiments did not show any effect of leptin treatment on Treg generation in culture. This discrepancy warrants further discussion.
3. Figure 4: For Th17 cell induction, leptin addition appeared to promote Th17 differentiation from WT controls (4.45% vs. 7.21%). In contrast, leptin addition markedly suppressed Th17 cell generation in cocultures with DC from Lepob/ob mice (46.3% vs. 26.8%). These results should be discussed along with recent findings on a role of leptin in enhancing Th17 response by Deng J et al. (Arthritis & Rheum, 2012; 64:3564) and Yu Y et al. (J Immunol, 2013; 190:3054). Furthermore, the total cell numbers of Th17 cells generated in co-cultures should be provided.

Minor points:

1. Abstract: line 11, “produced” could be replaced with “induced” as an appropriate wording.
2. For Fig 5C, the indication of statistical significance on different columns is confusing and needs to be revised.

Reviewer: 3

Comments to the Author

This report by Moraes-Viera et al. provides evidence that leptin deficient dendritic cells (DC) display impaired maturation and reduced ability to stimulate CD4+ T-cell proliferation and Th1 differentiation in vitro. Furthermore, the authors show that leptin deficiency in DC leads to increased induction of Tregs and Th17 cells in vitro. The subject area of metabolic immunity, which is the focus of this paper, is one of high interest, and the authors add to this field with interesting data on the ability of leptin deficient DC to induce helper T-cell subsets. However, some data are questionable and contradictory to the conclusions drawn. Furthermore, the manuscript is poorly written, both in terms of language and content, and will require substantial revision.

Major comments

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1. The authors use Lepob/ob mice to determine the role of leptin on DC maturation and function. These DCs are deficient in the production of leptin, rather than the recognition of leptin. Therefore, the results suggest that the inability of DCs to produce leptin impairs their maturation and promotes DC-dependent induction of Treg and Th17, rather than Th1, responses. However, the conclusions drawn mitigate DC capacity to respond to leptin as the reason for the observed results. A more accurate control to use throughout, in keeping with the conclusions, is the Lepdb/db mouse, which is deficient in the leptin receptor, instead of as a confirmatory control in only certain experiments. The capability of DCs to produce leptin should be examined.

2. The authors imply that the results observed are due to the effect of leptin on DCs. However, previous studies have shown that CD4+ T cells can produce leptin and express the leptin receptor, ObR (Lord, Nature, 1998; De Rosa, Immunity, 2007). Therefore, the authors need to prove that the DC:T cell co-culture results are not due to the direct effect of leptin on T cells.

3. The paper needs major revision for written English. Moreover, the content and specificity are weak. The Introduction is vague and lacks key references. Also, the Results section flows poorly, and needs to discuss the rationale behind the experiments presented. Finally, there are several examples where what is stated in the text is different from the data; in Fig 5E the authors state that leptin administration increased Tregs and Th17 cells in vivo, whereas the data depict a significant decrease in these populations following addition of leptin.

4. Figure 4E shows that co-cultures of DC from Lepob/ob mice and T cells have elevated levels of GATA-3. The authors should perform additional experiments to confirm that leptin-deficient DC also display enhanced ability to induce Th2 responses.

5. Figure 5 aims to confirm the findings that leptin-deficient DC promote Treg and Th17 responses in vitro also apply in vivo. However, there are discrepancies that raise questions about these data. For example, flow cytometry plots (5A) demonstrate that WT mice have a higher percentage of Tregs than Lepob/ob mice, which is in contrast to the bar graph summary in C and the text. This is also true of D, in which WT mice have higher IFN-γ and no difference in IL-17A in the flow cyotmetry plots, whereas the bar graph in E and the text state no difference.

6. The authors perform a DTH assay as a means to generate a Th1 response in vivo. However, Th17 cells are known to be involved in such responses. The authors should confirm that the increased DTH response observed in the rLep-treated mice was due to increased Th1 responses and not Th17 responses.

Specific comments

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1. The manuscript title is inaccurate, as the authors provide evidence that leptin deficiency in DC enhances Treg and Th17 cells.

2. The Abstract mentions that leptin inhibits Th2 and induces Th17 responses, but this is not referenced in the Introduction.

3. Fig. 1
Title is misleading as A, B are looking at DC isolated from lymph nodes.
A. Gate seems to cut through population. Would like to see negative control and explain gate setting.
D. Need to state what the data are normalized to.

4. Fig. 2
A, B. These figures are almost exact repeats of data presented by Lam et al. (EJI, 2006), but using Lepob/ob mice. The paper by Lam et al. (EJI, 2006) should be cited here.
B. The box and figure legends for the different mice are different. For example, the figure legend describes a red line for DC in the presence of leptin but the figure does not show this. It is difficult to obtain the result from this figure and so this needs to be presented in a clearer fashion.

E. Why are there fewer cells in Lep-/-?

F. What were the co-cultures stimulated with? Also, one could argue that not all of these are solely 'Th1-related cytokines' as stated in the text, but also Th17 associated cytokines, such as IL-6, and indeed Th associated cytokines in the case of IL-2.

5. Figs 1 and 2 are not well ordered and should be changed. For example, an improvement would be to separate the DC and T cell data in Fig. 2, perhaps creating a new figure for the T cell data.

6. Figures 1 and 2 show very similar results as have been previously shown (Lam, EJI, 2006; Mattiolo, JI, 2005; Macia, JI, 2006). These reports should be referenced accordingly.

7. Fig. 3
A. Should present flow plots of cells in the absence of TGF-b as negative control.
The conclusion that leptin addition does not have a direct affect on naïve T cells cannot be stated, as these cultures also contain leptin responsive DC.

8. The flow cytometry plot order changes in Fig 3 and 4 to WT – Lep-/-Lep – Lep-/-. This is different from Fig 2. To save confusion, the order should be uniform throughout.

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9. Fig. 4
Are cells stimulated with a-CD3?
A. What is the point behind the bottom 3 flow plots displayed (TGF-b + IL-6 + leptin)? These are unnecessary, are not described in the text and should be removed.
B. Could present negative control and include statistics (also for D and E).
E. They state that Th17 cells induced by Lepob/ob DC display reduced foxp3 mRNA, but this is not convincing from the graph. Need to prove with statistics.
The text states 'addition of rLep restored the capacity of Lepob/ob DC in inducing the differentiation of Th17 cells'. However, the converse is true.

10. Parametric statistical tests were used throughout, but it cannot be assumed that the data are normally distributed. Therefore, non-parametric tests, such as Mann-Whitney and Kruskal-Wallis tests, should be used.

11. Figure legends need to be written in a more concise manner. Gating strategies for flow cytometry plots need to be included.

12. Reference numbers 5 and 6 are the same.

13. Supplementary Figure 1.
A. Is there really a shift in these plots? These data are not convincing

14. The finding that leptin decreases DC ability to polarize Th17 responses is in contrast to a report by Deng et al. (Arthritis & Rheumatism, 2012), which shows leptin can directly enhance Th17 responses in vitro. This should be addressed in the discussion.

First Revision – authors' response – 26 July 2013

Reviewer: 1
Comments to the Author
The paper describes a comprehensive series of experiments that extend the effects of leptin on the inflammatory process.

Reviewer: 2
Comments to the Author

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The work by Moraes-Vieira et al. identified a unique phenotype of dendritic cells (DCs) derived from leptin-deficient ob/ob mice with potent function in enhancing Treg and Th17 cell generation in culture. This well performed study provides further insight in understanding the role of leptin in regulating T-cell response via modulating DC function.

A few points of concerns are mentioned below:

Major points:

Reviewer: Figure 1: CD11c was used as an exclusive marker for DC. Moreover, the gating for CD11c+ cells was not adequately set. Thus, co-staining with MHC-II should be performed for DC phenotypic analysis. Moreover, both frequency and total cell number of DCs from the spleen of WT and Lepob/ob mice should be provided to better assess the effect of leptin deficiency on DC generation.

Response: We thank the reviewer for all the comments. We agreed with all and revised the manuscript taking into account all the criticisms. We performed a new staining using both CD11c and MHCII. We considered DC the double positive population (Figure 1A). Moreover, we added a supplemental Figure with the total cell number of DC in the spleen and lymph node of Lep ob/ob and control mice.

Reviewer: Figure 3: The total number of Teg cells generated in co-cultures with BM-derived DCs from WT and Lepob/ob mice should be provided for comparison. In contrast to the findings on a direct function of leptin in suppressing Treg generation (De Rosa V et al, Immunity, 2007), current experiments did not show any effect of leptin treatment on Treg generation in culture. This discrepancy warrants further discussion.

Response: We added a supplemental Figure with Treg and Th17 cell number. De Rosa et al [1] demonstrated that leptin inhibition leads to Treg proliferation. They did not report inhibition in Treg generation. Here we show that Lep ob/ob DCs (generated with antilogous serum – no leptin) generate more Treg from a naïve precursor (CD4+CD62L+CD44-Foxp3gfp-). Moreover, since we sorted CD62L+CD44-Foxp3gfp- CD4 T cells, we do not have contamination with Tregs and memory CD4+CD44+ T cells since they express CD62L. In agreement with De Rosa et. al., when DC from Lep ob/ob mice were generated with Lep ob/ob medium supplemented with 500ng/mL of recombinant leptin, no proliferation of Treg was observed. Moreover, the addition of recombinant leptin during the Treg differentiation assay at a higher dose (1000ng/mL) than the dose used throughout the manuscript (500ng/mL) decreased Treg generation. We included this data as a new supplemental figure 6.

Reviewer: Figure 4: For Th17 cell induction, leptin addition appeared to promote Th17 differentiation from WT controls (4.45% vs. 7.21%). In contrast, leptin addition markedly suppressed Th17 cell generation in cocultures with DC from Lepob/ob mice (46.3% vs. 26.8%). These results should be discussed along with recent findings on a role of leptin in enhancing Th17 response by Deng J et al. (Arthritis & Rheum, 2012;

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64:3564) and Yu Y et al. (J Immunol, 2013; 190:3054). Furthermore, the total cell numbers of Th17 cells generated in co-cultures should be provided.
Response: We added a new supplemental Figure 7 with Treg and Th17 cell number. Also, we better discussed these differences in the discussion. Deng et al. used WT naïve CD4+CD62L+ cells. This population of cells is not pure naïve cells, since Tregs and central memory CD4 T cells express CD62L. When we compared WT DC-induced Th17 differentiation we recapitulated the degree of increase observed in both Deng et al., and Yu et. al.'s paper. Albeit as reviewer 3 suggested we removed this panel from the manuscript. Nevertheless, DC from Lep ob/ob mice, generated with autologous serum (no leptin) induces more Th17 cells. Importantly, when DCs from Lep ob/ob were generated with medium supplemented with recombinant leptin (Lep ob/obLep), this phenotype is reversed to that observed with WT DC. Another important point is that both Deng et al., and Yu et. al. used an antigen-free assay for Th17 induction while we used a APC-induced system, making any comparison difficult. Moreover, because the reviewer 3 found that the addition of leptin to the differentiation assay made it difficult to interpret and asked to remove the bottom panel, we took it out of the manuscript.

Minor points:
Reviewer: Abstract: line 11, "produced" could be replaced with "induced" as an appropriate wording.
Response: We modified the sentence.
Reviewer: For Fig 5C, the indication of statistical significance on different columns is confusing and needs to be revised.
Response: We adjusted the statistical significance in the figure.

Reviewer: 3
Comments to the Author
This report by Moraes-Viera et al. provides evidence that leptin deficient dendritic cells (DC) display impaired maturation and reduced ability to stimulate CD4+ T-cell proliferation and Th1 differentiation in vitro. Furthermore, the authors show that leptin deficiency in DC leads to increased induction of Tregs and Th17 cells in vitro. The subject area of metabolic immunity, which is the focus of this paper, is one of high interest, and the authors add to this field with interesting data on the ability of leptin deficient DC to induce helper T-cell subsets. However, some data are questionable and contradictory to the conclusions drawn. Furthermore, the manuscript is poorly written, both in terms of language and content, and will require substantial revision.
Response: we thank the reviewer for all the comments, agreed with all and revised the manuscript taking into account all the criticism.

Major comments

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Reviewer: The authors use Lepob/ob mice to determine the role of leptin on DC maturation and function. These DCs are deficient in the production of leptin, rather than the recognition of leptin. Therefore, the results suggest that the inability of DCs to produce leptin impairs their maturation and promotes DC-dependent induction of Treg and Th17, rather than Th1, responses. However, the conclusions drawn mitigate DC capacity to respond to leptin as the reason for the observed results. A more accurate control to use throughout, in keeping with the conclusions, is the Lepdb/db mouse, which is deficient in the leptin receptor, instead of as a confirmatory control in only certain experiments. The capability of DCs to produce leptin should be examined.

Response: We revised and described the experiments with Lep db/db as a control to validate our observations. We used Lep ob/ob BMDC to better evaluate the importance of leptin in DC generation. We can, using Lep ob/ob DC, generated bone marrow DC in the complete absence of leptin (with Lep ob/ob autologous serum) and determine the effect of the absence of leptin on DC generation. By adding recombinant leptin into this system (with Lep ob/ob autologous serum) we are just giving back leptin and by doing that the cell phenotype is reverted, behaving as WT DC. This clearly demonstrate that it is not the production of leptin that affect DC generation, but recombinant leptin reconstitution. Moreover, we added a new supplementary figure where we analyzed the amount of leptin secreted by iDC, mDC and CD4 T cell (supplemental Figure 5). The concentration of leptin secreted by both iDC, mDC and CD4 T cells (5-12pg/mL) are not able in modify DC phenotype as observed by a dose response assay where we added different doses of leptin into DC generated from Lep ob/ob mice with Lepob/ob autologous serum (no leptin) and included as a new supplemental Figure 6. Higher doses of leptin are needed to reverse the phenotype of BMDC than the amounts produced by the cells.

Reviewer: The authors imply that the results observed are due to the effect of leptin on DCs. However, previous studies have shown that CD4+ T cells can produce leptin and express the leptin receptor, ObR (Lord, Nature, 1998; De Rosa, Immunity, 2007). Therefore, the authors need to prove that the DC:T cell co-culture results are not due to the direct effect of leptin on T cells.

Response: We agree with the reviewer and we cannot completely role out the direct effect of leptin on CD4 T cells. Nevertheless, because we used WT naïve CD4 T cell (from WT C57BL/6 mice) in all experiments the direct effect on CD4 T cells would be the same. Even thought, DCs from Lep ob/ob mice still induce more Treg and Th17 than Lep ob/ob Lep and WT DC. Thus, since the T CD4 cells are a fixed parameter in all conditions (WT C57BL6 CD4 T cell), the differences observed can only be due to the action of leptin on DC, since these cells were generated without leptin. Moreover, the amount of leptin produced by the T CD4 cells is not capable in promote a quantitative change in Treg generation and DC phenotype as observed in conditions where no recombinant leptin was added.

Reviewer: The paper needs major revision for written English. Moreover, the content and specificity are weak. The Introduction is vague and lacks key references. Also, the Results section flows poorly, and

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needs to discuss the rationale behind the experiments presented. Finally, there are several examples where what is stated in the text is different from the data; in Fig 5E the authors state that leptin administration increased Tregs and Th17 cells in vivo, whereas the data depict a significant decrease in these populations following addition of leptin.
Response: We revised the English and sent to English review as showed by the certificated uploaded with the manuscript. Also, we completely modified the introduction section. We apologize for the confusion in Fig. 5E. We corrected it in the text.

Reviewer: Figure 4E shows that co-cultures of DC from Lepob/ob mice and T cells have elevated levels of GATA-3. The authors should perform additional experiments to confirm that leptin-deficient DC also display enhanced ability to induce Th2 responses.
Response: we performed this experiment and in shown in a new Supplemental Figure 9. As observed by Batra et al.[2], Lepob/ob DC generated in the lack of leptin (Lepob/obautologous serum) have enhanced ability to inducing Th2 cells.

Reviewer: Figure 5 aims to confirm the findings that leptin-deficient DC promote Treg and Th17 responses in vitro also apply in vivo. However, there are discrepancies that raise questions about these data. For example, flow cytometry plots (5A) demonstrate that WT mice have a higher percentage of Tregs than Lepob/ob mice, which is in contrast to the bar graph summary in C and the text. This is also true of D, in which WT mice have higher IFN-γ and no difference in IL-17A in the flow cytometry plots, whereas the bar graph in E and the text state no difference.
Response: We apologize for the confusion in Fig. 5. Fig 5A was misplaced and we corrected it. The Bar graph in Fig. 5C was correct. Also, Fig 5D was mislabeled and we corrected it.

Reviewer: The authors perform a DTH assay as a means to generate a Th1 response in vivo. However, Th17 cells are known to be involved in such responses. The authors should confirm that the increased DTH response observed in the rLep-treated mice was due to increased Th1 responses and not Th17 responses.
Response: We performed a new experiment. Although in the experiment showed in the manuscript we did not see a statistical difference regarding the increase of IFN-gamma, the p value was 0.0693. Moreover, Th17 cells were decreased in mice that received leptin, making it unlike that the increased DTH was due to Th17 response. In the new experiment we increased to number of mice to try obtaining statistical significance regarding this Th1 aspect. In this new experiment we observed a higher percentage and number of Th1 cells in the draining lymph node of mice that were immunized with MOG peptide plus recombinant leptin. This indicates that Th1 increase may account for the increased DTH. To further confirm this, we inject MOG peptide in the footpad one week after the immunization and 48hours after the challenge we harvest the foot, extracted RNA and analyzed the expression of the macrophage marker CD68. We observed an increase expression of CD68 in the footpad, indicating that the increased DTH

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was due to enhance Th1 response in mice that received leptin. These results were added in Figure 5. Moreover, we also immunized WT and Lep ob/ob mice with MOG peptide plus recombinant leptin. We observed that recombinant leptin reduced the levels of Treg in both WT and Lep ob/ob mice. Moreover, WT and Lep ob/ob mice immunized with recombinant leptin displayed increased CD11c percentage in the lymph node. Leptin leads to increased expression of MHCII, CD86 and CD 40 in CD11c+ cells of WT mice. Nevertheless, this increase was not observed in Lep ob/ob CD11c+ cells.

Specific comments

Reviewer: The manuscript title is inaccurate, as the authors provide evidence that leptin deficiency in DC enhances Treg and Th17 cells.
Response: We corrected mentioned this abovet.

Reviewer: The Abstract mentions that leptin inhibits Th2 and induces Th17 responses, but this is not referenced in the Introduction.
Response: We added this information to the introduction.

Reviewer: Fig. 1
Title is misleading as A, B are looking at DC isolated from lymph nodes.
A. Gate seems to cut through population. Would like to see negative control and explain gate setting.
D. Need to state what the data are normalized to.
Response: We corrected the title and perform a new experiment where we gated the double population CD11c+MHCII+ as suggested by reviewer 2 and include the flow cytometry negative results. D: we normalized to WT BM and added to the figure legend the information.

Reviewer:Fig. 2
A, B. These figures are almost exact repeats of data presented by Lam et al. (EJI, 2006), but using Lepob/ob mice. The paper by Lam et al. (EJI, 2006) should be cited here.
B. The box and figure legends for the different mice are different. For example, the figure legend describes a red line for DC in the presence of leptin but the figure does not show this. It is difficult to obtain the result from this figure and so this needs to be presented in a clearer fashion.
Response: We cited the paper where indicated by the reviewer. This was supposed to be a color figure; we apologize and corrected it in the manuscript.

Reviewer:E. Why are there fewer cells in Lep-/-?
Response: Although appears that there is less number of cells, when the graph in displayed in cell numbers, no difference is observed.

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Reviewer:What were the co-cultures stimulated with? Also, one could argue that not all of these are solely 'Th1-related cytokines' as stated in the text, but also Th17 associated cytokines, such as IL-6, and indeed Th associated cytokines in the case of IL-2.
Response: We revised and corrected the statement about Th1-related cytokines. The co-cultures were stimulated with anti-CD3, we added this information in the figure legends.

Reviewer:Figs 1 and 2 are not well ordered and should be changed. For example, an improvement would be to separate the DC and T cell data in Fig. 2, perhaps creating a new figure for the T cell data.
Response: We made the modification suggested and the T cell is now in a new figure.

Reviewer: Figures 1 and 2 show very similar results as have been previously shown (Lam, EJI, 2006; Mattiolo, JI, 2005; Macia, JI, 2006). These reports should be referenced accordingly.
Response: We referenced these manuscripts as suggested.

Reviewer: Fig. 3
A. Should present flow plots of cells in the absence of TGF-b as negative control.
The conclusion that leptin addition does not have a direct affect on naïve T cells cannot be stated, as these cultures also contain leptin responsive DC.
Response: We added the negative control as suggested. Although DC can respond to leptin, the amount produced by CD4 T cells (10ng/mL) is not sufficient to alter the DC phenotype. We added these results as a new supplementary figure as described above.

Reviewer:The flow cytometry plot order changes in Fig 3 and 4 to WT – Lep-/-Lep – Lep-/-. This is different from Fig 2. To save confusion, the order should be uniform throughout.
Response: We uniformed it throughout as suggested.

Reviewer:9. Fig. 4
Are cells stimulated with a-CD3?
A. What is the point behind the bottom 3 flow plots displayed (TGF-b + IL-6 + leptin)? These are unnecessary, are not described in the text and should be removed.
B. Could present negative control and include statistics (also for D and E).
E. They state that Th17 cells induced by Lepob/ob DC display reduced foxp3 mRNA, but this is not convincing from the graph. Need to prove with statistics.
The text states 'addition of rLep restored the capacity of Lepob/ob DC in inducing the differentiation of Th17 cells'. However, the converse is true.
Response:
A: cells were stimulated with andi-CD3 and we added this information in the manuscript.
B: We removed the suggested plots.

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E: We make the graph with the same Y axis. Therefore, is clear that we have induction of Rorc and not Foxp3. Also we added all the statistics as requested and correct the statement in the results.

Reviewer:Parametric statistical tests were used throughout, but it cannot be assumed that the data are normally distributed. Therefore, non-parametric tests, such as Mann-Whitney and Kruskal-Wallis tests, should be used.
Response: We revised all the statistical analysis and changed as suggested.

Reviewer:Figure legends need to be written in a more concise manner. Gating strategies for flow cytometry plots need to be included.
Response: we revised the figure legends as requested and changed accordingly.

Reviewer:Reference numbers 5 and 6 are the same.
Response: We apologize and corrected it.

Reviewer:Supplementary Figure 1.
A. Is there really a shift in these plots? These data are not convincing
Response: We agreed with the reviewer and added inside of the histogram he median fluorescence intensity. I all cases the expression of MHCII and co-stimulatory molecules is reduced in Lepdb/db BMDC compared to WT BMDC.

14. The finding that leptin decreases DC ability to polarize Th17 responses is in contrast to a report by Deng et al. (Arthritis & Rheumatism, 2012), which shows leptin can directly enhance Th17 responses in vitro. This should be addressed in the discussion.
Response: We added this section in the discussion as suggested by both reviewers.

Second Editorial Decision – 30 August 2013

Dear Dr. Camara,

My apologies for the delay in processing the review of your revised manuscript ID eji.201343592.R1 entitled "LEPTIN DEFICIENCY IMPAIRS DENDRITIC CELL MATURATION AND ENHANCES THE ABILITY OF DENDRITIC CELLS TO INDUCE REGULATORY AND TH17 T CELLS" which you had submitted to the European Journal of Immunology. One of the referees needed a bit longer to assess your revisions; however all opinions have now been received and the comments of the referees are included at the bottom of this letter.

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Unfortunately, referee 3 was not satisfied with the revisions made and further major revision is requested. You will see that referee 3's major concern is that although you have added data to substnatially improve the impact of your story, the data presentation and writing are still far from optimal. Therefore although no new data have been requested we request major revisions in data presentation and scientific writing along the lines of which are outlined by referee 3. You should know that the journal does not encourage multiple rounds of revision and you should fully address the concerns of the referee in this final round of revision. Of great importance, we strongly advise that you have your final revision proofread by a native English speaker for language and flow.

Please note that submitting a revision of your manuscript does NOT guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Karen Chu

On behalf of Dr. Andrea Cooper

Dr. Karen Chu
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com

****

Reviewer: 2
Comments to the Author
The authors have adequately addressed all of my questions.
As a result, the quality of the revised manuscript is substantially improved.

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Peer review correspondence

Reviewer: 3

Comments to the Author

The revised manuscript by Moraes-Vieira et al. adds to our knowledge on the function of leptin on the immune system by investigating the specific effect of leptin on dendritic cells (DCs) and their capacity to differentiate CD4+ T cells. The authors demonstrate that leptin-deficient DCs display impaired maturation and a reduced ability to induce Th1 differentiation, while enhancing Treg, Th17 and Th2 responses. Although these results are somewhat incremental, the data would be of importance to the field of metabolic immunity. However, despite some improvement over the first version, the manuscript suffers from substandard scientific writing, poor organization and flaws in data analysis.

Specific comments

1. The issue of DC culture in leptin-free serum is confusing. If WT DCs are cultured in leptin-free serum then the only leptin in the system produced is by the DCs themselves. The authors have shown that the levels of leptin produced by DCs (picogram range) are not sufficient to modulate the phenotype of DCs. By adding leptin (nanogram range) to leptin-/- DC cultures, the phenotype can be reverted back to WT. Why do WT DCs display a similar phenotype to leptin-/- DCs + rleptin when there should be significantly less leptin in these cultures?

2. As noted above, the standard of scientific writing remains very poor, falling below an acceptable threshold for publication. To list just one example, the sentence in the Abstract ‘Leptin has been shown to induce Th1 polarization, inhibit Th2 responses, induce Th17 responses, inhibit regulatory T cells (Tregs) and modulate autoimmune diseases’ is very long-winded and cumbersome. The writing throughout the paper is like this, making it very hard to slog through. The authors should revise the whole manuscript (particularly the Introduction) in a more concise style.

3. The organization of the figures is very problematic. The figure layouts are not well conceived and difficult to follow. The European Journal of Immunology allows for more figures in the main text than is current there, which the authors need to take advantage of. The authors should revise the figure layouts (including considering moving some supplementary figures into the main body of text) that provides a more logical flow. Specific suggestions follow below:

4. The figures need to be discussed in order of the text. For example, Fig. 4C is discussed after Fig. 4A followed by Fig. 4B. This is also true of several of the supplementary figures. For example, Supp. 6A and 6B,C are described completely separately from one another and should be split into two figures. The figures or text in this manuscript should be altered so that the manuscript follows a logical order. Similarly, figure panels that are related to one another do not follow on from each other. For example Fig. 3C displays summary graphs of Fig. 3A. Therefore, Fig. 3C should become Fig. 3B.

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5. The order in which the WT, Lepob/ob and Lepob/obLep data are presented changes from figure to figure, making it very frustrating to read. The authors should be consistent with the order throughout. The most logical order to me is WT then Lepob/ob then Lepob/obLep, as in Fig. 1. This is because leptin is added to Lepob/ob DCs and so is a follow on from this setting.

6. The rationale behind doing certain experiments is still unclear in many cases. For example, why were the particular cytokines measured in Fig. 3B chosen (e.g. due to the known association of leptin with the induction of Th1 responses).

7. The layout for Figs 1-2 is not logical and they appear repetitive. The authors should rearrange these figures. For example, the gene expression data should be in the same figure as the ELISA data (Fig. 2C inflammatory cytokines) and the flow cytometry data (Fig. 2B) as most of these cytokines/receptors were assessed by real time PCR also.

8. In figure 2, labels are missing from the flow plots (e.g. iDC and mDC). IN part B, the WT, Lepob/obLep and Lepob/ob histograms should be overlayed on one plot.

9. Fig 3- Gates depicting the % proliferation should be added to the flow plots. It is uncler where the values to create the graphs in Fig. 3C were obtained. Fig. 3B should become Fig. 3C and vice versa.

10. Fig 4- Gates depicting the % proliferation should be added to the flow plots.
The layout of this figure should be revised as it is currently large and difficult to follow. One suggestion is to separate the Treg and Th17 data into separate figures, as described in the text.

11. Fig 5- A and B. WT plots should be moved to the top to match the order depicted in the bar graphs.

12.Supp. 1- Panel B, The shift in STAT3 phosphorylation is extremely modest, so, statistics should be added to confirm the shift is significant (from at least 3 separately performed experiments, not from a single collection of FACS data). The figure legend does not include a description of each histogram. Also the authors cannot call the large FSC/SSC gate ‘monocytes’ without additional markers, as there may be activated lymphocytes in this gate also.

13 Supp. 3- Panel A is not convincing as it shows only very small shifts. Please provide statistics on 3 separately performed experiments. IN panel B, the labels missing on flow plots. Also, something has happened to the labels on the bar graphs.

14. Supp. 4-The authors conclude that there is no difference in the expression of IDO, PDL-1 or IL-10. The authors need to clarify which groups they are comparing, as some of the groups look highly different. More

importantly, there appears to be a significant difference between WT and Lepob/ob DC expression of IDO and PDL-1, which is in contrast to the authors conclusions. Statistics should be included to determine significance (again, from separately performed replicate experiments)

15. Supp. 9- Lepob/ob flow plot tail in cell population does not look like true staining and is likely to be an artifact. Gate should be set to exclude this so that results are not skewed.

Second Revision – authors' response – 8 October 2013

Reviewer: 3
Comments to the Author

Reviewer: The revised manuscript by Moraes-Vieira et al. adds to our knowledge on the function of leptin on the immune system by investigating the specific effect of leptin on dendritic cells (DCs) and their capacity to differentiate CD4+ T cells. The authors demonstrate that leptin-deficient DCs display impaired maturation and a reduced ability to induce Th1 differentiation, while enhancing Treg, Th17 and Th2 responses. Although these results are somewhat incremental, the data would be of importance to the field of metabolic immunity. However, despite some improvement over the first version, the manuscript suffers from substandard scientific writing, poor organization and flaws in data analysis.

Answer: The reviewer is correct and we apologize for the grammar errors and typos in the manuscript. A native English speaker has revised English grammar, and typos were thoroughly corrected.

Specific comments

Reviewer: 1. The issue of DC culture in leptin-free serum is confusing. If WT DCs are cultured in leptin-free serum then the only leptin in the system produced is by the DCs themselves. The authors have shown that the levels of leptin produced by DCs (picogram range) are not sufficient to modulate the phenotype of DCs. By adding leptin (nanogram range) to leptin-/- DC cultures, the phenotype can be reverted back to WT. Why do WT DCs display a similar phenotype to leptin-/- DCs + rleptin when there should be significantly less leptin in these cultures?

Answer: We believe that the DCs from WT progenitors came from an environment where leptin is endogenously present, and this pre-conditioning could have an impact on DC generation. Several factors could be involved on these similar phenotypes of WT and Lepob/ob + rLeptin, and on how leptin affect progenitor cells. Unfortunately, it is beyond the scope of the current manuscript and further studies need to be performed. Nevertheless, based on our results, WT DCs are able to produce leptin in a concentration

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up to 5ng/mL, which could be enough to maintain the phenotype of WT DCs. However, based on our results, this concentration is not sufficient to affect the phenotype of DC generated from leptin deficient mice. One possible hypothesis is that (as observed in the gene PCR-array) the expression of many genes were down regulated in DCs generated from leptin deficient mice, and the conversion of this phenotype was only possible by adding high concentrations of leptin. WT DCs did not have major changes in gene expression even in the presence of autocrine leptin production as well as in autologous serum which may be sufficient for WT DCs. Therefore, although WT DCs display a similar phenotype to leptin-/- DCs + rleptin, (where there should be lower amounts of leptin in these cultures), DCs generated from leptin deficient mice need higher doses, as demonstrated. This indicates that autologous secretion of leptin cannot account for the entire phenotype of DC and an exogenous source is needed.

Reviewer: 2. As noted above, the standard of scientific writing remains very poor, falling below an acceptable threshold for publication. To list just one example, the sentence in the Abstract 'Leptin has been shown to induce Th1 polarization, inhibit Th2 responses, induce Th17 responses, inhibit regulatory T cells (Tregs) and modulate autoimmune diseases' is very long-winded and cumbersome. The writing throughout the paper is like this, making it very hard to slog through. The authors should revise the whole manuscript (particularly the Introduction) in a more concise style.

Answer: We have re-edited the whole manuscript, which is now in a more cohesive format.

Reviewer: 3. The organization of the figures is very problematic. The figure layouts are not well conceived and difficult to follow. The European Journal of Immunology allows for more figures in the main text than is current there, which the authors need to take advantage of. The authors should revise the figure layouts (including considering moving some supplementary figures into the main body of text) that provides a more logical flow. Specific suggestions follow below:

Answer: We have revised the manuscript. As suggested by the reviewer we have added an extra figure to the main body of the manuscript, totaling 6 figures. The European Journal of Immunology allows more figures, in the authors instructions it is mentioned that the figure limit is 5. Nevertheless, we changed the order of the figures and now we have 6 total figures.

Reviewer: 4. The figures need to be discussed in order of the text. For example, Fig. 4C is discussed after Fig. 4A followed by Fig. 4B. This is also true of several of the supplementary figures. For example, Supp. 6A and 6B,C are described completely separately from one another and should be split into two figures. The figures or text in this manuscript should be altered so that the manuscript follows a logical order. Similarly, figure panels that are related to one another do not follow on from each other. For example Fig. 3C displays summary graphs of Fig. 3A. Therefore, Fig. 3C should become Fig. 3B.

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Answer: We have revised the order of the results section. We split the figures to provide better organization and flow to the manuscript. We also revised all figures to suit reviewer comments and suggestions.

Reviewer: 5. The order in which the WT, Lepob/ob and Lepob/obLep data are presented changes from figure to figure, making it very frustrating to read. The authors should be consistent with the order throughout. The most logical order to me is WT then Lepob/ob then Lepob/obLep, as in Fig. 1. This is because leptin is added to Lepob/ob DCs and so is a follow on from this setting.

Answer: We have reformulated the figure as suggested by the reviewer.

Reviewer: 6. The rationale behind doing certain experiments is still unclear in many cases. For example, why were the particular cytokines measured in Fig. 3B chosen (e.g. due to the known association of leptin with the induction of Th1 responses).

Answer: We have revised the experimental rational and included necessary changes in the manuscript.

Reviewer: 7. The layout for Figs 1-2 is not logical and they appear repetitive. The authors should rearrange these figures. For example, the gene expression data should be in the same figure as the ELISA data (Fig. 2C inflammatory cytokines) and the flow cytometry data (Fig. 2B) as most of these cytokines/receptors were assessed by real time PCR also.

Answer: We have included the PCR gene expression data (Figure 2B) as suggested by the reviewer.

Reviewer: 8. In figure 2, labels are missing from the flow plots (e.g. iDC and mDC). IN part B, the WT, Lepob/obLep and Lepob/ob histograms should be overlaid on one plot.

Answer: We have included the labels in the Figure 2A. Regarding the histograms, our coauthors preferred to maintain the histograms in the format it was previously shown, since it provides easier comparison. We thought the overlay made the graph looked crowded and confusing.

Reviewer: 10. Fig 4- Gates depicting the % proliferation should be added to the flow plots.The layout of this figure should be revised as it is currently large and difficult to follow. One suggestion is to separate the Treg and Th17 data into separate figures, as described in the text.

Answer: We have included the proliferation data as requested. We also rearranged the figure to a better layout. Now, as requested there is one individual figure for Treg and one for Th17.

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Reviewer: 11. Fig 5- A and B. WT plots should be moved to the top to match the order depicted in the bar graphs.

Answer: We have modified it in the figure as requested by the reviewer.

Reviewer: 12.Supp. 1- Panel B, The shift in STAT3 phosphorylation is extremely modest, so, statistics should be added to confirm the shift is significant (from at least 3 separately performed experiments, not from a single collection of FACS data). The figure legend does not include a description of each histogram. Also the authors cannot call the large FSC/SSC gate 'monocytes' without additional markers, as there may be activated lymphocytes in this gate also.

Answer: We have included the bar graph with the STATs results from different experiments. Because only few surface antibodies work with the PhosFlow technique (due to the harsh Permeabilization procedure), we did not have an marker available for monocytes in this technique. We agree with the reviewer that we have activated lymphocytes in this gate region. Therefore, we now called this region as enriched in monocytic-type cells, since it is the region, based on FSC and SSC, where monocytes are found.

Reviewer: 13 Supp. 3- Panel A is not convincing as it shows only very small shifts. Please provide statistics on 3 separately performed experiments. IN panel B, the labels missing on flow plots. Also, something has happened to the labels on the bar graphs.

Answer: We have included the STATs from the different experiments performed. The labels were modified due to PDF conversion and we apologize for that. Now we have carefully revised the figures after the PDF conversion.

Reviewer: 14. Supp. 4-The authors conclude that there is no difference in the expression of IDO, PDL-1 or IL-10. The authors need to clarify which groups they are comparing, as some of the groups look highly different. More importantly, there appears to be a significant difference between WT and Lepob/ob DC expression of IDO and PDL-1, which is in contrast to the authors conclusions. Statistics should be included to determine significance (again, from separately performed replicate experiments)

Answer: We have modified it in the text. We now state that the immature phenotype of iDC generated from Lepob/ob mice, in the lack of leptin, does not result in the up regulation of several well known anti-inflammatory molecules, that can be expressed by tolerogenic DC, such as IDO, PDL1 and IL-10. Indeed, the Lepob/ob DC express lower levels of IDO and PD-L1.

Reviewer: 15. Supp. 9- Lepob/ob flow plot tail in cell population does not look like true staining and is likely to be an artifact. Gate should be set to exclude this so that results are not skewed.

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Answer: We have performed a new experiment and we did not observe this “tail”, which guaranteed the absence of artifact.

Third Editorial Decision – 28 October 2013

Dear Dr. Camara,

Thank you for submitting your revised manuscript ID eji.201343592.R2 entitled "Leptin deficiency impairs dendritic cell maturation and enhances its ability to induce regulatory T and Th17 cells" to the European Journal of Immunology. Your manuscript has been re-reviewed and the comments of the referee are included at the bottom of this letter.

Although the referee has recommended publication, some revisions to your manuscript have been requested. Therefore, I invite you to respond to the comments of referee 3 and revise your manuscript accordingly.

You should also pay close attention to the editorial comments included below. *In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.*

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology. We look forward to receiving your revision.

Yours sincerely,
Karen Chu

on behalf of Dr. Andrea Cooper

Dr. Karen Chu
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com

European Journal of Immunology

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Reviewer: 3

Comments to the Author

The revised manuscript by Moraes-Vieira et al. adds to our knowledge on the function of leptin on the immune system by demonstrating that leptin deficient DCs display impaired maturation and reduced ability to induce Th1 differentiation, while enhancing Treg, Th17 and Th2 responses. These findings are of importance to the field of metabolic immunity and the data are of an acceptable quality for the EJI. Although the scientific writing has improved significantly, the standard still falls short. Importantly, the manuscript still does not flow well and the major conclusions drawn by the authors do not take in to account all of the data that they show.

Overall layout/structure

The data show that leptin deficiency decreases DC maturation and Th1 responses while increasing Th2, Th17 and Treg responses. The data are clear in demonstrating these findings. However, the manuscript is written in a way that unnecessarily complicates the data and at the same time ignores some major findings when concluding the data. The authors need to revise the layout of the manuscript to determine a better way to write and display their story. The manuscript contains a lot of data (15 supplementary figures), and this needs to be displayed in a more concise way. For example, several of the supplementary figures can be combined (e.g. S6 and S7, as well as S8 and S9).

Third Revision – authors' response – 8 November 2013

Reviewer: 3

Comments to the Author

The revised manuscript by Moraes-Vieira et al. adds to our knowledge on the function of leptin on the immune system by demonstrating that leptin deficient DCs display impaired maturation and reduced ability to induce Th1 differentiation, while enhancing Treg, Th17 and Th2 responses. These findings are of importance to the field of metabolic immunity and the data are of an acceptable quality for the EJI. Although the scientific writing has improved significantly, the standard still falls short. Importantly, the manuscript still does not flow well and the major conclusions drawn by the authors do not take in to account all of the data that they show.

Answer: We thank the reviewer for all comments and suggestions to improve the manuscript. We made the modifications accordingly.

Overall layout/structure

European Journal of Immunology

The data show that leptin deficiency decreases DC maturation and Th1 responses while increasing Th2, Th17 and Treg responses. The data are clear in demonstrating these findings. However, the manuscript is written in a way that unnecessarily complicates the data and at the same time ignores some major findings when concluding the data. The authors need to revise the layout of the manuscript to determine a better way to write and display their story. The manuscript contains a lot of data (15 supplementary figures), and this needs to be displayed in a more concise way. For example, several of the supplementary figures can be combined (e.g. S6 and S7, as well as S8 and S9).
Answer: We reviewed the manuscript and combined the figures as suggested.

Fourth Editorial Decision – 14 November 2013

Dear Dr. Camara,

It is a pleasure to provisionally accept your manuscript entitled "Leptin deficiency impairs maturation of dendritic cells and enhances induction of regulatory T and Th17 cells" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items (copyright forms etc.) are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Karen Chu

on behalf of Dr. Andrea Cooper

Dr. Karen Chu
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