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PD-L1 co-stimulation contributes to ligand-induced T cell receptor down-modulation on CD8+ T cells

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   22 June 2011

Dear Dr. Escors,

Thank you for the submission of your manuscript "PDL-1 co-stimulation provides the signal for ligand-induced T cell receptor down-modulation" to EMBO Molecular Medicine and please accept my apologies for the delayed reply.

We have now received two out of three referee reports on it and given that the review process so far has been lengthy, we would prefer to make a decision now. We will send you the third report as soon as it becomes available.

You will see that the reviewers find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

Importantly, reviewer #1 highlights that tumor-infiltrating lymphocytes should be analyzed while reviewer #2 feels that the conclusions regarding PD-1/PD-L1 in TCR downregulation should be toned down. In addition, this reviewer notes that FACS plots should be shown where possible.

On a more editorial note, please confirm that you received institutional ethics approval for the animal experiments and include a corresponding statement in the Material and Methods section.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can address the issues that have been raised within the time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless discussed differently with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

REFEREE REPORTS:

Referee #1 (Comments on Novelty/Model System):

The paper addresses an important issue in T cell physiology that has wide implications in Medicine, from autoimmunity to cancer immunotherapy. Moreover, it identifies a novel mechanism for ligand-mediated TCR down-modulation. Technically the work is well-controlled and overall very sound.

Referee #1 (Other Remarks):

Ligand-induced TCR down-modulation is a critical process in the regulation of T cell activation. While the current dogma is that TCR/ CD28 signals control this process, the manuscript by Karwacz and colleagues identifies the PD-L1/ PD-1 as a novel TCR "extrinsic" pathway that promotes ligand-induced TCR down-modulation. Interference with PD-L1/PD-1 signaling strongly inhibits TCR down-modulation leading to hyper-activated T cells in vitro and in vivo. Moreover, the authors show that RNAi silencing of PD-L1 accelerates anti-tumor immune responses. They further propose a mechanism by which PD-1 signaling induces the up-regulation of Cbl-b, previously shown to be required for TCR down-modulation.

Issues to be addressed by the authors:

1. The authors should make it clearer that the mechanism proposed applies specifically to CD8+ T cells (not to CD4+ T cells). This should be stated in the title and in the abstract.

2. The different results obtained with CD8+ and CD4+ T cells should be discussed in the Discussion. The full discussion could be longer and more incisive. It should, for example, comment further on the lack of curative effects of PD-L1 silenced DC vaccines in the absence of DC activators (Fig. 4d).

3. Page 3: the sentence: "BM-DCs ... activated proliferation of OVA-specific CD4 and CD8 T cells purified from transgenic OT-I and OT-II mouse strains, respectively" should be corrected to "from transgenic OT-II and OT-I mouse strains, respectively".

4. Same page: "the majority of CD8 T cells clustered around BM-DCs expressing IiOVA-p5 (Fig. 1b)" should be clarified to "around 90% of CD8 T cells..." to make a direct comparison with the 40% previously mentioned for the controls.

5. Besides Thy1.2 (Fig. 1d), the expression of additional surface markers (such as CD2 and CD28) should be analyzed and shown. The specific down-modulation of the TCR complex is a key point of this study.

6. Authors postulate that the drugs used in figure 5 would enhance the expression of co-stimulatory and adhesion molecules in DCs. However, they show no evidence for this. Have they analyzed CD80, CD86, CD70 or ICAM-1 expression in their system?

7. What is the evidence that the TCR down-modulation mechanism analyzed in figures 1-2 is involved in the in vivo data obtained in the tumor models of figures 4-5? Did the authors analyze tumor-infiltrating lymphocytes (particularly CD8+ T cells)? If not, they need to discuss the limitations of their mechanistic findings in the discussion.

Referee #2 (Comments on Novelty/Model System):

see remarks to the authors

Referee #2 (Other Remarks):

TCR downregulation in response to its own triggering has been reported first by Lanzavecchia's group in the 90s (Valitutti et al., Nature 375: 148, 1995) and many others since. The initial understanding was that a TCR molecule is degraded upon engagement of the pMHC complex which then triggers many TCR molecules consecutively. Though this serial triggering hypothesis fell out of favor when it was found that
- TCR molecules are continuously recycled and their previous engagement is not necessary to do so (San Jose et al., Immunity 12: 161, 2000; Liu et al., Immunity 13: 665, 2000),
- TCR molecules engineered with artificially high affinities to pMHC complexes are functional (Holler et al., J Exp Med 194: 1043, 2001),
- low TCR numbers per cell do not affect the cells' functionality (Labrecque et al., Immunity 15: 71, 2001), and
- the immunological synapse appeared to fix the TCR molecules engaged at the signaling interface between APC and T cell (Grakoui et al., Science 285: 221, 1999).
Since TCR signaling and the immunological synapse is now being seen as a highly dynamic events (Huppa et al., Nature 463: 963, 2010; Fooksman et al., Ann Rev Immunol 28: 79, 2010), the idea of serial triggering is currently resurrected and a deeper investigation of the mechanisms responsible for TCR downregulation is certainly warranted.

Karwacz et al. address this issue from an unexpected angle. They demonstrate by several means that TCR downregulation can be blocked partially by interfering with PD-1/PD-L1 interaction. While this data set is an interesting one, the text and some figures have to be revised before publication in EMBO Mol Med.

1) The results are overambitiously framed as though the PD-1/PD-L1 interaction were the only possible cause for TCR downregulation. This is certainly untrue: It has been shown many times that several anti-TCR or -CD3 mAbs alone can trigger it in the absence of APCs or any other signals. Actually, reference 6 establishes the link between TCR downregulation and c-cbl/cbl-b by triggering with an anti-CD3 mAb. We also observe it in the lab on a daily basis ourselves. None of the observations in the paper actually support the exclusivity claimed in title, abstract (third sentence), and discussion that PD-1/PD-L1 is THE signal for TCR downregulation. However, the paper's data support the notion that PD-1/PD-L1 interactions contribute to TCR downregulation and interference with them can possibly be useful in several clinical situations. The unnecessarily strong wording also blinds introduction and discussion for the well established work on the issue. Actually, none of the papers mentioned above is mentioned, not even alluded to in any of the cited reviews. This is unfortunate and should be thoroughly reworked as the current wording unnecessarily takes away from the paper's clear data and thrust.

2) It is stated several times that TCR downregulation is an immunoregulatory process necessary for the maintenance of tolerance. Though it was initially understood this way (ref. 8) and recently recovered (Holst et al., Nat Immunol 9: 658, 2008), I do not think this is universally true. There are several systems where T cells transferred into Ag-presenting hosts do not downregulate their TCR despite continuous triggering (e.g.. Choi & Schwartz, Immunol Rev 19: 140, 2007), unless Ag is presented by B cells (Knoechel et al., J Immunol 175: 21, 2005; Han et al., PNAS 107: 20453, 2010). I am also not aware of TCR downregulation in chronic infections by LCMV mutants, where PD-1 plays a very important role (Barber et al., Nature 439: 682, 2006; Wherry, Nat Immunol 12: 492, 2011).

3) Overall, the data are clearly explained and well presented. I recommend, however, that the authors include flow cytometry plots wherever possible to enhance credibility. This is badly missing in Figs. 1f, 1g, 3a and 3e.

DETAILED RESPONSES TO REVIEWER 1

Reviewer 1 considers that our results are very relevant, with wide implications in medicine. The Reviewer suggests several issues to solve to improve our manuscript.

Points 1 and 2:

The Reviewer remarks that the mechanism proposed applies specifically to CD8+ T cells, and that this should be stated in the title and in the abstract. In addition, the different results obtained with CD8 and CD4 T cells should be discussed fully in the discussion section.

We agree with the referee that the presented data mainly applies to CD8 T cells. As suggested by the Reviewer, we have modified the title as follows: "PDL-1 co-stimulation contributes to ligand-induced CD8 T cell receptor down-modulation". The abstract has been modified specifying CD8 T cells every time they are mentioned.

As the Reviewer suggested, we have also included the following: In Results, page 5, line 19: "By comparison, no differences were observed for DC-CD4 OT-II cell association, which was around 10% (results not shown)". In Discussion, from page 11, line 11: "Unexpectedly, we did not observe significant ligand-induced TCR down-modulation after three-four days co-culture of OVA-specific CD4 T cells with IiOVA-expressing BM-DCs, whether PD-L1 was silenced or not. Accordingly, we were unable to test in the mouse model if PD-L1/PD-1 co-stimulation was involved in CD4 TCR down-modulation. It is worth noting that unlike CD8 T cells, only a minor proportion of OVA-specific OT-II T cells strongly associated to IiOVA-expressing DCs. This is in agreement with other reports in which naïve CD4 T cells detach quickly from antigen-presenting cells, but they get fully activated later by multiple, short-lived contacts (Celli et al., 2005). However, it is likely that PD-L1/PD-1 interaction may also be involved in CD4 TCR down-modulation, as we did observe inhibition of TCR down-modulation in PD-1-silenced human CD4 T cells, but in the context of superantigen stimulation."

Point 2.

Reviewer 1 comments that the discussion could be longer and more incisive, for example commenting the lack of curative effects of PD-L1 silenced DC vaccines unless a DC activator is provided.

We fully agree with the referee. The discussion in the original manuscript was written for another journal with more space constraints. In fact, interference/blocking with PD-L1/PD1 alone is not as therapeutically effective as other strategies. Thus, we have included the following: Page 11 line 22: "PD-L1/PD-1 interaction is receiving an increasing interest as a therapeutic target. While most of the studies use systemic administration of blocking antibodies, a minor proportion have silenced B7 inhibitory molecules in DCs (Breton et al., 2009; Hobo et al., 2010). In any case, therapeutic outcomes vary considerably, and are quite dependent on the specific experimental system. In many instances, combination with other strategies has to be applied to achieve effective therapy (Breton et al., 2009; Curran et al., 2010; Hobo et al., 2010; Pilon-Thomas et al., 2010). In our experimental system, PD-L1 silencing in DCs alone did not improve overall cure rates compared to non-silenced DCs. PD-L1 silencing had to be combined with DC molecular activators to achieve increased therapeutic activities. Curiously, this phenomenon is observed in many other published work, and may account for the relative lack of efficacy of PD-L1/PD-1 blocking antibodies unless they are given in combination therapies. Thus, the anti-tumour efficacy of PD-L1/PD-1 blocking antibodies is enhanced by lentivector vaccination (Sierro et al., 2011; Zhou et al., 2010). In these two last reports, an increased recruitment of tumour infiltrating CD8 T cells was observed after systemic administration of PD-L1/PD-1 antibodies. In agreement with this, we also observed a tendency to increased intra-tumour CD8 T cell numbers in mice vaccinated with IiOVA-expressing PD-L1-silenced DCs. However, we could not demonstrate significance in relative numbers or TCR surface levels compared to vaccination with non-silenced DCs. In contrast to systemic administration of blocking antibodies, PD-L1 silencing in our experimental system is taking place in a limited number of DCs. Secondly, we could not differentiate a specific recruitment of effector

CD8 T cells from just simply an overall increase in CD8 T cell numbers (Figs 4 and 5). However, in light of previous reports, it is likely that also in our case, there is an enhanced, active intratumoural recruitment of CD8 T cells. Summarizing, our results demonstrate that PD-L1 silencing in DCs may speed-up T cell expansion and cytokine responses (which is important for tumour immunotherapy) but other factors also contribute to the effectiveness of the immune response, for instance the functionality of activated T-cells which is a direct consequence of a range of co-stimulatory signals during antigen presentation. Further co-stimulation could enhance the expression of cytotoxic molecules in effector CD8 T cells such as granzyme B and perforin, which could be responsible for the observed increase in therapeutic activity."

Point 3.

Page 5, line 16. The correction suggested by the Reviewer has been introduced in the sentence.

Point 4.

Page 5, line 18. The correction suggested by the Reviewer has been introduced in the text.

Point 5.

The Reviewer considers that besides Thy1.2, the expression of additional surface markers should be analysed and shown, because the specific down-modulation of the TCR complex is a key point of this study.

We don't agree with the referee in this point for the following reasons. The key point of this study is that the TCR complex does not internalise. We feel that there is no need to re-demonstrate ligand-induced TCR down-modulation, as it is well established (see comments from Reviewer 2), especially in antigen-specific presentation assays. Accordingly, we have modified the manuscript including further references to ligand-induced TCR down-modulation, as requested by Reviewer 2.

We have shown that TCR-CD3 is not internalised. The role of Thy1.2 in our experiments is nOT-Intended to demonstrate ligand-induced TCR down-modulation, but rather a way of identifying T cells using a maker that does not associate to TCRs. In addition, we have specifically inhibited TCR down-modulation with shRNA and blocking PD-L1/PD-1 antibodies, but not with transduction controls or isotype antibodies. This demonstrates that our inhibition of TCR down-modulation is specific and dependent on PD-L1/PD-1 interactions.

Point 6.

The Reviewer comments that we are postulating that our molecular activators shown in figure 5 would enhance expression of co-stimulatory and adhesion molecules in DCs, but we don't show evidence for it.

We sincerely apologise if we did not explain it clearly in the text. We don't postulate up-regulation of co-stimulatory/adhesion molecules because we have already published that in two previous publications (Escors et al. 2008; Arce et al. 2011) . That is the main reason why we have used them in our present study. In addition, we have observed up-regulation of the same markers in this study

To clarify this point in the text to the readers, we have modified the following sentence, page 9, line 13 as follows: "We chose these MAPK modulators because we have previously demonstrated that p38 activation and ERK inhibition using these modulators increase expression of co-stimulatory and adhesion molecules in DCs such as CD80, CD40 and ICAM I (Arce et al., 2011; Escors et al., 2008).".

Point 7.

Reviewer 1 suggested analysing tumour-infiltrating CD8 T cells, or in its defect, at least discussion of the limitations of our findings.

We fully agree with the Reviewer, and in fact we did analyse tumour infiltrating CD8 T cells, and we have included this information in the discussion (see point 2). We observed a tendency to increased intra-tumour CD8 T cell numbers but we could not demonstrate significance in our experimental system, in contrast to other studies using systemic administration of blocking antibodies (which we have accordingly cited in our revised manuscript). In addition, the relatively low numbers of isolated T cells from tumours did not allow us to draw any definitive conclusion regarding their TCR levels, and we made that also clear in the text (see point 2).

DETAILED RESPONSES TO REVIEWER 2

Reviewer 2 considers our data to be interesting and relevant for the field, although his/her main concern is with overstatement of our conclusions. Reviewer 2 comments that our current wording unnecessarily takes away from the paper's clear data and thurst. We completely agree with the Reviewer that our manuscript could be improved with a more tactful/realistic interpretation from the data, and we hope that in our revised manuscript we have achieved to do so. In addition, the Reviewer suggested several ways to improve the manuscript and cover key publications in the field.

Therefore, we have answered all of the Reviewers' concerns point by point as follows:

Point 1.

The Reviewer points out that the results are over ambitiously framed as though PD-L1/PD1 interaction was the only possible cause for TCR donwregulation. The Reviewer comments that this is untrue because it has been shown many times that several anti-TCR or CD3 mAbs alone can trigger down-modulation in the absence of other signals, and points to one of the cited references as an example, and also their particular experimental experience.

We overall agree with the referee, but at this point I would also call for careful interpretation, because PD-L1 is expressed both by naïve and activated T cells. In fact, this is exemplified by Miza et al, who reported increased TCR surface expression by anti-CD3 when PD-L1 was blocked with antibodies, in the absence of any APC. Fooksman et al (Ann Rev Immunol), points out that PD-L1/PD-1 interactions may take in homotypic (T cell-T cell) interactions, or even in cis between membrane projections from the same cell. The participation of PD-L1 expressed by T cells cannot be underestimated. To clarify this particular point, we have modified the discussion (see detailed modifications below):

Nevertheless, we agree that with our data we have only identified a contributor in one of the steps of TCR down-modulation. In fact, in our hands ligand-induced TCR down-modulation proceeds steadily, reaching a maximum around 3 to 4 days after antigen presentation. Afterwards, TCR surface levels recover slowly (which would probably suggest a change in "recycling" equilibrium, and possibly linked to the "second type" of down-modulation as proposed by San Jose et al?). During the revision, we realized that this timing might prove important in the context of this complex mechanism. We decided to include this data in the revised manuscript so that we could discuss it in the context proposed by the Reviewer (see points below).

All these issues have been clarified throughout the text, which has significantly benefited from the Reviewer's points of view. More specifically, we have introduced the following changes:

- We have removed the strong wording throughout the text. The title has been changed accordingly as follows: "PDL-1 co-stimulation contributes to ligand-induced CD8 T cell receptor down-modulation"

- Abstract, page 2 line 4, we have modified the sentence eliminating "alone", and modified it as follows: "Current understanding of this process is that intrinsic TCR/CD28 signal transduction leads to TCR down-modulation"

- Abstract, page 2, line 4, we have modified the sentence as follows: ". Here we show that the interaction between PD-L1 on dendritic cells (DCs) and PD-1 on CD8 T cells <u>contributes to</u> ligand-induced TCR <u>down-modulation</u>".

- Introduction: We have modified the second paragraph to give an overview of the down-modulation mechanism, including some of the key references provided by Reviewer 2. We have further discussed this in more detail in the Discussion (see "discussion" modifications below). The modified paragraph in the introduction is as follows (Page 3, line 27): "Ligand-induced TCR down-modulation is another regulatory process of T cell activation at the level of antigen presentation. TCRs are removed from the T cell surface shortly after activation, limiting signal transduction and avoiding excessive responses (Holst et al., 2008; Naramura et al., 2002; San Jose et al., 2000; Schonrich et al., 1991; Shamim et al., 2007). The current view of ligand-induced TCR down-modulation is that intrinsic TCR signalling following antigen recognition is sufficient for TCR down-modulation. However, the exact mechanism by which this occurs is still under extensive research. Ligand-induced TCR down-modulation is a complex, multi-mechanistic process (Lauritsen et al., 1998). It is well established that ligand-engaged TCR complexes are quickly internalised (Cai et al., 1997; Dietrich et al., 1998; Huppa et al., 2010; Lauritsen et al., 1998; Valitutti et al., 1995). Afterwards, other TCR complexes including non-engaged ones, down-regulate following signal transduction from the triggered TCRs (San Jose et al., 2000). Nevertheless, up-regulation of E3 ubiquitin ligases of the Cbl family in T cells contributes to ligand-induced TCR down-modulation, (Naramura et al., 2002; Shamim et al., 2007), as demonstrated by studies with Cbl knock-out (KO) mice. Cbl KO T cells have reduced TCR down-modulation following antigen presentation, leading to sustained signalling and hyper-activation (Chiang et al., 2000; Naramura et al., 2002; Shamim et al., 2007). Interestingly, to date, no additional extrinsic signals provided by DCs in the immunological synapse have been identified which are critical for ligand-induced TCR down-modulation. "

- Results: The introduced changes have been underlined, as follows:

  In page 6 line 11 we have modified the sentence as :" Collectively, our results demonstrate that DC-derived PD-L1 signaling <u>contributes</u> to ligand-induced TCR down-modulation after antigen presentation"

  Page 6 line 14: "Recent evidence demonstrates that expression of Cbl E3 ubiquitin ligases <u>significantly contributes</u> to ligand-induced TCR <u>down-modulation</u>."

  We have included a supplementary figure 2 showing the kinetics of TCR down-modulation with and without PD-L1 silencing at different time points. This data will clarify the step in which PD-L1/PD-1 contributes to TCR down-modulation. Therefore, we have included the following in results, Page 5 line 30: "It is worth noting that TCR down-modulation reached a maximum on day three to four, and TCR levels gradually recovered afterwards (supplementary Fig 2). PD-L1 silencing in DCs significantly delayed TCR down-modulation but did not completely abrogate it (supplementary Fig 2)."

  In Page 6 line 27 we have modified the sentence as: "To confirm that PD-L1/PD-1 co-stimulation <u>was a regulator</u> of TCR down-modulation and also exclude off-target shRNA-mediated mechanisms"

Page 7 line 4: "Collectively, these data demonstrate that the PD-L1/PD-<u>1 interaction contributes to ligand-induced </u>TCR down-modulation and <u>that it would depend on PD-1 expression </u>in T cells after activation, <u>in our experimental system"</u>

Page 7 line 13: "Accordingly, CD3 surface down-modulation was significantly inhibited following PD-1 silencing in stimulated GFP+ CD4 T cells, <u>although not completely abrogated </u>(results not shown)."

- Discussion: The first paragraph of the discussion has been modified extensively to include a more detailed background of TCR down-modulation in the context of our results:

Page 10, line 2: "We have provided evidence that PD-L1 in antigen presenting DCs contributes to ligand-induced TCR down-modulation. Ligand-induced TCR down-modulation is a complicated process in which more than one mechanism is involved (Lauritsen et al., 1998). Firstly, serially ligand-engaged TCRs internalise quickly (from seconds to hours) after ligand-MHC binding (Huppa et al., 2010; Valitutti et al., 1995). Secondly, non-engaged TCR complexes can also be down-regulated following signal transduction from previously engaged (and endocytosed) TCR complexes (San Jose et al., 2000). It is still under debate whether TCR down-modulation is caused by internalisation followed by degradation, or by preventing TCR recycling back to the membrane (Dietrich et al., 1998; San Jose et al., 2000; Valitutti et al., 1995). Interestingly, we have observed that TCR down-modulation progressively continues up to three days after initial antigen presentation by DCs. Subsequently, TCR surface levels recover again. This TCR down-modulation may be caused by either direct internalisation, or just a shift in recycling towards intracellular retention of TCR complexes (San Jose et al., 2000; Valitutti et al., 1995). Our results implicate PD-L1/PD-1 co-stimulation in the second, slower phase, and they are in agreement with the following working model for regulation of T cell activation during antigen presentation by DCs (Fig 8):"

Page 10, line 25: "Whether this removal is triggered by active internalization or a change in the dynamics of TCR recycling is yet unclear. Our data supports PD-1 as an early brake that fine-tunes T cell activation during antigen presentation after TCR signal transduction"

Page 10, line 29: "Firstly, TCR/CD28 signal transduction *per se* is not required <u>for full ligand-</u>induced TCR down-modulation, as observed in Cbl-b KO CD8 T cells (Shamim et al., 2007)."

Page 11 line 5: We have introduced the following: "This last observation would implicate PD-L1/PD-1 homotypic T cell interactions (T cell-T cell) either in *trans* or in *cis*, as suggested by others (Fooksman et al, 2010).".

Page 11, line 7: "The interpretation of all these results is therefore straightforward if PD-L1/PD-1 binding <u>contributes to ligand-</u>induced TCR down-modulation through Cbl-b expression."

- Figure 4. Now it is Figure 8 after the extensive manuscript reorganization. While in the original figure we pointed that Cbl-b led to "internalization", now we have changed to " internalization/recycling". We have further made it clearer in the figure legend, as follows: "C. both SHP and Cbl-b de-phosphorylate and inactivate by ubiquitination

key components of the TCR signal transduction machinery. D. <u>Cbl-b either triggers TCR down-modulation, or shifts the recycling equilibrium towards retention of ligand-engaged TCR complexes.</u> "

Point 2.

The Reviewer comments that we have stated several times in the text that TCR down-regulation is an immunoregulatory process necessary for the maintenance of tolerance.

We agree with the Reviewer that TCR down-modulation itself is not an immunoregulatory process as understood by induction of immunological tolerance (although it can participate in some circumstances). This is not what we wanted to state in our manuscript, and we accept the criticism. The message we wanted to give is that interference with TCR down-modulation by inhibiting PD-L1/PD-1 over-stimulates T cells, in the context of "physiological" antigen presentation by DCs. This is supported by several papers in addition to some others pointed out by the Reviewer (Liu et al, 2000, for example). This is the message that we wanted to transmit in our manuscript, rather than its linkage to tolerance. We are also aware and we accept that there are many mechanisms in which T cells are exhausted without down-regulating their TCR, such as in some chronic viral infections.

To clarify all these points to the reader we have modified the following:

- Abstract, first line, Page 2 line 2: We have changed the first line of the abstract as follows "T cell receptor (TCR) down-modulation after antigen presentation is a fundamental process that regulates TCR signal transduction ". In this way, "immunoregulatory process" doesn't appear any more in the revised manuscript, to avoid misinterpretation.

- The last paragraph of the discussion has been changed as follows: Page 12, line 20:"Our model highlights the contribution of PD-L1/PD-1 co-stimulation to ligand-induced TCR down-modulation during "physiological" antigen presentation by DCs. This is a very complex process that is regulated at multiple levels, and there are other pathways that inhibit effector T cell activities without modifying TCR levels (Barber et al, 2006; Choi & Schwartz, 2007; Wherry, 2011). Defining the mechanism by which PD-1 engagement leads to Cbl-b up-regulation and modulates TCR levels is a key question in T cell physiology and a potential target for therapeutic intervention"

Point 3

The Reviewer suggests that the data is clearly explained and well-presented, but flow cytometry plots for figures 1f, 1g, 3a and 3e are missing, and should be shown.

We agree with the Reviewer. Flow cytometry plots were not included originally because the manuscript was automatically transferred from another journal to EMBO mol med, which was more restrictive format. In addition, we have changed the figures' format (according to EMBO Mol Med's specifications) and included more figures to clarify the issues raised by the Reviewer.

- <u>Figure 1f:</u> In this case we had already provided the flow cytometry plot, which is present <u>in the same figure (1c in the original figure).</u> Possibly due to the "unconventional" organisation of the figure, and the lack of reference to it in the legend, the Reviewer has missed this. To avoid this, we have modified Figure 1 and its legend, so that flow plots and their corresponding bar graphs are placed one next to the other. The figure legend is more explicit now linking both of them, as follows, Page 21, line 7 : "C Surface expression of transgenic OVA TCR chains in OT-I cells co-cultured for four days with DCs transduced with the indicated lentivectors (top). Transgenic OT-I cells are $V\beta5.1.5.2^{high}$ and $V\alpha2.1^{high}$. D. As in (C), but plotting transgenic OVA-specific TCR surface expression in OT-I CD8 T cells co-cultured

with the indicated transduced DCs (bottom), as a bar graph with error bars (standard deviations).

- <u>Figure 1g.</u> Now it is part of Figure 2, due to the inclusion of FACS plots for a direct comparison between the different samples. We have included an additional figure legend and introduced all the changes in the text.

- <u>Figure 3a.</u> Now it is Figure 4. FACS plots have been included to show the gating on TCR-expressing OT-I cells, and the CFSE dilution in TCRhigh T cells comparing all the samples.

- <u>Figure 3e.</u> The Reviewer asked to provide the original FACS data. Therefore, the FACS plots are shown now in Figure 5 with its individual figure legend, as requested by the Reviewer. We have also added the following sentence in Results, Page 8 line 15: "Numbers of pentamer-positive CD8 T cells after vaccination with PD-L1-silenced IiOVA-DCs remained higher than in non-silenced vaccination controls, up to three weeks after vaccination, with high pentamer fluorescent intensities that probably reflect high TCR surface levels (Fig 5). These results suggested an accelerated or uncontrolled CD8 T cell activation/expansion, in agreement with our *in vitro* studies using OT-I cells."

Additional Note by Editor   01 July 2011

Third referee withdrawn.

2nd Editorial Decision   01 July 2011

Dear Dr. Escors,

Please find enclosed the final reports on your manuscript. We are pleased to inform you that your manuscript is accepted for publication and will be sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.

As you can see below, Reviewer 2 suggests to change the title of the study slightly and we would encourage you to do so. If you agree, please let us know and we will include this change in the file.

Please see below for additional IMPORTANT information and instructions regarding your article, its publication, and the production process.

Congratulations on your interesting work.

Yours sincerely,

Editor
EMBO Molecular Medicine

REFEREE REPORTS:

Referee #1 (Comments on Novelty/Model System):

Ligand-induced TCR down-modulation is a critical process in the regulation of T cell activation.
This work identified the PD-L1/ PD-1 as a novel pathway that promotes ligand-induced TCR down-

modulation. The authors successfully manipulated the expression of the key molecules in the pathway and provided important mechanistic insight to this phenomenon.

Referee #1 (Other Remarks):

The authors have adequately addressed my concerns and, with the further changes suggested by Ref 2, the revised manuscript is markedly improved.

Referee #2 (Other Remarks):

The authors have to be commended for swiftly revising text and figures according to this reviewer's requests. The solid data and their clear presentation and discussion now make this ms. a good catch for EMBO Mol Med. The only quibble I have are the revised and the running titles. "CD8 TCR" is not necessarily the TCR on CD8+ T cells, but can be misunderstood as the molecule CD8 also being downregulated. My suggestion: "PD-L1 co-stimulation contributes to ligand-induced T cell receptor down-modulation on CD8+ T cells".