European Journal of Immunology

Plasmacytoid dendritic cells induce tolerance predominantly by cargoing antigen to lymph nodes

Karan Kohli, Anika Janssen and Reinhold Förster

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Handling Executive Committee member: Sozzani

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 - 31-Mar-2016

Dear Dr. Forster,

My apologies for the delay in the peer review of your manuscript ID eji.201646359 entitled "Plasmacytoid dendritic cells induce tolerance predominantly by cargoing antigen" which you submitted to the European Journal of Immunology. There was a delay in one of the reports and again a delay due to the Easter holidays. Nevertheless all opinions have been received and 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. Should you disagree with any of the referees' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

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.*

European Journal of Immunology

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 referee(s) 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 Prof. Silvano Sozzani

Dr. Karen Chu
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Reviewer: 1

Comments to the Author
The major finding of this manuscript is that antigen-loaded resting pDC transferred i.l. do not home to the T cell zone of draining lymph nodes and do not interact with cognate T cells as do steady state trafficking cDC, as described in the literature. Furthermore, the authors nicely demonstrate that antigen loaded resting pDC delivered to the afferent lymphatic can inhibit delayed type hypersensitivity induced by intralymphatic delivery of activated, antigen-loaded conventional DC. They observe OT-II cell clustering in mice that received OVA loaded pDC; however this clustering is not associated with the transferred pDC. They suggest this indicates that pDC arriving from the lymphatics can transfer antigen to endogenous lymphatic cDC but this is not directly shown.

The authors should address the following major points:

In general, the authors compare the migration behavior and T cell priming ability of resting/unstimulated pDC to LPS-stimulated cDC. However they do not show the T cell priming ability or migration behavior of unstimulated cDC or stimulated pDC, which are important controls needed in order to make conclusive statements about the unique tolerogenic or immunostimulatory properties of either cell type within their experimental systems.

Figure 1:

1. The authors should demonstrate that the S1P receptors are not different on their adoptively transferred conventional and plasmacytoid dendritic cells. They should ensure that the difference seen in the OT-II proliferation kinetics seen between the groups cannot be explained solely by a difference in the S1P receptors expressed by the subsets of dendritic cells and therefor their retention time in the draining lymph nodes.
2. If the OT-II cells are undergoing “abortive proliferation,”• then the authors should demonstrate a difference in the percentage of proliferating OT-II cells on day 4 compared to day 6 and day 11 by efluor670 staining.

Figure 2:

1. The authors should show a “non-resting”• CpG-B stimulated pDC cocultured with OT-II cells to demonstrate viability and proper ratios of pDC to T cell of their in vitro assay (A&B).
2. In order to conclude that resting pDC do not prime CD4+ T cells but can process antigen, the authors should transfer bone marrow sorted pDC pulsed with OVA along with OT-II cells in the MHC II -/- host. This is essential to eliminate any artefact of the Flt3 overexpression system. The authors demonstrate in E and F that their in vitro derived “resting pDC”• are clearly different in their antigen processing ability than endogenous pDC shown in F, thus it is possible that their ability to prime CD4+ T cells may be different as well. The authors should either acknowledge this in the text, or perform the above experiment to clearly demonstrate their conclusion.

Figure 4:

1. The authors cannot conclude that the clustering of OT-II cells in mice that received transferred resting pDC is due to clustering around endogenous cDC. The authors should repeat the experiment using recipient mice that have endogenous cDC labeled with a fluorescent protein that can be distinguished from the pDC and CD4 T cells if they want to make this claim.

Figure 5:

1. This figure suggests that the phenomenon being described between MHC Class II deficient mice and the wild type mice may be artificial due to transfer of free antigen in with the loaded CD4+ T cells or pDC. The authors should demonstrate whether the OT-II response to CD4+ and pDC transfer into wt

mice differs from injecting OVA peptide alone into the afferent lymphatic.

Figure 6:

1. The authors should show a control of OVA s.c. injection without any previous cell transfer to demonstrate the baseline ear swelling associated this procedure.

minor points:

1. In the introduction, the authors may cite the original publications that suggested pDC, or interferon-producing cells, enter the blood stream via HEV (Cella, 2000 & Diacovo, 2005).
2. In the introduction, the authors should mention that pDC accumulate in skin during mechanical injury (Gregorio, 2010).
3. Line 102: Remove i.l. between that and received.
4. In figure 2, it is unclear how the pDC-Ova in (A) and DC-Ova+ LPS cells throughout the figure are derived. It is also unclear if the DC-Ova+LPS consists of a mixture of pDC and cDC or just cDC.
5. In figure 6, thickness is misspelled.

Reviewer: 2

Comments to the Author

In this study Kohli and collaborators attempt to demonstrate that plasmacytoid dendritic cells(pDCs) loaded with exogenous antigen could induce tolerance in mice when adoptively transferred via intra lymphatic. This issue is relevant because it can potentially elucidate the role of pDCs in tolerance induction. However, there are several methodological flaws, which make most of the statements not clearly supported by data. Also, there is no attempt to elucidate the mechanisms by which this transfer would occur.

Specific comments

A main problem of the study is the lack of any attempt to understand how these antigens are transferred. The manuscript should be revised describing the mechanism by which this transfer occurs. Antigen transfer between pDCs and other cells can occur by the acquisition of the whole peptide-MHC complexes as recently described (Bonnacorsi et al. J Immunol. 2014 Jan 15;192(2):824); but this should not be the case as demonstrated in MHCII-/- mice. Nevertheless, authors should comment on this issue. Alternatively, lymph-node resident APCs might engulf fragments from dead cells, including pDCs (e.g. Inaba et al. J Exp Med. 1998 Dec 7;188(11):2163).

To further investigate this point, it might be possible to perform a functional assay, i.e., presentation to OT-II cells by ex vivo isolated APC from wild type recipient mice lymph nodes after that OVA-loaded pDCs has been transferred via i.l. route.
Finally, the transfer of antigen from pDCs to resident APC do not provide any explanation regarding how resident APC could induce tolerance. Would it be the same whether other cells reaching lymph node from the periphery would transfer the antigen? Is there something special about antigen transfer by pDCs to confer tolerogenic properties to APC? Or Authors believe that resident APC should be tolerogenic merely because they are in steady state? In this case, what is the relevance of antigen transfer by pDCs. How can we exclude that pDCs can induce tolerance by presenting antigens in an abortive fashion? Whatever the case these issues should be investigated in deeper details.
To better elucidate the mechanism by which this transfer might occur, authors should check the viability of pDCs after adoptive transfer. It has been suggested that migratory DCs die in the T cell area and if this occur also to pDCs it is reasonable to hypothesize that pDC-fragments might be engulfed by resident DCs. This should be verified.
To demonstrate the localization of migrating pDCs in the lymph node, the authors followed the faith of GFP labelled pDCs after i.l. injection. Data suggest that pDCs do not enter the paracortex area. However, if pDCs do not enter the paracortex area where do they might encounter APCs to transfer their antigens? Also, a very limited number of pDCs are present in the lymph node after i.l. injection. Are those few cells adequate to transfer a suitable amount of antigens?
In any case, one should consider that the setting of this experiment does not resemble the physiological once since, in vivo, pDCs that are supposed to migrate via afferent lymph are those from peripheral tissues that might display a peculiar phenotype and functions if compared to those employed in this experiments. Although we understand the technical difficulties associated to pDCs isolation from peripheral tissues, this experimental setting might not allow a correct interpretation and therefore authors should at least discuss their data in this regard.

CD4+T cells seem able to transfer OVA similarly to pDCs (Fig.5). It is at least surprising that CD4+ T cells can transfer OVA since in figure 2E they do not appear able to internalize OVA. The evidence that also CD4+ T cells can transfer the antigens to resident APC raise questions regarding the biological relevance of this transfer by a rare population of cells such as pDCs.

First Revision – authors' response 01-Jul-2016

Point by point reply to reviewer's comments

European Journal of Immunology

Reviewer: 1

Comments to the Author
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The authors should address the following major points:

In general, the authors compare the migration behavior and T cell priming ability of resting/unstimulated pDC to LPS-stimulated cDC. However they do not show the T cell priming ability or migration behavior of unstimulated cDC or stimulated pDC, which are important controls needed in order to make conclusive statements about the unique tolerogenic or immunostimulatory properties of either cell type within their experimental systems.

Figure 1:
1. The authors should demonstrate that the S1P receptors are not different on their adoptively transferred conventional and plasmacytoid dendritic cells. They should ensure that the difference seen in the OT-II proliferation kinetics seen between the groups cannot be explained solely by a difference in the S1P receptors expressed by the subsets of dendritic cells and therefor their retention time in the draining lymph nodes.

> It is currently unknown what mechanisms exactly determine the retention time of pDCs and DCs in lymph nodes. We know from T cells that both CCR7 (as a retention factor) and S1P1 (as an egress factor) determine their dwell time in lymph nodes and it definitely would be worthwhile addressing this question experimentally. However, we think that this is of little relevance for the present study since we postulate that pDCs do not prime OT-II cells on their own but only by transferring the antigen to endogenous APCs in WT recipients. Thus, the extent of OT-II cell expansion is dependent on the T cell priming properties of endogenous APCs and not the transferred pDCs in wt recipients.
>
> We used activated DCs as a positive control group rather than a comparative group. The difference in the OT-II expansion kinetics could be attributed to many factors, which could include retention time, cell numbers in the lymph node positioning of cells, amount of antigen etc. In fact, we observe that 18 hours post i.l. transfer, the number of activated DCs in the LNs is more than the no. of activated pDCs. Thus, we concede that there could be many factors that could affect the “difference “in the expansion kinetics of OT-II cell observed in the two groups. However, the experiments done here show that transfer of cell-associated antigen from pDCs to LN-resident APCs leads to abortive proliferation of cognate CD4 T cells. They do not stress that two different DC types induce different expansion of OT-II cells.

2. If the OT-II cells are undergoing “abortive proliferation,” then the authors should demonstrate a difference in the percentage of proliferating OT-II cells on day 4 compared to day 6 and day 11 by efluor670 staining.

As, suggested by the reviewer we now show that even at day 6 and day 11 the proliferation profile is similar in both groups i.e. all OT-II cells have proliferated (supplemental Fig. 3) to the same degree. However, as already depicted in original Fig. 1, the number and frequency of OT-II cells increases from day 4 to day 6 upon transfer of DCs, they decrease upon transfer of resting pDCs . This indicates that the stimulus OT-II cells receive during the priming stage dictates their expansion.

Figure 2:

1. The authors should show a "non-resting" CpG-B stimulated pDC cocultured with OT-II cells to demonstrate viability and proper ratios of pDC to T cell of their in vitro assay (A&B).

As suggested by the reviewer we performed T cell proliferation experiments using stimulated pDCs both *in vitro* and *in vivo* with MHCII $^{-/-}$ recipients (revised Fig. 2). These results show that activated pDCs gain some ability to prime T cells but cannot induce robust expansion of T cells.

2. In order to conclude that resting pDC do not prime CD4^{+} T cells but can process antigen, the authors should transfer bone marrow sorted pDC pulsed with OVA along with OT-II cells in the MHC II -/- host. This is essential to eliminate any artefact of the Flt3 overexpression system. The authors demonstrate in E and F that their in vitro derived "resting pDC" are clearly different in their antigen processing ability than endogenous pDC shown in F, thus it is possible that their ability to prime CD4+ T cells may be different as well. The authors should either acknowledge this in the text, or perform the above experiment to clearly demonstrate their conclusion.

Unfortunately we could not perform the experiment suggested by the reviewer as currently we do not possess enough MHC II $^{-/-}$ mice due to breeding problems. Nonetheless, we used pDCs isolated from the BM of untreated wt mice, in the *in vitro* proliferation assay. .The results obtained with these pDCs isolated from the bone marrow were similar to that obtained with *in vitro* generated pDCs. This suggest that *in vitro* generated pDCs have similar T cell priming properties as those isolated freshly from BM. This experiment is now shown in Supplemental Fig. 4

Figure 4:

1. The authors cannot conclude that the clustering of OT-II cells in mice that received transferred resting pDC is due to clustering around endogenous cDC. The authors should repeat the experiment using recipient mice that have endogenous cDC labeled with a fluorescent protein that can be distinguished from the pDC and CD4 T cells if they want to make this claim.

Based on the reviewer's suggestion, we immunostained LN sections of wt mice which received GFP $^{+}$ ova-loaded pDCs and TAMRA labelled OT-II cells to identify endogenous DCs. We could identify clusters of OT-II cells around endogenous CD11c^{+} DCs. This experiment is shown in Supplemental Fig. 5

Figure 5:

1. This figure suggests that the phenomenon being described between MHC Class II deficient mice and the wild type mice may be artificial due to transfer of free antigen in with the loaded CD4+ T cells or pDC. The authors should demonstrate whether the OT-II response to CD4+ and pDC transfer into wt mice differs from injecting OVA peptide alone into the afferent lymphatic.

Injection of either ova-loaded CD4+ T cells or ova-loaded pDCs results in the transfer of ova to endogenous DCs and subsequent proliferation of OT-II cells. Thus, it is very likely that injection of free antigen would cause proliferation of OT-II cells. However it seems impossible for us to calculate the amount of soluble antigen, which should be injected to match the amount of antigen that is released by the transferred cells. Thus, to address the reviewer's comment i.e. if free antigen is passively releases by the transferred cells, we used $CD4^+$ T cells derived from transgenic mice that ubiquitously express a membrane-bound form of ova (act-m-ova). Interestingly, we observed some proliferation of cognate OT-II cells upon i.l. transfer of act-m-ova $CD4^+$ T cells. These results support our idea that cell-associated antigen can be transferred to and presented by endogenous APCs. This experiment is now shown in supplemental Fig. 6.

Figure 6:

1. The authors should show a control of OVA s.c. injection without any previous cell transfer to demonstrate the baseline ear swelling associated this procedure.

The experiment suggested had already been shown in figure 6 of the original manuscript. The 3rd column (L-R) in figure 6B shows the baseline swelling in the group of mice that received no cells at day 0 and at day 6 but only s.c. injection of OVA at day 12.

minor points:

1. In the introduction, the authors may cite the original publications that suggested pDC, or interferon-producing cells, enter the blood stream via HEV (Cella, 2000 & Diacovo, 2005).

As suggested, this has been done.

2. In the introduction, the authors should mention that pDC accumulate in skin during mechanical injury (Gregorio, 2010).

As suggested, this has been done.

3. Line 102: Remove i.l. between that and received.

i.l. here stands for intra-lymphatically

4. In figure 2, it is unclear how the pDC-Ova in (A) and DC-Ova+ LPS cells throughout the figure are derived. It is also unclear if the DC-Ova+LPS consists of a mixture of pDC and cDC or just cDC.

This is now mentioned in the M&M.

5. In figure 6, thickness is misspelled.

This has been corrected now.

Reviewer: 2

European Journal of Immunology

Comments to the Author

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To further investigate this point, it might be possible to perform a functional assay, i.e., presentation to OT-II cells by ex vivo isolated APC from wild type recipient mice lymph nodes after that OVA-loaded pDCs has been transferred via i.l. route.

> Before submitting the manuscript we made several attempts to address how the antigens are transferred. We had already performed the very experiment suggested by the reviewer i.e. culturing OT-II cells together with *ex vivo* isolated $CD11c^+$ DCs from wt mice after the transfer of ova-loaded $CD4^+$ cells. However, even the entire bulk of the isolated *ex vivo* DCs could not induce proliferation of OT-II cells. Alternatively, to track antigen transfer we loaded cells with either fluorescent conjugated ova or DQ-OVA, but could not detect any fluorescence in the endogenous APCs post transfer. These experiments suggest that the amount of antigen transferred is limited and is below the detection limits of the above experiments.
>
> Certainly death of cells that carry antigen could also contribute to the release of antigen and even though CD4 T cells are known to be long lived; at least a minor frequency would die post i.l. transfer. However experimentally it would be hard to determine if cell death is the only way antigen is transferred.

Finally, the transfer of antigen from pDCs to resident APC do not provide any explanation regarding how resident APC could induce tolerance. Would it be the same whether other cells reaching lymph node from the periphery would transfer the antigen? Is there something special about antigen transfer by pDCs to confer tolerogenic properties to APC? Or Authors believe that resident APC should be tolerogenic merely because they are in steady state? In this case, what is the relevance of antigen transfer by pDCs. How can we exclude that pDCs can induce tolerance by presenting antigens in an abortive fashion? Whatever the case these issues should be investigated in deeper details.

> Yes, we show in our *in vivo* proliferation assay that even non-APCs such as $CD4^+$ T cells could transfer antigen. However, since it is widely believed that resting pDCs induce tolerance with a lack of consensus about the mechanism, the importance of this study lies in the elucidation of the mechanism by which resting pDCs could induce tolerance in the steady state. Our study suggests that this could be achieved by antigen transfer and is independent of the T cell priming properties of pDCs and independent of physical contacts between resting pDCs and cognate $CD4^+$ T cells.

However, in the present study we also show that pDCs can acquire and process exogenous antigens while $CD4^+$ T cells cannot. This ability combined with the unique homing properties of pDCs could enable them to transfer peripheral antigens to the secondary lymphoid organs as well as primary lymphoid organs. Thus that ability to capture and transfer antigens, especially exogenous antigens is more pertinent for pDCs than other immune cells.

To better elucidate the mechanism by which this transfer might occur, authors should check the viability of pDCs after adoptive transfer. It has been suggested that migratory DCs die in the T cell area and if this occur also to pDCs it is reasonable to hypothesize that pDC-fragments might be engulfed by resident DCs. This should be verified.

We thank the reviewer for the suggestion. We concede that death of cells might result in the release of antigen. However experimentally it would be hard to test if cell death is the sole mechanism which results in antigen transfer. Nonetheless, based on reviewer's suggestion we attempted to assess the viability of pDCs post transfer. 16 hours post transfer 1% or less of the transferred and then re-isolated pDCs were annexin-V positive. These observations suggest that pDC cell death might be less important for their ability to induce tolerance.

To demonstrate the localization of migrating pDCs in the lymph node, the authors followed the faith of GFP labelled pDCs after i.l. injection. Data suggest that pDCs do not enter the paracortex area. However, if pDCs do not enter the paracortex area where do they might encounter APCs to transfer their antigens? Also, a very limited number of pDCs are present in the lymph node after i.l. injection. Are those few cells adequate to transfer a suitable amount of antigens?

Apparently, a few pDCs entering in the LNs and without even entering the paracortex area can transfer antigens to endogenous APCs. To address the reviewer's comment we immuno-stained of sections of LNs of wt mice that received ova-loaded pDCs i.l and OT-II cells i.v. and observed that OT-II cells form clusters around $CD11c^+$ DCs. These clusters localized in outer paracortex, however most clusters were also spatially proximal to the injected pDCs. This suggests that ova-loaded pDCs transfer the antigen to DCs close to them which causes the clustering of OT-II cells around endogenous pDCs. This experiment is now shown in supplemental Fig. 5

In any case, one should consider that the setting of this experiment does not resemble the physiological once since, in vivo, pDCs that are supposed to migrate via afferent lymph are those from peripheral tissues that might display a peculiar phenotype and functions if compared to those employed in this experiments. Although we understand the technical difficulties associated to pDCs isolation from peripheral tissues, this experimental setting might not allow a correct interpretation and therefore authors should at least discuss their data in this regard.

We agree and have also considered the artifacts that might be associated with *in vitro* generated pDCs. To this end we used *ex vivo* pDCs isolated from Flt-3L overexpressing tumor treated mice in the *in vivo* proliferation assay with $MHCII^{-/-}$ recipients. The phenotype of *in vitro* generated and *ex vivo* isolated pDCs, especially the expression of co-stimulatory molecules resembles that of pDCs found in the peripheral tissues and lymphoid organs (1). In fact, the phenotype is conserved across species as a study in which pDCs were isolated from the cannulated lymph of sheep also displayed a similar phenotype (2). Moreover, *in vitro* generated pDCs also exhibit prototypical functions associated with pDCs such as type 1 interferon secretion and CCR9-dependent migration to the thymus. Nonetheless, we appreciate that such pDCs are not purely physiological and discuss this in the revised manuscript.

Moreover, based on the reviewers' comments we performed *in vitro* proliferation assays using pDCs isolated from the BM compartment of untreated wt mice. As observed with *in vitro* generated pDCs, even BM-derived pDCs could not induce any proliferation of OT-II cells. This is now shown in supplemental Fig.4

$CD4^+$T cells seem able to transfer OVA similarly to pDCs (Fig.5). It is at least surprising that $CD4^+$ T cells can transfer OVA since in figure 2E they do not appear able to internalize OVA. The evidence that also CD4+ T cells can transfer the antigens to resident APC raise questions regarding the biological relevance of this transfer by a rare population of cells such as pDCs.

It is widely believed that resting pDCs induce tolerance. As mentioned in the Introduction, different models were used that suggest that resting pDCs are indispensable for tolerance induction. One suggested mechanism is that resting pDCs induce the generation of Tregs. The extant models and suggested mechanism indicate that resting pDCs induce initial priming of CD4 T cells, however in a tolerogenic fashion, but this has not been directly studied. The major conclusion of the present study is that resting pDCs cannot prime naïve $CD4^+$ T cells themselves but transfer antigen to induce abortive proliferation of cognate $CD4^+$ T cells and tolerance. Thus, the results of this study suggest that when resting pDCs are shown to induce tolerance they do so in an indirect manner. We and others have shown that resting pDCs can capture and process antigens and transport them to primary and secondary lymphoid organs. Thus even as a rare population, they can considerably contribute to the transport of antigen from peripheral organs and its dissemination to lymph nodes. Thus, antigen transfer becomes more relevant for resting pDCs considering their unique migratory properties within all DC subsets such as homing via HEV as well as via afferent lymph vessels).

1. Brawand, P., D. R. Fitzpatrick, B. W. Greenfield, K. Brasel, C. R. Maliszewski, and T. De Smedt. 2002. Murine plasmacytoid pre-dendritic cells generated from Flt3 ligand-supplemented bone marrow cultures are immature APCs. *J Immunol* 169: 6711-6719.
2. Pascale, F., V. Contreras, M. Bonneau, A. Courbet, S. Chilmonczyk, C. Bevilacqua, M. Epardaud, V. Niborski, S. Riffault, A. M. Balazuc, E. Foulon, L. Guzylack-Piriou, B. Riteau, J. Hope, N. Bertho, B. Charley, and I. Schwartz-Cornil. 2008. Plasmacytoid dendritic cells migrate in afferent skin lymph. *J Immunol* 180: 5963-5972.

Second Editorial Decision - 03-Aug-2016

Dear Dr. Forster,

It is a pleasure to provisionally accept your manuscript entitled "Plasmacytoid dendritic cells induce tolerance predominantly by cargoing antigen" 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 listed below 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,
Laura Soto Vazquez

on behalf of
Prof. Silvano Sozzani

Editorial Office
European Journal of Immunology
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www.eji-journal.eu