THE EMBO JOURNAL

Manuscript EMBO-2013-86369

Specific calcineurin-targeting in macrophages confers resistance to inflammation via MKP-1 and p38

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Review timeline:

Submission date:	23 July 2013
Editorial Decision:	08 August 2013
Revision received:	21 December 2013
Editorial Decision:	08 January 2014
Revision received:	20 January 2014
Accepted:	27 January 2014

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.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 08 August 2013

Thank you very much for submitting your study on calcineurin targeting to control macrophage polarization for consideration to The EMBO Journal editorial office. Having received two very consistent assessments, I am in a position to reach a final decision.

Both scientists judge the major message of anti-inflammatory targeting of macrophages as timely topic and of general relevance. However, for a rather molecular-oriented title, particularly ref#2 brings up critical points on

-better characterization of the macrophage functional state (major point 1b),

-differentiation of the therapeutic peptide from other means of calcineurin inhibition by a more global assessment of reprogramming;

-(presumably) transcriptional profiling to surface at the same time the importance of p38/MAPK

signaling as relevant and major underlying molecular mechanism.

-Lastly, further experiments should address possible directness of p38-function downstream of CN-inhibition.

Conditioned on such amendments, I would be delighted to assess a thoroughly revised study for publication in The EMBO Journal. I realize that these are demanding and time-consuming experiments.

We are therefore ready to offer time beyond the usual three months revision period, if required. Please do not hesitate to get in touch in case of further questions (preferably via E-mail).

Please be reminded that The EMBO Journal considers only one round of revisions and the ultimate decision on publication will dependent on the outline and strength of the revised manuscript.

REFEREE REPORTS:

Referee #1:

The manuscript reports that deletion of CnB or inhibition of calcineurin by expression of an LxVP peptide polarizes macrophages toward an anti-inflammatory state. This state is characterized by examining several markers for both anti-inflammatory and inflammatory macrophages, and the anti-inflammatory polarization is shown to depend on p38 activity. Lentiviral delivery of an LxVP peptide construct into macrophages, by injection in vivo, has therapeutic effects in zymosan-induced inflammation, oxazolone contact hypersensitivity, and collagen-induced arthritis.

The strengths of the work are a compelling biological story and a potential relevance to translational medicine. However, certain issues need to be addressed:

(1) The manuscript argues that the effect of CN inhibition is mediated, at least in part, by an increase in p38 activity, but provides no mechanism connecting CN to p38. It would be strengthened considerably by experiments testing the known mechanism, demonstrated in cardiac myocytes, in which CN negatively regulates p38 through increasing expression of MKP-1 (Lim et al., ref. 27). MKP-1 is also involved in regulating p38 activity in macrophages and macrophage-like U937 cells (Perdiguero et al., ref. 37; Comalada et al. (2012) Eur. J. Immunol. 42, 1938; Liu et al. (2013) Cell. Signal. 25, 1845), though in this case the contribution of CN remains to be determined. The experiments should test whether CN signaling increases expression of MKP-1; whether inhibiting CN with LxVP decreases expression of MKP-1; whether shRNAs targeting MKP-1 activate p38 in the absence of any manipulation of CN; and, in the latter case, whether inhibition of CN then has no further effect on p38.

(2) LxVP peptide inhibits CN, but it has seen limited use, and its off-target effects are unknown. It would be good pharmacological practice to use a second CN inhibitor from a distinct structural class, or targeting CN through a different site. Since neither CsA nor FK506 is suitable for this purpose, it would be worthwhile to test VIVIT peptide.

(3) The manuscript refers to a "CN-p38 axis", terminology that implies a principal signaling pathway around which macrophage polarization is organized. A more apt description might be "crosstalk". The manuscript presents no evidence against the simple interpretation that CN and p38 are in two separate pathways, independently driven, and active at the same time. Moreover, the

literature indicates that p38 can contribute to either inflammatory or anti-inflammatory function, conditioned on the balance of signaling in other pathways at a given moment. This malleability is inconsistent with a fixed "axis" controlling polarization.

(4) Perdiguero et al. (ref. 37) show that altering the timing of p38 activation interferes with the resolution of inflammation in their experimental model. One would not expect the authors to investigate additional models in the current manuscript, but the discussion should note the published evidence suggesting that the benefits of an intervention aimed at macrophage p38 activity might be tempered by unwanted negative effects on normal tissue repair processes.

(5) The CsA and FK506 data are a distraction, and are not needed to support any major conclusion of the manuscript.

(6) It is not unexpected that treatment with CsA or FK506 could inhibit p38 activation. As the authors note, CN-independent inhibition of p38 activation is a known effect of these compounds at pharmacological concentrations in T cells (Matsuda et al., ref. 6). The inhibitory action is upstream of p38, and inhibition is observed, or not, depending on the pathway through which p38 is activated. The current manuscript, however, does not establish clearly that CsA and FK506 have this effect in macrophages. In both Figure 1h and Figure 5a-b, the comparison is between cells treated acutely in culture with CsA or FK506 and cells in which CnB had been deleted or CN inhibited with LxVP in vivo. The comparison is flawed, because cells are likely to be exposed to a pattern of signals in vivo that is not replicated in culture. A full comparison of CsA, FK506, and LxVP in vitro would be an unnecessary digression, but if the authors want to carry out the experiments, the data would constitute a supplementary figure, with a citation of the Matsuda et al. paper as precedent.

Minor matters that need attention:

(7) The 10<0> label is misplaced in all the flow cytometry panels, except in Figure 2e, where all the other labels are misplaced.

(8) The statistical approach used to compare levels of mRNA in Figures 1c-e, 1h, 4b, and 5c-d should be explained. If the mean of technical replicates for the control condition is set to 1, and each individual measurement is expressed relative to the mean, there will be a variance/s.e.m. for the control condition that can be used in the t-test. Why does this variability not appear as error bars in the graphs? Otherwise, how was the test done?

(9) In Figure 2c-d, the differences in Arg1 and Mrc1 mRNAs are much less than the corresponding differences in Figure 1c-d; and in Figure 2b, IL-10 secretion by the CnB-deleted cells does not reach even the levels of the control in Figure 1b. It is not clear that this can be considered as "reproducing the phenotype" of Figure 1. And arguably, since the effects are small, a control of wildtype macrophages transduced with Cre lentivirus is needed to establish that similar small effects would not be produced in this case without deletion of CnB.

(10) The IL10 values are vastly different for the control conditions in Figure 4a (mutLxVP, ~0 pg/ml), Figure 2b (Mock, ~20 pg/ml), and Figure 1b (Control, ~200 pg/ml). Why?

(11) Cytokine secretion in Figure 4d is labeled as "relative units". Relative to what?

Referee #2:

The manuscript by Escolano et al addresses the potential of macrophages to be programmed towards an anti-inflammatory phenotype by targeting calcineurin (CN) with the so-called LxVP peptide that exerts its effect on calcineurin in a different fashion than the well-known pharmacological inhibitors of CN cyclosporine A or FK506. The manuscript establishes a potential link to the p38-MAPK pathway being responsible for the observed effects of the LxVP peptide. Moreover, in a serious of in vivo experiments the authors demonstrate that delivery of the LxVP peptide in form of lentiviral gene transfer can reduce the inflammatory response in three models of inflammation (Collagen-induced arthritis, Zymosan-induced acute inflammation, Oxazolone-Induced contact hypersensitivity).

This is a very interesting report on a very timely topic. Moreover, targeting macrophages in diverse settings of chronic inflammatory situations might open up additional immunological treatment options in addition to targeting other cell types such as T, B, NK and dendritic cells.

Despite the potential of the work, several issues will need further clarification.

Major comments:

1. In light of the therapeutic potential of the LxVP peptide important aspects of the work are a) to show differences to CSA and FK506, b) to demonstrate the anti-inflammatory (M2-like) state of the LxVP activated macrophages, and c) to demonstrate a central role of the p38-MAPK pathway. This is mainly based on differential expression of few well-known macrophage markers associated with M1 and M2 phenotypes.

a) For the therapeutic use of LxVP in contrast to CSA and FK506 it will be of utmost importance to clarify the global differences in effects as well as the possibility to predict for potential side effects of LxVP. A genome-wide assessment at least on transcriptomics level would allow the identification of biological processes that are differentially regulated by the two classes of molecules. More specific, instead of only using IL10, Arg1, Mrc1 or iNOS as readouts (Figures 1, 2, 4), the complete spectrum of differences would be revealed. This would also be informative in the light of previous publications suggesting e.g. that iNOS is reduced in macrophages in response to CsA (Eur J Pharmacol. 2002 Jul 19;448(2-3):239-44.) while in this study, no effect was seen (Fig 1h).

b) Global assessment would also directly address whether LxVP indeed turns macrophages into a typical alternatively activated M2-like macrophage, or much more exciting, into a newly defined and specific anti-inflammatory type of cell.

c) Similarly, a gene network analysis of transcriptional re-programming of macrophages in response to LxVP could directly assess whether the p38-MAPK pathway is the most important one for the proposed molecular mechanism turning these cells into anti-inflammatory macrophages. Although the presented data for p38 involvement are indicative, they would benefit from the following improvements: 1. Data presented in figure 5a and b are representative; a quantification of a statistically sound number of experiments should be added. Is the difference truly statistically different? Moreover, is the difference to CsA and FK506 also different over time? In other words, would it be possible that p38 phosphorylation for CsA or FK506 is only at a different time point? Figure 5c and d shows molecules associated with the anti-inflammatory phenotype (Mrc1, Arg1,

IL10), what about the other molecules shown in Figures 1, 2 and 4? The P-ERK data show two bands, are both specific? If the upper band is specific, P-ERK would also show a sustained activation by LxVP, as suggested for p38. Presentation of quantitative experiments from a statistically sound number of experiments would clarify potential differences. What happens, if ERK is blocked instead of p38? p38 is downstream of many exogenous mediators: is it possible that there is no direct link between LxVP-mediated CN inhibition and p38 activation, but rather an autocrine loop downstream of CN and if so, could this be one of the mediators shown to act via p38? E.g. TNF? The data provided in Figure 7d are also not yet sufficient to distinguish between a direct and an indirect effect. Since calcineurin has been previously shown to negatively regulate NFkB, MAPKs, and IRF activation, it would be very helpful, if NFkB and IRF activation could be ruled out in the LxVP model as well.

2. The lentiviral vector used seems to have an extraordinary tropism for F4/80+ CD11c+ CD11b+ cells. How can this be explained? Does this lentiviral vector transduce highly purified immune cells other than macrophages in vitro? Potentially, the virus is best taken up by macrophages in vivo. Some clarification towards those questions would greatly enhance plausibility of these exciting findings. Along these lines, in Supplementary Fig. S5a, there seem to be GFP+ cells that are not Mac3+. Are there some other cells positive? And if so, what kind of cells are these? Maybe some additional controls would substantiate the strong tropism.

3. In the myeloid field, there is some debate about the role of cell surface markers and the nomenclature of the cells (macrophages versus dendritic cells, versus MDSC and so on). Particularly in the in vivo experiments, it would strengthen the manuscript, if some newly suggested markers could be assessed as well: e.g. Mertk has been recently suggested to be a better macrophage marker than F4/80 or CD11c and CD11b. In the same context, the cells being activated by LxVP i.p seem to have an astounding tropism towards the inflamed site. What are the signals (chemokines) that these cells require to migrate towards the inflamed site? What is the distribution of these cells in other organs such as spleen, liver and lung? Since the manuscript makes no point towards the potential mechanisms that attracts these cells to the inflamed site, some additional information about potential candidates involved in this process would clearly enhance the message of this manuscript.

Minor comments:

1. In the introduction on page 3 (last sentence first paragraph) the authors state that the new LxVP peptide would lack side effects of CsA or FK506. While this might be correct, LxVP itself could have side effects - yet unknown - when used therapeutically. To make a more fair statement, this should be reflected here.

2. Figure 1e and 2e show iNOS expression data in the two KO strategies for CnB1. Show both RNA and protein expression data for both models.

3. The genetic background of the MKK knockout mice should be stated. Moreover, are there differences in the models of inflammation used in the different genetic backgrounds?

1st Revision - authors' response 21 December 2013

Referee#1:

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We thank the Reviewer for his/her careful reading of the manuscript, for the valuable comments on the relevance of our study and for raising important issues .We have taken into account all the suggestions to prepare the revised version of our manuscript.

Following the reviewer´s suggestion we investigated the effects of CN signaling on MKP-1 expression. The results show that CN deletion or inhibition by LxVP reduce MKP-1 protein levels in macrophages (**new Figure 5 A and B**), thus indicating that CN activity positively regulates MKP-1 expression in these cells. To address whether MKP-1 targeting in macrophages results in p38 activation without any manipulation of CN, and whether LxVP has further effects in this case, we used MKP-1 deficient mice instead of the suggested shRNA strategy. As previously described (Perdiguero et al, 2011 and Hu et al, Cellular Signalling 19,2, 393-400, 2007), MKP-1 deficiency in macrophages leads to p38 hyperphosphorylation (**new Figure 5C).** In this case, the hyperphosphorylation of p38 is comparable to the hyperphosphorylation obtained by LxVP treatment in wild type cells (**new Figure 5D**). Moreover, in these MKP1-deficient macrophages, LxVP did not have any further effect on p38 activation (**new Figure 5D**). These results indicate an essential role of MKP-1 in the CN-mediated regulation of p38 activation. In the Discussion (pg 14, 2nd paragraph), we now state that "Our results strongly suggest that MKP1 mediates the repression of p38 by CN" and summarize the supporting findings that "CN deletion or LxVP mediated inhibition

reduced MKP-1 levels, LxVP activated p38 to a similar extent as displayed by MKP-1-deficient macrophages, and LxVP did not further increase p38 activation in MKP-1-deficient macrophages."

(2) LxVP peptide inhibits CN, but it has seen limited use, and its off-target effects are unknown. It would be good pharmacological practice to use a second CN inhibitor from a distinct structural class, or targeting CN through a different site. Since neither CsA nor FK506 is suitable for this purpose, it would be worthwhile to test VIVIT peptide.

The reviewer raises an important issue. We have analyzed the effects on the macrophage phenotype of other modes of CN inhibition than LxVP. We tested the chemical compound gossypol, which has been reported to inhibit the phosphatase activity of CN. However, the data obtained with this drug were inconclusive due to its high toxicity in cell culture. A much more interesting tool is the VIVIT peptide suggested by the reviewer, which inhibits the binding of CN to NFATs and other CN-substrates containing PxIxIT sites. However, unlike LxVP, the PxIxIT peptide does not inhibit CN phosphatase activity (Aramburu et el, 1998). Our data show that VIVIT, despite efficiently inhibiting zymosan-induced NFAT-dependent transcription, does not have the same effect as LxVP on macrophage phenotype (**new Supplementary Figure S5 A-D**). This result suggests that the anti-inflammatory phenotype of macrophages induced by CN-targeting requires inhibition of CN enzyme activity, and not only inhibition of CN binding to PxIxIT-containing substrates. We now discuss this issue in the manuscript: "Surprisingly, the classical CN inhibitors CsA and FK506 did not reproduce the phenotype obtained upon CN-gene-deletion or LxVP inhibition, and this was also the case with the VIVIT peptide. VIVIT is a high-affinity improved version of the natural peptide PxIxIT that binds to CN and inhibits the binding to NFAT and other CN-substrates containing PxIxIT sites (Aramburu et al, 1998). However, LxVP but not the PxIxIT peptide inhibits the phosphatase activity of CN (Martinez-Martinez et al, 2006; Rodriguez et al, 2009). Thus, the LxVP peptide is expected to inhibit signaling mediated by all substrates regulated by CN activity, and not only CN signaling by substrates containing LxVP or LxVP-like sites. This difference between the PxIxIT/VIVIT and the LxVP motifs might be related to the selective ability of LxVP to induce an anti-inflammatory phenotype"

(3) The manuscript refers to a "CN-p38 axis", terminology that implies a principal signaling pathway around which macrophage polarization is organized. A more apt description might be "crosstalk". The manuscript presents no evidence against the simple interpretation that CN and p38 are in two separate pathways, independently driven, and active at the same time. Moreover, the literature indicates that p38 can contribute to either inflammatory or anti-inflammatory function, conditioned on the balance of signaling in other pathways at a given moment. This malleability is inconsistent with a fixed "axis" controlling polarization.

Although we have now experimental evidence connecting CN and p38, we have followed the reviewer's recommendation and have eliminated the term "axis" from the title.

(4) Perdiguero et al. (ref. 37) show that altering the timing of p38 activation interferes with the resolution of inflammation in their experimental model. One would not expect the authors to investigate additional models in the current manuscript, but the discussion should note the published evidence suggesting that the benefits of an intervention aimed at macrophage p38 activity might be tempered by unwanted negative effects on normal tissue repair processes.

The reviewer is right and we now indicate in the manuscript that potential undesired effects related to tissue repair processes shoud be taken into account. On pg 18, 2nd paragraph, we discuss the evidence that p38 hyperactivation in macrophages could lead to impaired tissue healing (Perdiguero et al, 2011): "Another possible undesired effect of LxVP might be impaired tissue healing due to hyperactivation of macrophage p38 (Perdiguero et al, 2011) and it will therefore be important to assess whether such effects counter the possible benefits of LxVP in the treatment of inflammatory disesases". We also point out that "further progress will be needed to establish the safety and advantages of LxVP treatment."

(5) The CsA and FK506 data are a distraction, and are not needed to support any major conclusion of the manuscript.

We agree that the three independent approaches used in the study to validate our observations (LxVP peptide and constitutive and inducible CN deletion) provide enough evidence for the key role of CN in macrophage polarization. However, the use of IS drugs for CN inhibition is very extended in the field, and many conclusions on CN function have been reached by this means. We present data showing that the high doses of these drugs used in many reports produce off-target effects that have led to erroneous conclusions. We believe that this information will be very useful for researchers working in the field of CN and immunosuppressant drugs.

(6) It is not unexpected that treatment with CsA or FK506 could inhibit p38 activation. As the authors note, CN-independent inhibition of p38 activation is a known effect of these compounds at pharmacological concentrations in T cells (Matsuda et al., ref. 6). The inhibitory action is upstream of p38, and inhibition is observed, or not, depending on the pathway through which p38 is activated. The current manuscript, however, does not establish clearly that CsA and FK506 have this effect in macrophages. In both Figure 1h and Figure 5a-b, the comparison is between cells treated acutely in culture with CsA or FK506 and cells in which CnB had been deleted or CN inhibited with LxVP in vivo. The comparison is flawed, because cells are likely to be exposed to a pattern of signals in vivo that is not replicated in culture. A full comparison of CsA, FK506, and LxVP in vitro would be an unnecessary digression, but if the authors want to carry out the experiments, the data would constitute a supplementary figure, with a citation of the Matsuda et al. paper as precedent.

Taking into account the referee's comments we have investigated whether the inhibition of p38 activation by CsA in macrophages is dependent on the nature of the activating stimulus, as described in T cells (Matsuda et al, 2000). We pre-treated macrophages with pharmacological doses of CsA and stimulated them with LPS, PMA+Io or hyperosmolar medium. Analysis of p38 phosphorylation revealed that CsA-mediated inhibition of p38 in macrophages is also dependent on the activating stimulus. Moreover, like in T cells, CsA was able to inhibit PMA+Io induced activation of p38 but not that induced by LPS or hyperosmolar treatment. We have incorporated these data in **new Supplementary Figure S13.**

The referee is right about the potential contribution of *in vivo* signals to the phenotype observed in CN-deleted or LxVP inhibited macrophages. However, a full comparison with CsA or FK506 treatment *in vivo* would not be conclusive since it would be difficult to control the *in vivo* levels of IS and therefore hard to conclude about the effects in macrophages. Moreover, *in vivo* administration of IS would lead to the inhibition of CN not only in macrophages but also in other cell types such as T cells which could result in a different effect in the phenotype of macrophages. However we compared LxVP and CsA treatment *in vitro* (**Figure 4A)**. We have now clarified this in the figure legend. Although we find a similar phenotype after *in vivo* or *in vitro* LxVP transduction, we do not discard a synergistic effect of *in vivo* signals after i.p LxVP administration.

Minor matters that need attention:

(7) The 10<0> label is misplaced in all the flow cytometry panels, except in Figure 2e, where all the other labels are misplaced.

Since in these panels the absolute numbers are not necessary to understand the results, we have simplified the figures by eliminating these labels.

(8) The statistical approach used to compare levels of mRNA in Figures 1c-e, 1h, 4b, and 5c-d should be explained. If the mean of technical replicates for the control condition is set to 1, and each individual measurement is expressed relative to the mean, there will be a variance/s.e.m. for the control condition that can be used in the t-test. Why does this variability not appear as error bars in the graphs? Otherwise, how was the test done?

In the figures indicated by the referee we have evaluated whether the fold induction between treatment and control conditions was statistically significant. We set the control to 1 and then performed a one-sample *t*-test to assess whether the values in the treatment condition were significantly different from 1. In this test all the inter-experimental variability is considered in the treatment condition.

(9) In Figure 2c-d, the differences in Arg1 and Mrc1 mRNAs are much less than the

corresponding differences in Figure 1c-d; and in Figure 2b, IL-10 secretion by the CnB-deleted cells does not reach even the levels of the control in Figure 1b. It is not clear that this can be considered as "reproducing the phenotype" of Figure 1. And arguably, since the effects are small, a control of wildtype macrophages transduced with Cre lentivirus is needed to establish that similar small effects would not be produced in this case without deletion of CnB.

We agree. Although the tendencies observed in the expression of anti-inflammatory and pro-inflammatory markers are reproduced in all three models of CN targeting, the absolute differences are not exactly reproduced. This may be due to differences between the CN targeting approaches used; for example the lentiviruses used in the LxVP model and the inducible deletion of CN or the different mouse strains.

Taking into account the referee's comments we have included a control experiment showing that CRE expression in wild type macrophages does not produce the anti-inflammatory phenotype observed after CRE-mediated CN deletion (**New Supplementary Figure S2A-D).**

(10) The IL10 values are vastly different for the control conditions in Figure 4a (mutLxVP, ~0 pg/ml), Figure 2b (Mock, ~20 pg/ml), and Figure 1b (Control, ~200 pg/ml). Why?

We observed variability in the expression of IL10 that could be explained, as above, by the differences in the strategies used to target CN. In the case of the study with LxVP and the inducible deletion of CN, a control lentivirus is used as a mock. This probably affects the levels of IL-10 expression. Also, macrophages in these approaches come from mice on different genetic backgrounds. In the case of constitutive CN deletion, control means basal conditions and this strategy is also carried out in a different genetic background.

(11) Cytokine secretion in Figure 4d is labeled as "relative units". Relative to what?

To represent the expression of all the cytokines analyzed in a single graph we normalized the expression levels to the mean of the different experiments.

Referee 2#:

The manuscript by Escolano et al addresses the potential of macrophages to be programmed towards an anti-inflammatory phenotype by targeting calcineurin (CN) with the so-called LxVP peptide that exerts its effect on calcineurin in a different fashion than the well-known pharmacological inhibitors of CN cyclosporine A or FK506. The manuscript establishes a potential link to the p38-MAPK pathway being responsible for the observed effects of the LxVP peptide. Moreover, in a serious of in vivo experiments the authors demonstrate that delivery of the LxVP peptide in form of lentiviral gene transfer can reduce the inflammatory response in three models of inflammation (Collagen-induced arthritis, Zymosan-induced acute inflammation, Oxazolone-Induced contact hypersensitivity).

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Despite the potential of the work, several issues will need further clarification.

Major comments:

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a) For the therapeutic use of LxVP in contrast to CSA and FK506 it will be of utmost importance to clarify the global differences in effects as well as the possibility to predict for potential side effects of LxVP. A genome-wide assessment at least on transcriptomics level would allow the identification of biological processes that are differentially regulated by the two classes of molecules. More specific, instead of only using IL10, Arg1, Mrc1 or iNOS as readouts (Figures 1, 2, 4), the complete spectrum of differences would be revealed. This would also be informative in the light of previous publications suggesting e.g. that iNOS is reduced in macrophages in response to CsA (Eur J Pharmacol. 2002 Jul 19;448(2-3):239-44.) while in this study, no effect was seen (Fig 1h).

b) Global assessment would also directly address whether LxVP indeed turns macrophages into a typical alternatively activated M2-like macrophage, or much more exciting, into a newly defined and specific anti-inflammatory type of cell.

c) Similarly, a gene network analysis of transcriptional re-programming of macrophages in response to LxVP could directly assess whether the p38-MAPK pathway is the most important one for the proposed molecular mechanism turning these cells into anti-inflammatory macrophages.

We thank the Reviewer for the positive comments about the potential and interest of our study. We have taken into account most of his/her suggestions to prepare the new version of our manuscript and consider that it has been significantly improved as a result.

We completely agree with the referee's comments about the biological and clinical relevance of a deeper analysis on the effects of LxVP in macrophages. Also, as he/she notes, it would be interesting to make a full comparison with the well-known IS drugs CsA and FK506, to better identify the benefits of LxVP treatment. However, to be conclusive and truly informative, this kind of analysis should provide detailed in-depth short-and long-term profiles of gene and protein expression together with an analysis of cell functionality after the different treatments. Such an analysis would require a significant investment in terms of additional experiments and, more importantly, time (We estimate that we would need more than one additional year to carry out this work). Even a less complex transcriptional profile analysis would require a major additional effort, but would essentially provide a static picture of the whole, complex biological process under study. With these arguments in mind, we believe that this kind of analysis, although undoubtedly interesting, would be premature at this stage.

Although we have not been able to satisfy the reviewer's suggestions/issues related to global transcriptomic assessments, we have performed alternative approaches aimed at clarifying most of these concerns.

a) We have performed experiments to clarify the differences between our results and previously published data about the effect of IS drugs on iNOS expression. We present new results that, on the one hand, confirm the previous published data, showing that high doses of CsA (3-10μg/ml) inhibit iNOS gene expression (**new Supplementary Figure S1A**). However, we also show that this inhibition is not associated with the inhibition of CN activity; lower doses of CsA (200ng/ml), which efficiently inhibit CN-NFAT signaling (**new Supplementary Figure S1B**), not only failed to inhibit iNOS expression but significantly increased it (**new Supplementary Figure S1A**). We think that the use of IS doses much higher than those required for CN inhibition has led people working in the field to misinterpret the actual role of CN in several physiological and pathological settings. We now discuss these issues (pg 13-16).

b) We have characterized additional phenotypic markers associated with a "standard" anti-inflammatory phenotype and include new results suggesting that the phenotype described here does not match any of the subgroups previously defined. Indeed, we show that LxVP or CN deletion do not upregulate the expression of the M2 markers Ym1 or TGFβ (**new Supplementary Figure S12**).

c) We have further explored the molecular mechanisms underlying the anti-inflammatory actions related to the CN-mediated regulation of p38 MAPK (a concern also raised by reviewer#1). This work has allowed us to identify an important signaling mediator and to study post-transcriptional modifications in components of the CN signaling pathway. We found that CN deletion or inhibition by LxVP in macrophages reduced the protein levels of MKP-1 (**new Figure 5 A and B**), thus indicating that CN activity positively regulates MKP-1 expression in these cells. We also addressed whether MKP-1 targeting in macrophages resulted in p38 activation without any manipulation of CN. MKP-1-deficient macrophages display a p38 hyperphosphorylation (**new Figure 5C and D**) of a similar magnitude to that induced by LxVP treatment in wild type macrophages (**new Figure 5D**). We also present new experiments showing that maintained p38 activation, induced by a constitutively active form of the p38 kinase MKK6 (MKK6E), induces a predominant anti-inflammatory phenotype (please see Figure 1 below). MKK6 increases the expression of Arg1 and IL10 and inhibits iNOS. In the case of Mrc1, we do not observe increased mRNA expression. We propose that both CN inhibition and p38 activation are necessary for Mrc1 upregulation, whereas p38 activation may be sufficient in the case of the other markers, Arg1, IL10 and iNOS.

We believe that these are relevant results as they identify MKP-1 as an essential signaling component involved in the CN-mediated regulation of p38 activation in macrophages.

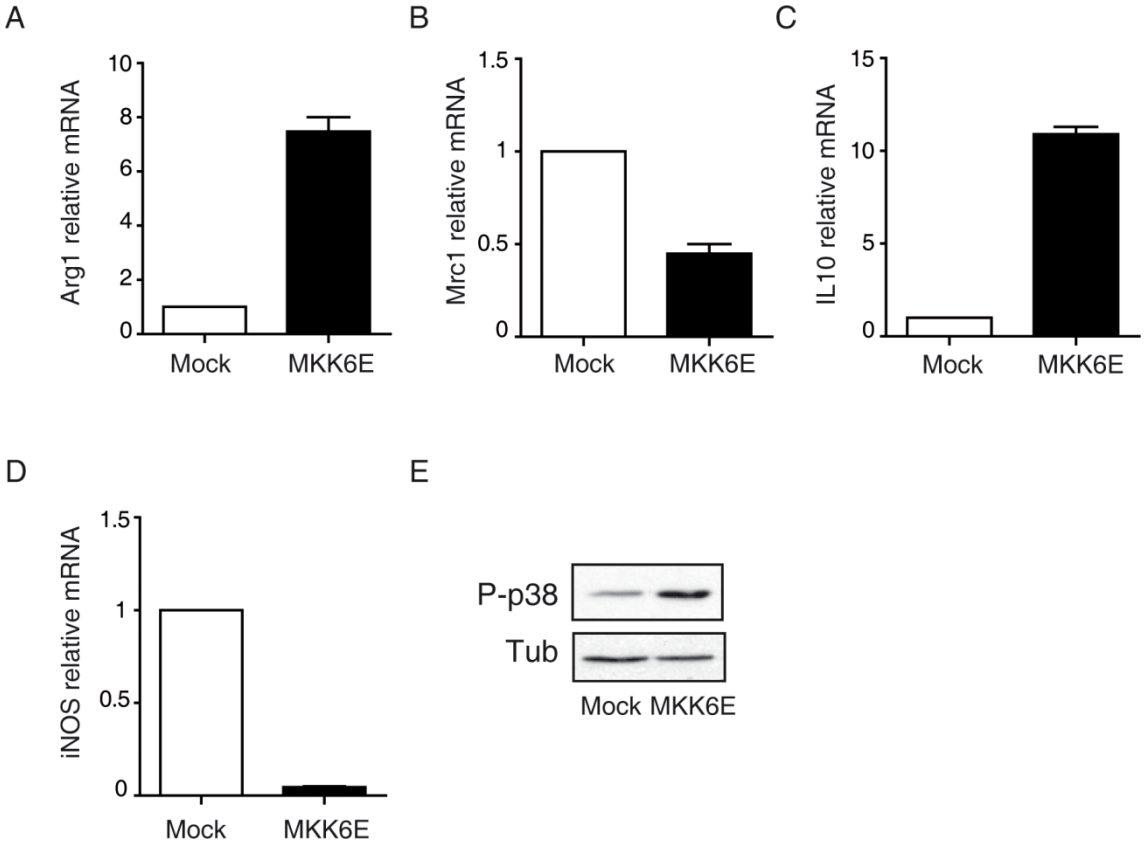

Figure 1. The expression of a constitutively active MKK6 induces anti-inflammatory properties in macrophages. (**A-D**) mRNA levels of Arg1 (**A**), Mrc1 (**B**), IL10 (**C**) and iNOS (**D**) in mock and MKK6E transduced peritoneal macrophages. (**E**) P-p38 expression in mock and MKK6E transduced peritoneal macrophages. Tubulin was used as a loading control.

Although the presented data for p38 involvement are indicative, they would benefit from the following improvements: 1. Data presented in figure 5a and b are representative; a quantification of a statistically sound number of experiments should be added. Is the difference truly statistically different? Moreover, is the difference to CsA and FK506 also different over time? In other words, would it be possible that p38 phosphorylation for CsA or FK506 is only at a different time point?

We have quantified the differences in p38 phosphorylation between LxVP or CN deletion and IS treatment, and show that these are statistically significant (**new Figure 4B and D**). We have also taken into account the referee's comments on the possibility that CsA or FK506 might induce p38 hyperphosphorylation at a different time point than LxVP or CN deletion. We mention in the discussion that a previous report shows that FK506, at doses higher than those required to inhibit CN, induces a peak of p38 activation in macrophages. We have also incorporated new experiments that reproduce these previously observed effects at high doses and further show that at pharmacological doses of CsA or FK506, able to inhibit CN, there is no observable induction of p38 activation at any time analyzed between 30 min and 5 days (**Figure 4A and C and new**

Supplementary Figure S7). In the Discussion we mention that the previously reported effects of IS using high doses are not actually related to CN signaling or activity.

Figure 5c and d shows molecules associated with the anti-inflammatory phenotype (Mrc1, Arg1, IL10), what about the other molecules shown in Figures 1, 2 and 4?

We now include data on SIGNR1 and iNOS expression in CN-deleted macrophages, on which p38 inhibition has no effect (**new Supplementary Figure S6**).

The P-ERK data show two bands, are both specific? If the upper band is specific, P-ERK would also show a sustained activation by LxVP, as suggested for p38. Presentation of quantitative experiments from a statistically sound number of experiments would clarify potential differences. What happens, if ERK is blocked instead of p38?

For the referee's interest, we include a quantification showing that LxVP does not activate ERK (please see Figure 2 below). But although ERK is not activated by LxVP, we do not rule out that other MAP kinases might contribute to the phenotype observed. We now include a statement that although "in our models p38-inhibition reduced the expression of several anti-inflammatory markers to basal levels, this does not exclude the contribution of a basal activity of other MAPKs or the involvement of other mediators in this phenotypic change mediated by CN-inhibition. However, the non-responsiveness of MKK3-/-6+/- mice to LxVP in the contact hypersensitivity model indicates that deficiency in p38 activation is sufficient to abrogate the anti-inflammatory action of LxVP-mediated CN-inhibition."

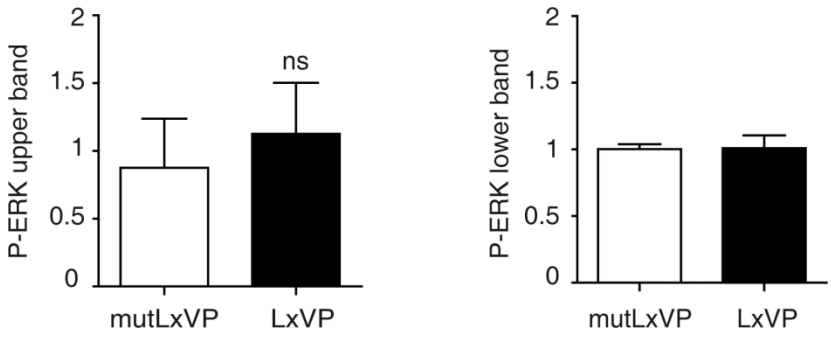

Figure 2. LxVP treatment does not induce ERK activation. (**A,B**) Quantification of the upper (**A**) and lower (**B**) bands of P-ERK expression in mutLxVP and LxVP treated peritoneal macrophages (mean ± s.e.m.; n=3).

p38 is downstream of many exogenous mediators: is it possible that there is no direct link between LxVP-mediated CN inhibition and p38 activation, but rather an autocrine loop downstream of CN and if so, could this be one of the mediators shown to act via p38? E.g. TNF? The data provided in Figure 7d are also not yet sufficient to distinguish between a direct and an indirect effect.

We now discuss the possibility that p38 activation could be the result of an autocrine effect after CN targeting in the context of the new data on MKP-1. We indicate that although MKP-1 is a functional link between CN and p38 activation, "additional mechanisms could also be involved in the activation of p38 that we have observed" and discuss (pg 14-15) that "Although we show that the expression of several cytokines (including TNF a and IL-1) is inhibited upon CN targeting, we cannot exclude that other secreted mediators might trigger autocrine actions that contribute to p38 activation. However, p38 activation by pro-inflammatory cytokines such as TNFα or IL1 can be discounted since CN targeting inhibits their secretion and the opposite effect on p38 would be expected."

Since calcineurin has been previously shown to negatively regulate NFkB, MAPKs, and IRF activation, it would be very helpful, if NFkB and IRF activation could be ruled out in the LxVP model as well

Following the referee's suggestion we have analyzed whether NFκB and IRF4 play a role in the anti-inflammatory phenotype induced in macrophages by LxVP (**new Supplementary Figure S14**). IRF4 activation has been associated with IL-4 and IL10 transcription via NFAT (Hu et al, 2002; Lee et al, 2009; Rengarajan et al, 2002) and with alternative macrophage polarization (Honma et al, 2005; Negishi et al, 2005; Satoh et al, 2010). However, we did not detect significant effects of LxVP on IRF4 gene expression or NFκB activation **(new Supplementary Figure S14).** As indicated by the reviewer, previous studies have shown that CN inhibitors can activate NFκB, and we have therefore tried to clarify this apparent discrepancy. We now explain that the previously reported effects of CsA and FK506 on NFκB activation are only achieved at high doses (CsA 50μg/ml and FK506 10μg/ml). We have reproduced these reported effects, but at lower, pharmacologically active doses that efficiently inhibit CN activity (CsA 200ng/ml or FK506 10ng/ml) activation of the NFκB pathway is not observed (**new Supplementary Figure S14**). In fact, this also appears to be the case for p38, which is only activated by high doses of IS that do not correlate with CN activity (**new Supplementary Figure S7**) and of iNOS expression, which is inhibited at high IS doses but is increased by a pharmacological dose (**new Supplementary Figure S1**). We anticipate that these results will be useful to researchers working in the field of CN and IS and help to resolve many discrepancies in the literature. We discuss this in the revised version of the manuscript and point out that in some studies, CN function has been assessed using doses of the IS-drugs, CsA and FK506, much higher than those required to inhibit CN signaling, concluding that "These findings indicate that it might be wise to revisit assertions about the involvement of CN signaling in physiological and pathophysiological settings when these are based on the use of high doses of IS."

2. The lentiviral vector used seems to have an extraordinary tropism for F4/80+ CD11c+ CD11b+ cells. How can this be explained? Does this lentiviral vector transduce highly purified immune cells other than macrophages in vitro? Potentially, the virus is best taken up by macrophages in vivo. Some clarification towards those questions would greatly enhance plausibility of these exciting findings. Along these lines, in Supplementary Fig. S5a, there seem to be GFP+ cells that are not Mac3+. Are there some other cells positive? And if so, what kind of cells are these? Maybe some additional controls would substantiate the strong tropism.

We agree that tropism of the lentiviral vectors in our model was not sufficiently clarified. We have now extended the discussion to better explain this important issue (pg17). We now indicate that we have used lentiviruses to transduce different types of cells. Although we show a clear selective tropism of lentiviruses for macrophages in the models used, we further emphasize that the route of administration is critical for determining the viral tropism. We explain that "In fact, lentiviruses systemically injected into the jugular vein are able to efficiently transduce the vascular wall (Esteban et al, 2011), and also successfully transduce primary cells *in vitro*, such as endothelial cells, T cell blasts and VSMC (Esteban et al, 2011; Urso et al, 2011)" In addition we discuss that "The macrophage tropism observed after i.p. inoculation might also reflect the metabolic activity of macrophages, which is much higher than that of other cell types enriched in peritoneal exudate such as T cells. In this regard, we have been unable to obtain lentiviral transgene expression in resting transduced T cells, but have successfully transduced primary T blasts (Urso et al, 2011), suggesting that T cells might be transduced by lentivirus but unable to express the transgene" .We also mention that the half-life of cells of the peritoneal cavity is another factor that could account for the selective expression of the transgene by macrophages and that granulocytes "might be transduced by lentiviruses, but viral integration and transgene expression likely requires longer that their lifespan. (Pillay et al, 2010)". We think that these observations provide clear possible reasons for the strong tropism observed.

We include some more pictures of the Mac3 staining to clarify that all the GFP^+ cells are macrophages (please see Figure 3 below). In **Supplementary Figure S11** the presence of cells at different focal planes explains that the Mac3 staining of one GFP^+ cell might not be detectable in the picture.

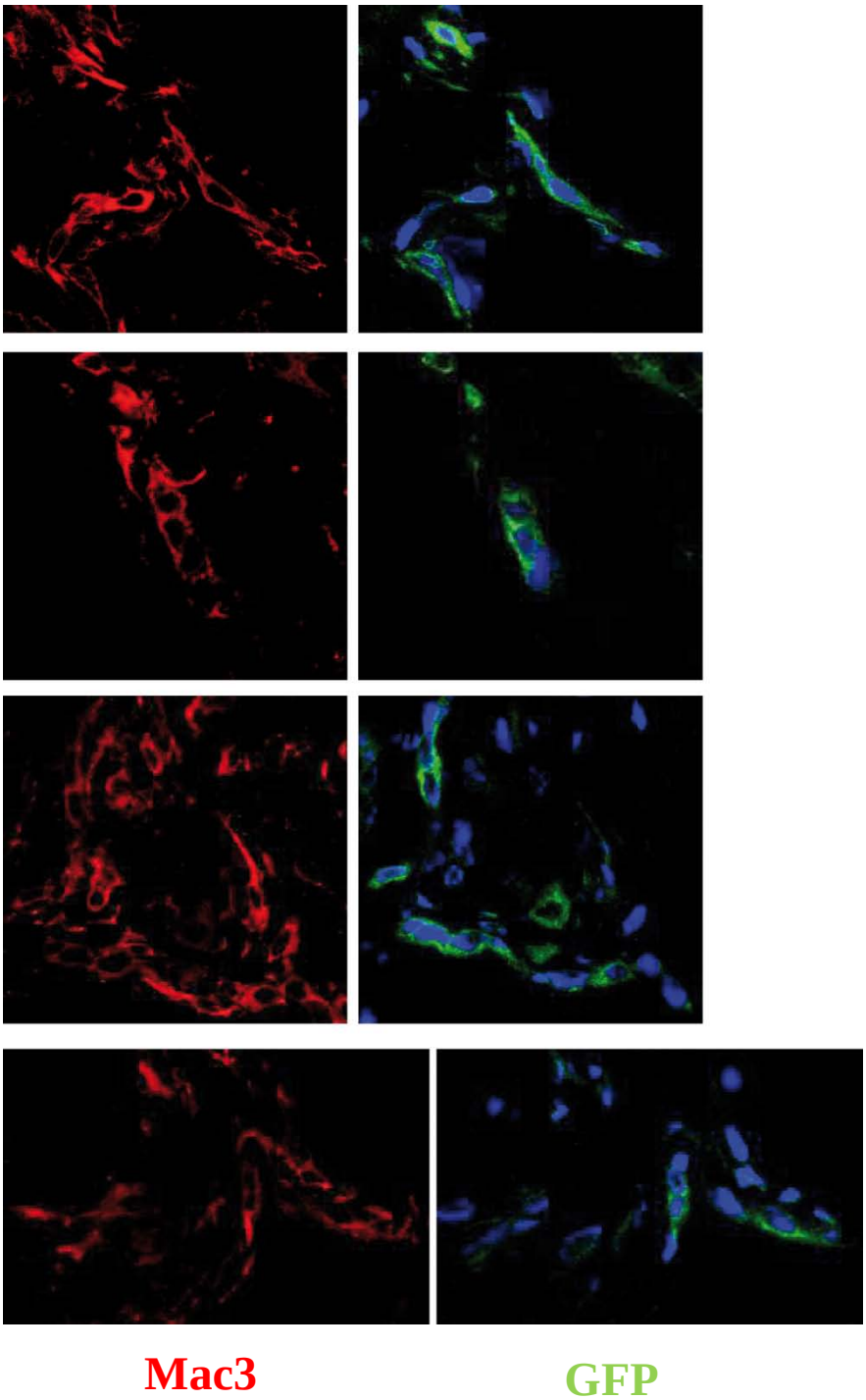

Figure 3. *In vivo* lentivirally transduced cells express Mac3. Images of Mac3 staining (red) and the corresponding GFP expression (green) in transduced cells. Nuclei are stained in blue.

3. In the myeloid field, there is some debate about the role of cell surface markers and the nomenclature of the cells (macrophages versus dendritic cells, versus MDSC and so on). Particularly in the in vivo experiments, it would strengthen the manuscript, if some newly suggested markers could be assessed as well: e.g. Mertk has been recently suggested to be a better macrophage marker than F4/80 or CD11c and CD11b. In the same context, the cells being activated by LxVP i.p seem to have an astounding tropism towards the inflamed site. What are the signals (chemokines) that these cells require to migrate towards the inflamed site? What is the distribution of these cells in other organs such as spleen, liver and lung? Since the manuscript makes no point towards the potential mechanisms that attracts these cells to the inflamed site, some additional information about potential candidates involved in this process would clearly enhance the message of this manuscript.

Yes, there is intense debate about the surface markers that are useful to differentiate macrophages and dendritic cells. Many markers are common to both cell types and sometimes only their expression levels allow a particular cell to be assigned as a macrophage or a dendritic cell. We have followed the referee's suggestion and sorted GFP^+ cells from peritoneal exudates after i.p. lentivirus administration. Size and complexity of these GFP-positive cells already indicate that they correspond to macrophages, and this is also corroborated by their expression of Mertk (**new Supplementary FigureS8**).

Taking into account the referee's comments about the potential mechanism driving macrophages specifically to inflammation sites, we measured in inflamed paws the levels of several chemokines reported to mediate macrophage migration in other models: MCP1, RANTES, MIP1α, MIP1β and CCL7. We show that the levels of all the chemokines analyzed were increased, particularly those of MIP1α and MIP1β (**new Supplementary Figure S15**).

As suggested by the reviewer, we have also checked for the presence of transduced cells in liver, spleen and lungs after i.p. lentivirus administration (**new Supplementary Figure S16**). We could detect GFP^+ cells in liver and spleen but not in the lungs. Although we did not further analyze the phenotype of these cells, their morphology and localization suggest that they could also be macrophages. In the case of the liver, no GFP^+ signal was detected in hepatocytes and the morphology of the transduced cells was similar to that of Kupffer cells. In spleen, GFP^+ cell were found in the marginal zone and the red pulp.

Minor comments:

1. In the introduction on page 3 (last sentence first paragraph) the authors state that the new LxVP peptide would lack side effects of CsA or FK506. While this might be correct, LxVP itself could have side effects - yet unknown - when used therapeutically. To make a more fair statement, this should be reflected here.

The reviewer is right. We have removed that statement and included this text in the Discussion: "Another possible undesired effect of LxVP might be impaired tissue healing due to hyperactivation of macrophage p38 (Perdiguero et al, 2011) and it will therefore be important to assess whether such effects counter the possible benefits of LxVP in the treatment of inflammatory disesases. However, unlike IS, LxVP induces its effects without binding to immunophilins (IP)(Martinez-Martinez et al, 2006), and is thus likely to lack the severe side effects associated with IS/IP complexes. Nevertheless, further progress will be needed to establish the safety and advantages of LxVP treatment".

2. Figure 1e and 2e show iNOS expression data in the two KO strategies for CnB1. Show both RNA and protein expression data for both models.

We now show iNOS protein data in both models of CN deletion (**Figures 1E and 2E**). We could not obtain an inhibitory effect on iNOS mRNA in the model of inducible CN deletion, most likely due to a matter of kinetics or mRNA stability. Even so, we consider that the protein data shown in the manuscript is biologically more relevant.

3. The genetic background of the MKK knockout mice should be stated. Moreover, are there differences in the models of inflammation used in the different genetic backgrounds?

We now indicate the genetic background of all mice used in the study.

2nd Editorial Decision 08 January 2014

Thank you very much for the revised study.

One of the original referees assessed your revised paper with the comments being enclosed below. While emphasizing paucity in macrophage molecular profiles, s/he would still support publication IF the relevant conclusions will be modified/ toned down to rather 'macrophage activation' throughout the text.

Conditioned on this (and the other requested amendments), I would be happy to proceed with formal acceptance/publication.

For this, please not the following further requirements:

-The EMBO Journal encourages the publication of source data, particularly for electrophoretic gels/blots, with the aim to make primary data more accessible and transparent to the reader. This entails presentation of un-cropped/unprocessed scans for KEY data of published work. We would be grateful for one PDF-file per figure with this information.

-Please also provide a minimal 2 up to 4 'bullet point' synopsis, as to highlight the major novelty/advance provided by your study.

-If you were to have an integrating figure as to visualize this in the format of 211 x 157 pixel, this would facilitate featuring your study on our homepage upon formal publication.

I am very much looking forward to your final amendments to the manuscript files and receipt of the above indicated additional items.

Please allow me already at this stage to congratulate you to this paper.

REFEREE REPORT:

Referee remarks:

The revised manuscript by Escolano et al has been greatly improved and most questions by the reviewers have been adequately addressed. Overall, this is a very interesting report on a very timely topic.

Yet, the authors left out an important chance to define the molecular profile of LxVP activated macrophages, which they suggest to be reported in further studies. This is certainly a possibility. However, at least the discussion should state this more clearly.

Major comment

1. For the major point by the reviewer to molecularly profile and define macrophages activated by LxVP the authors suggested to report this at a later time point. Taking the length of the manuscript into account, this is a legitimate suggestion. Nevertheless, the authors should make a very clear statement about this in the discussion. This is missing. In addition, without showing molecular profiles of LxVP activated macrophages, it is not really possible to put this into perspective with current models of macrophage polarization into M1 and M2 macrophages (currently, this is a general wrong doing in the literature and should be avoided here to improve the quality of the manuscript). I therefore suggest the following: Reduce any text in the introduction and the discussion concerning M1 and M2 polarization. Re-phrase 'macrophage polarization' with 'macrophage activation' throughout the text. Replace the paragraph in the discussion concerning macrophage polarization ("While p38 has been implicated in classical macrophage activation (Kang et al, 2007), its ....") with a paragraph suggesting that future work on a global level (molecular

profiling) will have to correlate LxVP activated macrophages with other well-described activation programs such as the M1 and the M2 polarization programs. In this context, it should be avoided to say that 'a link between CN and p38 in macrophage polarization has not been established before'. It could be that LxVP activation has nothing to do with M1 and M2 activation at all. In this respect, there might be no link whatsoever.

Minor comments

2. There is a typo in Figure 3F and some graphical issues in Figure 8F and G

3. Provide statistics of at least three individual experiments for Figure 5 data and present them as bar graphs next to the blots.

4. Change wording of subtitle in the result section: 'Systemically delivered LxVP targets macrophages, which migrate to sites of inflammation'. Since you describe two models, it should be plural.

5. Condense Fig S8 (MertK staining) into 6A. In 6A show also statistics as bar graphs of at least three independent experiments for each of the markers.

6. In Figure S11A: What are the cells that are GFP positive but not Mac3?

2nd Revision - authors' response 20 January 2014

Thank you very much for your e-mail expressing your interest in our revised study. We would also like to thank the referee for the helpful remarks and suggestions on the revised version. As you will see we have modified the manuscript according to these suggestions.

As suggested in the reviewer's major comments, we have reduced the text concerning macrophage polarization and have re-phrased "macrophage polarization" as "macrophage activation" throughout the manuscript. We have also replaced the term polarization with activation in the paragraph discussing the link between calcineurin and p38 (page 15 paragraph 2). In this way we retain the highlight on the relevance of our findings connecting p38 and calcineurin, but in relation to macrophage function (not polarization). Also following the reviewer's recommendations, we have included text in the Discussion (page 12 paragraph 2) indicating that "Future work on a global genomic and proteomic profiles will be needed to define the relationship of LxVP-treated (or CN-targeted) macrophages with other well-known activation programs such as those of M1 and M2."

We have amended the typos in Figs 3 and 8, provided statistical analysis of 3 independent experiments (as bar graphs) in Figure 5, and changed the wording of the subtitle on pg 9. Finally, we have included Supplementary Figure 8 (MertK staining) in Figure 6A, and for clarity, we have avoided the use of bar graphs (In 4 independent experiments 95-100% of cells expressed each of the macrophage markers analyzed). Finally, we now clarify that there are no GFP+ Mac3- cells in Figure S11A (S10 A in the current version of the manuscript). We indicate (Results pg 11, paragraph 1 that "…immunofluorescence analysis of footpad tissue sections for F4/80 and Mac3, which shows that all GFP+ cells were also F4/80+or Mac3+ (Figure 8C and Supplementary Figure S10A)."

We have included unprocessed scans for all the western blots shown in the manuscript and also provide a 4 bullet-point synopsis and a graphical abstract of our work. We include a clean PDF version of the manuscript and a word file showing the changes made after this revision.

I believe that the paper is much stronger as a result of the review process and hope that is now suitable for publication.

Let me thank you once more for your help and your professional editorial work.