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The isoenzyme of Glutaminyl Cyclase is an important regulator of monocyte infiltration under inflammatory conditions

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(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 19 April 2011

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. As you will see from the reports below, the referees find the manuscript potentially interesting and well written. However, they raise several concerns on your work, which should be convincingly addressed in a major revision of the present manuscript.

Although all referees are generally supportive, they have made clear suggestions. Should you address convincingly those points, the acceptance of the manuscript would entail a second round of review. Therefore acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor. Also, the length of the revised manuscript may not exceed 60,000 characters (including spaces) and, including figures, the paper must ultimately fit onto optimally ten pages of the journal. You may consider including any peripheral data (but not methods in their entirety) in the form of Supplementary information.

I look forward to seeing a revised form of your manuscript as soon as possible. Should you find that the requested revisions are not feasible within the constraints outlined below and choose, therefore, to submit your paper elsewhere, we would welcome a message to this effect.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

Cynis and colleagues report on the role of iso Glutaminyl Cyclase (isoQC) in modifying the N-terminus of monocyte chemoattractant protein 1 (CCL2; MCP-1) by formation of a pyroglutamate residue (pE1). Once modified, pE1-CCL2 protects the chemokine from degradation (thereby enhancing stability) and allows it to interact with its receptor, CCR2, and induce signaling. This interaction is important as it mediates the recruitment of monocytes to a site of inflammation. These monocytes may contribute to further damage at the site of injury by secreting proinflammatory cytokines. If CCL2 does not undergo pyroglutamate modification, the N-terminus is prone to degradation thereby suppressing the ability of this chemokine to recruit monocytes as shown by elegant studies here using THP-1 cells in a co-culture system. This well-written, data-loaded paper describes evidence for the role of isoQC as the key enzyme responsible for the maturation of CCL2 to pE1-CCL2 and its downstream effects (e.g., monocyte recruitment). *In vitro* studies demonstrate that isoQC is more effective than QC for the formation of pE1-CCL2 and that isoQC is localized to the Golgi where it may act as a housekeeping gene. *In vivo* studies, utilizing QC KO and isoQC KO mice as well as QC inhibitors were examined for their efficacy in multiple paradigms of inflammation [e.g., atherosclerosis ApoE3*Leiden mice with cuff placement), peritonitis (thioglycollate injection), and lung inflammation (LPS by oral gavage)]. The authors suggest that targeting pyroglutamate formation of the N-terminus of CCL2 using small molecule QC inhibitors will selectively and specifically reduce monocyte recruitment to sites of inflammation, which in turn, is expected to better control the inflammatory response that often ends up causing more injury to the inflamed tissue and its neighboring cells.

Comments:

1. The last sentence of the abstract is somewhat confusing. Please clarify.

2. Introduction P3: "N-terminal pE-residues have been described for a number of hormones and secreted proteins, such as.....TRH, GnRH, and fibronectin". What about amyloid-β protein? Should not this be included here, too?

3. A brief description of the difference between QC and isoQC would be helpful in the Introduction.

4. Were the other pE-modified hormones and secreted proteins mentioned in the Introduction (TRH, GnRH and fibronectin) examined in any of the *in vivo* studies in QC KO, isoQC KO and/or QC inhibitor studies? What was the effect of the compounds on these other substrates?

5. P6: bottom line: "primary cells from QC KO mice" - please define which cell types were examined.

6. Results: What are the main differences between the QC inhibitors, PQ50 and PQ529? Do both inhibit both QC and isoQC? Is one more selective for isoQC?

7. P9: bottom: Why did the neointima formation and accelerated atherosclerosis progress after 2 weeks? Does this mean 2 weeks after treatment with the QC inhibitor? Please clarify the section starting at: "At the later time point of 2 weeks..." to the end of the paragraph (P10).

8. Did the QC inhibitors affect the levels of other CCLs in the various inflammation studies *in vivo*?

Did they change CCR2 levels?

9. Were cytokine levels (e.g., IL-1, IL-6 and TNFα) measured in the inflamed mouse models after treatment with the QC inhibitors?

10. Did the glial primary cultures include microglia and astrocytes, or astrocytes only?

11. Is it known whether pE1-CCL2 levels are elevated in the context of atherosclerosis in humans? Is pE1-CCL2 focally present in the lesions?

12. P21, L15: "animal well fare" should be animal welfare.

13. What does the monocyte/macrophage marker, AIA31240, specifically recognize? What is the source of this reagent?

14. Figure 4: The title refers only to *in vivo* studies but the figure includes both *in vitro* (A, B) and *in vivo* studies (C-E).

15. Figure 5: isoQC is most highly expressed in lung tissue. Might the QC inhibitors have some benefit for chronic lung diseases such as asthma and/or COPD?

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

The manuscript by Cynis *et al*. describes the discovery that the N-terminal modification of CCL2 *in vivo* is mainly performed by the isoenzyme glutaminyl cyclase (isoQC). This was shown by using glutaminyl cyclase (QC) deficient mice and newly generated isoQC deficient mice. The authors demonstrated clearly that N-terminal pyroglutamate (pE1-) formation protects CCL2 as well as CCL7, CCL8 and CCL13 against proteolytic degradation. They also provide data indicating that pE1-CCL2 has a higher potency to internalize CCR2 receptors than non- pE1-modified CCL2 variants. Additionally the authors present the interesting idea that inflammatory diseases involving CCL2 may be dampened by inhibitors against QC and isoQC. To test this hypothesis several *in vitro* and *ex vivo* experiments have been performed. A newly developed specific pE1-CCL2 ELISA was used for that. The effects of QC/isoQC inhibitors have been shown also in different established inflammatory mouse models.

Referee #2 (Other Remarks):

In general, the manuscript is well written, extends our knowledge about post-translational modification of CCL2 and suggests an alternative for an intervention in CCL2 related disorders. However, I would like to comment on some of the experimental procedures in terms to their performance and/or interpretation:

Major points

1. The authors claim that pyroglutamic acid mediates activity of CCL2 (heading of Figure 1). I agree this might be the case for CCR2 receptor internalization but the data shown in Figure 1D does not support this. There is no significant difference seen in the chemotactic activity of pE1-CCL2 and Q1-CCL2, which are the two relevant CCL2 forms eligible for testing this hypothesis. Maybe a better-optimized migration assay could elucidate a difference between the activity of pE1-CCL2 and Q1-CCL2. As THP-1 cells are quite large, migration chambers with membranes of 8 µm pore size may result in a better performance of the migration assay. Furthermore, THP-1 cells normally grow in suspension under normal cell culture conditions and do not need to detach after migration through the pores. Were the migration chambers from Corning coated with anything? If not, why was it necessary to detach THP-1 cells from the membrane?

2. It is unusual that only 40% of the permanent cell line THP-1 is CCR2 positive. Have the cells been properly synchronized before the assays? Have they been checked for contaminations such as mycoplasma? Can it be excluded that they are cross contaminated by another cell type?

3. I highly appreciate that the authors have included an intranasal challenge model in their study (Figure 2E, F). However, it should be rigorously improved in several points: (a) A well established agent for intranasal challenge which can be given in a single dose should be used instead of consecutive stimulation, which may trigger desensitization of a receptor necessary to properly show the effect. Then it should be possible to detect CCL2, which precedes leukocyte infiltration. (b) I recommend determining the maximal influx of monocytes after the challenge by a kinetic study. (c) T cells and NK cells also express CCR2. Therefore, these cell types should be included in the analysis of cells obtained with the BAL. Additionally, a more detailed analysis of cell types especially monocytes should be performed. (d) The authors should indicate the absolute cell number of each individual cell type obtained in the BAL fluid in order to assure the comparability of their study with others in the field. (e) Inclusion of representative dot blots and showing, or at least explaining the gating of the cell populations would make the finding more reliable.

4. Is PQ529 a selective inhibitor for isoQC? If yes, this has to be shown by experimental data. However, according to the description in the Materials and Methods section the chemical compounds PQ50 and PQ529 inhibit both QC and isoQC. If yes, it should not be suggested that they selectively inhibit isoQC as stated in Figure 4 and 6, and page 10, line 5. In order to demonstrate the specificity of these compounds the total CCL2 protein concentration should also be determined in the experiments presented in Figure 4A, B as is done in Figure 2A, B. Since PQ50 and PQ529 are intended to selectively block QC/isoQC an analysis of CCL2 RNA should be included in order to demonstrate that there is no effect on transcriptional activation by LPS.

5. The statistically significant effect of PQ50 on the alleviation of atherosclerotic pathology is clearly shown. However the authors should discuss that their mouse model cannot simulate the long-term mechanisms, which trigger and promote atherosclerosis in man.

6. It should also be considered that QC/isoQC inhibitors may act not only in inflammatory diseases related to CCL2 and other MCPs as mentioned in the discussion but most probably would also have an effect on many other proteins modified by QC/isoQC. Therefore using QC/isoQC inhibitors may induce more side effects than novel drugs, which directly target CCL2.

7. Moreover, as we know today, CCL2 is also involved in recruitment of progenitor cells to the heart and the brain playing an important role in tissue regeneration. This should be fairly discussed.

8. Throughout the manuscript, the authors should clearly indicate which statistical test was applied to analyze the data for each experiment. Also, indicate whether standard deviation or standard error of the mean is given as the unit of variance.

Minor points:

1. Page 9, line 12, 13: Fig. 5 inset instead of Fig. 4 inset.

2. Page 14, line 4: Does (21) meant the citation (Buchholz et al, 2006)? Where does the PQ529 compound come from and how was it produced?

3. Page 19, line 12: Clarify "FACS-buffer CD16/32"

4. Page 20, Immunoblotting: A Western Blot is not described in Ref. (19). Was it exchanged with reference 20? But, there is also no WB described. How were anti-isoQC 5406 and anti-isoQC 5407 produced?

5. Page 21, Cuff-induced accelerated...: What constitutes "mildly"?

6. Figure 2: Indicate in the Figure Legend 2 that QC+/+ stands for wild type littermates.

Referee #3:

This is an interesting study on the role of glutaminyl cyclases in monocyte infiltration under inflammatory conditions.

The approach is multidisciplinary and involves genetic deletion and pharmacological inhibition of the enzyme. Murine as well as human cells and settings are used supporting the cross-species importance of the pathway.

Remarks:

- In view of the importance of specific quantification of pE1-CCL2 versus total CCL2, the validation of the in house developed ELISA should be included.
How was the specificity tested? What was the degree of crossreactivity of the antipE1 monoclonals with eg total CCL2? Did the authors test the crossreactivity of the capturing antibodies with other related chemokines?
What were the limit of detection (LOD) and the limit of quantification (LOQ) of the ELISA? What were the typical dilutions of plasma and other samples?

-Legend to Figure 1A could be adapted to improve clarity: eg immature, QP-CCL2, dislays cleavage sites for Aminopeptidase....

-Figure 1D: monocyte chemotaxis : highest efficacy for pE-CCL2, closely followed by Q-CCL2: is there a significant difference between both? Please mention.

-Pharmacological inhibition: the inclusion of the chemical structure of both used inhibitors would enhance transparence and future research possibilities for other researchers.

-Did the authors investigate LPS-induced inflammation in QC or isoQC negative mice? Were inhibitors administered to QC or isoQC deleted mice?

-p9 results on isoQC in atherosclerotic vessels. Fig 4 does not contain a western blot inset.

1st Revision - authors' response 11 May 2011

Referee #1:

Comments:

1. The last sentence of the abstract is somewhat confusing. Please clarify.

QC-inhibitors are, in contrast to macromolecules, such as antibodies and spiegelmers, orally available and could represent a step forward in treating CCL2-related disorders. Changes for clarification have been introduced in the respective sentence.

2. Introduction P3: "N-terminal pE-residues have been described for a number of hormones and secreted proteins, such as.....TRH, GnRH, and fibronectin". What about amyloid-β protein? Should not this be included here, too?

The pE-Abeta peptide in Alzheimer's disease appears to be a product of a side-reaction of QC, which could be of importance for AD initiation and progression. Since it is not a physiological substrate in the first place, it was omitted in the originally submitted manuscript; however, it is worth to be mentioned as a substrate in the light of the importance for AD. An additional sentence and reference has been introduced in the introduction.

3. A brief description of the difference between QC and isoQC would be helpful in the Introduction.

The basic similarities and differences between QC and isoQC have been included in the introduction.

4. Were the other pE-modified hormones and secreted proteins mentioned in the Introduction (TRH, GnRH and fibronectin) examined in any of the in vivo studies in QC KO, isoQC KO and/or QC inhibitor studies? What was the effect of the compounds on these other substrates?

The role of QC and isoQC on TRH and GnRH function but not on fibronectin function have been studied in QC and isoQC knock out animals by our group. A first report was published on the role of QC knock out on TRH and GnRH function in April 2011 in the *Journal of Biological Chemistry*. The topic was briefly touched in the discussion but the reference was missing. It has been added to the discussion and can be now also been found in the reference section (Schilling et al, 2011).
QC ko causes a mild hypothyreodism (HPT axis) by reducing T4 by about 20 % compared to wt littermates suggesting a mildly impaired TRH function. QC ko has no impact on GnRH function (HPG axis).

The impact of isoQC ko has not been published to date but data are already available. IsoQC ko has also no impact on GnRH function. It appears that isoQC ko causes a slight increase in T4 (10 %) in contrast to the slight decrease in QC ko. Therefore, the effect of a single knock out could be evened by application of non-selective QC/isoQC-inhibitors. Indeed, PQ50 and PQ529, which inhibit both QCs, do not affect hormones of the HPT and HPG axis.

5. P6: bottom line: "primary cells from QC KO mice" - please define which cell types were examined.

We have used primary macrophages, neurons and glia cells in this experiment. The respective paragraph has been specified.

6. Results: What are the main differences between the QC inhibitors, PQ50 and PQ529? Do both inhibit both QC and isoQC? Is one more selective for isoQC?

The inhibitors belong to different chemical classes. The chemical “warhead” of PQ50 is imidazole that of PQ529 is benzimidazole. Both inhibitors displace water at the 4th coordination site of the active site zinc ion in QC and isoQC. The inhibitors are not selective for QC or isoQC. To further improve clarity, also in the light, that reviewer 2 and 3 had similar questions; a sentence has been introduced on page 8. In addition, we have added a more detailed description of both inhibitors including the K_i-values for the human and murine enzymes in the materials and methods section.

7. P9: bottom: Why did the neointima formation and accelerated atherosclerosis progress after 2 weeks? Does this mean 2 weeks after treatment with the QC inhibitor? Please clarify the section starting at: "At the later time point of 2 weeks..." to the end of the paragraph (P10).

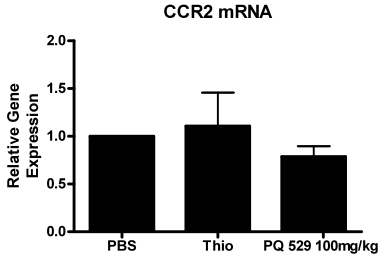
“Progressed” was used in the meaning of: the pathology has progressed from cell adhesion to accelerated atherosclerosis. Obviously this subordinate clause was confusing, so we have deleted it. The sentence is now: “In addition, at the later time point of 2 weeks, the overall CCL2 expression is lower compared to the early time point.”

8. Did the QC inhibitors affect the levels of other CCLs in the various inflammation studies in vivo? Did they change CCR2 levels?

We characterized the expression (mRNA) of CCL2 in LPS-stimulated THP-1 cells treated with PQ529. A down-regulation of CCL2 was not observed upon treatment with the inhibitor, suggesting that the transcriptional machinery is not directly targeted. A respective Figure has been included as Supplemental Figure 7A.

We investigated the expression levels (mRNA) of different proteins important for migration/inflammation, which might be regulated in cells present in the lavage fluid of the thioglycollate assay (CCL2, CCL7 and ICAM1). The data have been added as Supplemental Figure 7B-D. The figure shows the gene expression of CCL2, CCL7 and ICAM1 in cells from peritoneal lavage (further data from experiment depicted in Figure 4E). We did not observe a regulation of

CCL2, CCL7 and ICAM1 in this acute model. The expression of CCR2 was also assessed in thioglycollate-induced peritonitis. The expression level of CCR2 was not affected by PQ529 as shown in the figure below. The absence of CCR2 regulation is mentioned in the manuscript (page 9).



9. Were cytokine levels (e.g., IL-1, IL-6, and TNF-α) measured in the inflamed mouse models after treatment with the QC inhibitors?

We have included the expression levels of IL-6 and TNF-alpha in cells from peritoneal lavage after thioglycollate stimulation in Supplemental Figures 7E and 7F. IL-6 and TNF-alpha are up regulated by thioglycollate but are not regulated afterwards by inhibitor application.

10. Did the glial primary cultures include microglia and astrocytes, or astrocytes only?

The presented data were not generated with pure astrocyte cultures. We worked with mixed cultures of about 85 % astrocytes and 15 % microglia cells. The number of neurons in this preparation was negligible. Therefore we have chosen the term “glia culture” instead of “astrocytes”. We have revised one sentence in the method description to increase clarity (page 18).

11. Is it known whether pE1-CCL2 levels are elevated in the context of atherosclerosis in humans? Is pE1-CCL2 focally present in the lesions?

Under normal physiological conditions, CCL2 is completely N-terminally pyroglutamated (shown for instance in Figure 2A). Although previous analyses of circulating levels of CCL2 did not focus on the N-terminal residue, the CCL2 concentration was correlated with cardiovascular disease in several studies. The source of CCL2 in the circulation, however, might not be exclusively the atherosclerotic plaque, since such patients frequently suffer also from Diabetes or other metabolic impairments. For example, CCL2 is also released in adipose tissue or from kidneys in metabolic syndrome.
CCL2 has been indentified in atherosclerotic lesions. It is expressed by, e.g. macrophages or vascular smooth muscle cells and has been found, e.g. in the macrophage-rich areas neighboring the necrotic lipid core.

12. P21, L15: "animal well fare" should be animal welfare.

This has been corrected.

13. What does the monocyte/macrophage marker, AIA31240, specifically recognize? What is the source of this reagent?

AIA31240 is a polyclonal rabbit anti-mouse macrophage marker, which is frequently used in atherosclerotic mouse models to stain macrophages. The exact epitope is to the best of our knowledge not known. It was purchased from Accurate and Scientific Cooperation, Westbury, NY (cat. no. AIA31240). We have included the information in the materials and methods section.

14. Figure 4: The title refers only to in vivo studies but the figure includes both in vitro (A, B) and in vivo studies (C-E).

We have added "*in vitro*" to the figure title.

15. Figure 5: isoQC is most highly expressed in lung tissue. Might the QC inhibitors have some benefit for chronic lung diseases such as asthma and/or COPD?

Lung disorders such as asthma and COPD represent potential fields for application of QC-inhibitors. Dominant negative receptor antagonism to CCL2 has already been shown to be beneficial in lung fibrosis. We tried to approach this topic by intranasal LPS-challenge and found a reduction of infiltrating monocytes, which could be a starting point for in-depth analysis also suggested by reviewer 2.

Referee #2:

Major points
1a. The authors claim that pyroglutamic acid mediates activity of CCL2 (heading of Figure 1). I agree this might be the case for CCR2 receptor internalization but the data shown in Figure 1D does not support this. There is no significant difference seen in the chemotactic activity of pE1-CCL2 and Q1-CCL2, which are the two relevant CCL2 forms eligible for testing this hypothesis. Maybe a better-optimized migration assay could elucidate a difference between the activity of pE1-CCL2 and Q1-CCL2. As THP-1 cells are quite large, migration chambers with membranes of 8 µm pore size may result in a better performance of the migration assay.

We agree with the reviewer's statement, that in Figure 1D the difference between pE1- and Q1-CCL2 was not as pronounced as seen in the receptor internalization analysis. We already had addressed this problem by changing the method of cell count determination but didn't include the figure in the first submission. For explanation, in Figure 1D we originally used a standard cell counter system, which was sufficient to show the differences in migration between Q1/pE1 and the truncated D3-CCL2 variant but not between Q1 and pE1. We meanwhile determined the migration index using the quantitative method also used for the thioglycollate assay. There, the difference between pE1 and Q1 for attracting THP-1 cells at the low concentration could be quantified. The data provide evidence that Q1-CCL2 is less potent at 5-25 ng/ml concentration but exert similar potency to attract THP-1 at higher concentrations. We included a revised Figure 1D into the manuscript and made changes in the results and figure legend section.
Also please note, that under *in situ* conditions the N-terminal proteolytic turnover of CCL2 by aminopeptidases is relatively slow. In contrast, the *in vivo* concentration of circulation DP4 is about µM/L in humans, immediately turning over substrates such as Q1-CCL2 after release from secretory vesicles (irrespective of the theoretical *in situ* chemotactic potency of Q1-CCL2). Again figures 2 and 3 prove that almost all circulating CCL2 is pyroglutamated

1b. Furthermore, THP-1 cells normally grow in suspension under normal cell culture conditions and do not need to detach after migration through the pores. Were the migration chambers from Corning coated with anything? If not, why was it necessary to detach THP-1 cells from the membrane?

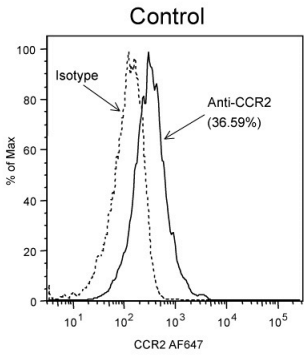
We used a protocol that included a detachment step with EDTA after the 2 h migration period. Although THP-1 cells are kept primarily in suspension, the cells exert some tendency to adhere to plastic surfaces, which requires, e.g. EDTA in FACS staining buffer (we use Miltenyi) to exclude attachment of cells in suspension to the wall of the tubes during analysis. The detachment step is in our opinion reasonable, because those cells which reached the down well but stick to the surfaces should be included into the analysis since they did respond to the migrational stimulus. We would agree that detachment might be not strictly required but since the cell count is higher it adds further robustness to the counting of cells.

2. It is unusual that only 40% of the permanent cell line THP-1 is CCR2 positive. Have the cells been properly synchronized before the assays? Have they been checked for contaminations such as mycoplasma? Can it be excluded that they are cross contaminated by another cell type?

We obtained the cells from the "German collection of microorganisms and cell cultures", which is a provider of original stocks of mammalian cell lines. These cells showed approx. 40 % CCR2 staining, which did not change upon passaging. Synchronizing the cells by serum deprivation for 24 h before CCR2-staining had no influence on the level of receptor found by FACS-analysis. A contamination with mycoplasma can be fairly ruled out. All cultures are regularly checked using the VenorGeM-qEP qPCR kit (Minerva). We have added this fact to the Materials and Methods section. We compared our results with those of other groups, who have published FACS data on CCR2 in THP-1 (Cullen JP *et al*. Atherosclerosis 2007 195(1) e125-133 or Phillips RJ *et al*. J Inflamm, 2005 2:14) (see picture obtained from Cullen *et al*.). Also these groups observed a fraction of about 40 % cells positive for CCR2.

3. I highly appreciate that the authors have included an intranasal challenge model in their study (Figure 2E, F). However, it should be rigorously improved in several points: (a) A well established agent for intranasal challenge which can be given in a single dose should be used instead of consecutive stimulation, which may trigger desensitization of a receptor necessary to properly show the effect. Then it should be possible to detect CCL2, which precedes leukocyte infiltration. (b) I recommend determining the maximal influx of monocytes after the challenge by a kinetic study. (c) T cells and NK cells also express CCR2. Therefore, these cell types should be included in the analysis of cells obtained with the BAL. Additionally, a more detailed analysis of cell types especially monocytes should be performed. (d) The authors should indicate the absolute cell number of each individual cell type obtained in the BAL fluid in order to assure the comparability of their study with others in the field. (e) Inclusion of representative dot blots and showing, or at least explaining the gating of the cell populations would make the finding more reliable.

We have included the absolute cell numbers in Supplemental Fig 5 and have added the marker for infiltrating monocytes ($7/4^{high}$ and $Ly6G^{low}$) in the result section to follow the suggestions 3c and d. We value the suggestions made by the reviewer, who is obviously experienced in models of lung inflammation, to improve the intranasal challenge model. However, we feel that those comprehensive analyses exceed the purpose of the current study, which is an introduction of a new mechanism to interfere with CCL2 activity in general without strict emphasis on a certain indication. The presented experiment was included to show convincingly that monocyte migration can be suppressed in different acute *in vivo* models, which are based on different stimuli, i.e. thioglycollate and LPS. An in-depth analysis as suggested by reviewer 2, including a kinetics of cell infiltration and differential effects on T and NK cells, is subject of subsequently planned studies which aims at evaluation of the strategy in distinct indications with emphasis on a translational validation of the approach to a human clinical situation in acute and chronic lung inflammation.



4a. Is PQ529 a selective inhibitor for isoQC? If yes, this has to be shown by experimental data. However, according to the description in the Materials and Methods section the chemical compounds PQ50 and PQ529 inhibit both QC and isoQC. If yes, it should not be suggested that they selectively inhibit isoQC as stated in Figure 4 and 6, and page 10, line 5.

This point was consistently raised among all reviewers. Obviously our previous description was inadequate. We have revised the compound description in the Materials and Methods section and included the K_i-values for all murine and human enzymes. PQ529 is not selective for one specific isoenzyme. The reviewer is right, the respective figure titles and the passages were misleading. We have changed the figure titles of Figures 4 and 6 to "QC/isoQC-inhibition" and have thoroughly revised the manuscript to avoid the suggestion of any isoform-specificity.

4b. In order to demonstrate the specificity of these compounds the total CCL2 protein concentration should also be determined in the experiments presented in Figure 4A, B as is done in Figure 2A, B. Since PQ50 and PQ529 are intended to selectively block QC/isoQC an analysis of CCL2 RNA

should be included in order to demonstrate that there is no effect on transcriptional activation by LPS.

We agree with the reviewer, that the demonstration of selectivity is important. Therefore, we have revised Figures 4A and B. We have replaced human and murine macrophages by murine glial cells, were we performed a much more comprehensive pE-CCL2 and total-CCL2 analysis. It is now demonstrated that there is a QC/isoQC-inhibitor dose-dependent reduction of pE-CCL2, the total-CCL2 level remains unaffected even at high concentrations of 40 µM of inhibitor (Fig 4A). For the same experiment we have analyzed CCL2 gene expression and have included these data as new Fig. 4B. In addition, the CCL2 gene expression in LPS-stimulated and PQ529-treated human THP-1 cells is presented in a new Supplemental Fig. 7A as also suggested by reviewer 1. Following these changes, the previous Fig. 4A and B showing the application of PQ529 to human and murine macrophages was moved to supplemental information (Supplementary Fig. 6A and B).

5. The statistically significant effect of PQ50 on the alleviation of atherosclerotic pathology is clearly shown. However the authors should discuss that their mouse model cannot simulate the long term mechanisms which trigger and promote atherosclerosis in man.

According to the suggestion, we have included a paragraph in the discussion section to highlight Pro's and Con's of the cuff-induced atherosclerosis model. The ApoE3*Leiden mouse line is a common model and well suited for a rapid testing of compounds. Of course, because it represents a transgenic line, it has definitely its limitations. The stage of plaque pathology represents rather early steps, however, the general process including involvement of CCL2 can be regarded as similar to the human situation and therefore the model owns some predictability for a translation of the results into a clinical situation.
For development, we consider other species and models in addition, such as restenosis after stenting of the iliac artery in rabbits.

6. It should also be considered that QC/isoQC inhibitors may act not only in inflammatory diseases related to CCL2 and other MCPs as mentioned in the discussion but most probably would also have an effect on many other proteins modified by QC/isoQC. Therefore using QC/isoQC inhibitors may induce more side effects than novel drugs which directly target CCL2.

The reduced conversion of different substrates by inhibition of QC and isoQC is potentially a cause of side effects. In order to evaluate the consequences, we generated and characterized QC and isoQC ko mice. Both lines have only mild if any phenotype and also the first double ko mice (QC and isoQC ko) are going to be analyzed. Moreover, regulatory toxicology in mice, rats, dogs and monkeys did not reveal side effects with the high doses used in these studies.
A potential reason is that N-terminal glutamine tends to form pE spontaneously at significant rates. Such an observation is for instance supported by Fig. 2B and Fig. 4A: Neither knock out nor inhibition reduces pE-CCL2 below LOQ.
Taken together, the data support that a (broad) therapeutic window exists for QC/isoQC inhibition that uncovers its full potential if the expression of target proteins is stimulated. We have added a paragraph in the discussion (page 13, last paragraph).
We agree, however, that a discussion and comparison of side effect potential is not appropriate at the current stage of development and therefore a subordinate clause was deleted, where we initially claimed that we will generate less side-effects than other molecules hitting directly CCL2. We see that this paragraph was offending and not sufficiently supported by published data.

7. Moreover, as we know today, CCL2 is also involved in recruitment of progenitor cells to the heart and the brain playing an important role in tissue regeneration. This should be fairly discussed.

We have added information on the important biological functions apart from disease in the discussion (page 13 last paragraph and page 14).

8. Throughout the manuscript, the authors should clearly indicate which statistical test was applied to analyze the data for each experiment. Also, indicate whether standard deviation or standard error of the mean is given as the unit of variance.

The unit of variance and the statistical analysis is now included in the figure captions.

Minor points:
1. Page 9, line 12, 13: Fig. 5 inset instead of Fig. 4 inset.

Corrected

2. Page 14, line 4: Does (21) meant the citation (Buchholz et al, 2006)? Where does the PQ529 compound come from and how was it produced?

Yes, Buchholz *et al*, 2006 was meant. As mentioned above, the QC-inhibitor section has been thoroughly revised. PQ529 was developed and produced by Probiodrug. The source is now indicated in the section. This is the first publication on PQ529.

3. Page 19, line 12: Clarify "FACS-buffer CD16/32"

Corrected in the revised manuscript. It is now: "CD16/32 in FACS-buffer (PBS, 5 % FCS) (F_c-Block)."

4. Page 20, Immunoblotting: A Western Blot is not described in Ref. (19). Was it exchanged with reference 20? But, there is also no WB described. How were anti-isoQC 5406 and anti-isoQC 5407 produced?

The correct reference was inserted (Cynis *et al*, 2008). The antibodies have been produced in rabbits by immunization with the N-terminal region of murine or human isoQC. The antibodies have been affinity purified using the immunization peptides after final bleeding. The immunoblotting section describes now in greater detail the generation of the new antibodies.

5. Page 21, Cuff-induced accelerated...: What constitutes "mildly"?

We clearly used a wrong wording here. It was our intention to say, that the elevation of cholesterol in the circulation is rather mild in this model. The precise composition of the hypercholesterolemic diet and the cholesterol levels usually found in ApoE3*Leiden mice have been added.

6. Figure 2: Indicate in the Figure Legend 2 that QC+/+ stands for wild type littermates.

Corrected in the manuscript

Referee #3:

Remarks :
- In view of the importance of specific quantification of pE1-CCL2 versus total CCL2, the validation of the in house developed ELISA should be included.
How was the specificity tested?

Cell clones, which potentially express pE1-CCL2 specific antibodies were initially screened using CCL2(pE1-38) and CCL2(D3-38) under ELISA conditions. Cell clones were selected which produce CCL2(pE1-38) specific antibodies.

At the next stage, we characterized the antibody specificity on PepSpot membranes. Among others, clone 2C9 was selected. The epitope comprises the first 4 amino acids of CCL2 (pE-P-D-A). The sequences pE-P-D-G/S present in human CCL8 and murine/rat CCL7 were also recognized, but with lower affinity. Therefore this antibody mainly recognizes CCL2 from human, murine, rat and other species. In addition, human CCL13 is equally recognized since it shares the same N-terminal sequence.
The intact N-terminus of CCL2 is absolutely crucial for detection by clone 2C9. Any elongation or truncation at the N-terminus leads to a loss of binding. For illustration a DotBlot has been included in the supplemental material (Supplemental Figure 4A).

Because the peptide concentration on PepSpots is high and might lead to false interpretation of affinities, we routinely use surface plasmon resonance (SPR, Biocore) analysis and isothermal titration calorimetry (ITC) to characterize the reactivity of our antibodies in a quantitative manner.
The cross-reactivity to other pE-containing peptides has been analyzed by Biacore analysis. The results have been included as Supplemental Table 1.

In addition we have included typical standard curves for the detection of human pE-CCL2/Q1-CCL2 and murine pE-CCL2/Q1-CCL2 on the newly developed ELISAs. Both total-CCL2 ELISA detect pE-CCL2 and Q1-CCL2 but the pE-specific ELISAs show a strong preference for pE-CCL2 only. We have included this information in the new Supplemental Figure 4B-E.

In addition, the methods for DotBlot and surface Plasmon resonance have been included in the Materials and Methods section.

What was the degree of crossreactivity of the antipE1 monoclonals with eg total CCL2?

Antibody 2C9 detects only pE-modified N-termini of CCL2 as described above. As soon as the pE-residue is absent or the chemokine is truncated, there will be no binding of the antibody to the respective variant.

Did the authors test the crossreactivity of the capturing antibodies with other related chemokines?

The capture antibodies, anti-murine JE(MCP-1) Antigen Affinity Purified Polyclonal Antibody (Peprotech) and anti-human CCL2/MCP-1 Antibody AF-279-NA (R&D Systems) are commercially available. According to the data sheet the antibody from R&D Systems does not cross-react with the related murine CCL2, human CCL8 and human CCL7.

For the murine capture antibody no cross-reactivity was available from the data sheet. However, due to the sandwich ELISA system using combinations of two target-specific antibodies, e.g. 2C9 antibody, it is very unlikely that additional chemokines are detected. In addition, the concentration of pE-CCL2 was usually in good agreement with the total-CCL2 concentration as shown, e.g. in Figure 2C and 2F, which would support the specificity of the ELISA.

What was the limit of detection (LOD) and the limit of quantification (LOQ) of the ELISA?

Human Total-CCL2 ELISA: LOQ=15 pg/ml
Human pE[1]-CCL2 ELISA: LOQ=30 pg/ml
Murine total-CCL2 ELISA: LOQ=20 pg/ml
Murine pE[1]-CCL2 ELISA: LOQ=20 pg/ml

The limit of detection was not determined, because the LOQ is in agreement with commercially available ELISAs and there was no need to quantify samples below the above stated values. The LOQ-values have been inserted for each ELISA in Supplemental Figure 4B-E.

What were the typical dilutions of plasma and other samples?

The ELISA have been validated for several body fluids, such as serum, CSF, cell culture supernatant, urine and are routinely used in our studies. A typical dilution for serum is 1:4 or 1:5. For, e.g. urine it would be 1:10.

-Legend to Figure 1A could be adapted to improve clarity: eg immature, QP-CCL2, dislays cleavage sites for Aminopeptidase....

We have revised Figure 1A and included the immature glutamine variants to improve clarity.

-Figure 1D: monocyte chemotaxis : highest efficacy for pE-CCL2, closely followed by Q-CCL2 : is there a significant difference between both? Pleasee mention.

This question was also raised by reviewer 2. We have changed Figure 1 by insertion of a novel Figure 1D. The difference between Q1-CCL2 and pE1-CCL2 is not as pronounced as suggested by

the CCR2 internalization assay in the old Figure 1D. We changed the cell count method to a more precise and quantitative method using FACS similar to the cell count in the thioglycollate assay and were able to better dissect the differences. By means of the improved quantification, significant differences between Q1- and pE1-CCL2 in attracting THP-1 cells could be observed. Figure 1D was replaced to better depict the differences.

-Pharmacological inhibition: the inclusion of the chemical structure of both used inhibitors would enhance transparence and future research possibilities for other researchers.

We have revised the QC-inhibitor part in the Materials and Methods section also as requested by reviewers 1 and 2. The IUPAC name for PQ50 and PQ529 was used to describe the compounds. The chemical name can be easily converted into a chemical structure by standard laboratory programs, such as ChemDraw.

-Did the authors investigate LPS-induced inflammation in QC or isoQC negative mice? Were inhibitors administered to QC or isoQC deleted mice?

Yes, we performed LPS-induced inflammation in QC and isoQC ko mice. The plasma concentration of pE-CCL2 was analyzed. The experiment is depicted in Figure 2C and D. We did not apply QC/isoQC-inhibitors to knock out mice, however, the LPS-induced inflammation is routinely performed in wildtype animals to profile QC-inhibitors. There, the QC/isoQC-inhibitors show a dose-dependent reduction of pE-CCL2 also in this model.

-p9 results on isoQC in atherosclerotic vessels. Fig 4 does not contain a western blot inset

This was a typo, which was corrected now in the revised manuscript. The Western Blot was shown in Figure 5 instead of Figure 4.

2nd Editorial Decision

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the referee that was asked to re-assess it. As you will see the reviewer is now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

Please pay a particular attention to points 1 and 4 of the referee's report that we feel need to be addressed.

In addition, we would kindly ask you to incorporate the Conflict of Interest statement within the main body of the text.

You may choose to take advantage of the fact that we allow the presentation of any peripheral data in the form of Supplementary information, to be published online only (materials and methods essential to the repetition of experiments described in the main body of the manuscript may not be presented in this way).

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

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

The revised manuscript by Cynis *et al*. properly addressed most of my recommendations to increase the reliability of the data presented and, to improve the manuscript. Now it is an impressive piece of work.

Referee #2 (Other Remarks):

I agree with the authors that a detailed analysis of their lung inflammation model exceeds the current study and that the use of the myeloid markers 7/4 (Ly-6B.2) and Ly6G is sufficient to characterize infiltrating monocytes.

Minor points:

1. The authors use newly generated isoQC knockout mice. The description of its generation and the genotyping protocol is missing and should be included in the manuscript.

2. The name of the mouse strain used is C57BL/6J and not C57/Bl6 or C57/Bl6J as used in the manuscript. This should be corrected.

3. Page 7, 8: The description "...using the markers 7/4high and Ly6Ghigh ..." is misleading. The name of the markers used in the study is 7/4 and Ly6G. The relative expression of these markers on the relevant cell population is high, medium or low. According to that the term 7/4high or medium or low, and Ly6Ghigh or medium or low is used to quantify the relative expression of these markers on the relevant cell population. This should be clarified.

4. Figure 2: Include a dot blot of the flow cytometry analysis similar to the analysis of cells from the thioglycollate induced peritonitis (Fig. 3 and Fig. 4).

5. Supplemental Figure 5: Show also the total cell number of monocytes.

6. Adjust lettering in Fig. 4A and 4B (CCL2 - Ccl2), and in supplemental Fig.7: (e.g. CCL2 in supplemental Fig. 7A and Ccl2 in supplemental Fig. 7B; Il6 to IL-6 or IL6).

2nd Revision - authors' response 01 June 2011

Referee #2

1. The authors use newly generated isoQC knockout mice. The description of its generation and the genotyping protocol is missing and should be included in the manuscript.

An additional paragraph describing the generation of isoQC ko mice and the genotyping protocol was introduced in the Materials and Methods section. In addition, we have added a reference for ENU mutagenesis, on which the generation of the mice is based on.

2. The name of the mouse strain used is C57BL/6J and not C57/Bl6 or C57/Bl6J as used in the manuscript. This should be corrected.

We have revised the manuscript and replaced the strain identification with “C57BL/6J”.

3. Page 7, 8: The description "...using the markers 7/4high and Ly6Ghigh ..." is misleading. The name of the markers used in the study is 7/4 and Ly6G. The relative expression of these markers on the relevant cell population is high, medium or low. According to that the term 7/4high or medium or low, and Ly6Ghigh or medium or low is used to quantify the relative expression of these markers on the relevant cell population. This should be clarified.

We have revised the respective paragraph to achieve clarity.

4. Figure 2: Include a dot blot of the flow cytometry analysis similar to the analysis of cells from the thioglycollate induced peritonitis (Fig. 3 and Fig. 4).
5. Supplemental Figure 5: Show also the total cell number of monocytes.

The intranasal application of LPS in isoQC ko mice was conducted by a contract research organization (Fraunhofer Institute for Toxicology and Experimental Medicine in Hannover, Germany). The dot blots for flow cytometry analysis and the total cell number of monocytes were not part of the reporting by the CRO. We have contacted the responsible study director but unfortunately, she is not available until mid of July. Therefore, it was not possible to include this data before the deadline to re-submit the revised manuscript.

6. Adjust lettering in Fig. 4A and 4B (CCL2 - Ccl2), and in supplemental Fig.7: (e.g. CCL2 in supplemental Fig. 7A and Ccl2 in supplemental Fig. 7B; Il6 to IL-6 or IL6).

For an easier discrimination between human and murine genes, human genes are frequently displayed using capital letters (e.g. CCL2) whereas the murine counterparts are depicted using a capital letter followed by small type (e.g. Ccl2). This was the case in Figures 4 and 7. However, to avoid confusion, we have changed all gene and protein descriptions to the capital letter code (e.g. CCL2).

The manuscript was again subjected to careful proofreading. Thereby, we noticed that a part of the results of the statistical comparison in Figure 1C was still lacking (i.e. the difference between pE-CCL2 and Q1-CCL2). Therefore, the Figure 1C was revised to include now the complete results, which provides further support for a significant difference between pE-CCL2 and Q1-CCL2 with regard to chemotactic potency.

Additional correspondence (editor) 03 June 2011

Thank you for submitting back your revised manuscript. I have read your point-by-point letter now and understand why you haven't provided the FACS scatterplot and total monocytes number. However, as I believe that points 4 and 5 required by Reviewer #2 should be included in the manuscript, I am ready to extend your deadline for resubmission in order for you to obtain the missing data from the research organization.

In your paper interest it would be great if you could arrange to obtain the data as soon as possible.

I am looking forward to hear back from you soon.

Yours sincerely,

Editor
EMBO Molecular Medicine

Additional correspondence (author) 06 June 2011

Thank you for the quick reply. We tried to reach our contact person and got the information that she will not be available for the upcoming 4 weeks. The colleagues told us that there is no substitute for her. Accordingly, we might be able to introduce the data within a 5-week timeframe. In order to avoid any misunderstanding, we cannot guarantee that we get the additional data to revise the figures entirely according to the recommendations.

