Author's response to reviews

Title: Modulation of RANTES expression by HCV core protein in liver derived cell lines

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Version: 3 Date: 21 November 2006

Author's response to reviews: see over
Dear Editor,

Thank you for consideration and review of the manuscript “Modulation of RANTES expression by HCV core protein in liver derived cell lines” by A. Ruggieri, M. Franco, I. Gatto, A. Kumar and M. Rapicetta. We appreciated the peer review process but we were surprised of the rejection for publication in BMC Gastroenterology since one of the reviewer gave positive comments, appreciated the accuracy of the methodology and conclusions of the study. Accepting his suggestions the authors tried to improve the manuscript addressing the concerns as indicated in the list included. In particular statistical analysis (that was one of the major compulsory requested also by the other reviewer) was included for Figs. 1, 2, 5, 7 and 8; meaning of the error bars was added in legends of Figs. 1, 2, 5, 7 and 8.

With regard to the other reviewer’s revision, little or no guidance was present and the observations seem not to expect a feedback in some cases. Nevertheless, the authors tried to improve the manuscript accepting some comments (especially major compulsory revisions in points: 1, 2, 3 and 9) and they answered to the observations in points 3, 4, 5 and 8) and corrected the X- and Y- axis label in Fig. 1 and 2. Statistical analysis, that is the main concern of the reviewers, was performed for Figs. 1, 2, 5, 7 and 8.

According to the above, the Authors kindly request the Editor to re-consider their manuscript for publication even by submitting it to a new peer reviewer that will provide a feed-back.

Looking forward to a positive reply the authors thank you.
Reviewer's report

Title: Modulation of RANTES expression by HCV core protein in liver derived cell lines

Version: 1 Date: 14 August 2006

Reviewer: Hans Dieter Nischalke

Reviewer's report:

Ruggieri et al. analysed the modulation of RANTES expression by HCV core protein in liver derived cell lines. They found that RANTES is differentially regulated by HCV core protein in HepG2 and CHL cell lines. RANTES expression was inhibited in core expressing HepG2 cells, by a reduction of RANTES promoter activity. Conversely, RANTES protein and mRNA were induced by the core protein in CHL cells, through the induction of the promoter. Analysis of the mechanism underlying the opposite modulation of RANTES by the core protein revealed that IRF-1 expression was induced by HCV core protein in HepG2 cells, whereas in CHL cells IRF-1 was not influenced by transfection with the core protein construct. The effect of the core protein on RANTES promoter was counteracted by co-transfection of same cell lines with NF90. The paper reports confirmatory in vitro data, highlighting substantial differences between the two cell lines. However, some concerns/questions remain to be resolved:

Major Compulsory Revisions:

1.) Figure 1: This figure is somewhat confusing. The data presented raise some doubt concerning specificity of HCV core-induced down-regulation of RANTES expression. First, it is unclear why the authors postulate that figure 1B indicates â€œpercentage of cells positive for RANTES expressionâ€_ (see legend to figure) but the X-axis is labeled â€œrelative fluorescence intensityâ€_. More importantly, how do the authors explain the clear reduction of RANTES expression in cells transfected with the control vector (Hepswx)? This should at least be discussed. As the authors are comparing the effects of HCV core in different cell lines it remains unclear why they do not provide data from transfections of HepG2 cells with vector containing core-E2-E1-NS2-NS3 as shown for the Chang liver cells (figure 2).

X-axis label in Fig 1B was changed to “Percentage of RANTES positive cells” and “fluorescence intensity” was the label of the X-axis in the histogram in Fig.1A. The reduction of RANTES expression in Hepswx was mentioned in Results section (pag 11, lane 203-206) where the authors added the sentence “Despite a reduction in RANTES expression was detected in Hepswx cells as compared to the parental cell line, suggesting a possible contribution of constitutive transfection, the differences in percentage of cells positive for RANTES expression among Hep39 as compared to Hepswx cell lines was statistically significant (p<0.05).”

2.) Figure 2: This figure is confusing. From figure 2A and the figure legend it remains unclear what the blue histogram represents. Is this from the untransfected cells? If this is the case it would be doubtful that HCV core has any effect on RANTES expression in their experiments. Therefore, this data should be given in both figures (2A and 2B) as has been done for the HepG2 cells (figure 1). Again, labeling of the X-axis in figure 2B.
does not match the legend (fluorescence intensity vs. percentage of cells). This should be corrected.

The histogram has been redrawn by Cell Quest software omitting the blue histogram, that actually referred to the CH827 cell line (expressing E1-E2), not to the un-transfected cell line as was hypothesized by the reviewer; those data are not scheduled in this manuscript and are object of further ongoing studies by the Authors.

Labeling of the X-axis in Fig.2B and of X- and Y-axis in Fig.2A has been corrected as in Fig.1.

3.) The pink histogram (Chang352) shows a second maximum and is clearly different from vector control (green) and the isotype control (black). In contrast, in figure 2B the corresponding percentage of RANTES positive cells are all about the same. It would be helpful to present the data as dot-plots and to give precise information on the position of the gates. How many experiments have been performed?

Number of experiments and the meaning of error bars were added in the legends of Figs. 1 and 2. The histogram in Fig.2A, as well as in Fig.1A are from one representative experiment out of three performed independently, whereas the bars plot in Fig.2B, as well as in Fig.1B, were drawn on the basis of the mean values from the three experiments, thus they could appear not exactly matching. The data from FACS analysis were analysed without gating.

4.) Figure 3: The authors state that transfection with their HCV core-containing vector (Hep39) results in downregulation of RANTES mRNA expression as compared to cells transfected with the empty vector. However, from the figure it seems that expression of b-Actin mRNA is also reduced in the HCV core-transfected cells. Thus, it remains unclear whether HCV core-induced reduction of RANTES mRNA is a specific phenomenon. Therefore, ratios between RANTES and b-Actin mRNA levels should be calculated by densitometry and presented in the manuscript.

If HCV core protein would modulate $\beta$-actin in Hep39 cell line one would expect a less reduction of the signal for RANTES than that resulted; therefore the authors do not agree with the reviewer’s interpretation of PCR results.

5.) Figure 6: The presented immunoblot shows an increased IRF-1 expression in HepG2 cells after transfection with HCV core. IRF-1 is a direct transcriptional activator for RANTES expression (Liu at al.2005). How do the authors explain that higher IRF-1 expression leads to a reduced production of RANTS in HepG2 cells?

As reported in discussion (pag. 16, lane 319), one possible explanation for the role of IRF-1 in RANTES expression in the cell systems analysed, is that the basal level of IRF-1, which is different in the two cell lines examined, and the different available level of IRF-1, as well as other possible undisclosed transcription factor, may contribute to the core mediated effect on RANTES. With regard to the cited study by Liu et al. it reports RANTES promoter regulation by IRF-1 upon interferon gamma treatment in mouse macrophages, that are quite different from liver derived cell lines of human origin examined in present study. In addition, the analysis in this manuscript has been performed in absence of interferon treatment but in response to HCV core protein expression.

6.) The authors state that Chang liver cells do not show any increase in IRF-1 expression after transfection. To my opinion there is an increase both after transfection with the control vector (CHwt) and after transfection with the core containing vector (CH39) in comparison to the untransfected cell line (CHL). It seems, that the vector alone exerts some influence on gene transcription independently from the insert.

No change has been done since no feedback is expected.
7.) Jurkat cell lysate (CTR) seem to be unsuitable as a positive control for IRF-1 expression because the specific band is virtually absent on the immunoblot. Maybe pre-stimulation of Jurkat cells is necessary.

The immunoblot of Jurkat cell lysate was omitted in Fig. 6 , given the clear cut specificity of the band identified .

8.) In the results section the authors describe that in cells expressing HCV proteins C-E1-E2-NS2-NS3 (CH352) the fraction of RANTES positive cells was similar to the controls, suggesting a possible role of HCV non-structural proteins (NS2, NS3) in counteracting the effects of structural HCV proteins in RANTES induction. In contrast to this interpretation Soo et al. showed in HeLa cells that the structural proteins E1 and E2 can abolish core-mediated induction of RANTES promoter constructs, whereas transfection with HCV non-structural proteins revealed activating effects (Soo et al. 2002). Thus, the possibility of HCV envelope proteins E1 and E2 counteracting HCV core-induced RANTES promoter activation must also be considered.

With regard to the contribution of E1 and E2 in counteracting the effect of HCV core protein on RANTES expression the sentence “… since the structural E1 and E2 proteins of HCV have been reported to induce RANTES secretion [20]” was added in results section (pag 11 lane 212). Since it has been recently reported by Nattermann et collaborators (J Viral Hepatitis 2004) that interaction of E2 protein with CD81 increased RANTES secretion by CD8+ lymphocytes from HCV patients, the authors did not consider the possibility that E1-E2 counteract the effect of core on RANTES in CH352 cell lines, as if this would be the case the envelope proteins should inhibit RANTES. However results from Soo et al. as well as from Nattermann et al. and from present study seem to underline that the effect of core on RANTES expression is cell specific.

9.) Statistical analysis should be given for the data presented in figure 5, 7 and 8

Statistical analysis was given for Figs. 1, 2, 5, 7 and 8.

Minor Essential Revisions:

1.) X-axis in figure 1A and 2A should be labelled.
   Done.

2.) In figure 2 and 5 labeling (A) and (B) is missing.
   Done.

What next?: Reject because scientifically unsound

Level of interest: An article of limited interest Acceptable

Quality of written English: Acceptable

Statistical review: No

Declaration of competing interests:
'I declare that I have no competing interests'
Reviewer's report

Title: Modulation of RANTES expression by HCV core protein in liver derived cell lines

Version: 1 Date: 28 August 2006

Reviewer: alfredo garzino demo

Reviewer's report:

General
In this manuscript, the authors report the results on their studies on the modulation of expression of RANTES by HCV core protein. To this end, they use two cell lines, HepG2 and CHL, transfected with an expression construct encoding for HCV core protein, and monitor RANTES expression by flow cytometry. Interestingly, RANTES expression was found to be reduced in association with HCV core in HepG2, while in CHL cells RANTES expression was enhanced (although constructs expressing HCV structural proteins did induce enhancement). The mechanism of RANTES modulation by HCV core was studied using by RT PCR for RANTES RNA signal, and by transfecting the same cell lines with reporter constructs encoding for luciferase driven by the RANTES promoter. These experiments showed that the levels of RANTES RNA signal and of RANTES promoter activity in the two cell lines paralleled the modulation of expression of RANTES by HCV core as detected by flow cytometry. As possible correlate of the modulation of promoter activity, the authors focused on IRF-1 and -7, finding that the former was increased in HepG2 but not in CHL cells, so that it can be hypothesized that differential expression of this factor could at least partially account for the differences in RANTES expression by these cell lines. Finally, the authors studied whether expression of NF90ctv was also associated with the modulation RANTES expression by HCV core. Their finding show that transducing NF90ctv in CHL cells abrogated RANTES activation by HCV core; in HepG2 cells expressing core proteins, however, NF90ctv was associated with a two-fold RANTES promoter induction. These results are potentially relevant to the ability of HCV to evade immune responses and persist in chronic infection. Overall, the methodology of this manuscript and its conclusion are accurate, but several points, indicated below, need to be addressed by the authors.

Major Compulsory Revisions

a) Most of the findings on RANTES expression are performed by flow cytometric analyses. While the findings on decreased RANTES production in HepG2 cells appear more clear-cut, the low levels of basal RANTES expression in CHL make it difficult to discern whether a true induction of RANTES occurs. Testing of RANTES concentration in supernatants of control and transfected cells would help corroborating these findings. Flow cytometric analysis provided a measure of RANTES protein produced by the cells, either as percentage of cells that express the protein as well as the level of RANTES inside the cells. This analysis was also comparative with respect to the previous similar study by Soo and co-workers that applied FACS analysis to detect variation in RANTES level by HeLa cells. For that reason we think that the ELISA test on secreted RANTES do not need to be performed.

b) Legends of figure 1 and 2 do not indicate number of experiments and/or replicates performed. Error Bars are used in several figures but the legends do not indicate what they stand for. Most of the data presented is rather clear-cut. However, the authors
should consider statistical testing of observed differences (a simple t-test would suffice).

The legends of Figs 1 and 2 were modified by the addition of the requested information about number of experiments. error bars that represent standard deviations; the t-test was also applied to measure significance of differences in percentages of RANTES positive cells among cell populations examined. Accordingly, the new paragraph “Statistical analysis” was introduced in Methods (see pag.10 line 184); in Results section (pag 11) we reported the ±SD value together with the value of percentage of RANTES positive cells. In addition, we included the following sentences for results of the statistical analysis “[..] the differences in percentage of cells positive for RANTES expression among Hep39 as compared to Hepswx cell lines was statistically significant (p<0,05). […] The differences observed between CH39 and control cell lines was significant according to the p value (<0,05) in a t Test’s.”.

c) The rationale for adding NF90ctv to these studies is not clear, and it might actually confuse the reader. The authors should try to present more clearly their case for including the study of this factor to their studies.

With regard to reported knowledge about NF90, in Background (pags 5-6, lanes 97-101 and 106-108) the authors added the following sentences: “NF90 belongs to a family of double-stranded-RNA binding proteins [27], which has been reported to regulate replication of the Pestivirus BVDV [28] (evolutionary related to the Flavivirus family to which HCV belongs) and when introduced into osteosarcoma cell line it induces resistance to HIV replication in cell culture [29] acting as a component of cell defense against viral infection by activation of some IFN-response genes [29]”. In addition, we investigated the role of NF90 transfection on the expression of RANTES in order to reveal its possible immunomodulatory function and its potential to counteract HCV core protein effect on RANTES.

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Minor Essential Revisions

a) The authors should be consistent throughout the manuscript with nomenclature and abbreviations. Once they define Chang Liver cells as CHL, there is no need to go back and forth between the name and its acronym. Similarly, NF90 is sometimes defined as such and other times as NF90ctv. These and all other names and abbreviations must be kept consistent.

The nomenclature and abbreviations of cell lines and of NF90 were converted in order to be consistent throughout the manuscript and in the legend of Figs. as suggested.

b) In the abstract there is insufficient explanation on the rationale for studying IRF-1 and -7. The same can be said for NF90ctv.

In pag.2 lane 39 of the Abstract section we introduced the sentence: “Based on the reported observation that HCV genome modulates IRF-1 and IRF7 in replicon system and IRF1, IRF3 and IRF7 have been reported to regulate RANTES promter in various cell systems,…” in order to explain the rationale for studying IRF-1 and IRF-7 expression. We did not add more about NF90 in the Abstract section because of space reason, but we did it in Background section (see above).

c) In the Background section, second paragraph, the authors state that “Initial inflammatory response to early viral infection is determined by RANTES […]” This statement seems to suggest causality; however, the references provided do not seem to prove causation, just association.
According to the reviewer suggestion we changed the sentence “Initial inflammatory response to early viral infection is determined by RANTES…” into: “Initial inflammatory response to early viral infection is associated to secretion of RANTES, […]”.

d) Still in the **background section**, third paragraph, the authors used the word “addiction”, but probably meant “addition”.
In the third paragraph (lane 85) of the background section the incorrect word “addiction” was changed to “addition”.

e) The **background section** could include a quick introduction of the relevance of IRF-1 and -7 to this study (and see comment above on NF90ctv).
In the background a brief introduction of the rationale for examining IRFs in this study was included (pag. 5, lane 93-96 and 103-105) by the sentence: “**RANTES activation by viral infection has been reported to be regulated by synergistic activity between interferon response factors, IRF-3, IRF-7 and NF-kB in various cell lines [32] and by IRF-1, which can bind to RANTES promoter in mouse macrophages [36]. Moreover, HCV genome has been reported to modulate IRF-1 and IRF-7 in replicon system. [30,31] […] The expression of IRF-1 and IRF-7 has been examined to reveal their possible role in RANTES modulation by the core protein**”.

f) There is no description in the **Methods section** on how NF90ctv is introduced in cells.
It is reasonable to assume that this factor was transfected as an expression vector, but this is not clearly stated nor a construct is described or quoted from previous studies.
In Methods section under “**Stable transfectants and expression constructs**” we mentioned the pCI-NF90 plasmid for expression of NF90 factor into the cell lines by transfection with the Fugene (pag.7 lane 125).

g) In the **first paragraph of the Discussion**, the authors state “Consistent with the results in a recent report […]”, but quote two studies.
In the first paragraph of the Discussion we eliminated the wrong bibliographic citation and we left the statement “**Consistent with recently reported observations[18]…**”.

h) In the **second paragraph**, the authors state “consistent with the data from Miller and co-workers (2004) […]” but provide no numeric reference.
In the second paragraph of the Discussion we added the number of reference omitted in the previous version of the manuscript that has been changed to :"**Consistent with the data from Miller and co-workers (2004) [29]…”

i) The **legend to Figure 2** refers to (A) and (B), which are not indicated on the figure itself.
The (A) and (B) letter were added in the two part of Figure 2.

Discretionary Revisions (which the author can choose to ignore)

**What next?:** Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** No
Declaration of competing interests:
I declare that I have no competing interests