Author's response to reviews

Title: Tumour Cell derived Effects on Monocyte/Macrophage polarization and function and modulatory potential of Viscum album lipophilic extract in vitro

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Author's response to reviews: see over
Dear Dr. Rowles

We were pleased about the opportunity to revise our paper entitled “Effects of Viscum album lipophilic extract and oleanolic acid on monocyte migration and macrophage polarization in vitro” (manuscript 1938897472149711).

We are thankful that the reviewers rated our manuscript very interesting and worth publishing. Their comments were helpful and we appreciated the constructive feedback on our original submission. Due to major compulsory remarks the revised title of our manuscript is: “Tumour Cell derived Effects on Monocyte/Macrophage polarization and function and modulatory potential of Viscum album lipophilic extract in vitro.” We have carefully considered all comments and suggestions and we addressed them point-by-point.

Thank you for the positive and helpful suggestions.

Sincerely,
Myriam Estko
(Corresponding author)
Reply to reviewer’s reports

**Reviewer:** Jaroslaw Czyz  
**Reviewer's report:**

In their paper, the authors estimated the impact of liophilic extracts from *Viscum album* containing pentacyclic triterpenes and purified oleanolic acid on the motile activity of monocytes and their polarization to M1 and M2 phenotypes in the in vitro conditions. Additionally, the estimated the effect of the media conditioned by cancer cells on the polarization pattern of monocytes. Overall, the idea and approach are original and sound, the story is very interesting and undoubtedly worth of publishing, however its construction is confusing for me.

Major compulsory revisions:

1. In general, in its present form the manuscript lacks the axis. The authors should decide whether they want to concentrate on the conditioned media effects (in this case, the data on VALE and OA should be presented as additional result and not described as a scaffold of the article). Alternatively, they could concentrate on the extracts and use conditioned media as an additional experimental design. Consequently, the whole manuscript should be rewritten to follow one line of reasoning.

   We rewrote the whole manuscript and decided to concentrate on the effects of conditioned tumour cell media on macrophage polarization complemented by the investigation of a potential modulatory impact of VALE/OA. The title was adapted as follows: “Tumour cell derived effects on monocyte/macrophage polarization and function and modulatory potential of *Viscum album* lipophilic extract in vitro”. All parts of the manuscript especially the abstract and the discussion were adapted to this concept.

2. As far as I can assess, several fragments require revising due to clumsy expressions and/or incomprehensible construction (see for example lines: 54-57; 76, 96-99; 164; 175-176; 255-256, etc.). Native speaker with experience in writing scientific papers should be consulted.

   The text fragments mentioned by the reviewer were revised according to standard scientific writing.

3. Additional data on the effects of VALE and OE on the transmigration of polarized monocytes would considerably enhance the message of the story. This would also help the authors to combine the data on conditioned media with the triterpene story. Or, if the authors think these experiments are pointless, they should clearly explain, why they think so?

   We do agree that data about effects of VALE/OA on transmigration of polarized MDM would have been advantageous. But initial experiments concerning this matter did not result in reasonable data and because volume and number of blood samples were limited, we decided to concentrate primarily on effects on polarization. The investigation of the influence of VALE
and OA on the function of polarized macrophages may be the topic of another prospective project.

4. The structure of the discussion part leaves much to be desired, the fragments concerning triterpenes are scattered among overextended considerations on monocyte polarization. This should be modified and made more concise.

We do agree that the discussion of our manuscript had to be amended. Therefore its structure was adapted to the altered concept and tightened up to clearly focus on subject and goal of our study.

Minor essential revisions:

1. With regard to the data on “monocyte migration”, the authors should clearly state that they analyzed the transmigration/chemotaxis of these cells. By the way, are 8 micro pores not too big for the analyses of transmigration of relatively small cells, like monocytes?

In our modified manuscript we made clear that we investigated effects on transmigration/chemotaxis of monocytes.

We knew that for monocytes (cell size 12-20 µm) a transmigration assay with 5 µm pores would have been optimal. Unfortunately this pore size was not available for our system. At first we attempted to establish our chemotaxis/transmigration assay using fluoroblock inserts with 3 µm pores but we could not detect any signal. Therefore we chose the next available pore size with the fluoroblock system, 8 µm. Now we got reasonable results, low spontaneous transmigration with untreated monocytes and elevated transmigration in response to the positive control MCP-1.

2. Biological significance of the tested concentrations should be at least shortly discussed.

We chose the concentrations for VALE and OA according to our previous experiences with in vitro studies on PBMCs, monocytes and fibroblasts. 25-50 µg/ml VALE or 2.5-5 µg/ml OA, respectively proved to be optimal regarding stimulating but not cytotoxic effects on treated cells.

We added following sentence to the discussion: “For this purpose we used concentrations of VALE and OA that were established in prior studies on PBMC, monocytes and fibroblasts and proved to be effective but not cytotoxic [47, 48].” (lines 348-349)
Reviewer: Roman Huber
Reviewer's report:

3 major shortcomings.

1. Dose dependency of the observed effects has not been shown. This has to be done.

*The final concentrations of VALE and OA were chosen according to prior experiments on PBMCs and monocytes [1]. In these experiments we observed that the effective stimulatory concentration range is very small and higher concentrations soon have cytotoxic effects. Therefore and because volume and number of blood samples were limited we decided to work with known effective concentrations and not to investigate dose dependency.*

2. To show that triterpene content is related to the observed effects a comparison with triterpene-free viscum album extract should have been performed. Either the authors add these experiments or they cancel the paragraphs in their manuscript which suggest a causal relationship between observed effects and triterpene content.

*Pentacyclic triterpenes have been shown to be pharmacologically active substances exhibiting immunomodulatory, wound-healing and anti-tumour effects [2-4]. The amount of the predominant triterpene oleanolic acid in our lipophilic mistletoe extract (VALE) is about 10%. In order to prove, if the observed immunomodulating effects of VALE can be referred to OA as one of the active compounds, we used pure OA at the corresponding concentration as positive control. In our current and previous experiments we achieved comparable effects with both VALE and OA, in our opinion suggesting an underlying causal relationship between OA and observed effects.*

3. To convincingly show an effect of VALE and OA on monocyte migration the experiments reported in figure 1 and figure 2 should have been performed analogous: at least VALE and OA should have been incubated without MCP-1 analogous to the experiments performed in figure 2.

*In our study we intended to investigate the effect of VALE/OA on monocyte chemotaxis/migration in response to MCP-1 or tumour cell conditioned media. In a first step we examined if VALE/OA alone did exert any chemotactic activity on monocytes. We included these results in figure 4 and adapted the results part as follows: “As shown in figure 4 VALE or OA alone did not act as chemotactic attractants for monocytes. Migration of monocytes towards medium supplemented with VALE was comparable to the spontaneous migration measured in samples with medium alone. Monocyte transmigration towards OA was significantly lower than the negative control (p<0.05).” (lines 274-277)*

*Additionally we included a new table (table 2) demonstrating the effect of VALE/OA on monocyte transmigration in response to tumour cell supernatants. (line 291)*
Minor:

- Number of experiments should be added in figure 2

As mentioned in the methods section and the legend of figure 3 (former Fig. 2), 3-5 independent experiments were performed.

- Reference for figure 4 in Results is missing.

The missing reference for figure 2 (former Fig. 4) is now included in the results section.

- TAM has not been explained when first mentioned in the Discussion

We inserted the following sentence to the discussion: “Macrophages within tumours are called TAMs (Tumour Associated Macrophages) and have often been associated with an M2 phenotype.” (lines 336-337)

- Absence of cytotoxicity of VALE and OA to monocytes and macrophages should be shown in a separate figure.

In figure 2 (former Fig. 4) as well as in table 1 we provided the viability data of monocytes/macrophages after treatment with VALE and OA. In our opinion this presentation is appropriate but if requested it is possible to replace the table by a figure.
This is certainly an interesting observation, but I have some objections:

1. Do authors have any informations on the purity of VALE and OA? Is a contamination with lectins or viscotoxins excluded with certainty and is there anything known in the literature about the effect of mistletoe lectins or viscotoxins on monocyte migration and macrophage differentiation? This should be at least mentioned in the discussion.

   *In the methods section (products investigated), we inserted following clarification: “Possible participation of contaminating mistletoe lectins and viscotoxins in VALE can be excluded as both toxic proteins are insoluble in lipophilic solvents. In addition the heat labile mistletoe lectins [5] and viscotoxins [6] are expected to be inactivated during the extraction procedure of VALE (at least 5 h at 70°C).”* (lines 110-113)

   *To not further provide confusing information we did not mention and discuss the potential impact of hydrophilic (but not lipophilic) mistletoe lectins and viscotoxins in our manuscript.*

2. Samples from how many blood donors were analysed?

   *The blood samples of 11 healthy donors were analysed. We added this number to the methods section (isolation of blood monocytes).* (line 116)

3. p7 line 175 ff: how were the amounts of IFN-gamma, LPS, IL-4 and IL-13 used for macrophage polarization determined?

   *In the methods section (Polarization of monocyte-derived macrophages (MDM) and treatment with VALE/OA) we added: “Monocytes were differentiated and polarized according to established protocols [7-9], with some modifications.”* (lines 147-148)

4. Did authors use other non-tumour cells as controls for analysis of monocyte migration?

   *No, we were especially interested in the relationship between monocytes/macrophages and tumour cells. As positive control for chemotaxis/migration we used the monocyte chemotactic protein 1. The inclusion of further cell types would have been beyond the scope of our project.*

5. In legend of figure 2 the cell lines should be explained once more

   *We modified the legend of Fig. 3 (former Fig. 2) as follows:*

   *Figure 3 - Migration of monocytes induced by tumour cell conditioned media*

   *The effect of conditioned media of human lung carcinoma cell line NCI-H460, breast carcinoma cell line HCC1143, prostate carcinoma cell line DU145 and pancreas*
adenocarcinoma cell line PA-TU-8902 on monocyte chemotaxis/transmigration in comparison with MCP-1 and medium (1% FCS) alone was measured in a chemotaxis assay. Each bar represents the mean fluorescence intensity ± SD (a.u. = arbitrary units) of 3-5 independent experiments for each cell line. *p<0.05, ***p<0.001 compared to the negative control (medium, 1% FCS), (LSD-test). (lines 669-676)

6. The legend of figure 3 is not quite clear; what does A, B, C exactly mean?

As it was difficult to display significances between 5 different polarization types graphically we decided to use another system. Columns labelled with distinct letters differ significantly from each other. Columns that share the same letter are not significantly different.

See legend of Fig. 1 (former Fig. 3): “Columns with distinct letters differ significantly from each other”. (lines 658-659)

References:

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