Author’s response to reviews

Title: HMG-CoA reductase inhibition aborts functional differentiation and triggers apoptosis in cultured primary human monocytes: a potential mechanism of statin-mediated vasculoprotection

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PDF covering letter
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**REPLY TO REVIEWERS’ COMMENTS**

We thank both reviewers for their time and effort to provide constructive criticism of our work. Following is a point-by-point reply that we hope will address the majority of your concerns.

**GENERAL COMMENT**

We agree with the reviewers that much remains to be clarified regarding the molecular mechanisms underlying our findings. Our work highlights a relatively unexplored aspect of statin action, namely its effects on monocyte survival and function. In their capacity as macrophage precursors, monocytes are clearly central to the pathology of vascular disease in general and atherogenesis in particular. Yet, only a handful of papers (all of them cited in our manuscript) have looked at this cell type in the context of statin therapy; and only one of these (Kaneider et al 2001) reported some findings relating to cerivastatin-associated apoptosis in monocytes.

Since completion of his PhD and preparation of this manuscript Dr Vamvakopoulos has moved to a new post, which has caused an unavoidable delay in resuming this work. Funding constraints have also been encountered, given that the current manuscript is our first contribution to the field.

However, we feel that our manuscript, in its present form, contains enough new data to warrant publication. We have been careful not to overinterpret our findings while still placing them in the context of a plausible and well-defined pathogenetic model. We believe that our experimental rationale, design and analysis have been solid; although we acknowledge that much more remains to be studied. We hope to soon be in a position to provide more answers to outstanding questions.

**REFEREE #1 (DR GUIJARRO)**

**GENERAL COMMENT**

Conducting these experiments with clinically used statins carries obvious advantages of which we are well aware. However, at the time this study was conducted we had no established clinical collaboration. Mevastatin was selected for this study because it is a patent- and prescription-free, commercially available, naturally occurring (i.e. non-synthetic) HMG-CoA reductase inhibitor. The newer, clinically used statins, especially synthetic ones (fluvastatin; atorvastatin; and the withdrawn cerivastatin), are much more potent inhibitors than mevastatin. This fact reinforces our view that our findings may indeed be applicable in the clinic.

**DO STATINS ABORT MONOCYTE DIFFERENTIATION?**

Differentiation is, by definition, the process of events leading to a more “mature”, specialized cellular phenotype. Currently, phenotype can be described in terms of gross cell morphology; cell function; immunophenotype (i.e. protein expression); or gene expression. Cell morphology alone is a fairly poor index of differentiation; however, we have observed that statin-treated monocytes tend to
remain in culture as round-shaped, poorly adherent cells in contrast to untreated cells, which adhere strongly, spread and migrate around the culture vessel. Immunophenotypic changes occurring during monocyte-to-macrophage differentiation are poorly characterised. To our knowledge, most data come from studies of leukemic cell lines (MonoMac; THP-1; U937) and are therefore highly suspect. Furthermore, surface marker expression is greatly dependent on the microenvironment (e.g. ECM composition, growth factors, inflammatory stimuli etc) where this process is taking place. If you have a specific immunophenotypic marker in mind, which has been proven in previous \textit{in vitro} studies, we would definitely be keen to evaluate it in future work.

Functional indices of macrophage differentiation (such as enhanced phagocytic potential; lipid sequestration; responsiveness to inflammatory stimuli) are highly relevant to the pathogenesis of vascular disease. In culture, monocyte-to-macrophage differentiation occurs spontaneously over several days. Since the pro-apoptotic effect of statin-treatment occurred fairly early (within 48 h), we chose to evaluate \textit{immediate-early markers of functional differentiation} in the same context. To our knowledge, reduced elaboration of IL-1β following LPS challenge is the best characterised and most consistent such marker, proven both \textit{in vitro} (cultured monocytes) and \textit{in vivo} (comparison of blood monocytes to alveolar macrophages). All relevant papers are cited in our manuscript and we repeatedly caution throughout our text that our findings apply to \textit{functional} monocyte differentiation.

Our relevant findings show that statin-treated monocytes retain their high IL-1β output following challenge with LPS, suggesting a delay / blockade in functional differentiation. Because soon thereafter these same cells undergo apoptosis we claim that this process is not merely delayed, but effectively aborted. The concept of arrested differentiation leading to apoptosis is not new and in our text we do NOT claim that statins are directly pro-apoptotic for monocytes. Instead, our observation that lymphocytes, which are largely dormant in culture, are relatively resistant to apoptosis in our model immediately suggests that monocyte apoptosis may occur as a result of the statin-imposed differentiation blockade. The fact that myeloid differentiation, in particular, seems to hinge on enhanced output from the mevalonate pathway may also explain the enhanced susceptibility of these cells.

- Our text has now been amended to further clarify this point.

**WAS MEVASTATIN ACTIVATED PRIOR TO USE?**

You are correct in pointing out that biological activity of all natural statins, including mevastatin, requires hydrolysis of their lactone ring. \textit{In vivo} this occurs predominantly in the liver and blood plasma via the action of various unidentified lactonases (carboxyesterases; ref. 1). For \textit{in vitro} studies, many authors working with cells of epithelial or mesenchymal origin have artificially activated these compounds by alkali treatment. We have NOT done this for several reasons. Firstly, there is no firm evidence to what extend (if any) this manipulation resembles physiological processes and how it may additionally modify / inactivate the drug. Secondly, there is now evidence that enzymes also expressed outside the liver, such as the paraoxonases (PON), also possess lactonase activity (ref. 2). PON enzymes are detoxifying and anti-oxidative and we reasoned that they would be present in monocytes; in addition, a monocyte carboxyesterase activity has been described (ref. 3) and may also function to activate statins. In the absence of any evidence from the literature, confirmation of this fact was empirical: we show in our
manuscript that the observed effects were prevented by exogenous supplementation of mevalonolactone, which proves that mevastatin was indeed activated and did exert its biological function. Though there is always the possibility that activation by this route was not optimal, this would only add more weight to our findings. Hence, we do not see any problem with our approach.

**Was statistical analysis conducted properly?**

Your description of proper statistical methodology is correct and corresponds precisely to our treatment of the data. We derived mean values for each individual tested and used these for further statistical calculations. This fact is implied in our usage of SEM, which is essentially a measure of the precision of the mean value and NOT a measure of spread of the raw data. We hope you will agree that our description of statistical methodology is both complete and concise and that no further changes are needed.

**Effect of GfN in our experimental system (p. 9)**

We understand your concern relating to the wording here; our text has been amended accordingly.

- **Page 9, para 2 has been rewritten.**

**Is apoptosis linked to aborted differentiation in our system?**

We have partly addressed this issue in earlier comments. We present here the first evidence that HMG-CoA reductase activity regulates monocyte functional differentiation: inhibition of this enzyme likely halts this process, while exogenous supplementation of mevalonolactone (in the presence of statin) does not merely overcome it, but appears to accelerate it. In this context monocytes, which spontaneously differentiate in culture, are far more susceptible to apoptosis than lymphocytes, which do not. Furthermore, mevalonolactone supplementation (in the presence of statin) does NOT fully prevent apoptosis. Finally, other authors have shown that HMG-CoA reductase is specifically up-regulated during monocyte differentiation (Yachnin et al 1984), suggesting increased utilization of the mevalonate pathway. Collectively these observations suggest to us that apoptosis occurs secondary to differentiation blockade in our system, probably due to depletion of mevalonic pathway products. However, this may well prove to be a chicken-and-egg issue. For this reason we are happy to accommodate your concerns and attenuate this conclusion.

- **Relevant text has been modified accordingly.**

**Discretionary revisions**

Your suggestions for future experiments are noted with thanks. We have also done our best to incorporate the additional points you mention in our Discussion without shifting its main focus or substantially increasing its length. In particular, we were very interested in the early findings you cite, regarding statin inhibition of M-CSF. Regrettably, however, with the possible exception of your review in
Miner Electrolyte Metab, we have been unable to unearth any published evidence of such findings. We would therefore be grateful if you could point us to the relevant original paper.

- Discussion section has been updated.

REFEREE #2 (DR KREUZER)

MAJOR POINTS

We have already outlined our rationale for assessing differentiation as we did in our reply to Dr Guijarro (please read above). In addition, we have cautioned at every opportunity that our results are applicable to functional monocyte maturation. We are keen to receive your suggestions for differentiation antigens, proven in culture, that we can evaluate in future studies.

With regard to your comment on the effect of mevalonolactone on apoptosis discussed in our paper, we do acknowledge that the mechanism outlined in the last paragraph of our Discussion is largely speculative and one we are actively researching. We note, however, that the Discussion section is there so that interesting new hypotheses, leading to new studies, can be voiced. In an effort to accommodate your concerns we have eliminated this part of our Discussion and have instead incorporated some elements, more clearly set out, elsewhere in this section.

Your comment on the effect of mevastatin on IL-1Ra release deserves some further explanation, which we originally omitted from our Discussion for reasons of brevity and coherence. Essentially your question boils down to this: given that both IL-1β and IL-1Ra are normally down-regulated during monocyte differentiation, why does mevastatin NOT prevent IL-1Ra down-regulation? The most likely explanation becomes apparent if one considers the different cellular pathways operating in IL-1β versus IL-1Ra release. Briefly, IL-1Ra is secreted via the classical pathway (i.e. via the Golgi apparatus); while IL-1β lacks a leader peptide and thus accumulates in the cytosol, being released through stimulation of the P2X7 receptor at the level of the cell membrane (ref. 4). We showed that apoptosis is rife among mevastatin-treated monocytes. Apoptosis is known to shut down Golgi function (ref. 5); hence IL-1Ra release, which is thus sensitive to apoptosis, will decline (in accordance with our observations). Conversely, IL-1β release only depends on events occurring at the level of the plasma membrane (micropore formation and microvesicle shedding) and is thus refractory to apoptosis. For this reason we believe that, in our system, IL-1β is a more accurate index of monocyte functional differentiation than IL-1Ra; and that our findings with IL-1Ra release are simply another manifestation of the increased apoptosis in these cells. Many authors have previously shown that statin treatment inhibits the release of various mediators; our findings with IL-1Ra vividly illustrate that these previous observations may have been confounded by cell death. We have now incorporated some relevant comment in our Discussion to emphasize this point; moreover, in the revised version of our Results we highlight the fact that apoptosis at 48 h and IL-1Ra down-regulation correlate, thus hinting that we consider the two interrelated. We thank you for bringing this point to our attention.
Last paragraph of Results has been amended; Discussion has been amended.

The question you pose as to whether mature macrophages subjected to statin treatment also undergo apoptosis is interesting. We are unaware of any relevant data in the literature and will be addressing this question in future experiments.

MINOR POINT

We concur that the term hydrophilicity, as used, should be qualified; we have now amended the relevant text to indicate that cerivastatin is relatively hydrophilic, compared to mevastatin.

Once more, we wish to thank both referees for their valuable comments.

ADDITIONAL AUTHOR REFERENCES