Author's response to reviews

Title: Echogenic perfluorohexane-loaded macrophages adhere in vivo to activated vascular endothelium in mice, an explorative study

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(adjusted title) Title: Echogenic perfluorohexane-loaded macrophages adhere in vivo to activated vascular endothelium in mice; an explorative study.

We are indebted to both reviewers for their encouraging and critical remarks. It certainly helped us to clarify the intricacies of the study. Below the remarks of the reviewers are given in plain text, while our responses are in italics. Modifications are given in bold characters, below and in the manuscript.

Reviewer #1: Francesco Stea
1.0 This paper presents a very interesting proof of concept for future applications. However, it has little to do with ultrasound, as the US part is marginal, it deals with echo from the blood only, and does not present numerical data. This is not a problem in itself, but do the authors believe that “Cardiovascular Ultrasound” is the right journal for the present paper?

The manuscript does not specifically deal with the echoreflectivity of blood but rather with the potential of PFH loaded macrophages to create site-specific echo signal enhancement. To clarify and stress the latter objective, we modified the text of the Abstract and Introduction. The identification of suitable contrast enhancers and practical consequences/limitations thereof is a major area of cardiovascular ultrasound research.

1.1 This paper is a proof of concept. (The paucity of numerical data makes even difficult defining it a “pilot”, “preliminary” or “feasibility” study.) This should be clearly reflected in the title.

As indicated in our reply to remark 1.0, we want to demonstrate stepwise that macrophages loaded with ultrasound contrast agents can be used to locate selectively activated endothelium. This is exactly what is indicated by the title, but we do agree with this reviewer that presently we did not yet consider the overall behavior, i.e., injection of contrast followed by ultrasound intensity assessment a few hours later. That is why we added to the title:

“; an explorative study”.

1.2 Page 13, In vivo interactions: standard deviations larger than the mean value are a clear further sign that the data distribution is not normal. This means that they can’t be expressed as mean/SD and the statistical tests should be non-parametric. Please revise Methods and Results accordingly.

*Indeed, the shape of the statistical distribution should be skewed. Accordingly we changed the text in Methods to indicate that the results are reported as median plus range and tested with the non-parametric Mann-Whitney U-test. As expected, the latter test resulted in slightly different significance levels but did not affect our conclusions. Actually, rolling and adhesion behavior of individual cells should be evaluated with respect to the total number involved. Accordingly we added on page 13: “(totaling 36 loaded and 30 unloaded BMM)”*

1.3 The Introduction section is very long. It should be shortened.

*We agree that the Introduction is rather long. However, we wanted to emphasize the complexity of site selective ultrasound signal enhancement with contrast agents, taking into account the specific physics of the contrast material (fluid, so no second harmonics) and adherence (resistance to local shear stress). These aspects are not always adequately dealt with. Therefore, we decided to leave the discussion on these aspects in the introduction and not to shorten it.*

1.4 Page 9, In vivo interactions…: TNF-alpha is not the only stimulation capable of provoking adhesion, so “necessity” is not the best term to be used here.

*You are right. We replaced “necessity” by “significance”.*

1.5 Page 25, Table 2: Rows and columns should be swapped

*As suggested we transposed the table, having now the physical parameter as entrance.*

1.6 Typo “macrophageendothelium” in Keywords.

*Thank you. We listed now “macrophage” and “endothelium” as separate Keywords*
Reviewer #2: Francesco Faita

2.0 This study aimed to investigate the potential of PFH-loaded BMM to act as ultrasound contrast agent targeted at vascular injuries. For this purpose, authors injected THF-alpha and PHF-loaded BMM in mice in different concentrations and they tested the detectability of the contrast agent at the level of the aorta, of the pulmonary artery and of the carotid artery. Moreover, they measured, by means of intravital microscopy, the degree of adhesion and the rolling behavior of the PHF-loaded BMM at the level of the carotid artery. Authors concluded that PFH-loaded BMM pass the pulmonary circulation and become detectable on the arterial side and that they roll and adhere selectively to activated endothelium under physiological shear stress. These findings would be of interest for those who are involved in study about ultrasound contrast agents.

Although minor revisions are desirable (as reported in subsequent “Minor Essential Revisions and Discretionary Revisions” section), in my opinion the manuscript is suitable for publication in Cardiovascular Ultrasound.

Thank you. No further comment.

Minor Essential Revisions and Discretionary Revisions:

2.1. The bolus used in order to obtain a detectable signal in the artery tree beyond the pulmonary circulation, was quite huge (15 millions 4%-loaded BMM). The authors should add some details about the process of suspending a similar amount in only 150 ul of RPMI-1640, which is indeed an appropriate total amount of contrast agent for single bolus.

The process is according to as phrased in the text: the loaded BMM were suspended in 150 ul of RPMI-1640 which implies that the actual bolus volume will be a few ul higher (for a large number of cells, the cell volume may even be in the order of 20 ul).

2.2. It is not clear the total number of mice involved in the echo study: in MATERIALS AND METHODS section, a total amount of 17 mice was claimed; however, results were reported only from 15 mice, in the corresponding section. Authors should clarify this.
Thank you for noting. The total number of mice for echo enhancement was not 17 but 15 (corrected on top of page 9). Actually we did not properly report about 4 mice (in one mouse we encountered problems with the anesthesia set-up; another mouse got accidently a minor dose injected while preparing the set-up. We rephrased the echo section (page 12-13) to clarify the observations:

In 3 mice we injected 5 to 7 million 2% loaded BMM. Of these three mice, one got unintentionally a spurious BMM injection, while for the other the experiment was terminated because of problems with the anesthesia set-up. In the third mouse (7 million BMM, slow infusion) we observed a notable transient echo enhancement in the pulmonary artery (Fig. 2).

4 Mice got an injection of 4% loaded BMM with a dose ranging from 2-15 million cells. Slow infusion of 2 million loaded cells did not induce echo enhancement in the aorta (mouse 1), but 7 million loaded cells (slow infusion) caused an increase in echo level of 0.8 dB in the pulmonary artery and of 0.2 dB in the aorta (mouse 2). Slow injection of a high dose of 15 million 4% loaded BMM (mouse 3) temporarily enhanced the blood echogenicity in the pulmonary artery with 9 dB (Figure 3, left panel) and induced a small, but detectable enhancement in the aorta (1 dB peak). Also a bolus injection of a large number (15 million) of 4% loaded BMM (mouse 4) caused a substantial, but transient blood echogenicity enhancement of 2 dB in the common carotid artery of one mouse (Fig. 3, right panel). The above results indicate that at least part of the injected BMM do pass the pulmonary circulation and arrive on the arterial side.

Accordingly the caption of figure 3 has been modified.

2.3. Absolute results in terms of added gain by PFH-loaded BMM is not so huge (1-2 db peak according to different sites and % loading factor). Authors should report more details about mean value and standard deviation of this gain after the contrast agent infusion. Moreover, they should add details on how they obtain and analyze B-mode images when evaluating this gain. In particular, they should specify;
# if a mechanical arm was used to keep the probe fixed;
# if ECG and/or respiratory gating was used;
We intended to demonstrate that with PFH-loaded BMM it was possible to achieve in vivo site-dependent echo enhancement. For that purpose the BMM should be able to pass the (lung) microcirculation to accumulate over time at the inflamed region. For this application the actual blood echo level should be low or absent; the latter is even more desirable to have a proper delineation of activated endothelium. We only injected high doses of PFH-loaded BB into the jugular vein to have proof that the contrast material indeed appeared at the arterial site despite the high background level of the blood pool in our experiments (fig.2). We refer to this aspect on page 15, second paragraph:

“even though the background level is quite high (Fig.2)”

Under normal conditions site-specific contrast enhancement will be obtained after slow infusion of smaller doses without generating blood signal enhancement because of the distribution over the blood pool. This aspect has now been stressed in the manuscript, specifically in the Abstract and Introduction. To avoid further confusion about the goal of injecting high doses, we replaced in the Conclusions of the Abstract “become detectable” by “appear”. Also we modified the text on page 16 (Discussion):

show only for extremely high doses administered in a short time a notable

In the revised manuscript (p9) we provide more specific details about the acquisition and processing procedure. As indicated, the probe was fixed, no gating was applied and all images, acquired at a frame rate of 25 frames per second, were analyzed. This is now indicated by:

Continuous B-mode recording were made at a video frame rate of 25 Hz, covering baseline, injection and distribution over the blood pool of loaded and unloaded BMM. Ultrasound movies were processed off-line using ImagePro software (Media Cybernetics, Silverspring, MD, USA) to extract the blood echo level for all frames at a single pixel situated in the pulmonary artery, aorta or carotid artery. In a final processing stage the intensity waveform was smoothed (window 10 seconds) and converted to dB with the average baseline level as reference.
For your information below, for fig 3 right (carotid artery), we provide the raw data over 150 seconds (3750 frames). We performed this experiment in a limited number of mice for a varying number of cells and loading conditions. However, we did not perform repeated experiments, but provided data based on single observations, although the original text erroneously created the illusion that this was the case.