Author's response to reviews

**Title:** Exosome derived from epigallocatechin gallate treated breast cancer cells suppresses tumor growth by inhibiting tumor-associated macrophage infiltration and M2 polarization

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**Author's response to reviews:** see over
Reviewer's report 1

[major modifications]

1. Some and largely unfocused; the Material and Methods are incomplete in some parts (see below); the Figures details are poorly defined (see below) : checked and corrected

   Abstract : rewriting
   Material and methods : complement the contents
   Introduction : delete unnecessary contents and reference
   Discussion : delete unnecessary contents and reference

   The experiments and the methods used appear generally adequate, although some points need to be clarified, which are the following.

   1. the experiments utilizing tumor derived exosomes need clarification about the methods used (cell culture conditions, the use of exosome-depleted FCS, the controls performed on exosome purification) and how they were performed. : p8 complement the contents (p7)

   2. the second paragraph of the Results (pg11) is particularly unclear and requires rewriting : checked and rewriting

   3. (1) the experiment with the miR-16 inhibitor (fig 2) should be detailed and explained : complement the contents (p 7)

   (2) In addition, the authors should evaluate TGF#, IL6, TNF# mRNA levels in TAM treated with exosomes isolated from 4T1 cells silenced for miR-16 expression in order to demonstrate the role of miR-16 in promoting M1 polarization of macrophages. : add the Fig 4C

   4. (1) the experiments in which macrophages are stimulated with tumor-derived exosomes should be detailed in the Methods section (how TAM stimulation with exosomes is performed, specifying the number of stimulated TAM and the quantity of exosomes) : checked and rewriting (p10)

   5. the use of anti-human CD68 and CD163 antibodies to stain murine macrophages should be amended. : complement the contents (p11)

   6. to improve clarity and ease the reader, the data from the in vivo experiments shown in fig5 should be moved at the beginning and presented as fig1: Fig 5 moved to Fig.1

   7. the diagram shown in fig6 should be better schematized to improve clarity (avoid repetitions and present a linear scheme). : checked

[Minor points]

1. In Material and Methods section, information is missing on 1. antibodies used for western blot analysis (pg 9, line 2); 2. reverse primers used to amplify miR-16 and U6 (pg 9, line 2) 3. database/software tools used to predict miR-16 targets. The two paragraphs about qPCR should be merged in a single one.

2. 4T1 murine breast tumor cell line growing upon sc injection in syngeneic BALB/c mice cannot be defined a ‘xenograft’ model. : checked and corrected
3. Authors should correct M1 with M2 and vice versa in the Abstract (line 11) and in the Results section (pg 13, line 18-19). checked and corrected

4. Legends to the figures 2, 3, 4 (pg 26) and the figures should consistently indicate if TAM used as control were treated with exosomes isolated from untreated 4T1 cells, and if 4T1 cells were stimulated or not with EGCG. checked and corrected (p29-32)

5. Figures 3 and 4 are inverted. Statistical analysis is missing. checked and corrected

6. In figures 3, 4 and 5 specify that results are expressed as 2-##Ct complement the contents (p8)

7. Figure 5, what is DW should be specified. The legend in Figure 5G explaining black and white bars is missing. checked and corrected

8. In Figure 6, IL10 is indicated instead of IL6. checked and corrected

Reviewer’s report 2

1. Abstract:, section’conclusion’: the AA state that they data indicate changes from M1 polarization to M2 polarization, w/o reporting in the ‘result’ section results supporting such conclusion: checked and corrected

2. Background:
(1) The first paragraph is too long with too many references. delete unnecessary contents and reference

(2) The second paragraph introduces the concept of exosomes and microvesicles in a confusing way. There is no need to describe microvesicles as only exosomes are considered in the study. checked and corrected (p8)

(3) Third paragraph: it is not clear why among miRNAs modulated by EGCG only miR-16 is studied? What is the rational for this choice? What are the other modulated miRNAs?. If EGCG regulates also proliferation as stated why did the AA not try to distinguish between the proliferation mediated effect and the effect mediated by macrophage recruitment/polarization? Why do the AA mention that ‘nobody studied the modulation of miR-16 by steroid hormones’ Is this relevant to the issue? complement the contents (p14)/ checked and corrected

(4) Paragraph 5, line 10: The sentence starting with ‘ TNF, IL-1, the macrophage growth ‘ is not understandable. The last sentence, about prostaglandins and COX2 is not relevant. checked and corrected

3. Methods :
(1) Quantification of miR-16 by RT-qPCR. Description of the ddCt calculation method refers to genes?? It should refer to miRNAs! No details are given on the miR-16 detection in exosomes, eg normalization, starting sample input in the qPCR assay. complement the contents (p8)

(2) Why did the AA use two different protein quantification assays, BCA in the case of exosome purification and Bio-Rad assay for Western blots. complement the contents (p7)

In vivo study (3) The description given for macrophage characterization is insufficient. E.g. citrate buffer [] are not reported, pH is similarly not reported. Times, temperatures and concentrations of antibodies used are not reported. complement the contents (p7)

4. Results
(1) The 100 uM concentration of EGCG is very high. How was it chosen? did the AA run experiment with lower concentrations? The rage is not problem for exosomes preparation and change of miRNA profile by EGCG is distinct
(2) First paragraph the last sentence is not clear at all, it seems part of the sentence is missing! : checked and corrected

(3) Second paragraph ‘miR16’ inhibitor is not described, no hints can be derived from the Figure and its legends!! Treatment of macrophages with exosomes is poorly described, e.g. no info is given on the amount of exosome used for the treatment! : complement the contents

(4) Third paragraph refers to Fig 3, but seem to be inconsistent! No p values are reported to support the claim of significance. : complement the contents

(5) Paragraph 4: Figure 3 should probably be Figure 4! : checked and corrected

(6) Paragraph 5 should report the main data supporting the hypothesis, but p values are not given, number of replicates is not specified.

5. Discussion

(1) Most part of the Discussion is not pertinent to the Results. : rewriting (p17-20)

(2) Second paragraph claims that ‘a selective subset of miRNAs is present within EGCG-treated exosomes, but no data are reported beyond miR-16, and the reader is left with the curiosity on the other miRNAs. : complement the contents (p14)

(3) Paragraph 3, again is not pertinent to the Results Paragraph 4 part of the sentences are not understandable : checked and corrected

6. Conclusion

(1) the AA simplt report what they did instead of reporting what they understood from their experiments REF no. 40 is not correctly reported (given names are reported instead of surnames) : corrected (ref 40 is changed 28)

(2) Fig. 4. In the title the AA claim the experiment is done using macrophages, whiuloe in the legend they say it refers to 4T1 cells. : checked and corrected