Author's response to reviews

Title: M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide

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The figures are enclosed in the attached PDF file.

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M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide

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We would like to thank the editor and the two reviewers for their helpful comments. As suggested, new analyses were performed and the new results were incorporated in the revised version of the manuscript. All these new results confirm the previous ones. We took into account all the comments in order to make the manuscript more clear, with a more precise interpretation of the results.

A point by point response to each of the comments is detailed here under.

Reviewer n°1

This report describes mostly on the characterization and differences between M1 and M2 macrophages and authors have done an extensive investigation to show that M1 and M2 are different. However, one of the hallmarks of macrophages is that they induce formation of nitric oxide via induction of iNOS. Authors have no data on iNOS and nitric oxide production in M1 and M2 macrophages. This is important as nitric oxide has been shown to regulate Etoposide toxicity in cells (Sinha et al 2013, 2014) and has also been implicated in Taxol cytotoxicity (Heincke et al 2014) in breast cancer cells. Since authors have used Etoposide as the drug to show that M1 and M2 regulate Etoposide cytotoxicity differently, it would be interesting and necessary to show nitric oxide production differs in M1 and M2 macrophages.
Sinha et al (J Pharmacol Exp Ther, 2013) showed that inducible nitric-oxide synthase (iNOS)–expressing human melanoma A375 cells display enhanced resistance to etoposide-induced cell death. Co-culturing A375 melanoma cells with LPS-induced macrophage RAW cells, which are murine macrophages, also significantly reduced etoposide cytotoxicity and DNA damage in A375 cells. In a previous study (Sinha et al, Chem Res Toxicol, 2013), they already showed that NO/NO-derived species react with etoposide in vitro and form products that show significantly reduced activity toward HL60 cells. All together, these results showed that NO oxidative chemistry can detoxify etoposide through direct nitrogen oxide radical attack.

Heinecke et al (PNAS, 2014) used a xenograph model of green fluorescent protein expressing MDA-MB-231 breast cancer cells implanted in the mammary fat pad of female nude mice. NOS2 inhibition reduced tumor growth and metastases. In vitro, NO drives MDA-MB-231 cell migration and cell resistance to taxol-induced cell death. In this study, NO is produced by the cancer cells and the authors did not study the role of macrophages.

These different studies evidence the importance of NO in regulating the sensibility of cancer cells to different anti-cancer agents. However, it has to be mentioned that iNOS and nitric oxide production is very different in murine versus human macrophages. While it is well accepted that murine M1 macrophages produce high amounts of NO (NOS2hi ARG1low) compared to murine M2 macrophages (NOS2low ARG1hi), this feature is not observed in human macrophages (Biswas, Allavena & Mantovani, Semin Immunopathol (2013) 35:585–600).

Furthermore, in the murine macrophages, M1 macrophages produce much more NO species than M2 macrophages. If NO would be involved in our human co-culture model, it would then be M1 macrophages that would be protective and not M2, as what is observed in our work. With all these considerations, we thus propose that NO is not involved in the protective effect of M2 macrophages observed in our work. This is now mentioned in the “Discussion” section of the revised version of the manuscript.

The figures are extremely busy and I had trouble viewing some of them; this extensive characterization of M1 and M2 is not necessary (at least to this reviewer) as most of these have been previously characterized.

As suggested, the figures have been redrawn to be much clearer.

On the other hand, the extensive characterization of M1 and M2 phenotype was kept because our model is the first model of M1 and M2 derived from THP-1 cells which used concentration of LPS low enough not to induce cell death and unspecific M2 marker expression in M1 macrophages. This easy, highly reproducible and well-characterized model may be of great interest for numerous researchers. It is thus important to make this characterization available to them.
Figure 5 is not necessary and can be deleted. Parts of Figure 6 can also be deleted.

As suggested, figure 5 as such has been deleted. All the figures have been redrawn to make the article clearer. New data have also been added in the new figures.

Manuscripts needs to be edited; heat decomplemented? inactivated. Quoted? called or described as or noted? Shacked? Reference 14: what is Blut? Please spell it out.

As suggested, these errors have been corrected in the revised version of the manuscript.

"Blut" is the full name of the journal in which was published the paper of Andreesen et al. (Ref. 15). "Blut" is the German word for blood.

Reviewer n°2

Major Compulsory Revisions

The study by GENIN et al titled as “M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide” reports that human monocytic cell line THP-1 derived M1 and M2 macrophages have different modulated effects on cancer cells when treated with chemotherapeutic agent etoposide. M1 and M2 macrophages were polarized from THP-1 and characterized by several markers and specific cytokines production. They used these two polarized macrophages studying their effects on cancer cell sensitivity to etoposide. The authors claimed THP-1 M2 macrophages inhibited but THP-1 M1 macrophages accelerated etoposide-induced cancer cell apoptosis.

Although it is important to study the role of TAMs in cancer cells sensitivity to chemotherapeutic agents, I don’t think the experiments in this manuscript sufficiently support the authors’ conclusions. When characterizing M1 and M2 macrophages, they used several markers, which were not significant to show the differentiation. The western blot results showing accelerating effects of M1 macrophages in etoposide- induced apoptosis were not obvious. The inhibition of cancer cell apoptosis is also just slightly affected by M2 macrophages. More evidences to show the apoptosis are needed here.

As suggested, several other markers of M1 and M2 macrophages have been studied and all the new results confirmed previous data. They are now incorporated in the revised version of the manuscript (please see details here under, comment n°1). Similarly, several other experiments using different experimental approaches have been performed in order to confirm data regarding the effects of M2 versus M1 macrophages on the etoposide-induced
apoptosis of HepG2 and A549 cells. They are now incorporated in the revised version of the manuscript (please see details here under, comment n°3).

Some special comments for authors:

1. In Fig 1, the imaging of differentiation markers is not showing in the same scale level between before and after differentiation. The differences seemed caused by scale differences.

The magnification for the pictures displayed in Figure has been carefully checked and this was correct. Actually, macrophages have a bigger size than non-adherent monocytes, which is what is observed in Figure 1. In order to confirm these observations, micrographs taken in phase contrast microscopy are shown here under (Figure R1).

Figure R1: Micrographs of THP-1 monocytes and M0 macrophages in phase contrast microscopy taken at the same magnification.

More markers need to be detected for the differentiation, like CD14 and CD68.

As suggested, the protein level of CD14 and CD68, two markers of macrophage differentiation, has been evaluated by immuno-labeling and confocal microscopy. As expected, the expression of CD68 was increased in M0 macrophages in comparison to monocytes while the expression of CD14 decreased. These new results have been added in the revised version of the manuscript in the new Figure 1.


As suggested, additional markers to characterize THP-1 derived M1 and M2 macrophages have been studied. The expression of four different markers has been quantified by RT-qPCR.

The expression of CCR7 did not increase in M1 macrophages in comparison to M0 (Figure R2) and thus was not included in the revised version of the manuscript.

Figure R2: CCR7 mRNA expression in M1 and M2 macrophages. THP-1 macrophages were incubated with IL-4 and IL-13 (20 ng/ml each) during 24, 48 or 72 hours and mRNA expression of CCR7 was studied by RT-qPCR and normalized by RPS9 expression. Results are expressed as means ± 1 S.D. (n=3).
On the other hand, the expression of CD80 increased in M1 in comparison to M2. Since this did not reach statistical significance, the expression level of another surface marker of M1 has been quantified, HLA-DR and was shown to markedly increase in M1 macrophages in comparison to M2 macrophages. These new results have been added in the new Figure 3 of the revised version.

Similarly, the expression of the M2 marker CD163 has been assessed. The expression of CD163 was shown to significantly increase in M2 macrophages in comparison to M1 macrophages. These new results have been added in the new Figure 4 of the revised version.

In conclusion, a more complete characterization of M1 and M2 THP-1-derived macrophages is now described in the revised version of the manuscript.

2. In Fig3 A, the authors showed TNF-α and IL-1β expression levels are also high in control M0 macrophages and decreased after 24 hours. Why their expression decreased after 24 hours? The IFN-γ alone or with LPS induced IL-1β was nearly comparable with M0 macrophages. In previous work, IFN-γ or LPS can induced much higher expression of IL-1β in M1 than in M0 macrophages, which was not observed in this manuscript. The author should give explanation to this (Tjiu JW et al, J Invest Dermatol 2009).

In the manuscript, we explained that PMA, as described in the literature, could induce the expression of some pro-inflammatory cytokines like TNF-α and IL-1β. Here is the quotation from our manuscript: "TNF-α and IL-1β were expressed in control M0 macrophages, but their expression decreased after 24 hours in control medium. This could be due to PMA used for monocyte-to-macrophage differentiation, which has been described to up-regulate their expression (Park et al., 2007)".

In the article of Tiju et al (Tjiu JW et al, J Invest Dermatol 2009), the authors used a LPS concentration much higher than the one used in our experiments. Indeed, they incubated THP-1 monocytes with 200 ng/ml of LPS. Moreover, they incubated monocytes with LPS + IFN-γ in the presence of PMA. Our differentiation kinetic is longer. We are waiting that monocytes are differentiated in macrophages (24 hours with PMA + 24 hours of rest) before the incubation with the M1 polarization cytokines. We also used a smaller LPS concentration to avoid LPS cytotoxicity on monocytes. These differences could explain why IL-1β expression in M1 macrophages was similar to the one measured in M0 macrophages and why the increase in TNF-α expression was not higher.

3. In Fig6 B and D, although the cleavages of procaspase-3 and PARP-1 are slightly inhibited in cancer cells co-cultured with THP-1 M2 macrophages, the cleavages are not significantly increased in the case of THP-1 M1 macrophages. More evidences about the different effects of THP-1 M1 and M2 macrophages in etoposide-induced cancer cell apoptosis are needed here. The molecular weight
of protein also needs to be labeled.

As suggested more experiments have been performed to support the data shown in the first version of the manuscript. Three set of data are now described in the revised version of the manuscript.

- Three independent replicates for the western blot for HepG2 and A549 cells have been performed and a histogram summarizing the data has been added in the new figure 9A (HepG2) and 10A (A549), with statistical analyses. Furthermore, as suggested, the molecular weight of proteins is now displayed on the western blot analyses.

- A caspase 3/7 activity assay with triplicates has been performed for the two cell lines. The results, with statistical analyses, are shown in the new figure 9B (HepG2) and 10B (A549).

- We also performed a third experimental approach for the two cell lines, HepG2 and A549 cells: annexin V – propidium iodine staining and FACS analyses. These new results are displayed in the new 10C for A549 cells.

It has to be noted that, for HepG2 cells, the results were not interpretable since the percentage of dead cells in controls was very high (around 30%) (Figure R3). This was probably due to the long trypinization that was needed to recover the cells for FACS analyses.

Figure R3: Modulation of cancer cell apoptosis induced by etoposide by co-cultured M0, M1 and M2 macrophages. Macrophages were co-cultured in indirect contact with HepG2 cells during 16 hours before incubation with or without 50 µM etoposide during 24 hours. After the incubation, cells were trypsinized, labeled with annexin V-FITC and propidium iodine (PI) and analyzed by FACS. Results are presented in for viable cells (annexin V-/PI-), apoptotic cells (annexin V+/PI-) and necrotic cells (PI+), as means (n=3).

All these new results confirm the previous data. All together, they show that M1 macrophages increased the etoposide-induced cell death in cancer cells while M2 macrophages were protective.

4. In Fig4 C, it’s better to show CD206 expression level using histogram. It is hard to see the real positive population using dots.

As suggested, CD206 expression level is now displayed using histogram (see new figure 5).

5. In line 38, “THP-1 deried macrophages”, should be “THP-1 derived macrophages”; In line 616, “western bloting” should be “western blotting”.
As suggested, these errors have been corrected in the revised version of the manuscript.