Author's response to reviews

Title: Murine CD4+CD25- cells activated in vitro with PMA/ionomycin and anti-CD3 acquire regulatory function and ameliorate experimental colitis in vivo

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Author's response to reviews: see over
Dear Dr Danese,

Please find enclosed our revised manuscript “Murine CD4+CD25- cells activated in vitro with PMA/ionomycin and anti-CD3 acquire regulatory function and ameliorate experimental colitis in vivo” to be considered for publication in BMC Gastroenterology. The comments and questions of the reviewers have been addressed and the text of the manuscript was modified accordingly.

This paper describes original research that has not been previously published nor is considered for publication elsewhere.

All authors agreed to submission of this paper and have declared their conflict of interest and funding.

We hope that you will consider the revised version of our manuscript for publication in BMC Gastroenterology.

On behalf of the authors,
Awaiting your answer,
Yours sincerely,

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Response to reviewers:

Reviewer: Franco Scaldaferrri
Reviewer's report:

- Potentially interesting paper, assessing the T regulatory capacity of subset of T cells manipulated in vitro in the experimental murine CD45RB transfer model of colitis. Well written, clear however it displays several major limitations.

- Authors state that murine cd4+cd25- cells activated in vitro with pma/ionomycin and anti-cd3 acquire regulatory function. However, even if they are associated to a “clinical amelioration” of colitis in mice, the causality could not be related so strictly to those “supposed” transferred regulatory T cells. Those cells, in fact, have not been fully characterized in vitro, in simplified system. Do they display regulatory properties in vitro? are they able to suppress proliferation of activated T cells in a dose dependent manner? Do they inhibit T cells cytokines production upon activation?
We acknowledge that it cannot be fully excluded that the amelioration of the disease phenotype observed in the mice treated with the TregPMA cell is not partially related to other cell types present in the cell suspension injected. We are aware that injection of increased numbers of CD45RB\(^{\text{high}}\) cells, that have no suppressive capacity, have been shown to induce a partial protection in the severity of colitis (6x10\(^6\) cells, reduction of the disease in 3 out of 5 animals), due to “competition with neighboring T cells for limited resources” that limits the activation of the T cells in the lymphopenic host (Barthlott, Kassiotis and Stockinger, J Exp Med, 2003). However, in this study, we used 1.2x10\(^6\) TregPMA cells, which is considerably less (5 times lower) than the number used by Barthlott and colleagues and from the final analysis, an amelioration of the severity of the disease was observed in 10 out of 10 animals.

However, to address the concern of the reviewer that the protective effect observed \textit{in vivo} could not be strictly due to the TregPMA administered cells, we discussed this point in the discussion of the revised manuscript.

The method of activation of CD4\(^+\)CD25\(^-\) cells by PMA/ionomycin was initially established on human cells. The functionality of the TregPMA cells generated was tested \textit{in vitro} in Mixed Lymphocyte Reaction (MLR). We demonstrated that the TregPMA cells were able to suppress the proliferation of responder cells in a dose and contact dependent manner. Those data were presented as a poster at the conference (2nd International Conference on Immune Tolerance, 23\(^{\text{rd}}\)-25\(^{\text{th}}\) October 2011, Amsterdam) and patented (application no. 11160727.1). After obtaining such convincing human data we found it not necessary to perform \textit{in vitro} MLR’s and we have decided to test this technique directly in an \textit{in vivo} setup with the use of a mouse model of IBD which we have described in a manuscript.

- **Do they stay alive in mice following the transfer?**

  We agree that the \textit{in vivo} follow-up of the TregPMA cells is an interesting issue in relation with cell survival and homing. However, the study described in this manuscript was designed, in first instance, to explore the functionality of TregPMA cells \textit{in vivo}. Further analysis would be definitively considered in follow-up studies.

- **Furthermore, authors state that it is the treatment with pma/ionomycin, by modulating ion channel function, that enhances the expression of cd25 on murine cd4+cd25- cells. A control group of mice receiving cd4+cd25- cells activated only with anti-CD3 and then cultured in IL2 supplemented medium should also be included in the paper.**

The activation of CD4\(^+\)CD25\(^-\) cells with anti-CD3 followed by culture in IL-2 supplemented medium resulted in a very low viability of the cells for both murine and human cells. Furthermore, those cells poorly expressed regulatory markers. By consequence, those cells could not be used for \textit{in vivo} experiment.
We mentioned this point in the revised manuscript as it confirms the importance of the additional activation step by PMA/ionomycin.

- **Finally introduction should contain more information about stimulation of T cells with (PMA)/ionomycin and anti-CD3. Aim of the study should be stated instead of anticipating, already in the introduction, results of the study and analysis.**
  We acknowledge the comment of the reviewer and adjusted the introduction accordingly.

- **In the discussion session more room should be dedicated to compare pma/ionomycin and anti-cd3-activated murine cd4+cd25- cells to other in vitro generated regulatory T cells. Limits of the paper should also be better presented.**
  We acknowledge the comment of the reviewer and adjusted the discussion accordingly.

Reviewer: Marinos Kallikourdis

Reviewer's report:
- **In this manuscript Majowicz and coworkers elegantly show that CD4+CD25- T cells activated in vitro via PMA/ionomycin express Treg-like markers and are able to reduce the disease severity of experimental colitis. The experimental methods are well-described and the experiments follow a logical sequence and are thoroughly executed and reported. The in vivo protective effect is studied from a variety of parameters and is convincing. There is one issue with the interpretation of the results, which I outline below.**

Discretionary Revisions:
- **The manuscript does not present data examining the in vitro suppressive capacity of these cells. Whilst this could be considered not necessary, given the convincing in vivo data, presenting in vitro suppression data could substantially strengthen the conclusions drawn by the in vivo studies.**

The PMA/ionomycin activation of CD4+CD25- cells technique was firstly established on the human cells. Obtained TregPMA cells were tested in *in vitro* Mixed Lymphocyte Reaction (MLR) and they suppressed the proliferation of responder cells in a dose and contact dependent manner. This was presented on a conference and patented. After obtaining such convincing human data we found it not necessary to perform *in vitro* MLR’s and we have decided to test this technique directly in an *in vivo* setup with the use of a mouse model of IBD which we have described in a manuscript.
Minor Essential Revisions:
- The number of CD45RB\textsuperscript{low} cells injected in “negative control” animals should be stated.

The number of CD45RB\textsuperscript{low} cells injected in “negative control” was $2 \times 10^5$ cells per mouse in 100 µl of PBS. This has been added to manuscript.

Major Compulsory Revisions:
- Experimental colitis, as performed by the authors and by many of the previous reports cited, is induced by administration of $4 \times 10^5$ CD45RB\textsuperscript{hi} T cells into a lymphopenic recipient. Administration of an equal number of CD45RB\textsuperscript{low} cells can protect from colitis, as indeed the authors and others have shown. However, as shown in Barthlott, Kassiotis and Stockinger, J Exp Med (2003), injection of increased numbers ($6 \times 10^5$) of CD45RB\textsuperscript{hi} cells, that have no suppressive capacity, is sufficient to show a reduction in the severity of colitis, due to “competition with neighboring T cells for limited resources” that limits the activation of the T cells in the lymphopenic host. The authors used $1.2 \times 10^6$ TregPMA cells, which is appreciably less than the number used by Barthlott and colleagues, though still 3 times more than the number of CD45RB\textsuperscript{hi} that causes the colitis. As a consequence, it is not possible to exclude that at least part of the protective effect observed in vivo is not due to the increased number of administered cells. This caveat should be made clear in the discussion.

We acknowledge that it cannot be fully excluded that the amelioration of the disease phenotype observed in the mice treated with the TregPMA cell is not partially related to other cell types present in the cell suspension injected. We are aware that injection of increased numbers of CD45RB\textsuperscript{hi} cells, that have no suppressive capacity, have been shown to induced a partial protection ($6 \times 10^6$ cells, reduction of the disease in 3 out of 5 animals) in the severity of colitis, due to “competition with neighboring T cells for limited resources” that limits the activation of the T cells in the lymphopenic host (Barthlott, Kassiotis and Stockinger, J Exp Med, 2003). However, in this study, we used $1.2 \times 10^6$ TregPMA cells, which is considerably less (5 times lower) than the number used by Barthlott and colleagues and from the final analysis, an amelioration of the severity of the disease was observed in 10 out of 10 animals.

However, to address the concern of the reviewer that the protective effect observed \textit{in vivo} could not be strictly due to the TregPMA administered cells, we discussed this point in the discussion of the revised manuscript.