Point-by-point answers to the reviewer's concerns

We appreciate the reviewer's positive responses, including characterizing the paper as "The manuscript is sound". In the following response, we address each calling for changes, indicating where relevant corrections have been added to the body of the manuscript and their locations. For reviewers' convenience all corrections are tracked in the main text file.

We thank to all reviewers for their comments and suggestions.

Response to the comments from the Reviewer #1

Comment 1

Part of the claimed interest of the manuscript is the short-term polyclonal stimulation procedure to obtain Tregs in vitro. It should be described sufficiently in the main text, especially in order to be able to understand how it differs from previous strategies to expand Tregs (in the current version only describes the presence of stimulating additives line 199). Looking at the methods, the procedure seems actually fairly classic, relying mostly on IL-2 and anti-CD3 and anti-CD28 coated beads and TGF-beta. Either there is a trick, and the reader would like to know what it is. Or it's just the classic way, and then there should be no misleading mention of a specific protocol (line 77). Otherwise, if the culture and differentiation of Tregs is similar or identical to what has been described and validated before, the figures 1 and 2 and even figure 3 do not make much sense as they do not show any new data or findings

Response:

The main idea of the present work is the characterization of cells during their maturation from naïve CD4+ T cells. According to our knowledge this is an original protocol which allows obtaining large amounts of Tregs within 7 days. All previously described protocols, represents the attempt to multiply ex vivo differentiated Tregs isolated from peripheral blood. We perform ex vivo maturation of Tregs from naïve CD4+ T cells and this determines the novelty and originality of our work.

Comment 2:

In figure 1 cells steadily expand and remain highly viable till day 9. By contrast, in most plots of fig. 2 there are clearly more cells at day 7 (and sometimes day 1). Can the author explain this discrepancy?

Response:

At the figure 1 the amount of cells during maturation is shown while in figure 2 the proportions of cells expressing a given cell marker are presented. There is no discrepancy between this two figures.

Comment 3:

The results of figure 3 should be discussed in the context of reference 7 (Epigenetic Control of the foxp3 Locus in Regulatory T Cells), which states that TGF-beta induced Tregs in vitro show a much lower degree of demethylation compared to naturally occurring peripheral CD25+CD4+ Tregs. Ref 7 should also be discussed in the context of figure 2, as it states that in vitro generated Tregs loose Foxp3 Expression upon restimulation.

Response:

Again, in our work we used naïve CD4+ T cells to develop Tregs, while matured CD4+CD25+ Tregs were used in referenced work 7. We believe that the difference in initial cells used in our study is the reason for discordance between the results of the works.
We included the discussion of this issue to discussion section.

Comment 4:
Results about the splice variant of IKAROS are essentially descriptive and provide little information Treg biology as stated in 328. Could the author show any data, or at least explain a bit more why the changes they observe in the population of splice variants of IKAROS contribute to Treg development? Same comment applies for the data on cell cycle regulation in figure 6.

Response:
We agree with the reviewer's concern about the descriptive manner of the data about the expression of splice variant and the involvement of cell cycle genes in the development of Tregs. However, be believe that this data are rather important for the integrity of the study, which is fully descriptive. It was previously shown that IKZF family members play roles in Tregs development and function (ref 49). Definitely, the role of each splice variant in the biology of Tregs remains to be investigated and we believe that in our study we for the first time demonstrated the changes of splice variants during Tregs maturation from CD4+ T cells.

Comment 5:
- Line 427 and beyond: the text says figure 6 instead of 7.

Response:
Corrected. Thank you very much!

Comment 6:
Figure 7. The figure legends states that it shows a representative experiment of a total of 5. It is understandable for A and C, but much less for B and D, which show ratios. All the 5 experiments should be pooled here, and the stat test should be performed on the means of all 5 experiments.

Response:
This is exactly what was done. Means of 5 experiments are shown at figs 7 B and D. Figure 7 legend was modified according to this concern.

Comment 7
Results of figure 7 seem to be in contradiction with reference 7 (where the loss of Foxp2 upon restimulation in absence of TGF-beta leads to a low suppression of proliferation) . Could the authors explain why briefly?

Response:

I must notice that this manuscript was edited for proper English language, grammar, punctuation, spelling, and overall style by American Journal Experts editing service. Below is the certificate.

Once again, we thank the reviewer and the editorial office for thorough review and hope that the corrections have improved this manuscript.

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Editing Certificate

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Phenotypical and Functional Characteristics of Human Regulatory T Cells During ex vivo Maturation from CD4+ T Lymphocytes

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