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Qualitative differences in T-cell activation by dendritic cell-derived extracellular vesicle subtypes

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23 November 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, the referees appreciate the topic of your work. However, they also think that your conclusions are not sufficiently supported by the data provided. They note issues regarding the standardization of the amount of EVs used for the different experiments (referee #1-3) and think that the observed effects are only subtle (referee #1) and currently without physiological relevance (referee #1 and #3). Importantly, referee #2 and #3 think that the advance provided by your findings is not sufficient for publication in The EMBO Journal.

Given these negative opinions I am afraid I have no other choice but to return your manuscript to you with the message that we cannot offer to publish it here.
Thank you in any case for the opportunity to consider this manuscript. I am very sorry that I couldn't bring better news this time, but I hope nevertheless that you will find our referees' comments helpful.

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REFEREE REPORTS

Referee #1:

This research group has recently reported that EVs released by human MoDCs could be separated in different subgroups by differential centrifugation. The characteristics of these subgroups were distinct but overlapping, both with regard to EV size and their molecular composition. In the current manuscript, they describe that both isolated large EVs and small EVs could activate freshly isolated allogeneic activated T cells but not naïve T cells. Large EVs from immature DCs particularly supported Th2 responses, while small EVs were more effective in stimulating Th1 and Th17 responses. Large and small EVs from matured DCs, however, could induce both Th1 and Th2 responses. In general this is a thorough study. It should be noted however that a considerable percentage (almost half) of the non-stimulated DCs were already activated (Figure 6A). Furthermore, the distinction of EVs in the 2K, 10K and 100K pellets is not absolute, with overlapping sizes (Figure 1 and fig S1) and overlapping protein content (Kowal et al 2016). Therefore, cross contamination of isolated vesicles types (small versus large, from immature versus mature DCs) can be expected. Nevertheless, distinct T cell responses could be detected. Effects of contaminating EVs can be expected particularly at high EV concentrations. In most assays only a fixed (arbitrary) concentration of EVs (released by 8 x 106 DCs) was used. For above mentioned reasons it would be appropriate to show the effects of increasing concentrations of EVs in at least some key experiment(s).
The flow cytometry data on isolated EVs is impressive. However, how do the authors explain the identical scatter profiles of large EVs (2K pellet) and small EVs (100K) in fig 1D?
Fig 2C is plotted as fold induction, with the control for each experiment set at 1. For many other figures (eg fig 3A 4B 5) data are plotted as absolute values rather than as "fold induction". I do agree that the latter way of presenting has the additional advantage of showing interexperimental variation of the control values, but the figures as a whole become messy and less clear (particularly when symbols are being used multiple times and are overlapping). I would advise to replot these figures (with control values set as 1, and with SD).
Replace text at x axis of fig 2D with "EVs from number of secreting cells (x 106)
In fig4C, the effects of EVs from the 2K pellet on T cells are very similar to the effects of DCs. Does that imply that the EVs from the 2K pellet are shed plasma membrane fragments? Mature DCs may shed more plasma membrane fragments (large EV) as a consequence of spike formation.
The data from fig 4A imply that DCs are 104 times more efficient in activating T cells as compared to the EVs that they release. What does that tell us about the potential importance of these EVs in this assay?

Referee #2:

The paper describes the effect of differently sized exosomes/vesicles (2K, 10K and 100K) on T cell proliferation and differentiation. The exosomes isolated from DC cultures and purified by different centrifugation steps 2K, 10K and 100K. The larger 2K EVs induced stronger proliferation than 10 and 100K EVs. The Th polarization analyses suggest that 2K EVs induce more Th2 and less Th1 than the smaller EVs. Next, the authors perform blocking experiments with DC-SIGN and CD40 suggesting that these molecules are involved in the Th1 induction as these were also observed more in the 100K fraction as opposed to the 10K fraction. Finally, no differences between Th differentiation were observed between differently-sized vesicles isolated from DC maturation by IFNy. The authors suggest that DCs spread different T cell polarization signals via EVs.

This manuscript shows that EVs have an effect on T cell activation and differentiation. However, the comparison of the three different pellets representing different sized EVs is less clear. Especially the amount of protein added to the T cells remains unclear and should be investigated in detail, as well as the amount/number of vesicles. The involvement of CD40 and DC-SIGN in Th polarization is weak and should be investigated further, especially since no clear data on presence in 2K pellets has been described for these molecules.

Major concerns

- Pellets are obtained after centrifugation and although the authors have analysed the size of vesicles

in the pellets, the purity remains unclear. Contaminations with cell-debris or protein complexes. might affect the Th polarization. Can these vesicles be purified by cell-sorting/selection or sucrose gradient isolation?

- The authors compare pellets isolated from the same amount of cells but this fails to show what number of vesicles and proteins are added to the T cells. It is possible that the observed effects are due to higher or lower number of vesicles and therefore higher or lower amount of cell-surface co-stimulatory molecules. This needs to be investigated in detail before any conclusions can be drawn on the T cell activation and polarization capacity of different pellets.

- The proliferation and Th differences are not very strong and positive controls are lacking in the Th polarization assays to understand the effect of strong polarizing factors.
- Fig 4. The authors have investigated effect of different concentrations DCs but need to investigate the effect of different amounts of vesicles in T cell polarization and proliferation.

- No analyses of DC-SIGN and CD40 expression has been performed on 2K pellet as well as no cell-surface expression of these molecules on the different EVs is provided. This should be investigated and would support the lower expression of these molecules on 2K vesicles.

- The differences observed with the antibodies are not very convincing as the differences are minor and also not observed for all cytokines. Do the authors have an explanation for this? If these molecules are involved in Th polarization, stronger effects would be expected. What is the variation within one donor and triplicates?

Minor comments:

Show FACS analyses of the activation markers on T cells with different EVs. It is unclear how high the expression is of CD69.

Fig 3B how is the ratio calculated and are the std dev taken in account before calculation? Please provide sufficient information on the statistics' calculation.

Referee #3:

The article by Tkach et al. describes the effect of different-sized DC-derived EVs on T cell proliferation and cytokine production. Using a differential centrifugation approach, the authors isolate EVs from immature and mature moDCs. Two different subsets of EVs are analyzed: either pelleted by high-speed centrifugation (small EVs, sEVs, 100K EVs) or collected using low speed centrifugation (large EVs, lEVs, 2K and 10K). The authors show that sEVs and lEVs obtained from mature moDCs induced T cell activation and Th1 polarization. However, 2K lEVs from immature moDCs promoted Th2 differentiation. These authors have characterized previously the content of EVs produced by human moDCs using proteomic and WB assays (Kowal et al., 2016). Indeed, this manuscript is mainly an extension of that previous work.

Major comment:
The main shortcoming of this study is the lack of physiological relevance and/or a molecular mechanism underlying the effects induced by moDCs-derived EVs. First, the authors separate different types of EVs by size based on purely arbitrary criteria. Then, they incubate these differently sized vesicles with T cells. It is important to point out that such a scenario would never take place, as all these types of vesicles are likely secreted simultaneously. Therefore, the observed effects may result from an artificial setting and may not have any real physiological relevance. As it is, this type of approach would require to be demonstrated in vivo or at least in a more physiologic context. This work does not constitute a major advance in the field of the function of exosomes. It is even unclear whether such a size-based segregation provides any functional advantage in a potential ex vivo therapeutic approach. Also, the concept that DC exosomes can activate T cells and the need of a pre-activated state is not novel. Finally, the authors observe the different effects of 2K and 100K pellets, but not between 10K and 100K pellets. However, they assume that the proteomic composition of 10K EVs reflects the proteomic composition of the 2K pellet, and attribute the

differential effect of 2K and 100K pellets to this fact, which is an unsound argument. If authors want to compare the differences in protein composition between the 2K and 100K pellets, they should perform these analyses and do not presume the results to explain their other observations.

Other comments
Throughout the manuscript, the authors normalize using the number of producer cells; however, they do not demonstrate that the number of EVs added in each case is always the same. One clear example appears in Fig. 1 B, in which it is clear that the number of particles produced is very variable.

Although the authors claim that all different vesicles show variable levels of MHC-II, representative histograms showed in Fig. 1D indicate higher levels of MHC-II in sEVs 100K, compared to lEVs. In all the figures in which geometric symbols are used, it is unclear whether each symbol corresponds to one experiment. Also, it is very important to show clearly that isotype mAb does not exert any effect.

In summary, although most of the experiments performed here are technically correct, they do not have physiological relevance and are not novel enough.

Appeal 25 November 2016

Thank you for having handled our manuscript so swiftly, and for the comments of the 3 reviewers. Although we understand the reasons that led to your negative decision, we would like to discuss a few points raised by the reviewers which, hopefully, could make you reconsider our article, for a future revised version.

The major issues you raise are the absence of physiological relevance, and the insufficient advance provided by our findings.
We respectfully disagree with this latter comment. EV research performed in the past decade has invariably focused on demonstrating functions of either « exosomes », recovered by ultracentrifugation ({greater than or equal to}70,000g), or « microparticles », recovered by high-speed centrifugation (10-40,000g), or a mixture of both, or of apoptotic bodies recovered by low-speed centrifugation from dying cells. But these studies never considered that the observed functions could be also displayed by other types of EVs simultaneously released by the cells, or could be differently associated to individual EVs in the heterogeneous bulk population analyzed. Our demonstration, based on thorough quantitative comparison of different EV types, that some functions (here: T cell proliferation and polarization) are in fact shared by all EVs released by mature DCs, have important consequences for future uses of EVs in immunotherapeutic approaches. Our approach also highlights a framework that any functional study of EVs should follow in the future, and which would greatly improve advancement of the field, both at the basic science level and for clinical applications. Although definition of « novelty » and « advance » are, by essence, subjective, we like to think that the scientific goals and specificities of EMBO J, as a non-for profit and academic journal, should make it value the level of advances provided by our work.

Concerning the physiological relevance of our study, we agree with reviewers that our results do not strongly argue for a physiological involvement of EVs in T cell-dependent immune responses, since our EVs are far less efficient than the secreting cells themselves. This is in reality a general feature of all published EV studies, which always rely on massive ex vivo concentration of EVs from cell culture conditioned medium, before use in functional assays. To us, the physiological relevance of EV functions is still not clearly established in any patho/physiological system, because demonstrating it requires a refined understanding of the heterogenity of EVs and their modes of biogenesis and secretion, which is only now starting to be comprehensively explored. Therefore, our point here is not to propose a physiological relevance of the EV function observed, but to demonstrate that comparing in a quantitative manner the different EVs simultaneously released, as well as their mother cells, is a crucial information required in any EV study. In fact, our choice of normalizing EV amount by the number of cells they come from, instead of by amount of proteins or particles/vesicles, was motivated by the wish to convey a feel of the actual efficiency of EVs as compared to their mother cells. This is an important feature of our work, which we probably did not explain clearly enough in our manuscript. If we were offered to provide a revised version and a

point-by-point response for our manuscript, we would defend our reasoning, but also provide informations on protein or vesicle numbers used in our assays to answer reviewers 2-3. In addition, we would also perform functional T cell activation assays in experimental conditions closer to the physiological situation, as asked by reviewer 3 (e.g. using conditioned medium of DCs without the EV concentration step).

All the other requests and comments of the reviewers can be addressed by rewriting and further discussion, by modification of some figures, and by inclusion of additional experiments. We propose, especially, to perform additional dose-response experiments (reviewers 1-2), to quantify expression of the molecules analysed in the different EV pellets (reviewers 2-3), to eliminate potential contaminants of EV pellets by density gradient floatation (reviewer 2), and to investigate further the molecules responsible for Th1 polarizing activity (reviewer 2). Such experiments should answer your concern that our « conclusions are not sufficiently supported by the data provided ».

We hope that these explanations will make you reconsider your rejection decision. If you wish, I can call you to discuss in more details the experiments to perform for a revised version.

2nd Editorial Decision 02 December 2016

Thank you again for your correspondence regarding our decision on your manuscript and for providing a point-by-point response upfront.
I have now carefully read your outlined revision and your response to the criticisms raised, and I appreciate your approaches. I can thus offer to run a resubmission by the referees again. Please note that I cannot predict how such a revision will be received by the referees and that I will need some very good support in order to move forward here.

You can use the link below to upload your manuscript once you have finalized the revision. Please get in touch at any point in case you would like to discuss the revision further with me.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

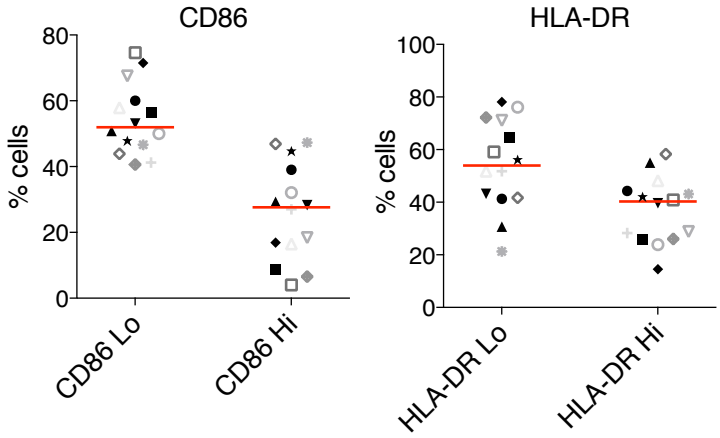
2nd Revision - authors' response 10 April 2017

Point-by-point response to the reviewers comments to EMBOJ-2016-96003, Tkach et al.

Referee #1:
This research group has recently reported that EVs released by human MoDCs could be separated in different subgroups by differential centrifugation. The characteristics of these subgroups were distinct but overlapping, both with regard to EV size and their molecular composition. In the current manuscript, they describe that both isolated large EVs and small EVs could activate freshly isolated allogeneic activated T cells but not naïve T cells. Large EVs from immature DCs particularly supported Th2 responses, while small EVs were more effective in stimulating Th1 and Th17 responses. Large and small EVs from matured DCs, however, could induce both Th1 and Th2 responses. In general this is a thorough study.
We thank reviewer 1 for his/her positive opinion on our work

It should be noted however that a considerable percentage (almost half) of the non-stimulated DCs were already activated (Figure 6A). Furthermore, the distinction of EVs in the 2K, 10K and 100K pellets is not absolute, with overlapping sizes (Figure 1 and fig S1) and overlapping protein content (Kowal et al 2016). Therefore, cross contamination of isolated vesicles types (small versus large, from immature versus mature DCs) can be expected. Nevertheless, distinct T cell responses could be detected. Effects of contaminating EVs can be expected particularly at high EV concentrations. In most assays only a fixed (arbitrary) concentration of EVs (released by 8 x 106 DCs) was used. For above mentioned reasons it would be appropriate to show the effects of increasing concentrations of EVs in at least some key experiment(s).

We agree with the reviewer that none of the pellets contain a pure population of EVs of a define size, this is clearly shown by the NTA analysis of figure 1a, in our EM-based analysis previously published (Kowal et al, 2016), and in the new SEM images provided as new figure 1C. It is true also that, depending on individual DC cultures, a variable percent of DCs become spontaneously mature, although the example shown in figure 6 is in the high range of spontaneous maturation, as observed by the high number of cells with high levels of CD86. In figure 1 for the reviewers, we show the percentage of cells with low and high levels of CD86 and MHC class II for several donors. The donor used for the original figure 6A (new figure 7A) is represented with a star.

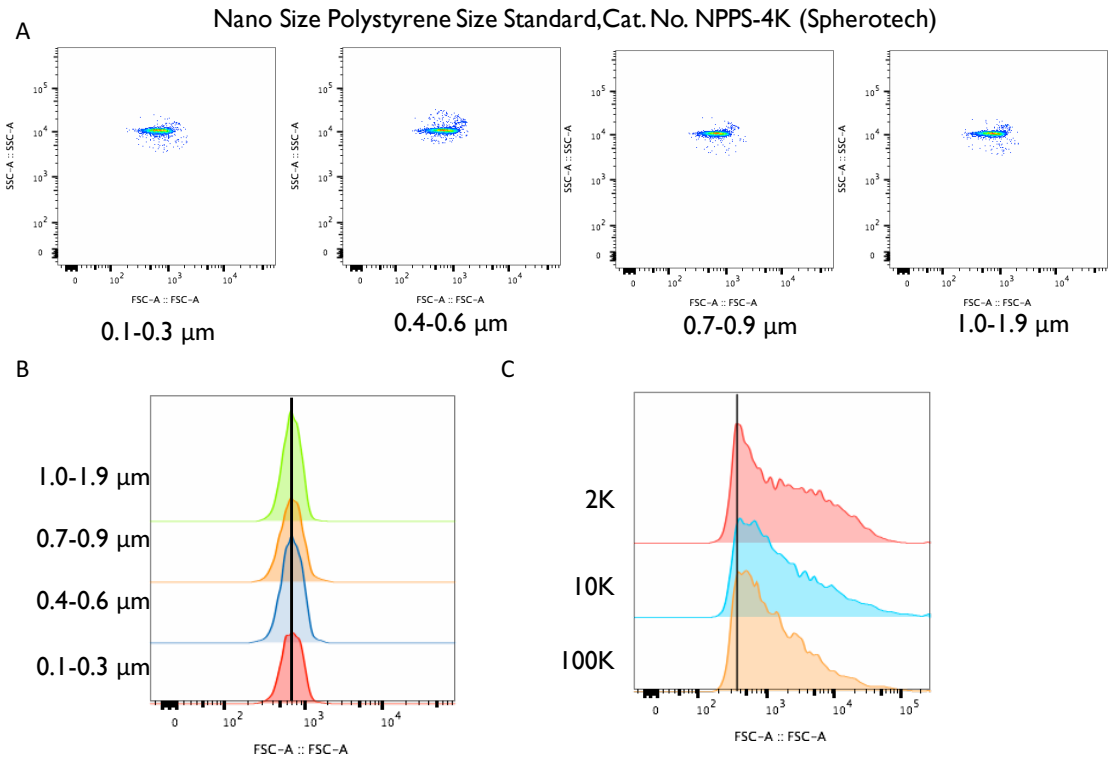


Reviewer figure 1.
Percentage of spontaneous maturation of moDCs in cell culture

The percentage of cells with low or high levels of CD86 and MHC class II is shown, corresponding to, immature and spontaneously mature DCs, respectively.

Nonetheless, as highlighted by the reviewer, we could still observe differences between the 2K and 10K-100K pellets, showing that the obtained enrichment in a given type of EVs was sufficient to allow observation of the functional differences. The fixed dose of EVs used in most of our assay had been chosen because the level of T cell proliferation achieved with this dose was comparable between the 3 pellets (figure 2D). To answer this reviewer's request, we show in the revised version the effect of four doses of EV pellets from immature DCs (1 to 8 x 10^6 cells) on induction of IFN-g and IL13 secretion (new figure 3A), and of EVs from either untreated (mostly immature) or IFN-g-matured DCs on T cell proliferation (new figure 6B). These dose-response experiments now show that the level of IFN-g, IL17 and IL13 secreted by T cells is maximal when EVs from 8x10^6 cells are used (plateau reached with EVs from, respectively, 4, 2 and 1x10^6 cells), which is consistent with the plateau of proliferation also observed (Figure 2C). We could not use higher amounts of EVs than those of 8x10^6 secreting cells, especially since the 2K pellet started inducing some cell death at higher doses (data not shown). We hope that these new figures answer the reviewer's comments.

The flow cytometry data on isolated EVs is impressive. However, how do the authors explain the identical scatter profiles of large EVs (2K pellet) and small EVs (100K) in fig 1D?
We have performed all flow cytometry analyses using the MACSQuant cytometer (Miltenyi Biotec). When analysing commercial polystyrene beads of different sizes (0.1 µm, 0.4 µm, 0.7 µm and 1 µm) we observed that all of them appear to have the same FSC and SSC profile on this cytometer, irrespective of their size (see enclosed figure 2 for the reviewer). This implies that the MACSQuant does not have enough resolution to resolve the size of the particles analysed. As specified in the text of our article, we do not interpret the flow cytometry data performed here as giving information on single EVs, but rather as another technic for bulk analysis of EVs, irrespective of their size. We have included a sentence p.6 line 164 explaining the specificity of the flow cytometer used.



Reviewer Figure 2.
Polystyrene Size Standard Beads on MACSQuant Cytometer

A-B Nano Size Polystyrene beads (Spherotech) were analysed by flow cytometry (MACSQuant). Beads were detected in a FSC/SSC in a logarithmic scale (A). Histograms show the FSC-A of the size standard beads, showing no appreciable differences among them (B).
C Histograms showing the FSC-A of the different DC-derived EV pellets (2K, 10K and 100K), showing a similar distribution among them (with the exception of the 2K which shows a higher number of events in 10^4 FSC-A intensity compared to the 10K and 100K).

Fig 2C is plotted as fold induction, with the control for each experiment set at 1. For many other figures (eg fig 3A 4B 5) data are plotted as absolute values rather than as "fold induction". I do agree that the latter way of presenting has the additional advantage of showing interexperimental variation of the control values, but the figures as a whole become messy and less clear (particularly when symbols are being used multiple times and are overlapping). I would advise to replot these figures (with control values set as 1, and with SD).
We thank the reviewer for this suggestion. We agree that some of these figures were difficult to read, and we may have mistakenly used some similar symbols for individual donors in some figures: this has now been corrected in all figures. We had decided to plot the experiments showing the quantitative values for each cytokine, in order to show their different secretion levels. As the reviewer points out, this type of representation makes it possible to evaluate the inter-experimental variation between donors, which is extremely high in this case since our assay relies on the activation of CD4+T cells in response to an histo-incompatibility, which is very variable because it depends on the mismatching between donors. Following the reviewer's suggestion, we have re-plotted most figures in the revised version to show the fold induction upon EV treatment compared to control cells: Figures 3B, 5B, 6D, 6F, but still show the actual quantitative values in the first occurrence of each type of experiment, to give an idea of the level of the response: Figures 2C-D, 3A, 5A, 5D, 7D (using different symbols for each donor once).

Replace text at x axis of fig 2D with "EVs from number of secreting cells (x 106)
Thank you for the suggestion, this is now done in all concerned figures (2D, 3A, 7D).

In fig4C, the effects of EVs from the 2K pellet on T cells are very similar to the effects of DCs. Does that imply that the EVs from the 2K pellet are shed plasma membrane fragments? Mature DCs may shed more plasma membrane fragments (large EV) as a consequence of spike formation.

The size of EVs in the 2K pellet (majority larger than 200nm, see illustration by SEM in new figure 1C and by TEM in figure extended view 1B) strongly suggests that these vesicles indeed come from the plasma membrane, rather than from intracellular multivesicular compartments, which contain mainly EVs smaller than 150 nm. Indeed, immature DCs observed by SEM present numerous large membrane ruffles, which we think are the source of these large EVs (see figure extended view 1A). We now explain more clearly this interpretation in the revised manuscript (page 5, 6, line 134-141). Moreover, we quantified by NTA the total amount of EVs in the 2K pellets of immature or IFN-g-matured DCs, and did not observe a consistent difference (new figure 7B). Interestingly, however, a higher proportion of EVs smaller than 200nm were recovered in the 2K pellets of mature DCs (Figure 7A). Notably, by SEM, a large proportion of mature DCs display a smooth surface, devoid of ruffles, thus consistent with secretion of fewer membrane-derived large EVs (new Fig EV4A). We hope this answer the reviewer's questions.

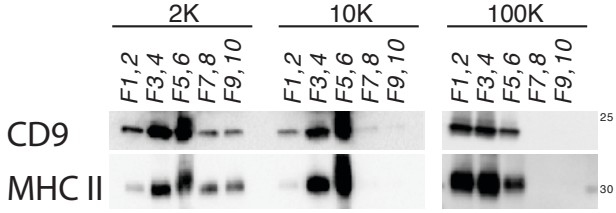
The data from fig 4A imply that DCs are 104 times more efficient in activating T cells as compared to the EVs that they release. What does that tell us about the potential importance of these EVs in this assay?
We agree with the reviewer that results presented in the previous version did not strongly argue for a physiological involvement of EVs in T cell-dependent immune responses, since isolated EVs are far less efficient than the secreting cells themselves. This is in reality a general feature of all published EV studies, which always rely on massive ex vivo concentration of EVs from cell culture conditioned medium, before use in functional assays. We suspect that the process of EV isolation is relatively inefficient, and that more EVs are actually secreted than those we succeed in isolating. To determine whether DC-derived EVs can activate T cells in more physiological conditions of DC/T cell ratio, we have now performed functional T cell activation assays upon co-culture with DCs at a distance, through transwell filters that prevent DCs from going to the T cell compartment, while allowing released EVs to reach T cells (new figure 5D). Importantly, in these conditions, 1 DC can significantly activate and polarize 1 T cell, and some level of cytokine secretion are even obtained when 8 T cells are exposed to the secretome of 1 DC, showing the physiological relevance of EV-mediated T cell activation. These experiments are now described p.11, line 300-306.

Referee #2:
The paper describes the effect of differently sized exosomes/vesicles (2K, 10K and 100K) on T cell proliferation and differentiation. The exosomes isolated from DC cultures and purified by different centrifugation steps 2K, 10K and 100K. The larger 2K EVs induced stronger proliferation than 10 and 100K EVs. The Th polarization analyses suggest that 2K EVs induce more Th2 and less Th1 than the smaller EVs. Next, the authors perform blocking experiments with DC-SIGN and CD40 suggesting that these molecules are involved in the Th1 induction as these were also observed more in the 100K fraction as opposed to the 10K fraction. Finally, no differences between Th differentiation were observed between differently-sized vesicles isolated from DC maturation by IFN-γ. The authors suggest that DCs spread different T cell polarization signals via EVs.
This manuscript shows that EVs have an effect on T cell activation and differentiation. However, the comparison of the three different pellets representing different sized EVs is less clear. Especially the amount of protein added to the T cells remains unclear and should be investigated in detail, as well as the amount/number of vesicles. The involvement of CD40 and DC-SIGN in Th polarization is weak and should be investigated further, especially since no clear data on presence in 2K pellets has been described for these molecules.
These points are developed below by the reviewers: see answers to each specific "major concern".

Major concerns

- Pellets are obtained after centrifugation and although the authors have analysed the size of vesicles in the pellets, the purity remains unclear. Contaminations with cell-debris or protein complexes might affect the Th polarization. Can these vesicles be purified by cell-sorting/selection or sucrose gradient isolation?
We are aware that cell debris and protein aggregates may be present in our EV preparations. Note that we cannot perform immuno-isolation for functional assays as suggested by the reviewer, since we do not know how to separate EVs from immuno-precipitating beads and/or antibodies without affecting their integrity. Thus, to eliminate potential contaminants of EV pellets we have

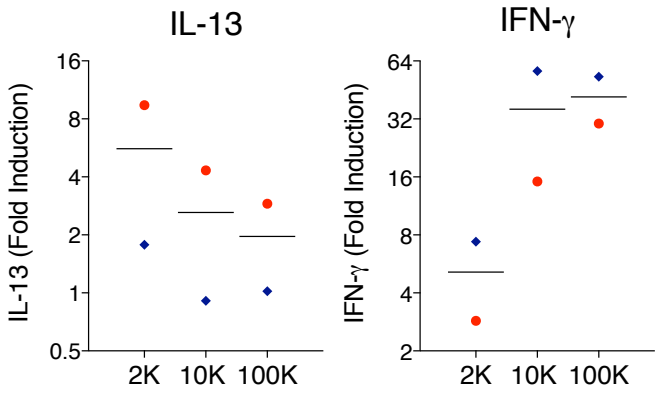
performed density gradient floatation of the three EV-containing pellets in iodixanol, as previously done in our group (Kowal et al. et al, 2016) and analysed the fractions in our functional assays: these results are shown in new figure 4. As shown in figure 4B-C the majority of the vesicles containing detectable levels of MHC class II by WB floated in the top three fractions of the density gradient (1.08-1.14 g/ml). We have observed that these three fractions were responsible for the majority of the IFN-g-induction in CD4+T cells. Interestingly, IL-13 was induced by all the 2K fractions of the density gradient, indicating that both fractions containing MHCII+ vesicles and other fractions can have an activity on CD4+T cells. The densest fractions (1.17-1.19 g/ml), were most efficient at inducing IL-13 secretion. With this separation technique, which is indicated for small vesicles, very large vesicles probably remain in the denser fractions. Indeed, when the same protocol was used by our colleagues (Hivroz et al, 2017) to separate intracellular vesicles, it showed the presence of several transmembrane proteins in fractions [7-10] (TGN46, TfR, CD45), which therefore also contain membrane-enclosed vesicles. In addition, we did detect some MHC II molecules in these fractions of 2K pellets in some DC preparations (Fig 4C, 2K panel, figure below for reviewers). We speculate that the number of MHC II on these big vesicles falls below the detection limit of WB, but is sufficient to activate T cells in conjunction with other yet unidentified components. These considerations are now included in the results section p10 lines 282-285, and in the discussion p16, lines 463-469.



Reviewer Figure 3: example of iodixanol separation of 2K pellets showing MHC-II and CD9 in the densest fractions.

- The authors compare pellets isolated from the same amount of cells but this fails to show what number of vesicles and proteins are added to the T cells. It is possible that the observed effects are due to higher or lower number of vesicles and therefore higher or lower amount of cell-surface co-stimulatory molecules. This needs to be investigated in detail before any conclusions can be drawn on the T cell activation and polarization capacity of different pellets.

As shown in figure 1B of this manuscript, and figure 1C of our previous article (Kowal et al. et al, 2016), when comparing the different pellets recovered by a single cell type in terms of total protein or vesicle number, we observe generally higher amounts of proteins and lower numbers of EVs in the 2K than the 100K pellet. Thus, if we chose to normalize our functional tests by amount of proteins given to the T cells, we would have to use a larger amount of the 100K pellet, whereas if we were normalizing to the total number of vesicles, we would have to use a larger amount of the 2K pellet. In both cases, interpreting the data in terms of relative efficacy of the pellets would potentially lead to contradictory conclusions! Therefore, here we chose to normalize EV amount by the number of cells they come from, i.e. same volume of conditioned medium, because 1) this reflects the physiological situation of a T cell encountering DC-derived EVs in their original respective proportion at the time of secretion, and 2) this setting allows to convey a feel of the actual efficiency of EVs as compared to their mother cells. This is an important feature of our work, which we probably did not explain clearly enough in our manuscript. We now explain our reasoning in the results section of the manuscript (see p. 7, line 179-188), in addition to providing information on average protein or vesicle numbers used in our assays (new table 1, showing particle number and protein amount of each pellet, according to secreting DC numbers). Of note, when analysing the cytokine secretion induced by different doses of our pellets (fig 3A), we have observed that lower amounts of the 2K (i.e. released by 2-4 million cells), with a comparable protein content of our 4-8 million cell-dose of the 10K and 100K (table 1) failed to promote the release of IFN-g On the contrary, a lower number of particles of the 100K pellet (i.e. released by 2-4 million cells) comparable to the highest doses of the 2K (4-8 million cells) were not able to promote IL-13 secretion. Moreover, to answer directly this reviewer's concern, we have cultured CD4+T cells with 5 ug of EVs of each pellet and observed that the 2K capacity to produce IL-13 as compared to the 10K and 100K pellet was retained, as well as the IFN-g induction upon 10K and 100K treatment (Reviewer figure 4).



Reviewer Figure 4.
Analysis of IL-13 and IFN-*g* release upon 6 d culture of 5 ug of the 2K, 10K and 100K pellet of DC-derived EVs with $CD4^+$ T cells.

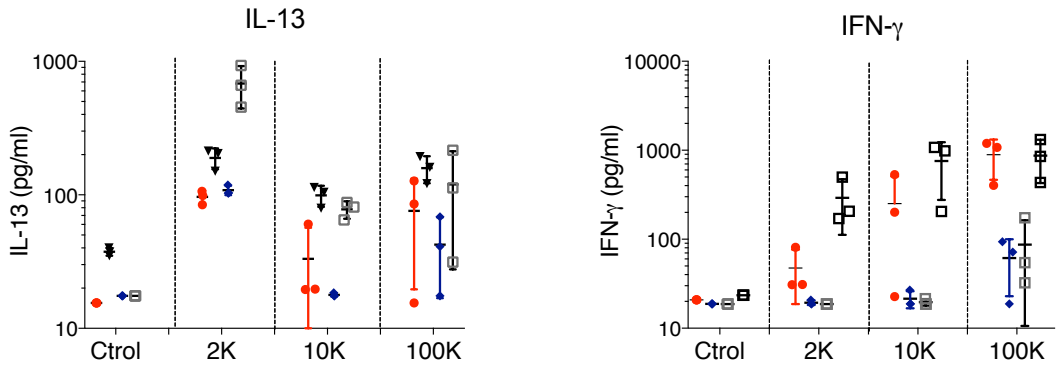
- The proliferation and Th differences are not very strong and positive controls are lacking in the Th polarization assays to understand the effect of strong polarizing factors.
We agree that the proliferation induced by EVs is not very strong, as compared to that induced by the mother DCs, but the level of cytokine secretion and of Th polarization observed are in the same range as those obtained from DCs in similar conditions of proliferation (former figure 4, now figure 5). We think that this figure 5 contains the relevant controls to our EV analysis. In our hands, DCs cultured in strong Th1 (LPS) or Th2 (R848) polarizing signals induced secretion of, respectively IFN-g and IL13 by T cells, in the same range as what we observed with EVs, when similar proliferation were induced (100-1000 pg/ml, data not shown). Assays used to show strong Th polarization involve re-culture of T cells after pre-priming in the presence of a strong TCR stimulus (anti-CD3/CD28), which then leads to much higher levels of cytokine secretion. When we performed such assays, the difference in Th polarization observed by first priming with EVs was not observed anymore after strong TCR activation. We do not think that such an experimental setting adds any information to our question, which was not to compare EVs to a strong Th1 or Th2 polarization signal, but to unravel the functional heterogeneity of EVs.

- Fig 4. The authors have investigated effect of different concentrations DCs but need to investigate the effect of different amounts of vesicles in T cell polarization and proliferation.
To answer this reviewer's and reviewer1's request, we have now included a dose response curve for IL-13, IFN-γ and IL-17 secretion upon induction by the EV-containing pellets (new figure 3A), and for induction of T cell proliferation by EVs from either untreated (mostly immature) or IFN-γ-matured DCs (former figure 6B, now figure 7D).

- No analyses of DC-SIGN and CD40 expression has been performed on 2K pellet as well as no cell-surface expression of these molecules on the different EVs is provided. This should be investigated and would support the lower expression of these molecules on 2K vesicles.
We agree with this reviewer, and we are now providing in the revised version information on amount of CD40 and DC-SIGN in the different EV pellets, analyzed by Western blotting (new Figure 6A and B): this analysis confirms that both DC-SIGN and CD40 are particularly enriched in the 100K pellets. This will also answer a request from reviewer 3.

- The differences observed with the antibodies are not very convincing as the differences are minor and also not observed for all cytokines. Do the authors have an explanation for this? If these molecules are involved in Th polarization, stronger effects would be expected. What is the variation within one donor and triplicates?
We agree that anti-CD40L and DC-SIGN antibodies only decrease, rather than abolish, the IFN-g-inducing activity of sEVs. Other molecules are thus probably involved, but we have not identified them here. We discuss a few, like mechanical properties, or density of MHCII, in the discussion, p.18, lines 509-519. We have clearly interpreted these data as showing a partial effect: p.4 line 107 ("we could assign part of the th1-promoting activity") and p12 lines 343-345 ("both partially explain their ability to polarize").

The strong variability of the Mixed Lymphocyte Reaction assay used here, due to the strongly variable histo-incompatibility of each DC and T cell donor, combined with the variability of relative proportion of large vs small EVs, and/or of CD40 and DC-SIGN expression on EVs secreted by each individual DC culture explain largely the apparent minor differences induced by the antibodies. We are afraid that our results are due to inherent biological variability of the assay. Due to this variability, we normally do not perform technical replicates for each individual EV sample, but instead we perform 2 biological replicates (see answer to minor point 2 below). In figure 5 for the reviewer are shown the results of several experiments using technical replicates (one EV donor with one T cell donor, in triplicate in different colors), to show the variability of the assay. This can be due to the fact that the response observed is due to an histo-incompatibility and the rate of response relies on the level of miss-match between the donors, and that the low number of responsive T cells (expected to be 2-5% of total CD4+T cells) results in potential variable number in the well. We overcome this problem by performing the assays using several DC-T cell combinations, as independent biological replicates.



Reviewer figure 5.
Technical triplicates for the culture of DC-derived EVs with total CD4^{+}T cells. Each color represents one EV donor with one T cell donor combination.

Minor comments:
Show FACS analyses of the activation markers on T cells with different EVs. It is unclear how high the expression is of CD69.
We now show the absolute value of % CD69-expressing cells in the experiments used to generate figure 2C (instead of the fold induction). We observed that around 2-3% of the total T cells are being activated in our system, which is in accordance to the number of expected reactive T cells upon an alloreaction (Suchin *et al,* **2001**).

Fig 3B how is the ratio calculated and are the std dev taken in account before calculation? Please provide sufficient information on the statistics' calculation.
Ratio of IL13 to IFN-g secretion was calculated for each individual DC-EV donor as the ratio of absolute pg of the two cytokines secreted by a given T cell donor exposed to these EVs. Each independent biological replicate (i.e. individual DC-EV donor) was used on T cells from two different donors, providing two biological replicates. No technical replicates were performed, since the high variability of the MLR reactions analysed here is strongly dependent on the histo-incompatibility of the DC/T cell combination, and thus, giving the limited amount of EVs we prioritized to perform biological replicates. Standard deviations are thus calculated from the represented individual biological replicates in figure 3B. This is now explained better in the revised manuscript (Figure legend 3B and C, page 35).

Referee #3:
The article by Tkach et al. describes the effect of different-sized DC-derived EVs on T cell proliferation and cytokine production. Using a differential centrifugation approach, the authors isolate EVs from immature and mature moDCs. Two different subsets of EVs are analyzed: either pelleted by high-speed centrifugation (small EVs, sEVs, 100K EVs) or collected using low speed centrifugation (large EVs, lEVs, 2K and 10K). The authors show that sEVs and lEVs obtained from mature moDCs induced T cell activation and Th1 polarization. However, 2K lEVs from immature

moDCs promoted Th2 differentiation. These authors have characterized previously the content of EVs produced by human moDCs using proteomic and WB assays (Kowal et al., 2016). Indeed, this manuscript is mainly an extension of that previous work.
Although the current manuscript indeed follows up on previous results published in our Kowal 2016 article, we respectfully disagree with this reviewer about the latter sentence: only protein composition and biochemical properties of the EV subtypes were analysed in the previous article, and no hints on the actual functions of the different EVs had been provided. More detailed answers are provided to the specific points raised below.

Major comment:
The main shortcoming of this study is the lack of physiological relevance and/or a molecular mechanism underlying the effects induced by moDCs-derived EVs.
The manuscript does provide some molecular mechanisms explaining, at least partially, the effect of the 100K pellet (DC-SIGN and CD40, former fig5, now figure 6).
See detailed responses to the comment on physiological relevance below

First, the authors separate different types of EVs by size based on purely arbitrary criteria.
We agree that separation of EVs by size is somehow arbitrary, but not "purely" arbitrary. Our rational is as follows: EVs formed inside endocytic multivesicular compartments (often called exosomes) must be of a similar size as intraluminal vesicles of these compartments, i.e. smaller than 150 nm in diameter (as observed in most EM pictures available in the literature, and in our own hands). Thus, EVs larger than 150 nm, which constitute more than 50% of the 2K pellet (figure 1A, new figure 1C, and figure 1 of Kowal et al, 2016), are most likely non-exosomal vesicles. This is now more clearly explained in the revised version: introduction p3, results p5-6, line 133-142.

Then, they incubate these differently sized vesicles with T cells. It is important to point out that such a scenario would never take place, as all these types of vesicles are likely secreted simultaneously. Therefore, the observed effects may result from an artificial setting and may not have any real physiological relevance. As it is, this type of approach would require to be demonstrated in vivo or at least in a more physiologic context.
Of course, in a physiological context, T cells would encounter simultaneously large and small EVs secreted by a DC: this consideration is the basis of our choice to normalize our comparative functional assays of 2K-10K-100K pellets by the number of secreting cells, and not by total number of vesicles or of proteins (see detailed explanations below). Since our goal was to determine whether these different EVs displayed different functions, the logical experimental process was to analyze their function separately, which we did here, and which allowed to demonstrate the differential Th polarization ability. This is obviously a reductionist approach, but necessary to answer the question asked.
To tackle the question of the physiological relevance, which was also a question of reviewer 1, we have now included a new figure 5D, where we have analyzed the effect of EV secretion without performing the "artefactual" process of separation/concentration of EVs before feeding to the T cells. For this, we have performed a co-culture of DCs and T cells through a transwell, allowing passage of EVs (and soluble cytokines), but preventing direct contact of the APC and T cells. We thus show that, at a DC:T cell ratio of 1:1 and even 1:8, i.e. conditions that could be found in a lymphoid tissue, T cells can be activated. Furthermore, they are polarized to both Th1 and Th2, as expected since both lEVs and sEVs are secreted, although Th2 polarization is less strong than when using isolated 2K pellets, and even less so when pore-size preventing passage of large EVs (0.4 um) is used. We believe that these novel results demonstrate that DC-derived EVs can activate and polarize T cells in conditions closer to the physiological situation, although we are aware that these results are purely obtained in vitro, which is an inherent limitation of human experimental systems.

This work does not constitute a major advance in the field of the function of exosomes. It is even unclear whether such a size-based segregation provides any functional advantage in a potential ex vivo therapeutic approach. Also, the concept that DC exosomes can activate T cells and the need of a pre-activated state is not novel.
Indeed, since we had previously demonstrated, in a mouse experimental system, that "exosomes" could only activate directly pre-activated T cells, confirmation of this observation in the current study is not claimed as novel. As stated in the discussion, p14 line 395-397 "However, our work is

the first to disclose the effect of human derived sEVs on primary $CD4^+$ T cells and to compare it to the effect induced by all the different EVs secreted by human DCs.".

We would like to explain better the type of advance our results provide. One major consequence of our work is the demonstration that comparing in a quantitative manner the different EVs simultaneously released, as well as their mother cells, is a crucial information required in any EV study.

EV research performed in the past decade (including by our group) has invariably focused on demonstrating functions of either « exosomes », recovered by ultracentrifugation (≥70,000g), or « microparticles », recovered by high-speed centrifugation (10-40,000g), or a mixture of both, or of apoptotic bodies recovered by low-speed centrifugation from dying cells. But these studies never considered that the observed functions could be also displayed by other types of EVs simultaneously released by the cells, or could be differently associated to individual EVs in the heterogeneous bulk population analysed. This question is clearly exposed in the introduction of our manuscript, line 71-77, page 3, 4. Our demonstration, based on thorough quantitative comparison of different EV types, that some functions (here: T cell proliferation and polarization) are in fact shared by all EVs released by mature DCs, has important consequences for future uses of EVs in immunotherapeutic approaches. Indeed, as proposed in the discussion of our manuscript, p15 lines 422-425, for some clinical approaches it could be more efficient to use a mixture of large and small EVs, rather than perform a complicated process of "exosome" isolation, to facilitate obtention of large amounts of injectable clinical materials. For other clinical goals, however, it is possible that, on the contrary, thoroughly isolating a given EV subtype may improve its therapeutic efficacy. This possibility is in fact suggested by the novel results provided in new figure 5B, where, to our surprise, we observed that the most efficient Th1-inducing sEV subtype was actually not the one containing exosomes (as defined in our previous work: (Kowal et al. et al, 2016)), but the less abundant subpopulation floating at 1.14 g/ml. Our approach thus highlights a framework that any functional study of EVs should follow in the future, and which would greatly improve advancement of the field, both at the basic science level and for clinical applications.

Finally, the authors observe the different effects of 2K and 100K pellets, but not between 10K and 100K pellets. However, they assume that the proteomic composition of 10K EVs reflects the proteomic composition of the 2K pellet, and attribute the differential effect of 2K and 100K pellets to this fact, which is an unsound argument. If authors want to compare the differences in protein composition between the 2K and 100K pellets, they should perform these analyses and do not presume the results to explain their other observations.

We agree with this reviewer, and this comment is similar to reviewer 2's comment above. In the revised manuscript, we now provide information on amount of CD40 and DC-SIGN in the different EV pellets, analyzed by Western blotting, which confirms their major enrichment in the 100K sEV pellet, and low abundance in the 2K lEV-enriched pellet (new figure 6A).

Other comments

Throughout the manuscript, the authors normalize using the number of producer cells; however, they do not demonstrate that the number of EVs added in each case is always the same. One clear example appears in Fig. 1 B, in which it is clear that the number of particles produced is very variable.

As pointed by this reviewer, the number of EVs added from the 2K or the 100K pellets are not necessarily the same, because cells do not secrete equal amounts of each EV type. But as stressed in the first major comment of this reviewer, "all these types of vesicles are likely secreted simultaneously***". Consequently, to match as much as possible a physiological situation of T cell encountering the EVs secreted by a given cell, we chose to normalize EV amount by the number of cells they come from, instead of by a total number of EVs, or a total amount of proteins. Indeed, as shown in figure 1B of this manuscript, and figure 1C of our previous article (Kowal et al. et al, 2016), and new table 1, when comparing the different pellets recovered by a single cell type, we observe generally higher amounts of proteins and lower number of vesicles in the 2K than the 100K pellet. Thus, if we chose to normalize our functional tests by amount of proteins given to the T cells, we would have to use a larger amount of the 100K pellet, whereas if we were normalizing to the total number of vesicles, we would have to use a larger amount of the 2K pellet. In both cases, interpreting the data in terms of relative efficacy of the pellets would***

potentially lead to contradictory conclusions! Therefore, here we chose to normalize EV amount by the number of cells they come from, because 1) this reflects the physiological situation of a T cell encountering EVs coming from a DC in their original respective proportion, and 2) this setting allows to convey a feel of the actual efficiency of EVs as compared to their mother cells. This is an important feature of our work, which we probably did not explain clearly enough in the previous version of our manuscript. We have now explained clearly our rational in the results section, p7 line 179-188, in addition to providing information on average protein and vesicle numbers used in our assays (new table 1).

Although the authors claim that all different vesicles show variable levels of MHC-II, representative histograms showed in Fig. 1D indicate higher levels of MHC-II in sEVs 100K, compared to lEVs.
Quantification of several individual donors is shown next to the example histograms of one given donor in figure 1E (former Fig 1D), confirming our claim that the level of MHC-II is variable in the different pellets. However, as justly noted by this reviewer, the 100K pellet is slightly enriched in HLA-DR, as compared to the 2K: this is now clearly specified, p. 6 line 170.

In all the figures in which geometric symbols are used, it is unclear whether each symbol corresponds to one experiment.
We apologize for having omitted to clearly specify in each figure legend that indeed each symbol corresponds to an individual EV-donor/T cell combination (biological replicate). This has been corrected in the revised version.

Also, it is very important to show clearly that isotype mAb does not exert any effect.
We now show, in figure 6D,6F, the levels of cytokine secretion by Th cells exposed to EVs in the presence of EV pellets and isotype mAbs, as compared to those obtained with EV pellets alone. In fact, presence of isotype mAbs tends to decrease the overall level of cytokines, suggesting some unspecific inhibitory activity, possibly due to binding of IgG the surface of either T cells or EVs, leading to steric hindrance for cognate binding of EV MHCII to T cells TCR. Despite this non-specific decrease, the anti-CD40 and anti-DC-SIGN antibodies both induce significant further decrease of Th1 polarization, as compared to the isotype controls (former figure 5, new figure 6). To simplify this figure, which was becoming very dense, we chose to show only IL13 induction by the 2K and IFNg induction by the 10K and 100K pellets, instead of the whole set of data. If reviewers and editors find it necessary, we can re-include these data as they were shown in former figure 5B,D.

In summary, although most of the experiments performed here are technically correct, they do not have physiological relevance and are not novel enough.
We thank the reviewer for acknowledging the technical quality of our work, and we hope that our explanations above answer the concerns about novelty and physiological relevance.

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Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, Dingli F, Loew D, Tkach M, Théry C (2016) Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proceedings of the National Academy of Sciences* 113: E968-E977

Suchin EJ, Langmuir PB, Palmer E, Sayegh MH, Wells AD, Turka LA (2001) Quantifying the Frequency of Alloreactive T Cells In Vivo: New Answers to an Old Question. *The Journal of Immunology* 166: 973-981

3rd Editorial Decision 11 May 2017

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. It had been seen by three referees whose comments are shown below again. Thank you also for already outlining to me during our pre-decision consultation how you would address the remaining concerns.

Referees #2 and #3 still raise strong concerns, and as I told you before, based on these reports I have not sufficient support from the referees to move forward with your manuscript here. Referee #3 kindly commented on the issues raised by referee #2 and thinks that it is crucial to address point 3 of referee #2 (physiological relevance for the fact that the differential polarizing effect is not seen upon DC maturation). Furthermore, as also pointed out in the report of referee #3, point 6 would need to be addressed (current weak evidence about molecules involved in Th1-polarizing activity by 100K EVs of immature DCs; and a lack of evidence on molecules underlying the Th2-polarizing effect of 2K EVs).

During pre-decision consultation you indicated that you will be most likely able to address the latter concern by adding some more mechanism in a further revision. You also clarified that the analyses using distinct EVs to induce Th polarization were meant to be rather proof-of-principle studies, illustrating that it is important to consider that different EV populations can have distinct effects.

Given your input, I have now discussed your work again within our team. I also asked referee #3 whether s/he would in principle be willing to re-review a version of your work that is revised as you outlined.
We concluded that we can offer to consider a further revised version, and I would thus like to ask you to address the remaining issues as outlined and to provide a point-by-point response to the referee concerns. Please note that the potential physiological significance of your findings should be made very clear, and I thus encourage you to re-write your manuscript and to consider (as you suggested yourself) a different title.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

This manuscript has been considerably revised and improved as compared to its first submission, and all of my previous concerns have been addressed adequately. The potential of extracellular vesicles in intercellular communication has drawn tremendous interest during the last decade in nearly all research areas of live sciences, accumulating in > 5.000 scientific publications to date on this topic. Certainly, the group of Thery has been at the forefront of demonstrating the importance of extracellular vesicles in immune regulation. The current work is an important extension of the work of her group, with the first demonstration that cells can release distinct classes of extracellular vesicles with different functions. This work not only bears significance for immunologists, but may also contribute to setting the stage for those researches that are studying the functions of extracellular vesicles from other cell types. I therefore fully support publication in EMBO J.

Referee #2:

The authors have performed several experiments to address the concerns. However, still important concerns remains. The selection of EVs remains unclear as it is based on an arbitrary method and this is also true for the decision to use EVs produced by DCs and not a specific amount/vesicles. The latter concern is addressed by a titration of vesicles but it remains unclear how many vesicles are compared in 2K to 100k EVs. The role for EVs in Th polarization by immature DCs (and role for DC-SIGN and CD40) is not convincing and the finding that this disappears after maturation is a major concern.

Major concern
1. Contaminations are still a problem and not addressed by the subfractionation. Data in Fig 4D are unclear.

2. The authors have partially addressed my concern, however, comparison between fractions based on the amount secreted remains rather non-specific as different concentrations are used of the EVs. The reviewer figure (how is the fold calculated?) does alleviate some of my concerns but this is only once example of two donors and the variation is rather large.
3. The controls concerning strong Th1 and Th2 polarizing conditions are lacking. The polarizing effect is compared with immature DCs alone (under normal conditions very bad inducers of polarization) , which is unusual in Th assays where Th polarizing PAMPs are usually taken along to determine whether the donors are indeed able to induce strong polarization. Intracellular Th stainings often help also determining the polarization efficiency. It is also a concern that re-stimulation of T cells which should only enhance differences negates the effect observed with EV priming.
4. The addition of a dose response curve shows that the effect for cytokines is indeed not dependent on the amount of EVs (within the range of 8 times more DCs).
5. This point has been addressed.
6. The authors provide a discussion why the antibodies are not efficient in inhibiting or affecting Th polarization. However, the data remain unconvincing for a role for CD40 and DC-SIGN. The problem remains that this could indeed be other molecules or contaminations (see 1).
With regard to the technical replicates, the differences are quite large and therefore it is necessary to include technical replicates in the assays. I am unclear why histo-incompatibility would affect the technical replicates, especially as 2 K from one donor has low variability whereas same donor 10 and 100K have quite extensive variability.

I have not addressed this point beforebut now it becomes more important with point 3. The authors use immature DC in their Th assays. Immature DCs are very bad inducers of polarization and this is also not physiological. Mature DCs induce T cell proliferation and polarization but immature DCs are thought to be less important. Thus, the importance of the EVs derived from immature DCs in inducing Th polarization is unclear. The difference between different EVs disappears upon maturation of the DCs. This should be explained.

Referee #3:

This study is technically well controlled and well performed, but it remains largely descriptive. The authors need to provide additional molecular insight into the different functional behaviour found with Th2-polarizing lEVs.
In the revised manuscript, the authors have addressed satisfactorily part of my main concerns and queries, including clarifications and additional experimentation.
The authors should address an additional issue to complete the picture of the molecular mechanism of Th1/2-polarizing effects of differently sized EVs. While the Th1 polarizing activity of MoDC-derived sEVs (100K) may be partly attributable to CD40 and DC-SIGN molecules, the authors need to identify the molecules underlying the Th2-polarizing effect of lEVs (2K), or at least provide an inkling of the molecular mechanism.

3rd Revision - authors' response 20 July 2017

Point by point response for Tkach et al, EMBOJ-2016-96003R1

Editor's comments

Referees #2 and #3 still raise strong concerns, and as I told you before, based on these reports I have not sufficient support from the referees to move forward with your manuscript here. Referee #3 kindly commented on the issues raised by referee #2 and thinks that it is crucial to address point 3 of referee #2 (physiological relevance for the fact that the differential polarizing effect is not seen upon DC maturation). Furthermore, as also pointed out in the report of referee #3, point 6 would need to be addressed (current weak evidence about molecules involved in Th1-polarizing activity by 100K EVs of immature DCs; and a lack of evidence on molecules underlying the Th2-polarizing effect of 2K EVs).

During pre-decision consultation you indicated that you will be most likely able to address the latter concern by adding some more mechanism in a further revision. You also clarified that the analyses using distinct EVs to induce Th polarization were meant to be rather proof-of-principle studies, illustrating that it is important to consider that different EV populations can have distinct effects.

Given your input, I have now discussed your work again within our team. I also asked referee #3 whether s/he would in principle be willing to re-review a version of your work that is revised as you outlined.
We concluded that we can offer to consider a further revised version, and I would thus like to ask you to address the remaining issues as outlined and to provide a point-by-point response to the referee concerns. Please note that the potential physiological significance of your findings should be made very clear, and I thus encourage you to re-write your manuscript and to consider (as you suggested yourself) a different title.

We thank you for giving us the opportunity to prepare a revised version of our manuscript, to address the last concerns of reviewers 2 and 3.

For the molecules underlying the Th2-polarizing effect of 2K EVs, we now provide several new panels in Figure 6, highlighting CD80 as one such molecule. Indeed, as opposed to CD40 and DC-SIGN which are clearly enriched in the 100K EVs, CD80 is present in the 2K pellets, and a blocking anti-CD80 antibody decreases secretion of IL-13 by CD4+ T cells exposed to 2K pellets, without affecting IL-13 secretion induced by the 10K and 100K pellets (new Figure 6H-L), nor IFN-g secretion induced by any type of EV pellets (new Figure EV4F). As shown in Figure 6G, we identified CD80 by a mini-screen analysing the effect in our IL-13/IFN-g secretion assay of blocking antibodies to several molecules known to be involved in DC-T cell interaction or T cell activation. Whereas several antibodies involved in T cell activation in general diminished both secretions, anti-CD80 was the only antibody inhibiting specifically the 2K-mediated IL-13-secretion. We think that these new results do provide the requested information on the molecular mechanisms that work in EV-induced T cell activation.

Concerning the physiological relevance of our observations, as discussed with you during the pre-decision consultation, we have now changed the title of our manuscript, and amended sentences in the abstract, results and discussion, to more clearly convey that the major message is not on the physiological implications of our observations, but rather on the proof-of-principle of the necessity to take into account the diversity of EVs in any functional studies. The T cell-polarization assay and the qualitative information it provides is principally used here as a read-out of this functional diversity. To preclude any overinterpretation on the physiological implications of our results, we now avoided as much as possible to use the terms “T cell - Th1/Th2 polarization”.
However, as it is still interesting to speculate on the physiological implications of our observations, we have also included at the end of the discussion a short paragraph proposing implications of the functional differences observed between EVs secreted by immature versus mature DCs. Indeed, the observation that all EVs secreted by matured DCs can promote Th1 polarization, whereas different EVs secreted by immature DCs have different T cell-orientation capacities, suggest that mature DCs could not only activate and polarize T cells by direct contact, but also via all their secreted EVs, while immature DCs would send contradictory messages via their EVs, resulting in inefficient T cell polarization.

We hope that you will find that this revised version does address the major remaining concerns on the previous version.

Referee #1:

This manuscript has been considerably revised and improved as compared to its first submission, and all of my previous concerns have been addressed adequately. The potential of extracellular vesicles in intercellular communication has drawn tremendous interest during the last decade in nearly all research areas of live sciences, accumulating in > 5.000 scientific publications to date on this topic. Certainly, the group of Thery has been at the forefront of demonstrating the importance of extracellular vesicles in immune regulation. The current

work is an important extension of the work of her group, with the first demonstration that cells can release distinct classes of extracellular vesicles with different functions. This work not only bears significance for immunologists, but may also contribute to setting the stage for those researches that are studying the functions of extracellular vesicles from other cell types. I therefore fully support publication in EMBO J.
We thank this reviewer for his/her very positive evaluation of our work, and for highlighting the importance of our results and experimental approach.

Referee #2:

The authors have performed several experiments to address the concerns. However, still important concerns remains. The selection of EVs remains unclear as it is based on an arbitrary method and this is also true for the decision to use EVs produced by DCs and not a specific amount/vesicles. The latter concern is addressed by a titration of vesicles but it remains unclear how many vesicles are compared in 2K to 100k EVs. The role for EVs in Th polarization by immature DCs (and role for DC-SIGN and CD40) is not convincing and the finding that this disappears after maturation is a major concern.
Answers to these comments are provided in answers to the detailed concerns below.

Major concern
1. Contaminations are still a problem and not addressed by the subfractionation. Data in Fig 4D are unclear.
We have performed gradient-based sub-fractionation on additional EV pellets from different donors for Figure 4D, and we confirmed that the IL-13-inducing activity of the 2K pellet is associated with materials that float, mainly in fractions 7-8 of the gradient (but also in lighter fractions 1-2), hence do not correspond to aggregated proteins that cannot float upward. Given the current technical state of the EV field, we do not see currently any other means to further distinguish what, in EVs' activity, comes from the EVs as they are formed within the secreting cell, or from additional components that may strongly/specifically associate to them after secretion.

2. The authors have partially addressed my concern, however, comparison between fractions based on the amount secreted remains rather non-specific as different concentrations are used of the EVs. The reviewer Figure (how is the fold calculated?) does alleviate some of my concerns but this is only once example of two donors and the variation is rather large.
Given the biochemical complexity of EVs (proteins, lipids, nucleic acids), it is impossible to quantify their concentration in a rigorous manner in terms of molarity, as is done for single molecules. Hence, we still believe that our choice of comparing their activity by equalizing the number of secreting cells was the best option. The above sentence has been added to the results section, to further explain our experimental choice.
We have provided in the previous version all requested additional comparisons showing the dose-response curves, and a table 1 showing amounts of proteins and particles present in each pellet: these data allow comparison of efficacy of the pellets per protein concentration or particle number if desired.
Finally, the previous Figure 4 for reviewers displayed the experiment requested by this reviewer: T cells stimulated with EVs pellets normalized by the amount of proteins. We apologize for not explaining what fold induction meant in this Figure. Fold induction was calculated as in the rest of the article: the ratio between the levels of cytokines secreted upon EV stimulation to the levels of cytokines secreted by control T cells not exposed to EVs. We have now performed this experiment with 3 additional donors, and the results are provided below for this reviewer.

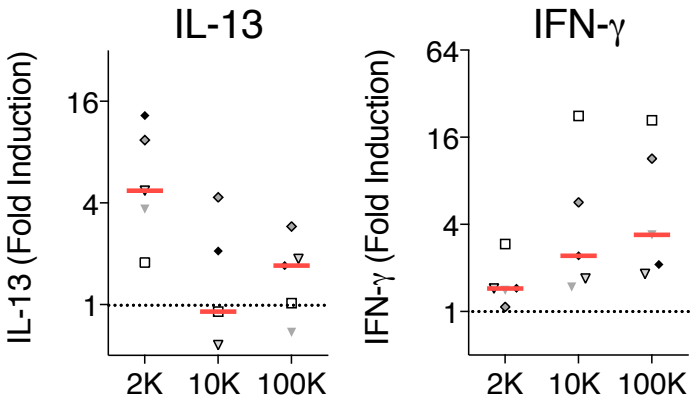

Figure for reviewer: Analysis of IL-13 and IFN-g release upon 6 d culture of 5 mg of the 2K, 10K and 100K pellet of DC-derived EVs with CD4+ T cells.

3. The controls concerning strong Th1 and Th2 polarizing conditions are lacking. The polarizing effect is compared with immature DCs alone (under normal conditions very bad inducers of polarization) , which is unusual in Th assays where Th polarizing PAMPs are usually taken along to determine whether the donors are indeed able to induce strong polarization. Intracellular Th stainings often help also determining the polarization efficiency. It is also a concern that re-stimulation of T cells which should only enhance differences negates the effect observed with EV priming.
The control this reviewer is asking for would be necessary if our message was to compare the physiological function of DCs versus EVs, like other works s/he is referring to are comparing the polarizing activity of e.g. different DC subsets or differently activated DCs. Here, however, the major message of our work is not on the actual physiological implications of EV secretion by DCs, but rather on the importance to consider the whole diversity of secreted EVs when performing functional studies, especially for downstream applications as therapeutic strategies. The T cell activation and polarization assay used here should be considered as a way to demonstrate a proof-of-principle that some functional activities are displayed equally by different types of EVs (i.e. induction of T cell proliferation, or Th1 polarization by all EVs from mature DCs), and some others are different in different EV types (i.e. induction of secretion of one or another type of cytokine by different EVs secreted by immature DCs). All internal controls required to interpret this assay are systematically performed throughout the manuscript. Note that both reviewers 1 and 3 highlighted the technical soundness of our work. We have also performed intracellular cytokine staining to detect IFN-g and IL-4, which was in the first version shown as main Figure 3, and in the first revised version and this one in Figure EV3 B-D.
To make our main message clearer, we have changed the title to *"Qualitative differences in T-cell activation by dendritic cell-derived extracellular vesicle subtypes"*, and amended the text, by avoiding use of the term "T cell-polarization", when it could sound like implying consequences in a physiological context, and we have included a clear statement in the last paragraph of the discussion on the interpretation of our work.

4. The addition of a dose response curve shows that the effect for cytokines is indeed not dependent on the amount of EVs (within the range of 8 times more DCs).
The dose-response curves of Figure 3A show that the activity of the 2K pellet reaches a plateau when materials secreted by only 1 million DCs are used, and the cytokine-inducing activity is strongly reduced when the amount of 2K pellet secreted by 0.5 million DCs is used. Thus, the activity is dependent on the amount of EVs. For the 10K and 100K pellet, the plateau is reached at either 4 (IFN-g-inducing activity) or 2 million DCs (IL-17-inducing activity), again dependent on the dose.

5. This point has been addressed.
Thank you

6. The authors provide a discussion why the antibodies are not efficient in inhibiting or affecting Th polarization. However, the data remain unconvincing for a role for CD40 and

DC-SIGN. The problem remains that this could indeed be other molecules or contaminations (see 1).
The effects of blocking antibodies are partial, indeed, but they are significant. Given the complexity of the composition of EVs, we are not surprised not to find a single molecule explaining the function observed. We do not exclude that other molecules than CD40 and DC-SIGN, or CD80 (new data of Figure 6, showing involvement of CD80 in the Th2-polarizing activity of the 2K pellet) participate also in the process, explaining why we cannot completely abolish the effects observed with any blocking antibodies. As shown in new Figures 6G, some other antibodies (like anti ICOSL or antiOX40L) did not show even the partial effects observed with the anti-CD40L and anti-DC-SIGN antibodies shown in Figure 6. In order to make our results more convincing, we added the results of four more DC-EV:T cell donors to the Figure (new Figure 6C and 6E).
Concerning contaminants, as answered to point 1, Th-polarizing activities of all pellets associate with fractions that float into a density gradient, hence, if contaminants, they are specifically associated to the floating materials. Given the current technical state of the EV field, we do not see currently any other means to further distinguish what, in EVs' activity, comes from the EVs as they are formed within the secreting cell, or from additional components that may strongly/specifically associate to them after secretion.

With regard to the technical replicates, the differences are quite large and therefore it is necessary to include technical replicates in the assays. I am unclear why histo-incompatibility would affect the technical replicates, especially as 2 K from one donor has low variability whereas same donor 10 and 100K have quite extensive variability.
As shown in previous Figure 5 for reviewer, the variability of technical replicates is similar to variability of biological replicates used throughout the article. Thus, performing technical replicates for each biological replicate would not increase the reliability of our assays. We are aware of the technical difficulty of using such variable assays as those observed with samples from human donors, and we compensated it by using much larger numbers of independent biological samples as done in more reproducible read-outs.

I have not addressed this point beforebut now it becomes more important with point 3. The authors use immature DC in their Th assays. Immature DCs are very bad inducers of polarization and this is also not physiological. Mature DCs induce T cell proliferation and polarization but immature DCs are thought to be less important. Thus, the importance of the EVs derived from immature DCs in inducing Th polarization is unclear. The difference between different EVs disappears upon maturation of the DCs. This should be explained.
As stated before, we want to stress that the major message of our work is not on the actual physiological implications of EV secretion by DCs, but rather on the importance to consider the whole diversity of secreted EVs when performing functional studies, especially for downstream applications to therapeutic applications. The T cell activation and polarization assay used here should be considered as a way to demonstrate a proof-of-principle that some functional activities are displayed equally by different types of EVs (i.e. induction of T cell proliferation, or Th1 polarization by all EVs from mature DCs), and some others are different in different EV types (i.e. induction of secretion of one or another type of cytokine by different EVs secreted by immature DCs).
The abstract and introduction of our article were clearly written in this orientation, although the title may have been possibly misleading. In this revised version, we have decided to change the title to reflect more clearly our goals, and we have changed some sentences in the abstract, introduction and results, to display more the read-out (cytokine secretion) than the physiological interpretation (Th1 vs Th2 polarization) of the experiments.
However, we have also included a very short speculative part at the end of the discussion on the physiological relevance of heterogeneous EV secretion by DCs. Indeed, as highlighted by this reviewer, immature DCs are not the most potent T cell polarizing cells, and we propose that this could be in part due to their ability to send contradictory messages in different EVs. Conversely, since mature DCs now only secrete EVs endowed with Th1 polarizing abilities, their EV secretion would instead enhance their efficacy, since all the messages they display, by direct contact but also by many different EVs spreading at a distance, concur to promote polarization of the encountered T cells towards Th1.

Referee #3:

This study is technically well controlled and well performed, but it remains largely descriptive. The authors need to provide additional molecular insight into the different functional behaviour found with Th2-polarizing lEVs.
In the revised manuscript, the authors have addressed satisfactorily part of my main concerns and queries, including clarifications and additional experimentation.
The authors should address an additional issue to complete the picture of the molecular mechanism of Th1/2-polarizing effects of differently sized EVs. While the Th1 polarizing activity of MoDC-derived sEVs (100K) may be partly attributable to CD40 and DC-SIGN molecules, the authors need to identify the molecules underlying the Th2-polarizing effect of lEVs (2K), or at least provide an inkling of the molecular mechanism.

we thank this reviewer for his/her fair assessment of our work. We are happy to be able to provide the requested molecular mechanisms in this revised version of our article; New Figure 6G-L and Figure EV4 shows that a mini-screen in search of molecules specifically involved in the Th2-polarizing activity of lEVs, highlighted CD80 as a candidate: we demonstrate in Figure 6H-L that CD80 is present in the 2K pellet, whereas, for instance CD40 and DC-SIGN are not, and that blocking CD80 specifically inhibits IL-13-inducing activity of the 2K pellet
Please note also that Figure 6 was revised to answer internal comments from other lab members, following presentation of the results of this article in an internal labmeeting. We have re-introduced some data/information that we had deleted in the previous revised version (with the goal to make the article less "dense" to read), but that our colleagues pointed as important for any reader to get a complete view of our results. We thus now indicate the ranges of actual cytokine concentrations measured in the control groups of all experiments where data are presented as ratios to control, and we show in Figure 6 and EV4 secretion of both cytokines (IL-13 and IFN-g in all experimental groups (all pellets ± blocking antibodies), instead of showing only the relevant cytokine/pellet combination.
We hope that this new set of data satisfactorily answers the last remaining concern of this reviewer.

4th Editorial Decision 07 August 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by referee #3 again who thinks that the revised version addresses the previous concerns and who supports publication. I am thus happy to accept your manuscript in principle for publication here.

REFEREE REPORT

Referee #3:

The authors have addressed in the revised manuscript my major remaining concern, by adding relevant new data on the differential involvement of CD80 molecule on the Th2 -polarizing activity of lEVs.
I am also satisfied with the re-writings, additions and modifications made to the manuscript, including the title, that now better convey the major findings of this study.
I therefore think the manuscript now deserves its publication in the EJ.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Clotilde Thery
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-96003

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size has not been chosen a priori
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Criteria to exclude samples: T cells that were not able to induce activation for any of the treatments assessed (i.e non-responder T-cell donnor) were excluded. This exclusion criteria was stablished at the beginning of the research project and applied all along this study.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For most analysis, non-paramteric tests were used that do not rely on distribution assumptions. Notewithstanding, parametric analysis were used in some instances and properly justified within the article.
Is there an estimate of variation within each group of data?	In all cases, all samples are shown (dot plots) and we thus avoided to compute any stimate of variation
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
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http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Clone number and brand is properly stated for all antibodies used in this work (Page 23, 24, 25 and 26)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	This study was conducted according to the Helsinki Declaration, with informed consent obtained from the blood donors, as requested by Institut Curie Review Board. (Page 21)
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	This study was conducted according to the Helsinki Declaration, with informed consent obtained from the blood donors, as requested by our Institutional Review Board. (Page 21)
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA