EMBO reports

Schistosomal extracellular vesicle-enclosed miRNAs modulate host T helper cell differentiation

Meningher Tal, Barsheshet Yiftah, Ofir-Birin Yifat, Gold Daniel,Brant Boris, Dekel Elya, Sidi Yechezkel, Schwartz Eli,Regev-Rudzki Neta, Avni Orly, and Avni Dror

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(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 18 March 2019

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think the manuscript is of interest, but requires major revisions to allow publication of the study in EMBO reports. Major points that need to be addressed in a revised manuscript to allow further consideration include to confirm the data using non-stimulated primary cells, to add necessary controls as indicated by the referees (in particular for the reporter assays), to show that you are really dealing with exosomes from schistosomes (at least using imaging and blocking EV uptake by antibodies demonstrating specificity), to add further insight on the miRNA content, and to clarify the focus on miR-10. As the reports are below, I will not further detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact me if a 3-months time frame is not sufficient so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main

HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature.

For more details please refer to our guide to authors:
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Important: All materials and methods should be included in the main manuscript file.

See also our guide for figure preparation:
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Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable. See:
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We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures)

Please also note that we now mandate that the corresponding author lists an ORCID digital identifier that is linked to his/her EMBO reports account!

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors study the involvement of extracellular vesicles released by schistosomes in the gradual decline of Th2 response. Using a transwell system they demonstrate that the secretome of schistosomes downregulates Th2 lineage differentiation. Subfractionation of the secretome by differential ultracentrifugation identifies extracellular vesicles. These extracellular vesicles contain miRNA that target Map3K7 which reduces NFkB activity and declines Th2 response.

Major comments
- In general extracellular vesicles should be studied in compliance with MISEV2018 guidelines. And experiments should be transparently reported in compliance with EV-TRACK. qPCR experiments should be conducted in compliance with MIQE guidelines.
- Based upon which data do the authors conclude that they specifically analyze exosomes? This requires specified analysis of the biogenesis of these vesicle structures.
- In general differential ultracentrifugation results in co-isolation of protein complexes/aggregates that can protect RNA. Correct quality control experiments should be performed to demonstrate that miRNA are specific to extracellular vesicles. Isolate extracellular vesicles using density gradient centrifugation and confirm the results (activity and miRNA content). Pereform incubation with supernatant of differential ultracentrifugation pellet to demonstrate that the supernatant does not harbor similar activities as extracellular vesicles. For RNA analysis, treat extracellular vesicles with protease and RNase to confirm presence of RNA inside extracellular vesicles. Perform functional experiments in dose reponse (incubate cells with different concentrations of extracellular vesicles).
- Please provide the complete list of identified proteins using proteomics. Add table of extracellular vesicles enriched proteins with peptide count and unique peptides to the main figures. Also perform an in-depth assessment of contaminating proteins. To address the purity of the samples.
- Extracellular vesicles are fluorescently labeled but remaining dye is not removed prior to incubation. This can impact the results of the experiment. Remove dye using a bottom-up density gradient prior to treatment.
- Why incubate cells with 10e7 extracellular vesicles? Why not study a dose reponse effect? Keep in mind that using differential ultracentrifugation, particles measured on Nanosight do not only correspond to extracellular vesicles but also protein complexes/aggregates.
- Describe material and methods into detail. Explain complete procedure of extracellular vesicle isolation and characterization. For NTA, report on instrument settings. No material and methods included on transwell assay. Explain isolation of extracellular vesicles; ....

Referee #2:

In their manuscript "schistosomes exosomal micro-RNAs: modulators of host T helper (CD4 +) cell differentiation" Meningher and colleagues demonstrate that schistosomes secrete extracellular vesicles that enter T cells and modulate their gene expression profile through micro-RNAs. Specifically they identify micro-RNA10 as an effector that down regulates NFkB, which should inhibit the expression of Th2 cytokines. The latter is not shown, only assumed. This is a carefully written manuscript around an interesting and certainly important story. The text is, however, at times too elaborate, as for example the introduction reads more like a review article, and hence should be more concise. The same is true for the first part of the abstract. Unfortunately the experimental design and execution has some serious shortcomings.

Gen. comments:
the paper does not include a model; however, I gathered that schistosomes secrete micro-RNA containing exosomes to target primarily T cells. There are several reasons to believe that this is not the case, at least not in vivo. First, resting T cells, and most of the T cells of the human body are resting, ingest very little exosomes. I guess it is for this reason, that the authors used the tumor cell line Jurkat to demonstrate vesicle uptake. Second, even though I think that their finding is correct, the functional effects in Jurkat cells was rather marginal. Pathogens usually induce prominent effects. Third, pathogens that have to survive in the host usually go for the more important cell, which is a dendritic cell or monocytes (which can develop into inflammatory dendritic cells). Inhibiting this cell population could efficiently inhibit a Th2 response. Indeed, monocytes rapidly ingest exosomes, much more than T cells. Another interesting target cell would be NK cells, which also ingest exosomes efficiently. To develop into DC, monocytes need NFkB. I would not only believe that this is the reason schistosomes target NFkB, I also think that the authors would see much more prominent effects using this target cell.
The experimental approach is rather simple. Monocytes develop into immature dendritic cells in the presence of IL-4 and GM-CSF. But monocytes that have ingested schistosomal exosomes likely fail to do so, if their principal finding is correct. That would be a really important result. I would therefore encourage the authors to do additional experiments in order to give their story in vivo

relevance.

Specific comments:
1) even though I would believe that the authors demonstrate exosomal effects, a direct evidence is missing. After incubation with schistosome vesicles in the transwell system, the authors should demonstrate vesicle uptake by confocal microscopy, or at least immunofluorescence, over several time points. For better journals an inhibition of this effect, for example by blocking antibodies, is usually required.

2) there is a lack of important controls throughout all experiments. For example, the authors concentrate on micro-RNA-10. To validate the specificity, they should compare the effect with control micro-RNAs (scrambled and an unrelated micro-RNA), block the effect by sponges and ideally mutate the target site in the 3ÙTR of MAP3K7. Furthermore, in their NFkB assays, they should have mutated the target sites in the promoter of their constructs .

3) in their current form the Western blots are not acceptable, particularly when the effects are very marginal. Positive and negative controls for MAP3K7 are missing. A dose-dependent effect is missing. In such cases first the whole Western blot with all controls has to be demonstrated. Then the same blot has to be stripped and blotted for control proteins.

4) Jurkat cells are not a very good or convincing system to demonstrate such effects. Although principal effects can be demonstrated, particularly when genes need to be transfected, the main function needs to be confirmed with a primary cell system (e.g. primary monocytes transfected with RNA). To obtain more prominent results in transfection experiments, the 293T system is better.

Referee #3:

This manuscript reports the effects of Schistosoma mansoni exosomal-like vesicles on T cell differentiation, with particular focus on Th2 signatures. A number of experiments were carried out which are nicely described. The authors firstly used transwell culture to show suppression of Th2 gene expression. They then focused on exosome release by the worms in vitro and characterized their proteome, as well as qPCR detection of specific miRNAs. Using Jurkatt cells, they showed uptake of labeled exosomes and presence of parasite miRNAs within cells. The authors focused on mir-10 and carried out a number of studies which suggested this miRNA can suppress expression of MAP3K7 to decrease NF kB signalling.

While the results are clearly described there are a number of points that should be addressed.

Major points:

The authors initially use niave CD4+ cells to demonstrate suppression of Th2 responses. However most subsequent experiments were carried out on Jurkatt cells, transformed T cells. My issue is how valid the data is from the Jurkatt cell studies to normal T cells during infection? Demonstrating uptake of labeled EVs by CD4+ cells (as used in the first experiment) would be very helpful in showing if primary cells can take up schistosome exosome-like vesicles. In addition, the work on MAP3K7 is all carried out using Jurkatts. The final experiment showing decreased levels of MAP3K7 after incubation with adult worms should be feasible in primary cells. I think carrying out these two experiments in niave CD4+ T cells is the minimum required to support effects on normal T cells, so that the conclusions are not based solely on the transformed Jurkatt cell data.

The authors speculate that the transwell studies indicate that parasite exosomes are involved in the parasite-host communication. Why the assumption that exosomes, rather than cell-free molecules, are the mediators? It is important to test medium from cultured worms that has been depleted of exosomes and compare what effects the parasite culture supernatant and purified exosomes have. Only in this way can the authors be sure that exosomes are required.

The authors carried out a global proteomic analysis of the purified exosomes, but to examine effects of miRNAs, focused only on three miRNAs. Mir-10 was selected, the authors state "since it

appeared as the most abundant miRNA in our analysis". But this is based on qPCR of only three miRNAs. Why not carry out miRNAseq of the purified exosomes? A global analysis of miRNAs present is needed to know if miR-10 is a highly abundant miRNA. Global miRNAseq would also be valuable in identifying what other miRNAs are present.

Better controls for the dual luciferase assay would be very useful in confirming the specificity of interactions - either mutation of the predicted miRNA binding sites or use of a control vector with a scrambled or reverse miRNA sequence, rather than empty plasmid.
The same applies to the MAPK-3'UTR plasmid - should use a plasmid with a non-target 3'UTR rather than just the psiCHECK-II plasmid alone as a control.

Would be very helpful to include more information to follow what is shown in Tables 1SA&B and 4S. In Table 1S, would be useful to show normalized read count data, then fold change, rather than showing control as 1. This would also show the level of gene expression. Relevant to this, was a sequence read cut off applied to determine the number of up- and down-regulated genes? This should be used to exclude genes with very low read counts, where fold change can be exaggerated.

In Table 4S, although the number of times a protein was identified in other studies is shown, it does not appear to be indicated for the current work. Should show number of hits and % of hits to specific proteins from the total number of hits.

Other points:

1. A few spelling errors need corrected eg Fig 5A
2. Introduction, last paragraph: "We assumed that part of the mechanism...." . Why assumed? Unless very strong evidence, better to change to hypothesized.
3. Introduction, background on T cells, is slightly simplistic:
e.g. "IFNγ exerts protective functions mostly in microbial infections" - what about in infection with intracellular parasites?
"The Th2 cytokines mostly play a role in response to parasitic infections" - should state in response to extracellular parasites.
"which in their turn, release granules containing toxic proteins or oxidizing molecules leading to helminth eradication" . This seems over-simplified. Could modify to contributes to helminth eradication.
4. P13, 1st sentence of last paragraph: "Since miRNAs can also regulate the expression at the translational levels, we decided to increase the screen beyond those target genes..". But the authors then focus on genes that are altered at the transcriptional level from RNASeq and focus on NFkB as a regulator of these. The sentence should be re-worded as not looking at translational effects of the miRNAs.
5. Figure 2, the arrows seem to be showing vesicles of very different sizes, in particular the last image.
6. Fig 4, y-axis bar for mir-10 should have specific values shown (rather than 0.000).
At these low levels of detection, how sure can the authors be of real changes?
7. In Figure 5B, why is there similar fluorescence for RPMI alone and with exosomes at 4oC. Would expect no background with RPMI if no exosomes present.
8. Discussion, 2nd paragraph, exposed to purified exosomes, rather than "clean" exosomes.
9. Discussion last paragraph, state "discovery of the mechanism of manipulation..." likely to be many mechanisms. Should re-word.
10. Same paragraph, wouldn't consider the hygiene hypothesis to be "novel".
11. Was Table S6 included?

1st Revision - authors' response 28 August 2019

Referee #1:

The authors study the involvement of extracellular vesicles released by schistosomes in the gradual decline of Th2 response. Using a transwell system they demonstrate that the secretome of schistosomes downregulates Th2 lineage differentiation. Subfractionation of the secretome by

differential ultracentrifugation identifies extracellular vesicles. These extracellular vesicles contain miRNA that targets MAP3K7 which reduces NFkB activity and declines Th2 response.

Major comments:

- In general extracellular vesicles should be studied in compliance with MISEV2018 guidelines. And experiments should be transparently reported in compliance with EV-TRACK. qPCR experiments should be conducted in compliance with MIQE guidelines.
- Based upon which data do the authors conclude that they specifically analyze exosomes? This requires specified analysis of the biogenesis of these vesicle structures.

As the Reviewer suggested, we read carefully the MISEV2018 guidelines. First, we do agree with the Reviewer's comment that we do not have biogenesis evidence demonstrating that the small particles that were isolated from the schistosomal-growing medium are indeed exosomes. However, we certainly present data, in compliance with MISEV2018, to strongly suggest that schistosomes secrete extracellular vesicles (EVs). Therefore, we changed throughout the manuscript the term exosomes into EVs.

As for the MISEV2018 guidelines' suggestions, more specifically:

1) "Report all details of the method(s) for reproducibility; centrifugation (g-force, rotor, ultracentrifuge, adjusted k-factor, tube type, adaptor if relevant, time, temperature)"
We added the information in the *Materials and Methods* in the paragraph entitled: Purification of schistosomal-EVs.

2) "Total starting volume of biofluid, or weight/volume/size of tissue"
The worms were grown in a 5ml medium. We changed the growing medium every three days. The collected medium was stored at -80^{o}C. EVs were isolated from 150-300ml of medium. Using this procedure, we obtained ~10^{11} EVs/ml. This information was added to the *Materials and Methods* in the paragraph entitled: Purification of schistosomal-EVs.

3) "use two different but complementary techniques, Techniques providing images of single EVs at high resolution, such as electron microscopy and related techniques, including atomic-Forcemicroscopy (AFM)"
We added AFM images to Fig. 2B together with the TEM images and the Nanoparticle Tracking Analysis (NTA). Now, there are three different techniques showing the presence of particles sized ~100nm in the schistosome-growing medium. The NTA and the AFM analysis also confirmed that these particles are absent in the control purified from an unused schistosome-medium.

4) " At least three positive protein markers of EVs, Transmembrane or membrane proteins-binding ability"
In our proteomic analysis, we identified the schistosomal homologs of two transmembrane proteins - the tetraspanins CD9 and CD63, the membrane proteins-binding ability annexin 2a, and the cytosolic proteins HSP70, GAPDH.

- In general, differential ultracentrifugation results in co-isolation of protein complexes/aggregates that can protect RNA. Correct quality control experiments should be performed to demonstrate that miRNAs are specific to extracellular vesicles. Isolate extracellular vesicles using density gradient centrifugation and confirm the results.
We agree with the Reviewer and performed this experiment: isolated EVs from the schistosomal-growing medium were loaded on the OptiPrep™ density sucrose gradient. Eight fractions were collected, and from each of them half was used for RNA extraction and subjected to TaqMan qRT-PCR for miRNA detection. The other half was used for Western Blot analysis. The main problem with this experiment is the fact that there are no available antibodies recognizing any schistosomal EV-proteins. Therefore, we used the anti-human HSP-70 which is 83% identical to the schistosomal-HSP-70. We add these results in *Supplementary data* as Fig. 3S.

- (activity and miRNA content).
Perform incubation with supernatant of differential ultracentrifugation pellet to demonstrate that the supernatant does not harbor similar activities as extracellular vesicles.
We performed the following experiments:

1) In all the experiments, the unused schistosomal-medium (containing RPMI, 1% (P/S), 1% L-glutamine and 10% EVs- free fetal bovine serum (FBS)) was processed precisely as the collected schistosomal-growing medium and used as a control.
2) The collected schistosomal-growing medium was filtered through a 0.1mm filter and placed on the trans-well above the primary Th cells. These experiments were done in parallel to new trans-well experiments in which live worms were placed. We did not detect schistosomal-miRNAs in Th cells that were exposed to the filtered medium, however, we did detect them in Th cells that were exposed to the worms as in the previous experiments. We added these new experiments as Fig. 5D-F.

- For RNA analysis, treat extracellular vesicles with protease and RNase to confirm presence of RNA inside extracellular vesicles.
We do believe that in the new version of the manuscript we have enough evidence to strongly suggest that the worms secrete EVs and that these EVs contain schistosomal-miRNAs.

- Perform functional experiments in dose response (incubate cells with different concentrations of extracellular vesicles).
We actually extended the functional experiments in the new version of the manuscript in other ways: (i) by assessing the effect of schistosomal-EVs on the *Il4* expression in highly purified Th cells versus combination of Th and APCs (Fig. 5) (ii) we demonstrated the effect of schistosomal-EVs on the expression of MAP37K in Th cells (Fig. 7).

- Please provide the complete list of identified proteins using proteomics
The complete list was added in Table 3SA-1 and 3SA-2

- Add table of extracellular vesicles enriched proteins with peptide count and unique peptides to the main figures.
We added a table with the 13 most common proteins that were identified in exosomes studies according to Vesicleped (Table 1). In this table, we added the list of peptides and the number of total unique reads mapped to each protein found in the schistosomal-EV proteomics that are mapped to the 13 most common proteins. In addition, in all the proteomics Tables - 3SA-1, 3SA-2 and 3S-B, the column # PSMs present the total number of reads of the identified peptide sequence.

- Also, perform an in-depth assessment of contaminating proteins.
~4% of peptides were unique schistosome proteins. Most identified peptides were from bovine serum. However, none of them were of either tetraspanin proteins (CD9 and CD63), annexin, aldolase or elongation factor 1-alpha (ef-1-alpha), which are known as the main hallmark proteins of EVs. In contrast, these schistosomal peptides were identified in the extract. We added this sentence to the manuscript. We also added to the *Supplementary data* Tables 3SA-1 and 3SA-2 that summarize the identified proteins from two proteomic analyses.

- Extracellular vesicles are fluorescently labeled but remaining dye is not removed prior to incubation. This can impact the results of the experiment. Remove dye using a bottom-up density gradient prior to treatment.
The fluorescently labeled dye was added to the EVs, as well as to the control medium after the supernatant was concentrated using a Vivaflow. Next, it was diluted into ~70ml of RPMI medium and ultracentrifuged at 150,000 × g overnight. If the dye was attached non-specifically on proteins or to any other components in the medium, then adding the dye to the control medium was also supposed to stain the cells. This is not the case. We extended the information on this procedure in the *Material and Methods*.

We added a new figure of Inverted Confocal Microscopy showing uptake of labeled EVs by the primary Th cells (Fig. 3). In most images (also in Fig. 2S), there are some cells, probably dead cells, which are strongly stained in the Control as well. These stained cells were not counted as EV-positive.

- Why incubate cells with 10e7 extracellular vesicles? Why not study a dose reponse effect? Keep in mind that using differential ultracentrifugation, particles measured on Nanosight do not only correspond to extracellular vesicles but also protein complexes/aggregates.
To demonstrate that the effect is not due to small aggregates, we added control of filtered medium in Fig 5D-F. Moreover, we demonstrated the specificity of the transfer of miRNAs into Th cells *in-*

vivo. We detected schistosomal miRNAs in Th cells isolated from lymph nodes of *Schistosoma*-infected mice. These miRNAs were found only in the gut-associated lymph nodes and not in the other lymph nodes or the enlarged spleen. To our understanding, this suggests that transferring of the miRNAs by the schistosome worms is very accurate, and therefore probably mediated by EVs and not as free RNA. We added this as Fig. 6 in the revised manuscript.

- Describe material and methods into detail. Explain complete procedure of extracellular vesicle isolation and characterization. For NTA, report on instrument settings.
We added the information regarding the NTA setting in the *Materials and Methods.*

- No material and methods included on transwell assay.
We added this information in the *Materials and Methods*.

- Explain isolation of extracellular vesicles
We added this information in the *Materials and Methods*.

- qPCR experiments should be conducted in compliance with MIQE guidelines.
We agree with the Reviewer and indeed performed the RT-PCR with compliance with most MIQE guidelines. The RNA extraction, reverse transcription, and PCR amplification were all done with commercial kits according to the manufactory protocols. Using commercial kits and working in accordance with their protocol, defined the RNA concentrations. All other components, like enzyme concentration, reaction volume, dNTPs concentration, buffer pH and many other data are listed in Table 1. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments (Clinical Chemistry 55:4 (2009)) are defined by the kit. For all kits used in the study, the manufacturer and its catalog number were specified. The Sequences of the Primers used in the study for each gene amplification are listed in Table 3 in *Materials and Methods*. The primers used for amplification of miRNAs are commercial and their catalog number is presented.

Referee #2:

- In their manuscript "schistosomes exosomal micro-RNAs: modulators of host T helper (CD4 +) cell differentiation" Meningher and colleagues demonstrate that schistosomes secrete extracellular vesicles that enter T cells and modulate their gene expression profile through micro-RNAs. Specifically they identify micro-RNA10 as an effector that down-regulates NFkB, which should inhibit the expression of Th2 cytokines. The latter is not shown, only assumed. This is a carefully written manuscript around an interesting and certainly important story. The text is, however, at times too elaborate, as for example the introduction reads more like a review article, and hence should be more concise. The same is true for the first part of the abstract. Unfortunately the experimental design and execution has some serious shortcomings.
Gen. comments:
the paper does not include a model; however, I gathered that schistosomes secrete micro-RNA containing exosomes to target primarily T cells. There are several reasons to believe that this is not the case, at least not in vivo. First, resting T cells, and most of the T cells of the human body are resting, ingest very little exosomes. I guess it is for this reason, that the authors used the tumor cell line Jurkat to demonstrate vesicle uptake.
We added a new Fig. 3 in which we demonstrated uptake of EVs by primary Th cells. The figure demonstrating uptake of EVs by Jurkat cells was moved into the *Supplementary data* (Fig. 4S). In addition, we demonstrated *in vivo* the presence of schistosomal-miRNAs in Th cells derived from the gastrointestinal associated lymph nodes, but not in Th cells derived from inguinal lymph nodes or spleen (Fig.6).

- Second, even though I think that their finding is correct, the functional effects in Jurkat cells was rather marginal. Pathogens usually induce prominent effects. Third, pathogens that have to survive in the host usually go for the more important cell, which is a dendritic cell or monocytes (which can develop into inflammatory dendritic cells).
We added new experiments in Figure 5D-F; Cells isolated from lymph nodes and spleen were sorted into CD4+CD11c- (Th cells) and CD4-CD11c+ (APCs). These sorted cells were seeded in 6-wells plates as Th and Th+APCs (5:1 ratio CD4:CD11c). In Trans-well above the cells, 25-30 live *S.*

mansoni adult worms were placed. We did not find any differences between these two conditions, as in both schistosomal-miRNAs were detected. The only minor effect was actually a slight inhibition of the miRNA uptake in the presence of the DCs.

- Inhibiting this cell population could efficiently inhibit a Th2 response. Indeed, monocytes rapidly ingest exosomes, much more than T cells. Another interesting target cell would be NK cells, which also ingest exosomes efficiently. To develop into DC, monocytes need NFkB. I would not only believe that this is the reason schistosomes target NFkB, I also think that the authors would see much more prominent effects using this target cell.
The experimental approach is rather simple. Monocytes develop into immature dendritic cells in the presence of IL-4 and GM-CSF. But monocytes that have ingested schistosomal exosomes likely fail to do so, if their principal finding is correct. That would be a really important result. I would therefore encourage the authors to do additional experiments in order to give their story in vivo relevance.
In the new version of the manuscript, we compared the effect of schistosomes on Th cells alone or in combination with APCs. The only minor effect was slight inhibition of the miRNA internalization in the presence of APCs.

- Specific comments:

1) even though I would believe that the authors demonstrate exosomal effects, a direct evidence is missing. After incubation with schistosome vesicles in the transwell system, the authors should demonstrate vesicle uptake by confocal microscopy, or at least immunofluorescence, over several time points. For better journals an inhibition of this effect, for example by blocking antibodies, is usually required.
As the Reviewer suggested, we performed the following experiment: primary Th cells were isolated from lymph nodes, placed on a dish covered with anti-CD3 and -CD28 antibodies. Fluorescently labeled isolated schistosomal-EVs were placed on these Th cells. Images were taken from the same slide by Inverted Confocal Microscopy continually after 3, 10, and 25 minutes (Fig. 3 and Fig. 2S). The statistics were calculated for each time point: counting the total number of cells in an image divided by the number of labeled cells in the same image.

The Reviewer suggested performing experiments in which we inhibit the entry of the EVs, for example by blocking antibodies. Indeed, it was shown that anti CD63 (a protein from tetraspanin family that is a major marker of EVs) can block EVs entry into cells. Obviously, we thought of doing such inhibitory experiments. However, to perform these experiments, we need either anti-CD63, anti-CD81, or antibody against any other schistosomal-tetraspanin or alternative schistosomal-EV- protein that was proven to block uptake of EVs. Unfortunately, these antibodies are not available yet.

2) there is a lack of important controls throughout all experiments. For example, the authors concentrate on micro-RNA-10. To validate the specificity, they should compare the effect with control micro-RNAs (scrambled and an unrelated micro-RNA), block the effect by sponges and ideally mutate the target site in the 3ÙTR of MAP3K7. Furthermore, in their NFkB assays, they should have mutated the target sites in the promoter of their constructs.
As the Reviewer suggested, we generated a plasmid mutated in the target site of miR-10 binding at the 3ÙTR of MAP3K7. We added this graph to the new Fig. 7 (all experiments with luciferase reporter plasmid were done in HEK-293 cells). As for the mutation in the promoter of NF-kB reporter, we used as control the pGL4-CMV- luciferase plasmid.

3) in their current form the Western blots are not acceptable, particularly when the effects are very marginal. Positive and negative controls for MAP3K7 are missing. A dose-dependent effect is missing. In such cases first the whole Western blot with all controls has to be demonstrated. Then the same blot has to be stripped and blotted for control proteins.
Throughout the manuscript, there are two Western blots. One of Jurkat cells over-expressing miR-10 compared to control cells over-expressing empty plasmid (Fig. 7C). The second Western blot is a new experiment in which proteins from primary Th cells were either exposed to live schistosomes or not, and then were subjected to Western blot analysis. In both we assayed the MAP3K7 and as control – normalizing protein, we used the GAPDH. Since the molecular mass of GAPDH is about 36 kDa and the molecular mass of MAP3K7 is about 67 kDa, we cut the membrane into two,

according to the molecular mass marker, and incubated each part with the designated antibody (therefore we did not need to strip and blot for the second antibody). We added the whole image of Western blot with three experiments of each in the *Supplementary data* as Fig. 6S and Fig. 7S

4) Jurkat cells are not a very good or convincing system to demonstrate such effects. Although principal effects can be demonstrated, particularly when genes need to be transfected, the main function needs to be confirmed with a primary cell system (e.g. primary monocytes transfected with RNA). To obtain more prominent results in transfection experiments, the 293T system is better.
In our previous version, unfortunately, we mistakenly wrote that the luciferase report assays were performed in Jurkat T cells. These experiments were actually performed in HEK-293 cells. As the effect of small RNA in primary cells, this experiment is technically challenging since the only way to transfect primary Th cells, even with small RNAs, is by electroporation. Instead, we performed a Western blot analyzing MAP3K7 expression in protein extract from primary Th cells that were either exposed to schistosomes or not. We added this as a new Fig.7F.

Referee #3:

This manuscript reports the effects of Schistosoma mansoni exosomal-like vesicles on T cell differentiation, with particular focus on Th2 signatures. A number of experiments were carried out which are nicely described. The authors firstly used transwell culture to show suppression of Th2 gene expression. They then focused on exosome release by the worms in vitro and characterized their proteome, as well as qPCR detection of specific miRNAs. Using Jurkat cells, they showed uptake of labeled exosomes and presence of parasite miRNAs within cells. The authors focused on mir-10 and carried out a number of studies which suggested this miRNA can suppress expression of MAP3K7 to decrease NF-kB signalling.
While the results are clearly described there are a number of points that should be addressed.

Major points:

The authors initially use naive CD4+ cells to demonstrate suppression of Th2 responses. However, most subsequent experiments were carried out on Jurkat cells, transformed T cells. My issue is how valid the data is from the Jurkat cell studies to normal T cells during infection? Demonstrating uptake of labeled EVs by CD4+ cells (as used in the first experiment) would be very helpful in showing if primary cells can take up schistosome exosome-like vesicles.
As the reviewer suggested, we performed an uptake assay of labeled EVs on isolated primary Th cells as seen in the new Fig. 3 and Fig. 2S. The results demonstrate a significant uptake.

In addition, the work on MAP3K7 is all carried out using Jurkats. The final experiment showing decreased levels of MAP3K7 after incubation with adult worms should be feasible in primary cells. I think carrying out these two experiments in naïve CD4+ T cells is the minimum required to support effects on normal T cells so that the conclusions are not based solely on the transformed Jurkat cell data.
As the Reviewer suggested, we performed a Western blot analysis on primary Th cells that were either exposed or not to adult schistosomes through trans-wells. As can be seen in the new Fig. 7F, the effect in primary Th cells was even stronger than in Jurkat T cells.

The authors speculate that the transwell studies indicate that parasite exosomes are involved in the parasite-host communication. Why the assumption that exosomes, rather than cell-free molecules, are the mediators? It is important to test medium from cultured worms that has been depleted of exosomes and compare what effects the parasite culture supernatant and purified exosomes have. Only in this way can the authors be sure that exosomes are required.
The Reviewer addresses the same comment as Reviewer #1. We added three experiments which strengthened the fact that the miRNAs are transferred to the cells in secreted EVs rather than as free molecules:

1) The medium was collected from the flask where the schistosomes grew-in. This medium was filtered through 0.1mm filter and placed on the trans-well, above the Th cells, in parallel to the trans-well in which live schistosomes were placed. We did not detect schistosomal miRNAs in the

cells that were exposed to filtered medium in contrast to the cells which were exposed to the worms. We added this new experiment as Fig. 5D-F.

2) EVs from the schistosomal-growing medium were loaded on a density sucrose gradient. We collected 8 fractions. From each fraction, half was used to extract RNA and subjected to TaqMan qRT-PCR to detect miRNAs. Half was used for Western blot analysis using markers of EVs. We used anti-human HSP-70, which is 83% identical to the *Schistosoma* HSP-70, because there are no available antibodies recognizing any schistosomal-EV proteins. The miRNAs and the HSP-70 deposited in the same fraction. If the miRNAs were aggregated in free protein complex, they were expected to appear in a different fraction that the EV markers. We added these results in the new Fig. 3S.

3) Finally, we detected schistosomal-miRNAs in Th cells isolated from lymph nodes of schistosome-infected mice. These miRNAs were found only in specific lymph nodes and not in the other lymph nodes or the spleen, which was enlarged. To our understanding this suggests that transferring of the miRNAs by this parasite is very accurate, and therefore probably through EVs and not as free RNA. These results strengthen our hypothesis that the miRNAs are transferred to the cells in secreted EVs rather than as free molecules.

The authors carried out a global proteomic analysis of the purified exosomes, but to examine effects of miRNAs, focused only on three miRNAs. Mir-10 was selected, the authors state "since it appeared as the most abundant miRNA in our analysis". But this is based on qPCR of only three miRNAs. Why not carry out miRNAseq of the purified exosomes? A global analysis of miRNAs present is needed to know if miR-10 is a highly abundant miRNA. Global miRNAseq would also be valuable in identifying what other miRNAs are present.
The Reviewer is right that we focused only on three miRNAs. He suggested performing miRNA-seq on EVs. Obviously, this was one of our aims. However, there are already three different publications which performed miRNA-seq of schistosomal-EVs. Therefore, we wanted to take our research a step further and understand how these miRNAs affect the host. Definitely, miR-10 and its ability to target MAP3K7 is just one example, and detailed study of the effects of other miRNAs should be performed in the future. Accordingly, we changed the paragraph and explained in detail why we focused on miR-10. The paragraph that was added:
"Then we looked for putative mRNA targets in Th cells. We decided to focus on the miR-10-targets based on several reasons: (i) In previous analyses of EVs, it appeared as the most abundant miRNA in *S. japonicum* [28] and one of the most abundant in *S. mansoni* [29, 61]; (ii) miR-10 was found in sera of rabbits and mice infected with *S. japonicum* [62]; (iii) Most importantly, miR-10 was the most abundant of the schistosomal-miRNAs among the selected miRNAs we examined in primary Th cells (Fig. 3; about 100 times more than miR-125 and 4 times more than Bantam); (iv) When we looked for predicted targets of selected schistosomal-miRNAs among the genes that were downregulated in schistosomal-exposed Th cells having a known Th2 function, using the bioinformatic tool Targetrank (http://hollywood.mit.edu/targetrank/) [63], we realized that miR-10 has the highest number of putative targets."

Better controls for the dual-luciferase assay would be very useful in confirming the specificity of interactions - either mutation of the predicted miRNA binding sites or use of a control vector with a scrambled or reverse miRNA sequence, rather than empty plasmid.
The same applies to the MAPK-3'UTR plasmid - should use a plasmid with a non-target 3'UTR rather than just the psiCHECK-II plasmid alone as a control.
As the Reviewer suggested, we generated a psiCHEHK-mut-MAP3K7, which contains a mutation in which 8 nucleoids in the seed miR-10 binding site were changed. A plasmid containing this mutation lost the inhibition effect that miR-10 had. We added these results to the new Fig. 7A and 7B.

Would be very helpful to include more information to follow what is shown in Tables 1SA&B and 4S. In Table 1S, would be useful to show normalized read count data, then fold change, rather than showing control as 1. This would also show the level of gene expression. Relevant to this, was a sequence read cut off applied to determine the number of up- and down-regulated genes? This should be used to exclude genes with very low read counts, where fold change can be exaggerated.

Table 1SA contains only genes whose expression was decreased in cells exposed to schistosomes, and whose expression was changed in all three experiments. Table 1SB contains only genes with increased expression in cells exposed to schistosomes and had their expression changed in all three experiments. As the reviewer suggested, we added to the *Supplementary data*, Tables 2SC and 2SB, which present the data as the read count normalized to counts per million mapped reads.

In Table 4S, although the number of times a protein was identified in other studies is shown, it does not appear to be indicated for the current work. Should show number of hits and % of hits to specific proteins from the total number of hits.
We updated Table 4S, and the number of peptides mapped to known EVs marker was added. Also, the percentage of the identified peptides cover the aligned protein was added. This table was added to the main manuscript as Reviewer 1 asked for it. In addition, in all proteomic tables - 3SA-1, 3SA-2 and 3S-B, the column # PSMs present the total number of reads to the identified peptide sequence.

Other points:

1. A few spelling errors need corrected eg Fig 5A
Have been edited

2. Introduction, last paragraph: "We assumed that part of the mechanism...." . Why assumed? Unless very strong evidence, better to change to hypothesized.
Has been changed as suggested

3. Introduction, background on T cells, is slightly simplistic:
e.g. "IFNγ exerts protective functions mostly in microbial infections" - what about in infection with intracellular parasites?
Has been changed as suggested

"The Th2 cytokines mostly play a role in response to parasitic infections" - should state in response to extracellular parasites.
Has been changed as suggested

"which in their turn, release granules containing toxic proteins or oxidizing molecules leading to helminth eradication". This seems over-simplified. Could modify to contributes to helminth eradication.
Has been changed as suggested

4. P13, 1st sentence of last paragraph: "Since miRNAs can also regulate the expression at the translational levels, we decided to increase the screen beyond those target genes..". But the authors then focus on genes that are altered at the transcriptional level from RNASeq and focus on NFkB as a regulator of these. The sentence should be re-worded as not looking at translational effects of the miRNAs.
From the Reviewer's comment, we understood that the claim we wanted to emphasize in the paragraph was not clear. We wanted to explain the rationale that brought us to study MAP3K7 as putative target of miR-10, despite the fact that its mRNA levels did not change between Th cells which were exposed to the parasite vs control Th in the RNA-seq analysis. We re-wrote this paragraph.

5. Figure 2, the arrows seem to be showing vesicles of very different sizes, in particular the last image.
We enlarged the figure, and the difference in the black line presenting 100nm in length is now clearer.

6. Fig 4, y-axis bar for mir-10 should have specific values shown (rather than 0.000).
We thank the Reviewer for pointing out this mistake. We changed the Y number in all three graphs in Fig.4 that allow now comparing the amount of each miRNA to the other two.

At these low levels of detection, how sure can the authors be of real changes?

The numbers are arbitrary units. They are very low because, the calculation is relative to mice endogenous none-coding RNA expression U6, which is expressed in much higher levels than the worms' miRNAs.

7. In Figure 5B, why is there similar fluorescence for RPMI alone and with exosomes at 4oC? Would expect no background with RPMI if no exosomes present.
First, we changed the figure and the previous figure was moved to the *Supplementary data* as Fig. 4S. The experiment performed at 4^{o}C is a control experiment to show that the penetration of the EVs into the cells is a biological process that does not occur at 4^{o}C.

8. Discussion, 2nd paragraph, exposed to purified exosomes, rather than "clean" exosomes.
Thanks for this comment, we changed accordingly.

9. Discussion last paragraph, state "discovery of the mechanism of manipulation..." likely to be many mechanisms. Should re-word.
Obviously, there are many mechanisms by which the parasite is manipulating the host immune system. However, we suggest ONE additional new mechanism - the transfer of EVs to the host.

10. Same paragraph, wouldn't consider the hygiene hypothesis to be "novel".
Thanks for this comment, we changed accordingly, and the word novel was deleted.

11. Was Table S6 included?
We thank the reviewer for noticing that we forgot to add Table 6S. We now added it in the revised manuscript.

2nd Editorial Decision 27 September 2019

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, all three referees have remaining concerns and further suggestions to improve the manuscript, we ask you to address in a final revised version of the manuscript. Please also provide a detailed point-by-point-response addressing these points.

Further, I have these editorial requests:

- Please provide a more comprehensive and shorter title (not more than 100 characters including spaces).

- The abstract is currently too long. Please shorten this to not more than 175 words.

- Please provide a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and legends for tables), but without the figures or tables included. Please make sure that any changes to previous versions are highlighted to be clearly visible. Legends should be compiled at the end of the manuscript text. Please upload figures and tables as separate files.

- Please add up to 5 key words to the title page.

- The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Thus, please select 5 EV figures from your Appendix and upload these as single figure files. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature. Please number tables and figures separately (Table S1, S2, S3 and Figure S1, S2, S3 ...). Then please check and/or update all call outs

in the manuscript text. Finally, please remove all legends or information regarding the Appendix or datasets from the main manuscript text (those presently found after the figure legends).

- Per journal policy, we do not allow 'data not shown', which is stated on page 16 the manuscript. All data referred to in the paper should be displayed in the main or Expanded View figures, or the Appendix. Thus, please add these data (or change the text accordingly, if these data are not important). See:
http://embor.embopress.org/authorguide#unpublisheddata

- In the manuscript text a Fig. 1C is mentioned. However, Fig. 1 does not have a panel C. Please check.

- Please call out the single panels of Fig. 3 in the text.

- Please add scale bars to all microscopic images (also on the Appendix) and remove any writing indicating their size from the scale bars. Please indicate the size only in the respective figure legend.

- We require that primary datasets produced in this study (i.e. the RNA-sequence and proteomics data) are deposited in an appropriate public database. See:
http://www.embopress.org/page/journal/14693178/authorguide#datadeposition

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. Please do that for your manuscript. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843
(https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

- We require that all corresponding authors supply an ORCID ID for their name. We will not proceed before this is done. Please find instructions on how to link their ORCID ID to their account in our manuscript tracking system in our Author guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

REFEREE REPORTS

Referee #1:

As response to my concern that the identified content and functions are not solely related to extracellular vesicles, the author use medium control, this is medium not conditioned by S. mansoni. However this does still not exclude that effects are due to soluble proteins or protein aggregates or extracellular vesicles released by S. mansoni, therefore the authors should perform an additional set of experiments.

The authors did perform a density gradient experiment in Figure 3S; this exactly confirms my concern that miR10 is not solely released by extracellular vesicles. Interesting would be to repeat functional experiments with these different fractions (fractions 2 and 3 presumably containing extracellular vesicles (when antibodies are lacking, EM can assist here) versus fractions 4, 5, 6 for example).

Overall I believe the authors have significantly improved the quality of the paper. I recognize that studying extracellular vesicles from S. mansoni may be hampered by the lack of antibodies etc. However from my point of view these concerns should at least be adequately addressed in the discussion section of the manuscript and the fractions of density gradient experiment should be more characterized (e.g. EM) and tested for their functional activity before reaching a final decision.

Referee #2:

The authors certainly improved their manuscript by adding a number of new data and overall the paper looks at least more acceptable for publication in EMBO Reports.
Although not all of my criticism have been addressed, only minor points remain:
1. The introduction is still too long and reads more like a section of a review article, but this is an editorial decision.
2. The authors now show uptake of labeled schistosomiasis-derived EV into primary Th cells. However, they should state that these cells were truly resting, or were they stimulated, e.g. by IL-2?
3. The authors somehow circumvented my question whether schistosomiasis-derived EV enter monocyte/dendritic cells (which they likely do) and exert their function there as well (inhibition of NFkB), probably to claim that these EV are taken up primarily by T cells. On the other hand they show evidence that lymph node derived T cells contain schistosomiasis-specific micro-RNA. Hence, this supports their case.
4. In figure 4, control and schistosomes are wrongly labeled.

Referee #3:

The authors have addressed some of the comments raised and carried out studies in primary Th cells in addition to Jurkat cells.

However I'm not convinced they have shown that miRNAs specifically in EV, rather than supernatant, are the mediators of the observed effect on Th cells. It is shown that the selected miRNAs (mir-10 and Bantam) are present in EVs but this does not rule out that any other miRNAs, or other mediators, are present in the schistosome growth medium supernatant. This needs to be shown using exosome-depleted, used growth medium on the Th cells, or alternatively the text needs to be modified to reflect this limitation.

The quality of the new images and data to support uptake of labeled EV into Th cells do not seem convincing nor accurate (Fig 3 and Fig S2). It is stated in the response that the strongly staining cells with the control, unused labeled medium are "probably dead cells" and were "not counted as EV-positive". On what basis are they considered to be dead cells and are dead cells also present in the Th cells incubated with labeled EV? If they are thought to be dead cells, this need to be demonstrated eg by co-staining with Trypan blue. As an alternative approach, cells could be fixed and counter-stained to score those live cells that have taken up labeled EV, for control and test.
In addition, the technique could be optimised - if there are no EV in the unused supernatant, what is

the staining in the control? Further washes of the incubated cells should remove non-specific binding of label to cells.
No size bars are shown in the images and the images in Fig S2 look to be of different magnification.

Despite the suggestions by the reviewers, an appropriate control for the NF-kB binding site reporter construct is not included (Fig 7E). Rather than mutation of the binding sites, a CMV promoter construct is used. As a proposed decrease in NF-kB activity is major conclusion of the work, evidence for this should be solid. Testing for reduced nuclear activity of NF-kB, by immunofluorescence and western blot of nuclear extracts would be alternative approaches to support this conclusion.

Description of Optiprep density gradient in Methods: was this carried out on used schistosome growing medium or "Schistosoma infected human sera" as stated? Confusing and not clear. And was schistosome-growing medium filtered or human serum? What was used for the miRNA qPCR and western blot with Hsp-70 antibody.

There seems to be no additional methods added for the purification of Th cells from Peyer's patches etc.

Appendix table numbers need corrected.

2nd Revision - authors' response 10 October 2019

Response to the Reviewers:

Referee #1:
As response to my concern that the identified content and functions are not solely related to extracellular vesicles, the author use medium control, this is medium not conditioned by S. mansoni. However this does still not exclude that effects are due to soluble proteins or protein aggregates or extracellular vesicles released by S. mansoni, therefore the authors should perform an additional set of experiments.

Reviewer 1 addressed this issue in the previous review. Therefore, we performed the experiments that are shown in Fig. 5 (5D and 5E). Obviously, our explanation was unclear and we re-wrote the paragraph: "Live worms were placed in a trans-well system as described above. In parallel, schistosomal-growth medium (used supernatant), was filtered through 0.1mm membrane to deplete schistosomal-EVs, and placed on top of a trans-well above Th cells. As expected, the schistosomal-miRNAs were detected in the Th cells that were exposed to the live schistosomes across the trans-wells, but were undetected in Th cells that were exposed to the filtrated schistosomal-supernatant (supernatant column; Fig. 5 D-F), excluding the effect of small protein/RNA aggregates."

The authors did perform a density gradient experiment in Figure 3S; this exactly confirms my concern that miR10 is not solely released by extracellular vesicles. Interesting would be to repeat functional experiments with these different fractions (fractions 2 and 3 presumably containing extracellular vesicles (when antibodies are lacking, EM can assist here) versus fractions 4, 5, 6 for example).
Overall I believe the authors have significantly improved the quality of the paper. I recognize that studying extracellular vesicles from S. mansoni may be hampered by the lack of antibodies etc. However, from my point of view these concerns should at least be adequately addressed in the discussion section of the manuscript and the fractions of density gradient experiment should be more characterized (e.g. EM) and tested for their functional activity before reaching a final decision.

As the Reviewer previously suggested, we did perform a density gradient experiment. Determining whether the worms' miRNAs are packed inside EVs is an important question since there are reports demonstrating excretion of miRNA from cells in a complex with the Ago-protein rather than in EVs. However, this is probably not the case since the Ago/miRNA complexes are found at different fractions than EVs (Jan Van Deun et al. J Extra cell Vesicles. 2014 Sep 18;3. doi: 10.3402/jev.v3.24858).

The Reviewer claims that there are fractions were the miRNA was detected without the HSP70 protein, and this is the proof that miR-10 is not solely released by EVs. Indeed, in fractions 1 and 6 we detect

miRNAs without HSP70. However, we believe that the reason is that the qRT-PCR is a much more sensitive assay than the Western blot, and therefore in fractions 1 and 6, where the miRNAs are fewer, HSP70 protein was below the detection limit. We also think that throughout the manuscript we provided other evidence strongly support the idea that the worms' miRNAs are packed and transferred inside EVs. However, since we cannot exclude completely the possibility that the miR-10 is also released by other mechanisms, we stressed it the current version of the discussion: "… The restricted delivery of the miRNAs by the *Schistosoma* strengthens the hypostasis that it mediated through EVs rather than free RNA, but other mechanisms cannot be excluded."

Referee #2:

The authors certainly improved their manuscript by adding a number of new data and overall the paper looks at least more acceptable for publication in EMBO Reports.
Although not all of my criticism has been addressed, only minor points remain:
1. The introduction is still too long and reads more like a section of a review article, but this is an editorial decision.
This work combines four scientific areas: parasitology of schistosome, immune response, EVs, and miRNAs. Therefore, we tried to include in the Introduction relevant information in all these aspects.

2. The authors now show uptake of labeled schistosomiasis-derived EV into primary Th cells. However, they should state that these cells were truly resting, or were they stimulated, e.g. by IL-2?
We apologize for not being clear enough. In this experiment, as in all the other experiments in the manuscript, Th cells were isolated from lymph nodes and stimulated in the presence of anti-CD28 and -CD3 antibodies without cytokines (Th0). In this specific experiment (Fig. 3), the labeled EVs were added 10 minutes after the beginning of the stimulation of the naïve Th cells for the indicated time points. We clarified it now in the figure legend.

3. The authors somehow circumvented my question whether schistosomiasis-derived EV enter monocyte/dendritic cells (which they likely do) and exert their function there as well (inhibition of NFkB), probably to claim that these EVs are taken up primarily by T cells. On the other hand they show evidence that lymph node derived T cells contain schistosomiasis-specific micro-RNA. Hence, this supports their case.
The Reviewer claims that schistosomiasis-derived EVs enter monocyte/dendritic cells. We perform the experiments where we expose Th to schistosomes in the present or not of dendritic cells (figure 5D-E). As seen, there are schistosomal miRNAs even in the absent dendritic cells. Obviously, we don't claim that schistosmal-EVs might enter other immune cells as well. We added therefore the following sentences at the end of the Discussion: " However, further studies are necessary to reveal the full scope of schistosomal-EV manifestations and their communication with the immune system."

4. In figure 4, control and schistosomes are wrongly labeled.
We thank the Reviewer for pointing out this mistake.

Referee #3:

The authors have addressed some of the comments raised and carried out studies in primary Th cells in addition to Jurkat cells. However, I'm not convinced they have shown that miRNAs specifically in EV, rather than supernatant, are the mediators of the observed effect on Th cells. It is shown that the selected miRNAs (mir-10 and Bantam) are present in EVs but this does not rule out that any other miRNAs, or other mediators, are present in the schistosome growth medium supernatant. This needs to be shown using exosome-depleted, used growth medium on the Th cells, or alternatively the text needs to be modified to reflect this limitation.

see comment 1 to reviewer number 1

The quality of the new images and data to support the uptake of labeled EV into Th cells do not seem convincing nor accurate (Fig 3 and Fig S2). It is stated in the response that the strongly staining cells with the control, unused labeled medium are "probably dead cells" and were "not counted as EV-positive". On what basis are they considered to be dead cells and are dead cells also present in the Th

cells incubated with labeled EV? If they are thought to be dead cells, this needs to be demonstrated e.g. by co-staining with Trypan blue. As an alternative approach, cells could be fixed and counter-stained to score those live cells that have taken up labeled EV, for control and test. In addition, the technique could be optimised - if there are no EV in the unused supernatant, what is the staining in the control? Further washes of the incubated cells should remove non-specific binding of label to cells.

The Reviewer is correct, we did not confirm that the strongly staining cells are "dead" cells. However, this non-specific strongly staying is found in both cells incubated with schistosomal-labelled EVs, but also in control cells staining only with medium containing the Thiazole Orange. The protocol that was used to stain the EVs with the Thiazole Orange is written in the *Materials and Methods*. To avoid a high background staining, after the labeling, the EVs were washed in ~70 ml RPMI. Still there are trances of non-EVs color residues in the control that probably stained cells with a defective cytoplasmatic membrane that looks totally different from EV-staining cells. Obviously, these cells were not counted.
No size bars are shown in the images and the images in Fig S2 look to be of different magnification.
We added the scale bars.

Despite the suggestions by the reviewers, an appropriate control for the NF-kB binding site reporter construct is not included (Fig 7E). Rather than mutation of the binding sites, a CMV promoter construct is used. As a proposed decrease in NF-kB activity is major conclusion of the work, evidence for this should be solid. Testing for reduced nuclear activity of NF-kB, by immunofluorescence and western blot of nuclear extracts, would be alternative approaches to support this conclusion.
We found that the Schistosoma miR-10 can regulate the expression of MAP3K7, one of the first kinases for NF-kB activation. To find whether miR-10 controls NF-kB activity through regulating MAP3K7, we used a plasmid that measures the bottom-line activity of NF-kB, which is regulation of gene transcription (Luciferase activity).
Obviously, additional experiments showing that miR-10 inhibits the transfer of NF-kB to the nucleus by immunofluorescence and western blot will add the information that miR-10 affects the signaling of NF-kB. However, our approach using the reporter plasmid, a well-characterized system to identify and quantify NF-kB activity in cells (*Nature. 1987 Apr 16-22; 326(6114):711-3*), allows a direct assessment of NF-kB activity. Therefore, we do not think that adding experiments showing localization of NF-kB will add much to the finding that miR-10 inhibits transcription of genes regulated by NF-kB.

The Reviewer also suggested generating a mutation in the NF-kB binding sites, as a control rather than using the CMV promoter construct. We show that overexpression of miR-10 inhibits the activity of a reporter that is dependent on NF-kB activity. It was shown by Nabel, G. et al (Nature. 1987 Apr 16-22; 326(6114):711-3.), that mutations in the binding sites of NF-kB, as the reviewer suggested, completely silences the promoter activity of the reporter. Therefore using a "dead" promoter might not add a significant information to our understanding that overexpression of miR-10 inhibits NF-kB activity.

Description of Optiprep density gradient in Methods: was this carried out on used schistosome growing medium or "Schistosoma infected human sera" as stated? Confusing and not clear. And was schistosome-growing medium filtered or human serum? What was used for the miRNA qPCR and western blot with Hsp-70 antibody?
From the Reviewer's comment, we understand that we wrote this in a confusing way.
Therefore, we left the description of the human-HSP70 only in the figure legend.
As we wrote isolated EVs from the schistosomal-growing medium were place on top of the OptiPrep™ density sucrose gradient. To detect the miRNAs we used specific schistosome miRNAs primers. However to detect EVs protein, we used the anti-human HSP70 antibodies. This is because there are no Schistosomal-antibodies to any of the known EVs proteins.

There seems to be no additional methods added for the purification of Th cells from Peyer's patches etc.
The method used to isolate Th cells from specific lymph nodes was exactly the same as in all other experiments, and is described in the *Materials and Methods* in the purification and differentiation of Th cells paragraph.

Appendix table numbers need to be corrected.
Has been corrected

3rd Editorial Decision 8 November 2019

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, referee #3 has a few points to improve the manuscript, I ask you to address in a further revised version of your manuscript.

Further, I have these editorial requests:

- Please add clearly visible scale bars of unique style (and colour) to all microscopic images (also on the Appendix). Do not show any writing indicating their size in the figure. Please indicate the size only in the respective figure legend.

- Please provide the bar diagrams in a unique style (same colours and thickness of axes, same size and style of font).

- Please put the heading of the data availability section in bold (without the colon), as all the other headings in the methods section.

- Please remove the legends for the datasets from the main manuscript text. Please provide these on the first TAB of the dataset excel files.

- I would suggest rearranging the Appendix, as it looks rather messy. Please add a title and the title of the paper first (Appendix for ...), then the TOC (but not as a table) with page numbers. Then first show all the Appendix figures, then the Appendix tables, then the references.

- We require that all corresponding authors supply an ORCID ID for their name. This is still missing for Neta Regev-Rudzki and Eli Schwartz. We will not proceed before this is done. Please find instructions on how to link their ORCID ID to their account in our manuscript tracking system in our Author guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

REFEREE REPORTS

Referee #1:

All comments have been adequatey addressed.

Referee #3:

The authors have addressed the main concerns and the wording of the text now clarifies the main points raised. Overall, a lot of work has been performed and the results are important in furthering understanding of host-parasite interactions.

There are some minor points that I think it would still be useful to address :

I queried this before: The Optiprep method states "500µl of Schistosoma infected human sera was overlaid onto the top of the gradient, and centrifuge at 100,000g for 22h at 4oC.....". Was it human sera that were used? This seems inconsistent with the results text and Appendix Figure S3. Would be good if authors can check the wording.

I appreciate that there may have been dead cells in both the control and labeled EV incubation samples. The different panels shown in Fig 3 in the main text don't raise any queries on this, but Appendix Figure S2 shows stained cells but then reports 0% staining for some samples with red cells eg RPMI 3 min, 10 min.
Without any explanation in the Appendix figure legend, this is quite confusing. I think that the last two few sentences given by the authors in their response to reviewers should also be included in the Appendix Figure legend.

Spelling:
In Discussion should it be "hypothesis" rather than "hypostasis"?

3rd Revision - authors' response 8 November 2019

Response to the Reviewer:

Referee #3:

I queried this before: The Optiprep method states "500µl of Schistosoma infected human sera was overlaid onto the top of the gradient, and centrifuge at 100,000g for 22h at 4oC.....". Was it human sera that were used? This seems inconsistent with the results text and Appendix Figure S3. It would be good if the authors can check the wording.

We thank the reviewer for pointing our mistake which he already wrote in the previous letter. Obviously, it is a mistake (cut and paste of method from previous paper). Just to clarify this point, 500ml of Schistosoma extracellular vesicle isolated from 150ml medium the worms grow-in, overlaid onto the top of the gradient. No human serum was used in this work. Obviously, we change it in the methods.

I appreciate that there may have been dead cells in both the control and labeled EV incubation samples. The different panels shown in Fig 3 in the main text don't raise any queries on this, but Appendix Figure S2 shows stained cells but then reports 0% staining for some samples with red cells eg RPMI 3 min, 10 min.
Without any explanation in the Appendix figure legend, this is quite confusing. I think that the last two few sentences given by the authors in their response to reviewers should also be included in the Appendix Figure legend.

As the reviewer suggested we added in the Appendix figure legend the explanation of which cells were excluded from counting. We added an arrow pointing to an example of a cell that was not counted

Spelling:
In Discussion should it be "hypothesis" rather than "hypostasis"?
Again we thanks the reviewer, it was changed.

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: Avni Dror
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Manuscript Number: EMBOR-2019-47882

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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?	For each experiments the statistics tests were basted on sample size of at list three individual unrelated experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The only experiment with animals was to detect the miRNAs of the parasites in the T cells isolated from specific lymph nodes. In these experiments four infected mice, and four control un infected mice were used
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not relevant
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.	Not relevant
For animal studies, include a statement about randomization even if no randomization was used.	Mice were taken from two different infection cycles with the parasite. The difference between the two infection cycles was four weeks. From each group of five mice, 2 mice were randomly taken to isolate T cells from lymph nodes. To each two infected mice two control uninfected mice were used.
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.	Not relevant
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not relevant
5. For every figure, are statistical tests justified as appropriate?	In all figures t-test were used, because we compared the effect of the worms to control on one individual factor. In most tests we used pair Two-tailed tests. If the number of samples in the compared groups were not equal unpair test was used. All statistics tests were done in GraphPad prism program. Only for analyzing the RNA-seq seq-monk for differential expression was used.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	All statistics tests were done in GraphPad prism program. Only for analyzing the RNA-seq seq-monk for differential expression was used.
Is there an estimate of variation within each group of data?	Not relevant
Is the variance similar between the groups that are being statistically compared?	yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

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).	Antibodies used in this study anti-TAK1 (MAP3K7) antibody (abcam [EPR5984], Cat. No. ab109526), anti-GAPDH (Cell-Signaling, Cat. No. 2118) anti-HSP70 (B-6) (Santa Cruz Biotechnology Inc., Cat. No. sc-7298). EasySep™ Mouse CD4+ T Cell Isolation Kit (Stemcell Technologies, Cat. No. 19852A). anti CD28 Hamster Anti-Mouse Clone 37.51 Catalog No.553297 (Pharmingen (San Diego, CA)) anti-CD3ε (145.2C11, hybridoma supernatant). goat anti-hamster antibodies (Whole Molecule Polyclonal Secondary Antibody, MP Biomedicals Catalog No. ICN56984 MP Biomedicals, Inc Supplier Diversity Partner 0856984)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK-293 and Jurkat from the ATCC. Free of mycoplasma

* 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.	Six-week-old male ICR mice were purchased from (Harlan Biotech, Rehovot, Israel) Mice were routinely infected by injection of about 200 cercariae each. the infected mice cycles were performed at Tel Aviv University (Tel Aviv University ethical committee number 01-13-076). CD4+ cells were isolated from female BALB/c mice that were purchased from (Harlan Biotech, Rehovot, Israel) and maintained under SPF conditions in the animal facility of The Faculty of Medicine in Safed. All experiments were approved by the ethics committee of Bar-Ilan University, Ethics number 15-03-2015
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Tel Aviv University ethical committee number 01-13-076 And Bar-Ilan University, Ethics number 15-03-2015
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.	Confirm compliance with the ARRIVE Guidelines in the relevant experiments

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not relevant. No Human subject were used in this research
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.	Not relevant. No Human subject were used in this research
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not relevant. No Human subject were used in this research and therefore no patients' photos are present in the manuscript
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not relevant. No Human subject were used in this research and therefore no human data are present in the manuscript
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not relevant.
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.	Not relevant.
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.	Not relevant.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD012525 The RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number; E-MTAB-7658
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).	In the Supplementary data we added Table 1SA Genes in Th cells whose expression was decreased after the exposure to the schistosomes (present as a percentage from control in pair experiments). Table 1SB: Genes in Th cells that their expression was increased after the exposure to the schistosomes (present as a percentage from control in pair experiments) Table 1SC: Genes in Th cells whose expression was decreased after the exposure to the schistosomes (present as normalized reads CPM (counts per million)). Table 1SD: Genes in Th cells that their expression was increased after the exposure to the schistosomes (present as normalized reads CPM (counts per million)). Table 3SA-1: Summary of all proteins mapped to the peptides that were identified in the proteomic of extracted EVs (experiment number 1) Table 3SA-2: Summary of all proteins mapped to the peptides that were identified in the proteomic of extracted EVs (experiment number 2) Table 3SB: Summary of schistosomal-peptides detected in the EV-proteomic analysis
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).	Not relevant. No Human clinical or human genomic were used in this research.
21. 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.	Not relevant. No computational model was used in this study

G- Dual use research of concern

22. 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.	Best on the list publish in HHS and USDA Select Agents and Toxins list, none of these biological agents or toxins have been used in our study