molecular systems biology

PLAU inferred from a correlation network is critical for suppressor function of regulatory T cells

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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 20 July 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees raise substantial concerns on your work, which, I am afraid to say, preclude its publication.

Overall, the reviewers recognized the ability of the time-series transcriptomic data, and subsequent network analysis, to identify known genes involved in Treg biology, and to provide new candidates. They were, however, not convinced that the experimental results exploring PLAU and FAM13A1 yet provided conclusive evidence supporting their relevance for Treg development or function. All three reviewers also noted the lack of a mechanistic explanation for PLAU's gene expression regulation in Treg cells, and reviewer #1 in particular seems to feel that additional experiments are needed in this regard. In addition, the reviewers each had a series of more specific concerns, including additional controls and/or experiments that they felt would be needed to support the conclusions in this work. Overall, the reviewers were not convinced that this work was suitable for publication in Molecular Systems Biology (rating publication suitability as "medium", "low," and "low").

Given that these experimental results provide the main support for the ability of network analysis to identify novel genes relevant to Treg biology, and the low-level of support expressed by the reviewers, we feel we have no choice but to return this manuscript with the message that we cannot offer to publish it.

Nevertheless, the reviewer did express some interest in this work, and we note that they provide a

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series of constructive suggestions regarding additional experiments needed to address their concerns. As such, we may be willing to reconsider a new submission based on this work that includes substantial new evidence. Any new submission, at minimum, should provide additional experimental data supporting and exploring the role of PLAU in Treg cells, in addition to addressing the important technical concerns raised by each reviewer.

A new submission would have a new number and receipt date, and please understand that we can give no guarantee about its eventual acceptability. If you do decide to follow this course, then it would be helpful to enclose with your re-submission an account of how the work has been altered in response to the points raised in the present review.

I would also like to acknowledge that the review process took somewhat longer than usual in this case because of significant delays in receiving the reviewers' reports. Thank you for your patience.

I am sorry that the review of your work did not result in a more favorable outcome on this occasion, but I hope that you will not be discouraged from sending your work Molecular Systems Biology in the future.

Thank you for the opportunity to examine this work.

Yours sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Reviewer #1 (Remarks to the Author):

The authors developed a strategy to identify the key genes for Treg function from the gene network reconstructed from the high-time-resolution transcriptome. They identified two new key genes PLAU and FAM13A1, which are important for the suppressor function of both human and murine Tregs. The network strategy based on high-time-resolution data would have potential for identifying novel key genes, and the data presented here show that PLAU plays a role in certain aspects of Treg function. In this reviewer's opinion, however, there still remains a concern regarding the functional significance of PLAU in Treg function.

Major comments,

1. In contrast with the data from human samples, functional significance of PLAU in Plau-KO mice is obscure. The authors need to address the relevance between PLAU and the expression of the Treg-associated molecules in mice. siRNA-based knockdown and overexpression of PLAU in mouse peripheral T cells may be useful to address the issue.

2. The authors identified PLAU as a new hub key gene for the Treg function, and they claimed that PLAU regulates the expression of Foxp3 and other Treg-associated molecules. However, PLAU is a serine protease that catalyzes the conversion of plasminogen to plasmin. Involvement of the serine protease in the transcriptional regulation needs to be addressed.

3. In the experiment in Fig. 5, the authors sorted PLAUlow and PLAUhigh Tregs, and expanded them by re-stimulation. It is required to show the stableness of the PLAU expression levels after the expansion.

4. The effect of PLAU on the expression of Treg-associated molecules was detected in Foxp3+ Tregs. It is needed to clarify whether the effect of PLAU is dependent on Foxp3. In addition, in Fig. 3, Foxp3negativePLAUhigh population was observed. The characteristics of the population is also required.

Minor comments,

5. For the HTR genome-wide gene expression analysis, the authors sorted CD4+CD25high T cells as Tregs. It is required to show the purity of Tregs.

6. In the siRNA experiments in Fig. 6, knockdown of FAM13A1 should be confirmed.

Reviewer #2 (Remarks to the Author):

In this report the authors use serial arrays following activation to generate a network of gene expression patterns. Many of the genes found by this method, and scored as "hubs", were known to be involved in Treg development and/or function (e.g., GARP), although it is surprising that FOXP3 was not found. Some of the other 'hub' genes were quite surprising, and require some explanation. One of these surprising genes, PLAU, is the focus of the biological section of the paper. The authors show that PLAU expression is increased in Tregs following activation to a higher extent that in Teff cells, although the increases seen in Tregs are modest at best. They also use siRNA knockdown to test its role in Treg function. Again, they only show very modest affects on the expression of a handful of Treg genes-possibly due to having rather inefficient knockdown of PLAU. Finally, they sort Tregs into PLAUlo and PLAUhi, and compare them functionally. The interesting aspect of this data is that the PLAUhi Tregs are more suppressive (although this data will need to be repeated using a CFSE dilution protocol). Again, the differences in the expression of other genes is very modest.

WHile somewhat interesting, there are several aspects of this work that need to be addressed before any consideration for publication can be made

1. The authors mention that the Tregs and Teffs used are alloantigen specific, but give no details. They need to provide more experimental detail as to how the Tregs were isolated and where they come from.

2. They really need to at least speculate on how PLAU (and its receptor) can regulate Treg function.

3. They need to determine whether the PLAUlo/hi Tregs represent natural and induced Tregs

4. There is not indication in the siRNA experiments as to cell viability

5. Figure 6 needs to be split into 2 figures (A-E in one, and F-I in the other)

6. The Fam13 data needs to either be examined in greater detail or removed from the manuscript.

7. All the differences they see in gene expression following siRNA knockdown with either gene are the same. This suggests that the differences are gene independent-they need to knockdown a broadly expressed gene to see if the effect on gene expression is the same as that seen for PLAU and FAM

Reviewer #3 (Remarks to the Author):

This manuscript reports on a time-resolved transcriptome study of human regulatory (Treg) and effector (Teff) CD4+ T cells. Using a heuristic, correlative approach for deriving 'gene networks' from the data, two genes - PLAU and FAM13A1 - are identified that have so far not been described in the context of Treg function. The function of these two genes is then investigated further using genetic tools. PLAU is shown to affect the suppressive capacity of Treg cells, albeit in a somewhat complicated fashion: Low PLAU levels reduce suppression of Teffs in humans and older mice, whereas PLAU-/- Treg cells from younger mice have better suppressive capacity. No role of FAM13A1 for suppression by Tregs is shown, although its knockdown seems to affect a few other Treg-relevant genes. As the suppression assay is standard, one is led to suspect that FAM13A1 does not have much of a role here.

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Overall, this is an interesting effort that goes a very considerable way from computational analysis of time-resolved transcriptome data to the experimental characterization of a new candidate gene (PLAU) implicated in Treg function. The mechanism(s) by which PLAU affects Treg activity are not investigated.

Critique:

(1) Regarding the bioinformatic analysis, it is sometimes difficult to understand in detail what has been done. Fig. 1 is helpful as an outline of the general procedure, which appears to be sound. However, the description in the text must be improved. In particular, the Methods section on Functional Network Reconstruction ... (p. 15) is nearly incomprehensible. Consulting a native English speaker might help. In the following, I list a few of the unclear points. Some of these points are minor, but as they accumulate in places they make the text difficult to read.

- The term "highly reliable ... gene networks" (p. 5) is misguided, as it would be exceedingly difficult to establish in which sense a 'gene network' generated by this heuristic analysis is biologically correct (assuming that this is meant with "reliable"). What the method could do is filter out interesting candidates for further experimental study; this is, of course, how it is used here.

- A combination of Local clustering (by Qian et al.) and Trend correlation (by two of the authors of this manuscript) was used in combination. Why?

- In the Methods section, P_LC1 and P_LC2 are not explained.

- Methods: "For the TC method, we introduced another adapted P-value (P_TC2) calculation based on the resulted sc and cc scores." What has actually been done?

- What sense does it make to combine the P_TC and P_LC values in the min criterion (last lines, p. 15)?

- p. 5: "... a robust correlation between the extent of expression correlation ..." Do the authors really mean a correlation of correlations?

- p. 5: The significance of the fact that a handful of T-cell related GO categories are among the 212 enriched ones is not clear.

- In Table 4, it is not clear whether a putative GARP-dependent gene that is upregulated (or downregulated) in Tregs vs Teffs is also upregulated (downregulated) in GARP-overexpressing Teffs. The table only records whether the gene's expression was changed in GARP-overexpressing Teffs, so it could be changed in the opposite way to its expression in Treg cells. The full information should be given.

- p6: The wording "under transcriptional control of GARP" is misguided as GARP appears to be a plasma membrane protein and has not been described as a regulator transcription.

- The 'combinatorial' time-point-wise way to establish expression differences between Treg and Teff cells is explained for two time points (p. 16). How has this been applied to the entire time series? The authors will be certainly aware of established statistical tests of whether two time series are different that operate on the entire time course at once. Why has this point-wise approach been used here? What motivates the equation for P_e (p. 16)? It appears that the issue of whether two time series are different given a certain measurement error is not given proper attention in the manuscript.

- Equations and inequalities in the Methods section should be typed as separate items (and not within the text) to enhance readability.

- Figure 1. The legend to part d needs revising.

(2) For the Teff proliferation assay, CFSE staining and detection by flow cytometry should be used instead of the H3-thymidine incorporation assay. The former is standard in the field and will allow comparison of the Treg suppression assays in the manuscript with published data. In particular,

CFSE staining allows inferring the fraction of dividing cells and the extent of division.

(3) For comparing two flow-cytometry distributions, strictly speaking they must be normalized to have the same areas under the curves. In many cases, this is not necessary to see differences (e.g., Fig. 4b). However, it is vital in Fig. 5e. Contrary to the claim in the manuscript, I suspect that normalization will reveal no difference between the two FoxP3 distributions.

(4) Contrary to the claim on p.4, there is no substantial downregulation of genes in the TCR pathway visible in Suppl. Fig. 1. Some important genes (e.g. CD28) are even higher in Tregs. It would be good to see a list of which genes are supposed to be downregulated in Tregs alongside with the time-series plots.

Minor points:

(5) Throughout the paper, it was never quite clear whether unstimulated Teffs and Tregs were included as time point 0. The resolution of Suppl. Fig. 2 is very poor, but there seems to be no time point 0.

(6) Why was there no attempt to distinguish between naÔve Teff cells and memory cells (which could be done by sorting)? Very likely, there are memory cells in the culture and so it is not surprising - but rather expected - to see fast expression of effector cytokines (see p. 4 "..surprisingly, it is within 1h but not after days ..."). Also, Treg cells do express cytokines (e.g., IL-10).

(7) The authors should discuss carefully what is known about the role of antigenic stimulation for the suppressive activity of Treg cells.

Re-submission 19 July 2012

We have considered the constructive suggestions and comments from the reviewers and the editor, and have performed a substantial amount of new experiments to address all concerns. In the new version, we have significantly improved the manuscript by the following measures:
1, we performed several additional experiments to investigate the molecular mechanisms through which *PLAU* mediates Treg suppressive function.
2, we performed additional experiments to address the difference between younger and older mice.
3, we performed additional experiments such as CFSE measurements to clarify the technical and control issues raised by the reviewers.
4, we further revised and clarified unclear points that might lead to misunderstandings.
Please also find enclosed a reply to the reviewers´ comments, point-by-point.
We hope that the revisions and the improvement of the manuscript will meet the publication criteria for *Molecular Systems Biolog*y and are looking forward to your response.

The following parts including point-by-point responses to reviewers' comments and the editor response letter for the previous manuscript.

Reviewer #1 (Remarks to the Author):

The authors developed a strategy to identify the key genes for Treg function from the gene network reconstructed from the high-time-resolution transcriptome. They identified two new key genes PLAU and FAM13A1, which are important for the suppressor function of both human and murine Tregs. The network strategy based on high-time-resolution data would have potential for identifying novel key genes, and the data presented here show that PLAU plays a role in certain

aspects of Treg function. In this reviewer's opinion, however, there still remains a concern regarding the functional significance of PLAU in Treg function.

Major comments,

1. In contrast with the data from human samples, functional significance of PLAU in Plau-KO mice is obscure. The authors need to address the relevance between PLAU and the expression of the Treg-associated molecules in mice. siRNA-based knockdown and overexpression of PLAU in mouse peripheral T cells may be useful to address the issue.
Reply: The obscureness of the functional significance of Plau in Plau-KO mice has been addressed by several new experiments in the new version (page 11-12). CFSE dilution results show that the Tregs from spleens in 20-week-old *Plau*$^{-/-}$ mice had a significantly lower suppressor potential than WT-mice. But the Tregs isolated from 3-week-old *Plau*$^{-/-}$ or WT mice splenocytes showed no significant difference in suppressive potential by CFSE results (Note that, in Fig. 6 of our previous version, the cpm value measured by H3 thymidine uptake was too small due to a very low proliferation of Teffs from 3-week-old mice independent of *Plau*$^{-/-}$ or WT mice attributing to ineffectiveness and immaturities of APC cells also from 3-week-old mice (Dakic et al, 2004) (page 14). Although those results had indicated that Plau$^{-/-}$ Tregs had higher suppressive function than WT Tregs, those P-values shown were from comparisons between very low cpm values and they are therefore not reliable). We found that the bases of the difference between younger and older mice were due to the differences in the suppressive function between two Tregs subsets Cd44^{high}Cd62L^{low} and Cd44^{low}Cd62L^{high} Tregs in WT and *Plau*-KO mice.

We found that the the frequency of Cd44^{high}Cd62L^{low} Tregs in secondary lymphoid organs was significantly higher in relatively older mice compared to 3-week-old mice regardless of WT or *Plau*-KO mice. Furthermore, as demonstrated by both division percentages and division numbers indicated by CFSE dilution peaks, the expression of Plau mainly promotes the suppressive function of the Cd44^{high}Cd62L^{low} Tregs but not the Cd44^{low}Cd62L^{high} Tregs. The high percentages of Cd44^{high}Cd62L^{low} Tregs among the total Tregs in older mice contribute to the phenotypic differences of the total Tregs between WT and *Plau*$^{-/-}$ mice. The effects of *Plau* on total Tregs in older mice coincide very well with that in cultured human Tregs. This is due to the fact that Cd44^{high}Cd62L^{low} Tregs, namely, the ‘memory-like’ Tregs are the major population of Tregs in older mice while almost all the cultured human Tregs are CD45RO^{+} memory Tregs after several-round exposures to irradiated allogeneic Epstein-Barr-virus (EBV)-transformed B cells (EBV-B cells).
We did not perform siRNA experiments for murine Tregs because in-vitro cultured sorted murine Cd4^{+}Cd25^{+} Tregs are unstable and Foxp3 expression decreases steeply even in a few hours following stimulation in our laboratory. The instability of cultured murine Cd4^{+}Cd25^{+} T cells has also been reported by other groups (Chai et al., J. Immunol. Jan 15; 2008, 180:858-90).

2. The authors identified PLAU as a new hub key gene for the Treg function, and they claimed that PLAU regulates the expression of Foxp3 and other Treg-associated molecules. However, PLAU is a serine protease that catalyzes the conversion of plasminogen to plasmin. Involvement of the serine protease in the transcriptional regulation needs to be addressed.

Reply: We tried to address the mechanisms through which PLAU mediates the suppressive functions of Tregs and added a new section ‘PLAU mediates Treg suppressor function via STAT5 and ERK signaling pathways’ (page 10-11) and the corresponding Fig. 6 and Supplementary figures S5 to S9. We investigated the two defined domains of PLAU, the N-terminal domain with receptor binding capability as well as the C-terminal domain with catalytic activity. We found that the suppressive capability of Tregs pretreated with the specific catalytic inhibitory antibody was significantly impaired as shown by CFSE dilution measurement of co-cultured Teffs. In order to identify the signaling pathways through which the PLAU catalytic domain mediates Treg suppressor function, we assessed STAT1, STAT3, STAT5, ERK1/2, AKT and JNK1/2 as potential PLAU targeted signaling pathways suggested by molecular pathway databases or literatures. We did not observe any significant difference in the phosphorylation of three proteins STAT3, AKT and JNK1/2 in the presence or absence of the catalytic inhibitory antibody. We cannot observe apparent STAT1 phosphorylation in the first 90 mins of stimulation neither in the presence nor in the absence of the catalytic inhibitory antibody. However, Tregs pretreated with the catalytic inhibitory antibody showed much less phosphorylation of STAT5 and ERK1/2 proteins compared with untreated control Tregs. Activation of STAT5 is required for upregulation of FOXP3 expression in both human and

murine Tregs (Murawski et al, 2006). Human Tregs display a greater capacity to phosphorylate ERK compared with Teffs upon TCR-mediated activation (Crellin et al, 2007). We therefore conclude that PLAU mediates Treg suppressor function via STAT5 and ERK signaling pathways.
We have also investigated whether the receptor binding domain of PLAU plays a role in controlling the suppressor function of Tregs towards Teffs. But detailed analyses rules out the possibility that the membrane-bound PLAU on Tregs promotes the suppressive function of Tregs via binding to PLAUR on Teffs. We therefore conclude that PLAU mediates the Treg suppressor function intracellularly rather than via binding to PLAUR on Teffs.

3. In the experiment in Fig. 5, the authors sorted PLAUlow and PLAUhigh Tregs, and expanded them by re-stimulation. It is required to show the stableness of the PLAU expression levels after the expansion.

Reply: We have performed further experiments in the new version. After staining PLAU antibodies, $PLAU^{high}$ and $PLAU^{low}$ Tregs were sorted from Tregs by using larger gap between $PLAU^{high}$ and $PLAU^{low}$ sorting gates in the histogram compared with that in the old version. As shown in Figure 5d, we have clearly shown the stableness of the PLAU expression levels after several-round expansions. We have also shown a clear higher FOXP3 and GARP expression in $PLAU^{high}$ *vs.* $PLAU^{low}$ Tregs.

4. The effect of PLAU on the expression of Treg-associated molecules was detected in Foxp3+ Tregs. It is needed to clarify whether the effect of PLAU is dependent on Foxp3. In addition, in Fig. 3, Foxp3negativePLAUhigh population was observed. The characteristics of the population is also required.

Reply: Our siRNA experiments have already shown that knockdown of PLAU in Tregs can significantly downregulate the expression of FOXP3 and several other important Treg genes. The new experiments about signaling pathways analyses show that PLAU mediates Treg suppressor function via STAT5 and ERK signaling pathways (Fig. 6). It is already known that STAT5 is required for FOXP3 expression in both human and murine Tregs. This supports the conclusion that the effect of PLAU on Treg suppressor function is dependent on FOXP3 and also several other important Treg genes via STAT5 signaling pathway.
In Figure 3d, only 2.38% of Tregs are $PLAU^{high}FOXP3^{negative}$ Tregs at day 3 post restimulation and only as few as 0.12% of Tregs are $PLAU^{high}FOXP3^{negative}$ Tregs at day 7 post restimulation by EBV-B cells. The reason why there are a relatively higher percentage of $FOXP3^{negative}$ Tregs at day 3 post restimulation by EBV-B cells is due to the presence of a small number of EBV-B cells which are in the same analyzed FSC and SSC gate as Tregs. The EBV-B cells also express PLAU. But at day 7, most of the irradiated EBV-B cells are dead and cannot be observed by FACS staining used here.
Furthermore, stable Tregs derived from sorted human $CD4^{+}CD25^{high}$ T cells and alloantigen-specific Teffs derived from sorted $CD4^{+}CD25^{-}$ T cells were from the same batch of Tregs and Teffs from our recent work (Probst-Kepper et al. 2009). The characterization and the high purity has been shown in our recent published work (Probst-Kepper et al., J Cell Mol Med. 2009 Sep;13(9B):3343-57). We have also further characterized the cells by some of the surface proteins such as CD127, CD45RO, IL1R1, GARP and so on as shown in Supplementary Figure S13.

Minor comments,

5. For the HTR genome-wide gene expression analysis, the authors sorted CD4+CD25high T cells as Tregs. It is required to show the purity of Tregs.

Reply: We have addressed this concern in the third paragraph of the response to point 4 raised by the current reviewer.

6. In the siRNA experiments in Fig. 6, knockdown of FAM13A1 should be confirmed.

Reply: In order to focus on discussing PLAU as suggested by the other reviewers, we have now removed the part of FAM13A1 and the corresponding figures.

Reviewer #2 (Remarks to the Author):

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In this report the authors use serial arrays following activation to generate a network of gene expression patterns. Many of the genes found by this method, and scored as "hubs", were known to be involved in Treg development and/or function (e.g., GARP), although it is surprising that FOXP3 was not found. Some of the other 'hub' genes were quite surprising, and require some explanation. One of these surprising genes, PLAU, is the focus of the biological section of the paper. The authors show that PLAU expression is increased in Tregs following activation to a higher extent that in Teff cells, although the increases seen in Tregs are modest at best. They also use siRNA knockdown to test its role in Treg function. Again, they only show very modest affects on the expression of a handful of Treg genes-possibly due to having rather inefficient knockdown of PLAU. Finally, they sort Tregs into PLAUlo and PLAUhi, and compare them functionally. The interesting aspect of this data is t
hat
the PLAUhi Tregs are more suppressive (although this data will need to be repeated using a CFSE dilution protocol). Again, the differences in the expression of other genes is very modest.

Reply: We agree with the reviewer that the expression difference for PLAU between Tregs and Teffs is not huge. But the difference is still statistically significant. One of the major advantages with the proposed network strategy is that we are able to identify important genes that do not necessarily stand out by only comparing the mRNA and/or protein expression differences (page 7, 8, 12).
We also agree with reviewer that the effects of a knockdown of PLAU on the expression of some important Treg genes is not dramatically big and is not turning black (0) to white (1) or *vice versa*. However, with only around 40% of PLAU expression knockdown, we can already observe around 30-40% downregulation of several important Treg genes. These effects are not huge but still significant as shown by *t*-test. Nevertheless, the combination of modest downregulation of several important Treg genes has eventually caused the functional change as shown in the old version and the current version.
In this work, we want to highlight that a combination of modest effects on several relevant genes might cause a significant functional or phenotypic effect. We also would like to point out that it might be also very important to look at the genes, e.g., PLAU with quantitative expression differences between Tregs and Teffs, rather than only to focus on the genes either expressed or not (1or 0).
In the old version of the manuscript, the difference in suppressor capability is significant but not very huge as shown by the [H3]thymidine uptake experiments of sorted $PLAU^{high}$ and $PLAU^{low}$ Tregs. But by extending the co-culturing time and by checking specifically the proliferation of Teffs, the CFSE dilution experiments show a more clear and significant result. Furthermore, by using a specific PLAU inhibitory antibody, we have also demonstrated a clear impairment of Treg suppressor function as shown by CFSE results. In short, by modulating PLAU activity, we indeed observe a clear and significant effect on Treg suppressor function.

WHile somewhat interesting, there are several aspects of this work that need to be addressed before any consideration for publication can be made

1. The authors mention that the Tregs and Teffs used are alloantigen specific, but give no details. They need to provide more experimental detail as to how the Tregs were isolated and where they come from.

Reply: To further clarify alloantigen-specific information of Tregs/Teffs, we have made changes in the section 'Generating HTR dynamic transcriptome' (page 4) of the new version. In the old version, our writing might cause a misunderstanding. In fact, stable Tregs derived from sorted human $CD4^{+}CD25^{high}$ T cells and alloantigen-specific Teffs derived from sorted $CD4^{+}CD25^{-}$ T cells were from the same batch of Tregs and Teffs from our recent work (Probst-Kepper et al. 2009). The long-term responses of these T cells following activation after days have already been characterized (Probst-Kepper et al. 2009). The characterization and the high purity has been shown in our recent published work (Probst-Kepper et al., J Cell Mol Med. 2009 Sep;13(9B):3343-57). All the stable Tregs and Teffs were expanded and restimulated by using irradiated allogeneic Epstein-Barr-virus (EBV)-transformed B cells (EBV-B cells). We have also mentioned EBV-B cells in the main text and the Methods in the new version, although all the details have already been given in the above cited work. Furthermore, we have also characterized the cells by some of the surface proteins such as CD127, CD45RO, IL1R1, GARP and so on as shown in Supplementary Figure S13. Note that we

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have additionally isolated fresh Teffs for the suppression assay purpose as indicated in the manuscript (see Methods, page 13).

2. They really need to at least speculate on how PLAU (and its receptor) can regulate Treg function.

Reply: In the new version, we have intensively addressed the mechanisms through which PLAU mediates Treg suppressor function. For details, please see the main text and figures (Fig. 6 and Supplementary Figs. S5-S9, page10-13,) and also the detailed responses to point 2 of reviewer 1.

3. They need to determine whether the PLAUlo/hi Tregs represent natural and induced Tregs

Reply: The Tregs used in this work are from the same batch of Tregs from our recent work (Probst-Kepper et al., J Cell Mol Med. 2009 Sep;13(9B):3343-57).
All of our Tregs are naturally derived Tregs rather than induced Tregs (iTregs) because our Tregs are GARP positive, which do represent naturally derived Treg but not iTreg (J Cell Mol Med. 2009 Sep;13(9B):3343-57, Eur J Immunol. 2009;39:3315–22, and Proc Natl Acad Sci U S A. 2009;106:13439–44). Furthermore, GARP positive Tregs are demethylated on the so-called Treg-specific demethylated region (Transfus Med Hemother. 2011 Oct;38(5):287-291, and Genome Res. 2009;19:1165–74).

4. There is not indication in the siRNA experiments as to cell viability

Reply: When we performed the FACS analysis, we set up the gates based on the FSC and SSC area of viable Treg cells (after removal of B cells). Furthermore, additional experiments show that more than 99.8% of the analyzed gated Tregs are viable at day 3 post siRNA electroporation treatment (page 8) (Supplementary Fig. S4).

5. Figure 6 needs to be split into 2 figures (A-E in one, and F-I in the other)

Reply: In order to focus on the PLAU story, we have removed the FAM13A1 parts from the text and the corresponding figures as suggested.

6. The Fam13 data needs to either be examined in greater detail or removed from the manuscript.

Reply: We have removed FAM13A1 data from the current manuscript.

7. All the differences they see in gene expression following siRNA knockdown with either gene are the same. This suggests that the differences are gene independent-they need to knockdown a broadly expressed gene to see if the effect on gene expression is the same as that seen for PLAU and FAM

Reply: As shown in Figs. 4 and 5, and in the main text (page 9), we observed only 5 or 6 out of the 11 analyzed genes showing a significant downregulation. Furthermore, we have actually knocked down a non-candidate gene for other purposes but did not observe a significant and reproducible effect on even 1 gene out of the analyzed 11 genes except for the target gene to be knocked down. The non-candidate genes are those not ranked as top 200 candidates according to our network strategy.

Reviewer #3 (Remarks to the Author):

This manuscript reports on a time-resolved transcriptome study of human regulatory (Treg) and effector (Teff) CD4+ T cells. Using a heuristic, correlative approach for deriving 'gene networks' from the data, two genes - PLAU and FAM13A1 - are identified that have so far not been described in the context of Treg function. The function of these two genes is then investigated further using genetic tools. PLAU is shown to affect the suppressive capacity of Treg cells, albeit in a somewhat complicated fashion: Low PLAU levels reduce suppression of Teffs in humans and older mice, whereas PLAU-/- Treg cells from younger mice have better suppressive capacity. No role of FAM13A1 for suppression by Tregs is shown, although its knockdown seems to affect a few other Treg-relevant genes. As the suppression assay is standard, one is led to suspect that FAM13A1 does not have much of a role here.

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Overall, this is an interesting effort that goes a very considerable way from computational analysis of time-resolved transcriptome data to the experimental characterization of a new candidate gene (PLAU) implicated in Treg function. The mechanism(s) by which PLAU affects Treg activity are not investigated.

Reply: Thanks for pointing out the issue of FAM13A1. The reason why we did not perform suppression assays to check the role of FAM13A1 gene on the suppressive function of Tregs by siRNA experiments is due to the in-born effects of siRNA electroporation transfection on Treg suppressor function. We showed the effects of PLAU on Treg suppression by other experimental techniques, also rather than by siRNA treatment. PLAU is a membrane-bounded protein in Tregs with well-established (inhibitory or detecting) antibodies and therefore we have more loss-of-function options. We have now removed FAM13A1 data from the current manuscript to focus on the PLAU story. We have also performed a substantial amount of new experiments to explore the mechanisms through which PLAU mediates Treg suppressor function.

Critique:

(1) Regarding the bioinformatic analysis, it is sometimes difficult to understand in detail what has been done. Fig. 1 is helpful as an outline of the general procedure, which appears to be sound. However, the description in the text must be improved. In particular, the Methods section on Functional Network Reconstruction ... (p. 15) is nearly incomprehensible. Consulting a native English speaker might help. In the following, I list a few of the unclear points. Some of these points are minor, but as they accumulate in places they make the text difficult to read.

Reply: We have tried to revise the section 'Functional network reconstruction' into a more comprehensible way (page 16-17).

- The term "highly reliable ... gene networks" (p. 5) is misguided, as it would be exceedingly difficult to establish in which sense a 'gene network' generated by this heuristic analysis is biologically correct (assuming that this is meant with "reliable"). What the method could do is filter out interesting candidates for further experimental study; this is, of course, how it is used here.

Reply: We agree with the reviewer that the term 'highly reliable' is not so accurate. In the mentioned sentence (page 5) and the first paragraph of the section 'Discussion', we removed the term 'highly reliable'.

- A combination of Local clustering (by Qian et al.) and Trend correlation (by two of the authors of this manuscript) was used in combination. Why?

Reply: We'd like to utilize the advantages of both methods because none of the methods with different detecting principles have the full power to identify all the potential functional linkages between genes. We had shown the complementation of the two methods in the Trend correlation methodology paper (He and Zeng, 2006). A combination of the two methods can identify the potential functional linkages over time-series data which can not be found by one of them but might be found by another one.

- In the Methods section, P_LC1 and P_LC2 are not explained.

Reply: In this version, we have explained the symbols 'P_LC1' and 'P_LC2' (page 17).

- Methods: "For the TC method, we introduced another adapted P-value (P_TC2) calculation based on the resulted sc and cc scores." What has actually been done?

Reply: As required, we have explained the P_TC2 in a more detailed way in Supplementary figure S16 in order to simplify the main text. At the same time, we have also put one sentence 'Briefly speaking, the reason to reorder the distribution table is that we found that some of the pairs of two genes, which have a very high cc score but the sc score is not very high, might still have a high chance to be potentially functionally linked' in the main text (page 16) to explain the rational why we do so.

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- What sense does it make to combine the P_TC and P_LC values in the min criterion (last lines, p. 15)?
Reply: It might be easier to answer this question if one looks at the whole equation which describes several following conditions:
1, the terms P_TC<=0.05 OR P_LC<0.05 are to make sure that at least one of the two methods show a significant correlation between a pair of two genes.
2, the terms P_TC1 (P_LC1)<=0.05 AND P_TC2(P_LC2)<=0.05 are to make sure the reproducibility of the results among the two repeated time-series data.
3, the term combining P_TCs and P_LCs <=1E-9 is to make sure that we can utilize the advantages of both methods. When the P-values from one of the two methods are not very small with a certain limitation of the method but the P-values from another method might be very small, multiplication of these P-values can reach a very significant P-value and therefore indicate the high possibility that there is a certain functional association between the two genes. This is why we combine P_TC and P_LC values.

- p. 5: "... a robust correlation between the extent of expression correlation ..." Do the authors really mean a correlation of correlations?

Reply: Thanks for pointing out this mistake. We have revised it into 'a robust correlation between the expression of genes over time' (page 5).

- p. 5: The significance of the fact that a handful of T-cell related GO categories are among the 212 enriched ones is not clear.

Reply: We'd like to show the co-occurrence of two genes in the annotated same biological processes for predicted functional linkages (Page 5). We have found the co-occurrence was significant in 212 GO categories and several T-cell related pathways were among these categories. The purpose to show a handful of T-cell related GO categories is to demonstrate that the expected pathways (T-cell pathways) were found by us. But there are many other more general immune response related pathways, such as inflammatory response, which also show significance in Supplementary Table 2. Many other pathways, eg., cell cycle regulation, regulation of cell shapes, were also activated during the Treg stimulation process and have been found by us. These general pathways might be activated in the activation process of any dividing cell type. We do not expect to see that most of the significantly-enriched pathways are T-cell related GO categories because a significant correlation among two genes in any activated pathway, not necessarily restricted to T-cell related pathways, will be found by our strategy.

- In Table 4, it is not clear whether a putative GARP-dependent gene that is upregulated (or downregulated) in Tregs vs Teffs is also upregulated (downregulated) in GARP-overexpressing Teffs. The table only records whether the gene's expression was changed in GARP-overexpressing Teffs, so it could be changed in the opposite way to its expression in Treg cells. The full information should be given.

Reply: We agree with the reviewer that it is unclear whether a putative GARP-dependent gene that is upregulated (or downregulated) in Treg vs. Teffs is also upregulated (downregulated) in GARP-overexpressing Teffs. But the purpose of that section is to show whether those genes linked to GARP were GARP-dependent. We therefore just need to check whether those genes linked to GARP were up or down regulated. An upregulation (or downregulation) of genes in GARP-overexpressing Teffs not necessarily coincides with an upregulation (or downregulation) of the same genes in Tregs vs. Teffs. This is due to the fact that the GARP-overexpressing Teffs, although acquiring some Treg characteristic, are quite different from Tregs, and therefore the system GARP-overexpressing Teffs *vs.* Teffs and the system Tregs *vs.* Teffs have no direct relationship. Upon acceptance, a complete list of the significantly-upregulated and/or -downregulated genes in Tregs *vs.* Teffs and the other related information will be all available in our webpage and/or the corresponding GEO datasets.

- p6: The wording "under transcriptional control of GARP" is misguided as GARP appears to be a plasma membrane protein and has not been described as a regulator transcription.

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Reply: We have changed this misleading term into 'dependent on GARP expression' (page 6).

- The 'combinatorial' time-point-wise way to establish expression differences between Treg and Teff cells is explained for two time points (p. 16). How has this been applied to the entire time series? The authors will be certainly aware of established statistical tests of whether two time series are different that operate on the entire time course at once. Why has this point-wise approach been used here? What motivates the equation for P_e (p. 16)? It appears that the issue of whether two time series are different given a certain measurement error is not given proper attention in the manuscript.

Reply: We might not write this issue clearly, which might cause a misunderstanding. The expression differences between Tregs and Teffs were never explained for two time points (page 17 in the new version). We used paired Student's *t*-test to check whether the overall gene expression was different between Tregs and Teffs during the measured period. We have generated Student's *t*-test P-values, Pt1, Pt2, Pt3 and Pt4 for the four comparison pairs, Treg1 and Teff1, Treg1 and Teff2, Treg2 and Teff1, Treg2 and Teff2, respectively. At the same time, we have also compared the mean expression between the four pairs. A significantly-differential expression is defined for one gene only if all the four P-values are significant and all the four comparison pairs between average expression levels show the same relationship (> or <) as well as the number of absent calls in each of the two repeated time-series data (Treg or Teff data, dependent on up or down regulation) is equal to or less than 10. Another required criterion is that the average expression of the given gene in each of the two repeated time-series data (Treg or Teff data, dependent on up or down regulation) need to be higher than 50 to avoid the case that the gene expression level in both Tregs and Teffs are very low but still show differences. Based on such strict combinatorial criteria, we have high confidences in finding significantly differentially-expressed genes. Because the two repeated time-series datasets for Tregs (or Teffs) are already different in the beginning when the activation process cannot start at the exact same stages and situations for Tregs (or Teffs), we do not think it is very reasonable to average the two time series data, calculate standard deviations and then compare them by considering measurement errors. From this point of view, we believe a pair-wised comparison over the whole time period between a limited number of different time-series datasets by a statistic test is a more suitable strategy which has actually been quite successful as shown in this work (the last paragraph of section 'generating HTR dynamic transcriptome', even not mentioning the discovery of PLAU).

- Equations and inequalities in the Methods section should be typed as separate items (and not within the text) to enhance readability.

Reply: We have already revised them in the current version as suggested.

- Figure 1. The legend to part d needs revising.

Reply: We have revised it in this version.

(2) For the Teff proliferation assay, CFSE staining and detection by flow cytometry should be used instead of the H3-thymidine incorporation assay. The former is standard in the field and will allow comparison of the Treg suppression assays in the manuscript with published data. In particular, CFSE staining allows inferring the fraction of dividing cells and the extent of division.

Reply: Thanks for this constructive suggestion. In this version, we have performed CFSE experiments and the major results still remain the same (for details, see Methods and Figs. 5, 6 and 7 and the text in page 9-12). Although the established cultured human Teffs are quite homogenous and have similar cell cycle stage and consequently show much less clear peak divisions, the fresh murine T cells are heterogeneous and shows very clear CFSE division peaks.

(3) For comparing two flow-cytometry distributions, strictly speaking they must be normalized to have the same areas under the curves. In many cases, this is not necessary to see differences (e.g., Fig. 4b). However, it is vital in Fig. 5e. Contrary to the claim in the manuscript, I suspect that normalization will reveal no difference between the two FoxP3 distributions.

Reply: After normalizing them into the same unit area, we still see a difference in FOXP3 expression between the two subsets of Tregs. In this version, we additionally performed a

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substantial amount of experiments and sorted PLAU^{high} and PLAU^{low} Tregs from Tregs by increasing the gap between PLAU^{high} and PLAU^{low} sorting gates in the histogram. As shown in Figure 5d, we have clearly shown the stableness of the PLAU expression levels in PLAU^{high} vs. PLAU^{low} Tregs after several expansions. We have also shown a clear higher FOXP3 and GARP expression in PLAU^{high} Tregs vs. PLAU^{low} Tregs in the current version.

(4) Contrary to the claim on p.4, there is no substantial downregulation of genes in the TCR pathway visible in Suppl. Fig. 1. Some important genes (e.g. CD28) are even higher in Tregs. It would be good to see a list of which genes are supposed to be downregulated in Tregs alongside with the time-series plots.

Reply: In the new version, we have included the list of downregulated genes in the figure legend of Supplementary figure S1. Note that, some of the official gene names in the legend are not identical to the gene symbols indicated in Supplementary figure S1. Meanwhile, the names of differentially-expressed genes also displayed nearby the time-series expression patterns of the corresponding places of the signaling pathway of Figure S1. We cannot explain why some important genes such as CD28 show higher expression in Tregs than Teffs. However, it is acceptable that a down or upregulation in a pathway not necessarily happens to every gene in the pathway in order to achieve a cellular function and a much more complicated rather than unified pattern occurs. In the text, we claimed that the downregulated genes were significantly enriched in TCR pathway but we admitted that not each gene in TCR pathway was downregulated in Tregs vs. Teffs.

Minor points:

(5) Throughout the paper, it was never quite clear whether unstimulated Teffs and Tregs were included as time point 0. The resolution of Suppl. Fig. 2 is very poor, but there seems to be no time point 0.

Reply: Thanks for pointing out this mistake. We have now revised it and stated it clearly in Figure legends in the new version. In all our figures, time point 1 is the activation starting time zero.

(6) Why was there no attempt to distinguish between naïve Teff cells and memory cells (which could be done by sorting)? Very likely, there are memory cells in the culture and so it is not surprising - but rather expected - to see fast expression of effector cytokines (see p. 4 "..surprisingly, it is within 1h but not after days ..."). Also, Treg cells do express cytokines (e.g., IL-10).

Reply: We have made this attempt and found that almost all the cultured Tregs and Teffs become CD45RO^{+} memory T cells after several-round exposures to antigen presenting cells which are EBV-B cells in our work (Page 4 of the text) (Supplementary Fig. S13a).
We agree with the reviewer that memory cells respond much faster than naïve T cells. However, according to our knowledge, the immediate fast response of those effector cytokines has never been shown in the publication by a high-time-resolution time-series mRNA analysis. Interestingly, the immediate response of some cytokines seems to only happen to Teffs but not Tregs although they are all memory T cells. This is why the difference immediately showed in 1 h.

(7) The authors should discuss carefully what is known about the role of antigenic stimulation for the suppressive activity of Treg cells.
Reply: There are two different situations, 1) antigen (TCR) stimulation of naive Tregs does represent a prerequisite to induce their suppressor activity (like in case of ex vivo Treg cell activation; J Immunol. 2000 Jan 1;164(1):183-90;Mayer et al., Plos One 2012;7(1):e29355); 2) in case of antigen-experienced Treg cells (like a Treg line or Treg clone), TCR stimulation still induces a "suppressor program/signature". Thus it is still necessary to enhance their suppressor activity. The allogeneic B cells represent the antigenic stimulation, since the Tregs are alloantigen-specific towards these EBV-B cells. In-vitro culture of any T cells does need a serial restimulation of the T cells with their cognate antigen (in our case they are allogeneic EBV-B cells) for survival and expansion, which leads to the up-regulation of Treg activation markers like GARP or CD69 or HLA-DR and down-regulation thereafter in a periodical way (Cancer Res. 2009 May 15;69(10):4335-45, J Cell Mol Med. 2009 Sep;13(9B):3343-57).
We did not discuss this point in the text because we have already addressed the issues in our recent published work (J Cell Mol Med. 2009 Sep;13(9B):3343-57). Furthermore, it has been already

known that resting Tregs must be activated to acquire functional suppressor activity (see review, J. Immun. 2011, Apr 15;186(8), 4535-40).

2nd Editorial Decision 12 September 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who agreed to evaluate this resubmitted study. As you will see the referees were somewhat split in their assessment. The second reviewer raises some important concerns, which we feel are sufficient to preclude publication of this work at present.

Reviewer #1 (previously Reviewer #2), an expert in Treg biology, feels that the new experimental evidence and other revisions had substantially improved this work, and s/he is now clearly supportive of publication. The other previous reviewers were not available to evaluate this work.

This work was also evaluated by a new referee (Reviewer #2), a bioinformatics expert. His/her appraisal was less positive, but we feel that the points raised, while clearly important, may be addressable with some conceptual clarification and additional computational analysis. Broadly this reviewer seemed to have two main concerns:

1. The distinction between correlation-based coexpression networks vs more mechanistic gene regulatory networks.

In this regard the reviewer took issue with the frequent use of the word "functional" when describing the networks. Clearly, coexpression networks can be used to derive functional insights, as evidenced by the PLAU-related experiments in this work. Nonetheless, we agree that this is a valid and potentially important conceptual distinction, which should be made more clear to readers. Specifically, linkage between genes in the network do not necessarily indicate direct mechanistic regulatory connections. This issue is somewhat semantic, but is non-trivial in regards to how future scientists might use these networks.

2. Seemingly arbitrary thresholds, parameters, or network construction choices.

While the customized nature of the network construction methods do not reduce the value of the biological insights derived from this analysis, these points are important for future scientists that might wish to apply this methodology in their own settings, or who may wish to perform a deeper analysis of these networks. Both are important for the scope of Molecular Systems Biology. Some additional effort should be made to determine how changing parameters and thresholds might affect the resulting networks and biological conclusions (i.e. robustness), and to better justify the network construction methods and parameters.

Given the positive evaluation of the experimental component of this work, we are hopeful that these points may be potentially addressable, and as such we would like to offer you another opportunity to respond to these issues and to prepare a final revised manuscript.

Please also see our journal's policies on data release (<http://www.nature.com/msb/authors/index.html#a3.5>). In particular, all new high-throughput datasets should be deposited in an appropriate public repository, and the resulting accession numbers and confidential reviewer logins should be incorporated into the Methods section of this work.

Please resubmit your revised manuscript online, with a covering letter listing amendments and responses to each point raised by the referees. Please resubmit the paper **within one month** and ideally as soon as possible. If we do not receive the revised manuscript within this time period, the file might be closed and any subsequent resubmission would be treated as a new manuscript. Please use the Manuscript Number (above) in all correspondence.

In addition, I would like to acknowledge that this decision took somewhat longer than usual, due to

some delays in finding available peer referees. I apologize for the delay. Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed the issues raised by the previous submission and the manuscript is now acceptable for publication.

Reviewer #2 (Remarks to the Author):

In this review I want to confine myself to the computational aspects of the manuscript.
The authors use a crude heuristic procedure that is based on expression associations that are different in Treg and Teff cells. A "network" of Treg specific associations is constructed by thresholding and hub genes are identified among them PLAU the gene that is later followed up in biological evaluation experiments.

Overall I do not feel that the computational aspects are a strength of the manuscript.

- The method is solely based on pair wise dependencies of genes expression values (coexpression). No network reflecting the full multivariate dependencies is constructed.
Coexpression is measured with non-standard measures developed by some of the authors, nevertheless also these measures can only reflect that the genes are coexpressed. Functional interpretations are highly speculative and the excessive use of the term "functional" has no theoretical justification in this method.

- The network is build by thresholding pair wise associations scores (p-values). This discretization discards information relative to the matrix of pair wise scores. It appears that it is done solely for being able to draw networks. A biological interpretation of the networks is difficult.

- Building the "T-reg specific" network by collecting edges only found in the Treg network is sub-optimal. It is well possible that many of the edges are based on data that is very similar in both cell types. This is the case if the Treg score was slightly above the threshold and the Teff score slightly below. It appears better to test whether the differences of association is significant between the two cell types.

- The authors used many criteria and parameter settings without giving any justifications (page 17 is large collection of such arbitrary choices)

1st Revision - authors' response 01 October 2012

We have considered the constructive suggestions and comments from the reviewers and the editor, and have performed some additional computational analyses to address all concerns. In the new version, we have improved the manuscript by the following measures:

1, We performed additional robustness analyses to demonstrate that the choice of thresholds and criteria is not arbitrary. We actually show that our strategy is quite insensitive to the choice of P-value thresholds.

2, We performed additional analyses to show that the difference of correlation degree for almost all of the interactions in Treg-specific correlation network is very large between Tregs and Teffs.

3, We have rewritten the section of “correlation network construction and analysis” in the Methods to describe the rationale and to give clear justifications of the criteria and parameter setting. We have also systematically changed the term ‘functional network” into ‘correlation network’ as already shown in title. In addition, we have added a paragraph and references in the Introduction to discuss multivariate dependencies and transcription regulatory networks in order to clarify the concept we used.

You also find enclosed a reply to the reviewers´ comments, point-by-point. A confidential review login link has been provided in page 20 of the section ‘data availability’.

We hope that the revised version of the manuscript will now meet the publication criteria for *Molecular Systems Biolog*y and are looking forward to your response.

Point-by-point responses to reviewers’ comments.

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed the issues raised by the previous submission and the manuscript is now acceptable for publication.

Reviewer #2 (Remarks to the Author):

In this review I want to confine myself to the computational aspects of the manuscript.
The authors use a crude heuristic procedure that is based on expression associations that are different in Treg and Teff cells. A "network" of Treg specific associations is constructed by thresholding and hub genes are identified among them PLAU the gene that is later followed up in biological evaluation experiments.

Overall I do not feel that the computational aspects are a strength of the manuscript.

Reply: We agree with the reviewer that the computational aspects are not the focus of this manuscript. The major focus of this work is on the experimental side, to validate experimentally-testable key candidate genes inferred by using a simplified but highly efficient computational approach, which cannot be easily obtained by standard experimental differential expression analysis.

- The method is solely based on pair wise dependencies of genes expression values (coexpression). No network reflecting the full multivariate dependencies is constructed.
Coexpression is measured with non-standard measures developed by some of the authors, nevertheless also these measures can only reflect that the genes are coexpressed. Functional interpretations are highly speculative and the excessive use of the term "functional" has no theoretical justification in this method.

Reply: We appreciate that the reviewer helped us to point out that the term “functional” might be misleading. We have now systematically changed the term “functional network” into “correlation network”. Only in a few places we used another term “functional association network”, which also indicates significant correlation among genes as some of our coauthors and many others demonstrated (Lee et al, 2004; Basso et al, 2005; He&Zeng 2006; Huttenhower et al, 2006; Lee et al, 2007; Langfelder&Horvath 2008; Ruan et al, 2010).

The reviewer commented that we measured coexpression with non-standard measures. In this work, we have not only used our own method (the trend correlation (TC) method) but also combined another well-established method (the local clustering (LC) method from Mark Gerstein group, (Qian

et al, 2001)). The later has already been widely used for time-series analysis and well appreciated as recently reviewed by Bar-Joseph et al (Nature Review Genet, 2012, 13: 552-564) (Bar-Joseph et al, 2012). The TC method has also been demonstrated to be useful in identifying potential functional association from time-series data and to be complementary to the LC method based on extracting main features of the change trend and the change level of gene expression between consecutive time points (He&Zeng 2006; Bergmann 2010). The TC method has also been appreciated as 'a precursor to more formal statistical models' (Bergmann 2010). Due to different principles behind the LC and the TC methods, we here therefore combined both methods to complement each other in order to identify more complete potential functional associations between genes (paragraph 2 of page 17). We have now added a new paragraph in the section of "Correlation network construction and analysis" in the new version (paragraph 2 of page 17) in order to explain why we used both methods.

We are aware of more sophisticated approaches for inferring gene association networks by taking into account multivariate stochastic dependencies among genes, for instance via partial correlation (also known as covariance selection) (Waddell&Kishino 2000; Toh&Horimoto 2002; de la Fuente et al, 2004; Schafer&Strimmer 2005). However, the corresponding concepts do not easily transfer to our case of time-series data, to the best of our knowledge, at least not in a sense that would be helpful to our work here (e.g., by partial autocorrelation function (Box et al, 2008) and Granger causality test). In addition, some efforts have also been made to identify synergistically interacting genes by multivariate dependency analysis (Anastassiou 2007). However, these interactions form undirected correlation networks, which also cannot provide direct 'functional' evidence.

As for directed causal transcription regulatory networks, multiple regulators might function synergistically in controlling the same target genes (Margolin et al, 2006; Huynh-Thu et al, 2010; Margolin et al, 2010). However, to identify highly accurate experimentally-testable transcription regulatory networks in mammalian cells purely based on expression data is very challenging (Walhout 2011; Bar-Joseph et al. 2012; Della Gatta et al, 2012; Quo et al, 2012; Stelniec-Klotz et al, 2012) and is not the focus and the purpose of the current work.

We are, nevertheless, planning to pursue such an advanced methodological analysis including multivariate analysis and time series data (and possibly also causality) in future work, but this is beyond the current paper which has a strong experimental flavor as we argued above.

While multivariate dependency indeed is a very important but challenging issue in the field of network construction, simplified correlation networks have already demonstrated to be very useful (Basso et al. 2005; Lee et al. 2007). Furthermore, our analysis demonstrates the power of pairwise correlation networks as evidenced by the experimental results of PLAU.

We have now added one paragraph and some other sentences in the Introduction to discuss 'multivariate dependencies' and some recent advances to further clarify that 'we are constructing a pairwise correlation network' (paragraph 3 of page 3, the second sentence of paragraph 2 of page 4). We have also clarified this in title, abstract, Methods (page 17), Figure 1 and the legend of Figure 1 (page 25).

- The network is build by thresholding pair wise associations scores (p-values). This discretization discards information relative to the matrix of pair wise scores. It appears that it is done solely for being able to draw networks. A biological interpretation of the networks is difficult.

Reply: We agree with the reviewer that we built up the network by thresholding pair wise association scores (P-values). However, we do not fully understand what the reviewer suggested by "matrix of pairwise scores", but presumably s/he referred to an approach that takes into account multivariate dependencies along the lines discussed in the above point, such as partial correlation (which requires inverting the correlation matrix). It would have been indeed straightforward to apply such a (linear) correlation-based method for detecting pairwise gene associations with higher precision (and presumably with less false positives). However as we argued above, the focus of this work is on experimental side and we decided to choose simpler approaches rather than more sophisticated multivariate analysis techniques. In addition, in this work, we are providing other means for dealing with potential large false positive rates, such as very stringent combinatory thresholds, requiring reproducibility among replicates and so on as detailed in text (Supplementary

Figure 2, the last paragraph of page 5, paragraph 3 of page 17 and paragraph 1 of page 18). The accuracy of the constructed network has been shown by comparing our network with pathway databases and additional validation experiments for GARP (paragraph 1-4 of page 6). The successful identification of PLAU and other top hub genes is the most convincing evidence supporting the accuracy of the constructed network (the last two paragraphs of page 7 and the first two paragraphs of page 8). We believed that a simple correlation based pruning algorithm would be sufficient for our goal to predict novel key genes from pairwise correlation networks.

The reviewer commented that "it is done solely for being able to draw networks". The purpose of our work is not just to draw networks, but to utilize the constructed correlation network as a hypothesis generator to come up with new testable ideas concerning the function of individual genes in Tregs. The prediction of the novel key gene PLAU as described in this work is a nice example that our work is beyond just drawing a network (page 6-8, the section "Identifying PLAU…").

The reviewer also raised the concern that the biological interpretation of the networks might be difficult. The most straightforward biological interpretation is that the genes directly linked in our correlation network might be functionally associated. This is clearly visible by comparing our network with GO terms (paragraph 1 of page 6). In addition, genes surrounding the hubs in specific subnetworks are likely to be involved in similar biological processes or pathways as the hub genes. This has been demonstrated for the subnetworks of FOXP3 and GARP (paragraph 2-4 of page 6). Furthermore, applying our strategy (page 6-8) to different sets of genes (rather than the set of 400 genes used in this work) related to diseases in which Tregs might be involved, will also allow the identification of new key genes related to those diseases.

- Building the "T-reg specific" network by collecting edges only found in the Treg network is sub-optimal. It is well possible that many of the edges are based on data that is very similar in both cell types. This is the case if the Treg score was slightly above the threshold and the Teff score slightly below. It appears better to test whether the differences of association is significant between the two cell types.

Reply: Thanks for raising this interesting issue, which helped us to design further analyses.

If we simply test whether the differences of association is significant between the two cell types, the pairs of genes, which are not significantly correlated in both cell types, e.g., with no significant correlation in Tregs and far less significant correlation in Teffs, will still show a significant difference. This type of linkages (pairs) are clearly not what we need since we are trying to construct a Treg-specific correlation network and all the interactions need to be significantly correlated in Tregs. Due to this problem, we cannot simply generate a distribution curve of correlation differences (delta) to define linkages according to a simplified confidence level. We are therefore keen to use the combinatorial thresholding method to construct the Treg-specific correlation network in this work although it might be not perfect.

Furthermore, we did additional analyses to check whether the cases mentioned by the reviewer were rare or not and added some sentences to address this issue (paragraph 1 of page 18). As shown in Supplementary Figure 17 (page 25 in supplementary file), more than 99% and 95.5% of all the Treg-specific correlation linkages show around 3- and 10-fold less P-value, respectively, in average for each of the four individual P-values in Tregs than in Teffs (for more details, see paragraph 1 of page 18). This means that most of the linkages identified in Treg-specific correlation network are very significantly correlated in Tregs but are far less significant in Teffs.

For the remaining small percentage (<1%) of Treg-specific correlation linkages, only one of the two replicates showed a very significant correlation in Teffs but another one showed no significance, which still causes a very significant final product of P-values integrated from replicates of Teffs. These interactions were included in Treg-specific correlation network as they did not show reproducible results between replicates in Teffs (see paragraph 1 of page 18). The occurrence of this small fraction is an additional argument for not simply testing differences of association degree, which will reject this small fraction.

We have now revised the section of network construction to make it more clear (see the last paragraph of page 17 and paragraph 1 of page 18). In fact, in addition to the criteria of integrated P-

values, we only considered the pairs, which reproducibly showed a significant correlation in all the replicates of Tregs but not in Teffs, as Treg-specific correlation linkages.

- The authors used many criteria and parameter settings without giving any justifications (page 17 is large collection of such arbitrary choices)

Reply: We have now re-written the whole section "Correlation network construction and analysis" to describe the logic and to give clear justifications of the criteria and parameter settings (page 17-19 in the new version, with 2 more pages than the older one). In fact, we used standard well-established P-value cutoffs and criteria, which were not described clearly in the old version.

To further justify the choice of the thresholds and to check threshold robustness, we performed additional robustness analyses to examine whether a reasonable variation of the P-value thresholds would cause significant changes in the total number of correlation linkages of the Treg-specific correlation network and in the predicted functional importance of key candidate genes (see paragraph 2 of page 18). As shown in Supplementary Figure 18 (page 26 in supplementary file), we found that an order of magnitude variation of thresholds [0.01, 0.1] did not significantly change the resulting network and the biological conclusions with respect to the predicted functional importance of PLAU and GARP (for details, see paragraph 2 of page 18).

In summary, the choice of criteria and thresholds is not arbitrary (page 17-19, the whole section of the new version). In fact, our strategy is quite robust to the choice of P-value thresholds (paragraph 2 of page 18).

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References

Anastassiou D (2007). Computational analysis of the synergy among multiple interacting genes. Mol Syst Biol 3: 83.

Bar-Joseph Z, Gitter A and Simon I (2012). Studying and modelling dynamic biological processes using time-series gene expression data. Nat Rev Genet 13: 552-564.

Basso K, Margolin AA, Stolovitzky G, Klein U, Dalla-Favera R and Califano A (2005). Reverse engineering of regulatory networks in human B cells. Nat Genet 37: 382-390.

Bergmann D. (2010). "Genetic network modelling and inference [Doctoral dissertation]." from http://etheses.nottingham.ac.uk/1209/.

Box GEP, Jenkins GM and Reinsel GC (2008). Time series analysis : forecasting and control. Oxford, Wiley.

de la Fuente A, Bing N, Hoeschele I and Mendes P (2004). Discovery of meaningful associations in genomic data using partial correlation coefficients. Bioinformatics 20: 3565-3574.

Della Gatta G, Palomero T, Perez-Garcia A, Ambesi-Impiombato A, Bansal M, Carpenter ZW, De Keersmaecker K, Sole X, Xu L, Paietta E, Racevskis J, Wiernik PH, Rowe JM, Meijerink JP, Califano A and Ferrando AA (2012). Reverse engineering of TLX oncogenic transcriptional networks identifies RUNX1 as tumor suppressor in T-ALL. Nat Med 18: 436-440.

He F and Zeng AP (2006). In search of functional association from time-series microarray data based on the change trend and level of gene expression. BMC Bioinformatics 7: 69.

Huttenhower C, Hibbs M, Myers C and Troyanskaya OG (2006). A scalable method for integration and functional analysis of multiple microarray datasets. Bioinformatics 22: 2890-2897.

Huynh-Thu VA, Irrthum A, Wehenkel L and Geurts P (2010). Inferring regulatory networks from expression data using tree-based methods. PLoS ONE 5.

Langfelder P and Horvath S (2008). WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 9: 559.

Lee I, Date SV, Adai AT and Marcotte EM (2004). A probabilistic functional network of yeast genes. Science 306: 1555-1558.

Lee I, Li Z and Marcotte EM (2007). An Improved, Bias-Reduced Probabilistic Functional Gene Network of Baker's Yeast, Saccharomyces cerevisiae. PLoS ONE 2: e988.

Margolin AA, Nemenman I, Basso K, Wiggins C, Stolovitzky G, Dalla Favera R and Califano A (2006). ARACNE: an algorithm for the reconstruction of gene regulatory networks in a mammalian cellular context. BMC Bioinformatics 7 Suppl 1: S7.

Margolin AA, Wang K, Califano A and Nemenman I (2010). Multivariate dependence and genetic networks inference. IET Syst Biol 4: 428-440.

Qian J, Dolled-Filhart M, Lin J, Yu H and Gerstein M (2001). Beyond synexpression relationships: local clustering of time-shifted and inverted gene expression profiles identifies new, biologically relevant interactions. J Mol Biol 314: 1053-1066.

Quo CF, Kaddi C, Phan JH, Zollanvari A, Xu M, Wang MD and Alterovitz G (2012). Reverse engineering biomolecular systems using -omic data: challenges, progress and opportunities. Brief Bioinform 13: 430-445.

Ruan J, Dean AK and Zhang W (2010). A general co-expression network-based approach to gene expression analysis: comparison and applications. BMC Syst Biol 4: 8.

Schafer J and Strimmer K (2005). An empirical Bayes approach to inferring large-scale gene association networks. Bioinformatics 21: 754-764.

Stelniec-Klotz I, Legewie S, Tchernitsa O, Witzel F, Klinger B, Sers C, Herzel H, Bluthgen N and Schafer R (2012). Reverse engineering a hierarchical regulatory network downstream of oncogenic KRAS. Mol Syst Biol 8: 601.

Toh H and Horimoto K (2002). Inference of a genetic network by a combined approach of cluster analysis and graphical Gaussian modeling. Bioinformatics 18: 287-297.

Waddell PJ and Kishino H (2000). Cluster inference methods and graphical models evaluated on NCI60 microarray gene expression data. Genome Inform Ser Workshop Genome Inform 11: 129-140.

Walhout AJ (2011). What does biologically meaningful mean? A perspective on gene regulatory network validation. Genome Biol 12: 109.

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