The MHC I immunopeptidome conveys to the cell surface an integrative view of cellular regulation

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1st Editorial Decision 21 April 2011

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.

The reviewers raised a series of important concerns, of which we find two particularly important:

1. They remained substantially unconvinced that the immunopeptidome was genuinely presenting a faithful representation of intracellular signaling and metabolism. Indeed, the first reviewer indicated that is was not clear whether the observed changes were specific results of changes in mTOR signaling, or a more general result of cellular stress.

2. The reviewers found the experiments supporting the antigenicity of these immunopeptidome changes largely unconvincing.

Given that these concerns raise some doubt about the key claims and conclusions of this work, we feel that we have no choice but to return this work with the message that we cannot offer to publish it.

Nevertheless, the reviewers expressed interest in the topic of this work, and noted that it could potentially make some important contributions to the field. Therefore, we would like to offer that we

may be willing to reconsider a substantially expanded new submission based on this work. We recognize that this would involve further experimentation and analysis. We feel that the reviewers make constructive suggestions in this regard, including testing immunopeptidome changes under other stress conditions and performing more appropriate tests for antigenicity. Since these additional experiments remain very substantial, we would understand if you decide to submit this work instead to another journal.

Any resubmission would have a new number and receipt date, and we can give no guarantee about its eventual acceptability. However, 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 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

REFEREE REPORTS

Reviewer #1 (Remarks to the Author):

This manuscript is a comprehensive description of the peptides presented by MHC class I molecules upon treatment of cells with the drug rapamycin. Caron et al use high throughput mass spectrometry to sequence the peptides eluted from the surface of EL4 cells treated with rapamycin, and use bioinformatic tools and comparison with b2m-deficient EL4 cells to assign the eluted peptides to specific MHC class I molecules. They found a large number of peptides that were increased in abundance, and only six peptides that were entirely novel, suggesting that treatment with this drug leads to the increased expression of peptide-MHC I complexes that were already present at steady-state.

They also performed a microarray analysis of rapamycin-treated EL4 cells to determine which genes were differentially expressed upon rapamycin treatment. The source genes for the most upregulated peptides and the most-upregulated transcripts were then assigned to different cellular processes based on analysis of gene ontology terms. Six cellular processes were found to be "enriched", and to account for 70 upregulated genes and 38 upregulated peptides. The subsequent analysis focussed on this subset of upregulated genes and peptide source genes.

When the subset of 70 upregulated genes and subset of transcripts for 38 peptides were compared, a striking pattern emerged: none of the peptides analyzed came from the upregulated genes. However, systems based mathematical analysis of both sets of genes revealed that the peptide-source genes were very tightly-connected to the upregulated genes; and that peptide-source genes and upregulated genes were within the same signaling/functional cellular protein networks. In other words, while the MHC I-associated peptides did not originate directly from differentially-expressed genes, they do originate from genes that are very closely functionally connected to differentially expressed genes, suggesting that the peptide MHC I repertoire is a functional , if not a direct, representation of the transcriptional state of a cell.

The authors then go on to show that the increased expression of these 38 peptides by rapamycin-treated cells was not due to increased transcription or translation. Instead, they hypothesize that these peptides originate from the increased availability of proteasomal degradation products due to increased numbers of defective ribosomal products (DRiPs). Using one peptide source protein,

rictor, as model, they show that increased amounts of polyubiquitinated rictor are detected in rapamycin-treated cells when the proteasome is inhibited. Using two model peptides the authors then show that WT mice immunized with these peptides can kill rapamycin-treated targets, suggesting that the peptides induced by rapamycin treatment could be immunogenic.

This study is technically sophisticated, and offers a fresh approach to the analysis of the modulation of the repertoire of peptides presented by MCH class I molecules. However, there are some concerns.

1. The authors use peptides eluted from b2m-deficient EL4 cells to eliminate non-specific peptides from their mass spectroscopy analysis. Were these b2m-deficient EL4 cells rapamycin-treated? If not, this is a serious omission and must be rectified.

2. The striking observation that subsets of differentially expressed genes and peptide source genes are entirely different is confined to an analysis of the peptides and genes that cluster in certain cellular processes. Does this observation still apply when all 98 peptide source genes are considered? If there were some overlap between differentially expressed genes and the remaining source genes for peptides, would that weaken their conclusions?

3. The authors emphasize that genes from certain cellular processes are enriched in their analyses. However, this is when compared to untreated cells. The processes listed-protein transport, cell cycle, DNA replication and transcription-are fundamental cellular processes and could be affected by any cellular stress. Therefore a more appropriate control would be EL4 cells treated with a different drug or cellular stressor. If it was found that genes within the mTOR network formed sources for MHC I peptides specifically in response to rapamycin treatment and are not upregulated in response to other cellular stresses, the conclusion that "the immunopeptidome projects at the cell surface a faithful representation of the biochemical networks and metabolic events regulated at multiple levels inside the cells" would be significantly stronger.

4. It would be good to include control experiments in which antibodies to CD8 or MHC class I could block the immune responses seen in Fig 7C and D.

5. It may be that novel, or "foreign" peptides tested in Fig 7C and D are significantly more immunogenic than relatively increased peptides. Can the novel peptides be compared to those peptides whose abundance has increased in analyses such as in Fig. 7C and D, to assess their relative contributions to the immune response, as the authors themselves bring up in the final paragraph of the results section?

In summary, Caron et al have produced an elegant study that combines biochemical and systemic approaches to show convincingly that the repertoire of peptides presented by MHC class I can be modulated by drug treatment. However, the data does not yet warrant the conclusion that this altered peptide repertoire specifically represents the biochemical pathway that has been perturbed and is not a general consequence of cellular stress.

Reviewer #2 (Remarks to the Author):

The authors have conducted a rather extensive proteomic analysis of peptides bound to mouse MHC class I molecules in tumor cells (EL4) cultured normally or in the drug rapamycin. They find substantial changes to the set of peptides bound to MHC CI (although in truth, as they note, only a modest number of changes are of more than minimal abundance and the actual number of such altered peptides being displayed is a small fraction of the several thousand peptides bound by the overall class I pool of these cells). They do a good job of informatically processing the data in conjunction with the know target of rapamycin and the connected pathways involving this mTOR target, from which they conclude that many of the new peptides arise from components of this large network of interacting molecules. Interestingly, they find little association between mRNA changes and peptides bound to MHC class I molecules in drug-treated cells, and further biochemical study reveals that protein abundance or bulk lifetime does not correlate with MHC CI loading. This leads the authors to conclude that post-translational modifications to a small fraction of the protein made by certain mRNAs is responsible for most of the processed antigens. They claim throughout that the

data indicate that identification of the immunopeptidome of cells can be informative about the alternations in cellular biochemistry resulting from drug treatment or other insults to a cell.
Major issues:
1. In the abstract, the authors claim "Very little is known about the origin, composition and plasticity of the immunopeptidome." This is not correct; there are many mass spectrometry studies looking at the diversity and composition of peptides bound to MHC class I molecules (Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Hunt DF, Henderson RA, Shabanowitz J, Sakaguchi K, Michel H, Sevilir N, Cox AL, Appella E, Engelhard VH. Science. 1992 Mar 6;255(5049):1261-3.
Mass spectrometric analysis of peptides associated with the human class I MHC molecules HLA-A2.1 and HLA-B7 and identification of structural features that determine binding. Engelhard VH, Appella E, Benjamin DC, Bodnar WM, Cox AL, Chen Y, Henderson RA, Huczko EL, Michel H, Sakaguichi K, et al. Chem Immunol. 1993;57:39-62. Review. No abstract available.
J Immunol Methods. 2002 Apr 1;262(1-2):5-19. Electrospray mass spectrometry for the identification of MHC class I-associated peptides expressed on cancer cells. Bonner PL, Lill JR, Hill S, Creaser CS, Rees RC.
Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry. Weinzierl AO, Rudolf D, Hillen N, Tenzer S, van Endert P, Schild H, Rammensee HG, Stevanović S. Eur J Immunol. 2008 Jun;38(6):1503-10.
Eur J Immunol. 2000 Aug;30(8):2216-25. Identification of tumor-associated MHC class I ligands by a novel T cell-independent approach. Schirle M, Keilholz W, Weber B, Gouttefangeas C, Dumrese T, Becker HD, Stevanović S, Rammensee HG.
among others) and labs such as those of N. Shastri and J. Yewdell have published dozens of papers on how MHC class I associated peptides are generated and their source proteins in the cell.
2. There are also previous studies showing changes in the MHC class I or class II-associated peptidome in infected cells, which can be considered analogous to using rapamycin to change intracellular biochemistry (Hum Immunol. 2003 Jan;64(1):44-55. Autoreactivity against induced or upregulated abundant self-peptides in HLA-A*0201 following measles virus infection. Herberts CA, van Gaans-van den Brink J, van der Heeft E, van Wijk M, Hoekman J, Jaye A, Poelen MC, Boog CJ, Roholl PJ, Whittle H, de Jong AP, van Els CA.
J Immunol Methods. 2005 Feb;297(1-2):153-67. Identification of HLA-DRB1-bound self-peptides following measles virus infection. Ovsyannikova IG, Johnson KL, Naylor S, Poland GA.).
3. On p. 9 the authors write "Thus, the immunopeptidome projects at the cell surface a faithful representation of intracellular metabolic processes regulated at the transcript level." This statement is rather confusing; given that the DEM and DEG are completely distinct, as stated just above in this section of the manuscript, this claim that the MIPs at the cell surface represent a faithful representation of metabolism regulated at the gene level is at best disingenuous if not misleading. It is true that networks/pathways with connected nodes include both DEM and DEG, but there is a clear dichotomy between changes in DEG (and presumably their protein products) and the DEM, so there is no direct projection of the network component changes in the MIPs.

4. On. P. 10, the statement "Thus, this systems level analysis demonstrates that rapamycin-induced variations in the immunopeptidome arise from biochemical networks specifically connected to the target of rapamycin (i.e. mTOR)." must be the case if the claim made above about the network overlap between DEM and DEG is true. Thus, it is a necessary and not an unsuspected finding.

Later in this same section, the authors discuss biochemical networks. The rationale for this focus on pathway mapping is unclear. Without achieving insight into which specific gene products are feeding into the DEM, merely classifying the DEM within modules that per force are most affected by the drug treatment is not a very insightful set of findings.

5. On p. 11, they state "In contrast, we observed that 36% (12 out of 33) of the source genes were upregulated at the transcript level." I do not understand this statement. Just above they say that .."there is no overlap between DEM and DEG.." but here they say that 36% of the source genes for DEM are upregulated at the transcript level, which means they should come from DEG.

6. On p. 13, there is the claim "Collectively, our data indicate that changes in the immunopeptidome integrate events occurring at the transcriptional and co- or

post-translational level." Because which proteins show greater or lesser changes of this type are not predictable, this beggars the claim of the authors that they can use their study to infer changes in intracellular biochemistry from analysis of MIPs.

7. The experiments using immunized mice and in vitro killing assays do not address immunogenicity but rather antigenicity. Further, they only show that exposure to a supraphysiologic level of a specific peptide in the form of synthetic peptide-pulsed dendritic cells can elicit T cells unable to respond functionally to the low levels of a peptide normally expressed. This is consistent with expectations from quantitative tolerance mechanisms, as is the fact that such T cells are able to respond to APCs with higher levels of the ligand. At a minimum, the proper experiment is to use the rapamycin-treated EL4 cells as immunogens and see if a response can be detected, not peptide-pulsed DCs whose level of the bound peptides has not been adjusted to the level seen on EL4 cells treated with drug.

8. The claim "In other words, they suggested that, at the organismal level, the immune self was practically invariant." made on p. 15 is not correct - what was stated in these early reports is that the MOST ABUNDANT MHC CI bound peptides come from highly abundant housekeeping proteins that are common to many cell types, but that as many as 10000 different peptides are presented when one considers levels of as few as 10 copies per cell, which suggested that a large fraction of the proteome of a cell would be represented in MIPs.

Methodological issues:
1. It is critical to know what fraction of detected peptides were excluded by using a motif filter, as the predictive algorithms for MHC class I binding, while quite good, are nonetheless not fully reliable and many MIPs might be excluded by this approach.

2. It is unclear how relative abundance was actually calculated for the untreated vs. treated cells, given the size, and thus, what is certainly a total protein difference. It is unclear if what matters most if the fractional loading of available MHC CI or the total number of loaded class I molecules.

Reviewer #3 (Remarks to the Author):

The manuscript by Caron et al. is well written and timely. The authors followed the effect of the mTOR inhibitor rapamycin on the immunopeptidome of EL4 murine cells as a way of gauging the effects of this inhibitor on cellular processes of protein degradation and presentation of MHC peptides. This way, they aimed to expand their previous proteomics studies on mTOR signaling by studying its effects from the point of view of the immunopeptidome. They first evaluated if the inhibitor is indeed effective in these cells and found out that it is. Also, they demonstrate that rapamycin did not inhibit or modify significantly the known components of the MHC peptide loading machinery.
The authors have followed the levels of expression of MHC peptides at the cell surface using the mild acid elution method and have detected an increase in many classical MHC class I peptides but not in non-classical MHC peptides. They assigned the peptides to each of these MHCs using their consensus sequence motifs. Since the mild acid elution method release many non-MHC peptides, the authors have defined as MHC peptides only those that were eluted from cells containing the b2m and not from those lacking it (suggest mentioning this fact in the Results since this is an important issue that may be overlooked in the Methods). In my opinion this methodology is the weakest point in this otherwise very good manuscript. The problem is that the association of the detected peptides is inferred from the assay rather than being purified by immunoaffinity chromatography with anti-MHC mAb, which should result in relatively pure preparations of peptides and in about tenfold more identified and quantified peptides from the number of cells used for this study. Therefore, the authors base their results on a relatively small number of about 400 identified peptides, which may be considered somewhat small for a significant pathway and cellular function analysis. The problem is also related to the fact that this way the authors can only identify and quantify only the most abundant peptides or easily detected, which may not represent as well the entire MHC immunopeptidome.
They next went ahead and compared the transcriptome of the same type cells treated with rapamycin (in a separate experiment) with the immunopeptidome. Since the correlation observed was not significant between the MHC peptides and transcripts that changed in their amounts due to the

treatment, the authors have expanded it by looking at shared genes/proteins participating in the same pathways and functional complexes as do the detected MHC peptides that changed in their levels. So, instead of finding strong correlation between the exact transcripts and MHC peptides derived from them, they found correlation with other members of the same functional groups of proteins and this way inferred that similar pathways are affected at the level of the transcriptome and the immunopeptidome. The most effected transcripts and MHC peptides were related to mTOR signaling and function, thus validating the relevance of the study.
The groups of MHC peptides changing in their levels were most significantly associated with transcriptional and translational functions of the cellular processes. This is an interesting observation, but not that surprising when looking at immunopeptidomes in general since these pathways are often composed of rapidly turning-over proteins which are likely to contribute more to the immunopeptidome.
The argument in the first sentence of the discussion "Early proteomic studies of the immunopeptidome have led scientists to believe that MIPs were derived from housekeeping genes and presented minimal if any differences among different cell types" is not completely accurate, since early studies on MHC peptides relied on rather insensitive instruments and identified as a results only the more abundant MHC peptides, which were obviously often produced from housekeeping genes. The newer experiments, in most studies relying on more modern mass spectrometers, identify more balanced repertoires of peptides from more cellular processes. Furthermore, studies that looked at the MHC peptidomes associated with just one immunoaffinity purified MHC allele produce more similar patterns of peptides, while studies relying on the pools of peptides carried by all the MHC allomorphs in cells differing in their haplotypes, obviously did not claim that the peptides repertoire are similar between cells. This issue is better clarified in the discussion.

The author eventually checked that some of the identified peptides could elicit T cell response indicating that some mTOR induced MHC peptides are possibly immunogenic. This part of the study does not contribute so much to its systems biology conclusions, since anyways, many peptides can elicit immune response, but this does not indicate that particular pathways and cellular processes generate MHC peptides more likely to be immunogenic, as might be concluded from the claims in this manuscript.
I suggest accepting this manuscript with minor revisions, since as I stated above, I think that it is an important manuscript, with vast implication to the study of systems biology through the use systems immunology. It also provides a totally fresh point of view, not only on the immunopeptidome, but also on the cellular proteome and degradome.
The figures and tables in the article are needed and useful. As stated above, I am not sure if the CTL assays contribute much to the manuscript, but it can be left in.
The length of the article is adequate.
The methods part is clear enough.

Resubmission 8 June 2011

Reviewer #1 (Remarks to the Author):

This manuscript is a comprehensive description of the peptides presented by MHC class I molecules upon treatment of cells with the drug rapamycin. Caron et al use high throughput mass spectrometry to sequence the peptides eluted from the surface of EL4 cells treated with rapamycin, and use bioinformatic tools and comparison with b2m-deficient EL4 cells to assign the eluted peptides to specific MHC class I molecules. They found a large number of peptides that were increased in abundance, and only six peptides that were entirely novel, suggesting that treatment with this drug leads to the increased expression of peptide-MHC I complexes that were already present at steady-state.

They also performed a microarray analysis of rapamycin-treated EL4 cells to determine which genes were differentially expressed upon rapamycin treatment. The source genes for the most upregulated peptides and the most-upregulated transcripts were then assigned to different cellular processes based on analysis of gene ontology terms. Six cellular processes were found to be

"enriched", and to account for 70 upregulated genes and 38 upregulated peptides. The subsequent analysis focussed on this subset of upregulated genes and peptide source genes.

When the subset of 70 upregulated genes and subset of transcripts for 38 peptides were compared, a striking pattern emerged: none of the peptides analyzed came from the upregulated genes. However, systems based mathematical analysis of both sets of genes revealed that the peptide-source genes were very tightly-connected to the upregulated genes; and that peptide-source genes and upregulated genes were within the same signaling/functional cellular protein networks. In other words, while the MHC I-associated peptides did not originate directly from differentially-expressed genes, they do originate from genes that are very closely functionally connected to differentially expressed genes, suggesting that the peptide MHC I repertoire is a functional , if not a direct, representation of the transcriptional state of a cell.

The authors then go on to show that the increased expression of these 38 peptides by rapamycin-treated cells was not due to increased transcription or translation. Instead, they hypothesize that these peptides originate from the increased availability of proteasomal degradation products due to increased numbers of defective ribosomal products (DRiPs). Using one peptide source protein, rictor, as model, they show that increased amounts of polyubiquitinated rictor are detected in rapamycin-treated cells when the proteasome is inhibited. Using two model peptides the authors then show that WT mice immunized with these peptides can kill rapamycin-treated targets, suggesting that the peptides induced by rapamycin treatment could be immunogenic.

This study is technically sophisticated, and offers a fresh approach to the analysis of the modulation of the repertoire of peptides presented by MCH class I molecules. However, there are some concerns.

Response: We truly appreciate the clarity of the Reviewer in summarizing our work. We also thank the Reviewer for highlighting the novelty of our approach.

1. The authors use peptides eluted from b2m-deficient EL4 cells to eliminate non-specific peptides from their mass spectroscopy analysis. Were these b2m-deficient EL4 cells rapamycin-treated? If not, this is a serious omission and must be rectified.

Response: As in our previous studies, peptides eluted from β2m-deficient EL4 cells were considered contaminant (non MHC I-associated) peptides. As now specified in Materials and Methods (p. 24), we eluted and analyzed peptides from both untreated and rapamycin treated (for 48h) β2m-deficient EL4 cells. Thus, the 422 peptides analyzed in this report are genuine MIPs. They do not include peptides found in eluates from untreated or rapamycin treated β2m-deficient EL4 cells. The raw data for untreated and rapamycin treated β2m-deficient EL4 cells are now presented in new Supplementary Table (Supplementary Table S1).

2. The striking observation that subsets of differentially expressed genes and peptide source genes are entirely different is confined to an analysis of the peptides and genes that cluster in certain cellular processes. Does this observation still apply when all 98 peptide source genes are considered? If there were some overlap between differentially expressed genes and the remaining source genes for peptides, would that weaken their conclusions?

Response: This point is very well taken and we performed the analysis suggested by the Reviewer. We compared differentially expressed transcripts (DEGs; n = 1353) with all 98 peptide source genes coding for the most differentially expressed MIPs (Figure 3A and B). We observed an overlap for only two genes. Therefore, we have expanded this section of the manuscript. We now mention (p.8, 2nd paragraph): "These genes are hereafter referred to as differentially expressed genes (DEGs). We next compared the 1353 DEGs with 98 unique source genes coding for the most differentially expressed MIPs (DEMs) [fold change > 2.5; $P < 0.05$: as described in (Fortier et al., 2008)] (Figure 3B and Supplementary Table S2). Based on microarray gene expression data, only two DEM source genes (*Tmod1* and *Dhcr7*) were found to be differentially expressed at the transcript level (Figure 3A and B). Interestingly, the only DEM that was less abundant was also found to be downregulated at the transcript level. Thus, these results indicate that only a minority of DEMs originate from DEGs."

In the initial manuscript, we stated that the overlap between DEGs and a subset of 38 DEMs was 0%. The new analysis, which includes all 98 DEMs shows that the overlap is about 2%. Our conclusion therefore remains unchanged: almost no DEMs originate from DEGs and most DEM source genes are regulated post-transcriptionally. This point is further discussed in the new version of the manuscript. We have therefore adopted the analytical strategy proposed by the Reviewer are grateful for his suggestion.

3. The authors emphasize that genes from certain cellular processes are enriched in their analyses. However, this is when compared to untreated cells. The processes listed-protein transport, cell cycle, DNA replication and transcription-are fundamental cellular processes and could be affected by any cellular stress. Therefore a more appropriate control would be EL4 cells treated with a different drug or cellular stressor. If it was found that genes within the mTOR network formed sources for MHC I peptides specifically in response to rapamycin treatment and are not upregulated in response to other cellular stresses, the conclusion that "the immunopeptidome projects at the cell surface a faithful representation of the biochemical networks and metabolic events regulated at multiple levels inside the cells" would be significantly stronger.

Response: The Reviewer brings up an important point. We agree that the cellular processes perturbed by rapamycin (i.e. protein transport, cell cycle, DNA replication and transcription) could be affected by many other cellular stresses and not only by rapamycin treatment. Thus, we understand the concern that changes in the immunopeptidome in rapamycin-treated cells might be stereotypical stress-induced changes. On the other hand, gene expression profiling of mammalian cells exposed to various types of cell stress (heat shock, ER stress, oxidative stress, and crowding or starvation) has shown that stress responses are much more distinct and specific to the individual agents than the corresponding responses in yeast (Murray et al., 2004).

Two elements strongly support our contention that rapamycin-induced changes in the immunopeptidome are connected to the mTOR network. First, we found that the connectivity between DEM source genes and mTOR network components was amazingly strong ($P < 10^{-5}$). A bootstrap procedure (500,000 iterations) failed to reveal a single set of peptide source genes that were so tightly connected to component of the mTOR network (Figure 4C). Second, as now mentioned in the revised manuscript, we have evaluated the impact of other types cell stress (tunicamycin, palmitate or glucose deprivation) on expression of MHC-peptide complexes at the surface EL4 cells (the cell line used the present study) (Granados *et al.*, 2009). We found that cell stress induced by tunicamycin, palmitate and glucose deprivation led to a decrease in the generation of MIPs and in the expression of MHC I molecules. These effects are the exact opposite of what we found upon treatment of EL4 cells with rapamycin: overexpression of MHC I molecules (Supplementary Figure S2B) and increased abundance of cell surface MIPs (Figure 2B). In our opinion, it is therefore sound to conclude that rapamycin-induced variations in the immunopeptidome i) arise from biochemical networks whose components are highly connected to the target of rapamycin (i.e. mTOR) and ii) are not unspecific changes found with any type of cell stress.

However, in line with the Reviewer comment, we cannot formally state that i) changes in the MIP repertoire reflect the activity of all signalling modules perturbed by rapamycin, or that ii) perturbation of mTOR by other agents or in other contexts would bring the same changes in the MIP repertoire. Hence, we agree with the Reviewer that the use of the following terms was sometimes inappropriate or needed to be qualified in the initial version of our manuscript: faithful, specific and selective. Therefore, we have rewritten an important paragraph in the result section (p.9-10). In addition, we have expanded the discussion section to put into proper context the issue of specificity raised by the Reviewer.

In the revised manuscript, the heading title of the section in p.10 is now: “DEMs arise from biochemical networks connected to mTOR”. This section is now as follows: “The above results suggested that DEMs originated from genes connected to the mTOR network. However, considering that mTOR has a pervasive role in protein synthesis and degradation (Caron *et al.*, 2010), we could not discard the possibility that DEMs originate from some non-specific generic effect of rapamycin on protein metabolism. In the latter case, DEM-coding genes would not be tightly connected to the mTOR network. Hence, in order to further evaluate the relationship between DEMs and mTOR, we first conducted an analysis on the 98 DEM source genes (Figure 3B and Supplementary Table S2)

using the interaction network database STITCH (Kuhn *et al.*, 2010). This analysis uncovered a network containing 30 DEM source genes that were interconnected and organized within discrete functional modules (Supplementary Figure S4). Strikingly, the network included the chemicals rapamycin and everolimus (rapamycin analog) in addition to components (e.g.: Rictor, Sgk1) and modules (e.g.: mTOR signalling, translation, lipid biosynthesis) known to be directly regulated by mTOR (Caron *et al.*, 2010). Therefore, we reasoned that rapamycin-mediated changes in the immunopeptidome might originate from genes that are very closely connected to components of the mTOR signalling network. To systematically evaluate this assumption, we measured the connectivity score between the 30 DEM source genes and components extracted from a comprehensive map of the mTOR interactome and signalling network [Supplementary Table S5 based on (Caron *et al.*, 2010)]. By using the all-pair-shortest-path matrix described above, we calculated that the 30 DEM source genes were strongly interconnected to the mTOR network components relative to random assignments (bootstrapping, $P < 10^{-5}$) (Figure 4C). This systems-level analysis demonstrates that rapamycin-induced variations in the immunopeptidome arise from biochemical networks whose components are highly connected to the target of rapamycin (i.e. mTOR). Thus, this finding reinforces the notion that the immunopeptidome projects a functional representation of intracellular metabolic changes to the cell surface. Further immunopeptidomic studies are needed to evaluate whether the mTOR network regulates the repertoire of MIPs in other contexts."

We have also included the following paragraph in the discussion (p.16): "An important question is whether changes in the MIP repertoire induced by rapamycin would be found in response to any type of cell stress. Two elements strongly support our contention that rapamycin-induced changes in the immunopeptidome are connected to the mTOR network. First, we found that the connectivity between DEM source genes and mTOR network components was amazingly strong ($P < 10^{-5}$). A bootstrap procedure (500,000 iterations) failed to reveal a single set of peptide source genes that were so tightly connected to component of the mTOR network (Figure 4C). Second, we have previously evaluated the impact of other types cell stress (tunicamycin, palmitate or glucose deprivation) on expression of MHC-peptide complexes at the surface EL4 cells (the cell line used the present study) (Granados *et al.*, 2009). We found that cell stress induced by tunicamycin, palmitate and glucose deprivation led to a decrease in the generation of MIPs and in the expression of MHC I molecule. These effects are the exact opposite of what we found upon treatment of EL4 cells with rapamycin: overexpression of MHC I molecules (Suppl. Fig. S2B) and increased abundance of cell surface MIPs (Fig. 2B). In our opinion, it is therefore sound to conclude that rapamycin-induced variations in the immunopeptidome i) arise from biochemical networks whose components are highly connected to the target of rapamycin (i.e. mTOR) and ii) are not unspecific changes found with any type of cell stress. However, mTOR senses and integrates multiple environmental cues and mTOR network components interact with many signalling pathways, such as the Wnt pathway (Caron *et al.*, 2010;Inoki *et al.*, 2006). Therefore, further studies will be needed to determine whether perturbation of mTOR by other agents or in other contexts would bring the same changes in the MIP repertoire as those induced by rapamycin."

In the abstract, we have removed the term "faithful". We now state: "Moreover, we provide systems-level evidence that the immunopeptidome projects at the cell surface a representation of biochemical networks and metabolic events regulated at multiple levels inside the cells". We believe that this is an accurate conclusion that incorporates our main findinds: 1) the immunopeptidome is a functional, if not a direct, representation of the transcriptional state of a cell, 2) rapamycin-induced variations in the immunopeptidome arise from biochemical networks whose components are highly connected to the target of rapamycin (i.e. mTOR), and 3) changes in the immunopeptidome integrate events occurring at the transcriptional and co- or post-translational level.

Finally, in p. 15 (2nd paragraph), we have removed "rapamycin-specific" and have replaced it by "novel".

4. It would be good to include control experiments in which antibodies to CD8 or MHC class I could block the immune responses seen in Fig 7C and D.

Response: Thank you for this suggestion. Control cytotoxicity experiments were performed by using i) an antibody against CD8β and ii) β2m-deficient EL4 cells. Results from these new experiments

are now reported in a new Supplementary Figure (Supplementary Figure S5). These new data demonstrate that cytotoxicity against VNTHFSHL and KALSYASL peptides is MHC I-restricted.

5. It may be that novel, or "foreign" peptides tested in Fig 7C and D are significantly more immunogenic than relatively increased peptides. Can the novel peptides be compared to those peptides whose abundance has increased in analyses such as in Fig. 7C and D, to assess their relative contributions to the immune response, as the authors themselves bring up in the final paragraph of the results section?

Response: We randomly selected the SQAVNKQQI peptide, which is encoded by Ccnf to perform the experiment suggested by the Reviewer. The SQAVNKQQI peptide is presented at the cell surface under steady state condition and its abundance was increased by 6 fold upon rapamycin treatment (Supplementary Table S2). We immunized C57BL/6 mice with dendritic cells coated with SQAVNKQQI synthetic peptide. Splenocytes from immunized mice showed a modest but significant cytotoxicity for rapamycin-treated EL4 cells but not for untreated EL4 cells (see Figure 1 for Reviewers). This result shows that the SQAVNKQQI peptide can elicit a cytotoxic T-cell response *in vitro*. This suggests that MIPs that are presented at the cell surface under steady state condition and presented in excessive amounts after rapamycin treatment can be antigenic. To globally and cogently evaluate this concept, it would be important to perform this assay with many more peptides and to assess the polyfunctionality profile of peptide-specific T cells. That would practically represent a project by itself. Nonetheless, as underscored by Reviewer #3, the CTL assays did not contribute importantly to the systems biology conclusions of the manuscript. Since evaluation of peptide immunogenicity is not the main purpose of the study, we have decided to not pursue this experiment with additional synthetic peptides. We also feel that it would be preferable not to include Figure 1 for Reviewers in the revised manuscript.

In summary, Caron et al have produced an elegant study that combines biochemical and systemic approaches to show convincingly that the repertoire of peptides presented by MHC class I can be modulated by drug treatment. However, the data does not yet warrant the conclusion that this altered peptide repertoire specifically represents the biochemical pathway that has been perturbed and is not a general consequence of cellular stress.

Response: We are thankful to the Reviewer for his/her rigorous assessment of our manuscript. We believe that our response to Reviewer's point 3 satisfactorily addresses his/her main concern.

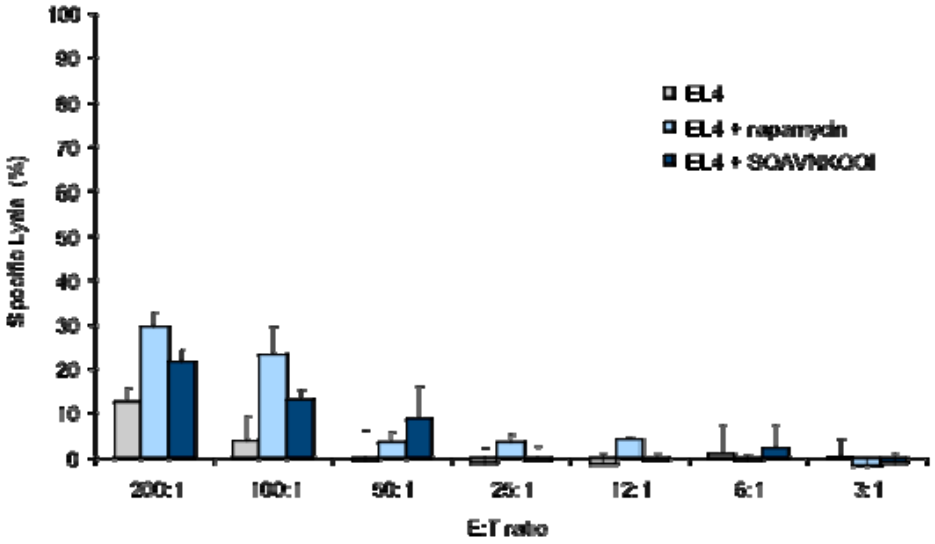

Figure 1 for reviewers. Mice were immunized with dendritic cells coated with SQAVNKQQI peptide. Splenocytes from primed mice were tested for cytolytic activity against CFSE-labeled target EL4 cells at different E/T ratios. EL4 cells coated with SQAVNKQQI were used as positive control. Data represent the mean ± SD for three mice per group.

Reviewer #2 (Remarks to the Author):

The authors have conducted a rather extensive proteomic analysis of peptides bound to mouse MHC class I molecules in tumor cells (EL4) cultured normally or in the drug rapamycin. They find substantial changes to the set of peptides bound to MHC CI (although in truth, as they note, only a modest number of changes are of more than minimal abundance and the actual number of such altered peptides being displayed is a small fraction of the several thousand peptides bound by the overall class I pool of these cells). They do a good job of informatically processing the data in conjunction with the know target of rapamycin and the connected pathways involving this mTOR target, from which they conclude that many of the new peptides arise from components of this large network of interacting molecules. Interestingly, they find little association between mRNA changes and peptides bound to MHC class I molecules in drug-treated cells, and further biochemical study reveals that protein abundance or bulk lifetime does not correlate with MHC CI loading. This leads

the authors to conclude that post-translational modifications to a small fraction of the protein made by certain mRNAs is responsible for most of the processed antigens. They claim throughout that the data indicate that identification of the immunopeptidome of cells can be informative about the alternations in cellular biochemistry resulting from drug treatment or other insults to a cell.

Response: We thank the Reviewer for his/her kind evaluation of our work. The Reviewer has raised important issues which are carefully addressed below.

Major issues:

1. In the abstract, the authors claim "Very little is known about the origin, composition and plasticity of the immunopeptidome." This is not correct; there are many mass spectrometry studies looking at the diversity and composition of peptides bound to MHC class I molecules

(Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Hunt DF, Henderson RA, Shabanowitz J, Sakaguchi K, Michel H, Sevilir N, Cox AL, Appella E, Engelhard VH. Science. 1992 Mar 6;255(5049):1261-3.

Mass spectrometric analysis of peptides associated with the human class I MHC molecules HLA-A2.1 and HLA-B7 and identification of structural features that determine binding. Engelhard VH, Appella E, Benjamin DC, Bodnar WM, Cox AL, Chen Y, Henderson RA, Huczko EL, Michel H, Sakaguichi K, et al. Chem Immunol. 1993;57:39-62. Review. No abstract available.

J Immunol Methods. 2002 Apr 1;262(1-2):5-19. Electrospray mass spectrometry for the identification of MHC class I-associated peptides expressed on cancer cells. Bonner PL, Lill JR, Hill S, Creaser CS, Rees RC.

Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry. Weinzierl AO, Rudolf D, Hillen N, Tenzer S, van Endert P, Schild H, Rammensee HG, Stevanović S. Eur J Immunol. 2008 Jun;38(6):1503-10.

Eur J Immunol. 2000 Aug;30(8):2216-25. Identification of tumor-associated MHC class I ligands by a novel T cell-independent approach. Schirle M, Keilholz W, Weber B, Gouttefangeas C, Dumrese T, Becker HD, Stevanović S, Rammensee HG. among others) and labs such as those of N. Shastri and J. Yewdell have published dozens of papers on how MHC class I associated peptides are generated and their source proteins in the cell.

Response: A) Large-scale analysis of MIPs. We agree with the Reviewer that many mass spectrometry studies have looked at the diversity and composition of peptides associated to MHC class I molecules. Pioneering work on large-scale analysis of the MIP repertoire has been performed in particular by the Rammensee-Stevanovic and Hunt-Engelhard teams. Moreover, a comprehensive review of this subject has been published by Mester *et al.* after the submission of our initial manuscript. In the introduction of the revised manuscript, we have expanded our summary of large-scale analyses of the MIP repertoire and now cite all references mentioned by the Reviewer (p. 3-4): "Moreover, progresses in MS have allowed increasingly sophisticated and comprehensive large-scale analyses of MIPs (Mester *et al.*, 2011). Large-scale analyses have yielded unprecedented insights into the peptide specificities and motifs of MHC molecules and the diversity of the MIP repertoire (Bonner *et al.*, 2002;Engelhard *et al.*, 1993;Hunt *et al.*, 1992;Weinzierl *et al.*, 2008). They have also demonstrated that MIPs derive from all cell compartments and that the MIP repertoire can be modified by neoplastic transformation (Fortier *et al.*, 2008;Hickman *et al.*, 2004;Schirle *et al.*, 2000;Weinzierl *et al.*, 2007). Nevertheless, we still know very little about the genesis and molecular composition of the immunopeptidome: why do proteins such as STT3B yield abundant MIPs while other do not (Perreault, 2010)? MS studies have also revealed that the immunopeptidome is not a random sample of the proteome: many abundant proteins do not generate MIPs, while some low-abundance proteins generate large amounts of MIPs (Milner *et al.*, 2006). Furthermore, large-scale analyses have yielded conflicting results on the relation between the transcriptome and the MIP repertoire (Fortier *et al.*, 2008;Mester *et al.*, 2011;Weinzierl *et al.*, 2007). Therefore, further systematic studies based on high throughput technologies and integrative approaches are needed in order to elucidate the mechanisms that mould the immunopeptidome. In-depth mechanistic understanding of the immunopeptidome biogenesis would allow prediction of its molecular

composition and would therefore be highly relevant to the development of immunotherapies (Sette and Rappuoli, 2010;Zarling *et al.*, 2006)."

B) The processing of MIPs. We also agree with the Reviewer that N. Shastri and J. Yewdell have published many papers on how MHC class I peptides are generated from their source genes. These elegant studies were based on classical reductionist approaches. We make numerous references to their work in the introduction (p. 3) and when we discuss the concept of DRiPs (p.12-14).

2. There are also previous studies showing changes in the MHC class I or class II-associated peptidome in infected cells, which can be considered analogous to using rapamycin to change intracellular biochemistry

*(Hum Immunol. 2003 Jan;64(1):44-55. Autoreactivity against induced or upregulated abundant self-peptides in HLA-A*0201 following measles virus infection. Herberts CA, van Gaans-van den Brink J, van der Heeft E, van Wijk M, Hoekman J, Jaye A, Poelen MC, Boog CJ, Roholl PJ, Whittle H, de Jong AP, van Els CA.*

J Immunol Methods. 2005 Feb;297(1-2):153-67. Identification of HLA-DRB1-bound self-peptides following measles virus infection. Ovsyannikova IG, Johnson KL, Naylor S, Poland GA.).

Response: We thank the Reviewer for pointing this out. Indeed, Ovsyannikova *et al*. identified MHC class II peptides that were down- and up-regulated 5 days after measles virus (MV) infection. Herberts *et al*. identified two abundant self MIPs, IFI-6-16_{74-82} and $Hsp90\beta_{570-578}$ that were induced or upregulated, respectively, 48h after MV infection and were immunogenic. These reports (now cited on p. 19 of our manuscript) demonstrate that viral infection can impinge on the self MIP repertoire. Furthermore, the report from Herberts *et al*. supports the notion that overexpressed MIPs can be immunogenic. However, the scale of these studies, and in particular the one on MHC class I-associated peptides (Herberts *et al*.) are not commensurate with the scale of the present study. To evaluate convincingly whether the MHC I immunopeptidome is plastic, we used a quantitative high-throughput mass spectrometry-based approach to profile in a time-sequential manner the abundance of 422 MHC I-associated peptides. We therefore believe that the present report presents the most comprehensive evaluation of the plasticity of the immunopeptidome.

3. On p. 9 the authors write "Thus, the immunopeptidome projects at the cell surface a faithful representation of intracellular metabolic processes regulated at the transcript level." This statement is rather confusing; given that the DEM and DEG are completely distinct, as stated just above in this section of the manuscript, this claim that the MIPs at the cell surface represent a faithful representation of metabolism regulated at the gene level is at best disingenuous if not misleading. It is true that networks/pathways with connected nodes include both DEM and DEG, but there is a clear dichotomy between changes in DEG (and presumably their protein products) and the DEM, so there is no direct projection of the network component changes in the MIPs.

Response: We agree with the Reviewer that this statement was confusing. In the new version of the manuscript, we have removed this statement.

4. On. P. 10, the statement "Thus, this systems level analysis demonstrates that rapamycin-induced variations in the immunopeptidome arise from biochemical networks specifically connected to the target of rapamycin (i.e. mTOR)." must be the case if the claim made above about the network overlap between DEM and DEG is true. Thus, it is a necessary and not an unsuspected finding.

Response: We thank the Reviewer for this comment. However, the observation that variations in the immunopeptidome are so tightly connected to mTOR ($p < 0.00001$) remains surprising and important. In fact, this result is a key finding because it reinforces the notion that the immunopeptidome projects a functional representation of intracellular metabolic changes to the cell surface.

This being said, we fully agree with the Reviewer that some level of connectivity between the immunopeptidome and the mTOR network could be considered plausible *a priori*. Plausible concepts nevertheless need to be demonstrated. Following the Reviewer's comment, we have changed the wording of this section. We now state (p.10, 2nd paragraph): "The above results suggested that DEMs originated from genes connected to the mTOR network. However, considering that mTOR has a pervasive role in protein synthesis and degradation (Caron *et al.*, 2010), we could not discard the possibility that DEMs originate from some non-specific generic effect of rapamycin on protein metabolism. In the latter case, DEM-coding genes would not be tightly connected to the mTOR network. Hence, in order to further evaluate the relationship between DEMs and mTOR, we first conducted an analysis on the 98 DEM source genes (Figure 3B and Supplementary Table S2) using the interaction network database STITCH (Kuhn *et al.*, 2010). This analysis uncovered a network containing 30 DEM source genes that were interconnected and organized within discrete functional modules (Supplementary Figure S4). Strikingly, the network included the chemicals rapamycin and everolimus (rapamycin analog) in addition to components (e.g.: Rictor, Sgk1) and modules (e.g.: mTOR signalling, translation, lipid biosynthesis) known to be directly regulated by mTOR (Caron *et al.*, 2010). Therefore, we reasoned that rapamycin-mediated changes in the immunopeptidome might originate from genes that are very closely connected to components of the mTOR signalling network. To systematically evaluate this assumption, we measured the connectivity score between the 30 DEM source genes and components extracted from a comprehensive map of the mTOR interactome and signalling network [Supplementary Table S5 based on (Caron *et al.*, 2010)]. By using the all-pair-shortest-path matrix described above, we calculated that the 30 DEM source genes were strongly interconnected to the mTOR network components relative to random assignments (bootstrapping, $P < 10^{-5}$) (Figure 4C). This systems-level analysis demonstrates that rapamycin-induced variations in the immunopeptidome arise from biochemical networks whose components are highly connected to the target of rapamycin (i.e. mTOR). Thus, this finding reinforces the notion that the immunopeptidome projects a functional representation of intracellular metabolic changes to the cell surface. Further immunopeptidomic studies are needed to evaluate whether the mTOR network regulates the repertoire of MIPs in other contexts."

Later in this same section, the authors discuss biochemical networks. The rationale for this focus on pathway mapping is unclear. Without achieving insight into which specific gene products are feeding into the DEM, merely classifying the DEM within modules that per force are most affected by the drug treatment is not a very insightful set of findings.

Response: We have articulated more clearly the rationale for this section. We have removed the following statement (p.11, 2nd paragraph): "To gain deeper insights about the origin of rapamycin-induced variations in the immunopeptidome, we integrated…", and have replaced it by the following: "To visualize the organization of relationships between DEM source genes, the transcriptome and the mTOR network, we integrated…".

Also relevant to this section, visualization of integrated networks can provide insightful findings (Germain et al., *Annu. Rev. Immunol.*, 2011). In this study, network integration and visualization allowed us:

1) to identify two modules that were composed of DEM source genes but no DEGs: the proteasome and the core mTOR signalling modules. In other words, changes in the immunopeptidome can provide unique biological insights that are not observed from microarray data analysis.

2) to observe that 82% of the DEM source genes (27 of 33) were localized within subnetworks that were transcriptionally perturbed by rapamycin. This is an insightful finding, which is now emphazised in the new version of the manuscript (p.12, 1st paragraph): "Collectively, these data indicate that rapamycin-induced variations in the immunopeptidome arise mostly from transcriptionally perturbed subnetworks that are connected to components of the mTOR network.". This result led us to propose that genes involved in transcriptionally perturbed subnetworks, although not necessarily differentially expressed at the transcript level, participate in modulating the composition of the immunopeptidome. Interestingly, reorganisation of transcriptional subnetworks is frequently observed in response to cell stress [i.e. pathogen infection (Kumar et al., *Cell*, 2010)]. Consequently, identification of components within transcriptionally perturbed subnetworks might

enable prediction of peptide source genes modulating the immunopeptidome's composition. This point is discussed in the new version of the manuscript (p. 18).

5. On p. 11, they state "In contrast, we observed that 36% (12 out of 33) of the source genes were upregulated at the transcript level." I do not understand this statement. Just above they say that .."there is no overlap between DEM and DEG.." but here they say that 36% of the source genes for DEM are upregulated at the transcript level, which means they should come from DEG.

Response: The Reviewer is correct. We have expanded the discussion to clarify this discrepancy (p. 17, 2nd paragraph): "We found that variations in the immunopeptidome functionally reflected perturbations of rapamycin-sensitive metabolic processes regulated in the transcriptome. More precisely, we observed that changes in the MIP repertoire originate from genes that are very closely functionally connected to DEGs. We observed that only 2 % of DEMs did originate directly from DEGs as measured by DNA microarray experiments. In contrast, quantitative PCR revealed that 36% of the DEM source genes were slightly upregulated at the transcript level. In fact, 8 DEM source genes (*Bptf, Bub1b, Ccnf, Chaf1b, Dtl, Mnat1, Myb* and *Tfdp2*) in Figure 3 come from DEGs that were identified by quantitative PCR (fold change between 1.2 and 3.1; Supplementary Table S6) but missed by microarrays. Differences in the sensitivity of both techniques are likely to explain this discrepancy. Indeed, whereas microarrays permit genome-wide profiling of mRNA expression levels, traditional quantitative PCR is by far more sensitive (Germain *et al.*, 2011). Therefore, genome-wide quantitative PCR would have revealed a more significant overlap between DEM source genes and DEGs. Although the overlap between DEM source genes and DEGs was underestimated from microarray data, altogether our data indicate that changes in MIP abundance originate mostly from post-transcriptional mechanisms."

6. On p. 13, there is the claim "Collectively, our data indicate that changes in the immunopeptidome integrate events occurring at the transcriptional and co- or post-translational level." Because which proteins show greater or lesser changes of this type are not predictable, this beggars the claim of the authors that they can use their study to infer changes in intracellular biochemistry from analysis of MIPs.

Response: We agree with the Reviewer that we cannot predict at which level peptide source genes are regulated based solely on immunopeptidomic data. That is where we want to go but we are not there yet. What we can predict however is the nature of MIP source genes that might be regulated at the transcriptional and co- or post-translational level. Our study provides the first systems-level evidence that the immunopeptidome projects at the cell surface a representation of biochemical networks and metabolic events regulated at multiple levels inside the cell. Therefore, our work provides a rationale for investigating systematically at which level MIP source genes are regulated in response to intracellular metabolic changes. By deciphering the complex relationships between the transcriptome, the translatome, the degradome and the immunopeptidome, one can expect to ultimately infer changes in intracellular biochemistry from immunopeptidome data.

7. The experiments using immunized mice and in vitro killing assays do not address immunogenicity but rather antigenicity. Further, they only show that exposure to a supraphysiologic level of a specific peptide in the form of synthetic peptide-pulsed dendritic cells can elicit T cells unable to respond functionally to the low levels of a peptide normally expressed. This is consistent with expectations from quantitative tolerance mechanisms, as is the fact that such T cells are able to respond to APCs with higher levels of the ligand. At a minimum, the proper experiment is to use the rapamycin-treated EL4 cells as immunogens and see if a response can be detected, not peptide-pulsed DCs whose level of the bound peptides has not been adjusted to the level seen on EL4 cells treated with drug.

Response: We thank the Reviewer for pointing out this inaccuracy. In the new version of the manuscript, we have substituted "immunogenicity" for "antigenicity". The experiment proposed by the Reviewer is logical and would indeed allow us to determine whether our MIPs are immunogenic. However, we are unable to perform this experiment because of the following experimental constraints: i) MIPs upregulated after treatment of EL4 cells with rapamycin for 48h rapidly disappear in the absence of rapamycin (i.e., after in vivo injection) and ii) we cannot treat immunised mice with rapamycin because rapamycin is immunosuppressive and inhibits T-cell responses.

8. The claim "In other words, they suggested that, at the organismal level, the immune self was practically invariant." made on p. 15 is not correct - what was stated in these early reports is that the MOST ABUNDANT MHC CI bound peptides come from highly abundant housekeeping proteins that are common to many cell types, but that as many as 10000 different peptides are presented when one considers levels of as few as 10 copies per cell, which suggested that a large fraction of the proteome of a cell would be represented in MIPs.

Response: We agree that our previous statement was inaccurate and we thank the Reviewer for his/her suggestion. Accordingly, we have rewritten this section of the discussion. We now state: (p.16, 1st paragraph): "Early proteomic studies were conducted with analyzers whose sensitivity (dynamic range) and accuracy were orders of magnitude inferior to that of MS analyzers that are now available (Depontieu et al., 2009; Nilsson et al., 2010; Yates et al., 2009). As a result, early studies on the immunopeptidome identified only the more abundant MIPs. Those peptides were found to derive from highly abundant housekeeping proteins that are common to many cell types (Barnea et al., 2002; Engelhard et al., 2002; Hughes and Hughes, 1995; Marrack et al., 1993). More recently, high-throughput MS-based analyses have shown that immunopeptidome conceals a cell type-specific signature."

Methodological issues:

1. It is critical to know what fraction of detected peptides were excluded by using a motif filter, as the predictive algorithms for MHC class I binding, while quite good, are nonetheless not fully reliable and many MIPs might be excluded by this approach.

Response: We thank the Reviewer for pointing this out. Indeed, identified peptides were filtered using an MHC motif filter to select $H2D^b$-, $H2K^b$- and Qa1/2-associated peptides. We have previously reported that this filtering excludes about 3% of MIPs (Fortier *et al.*, 2008). In supplementary methods, we provided the PHP script that we used for filtering identified peptides.

2. It is unclear how relative abundance was actually calculated for the untreated vs. treated cells, given the size, and thus, what is certainly a total protein difference. It is unclear if what matters most if the fractional loading of available MHC CI or the total number of loaded class I molecules.

Response: The relative abundance was calculated based on the total number of loaded class I molecules. In supplementary method, we provided additional details of the label-free quantitative proteomics approach used to profile the abundance of MHC class I peptides across conditions and replicates. Briefly, MS/MS spectra were processed using Mascot Distiller v2.1.1 (Matrix Science), and centroided MS/MS data were merged into single peak list file and searched with the Mascot search engine v2.2 (Matrix Science) against the combined forward and reversed IPI mouse database (version 3.23 containing 51,536 forward protein sequences with a mass precursor tolerance of ± 0.05 Da and a fragment tolerance of ± 0.5 Da. Search conditions included: trypsin with 1 missed cleavage and the following modifications: deamidation (NQ), oxidation (M), phosphorylation (STY) as variable modifications. False discovery rate (FDR) was obtained using a decoy database and peptides assigned to proteins with a $p < 0.05$ significance threshold and provided a FDR below 2%. Redundant MS/MS spectra were removed keeping only unique identification with the highest Mascot score. Identified peptides were filtered using an MHC motif filter based on the predicted mouse MHC I allele motifs (see PHP script in supplementary methods). Label-free quantitative proteomics analyses were performed using ProteoProfile (an in-house software that enables peptide detection), data clustering (sample replicates and conditions), and validation of assignments. Peptides displaying changes in abundance were validated manually and low signal/noise ratios, or overlapping peaks were not included for quantitative purposes.

Reviewer #3 (Remarks to the Author):

The manuscript by Caron et al. is well written and timely. The authors followed the effect of the mTOR inhibitor rapamycin on the immunopeptidome of EL4 murine cells as a way of gauging the effects of this inhibitor on cellular processes of protein degradation and presentation of MHC peptides. This way, they aimed to expand their previous proteomics studies on mTOR signaling by

studying its effects from the point of view of the immunopeptidome. They first evaluated if the inhibitor is indeed effective in these cells and found out that it is. Also, they demonstrate that rapamycin did not inhibit or modify significantly the known components of the MHC peptide loading machinery. The authors have followed the levels of expression of MHC peptides at the cell surface using the mild acid elution method and have detected an increase in many classical MHC class I peptides but not in non-classical MHC peptides. They assigned the peptides to each of these MHCs using their consensus sequence motifs. Since the mild acid elution method release many non-MHC peptides, the authors have defined as MHC peptides only those that were eluted from cells containing the b2m and not from those lacking it (suggest mentioning this fact in the Results since this is an important issue that may be overlooked in the Methods).

Response: We added the following sentence in the result section (p.7, 2nd paragraph): "Subtraction of contaminant peptides eluted from β2m- mutant EL4 cells allowed specific identification of genuine MIPs (de Verteuil *et al.*, 2010;Fortier *et al.*, 2008) (de Verteuil *et al.*, 2010;Fortier *et al.*, 2008)."

In my opinion this methodology is the weakest point in this otherwise very good manuscript. The problem is that the association of the detected peptides is inferred from the assay rather than being purified by immunoaffinity chromatography with anti-MHC mAb, which should result in relatively pure preparations of peptides and in about tenfold more identified and quantified peptides from the number of cells used for this study. Therefore, the authors base their results on a relatively small number of about 400 identified peptides, which may be considered somewhat small for a significant pathway and cellular function analysis. The problem is also related to the fact that this way the authors can only identify and quantify only the most abundant peptides or easily detected, which may not represent as well the entire MHC immunopeptidome.

Response: We thank the Reviewer for his/her positive assessment of our work. Considering the high complexity of the immunopeptidome, we agree with the Reviewer that 422 peptides is a relatively small number of peptides. Nevertheless, to the best of our knowledge, our dataset contains the largest number of sequenced MIPs ever reported from a single cell type. Furthermore, other methods allow for evaluation of MIPs associated with only one MHC molecule and are fraught with significant caveats. Limitations of standard immunoaffinity chromatography with anti-MHC mAb are a low peptide yield, preferential loss of peptides with low affinity for MHC I and contamination of the MHC molecules by cellular debris and detergents (Admon *et al.*, 2003;Gebreselassie *et al.*, 2006). A much higher peptide yield can be obtained by cell transfection with expression vectors coding soluble secreted MHCs (lacking a functional transmembrane domain) and elution of peptides associated with secreted MHCs (Barnea *et al.*, 2002;Hickman *et al.*, 2004). This interesting approach nevertheless has two limitations: i) cell transfection per se may perturb the MIP repertoire (Hickman *et al.*, 2003) and ii) the MIP repertoire associated with soluble MHC corresponds to the repertoire of peptides that can bind the transfected MHC allele (what "can be presented") but not necessarily to peptides that are normally presented at the cell surface (what "is presented").

They next went ahead and compared the transcriptome of the same type cells treated with rapamycin (in a separate experiment) with the immunopeptidome. Since the correlation observed was not significant between the MHC peptides and transcripts that changed in their amounts due to the treatment, the authors have expanded it by looking at shared genes/proteins participating in the same pathways and functional complexes as do the detected MHC peptides that changed in their levels. So, instead of finding strong correlation between the exact transcripts and MHC peptides derived from them, they found correlation with other members of the same functional groups of proteins and this way inferred that similar pathways are affected at the level of the transcriptome and the immunopeptidome. The most effected transcripts and MHC peptides were related to mTOR signaling and function, thus validating the relevance of the study.

Response: We thank the Reviewer of his/her positive assessment of our work.

The groups of MHC peptides changing in their levels were most significantly associated with transcriptional and translational functions of the cellular processes. This is an interesting observation, but not that surprising when looking at immunopeptidomes in general since these pathways are often composed of rapidly turning-over proteins which are likely to contribute more to the immunopeptidome.

Response: We agree with the Reviewer. Under steady state condition, proteins that regulate transcription, translation, and cell cycle progression are overrepresented in the immunopeptidome (Perreault, 2010). The surprising finding here is that DEM source genes are highly functionally interconnected to DEGs. In other words, it suggests that the immunopeptidome is a functional, if not a direct representation of the transcriptional state a cell. This statement is now highlighted in p.19 (1st paragraph).

The argument in the first sentence of the discussion "Early proteomic studies of the immunopeptidome have led scientists to believe that MIPs were derived from housekeeping genes and presented minimal if any differences among different cell types" is not completely accurate, since early studies on MHC peptides relied on rather insensitive instruments and identified as a results only the more abundant MHC peptides, which were obviously often produced from housekeeping genes. The newer experiments, in most studies relying on more modern mass spectrometers, identify more balanced repertoires of peptides from more cellular processes. Furthermore, studies that looked at the MHC peptidomes associated with just one immunoaffinity purified MHC allele produce more similar patterns of peptides, while studies relying on the pools of peptides carried by all the MHC allomorphs in cells differing in their haplotypes, obviously did not claim that the peptides repertoire are similar between cells. This issue is better clarified in the discussion.

Response: We agree with the Reviewer that our previous statement was inaccurate. We have rewritten this section of the discussion. We now clearly state: (p.16, 1st paragraph): "Early proteomic studies were conducted with analyzers whose sensitivity (dynamic range) and accuracy were orders of magnitude inferior to that of MS analyzers that are now available (Depontieu *et al.*, 2009;Nilsson *et al.*, 2010;Yates *et al.*, 2009). As a result, early studies on the immunopeptidome identified only the more abundant MIPs. Those MIPs were found to derive from highly abundant housekeeping proteins that are common to many cell types (Barnea *et al.*, 2002;Engelhard *et al.*, 2002;Hughes and Hughes, 1995;Marrack *et al.*, 1993). More recently, high-throughput MS-based analyses have shown that immunopeptidome conceals a cell type-specific signature."

The author eventually checked that some of the identified peptides could elicit T cell response indicating that some mTOR induced MHC peptides are possibly immunogenic. This part of the study does not contribute so much to its systems biology conclusions, since anyways, many peptides can elicit immune response, but this does not indicate that particular pathways and cellular processes generate MHC peptides more likely to be immunogenic, as might be concluded from the claims in this manuscript.

Response: We thank the Reviewer for this comment. We now clearly state (p.15, 2nd paragraph): "Further studies are needed to evaluate 1) the proportion of novel or overabundant MIPs that are antigenic and 2) whether particular pathways and cellular processes generate MIPs that are more likely to be antigenic.".

I suggest accepting this manuscript with minor revisions, since as I stated above, I think that it is an important manuscript, with vast implication to the study of systems biology through the use systems immunology. It also provides a totally fresh point of view, not only on the immunopeptidome, but also on the cellular proteome and degradome. The figures and tables in the article are needed and useful. As stated above, I am not sure if the CTL assays contribute much to the manuscript, but it can be left in. The length of the article is adequate. The methods part is clear enough.

Response: We thank the Reviewer for his/her positive assessment of this work.

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2nd Editorial Decision 21 July 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the referee who agreed to evaluate this revised study. As you will see, this referee is now largely supportive of publication, although s/he has a few minor suggestions for modifications. In addition, we also note some editorial issues regarding formatting and data availability. We ask you to carefully address these points in a final revision of the present work.

1. It will be important that the proteomic data described here is publicly released with this work. The editor notes that the list of MIPs was submitted to The Immune Epitope Database, but it was not clear whether the proteomic data for this study could be directly downloaded from this site. Ideally, we would ask you to submit the proteomic data to a community repository like PRIDE or Tranche. Please see our Instructions for Authors for more details on our data deposition policies, and feel free to contact us if you have any questions (http://www.nature.com/msb/authors/index.html#a3.5).

2. Reviewer #1 did agree that Figure 1 For Reviewers did not need to be included in the manuscript. We can include this Figure in the public Review Process File if you wish, which may help readers that want to fully understand the review process, but we would fully understand if you would rather leave this Figure out (for instance, if you are concerned about your ability to publish these results in another subsequent work).

3. Please note, that in addition to our capacity to host datasets in our supplementary information section, we provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for Figures 6 and 7. Please see our Instructions for Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>).

4. Please provide any Supplementary Tables with more than 50 rows as separate excel or text-table (tab-delimited or csv) files, and remove them from the Supplementary Information pdf. All supplementary figures and tables should still be listed at the beginning of the Supplementary Information pdf.

5. With your revised work please provide the following items:
-- a letter with a detailed description of the changes made in response to the referee. Please specify clearly the exact places in the text (pages and paragraphs) where each change has been made in response to each specific comment given.
-- three to four 'bullet points' highlighting the main findings
-- a 'standfirst text' summarizing the study in one or two sentences (approx. 250 characters)
-- a "thumbnail image" (width=211 x height=157 pixels, jpeg format), which can be used to highlight your paper on our homepage.

Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor
Molecular Systems Biology

REFEREE REPORT

Reviewer #1 (Remarks to the Author):

The authors have addressed the questions of control experiments satisfactorily. The extension of their analysis of the source genes for differentially expressed MHC I peptides to include all 98

peptides certainly strengthens their conclusions as well. The more nuanced wording of the manuscript and abstract is also appreciated.

Overall, I think this manuscript would be a good addition to the literature, and support its publication in your journal.

A few minor concerns:

1. During their analysis of the subset of DEM source genes that are connected to the mTOR network on pg 10, they once again state that "The systems-level analysis demonstrates that rapamycin-induced variations in the immunopeptidome arise from biochemical networks whose components are highly connected to the target of rapamycin." This sentence should be reworded to indicate that a percentage (30/98) or fraction of rapamycin-induced variations in the immunopeptidome arise from biochemical networks whose components are highly connected to the target of rapamycin.

2. The antigenicity experiments are interesting, and I agree with the authors that they do not need to be included in the final manuscript.

3. I would also suggest that the authors clarify in the main body of the text that they use bootstrapping as a statistical sampling method: there are many interpretation of the term "bootstrapping", some of which can be applied to informatic analyses under other circumstances. Clarifying this term would make the manuscript more accessible to a multi-disciplinary audience.

Again, I would like to reiterate that the statistical and informatic analyses applied to the analysis of the peptide MHC I repertoire are useful and represent a fresh approach to this problem. It may seem logically obvious that the peptide MHC I repertoire reflect the metabolic state of the cell, but rigorous analysis to show that this is actually the case is necessary. The authors make a good first step down this road, and with the more nuanced wording in this version, show some strong evidence for their conclusions.

1st Revision - authors' response 19 August 2011

Editor ‘s comments

1. It will be important that the proteomic data described here is publicly released with this work. The editor notes that the list of MIPs was submitted to The Immune Epitope Database, but it was not clear whether the proteomic data for this study could be directly downloaded from this site. Ideally, we would ask you to submit the proteomic data to a community repository like PRIDE or Tranche. Please see our Instructions for Authors for more details on our data deposition policies, and feel free to contact us if you have any questions (http://www.nature.com/msb/authors/index.html#a3.5).

Response: As now indicated on p. 24, our MS/MS data have been deposited in both Pride and ProteoConnections. For review of MS/MS data, a special account was created at Pride. To access these data reviewers can use the following information. Please note that we had to split the dataset in two because of file size limitations:

Accession numbers: 18855 (EL4 control T=0) and 18856 (EL4 stimulated with rapamycin, T=6, 12, 24, 48h)

2. Reviewer #1 did agree that Figure 1 For Reviewers did not need to be included in the manuscript. We can include this Figure in the public Review Process File if you wish, which may help readers that want to fully understand the review process, but we would fully understand if you would rather leave this Figure out (for instance, if you are concerned about your ability to publish these results in another subsequent work).

Response: We agree to include this Figure in the public Review Process.

3. *Please note, that in addition to our capacity to host datasets in our supplementary information section, we provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for Figures 6 and 7. Please see our Instructions for Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>).*

Response: Linked data files are provided for Figures 6 and 7.

4. Please provide any Supplementary Tables with more than 50 rows as separate excel or text-table (tab-delimited or csv) files, and remove them from the Supplementary Information pdf. All supplementary figures and tables should still be listed at the beginning of the Supplementary Information pdf.

Response: Supplementary Tables S1, S2, S4 and S5 are provided in separate Excel files

5. With your revised work please provide the following items:
-- a letter with a detailed description of the changes made in response to the referee. Please specify clearly the exact places in the text (pages and paragraphs) where each change has been made in response to each specific comment given.
-- three to four 'bullet points' highlighting the main findings
-- a 'standfirst text' summarizing the study in one or two sentences (approx. 250 characters)
-- a "thumbnail image" (width=211 x height=157 pixels, jpeg format), which can be used to highlight your paper on our homepage

Response: We have included separate files containing i) a thumbnail image, and ii) the bullet points and standfirst text. The license to publish has been signed and faxed to the Nature Publishing Group Production Department.

Reviewer #1 (Remarks to the Author):

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Response: We agree and have reworded this sentence (p. 11): “This systems-level analysis demonstrates that a substantial fraction of rapamycin-induced variations in the immunopeptidome arise from biochemical networks whose components are highly connected to the target of rapamycin (i.e. mTOR).”

2. The antigenicity experiments are interesting, and I agree with the authors that they do not need to be included in the final manuscript.

Response: Thank you.

3. I would also suggest that the authors clarify in the main body of the text that they use bootstrapping as a statistical sampling method: there are many interpretation of the term "bootstrapping", some of which can be applied to informatic analyses under other circumstances. Clarifying this term would make the manuscript more accessible to a multi-disciplinary audience.

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Response: We agree and now state on p. 10: “Then, bootstrapping was used as a statistical sampling method to calculate control connectivity scores from 10^5 sets of 38 randomly selected MIP source genes from a database of 891 unique source genes encoding $H2^b$-associated peptides”.

