



## RESEARCH NOTE

**REVISED** **Antigenicity of peptides comprising the immunosuppressive domain of the retroviral envelope glycoprotein [version 2; referees: 2 approved]**

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**Abstract**

To achieve persistent infection of the host, viruses often subvert or suppress host immunity through mechanisms that are not entirely understood. The envelope glycoprotein of several retroviruses is thought to possess potent immunosuppressive activity, mapped to a 17-amino acid residue conserved domain. Synthetic peptides corresponding to this immunosuppressive domain can inhibit lymphocyte activation, whereas mutation of key domain residues can increase the lymphocyte response to linked antigenic epitopes. Using three T cell receptors (TCRs) of defined specificity, we examine the effect of the immunosuppressive domain on the T cell response to their respective antigenic peptides. We find that fusion of a T cell epitope to the immunosuppressive domain can greatly modulate its potency. However, the effects heavily depend on the particular combination of TCR and peptide-major histocompatibility complex class II (pMHC II), and are mimicked by sequence-scrambled peptides of similar length, suggesting they operate at the level of pMHC formation or TCR-pMHC interaction. These results offer an alternative explanation for the immunogenicity of T cell epitopes comprising the putative immunosuppressive domain, which is more consistent with an effect on peptide antigenicity than true immunosuppressive activity.

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report



report

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**REVISED Amendments from Version 1**

The following changes were made in response to the Reviewers' comments:

1. The following sentence in the Abstract:

"However, the effects heavily depend on the particular combination of TCR and peptide-major histocompatibility complex class II (pMHC II), and are mimicked by sequence-scrambled peptides of similar length, suggesting they operate at the level of TCR-pMHC interaction."

now reads:

However, the effects heavily depend on the particular combination of TCR and peptide-major histocompatibility complex class II (pMHC II), and are mimicked by sequence-scrambled peptides of similar length, suggesting they operate at the level of pMHC formation or TCR-pMHC interaction.

2. The following sentence was added to the Methods/Peptides encompassing the immunosuppressive domain:

"Scrambled sequences represent permutation of the original peptide sequences and were constructed using the peptide manufacturer's algorithms. All peptides were sufficiently hydrophobic..."

3. Shaded parts of [Table 1](#) were replaced with red colour text for enhanced contrast. This is also indicated in the [Table 1](#) legend.

4. The following sentence in the last paragraph of Results and Discussion:

"In contrast, neither peptide fusion affected the response of the DO11.10 clone ([Figure 2B](#)), indicating that effects of the immunosuppressive peptides can be observed in some TCR-pMHC pairs, but not others."

now reads:

"In contrast, neither peptide fusion affected the response of the DO11.10 clone ([Figure 2B](#)), indicating that effects of the immunosuppressive peptides can be observed in some pMHC combinations or TCR-pMHC pairs, but not others."

**See referee reports**

## Introduction

Several studies over the last three decades have provided evidence to suggest that the envelope glycoprotein of certain retroviruses is immunosuppressive *in vitro* and *in vivo* ([Cianciolo et al., 1985](#); [de Parseval et al., 2001](#); [Denner, 2014](#); [Dupressoir et al., 2012](#); [Haraguchi et al., 1997](#); [Kudo-Saito et al., 2014](#); [Mangeny et al., 2007](#); [Schlecht-Louf et al., 2010](#)). Although the precise molecular mechanism remains unresolved, immunosuppressive activity has been pinpointed at a short region of the transmembrane polypeptide that, together with the surface unit (SU), constitutes one part of the trimeric envelope glycoprotein ([Cianciolo et al., 1985](#); [Dupressoir et al., 2012](#); [Schlecht-Louf et al., 2010](#)). For example, synthetic peptides corresponding to a region of 17-amino acid residues from the murine leukemia virus (MLV) envelope glycoprotein inhibit immune function in a variety of assays. These assays measure distinct aspects of immune responsiveness. Some measure non-specific lymphocyte or myeloid cell activation in response to mitogens or other generic stimuli ([Denner, 2014](#); [Denner et al., 1996](#); [Haraguchi et al., 1993](#); [Haraguchi et al., 1995](#); [Mitani et al.,](#)

[1987](#); [Tolosa et al., 2012](#)). Others measure antigen-specific T and B cell responses to immunization with synthetic peptides, recombinant envelope domains, and to infection with MLVs ([Morozov et al., 2012](#); [Schlecht-Louf et al., 2014](#); [Schlecht-Louf et al., 2010](#)). Use of different assays, many of which are multilayered, has hindered direct comparison of results from different studies, or definition of the specific step in immune function that may be suppressed. Here, we examine the properties of T cell epitopes linked to the immunosuppressive domain and provide an alternative interpretation for the effects.

## Materials and methods

### Mice

All animal experiments were approved by the Ethical Committee of the Francis Crick Institute, and conducted according to local guidelines and UK Home Office regulations under the Animals Scientific Procedures Act 1986. Inbred C57BL/6J and BALB/cJ mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and subsequently maintained at the Francis Crick Institute's animal facilities, under specific pathogen-free conditions. Male or female mice, between 8 and 12 weeks of age, were used for the isolation of macrophages. T cell receptor (TCR)-transgenic OT-II ([Barnden et al., 1998](#)) and DO11.10 ([Murphy et al., 1990](#)) mice were kept on the C57BL/6J and BALB/cJ genetic backgrounds, respectively, and were additionally crossed to *Rag1*<sup>-/-</sup> mice to prevent rearrangement of endogenous TCR loci.

### Macrophages

Macrophages were isolated from the peritoneal cavity of naïve euthanized C57BL/6J or BALB/cJ mice (2–6 mice per experiment). Peritoneal cells (containing ~50% macrophages) were plated in flat-bottomed 96 well-plates at  $4 \times 10^5$  per well the day before their use, in Iscove's Modified Dulbecco's Medium (IMDM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA USA), 2mM L-glutamine, 100U penicillin and 0.1mg/ml streptomycin, and cultured at 37°C in a 5% CO<sub>2</sub> atmosphere. Following overnight culture, macrophages were enriched by the removal of non-adherent cells, and were used for the assays.

### T cell hybridomas

The env<sub>124–138</sub>-reactive H5 and H18 CD4<sup>+</sup> T cell hybridomas have been previously described ([Young et al., 2012](#)). The ova<sub>323–339</sub>-reactive OT-II and DO11.10 hybridomas were generated by fusion of ova<sub>323–339</sub>-stimulated primary splenic CD4<sup>+</sup> T cells from single TCR-transgenic OT-II and DO11.10 mice, respectively, with TCRαβ-negative BW5147 thymoma cells, using established techniques ([Young et al., 2012](#)). In at least two experiments, primary CD4<sup>+</sup> T cells from TCR-transgenic mice were also used with comparable results.

### Peptides encompassing the immunosuppressive domain

Peptides were synthesized by Insight Biotechnology Ltd., Wembley, UK, at >98% purity. Scrambled sequences represent permutation of the original peptide sequences and were constructed using the peptide manufacturer's algorithms. All peptides were sufficiently hydrophobic and were dissolved in phosphate-buffered saline prior to use ([Table 1](#)).

**Table 1. Peptides used in this study.** The following peptides were synthesized and used as indicated in the text. The underlined amino acid residues indicate the E561R and A567F double mutation (Schlecht-Louf *et al.*, 2010). Red coloured sequences correspond to the immunosuppressive peptide.

Peptide name	Sequence
env <sub>124-138</sub>	PLTSLTPRCNTAWN <sup>R</sup>
env <sub>548-567</sub>	<u>LQNRRLDLLFLKEGGLCAA</u>
env <sub>548-567:E561R/A567F</sub>	<u>LQNRRLDLLFLK<u>R</u>GGLCA<u>F</u></u>
env <sub>124-138</sub> -env <sub>548-567</sub>	PLTSLTPRCNTAWN <sup>R</sup> LQNRRLDLLFLKEGGLCAA
env <sub>124-138</sub> -env <sub>548-567:E561R/A567F</sub>	PLTSLTPRCNTAWN <sup>R</sup> LQNRRLDLLFLK <u>R</u> GGLCA <u>F</u>
env <sub>124-138</sub> -env <sub>548-567:SCRAMBLED</sub>	PLTSLTPRCNTAWN <sup>R</sup> AGFAKGLRDGLRQLNELCLL
env <sub>124-138</sub> -env <sub>548-567:E561R/A567F:SCRAMBLED</sub>	PLTSLTPRCNTAWN <sup>R</sup> FGFAKGLRDGLRQLNRLCLL
env <sub>124-158</sub>	PLTSLTPRCNTAWN <sup>R</sup> LKLDQVTHKSSEGFYVCPGS
ova <sub>323-339</sub>	ISQAVHAAHAEINEAGR
ova <sub>323-339</sub> -env <sub>548-567</sub>	ISQAVHAAHAEINEAGR <u>LQNRRLDLLFLKEGGLCAA</u>
ova <sub>323-339</sub> -env <sub>548-567:E561R/A567F</sub>	ISQAVHAAHAEINEAGR <u>LQNRRLDLLFLK<u>R</u>GGLCA<u>F</u></u>

### T cell stimulation

T cell hybridomas were stimulated by the indicated peptides presented by primary macrophages. T cell clones restricted by H2-A<sup>b</sup> (H5, H18 and OT-II) were stimulated by C57BL/6J-derived macrophages, whereas the H2-A<sup>d</sup>-restricted clone (DO11.10) was stimulated by BALB/cJ-derived macrophages. Similar results were also obtained when primary splenic B cells or bone-marrow-derived dendritic cells were used for presentation. Approximately,  $1.5 \times 10^5$  T cells per well were used in flat-bottomed 96 well-plates in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal calf serum. The following day, T cell activation was assessed by flow cytometric detection of CD69 induction, using a fluorescein isothiocyanate-conjugated monoclonal anti-mouse CD69 antibody (armenian hamster; clone H1.2F3; eBioscience, San Diego, CA, USA; cat. no. 11-0691-85) at 1 in 200 dilution. At least 50,000 cells were acquired on LSR-Fortessa X-20 or FACSCanto II cytometers (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo v10 (Tree Star Inc., Ashland, OR, USA). Unstimulated hybridoma cells were included as CD69-negative controls, based on which the gating of CD69-positive cells was drawn.

### Transcriptional profiling by RNA sequencing

Transcriptional profiles of primary macrophages incubated with the env<sub>124-138</sub>-env<sub>548-567</sub> and env<sub>124-138</sub>-env<sub>548-567:E561R/A567F</sub> peptides were obtained by RNA sequencing. Briefly, duplicate cultures of peritoneal macrophages from C57BL/6 mice were stimulated overnight with 10 μM concentration of either peptide or were left unstimulated. RNA was then extracted using the RNeasy Mini QIAcube kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and subjected to RNA sequencing (GENEWIZ, Inc., South Plainfield, NJ, USA). Reads were assessed for quality and contamination with FastQC v0.11.2 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and preprocessed with Trimmomatic v0.32

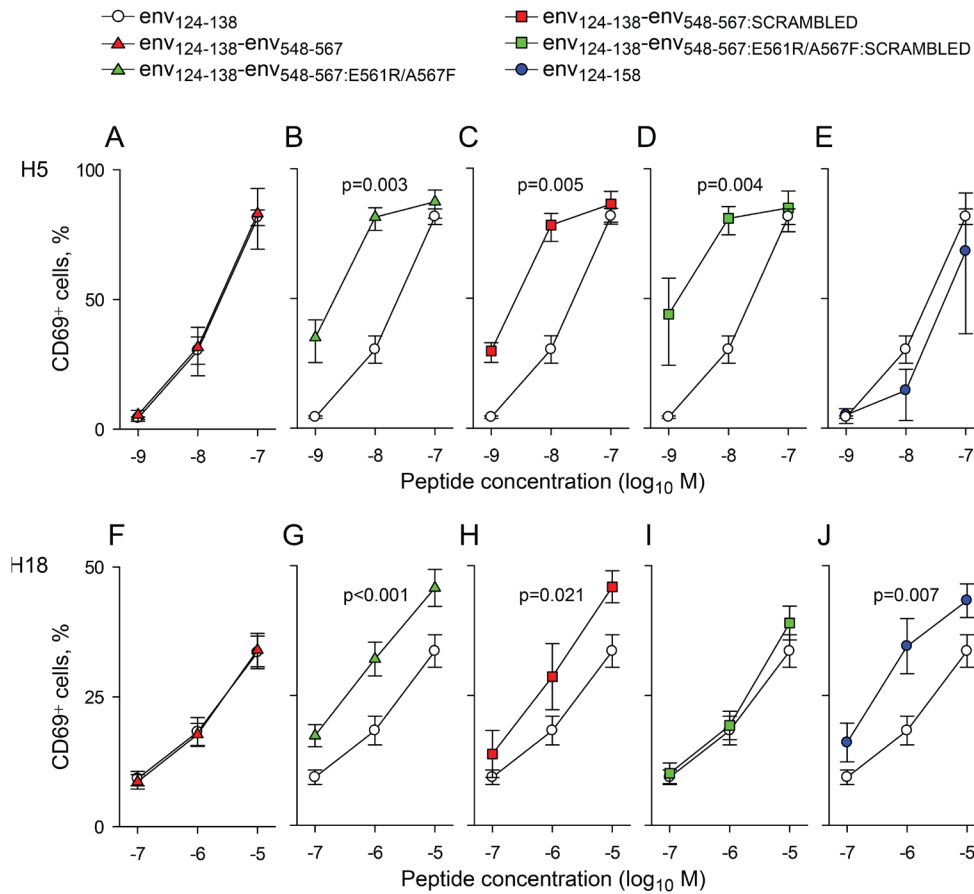
(<http://www.usadellab.org/cms/?page=trimmomatic>) to remove the identified adapters, low quality read tails and to filter for length, and were then assessed for differential expression with DESeq2 v1.6.3 (<http://bioconductor.org/packages/release/bioc/html/DESeq.html>) within R v3.1.3. Data for all samples were normalized and rlog transformed and were then analyzed using GeneSpring v12.1 GX (Agilent, Santa Clara, CA, USA).

### Statistical analysis

Statistical comparisons were made using SigmaPlot 13.0 (Systat Software Inc., Germany). Parametric comparisons of normally-distributed values that satisfied the variance criteria were made by unpaired Student's t-tests. Data that did not pass the variance test were compared with non-parametric two-tailed Mann-Whitney Rank Sum tests.  $P < 0.05$  were considered significant.

### Results and discussion

We used synthetic peptides corresponding to the conserved env<sub>548-567</sub> region of the Friend MLV envelope precursor gPr80 (LQNRRLDLLFLKEGGLCAA), which contains the originally described 17-amino acid residue immunosuppressive peptide (Cianciolo *et al.*, 1985). As controls, we introduced two amino acid substitutions (E561R and A567F), previously shown to abrogate the immunosuppressive activity of this region (Schlecht-Louf *et al.*, 2010), and also used scrambled sequences for both peptides (Figure 1). These peptides were tested for their effects on the antigenicity of the H2-A<sup>b</sup>-restricted env<sub>124-138</sub> CD4<sup>+</sup> T cell epitope in the SU of F-MLV (PLTSLTPRCNTAWN<sup>R</sup>). As immunosuppressive peptides have generally been found active only as part of larger polypeptides (Denner, 2014), we used peptide fusion of the T cell epitope and the immunosuppressive peptide into a single molecule (env<sub>124-138</sub>-env<sub>548-567</sub>). Fusion with the immunosuppressive peptide env<sub>548-567</sub> had no measureable effect on the ability of the env<sub>124-138</sub> epitope to stimulate two env<sub>124-138</sub>-specific CD4<sup>+</sup> T cell



**Figure 1.** *In vitro* responses of A<sup>b</sup>-restricted env<sub>124-138</sub>-reactive CD4<sup>+</sup> T cell hybridomas H5 (top row) and H18 (bottom row), to stimulation with the indicated peptides. Plotted are the frequencies of T cells that express CD69 following overnight stimulation. Data are the mean (±SEM) of 2–5 individual experiments.

clones (Figure 1A and F) that differ in functional avidity (Young *et al.*, 2012). CD4<sup>+</sup> T cell responses to env<sub>124-138</sub> were similarly unaffected by the addition in the cultures of the immunosuppressive peptide env<sub>548-567</sub>, either as a separate entity or fused with the unrelated H2-A<sup>b</sup>-restricted ova<sub>323-339</sub> epitope from chicken egg ovalbumin (ISQAVHAAHAEINEAGR) (Supplementary Figure 1). In contrast, fusion with either the doubly point-mutated (Figure 1B and G;  $p=0.003$  and  $p<0.001$  for clones H5 and H18, respectively) or sequence-scrambled immunosuppressive peptide (Figure 1C and H;  $p=0.005$  and  $p=0.021$  for clones H5 and H18, respectively) significantly increased the antigenicity of the env<sub>124-138</sub> epitope for both clones by ~10-fold. Increased antigenicity of the env<sub>124-138</sub> epitope as a result of mutations at the two residues or sequence scrambling of the fused immunosuppressive peptide could be interpreted as reversal of immunosuppression due to these modifications. However, comparison with the env<sub>124-138</sub> epitope on its own revealed that fusion with the index immunosuppressive peptide was

in fact neutral (Figure 1A and F), whereas fusion with the modified immunosuppressive peptides improved antigenicity.

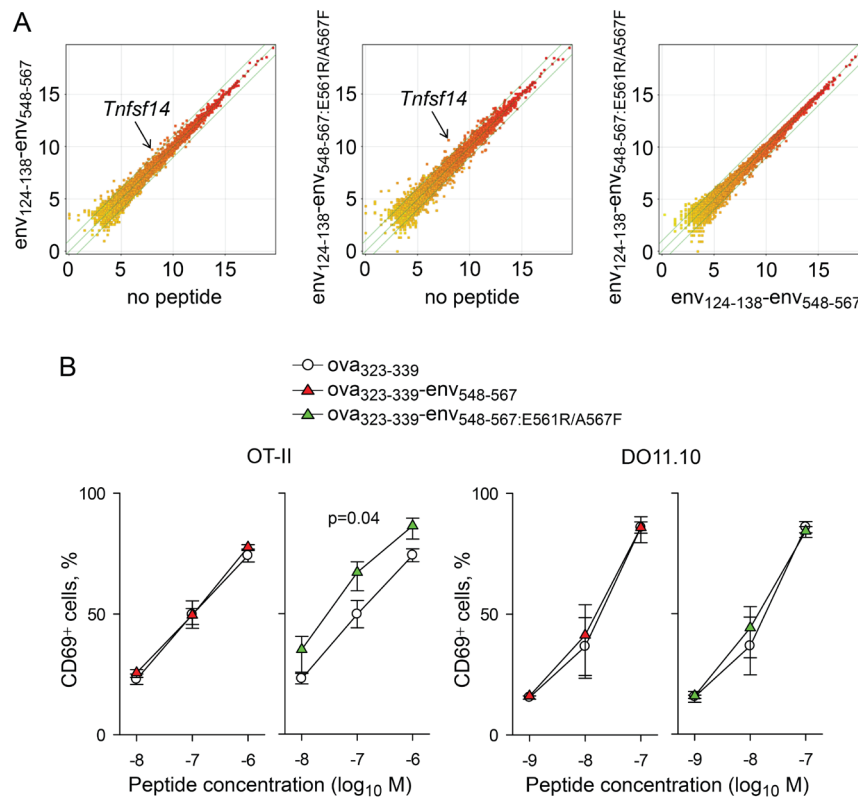
Peptide length is an important contributor to the antigenicity of MHC II-restricted epitopes. Indeed, N-terminal extensions of the env<sub>124-138</sub> epitope have been shown to increase antigenicity (Young *et al.*, 2012), in line with findings in other systems (Carson *et al.*, 1997; Holland *et al.*, 2013). The open structure of the MHC II groove allows presentation of peptides that extend at either end of the core epitope, and these epitope-flanking residues can contribute to T cell stimulation (Holland *et al.*, 2013). Importantly, the involvement of epitope-flanking residues critically depends on the particular pair of TCR and peptide-MHC II (pMHC) complex, as contact between particular epitope-flanking residues and residues in the TCR is necessary (Holland *et al.*, 2013). To examine whether the observed effect of the index or modified immunosuppressive peptides was TCR- and peptide sequence-dependent, we used

additional fusion peptides. Notably, a peptide that carried the E561R/A567F substitutions and was additionally sequenced-scrambled enhanced antigenicity of the env<sub>124-138</sub> epitope for the H5 clone ( $p=0.004$ ), but not the H18 clone (Figure 1D and I). Moreover, extending the length of the env<sub>124-138</sub> epitope with the sequence that naturally occurs in the F-MLV SU (env<sub>124-158</sub>) enhanced activation of the H18 clone ( $p=0.007$ ), but not of the H5 clone (Figure 1E and J). These two clones share the same TCR $\beta$  chain, but use different TCR $\alpha$  chains, which are responsible for the difference in functional avidity (Young *et al.*, 2012). The disparate behaviour of the two clones in response to the last two fusion peptides demonstrate that changes in antigenicity depend on the particular TCR. This finding argues against general immunosuppression caused by the env<sub>548-567</sub> peptide.

Also arguing against immunosuppressive ability, env<sub>124-138</sub> fusion peptides containing the index immunosuppressive peptide or the E561R/A567F mutant induced comparable transcriptional changes to the APCs used in these assays (Figure 2A). Incubation of primary macrophages with either of the two env<sub>124-138</sub> fusion peptides altered their transcriptional signature in comparison with the absence of peptide, with *Tnfsf14* (also known as

HVEM-L or LIGHT) most strongly induced (between 3- and 4-fold) (Figure 2A). However, the transcriptional signatures induced by the two peptides were effectively identical (Figure 2A).

We next examined if our findings with env<sub>124-138</sub>-specific CD4<sup>+</sup> T cell clones extended to other combinations of TCR and pMHC. To this end, we used the OT-II and DO11.10 clones, which react with the same ova<sub>323-339</sub> epitope presented by H2-A<sup>b</sup> and H2-A<sup>d</sup>, respectively. Fusion of the ova<sub>323-339</sub> epitope with the env<sub>548-567</sub> E561R/A567F mutant, but not the index immunosuppressive peptide enhanced the response of the OT-II clone (Figure 2B) ( $p=0.04$ ). In contrast, neither peptide fusion affected the response of the DO11.10 clone (Figure 2B), indicating that effects of the immunosuppressive peptides can be observed in some pMHC combinations or TCR-pMHC pairs, but not others. Thus, this pattern of changes in T cell activation, depending on the particular TCR-pMHC pair, is more consistent with an effect mediated by epitope-flanking residues than genuine immunosuppression. The potential of the immunosuppressive peptide, and modifications thereof, to alter TCR or BCR recognition of linked epitopes should, therefore, be considered when interpreting the antigenicity or immunogenicity of the retroviral envelope glycoprotein.



**Figure 2.** *In vitro* responses of peritoneal macrophages or hybridomas OT-II and DO11.10 to stimulation with the indicated peptides. (A) Transcriptional comparison of primary macrophages incubated overnight in the absence of peptide or in the presence of 10  $\mu$ M of the indicated peptide. Each dot represents the normalized counts for individual transcripts and green diagonal lines enclose changes in relative transcript abundance of <2-fold in each comparison. (B) *In vitro* responses of A<sup>b</sup>-restricted and A<sup>d</sup>-restricted ova<sub>323-339</sub>-reactive CD4<sup>+</sup> T cell hybridomas OT-II and DO11.10, respectively, to stimulation with the indicated peptides. Data are the mean ( $\pm$ SEM) of 2–3 individual experiments.

## Data availability

RNA-sequencing data reported in this paper are available at: [www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-5260](http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-5260) (accession number, E-MTAB-5260).

Raw values for T cell responses shown in [Figure 1](#), [Figure 2B](#) and [Supplementary Figure 1](#) are available from the Open Science Framework: <https://osf.io/8y8h4/>; doi, [10.17605/OSF.IO/8Y8H4](https://doi.org/10.17605/OSF.IO/8Y8H4) ([Kassiotis, 2016](#)).

## Author contributions

GK conceived the study. GK, BJ, UE and GY designed the experiments. BJ, UE and GY carried out the research. GK, BJ, UE and GY contributed to the preparation of the manuscript. All authors

were involved in the revision of the draft manuscript and have agreed to the final content.

## Competing interests

No competing interests were disclosed.

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*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

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## Supplementary material

**Supplementary Figure 1: *In vitro* responses of Ab-restricted env124-138-reactive CD4+ T cell hybridoma H18, to stimulation with the indicated combination of peptides.** The X-axis denotes the concentration of the env124–138 peptide. All other peptides were given at constant 10–6 M concentration. Plotted are the frequencies of T cells that express CD69 following overnight stimulation. Data re the mean ( $\pm$ SEM) of 1–6 individual experiments.

[Click here to access the data.](#)

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## Version 1

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This work gives insights into the immunosuppressive role of the envelope glycoprotein of certain retrovirus. They present the T cell response of 3 TCRs stimulated with different peptides encompassing both the T cell epitope and the immunosuppressive domain. The research note is of interest, clear and well written, and the final conclusion seems appropriate.

### Some section could be developed or clarify:

1. Abstract: "The effects heavily depend on the particular combination of TCR and peptide-major histocompatibility complex class II, and are mimicked by sequence-scrambled peptides of similar length, suggesting they operate at the level of TCR-pMHC interaction". The addition/substitution within the peptides could also influence the peptide stability within the MHC-II, or half-life of the pMHC-II complex, and therefore change the T cell stimulation, this should probably be discuss, or acknowledge that the TCR-pMHC-II interactions might not explain everything.
2. Methods. A brief explanation about "scrambled" peptides would be helpful, as would a sentence explaining why peptides were reconstituted in PBS rather than DMSO.
3. Conclusion. I would like a bit more explanation for the unexpected results. As well as maybe considering taking into account the comment from the abstract to re-word the strong conclusion "*In contrast, neither peptide fusion affected the response of the DO11.10 clone, indicating that effects of the immunosuppressive peptides can be observed in some TCR-pMHC pairs, but not others*".

### Minor comments:

1. Title: I would suggest changing "comprising" by "within".
2. Table 1: the shade section is not visible, either the colour needs to be darker or maybe bold the section.

3. The peptides env548-567 and env548-567-E561R-A567F are in Table 1 but don't seem to be used in the exp? Were they used as control?
4. The env124-138 + ova323-339-env548-567 used in the supplemental figure are not listed in this table.

**We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

**Competing Interests:** No competing interests were disclosed.

Author Response 14 Feb 2017

**George Kassiotis**, The Francis Crick Institute, UK

We wish to thank the Reviewers for their astute and constructive comments. Below is a point-by-point reply and a list of changes we have made to the manuscript in response to these comments:

**Major comments:**

1. We agree with the Reviewer that peptide alterations could affect the peptide-MHC-II complex (pMHC) itself, not only TCR binding to it. Our definition of TCR-pMHC interaction was meant to be broad enough to include effects on pMHC formation. Obviously, anything that affects pMHC formation or stability will also affect TCR-pMHC interaction, by reducing ligand availability. All these alternatives are on the same side of the argument, the other side being biological activity of the immunosuppressive domain. We have modified this sentence to read: "...at the level of pMHC formation or TCR-pMHC interaction."

2. "Scrambled" peptides are permutation of the original peptide sequence. The rationale for using them is that they would retain the physico-chemical characteristics (molecular mass, polarity, acidity, etc), but not any sequence-dependent domain, structure or recognition motif. Practically, these were constructed using the peptide manufacturer's (GeneScript) algorithms. The peptides were all sufficiently hydrophobic to dissolve in PBS. This was chosen in order to avoid any effect of organic solvents.

3. Again we agree with the Reviewer and rephrased this sentence to read: "...in some pMHC combinations or TCR-pMHC pairs, but not others". Given what is known about peptide-flanking residues and their effect on pMHC formation and stability and TCR binding, the results might not be unexpected. In fact, we believe this is the most parsimonious explanation for the effects of the putative immunosuppressive domain, one that needs not invoke any new biological activity or mechanism.

**Minor comments:**

1. The word "comprising" in this context was meant to be synonymous with "including" or "containing". The word "within" would have the opposite meaning. The immunosuppressive domain is within the antigenic peptides, not the reverse.

2. The shading has been replaced with red colour text for enhanced contrast.

3. These peptides were used for the experiments described in Supplementary figure 1.

4. The env124-138 and ova323-339-env548-567 peptides were used as separate entities, not as a fusion. They are listed in rows 1 and 10 of Table 1, respectively.

**Competing Interests:** No competing interests were disclosed.

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**Jean-Marie Saint-Remy**

Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium

I think this paper addresses a major issue with consequences for basic research and for the design of new forms of immunotherapy. The concept of “reversal of immunosuppression” is fundamental and stands, luckily enough, in contrast with the overwhelming view that every bit of immunological studies should be considered as eliciting “active immunosuppression”.

This paper is therefore novel and susceptible to shed new light in the field.

Experiments are neatly planned and well described.

The discussion is well equilibrated.

I therefore strongly recommend the indexing of these new findings.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

**Competing Interests:** No competing interests were disclosed.

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