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Vaccination with RSV $M_{209-223}$ peptide promotes a protective immune response associated with reduced pulmonary inflammation

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ABSTRACT

Respiratory syncytial virus (RSV) is the most common etiologic agent in severe infections of the lower respiratory tract in children with a high mortality rate. However, there are still no licensed vaccines for RSV. In this study, we investigated a putative vaccine based on $M_{209-223}$ peptide. Mice vaccinated with $M_{209-223}$ peptide expanded $M_{209-223}$-specific effector $CD4^+$ T cells upon infection. Vaccination resulted in increased numbers of regulatory T cells (Treg) and Th1 cells, and decreased numbers of Th2 cells. In addition, vaccination with $M_{209-223}$ peptide, protected mice from infection and prevented lung inflammation, leading to increase in IL-10 and IFN-γ production by lung $CD4^+$ T cells. Treg depletion with anti-CTLA4 antibodies abrogated protection induced by peptide vaccination. Our results support vaccination with $M_{209-223}$ peptide as an important strategy to generate protection, both systemic and local, by memory RSV-specific $CD4^+$ T cells in mice. Contrarily to inactivated RSV particles, $M_{209-223}$ peptide vaccination is capable of not only promoting viral clearance, but also reducing inflammatory processes in lungs upon infection.

1. Introduction

The respiratory syncytial virus (RSV) is the most common etiologic agent in severe infections of the lower respiratory tract in children. RSV infection is the main cause of hospitalizations for respiratory diseases worldwide, constituting a major problem in public health systems (Monto, 2002; Rudraraju et al., 2013).

RSV-related acute lower respiratory tract infection is an important cause of death in young children (aged younger than 5 years), approximately 74.500 children in this age group died in hospital with the condition in 2015 (Shi et al., 2017).

Prevention of RSV complications in premature children is managed with the use of an anti-RSV F protein monoclonal antibody (palivizumab), however this type of treatment is expensive and not widely available (Fernández et al., 2010).

The search for RSV vaccine development started in the 1960's. Studies have shown that these vaccines generated antibodies, but these were not efficiently neutralizing, and in some cases presented strong adverse effects (Fulginiti et al., 1969; Hurwitz, 2011; Kapikian et al., 1969). Currently, there is no licensed vaccine for RSV (Higgins et al., 2016) and experimental approaches based on modulation of T cells need to be further explored. RSV reinfections are often accompanied by severe lung disease characterized by an exacerbated Th2 profile (Anderson et al., 2013; Cohn et al., 2001; Waris et al., 1996), which occurs mostly in children, pregnant women, elderly people and immunosuppressed individuals. Children infected with RSV present an increased production of IL-4 in nasal secretions, and a decreased number of Treg in peripheral blood (Christiaansen et al., 2016). These

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findings indicate a Th2 response as a harmful, rather than helpful, one, suggesting that the induction of Treg cells during RSV immunization might be beneficial.

Liu et al. have demonstrated that RSV M protein contains CD4 T-cell epitopes. These epitopes associate with the major histocompatibility complex (MHC) class II molecule I-Ab. Fluorochrome-conjugated peptide–IA^b molecule tetrameric complexes can identify RSV M-specific $CD4^+$ T cells from C57BL/6 mice following RSV infection. It was also observed that $CD4^+$ T cells specific for the $M_{209-223}$ peptide differentiate into RSV-specific Treg cells (Liu et al., 2009). Tregs suppress inflammatory processes and also have been shown to promote the maturation of memory $CD8^+$ T cells (Laidlaw et al., 2015). We hypothesized that vaccinating with the $M_{209-223}$ RSV peptide would expand RSV specific Tregs, which would prevent airway inflammation as well as stimulate CD8 T cell activity in the lungs.

In this context, we developed a vaccine against RSV based on $M_{209-223}$ peptide. To evaluate the RSV-specific $CD4^+$ T cell populations, we used the C57BL/6 mice to take advantage of the RSV-specific MHC class II tetramer specific for this mouse strain. We found that mice vaccinated with $M_{209-223}$ peptide expanded a $M_{209-223}$-specific effector $CD4^+$ T cell population upon infection, and were protected from RSV challenge, showing decreased lung inflammation, dependent on RSV specific Treg generation. Our results provide evidence that vaccination with $M_{209-223}$ peptide could be a useful strategy to generate protection, both systemic and local, against RSV infection.

2. Materials and methods

2.1. Mice

C57BL/6 mice were obtained from a colony maintained at Centro de Modelos Biológicos e Experimentais (CEMBE)-PUCRS. Six to eight-week-old female animals weighing 15–20g were used for experiments and were housed at the CEMBE animal facility with water and food ad libitum. All animal procedures were performed in accordance to protocols approved by the University's Ethics Committee.

2.2. Cells and viruses

Vero cells (ATCC CCL81) were cultured in Dulbecco's modified Eagle's medium (DMEM - Gibco, USA), containing 10% fetal bovine serum (FBS - Gibco, USA), and gentamicin (0.08 mg/ml, NOVAFARMA) at 37 °C with 5% of CO_2. RSV A strain (line A2) was kindly provided by Prof. Fernando Polack, from Fundación Infant (Buenos Aires, Argentina). RSV was propagated in Vero cells using DMEM, with 2% FBS. Viral titration was performed using Vero cells staining the virus plaque with a mouse anti-RSV monoclonal antibody (MAB858-4, EMD-Millipore). To perform inactivation of the virus, each virus aliquot with 10^6 PFU was ultraviolet (UV)-irradiated for 30 min inactivation was confirmed by plaque assay.

2.3. Peptides and tetramers

The RSV peptide $M_{209-223}$ is 15 amino acids long (NKGAFKYIKPQSQFI) and was purchased from Biomatik, USA. The PE-I-A^bRSV$M_{209-223}$ tetramer was provided by the National Institutes of Health (NIH Tetramer Core Facility, Atlanta, GA).

2.4. Vaccination and RSV infection

Three groups of mice (n = 5) were vaccinated on day 0 and boosted on day 7 by subcutaneous (s.c.) injections, using 100 μg of $M_{209-223}$ peptide (group 2) as previously described by Liu et al. (2010), or 10^6 PFU UV-RSV (group 3) as previously described by (Derscheid et al., 2013; Johnson et al., 2014; Wang et al., 2017). Controls were injected with PBS (group 1), and all vaccinations contained 5 μg of Poly-IC (InvivoGen, USA). For infection, mice were anesthetized on day 14 intraperitoneally with ketamine (Cristalia, Brazil) (100 mg/kg) and xylazine (Syntec, Brazil) (10 mg/kg), and later inoculated intranasally with 10^7 PFU of live RSV in 30 μL of PBS. On day 14 post-immunizations (uninfected groups), or day 5 post-challenge (infected groups), mice were euthanized, and lung and secondary lymphoid organs were harvested.

2.5. Enrichment and staining of RSV $M_{209-223}$-specific $CD4^+$ T cells

Enrichment of specific T cells by tetramer pulldown was employed for analysis of the immune response to the vaccine (section 3.1, after 14 days of the first immunization), and for analysis of response upon viral challenge (section 3.3, after 5 days of challenge of vaccinated animals with RSV). In both experiments, animals were anesthetized and euthanized for harvest of spleen and lymph nodes (cervical, axillary, brachial, mesenteric, inguinal and periaortic). A single cell suspension from all lymphoid organs of each mouse was prepared in Fc block (2.4G2 supernatant + 2% mouse serum, 2% rat serum and 0.1% NaN_3). PE-conjugated MHC-peptide tetramers were added at a concentration of 10 nM for the analysis of peptide RSV$M_{209-223}$-specific T cells, and cells were incubated at room temperature for 1 h. Cells were then resuspended and mixed with anti-PE antibody conjugated magnetic microbeads (Miltenyi Biotec, USA) followed by two washes, resuspended in staining buffer (PBS + 2% FBS + 0.1% NaN_3) and passed over a magnetized LS column (Miltenyi Biotech). Bound cells were obtained by pushing 5 ml of staining buffer through the column and labeled for flow cytometry.

2.6. Lung cells isolation and in vitro stimulation

Lymphocytes were isolated from lung tissue using collagenase D (0,1 mg/ml) for 1 h at 37 °C. For the cytokine analysis, cells were stimulated with the $M_{209-223}$ peptide at 2 μg/ml for 6 h at 37 °C in the presence of GolgiStop (BD Pharmingen, USA) at 1 μg/ml, together with 1 μg/ml of costimulatory antibodies and with anti-CD28 and anti-CD49d (BD Biosciences), as previously described (Liu et al., 2009) and cells were labeled for flow cytometry.

2.7. Flow cytometry

Fluorochrome-conjugated antibodies used to study cell phenotype and cytokine production with flow cytometry were anti-CD11b (APC-Cy7), anti-CD11c (APC-Cy7), anti-CD11c (FITC) anti-CD19 (APC-Cy7), anti-B220 (FITC), anti-CD4 (PE-Cy7), anti-CD4 (APC), CD4 (APC-Cy7), anti-CD8 (PE-Cy7), anti-CD44 (PerCP) and anti-62L (FITC), anti-GATA3 (Alexa Fluor 647), anti-IFN-γ (FITC), anti-IL-4 (PerCP) and anti-IL-10 (APC) BD Biosciences (San Jose, CA) and anti-Foxp3 (Alexa fluor 647) eBioscience. Samples were acquired on a FACSCanto II flow cytometer (BD Biosciences, USA). Data analysis was performed using FlowJo software (version 7.5, Tree Star Inc., Ashland, US). All gating strategies are specified in the Supplementary Fig. S1.

2.8. Real-time PCR

Total RNA was extracted from lungs of infected animals, using viral RNA/DNA Mini Kit (PureLink®-Invitrogen), following the manufacturer's instructions. cDNA was synthesized with random primers using Sensiscript® Reverse Transcription kit (QIAGEN®). The quality of cDNA for each sample and Quantitative real time PCR (qPCR) was performed as previously described (Freitas et al., 2016). For the standard curve, ten-fold serial dilution of 6×10^7 copies of a plasmid with RSV F protein sequence were added to the same plate of qPCR in duplicate. The results were used for further quantification of viral load.

2.9. Histopathologic analysis

The histological analysis of the lungs of vaccinated mice was performed 5 days after RSV challenge. The animals were euthanized, the lungs removed and perfused with 10% buffered formalin on a gravity column (20 mmHg). The specimens were embedded in paraffin blocks, cut into 4 µm sections, stained with haematoxylin and eosin (HE) in order to assess cells infiltrates. Identification of mucus-secreting goblet cells we performed using Alcian blue staining. All samples were examined by light microscopy (Olympus, BX51).

2.10. In silico binding prediction

$M_{209\text{-}223}$ peptide passed through scrutiny of prediction programs to assess its binding capacity with a reference set of 27 HLA-II alleles (99% of the population HLA class II coverage) (Greenbaum et al., 2011). The analysis was performed using T cell epitope prediction tools from IEDB (Wang, et al., 2008; Wang et al., 2010). Because $M_{209\text{-}223}$ triggers a T cell response in mice carrying the MHC class II H2-IA^b (Liu et al., 2014, 2010; 2009; Rutigliano et al., 2005) we also performed a peptide binding prediction with this allele as a positive control.

2.11. Treatment with anti-CTLA-4

The animals were treated with 500 µg of CTLA-4-Ig (catalog BP0164, BioXcell) or isotype control (catalog BE0089, BioXcell), 4 h before infection, and a boost was performed on the 3rd day after infection, following a protocol employed in Kurup et al. (2017). Flow cytometry analysis and histopathology were performed on the 5th day after infection.

2.12. Statistical analysis

Data were tested for normal distribution by Kolmogorov-Smirnov test. Data were submitted to analysis of variance ANOVA, followed by the Tukey test, comparing the differences between the groups, using GraphPad Prism software San Diego, CA, USA. A confidence limit of 95% was established for significance of the results. Results were considered significant when p value less than 0.05 ($p < 0.05$).

3. Results

3.1. Vaccination with the UV-RSV or $M_{209\text{-}223}$ peptide generates specific $CD4^+$ T cell memory precursors in lymphoid organs

We characterized the phenotype and evaluated the expansion of effector $M_{209\text{-}223}$-specific $CD4^+$ T cells following three different vaccination strategies. All groups received a boost 7 days after the first vaccination (Fig. 1A). In the secondary lymphoid organs (SLOs), vaccination with $M_{209\text{-}223}$ peptide and with UV-RSV showed a substantial increase of effector $CD4^+$ T cells specific for $M_{209\text{-}223}$ peptide compared to control (Fig. 1B and C). Both vaccinated groups also presented a significant increase in $M_{209\text{-}223}$-specific Foxp3+ $CD4^+$ T cells and IFN-γ + $CD4^+$ T cells compared to animals injected with PBS plus Poly-IC (Fig. 1D and E). Upon immunization, peptide-specific central memory precursors expanded approximately 21- and 25-fold in groups vaccinated with $M_{209\text{-}223}$ peptide and UV-RSV, respectively (Supplementary Fig. S2).

3.2. Vaccination with the UV-RSV or $M_{209\text{-}223}$ peptide generates specific effector $CD4^+$ T cells in the lung

To analyze the expansion of peptide-specific T cells in lungs, lymphocytes were isolated on day 14 from lungs of previously vaccinated mice and were re-stimulated *in vitro* with $M_{209\text{-}223}$ peptide. Animals vaccinated with $M_{209\text{-}223}$ peptide showed a significant increase in the frequency of $M_{209\text{-}223}$ peptide-specific $CD4^+$ T cells (approximately 5-fold higher than control and UV-RSV vaccinated groups) (Fig. 2A). When these cells were analyzed for cytokine production, and increase of ~11-fold in frequency of $M_{209\text{-}223}$ peptide-specific IL-10-producing $CD4^+$ T cells in animals vaccinated with the $M_{209\text{-}223}$ peptide was observed (Fig. 2B). The frequency of IFN-γ^+ T cells specific to the $M_{209\text{-}223}$ peptide was approximately 4.3-fold higher (Fig. 2C) when compared to the control group and vaccinated with UV-RSV. Interestingly, we observed that, upon re-stimulation with the peptide, the frequency of $CD8^+$ T cells secreting IFN-γ was significantly higher in the group vaccinated with $M_{209\text{-}223}$ peptide (~4.6-fold) if compared to control and UV-RSV vaccinated (Supplementary Fig. S3). Thus, as expected, vaccination with the peptide induces a more robust population of specific $CD4^+$ T cells than vaccination with UV-RSV at the site of infection. Moreover, the expansion of $CD8^+$ T cells correlated with this increase of peptide specific $CD4^+$ T cells in the lung.

3.3. Mice vaccinated with UV-RSV, but not with $M_{209\text{-}223}$ peptide, expand a large population of Th2 specific T cells in the lymphoid organs upon challenge with live virus

We next verified the $CD4^+$ T cell-mediated cellular response of vaccinated groups upon challenge with live virus, enumerating total specific T cells using tetramer pulldown. We observed that total numbers of peptide-specific T cells in each group was not greatly expanded upon challenge when compared to what was previously observed after vaccination alone (Figs. 3B and 1B, respectively). $M_{209\text{-}223}$ vaccinated group presented a ~6.3-fold increase in polyclonal effector memory ($CD62L^-CD44^+$) tetramer positive T cells compared to controls (Fig. 3C), while UV-RSV vaccinated mice showed a 22–fold expansion. Central memory cells ($CD44^+CD62L^+$) were also expanded in $M_{209\text{-}223}$ (6-fold) and UV-RSV (12-fold) groups (Supplementary Fig. S4). When we analyzed the different phenotypes of these cells, a 27.5-fold expansion of $M_{209\text{-}223}$-specific Foxp3 + $CD4^+$ T cells was observed in mice primed with the peptide, versus 39.8-fold in mice primed with UV-RSV, compared to the control group (Fig. 3D). In memory $M_{209\text{-}223}$-specific $CD4^+$ T cells producing IFN-γ, we observed an expansion of 6.3 and 10.5-fold when compared to the control group, in animals vaccinated with $M_{209\text{-}223}$ peptide and with UV-RSV, respectively (Fig. 3E). Upon challenge, the number of effector memory, $Foxp3^+$ and IFN-γ^+ $M_{209\text{-}223}$-specific $CD4^+$ T cell memory precursors was not different between mice vaccinated with peptide or inactivated virus. An early study from Graham and collaborators reported that immunization with inactivated RSV induced a predominantly Th2 pattern of cytokines in the lung upon challenge, as analyzed by total mRNA expression (Graham et al., 1993). We then analyzed the peptide-specific Th2 cells and observed that while mice primed with $M_{209\text{-}223}$ peptide showed a modest (3-fold) increase in $GATA3^+$ peptide-specific cells compared to controls, mice primed with UV-RSV showed a much greater expansion of these cells: a 42.2-fold increase in relation to the unvaccinated controls, and 15-fold compared to $M_{209\text{-}223}$ immunized mice (Fig. 3F).

3.4. Vaccination with $M_{209\text{-}223}$ peptide protects animals from RSV infection

To evaluate the protective effect of each immunization, we first quantified virus particles in lungs five days after infection - which is when viral load is typically peaking in lungs of infected animals (Chavez-Bueno et al., 2005). While RSV particles could be abundantly detected in lungs of unimmunized mice, they were undetectable in mice vaccinated with $M_{209\text{-}223}$ peptide (Fig. 4A). Viral particles could still be detected in lungs of mice primed with inactivated virus, although in significantly reduced amounts compared to controls. Another good indicator of disease progression is weight loss. Interestingly, only mice vaccinated with the peptide gained or maintained their weight after challenge (Fig. 4B), while both unimmunized and UV-RSV immunized mice significantly lost weight after infection, compared to peptide

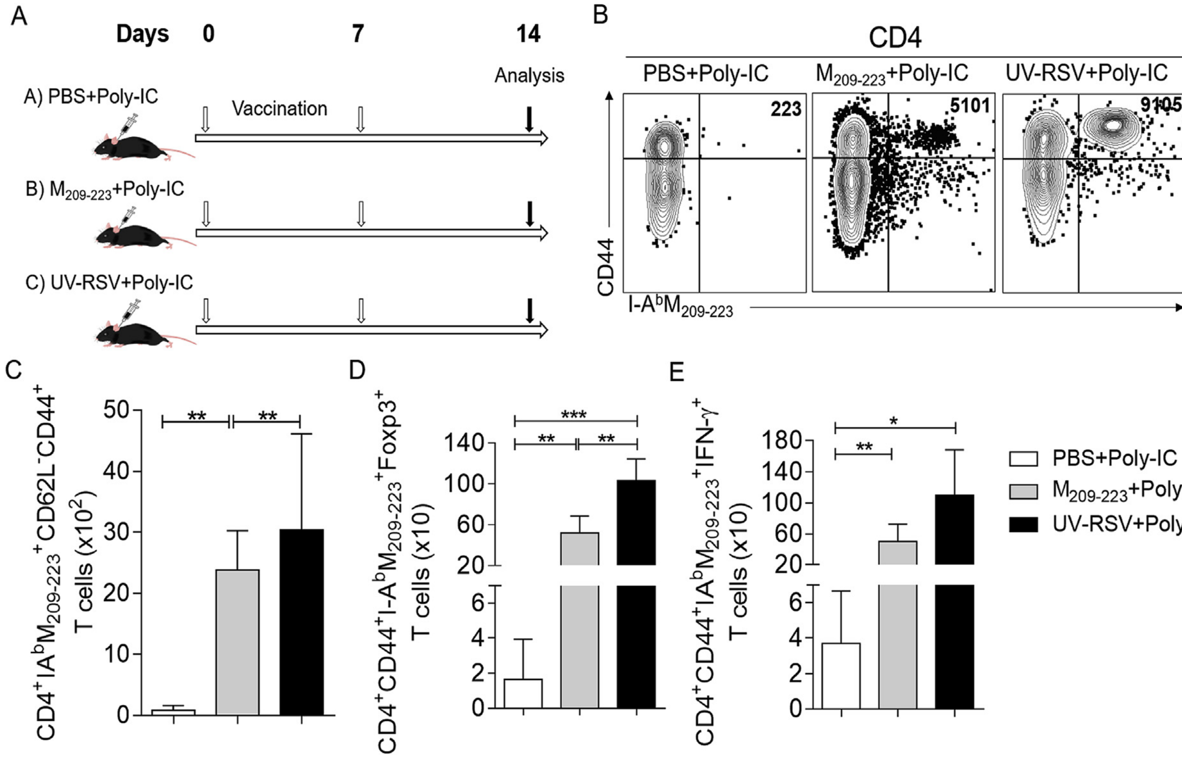

Fig. 1. Immunization schedule of mice and total CD4$^+$ T cell responses to RSV $M_{209\text{-}223}$ peptide and UV-RSV before challenge with RSV. Immunization schedule of mice: Three groups of mice (5 animals in each group) were subcutaneously injected with either PBS plus Poly-IC, 100 µg of $M_{209\text{-}223}$ peptide mixed with Poly-IC or 10^6 PFU RSV with Poly-IC previously inactivated by ultraviolet light (UV-RSV) on day 0 and boost on day 7. All groups received 5 µg of Poly-IC. Secondary lymphoid organs were harvested 14 days post-immunization for analysis (A). All secondary lymphoid organs were isolated at day 14 post-immunization and stained for tetramers and phenotyping antibodies to identify specific CD4$^+$CD44$^+$ T cells (B), effector memory specific CD4 T$^+$ Cells (C), Treg-specific CD4$^+$CD44$^+$ T Cells (D) and specific CD4$^+$CD44$^+$ T cells producing IFN-γ (E). Data (mean ± SD, n = 5/group) are represented as bars from different colors: PBS + Poly-IC (white bars), $M_{209\text{-}223}$ peptide + Poly-IC (gray bars) and UV-RSV (black bars). Data are representative of three independent experiments and compared by using the One-way ANOVA test with Tukey post-test. The significance of the differences between groups are shown as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

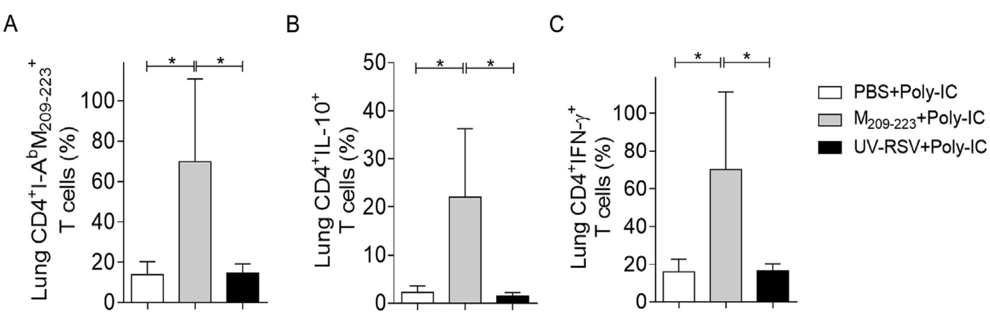

Fig. 2. Lung T cells producing different cytokines in response to peptide vaccination. Cells were stained with fluorochrome-conjugated antibodies for CD4 and MHC-peptide tetramers (I-A^b$M_{209\text{-}223}$) (A). Mice lung lymphocytes were cultured with the $M_{209\text{-}223}$ peptides for 6 h at 37 °C. Cells were stained with fluorochrome-conjugated antibodies for anti-CD4 and anti-IL-10 (B), anti-CD4 and anti-IFN-γ (C). Treatments' data (mean ± SD, n = 5/group) are represented as bars from different colors: PBS + Poly-IC (white bars), $M_{209\text{-}223}$ peptide + Poly-IC (gray bars) and UV-RSV (black bars). Data are representative of three independent experiments and compared by using the One-way ANOVA test with Tukey post-test. The significance of the differences between groups are shown as *$p < 0.05$.

vaccinated mice.

3.5. Vaccination of $M_{209\text{-}223}$ peptide protects lung tissue from inflammation caused by RSV infection

We evaluated the lungs of animals infected with RSV after five days by histology, analyzing the presence and/or composition of inflammatory infiltrates. No cell infiltrates were detected in the lungs of non-challenged animals that received only PBS plus Poly-IC (Fig. 5-A1). However, in animals that were primed only with PBS plus poly-IC, and were later infected with RSV, infiltrates were present in a large part of the lung tissue (Fig. 5-A2). Lungs of mice vaccinated with UV-RSV also had infiltrating cells in the lungs (Fig. 5-A4). Remarkably, animals vaccinated with $M_{209\text{-}223}$ peptide presented lungs with minimal cell infiltration, very similar to uninfected controls (Fig. 5-A3). We also evaluated the presence of mucus in bronchi epithelial cells. No mucus was observed in uninfected animals, and the presence of mucus (stained in blue) can be clearly visualized in mice that were not immunized, but were infected with RSV, respectively (Fig. 5-B1 and B2). Comparing the presence of mucus between the animals vaccinated with $M_{209\text{-}223}$ peptide and with UV-RSV, a higher production was observed in animals vaccinated with UV-RSV, respectively (Fig. 5-B3 and B4). Our results indicated that, although both the $M_{209\text{-}223}$ and UV-RSV immunization could expand a larger number of effector T cells in lymphoid organs, UV-RSV immunization not only failed to generate adequate protection, but also induced lung inflammation, contrarily to $M_{209\text{-}223}$ immunization.

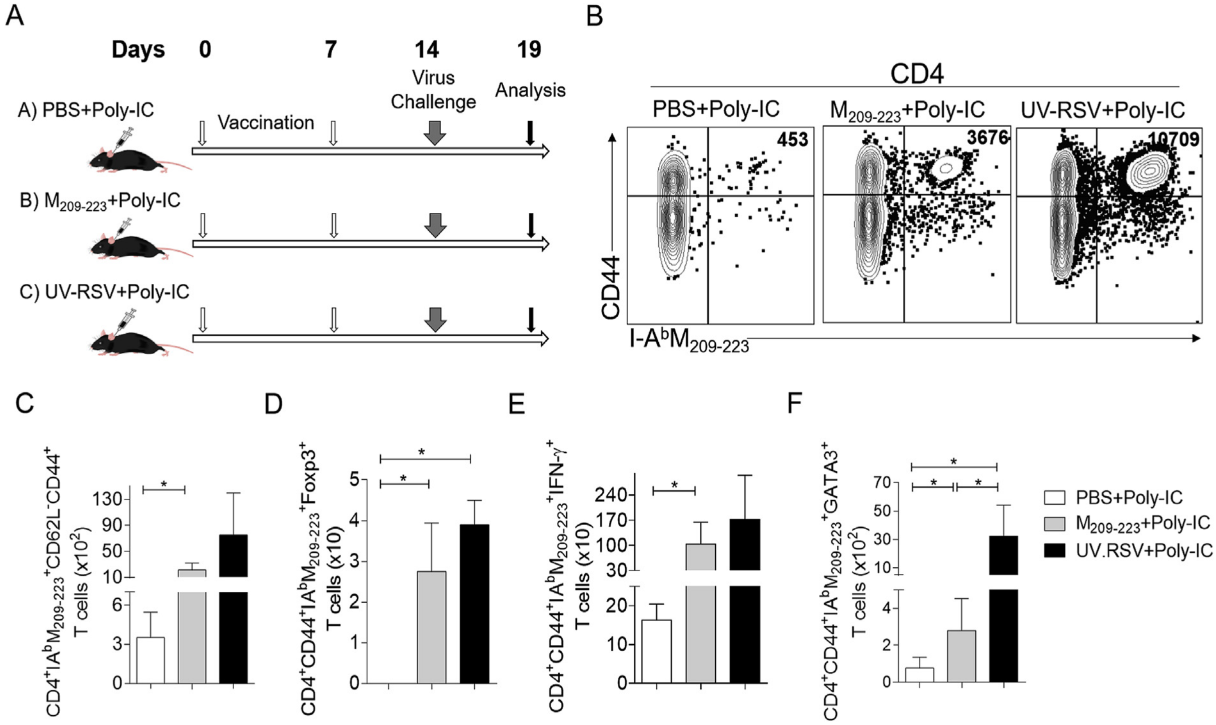

Fig. 3. Immunization schedule of mice and total CD4$^+$ T cell responses to RSV M$_{209-223}$ peptide and UV-RSV after challenge with live virus. Three groups of mice (5 animals in each group) were subcutaneously injected with PBS, 100 µg of M$_{209-223}$ peptide or 10^6 PFU RSV previously inactivated by ultraviolet light (UV-RSV) on day 0 and boost on day 7. All groups received 5 µg of Poly-IC as adjuvant. Mice were challenged with RSV at day 14 post-immunization. (A). All secondary lymphoid organs were isolated at day 5 post-challenge (day 19) and stained with tetramers and phenotyping antibodies to identify specific CD4$^+$CD44$^+$ T cells (B), effector memory specific CD4$^+$ T Cells (C), Treg-specific CD4$^+$CD44$^+$ T Cells (D), specific CD4$^+$CD44$^+$ T cells producing IFN-γ (E) and specific GATA3$^+$CD4$^+$CD44$^+$ T Cells (F). Data (mean ± SD, n = 5/group) are represented as bars from different colors: PBS + Poly-IC (white bars), M$_{209-223}$ peptide + Poly-IC (gray bars) and UV-RSV (black bars). Data are representative of three independent experiments and compared by using the One-way ANOVA test with Tukey post-test. The significance of the differences between groups are shown as *$p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.6. *Vaccination with the M$_{209-223}$ peptide expands the peptide-specific Treg and Th1 cells populations, and decreases Th2 responses in lungs 5 days after RSV challenge*

We next hypothesized that differences in the type of effector cells generated by each immunization could be responsible for the differences in protection observed, and that would impact inflammation in the lungs of infected animals. Remarkably, five days after infection, after *in vitro* re-stimulation with the M$_{209-223}$ peptide, expansion of lung peptide-specific T effector cells was significantly greater upon infection in mice previously immunized with the peptide and poly-IC, compared to UV-RSV (Fig. 6A). Likewise, M$_{209-223}$ vaccinated group had a 2-fold higher frequency of IL-10 and IFN-γ-producing CD4$^+$ T cells as compared to control and UV-RSV vaccinated groups, respectively (Fig. 6B and C). The percentage of lung regulatory CD4$^+$ T cells in the non-immunized animals was significantly higher compared to UV-RSV group (Fig. 6D). Animals vaccinated with UV-RSV had a 2-fold higher frequency of GATA3$^+$ cells compared to M$_{209-223}$ peptide immunized and non-immunized groups (Fig. 6E). Moreover, the percentage of IL-4 producing CD4$^+$ T cells in lungs of UV-RSV vaccinated mice was significantly higher than in the other two groups (Supplementary Fig. S5A), while INF-γ$^+$ and IL-10$^+$ cells were higher in the M$_{209-223}$ vaccinated group (Supplementary S5B and C).

Treg cells are often found to respond to infection and inflammation.

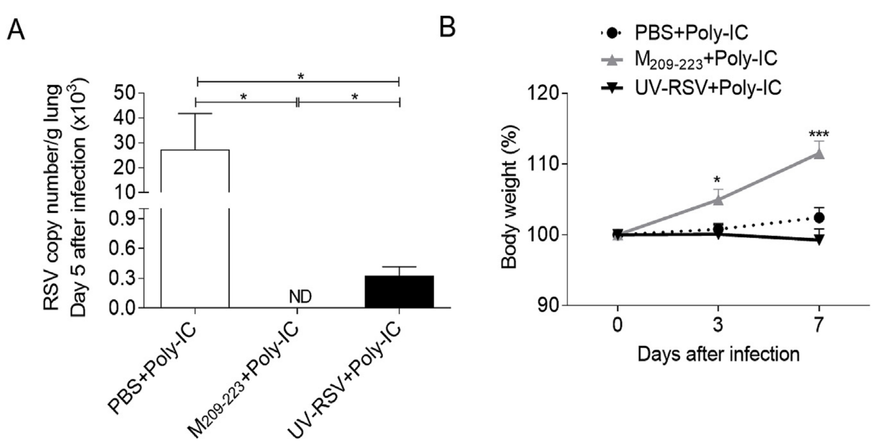

Fig. 4. Viral load and weight loss after RSV challenge following immunization and boost. Viral load in lung after 5 days of virus challenge (A). Mice were weighed on days 0, 3 and 7 after virus inoculation (challenge day = day 0). Shown are the percentages of baseline weight (B). The data (mean ± the SD, n = 5/group) are representative of three independent experiments and compared by using the One-way ANOVA test with Tukey post-test. The significance of the differences between groups is shown as *$p < 0.05$ and ***$p < 0.001$.

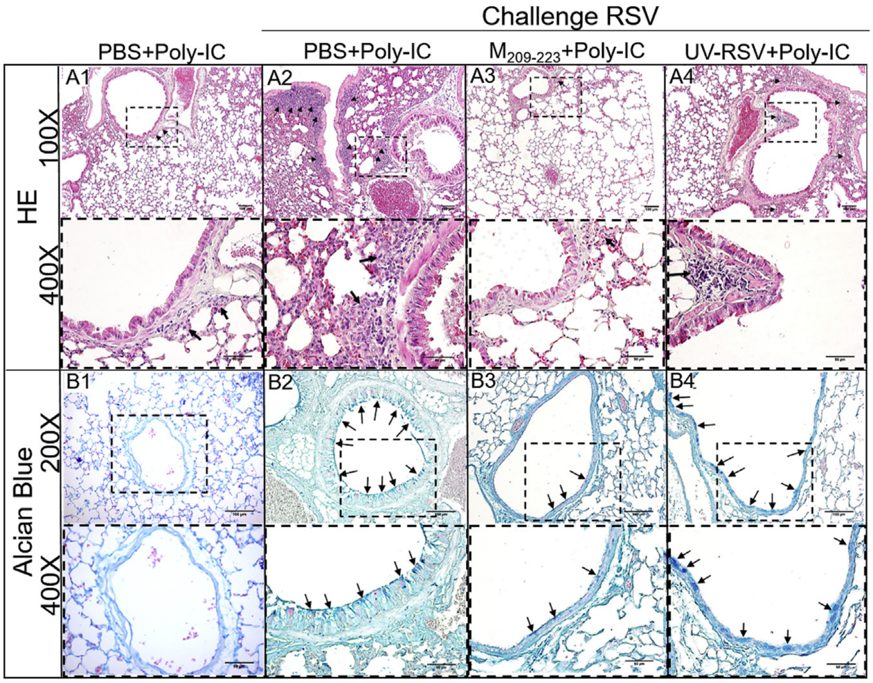

Fig. 5. Histological analyses of the lung in vaccinated mice five days after challenge with RSV. Lung tissue histological sections stained with haematoxylin and eosin (HE). Arrows indicate the presence of inflammatory cell infiltration (A). Mucus-producing cells (arrows) in airways were identified by Alcian Blue staining in RSV infected and control mice (B).

Nevertheless it has been reported that these cells increase influx of $CD8^+$ T Cells in the lungs of RSV infected animals. (Belkaid and Rouse, 2005; Ruckwardt et al., 2009; Veiga-Parga et al., 2013). In agreement with those studies, we observed that vaccination with the peptide increases the frequency of $CD8^+$ T cells producing IFN-γ (during priming/vaccination) and IL-10 (upon challenge), which could respectively help decrease viral load and inflammation, respectively (Supplementary Fig. S3 and Supplementary Fig. S6B).

3.7. In silico prediction shows that $M_{209\text{-}223}$ peptide can bind human HLA class II alleles

Based on our results, which were performed using a murine model, we decided to analyze the binding capacity of $M_{209\text{-}223}$ peptide against a reference set of 27 HLAs class II alleles and against the murine MHC class II allele H2-IA^b (positive control). The prediction values are expressed as a percentile rank, where lower values indicate good binders. Compared to murine MHC class II, $M_{209\text{-}223}$ presented better binding values for HLA class II, indicating the possible peptide binding with these alleles (Table 1). Our results suggest specific binding to DRB1, DRB3 and DRB5 alleles, with a predominance of DRB1.

4. Discussion

Disease progression during RSV infection is commonly associated with severe lung inflammation. It is believed that lung injury promoted by inflammation is related to pathogen elimination, however this remains undetermined (Liu et al., 2010).

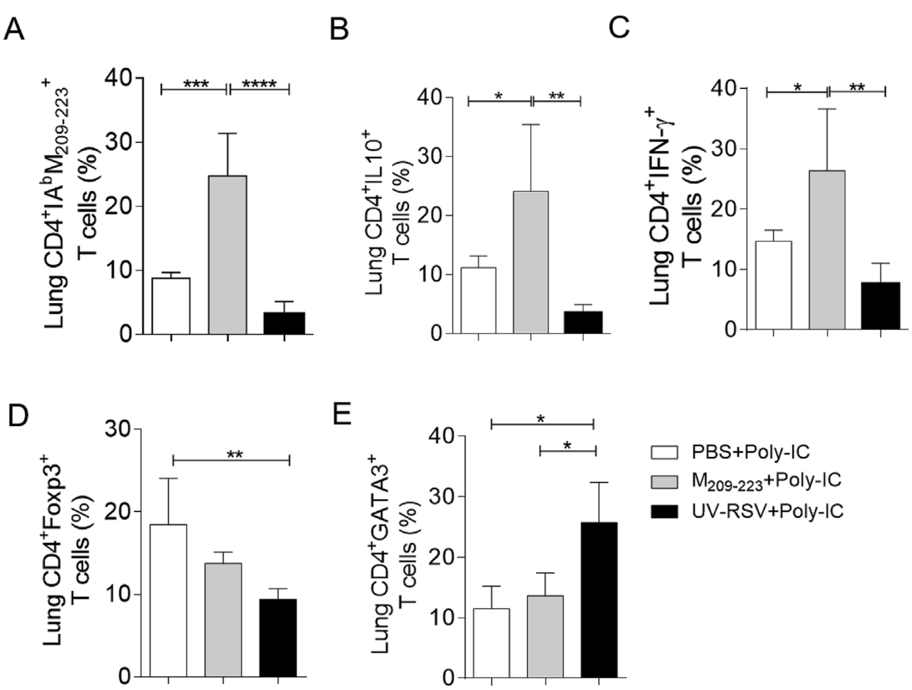

Fig. 6. Lung T cells positive for cytokines and transcription factors upon virus infection. Mice immunized with PBS + Poly-IC (white bars), $M_{209\text{-}223}$ peptide + Poly-IC (gray bars), or UV-RSV (black bars) and challenged with RSV for 5 days. Lymphocytes from the lung of infected mice were isolated and cultured with the $M_{209\text{-}223}$ peptides for 6 h at 37 °C. Cells were stained with fluorochrome-conjugated antibodies for anti-CD4 and MHC-peptide tetramers (I-$A^bM_{209\text{-}223}$) (A); anti-CD4 and anti-IL-10 (B), anti-CD4 and anti-IFN-γ (C), anti-CD4 and anti-Foxp3 (D), anti-CD4 and anti-GATA3 (E). Treatments are represented as bars with mean ± the SD (n = 5/group). Representative of three independent experiments. Statistics using the One-way ANOVA test with Tukey post-test. The significance of the differences between groups are shown as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

Table 1
$M_{209-223}$-MHC-II binding prediction calculated with T cell epitope prediction tools from IEDB.

Organism	MHC-II allele	Method used	Percentile Rank
Murine	H2-IAb	Consensus (smm/nn)	5.8
Human	HLA-DRB5*01:01	Consensus (smm/nn/sturniolo)	0.24
	HLA-DRB1*04:05	Consensus (smm/nn/sturniolo)	0.6
	HLA-DRB1*07:01	Consensus (comb.lib./smm/nn)	0.68
	HLA-DRB1*11:01	Consensus (smm/nn/sturniolo)	0.89
	HLA-DRB1*08:02	Consensus (smm/nn/sturniolo)	1.43
	HLA-DRB1*04:01	Consensus (smm/nn/sturniolo)	1.89
	HLA-DRB3*02:02	NetMHCIIpan	2.88
	HLA-DRB1*01:01	Consensus (comb.lib./smm/nn)	3.02

A Th2 response has been previously shown to induce chronic disease and inflammation in different models, including RSV infection (Christiaansen et al., 2014; Jiminez et al., 2015; Waris et al., 1996). An early study by Graham et al. (1993) observed that priming mice with inactivated RSV or protein F triggers a Th2 response upon challenge with live RSV. Another study shows that RSV infection induces GATA3$^+$, a transcription factor of Th2 cells, and IL-4 expression, increasing susceptibility in a murine allergic asthma model (Krishnamoorthy et al., 2012). In infants, RSV-induced bronchiolitis is accompanied by a greater IL-4/IFN-γ ratio in respiratory secretions, indicating Th2 polarization (Caballero et al., 2015). Thus, vaccination strategies that induce Th2 responses would not be ideal to prevent the inflammatory damage that follows infection by RSV.

Our findings are in agreement with these studies. When we vaccinated with UV-RSV and poly-IC, higher frequencies of GATA3$^+$ and IL4$^+$ expressing cells, both before and after challenge with RSV, were observed. This Th2 response correlated with an increased lung infiltration by inflammatory cells, mucus production and weight loss in infected mice - even though C57/BL6 are not as susceptible to RSV disease as other strains of mice (Taylor, 2017). These results indicated that our UV-RSV vaccination formulation containing the many viral epitopes and TLR ligands present in inactivated RSV tended to yield an inflammatory, Th2 response, confirming previous observations in the Graham study, that used a different inactivated RSV formulation.

In works performed by Dabbagh et al. and Cohn et al. it was observed that the airway epithelial cell mucin gene expression and mucous glycoconjugate production are induced by IL-4, a hallmark of the Th2 response (Cohn et al., 1997; Dabbagh et al., 1999). It is also possible that IL-13 has a role in this response. A previous work by Tekkanat et al. (2001) showed that in BALB/c mice IL-13 is also induced along with IL-4 and mucus in airway hypersensitivity responses during RSV infection - and that neutralization of IL-13, but not IL-4, is enough to induce IL-12 and decrease airway inflammation. In our study, the animals vaccinated with $M_{209-223}$ peptide showed that these mice had fewer IL-4 producing CD4$^+$ T cells, more IFN-γ and IL-10 producing CD4$^+$ T cells. Moreover, inflammatory infiltration or mucus production in lungs were not present. Importantly, $M_{209-223}$ RSV peptide expanded RSV-specific Th1 and Treg cells, resulting in a response that not only protected, but also prevented exacerbated inflammation. Poly-IC is known to stimulate innate signals that skew T cell differentiation to the Th1 type (Coffman et al., 2012; Longhi et al., 2009). Our strategy while using the $M_{209-223}$ RSV peptide was to intentionally skew the antiviral response to a Treg type - that would suppress Th2 inflammation during infection.

We attempted to discriminate between Treg cells effects and non-Treg effector cells that would be induced by vaccination with $M_{209-223}$ peptide. We hypothesized that if the main effect of $M_{209-223}$ peptide vaccination was to stimulate Tregs, and that these were the ones responsible for lung protection, a treatment with anti-CTLA-4 would not only inhibit Treg cells function, but also would deplete Tregs (Simpson et al., 2013). The data obtained showed that anti-CTLA-4 treatment in vaccinated animals results in extensive lung infiltration by immune

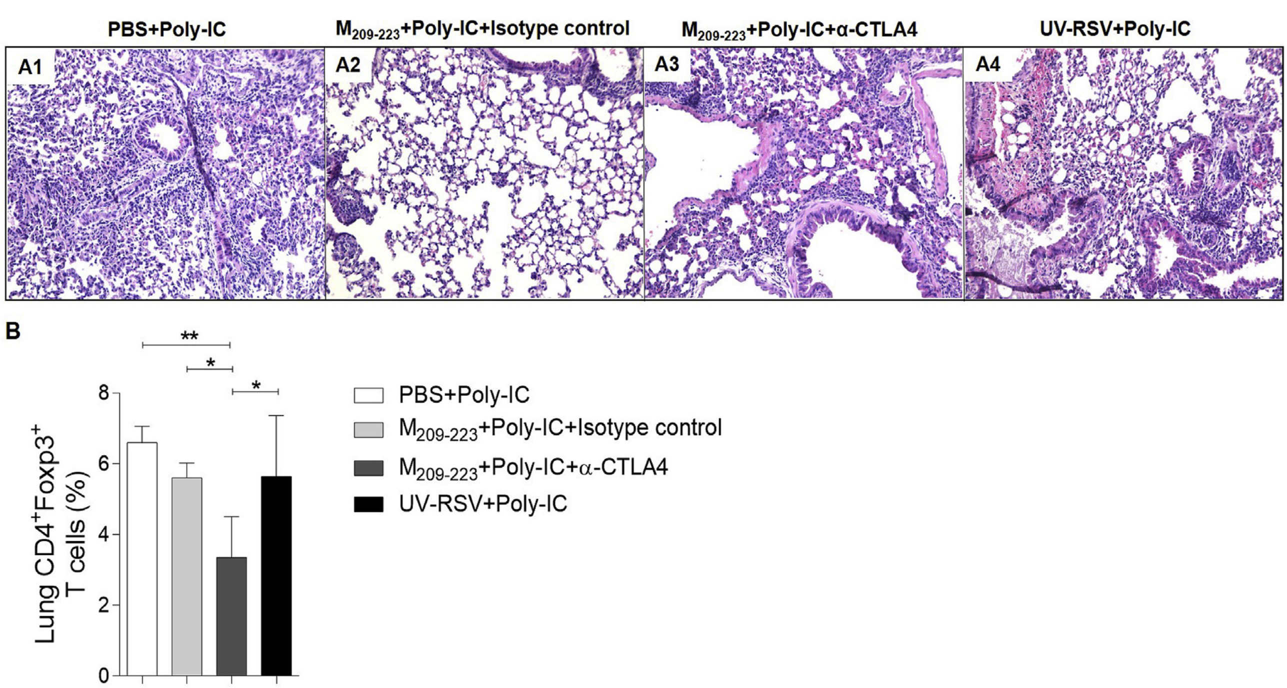

Fig. 7. Treatment anti-CTLA-4 depletes Treg cells and abrogates peptide induced protection. Mice immunized with PBS + Poly-IC, $M_{209-223}$ peptide + Poly-IC (Treated with anti-CTLA-4 and control isotype), or UV-RSV and challenged with RSV for 5 days. Lung tissue histological sections stained with haematoxylin and eosin (HE) (A1-A4). Lymphocytes from the lung of infected mice were isolated and cultured with the $M_{209-223}$ peptides for 6 h at 37 °C. Cells were stained with fluorochrome-conjugated antibodies for anti-CD4 and anti-Foxp3 (B). Treatments are represented as bars with mean ± the SD (n = 5/group). Representative of three independent experiments. Statistics using the One-way ANOVA test with Tukey post-test. The significance of the differences between groups are shown as *p < 0.05 and **p < 0.01.

cells upon infection, resembling the phenotype observed in animals that were infected, but not vaccinated (Fig. 7 - A1-4). Flow cytometry analysis confirms that treatment did eliminate about two thirds of the peptide specific Tregs in lungs (Fig. 7B). Our anti-CTLA-4 treatment results suggest that Treg cells are crucial to modulate lung inflammation caused by RSV infection (Durant et al., 2013).

More recently, Treg cells have been demonstrated to aid in the development of a $CD8^+$ T effector response, which is crucial for viral control of RSV (Fulton et al., 2010; Liu et al., 2010), dengue (Chandele et al., 2016), HIV (Gulzar and Copeland, 2004) and influenza (Ingulli et al., 2009). Priming with the peptide and poly-IC did not affect the percentage of IFN-γ^+ $CD8^+$ T cells in the lung of infected mice (Supplementary S6A), confirming previous observations by (Liu et al., 2010). However, it did result in a higher frequency of IL-10^+ $CD8^+$ T cells (Supplementary S6B), compared to UV-RSV primed mice. One study has linked IL-10 producing effector $CD8^+$ T cells to protection from influenza (Sun et al., 2009). The mechanisms underlying the relative contribution of the $M_{209-223}$ specific Th1 versus Treg populations for effective clearance of the infection as well as prevention of inflammation need to be further characterized. For example, a previous study Graham et al. It would be interesting to investigate if this peptide-specific Treg population could cooperate with resident memory CD8 T cells in the lung. Also, it would be important to determine antigen specificity of these local $CD8^+$ T cells (Graham et al., 2014).

Our *in silico* analysis shows that $M_{209-223}$ has the ability to bind DRB1, DRB3 and DRB5 alleles. HLA-DRB1 alleles were the most frequent in our analysis. In fact, a correlation between HLA-DR1 alleles and protection from autoimmune disorders triggered by Treg cells was already observed by Ooi et al. (2017). Also, the first ranked allele of our analysis (HLA-DRB5) was shown by Caillier et al. important to attenuate the severity of multiple sclerosis (MS), an autoimmune disorder that can take place when Treg cells fail to respond (Stacy J. Caillier et al., 2008).

Although peptide vaccines have not had a very successful track record in humans, data from a vaccine based on a peptide of RSV F protein, currently undergoing a phase 2 clinical trial (Higgins et al., 2016) showed promising results. We believe there might be value in exploring peptide $M_{209-223}$ for vaccination in humans, perhaps in combination with other viral peptides and TLR3 agonists.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.antiviral.2018.07.007.

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