Aalborg Universitet

AALBORG UNIVERSITY
DENMARK

Novel nucleotide-packaging vaccine delivers antigen and poly(I:C) to dendritic cells and generate a potent antibody response in vivo

Bruun, Natasja; Laursen, Marlene F; Carmelo, Rita; Christensen, Esben; Jensen, Trine S; Christiansen, Gunna; Birkelund, Svend; Agger, Ralf; Kofod-Olsen, Emil

Published in:
Vaccine

DOI (link to publication from Publisher):
10.1016/j.vaccine.2024.03.058

Creative Commons License
CC BY 4.0

Publication date:
2024

Document Version
Publisher's PDF, also known as Version of record

Link to publication from Aalborg University

Citation for published version (APA):
Bruun, N., Laursen, M. F., Carmelo, R., Christensen, E., Jensen, T. S., Christiansen, G., Birkelund, S., Agger, R., & Kofod-Olsen, E. (2024). Novel nucleotide-packaging vaccine delivers antigen and poly(I:C) to dendritic cells and generate a potent antibody response in vivo. *Vaccine*, *42*(11), 2909-2918. https://doi.org/10.1016/j.vaccine.2024.03.058

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal -

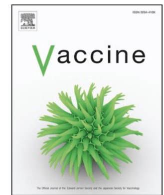
Take down policy
If you believe that this document breaches copyright please contact us at vbn@aub.aau.dk providing details, and we will remove access to the work immediately and investigate your claim.

Vaccine 42 (2024) 2909–2918

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Novel nucleotide-packaging vaccine delivers antigen and poly(I:C) to dendritic cells and generate a potent antibody response *in vivo*

Natasja Bruun, Marlene F. Laursen, Rita Carmelo, Esben Christensen, Trine S. Jensen, Gunna Christiansen, Svend Birkelund, Ralf Agger, Emil Kofod-Olsen *

Aalborg University, Department of Health Science and Technology, Denmark

A R T I C L E I N F O

Keywords:
Dendritic cell-targeting vaccine
Nucleotide adjuvant
Antigen-adjuvant co-delivery

A B S T R A C T

An issue with many current vaccines is the dependency on broadly inflammatory adjuvants, such as aluminum hydroxide or aluminum salts that affect many immune- and non-immune cells. These adjuvants are not necessarily activating all antigen-presenting cells (APCs) that take up the antigen and most likely they also activate APCs with no antigen uptake, as well as many non-immune cells.

Conjugation of antigen and adjuvant would enable the use of smaller amounts of adjuvant and avoid unnecessary tissue damage and activation of bystander cells. It would ensure that all APCs that take up the antigen would also become activated and avoid that immature and non-activated APCs present the antigen to T cells without a co-stimulatory signal, leading to tolerogenesis.

We have developed a novel vaccine that co-deliver antigen and a nucleotide adjuvant to the same APC and lead to a strong activation response in dendritic cells and macrophages. The vaccine is constructed as a fusion-protein with an antigen fused to the DNA/RNA-binding domain from the Hc2 protein from *Chlamydia trachomatis*. We have found that the fusion protein is able to package polyinosinic:polycytidylic acid (poly(I:C)) or dsDNA into small particles. These particles were taken up by macrophages and dendritic cells and led to strong activation and maturation of these cells. Immunization of mice with the fusion protein packaged poly(I:C) led to a stronger antibody response compared to immunization with a combination of poly(I:C) and antigen without the Hc2 DNA/RNA-binding domain.

1. Introduction

Vaccination against pathogenic microorganisms have had tremendous impact on human health and is one of the most important medical achievements. According to the World Health Organization, vaccination against infectious diseases such as diphtheria, tetanus, whooping cough and influenza effectively prevents the untimely death of 4–5 million people annually [1]. Subunit and toxoid vaccines, as those mentioned above, are widely used, since they are easy to produce and present no risk of introducing disease [2]. However, such vaccines normally need the addition of an adjuvant in order to induce a sufficient immune response.

Adjuvants induce an early state of inflammation and prevent development of tolerance towards the antigen. The most commonly used adjuvants are salts or hydroxide of aluminum [3]. Mixed with antigen, these aluminum compounds induce activation of antigen presenting cells (APC) and forms a local depot to ensure a prolonged exposure to the antigen [4]. Many experimental adjuvants being investigated are agonists of pattern-recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), and activate APCs to support a potent adaptive immune response [5,6]. These include Toll-like receptor (TLR) agonists such as polyinosinic:polycytidylic acid (poly(I:C)), synthetic CpG oligodeoxynucleotides (ODN), and imiquimod. Poly(I:C) is a synthetic double-stranded RNA and a known ligand for TLR3 [7]. Poly(I:C) and its derivatives have been evaluated in clinical trials, but so far, they have not received approval as vaccine adjuvants [6]. Several other TLR agonists are being investigated for their potential as adjuvants, in particularly TLR9 agonists [8].

Typically, antigen and adjuvant are injected together. The amount of adjuvant must be sufficient to activate local dendritic cells even though some of the adjuvant will diffuse away from the site of injection [6,9].

* Corresponding author.
E-mail address: ekol@hst.aau.dk (E. Kofod-Olsen).

https://doi.org/10.1016/j.vaccine.2024.03.058
Received 3 November 2023; Received in revised form 10 March 2024; Accepted 22 March 2024
Available online 27 March 2024
0264-410X/© 2024 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

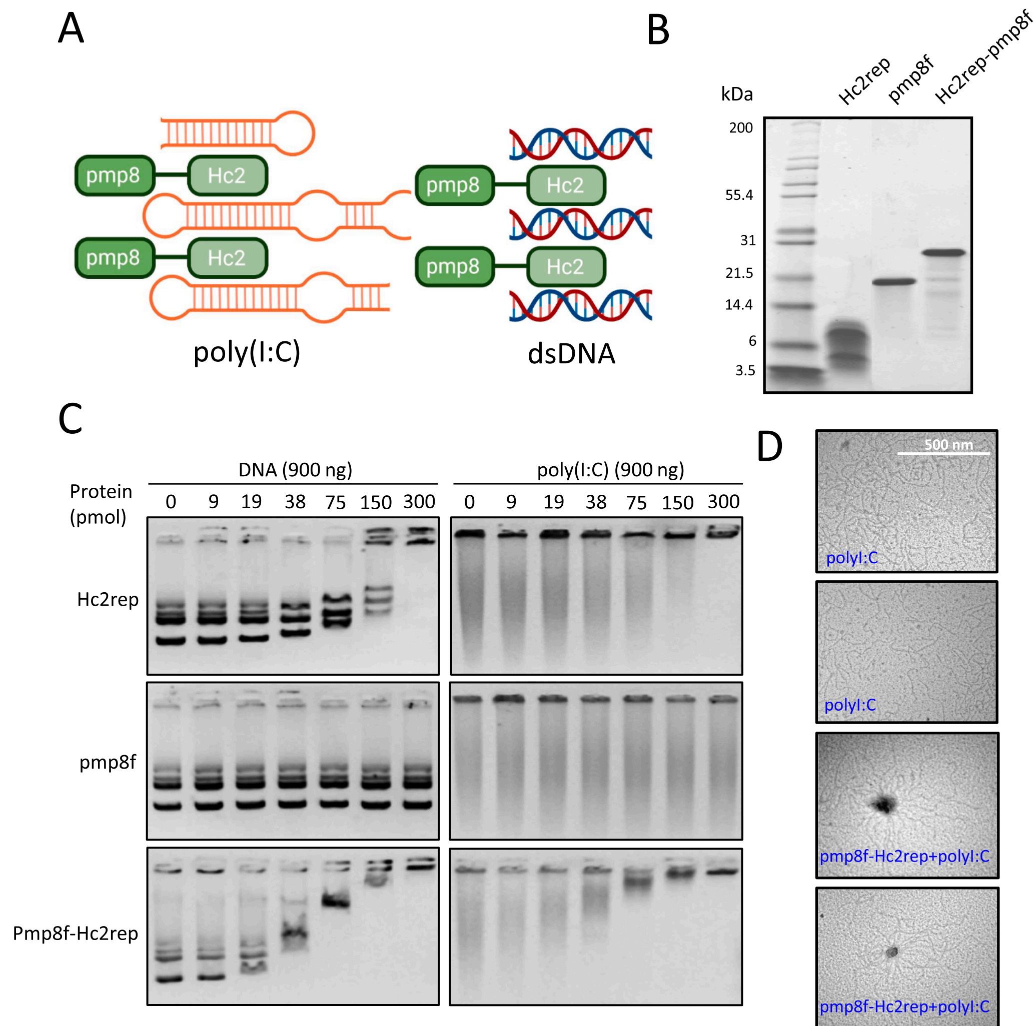


Fig. 1. Pmp8f-Hc2rep binds DNA or poly(I:C) to form small particles. (A) Visual representation pmp8f-Hc2rep fusion construct with DNA or poly(I:C) bound. Generated using BioRender. (B) SDS-PAGE gel loaded with Hc2rep, pmp8f, and pmp8f-Hc2rep and stained with Coomassie blue. The expected sizes were 3.8 kDa (Hc2rep), 15.6 kDa (pmp8f), and 19.4 kDa (pmp8f-Hc2rep). (C) Electrophoretic mobility shift assay. A fixed amount of DNA or poly(I:C) was mixed with increasing amounts of protein and loaded on an agarose gel. (D) Electron microscopy of poly(I:C) and pmp8f-Hc2rep-poly(I:C) complexes. Two out of five representative images are shown. Scale-bar represent 500 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

This can cause excessive local tissue damage from toxic effects of the adjuvant on non-immune cells. The adjuvant will provide a maturation signal to local dendritic cells that will begin to migrate towards the draining lymph node, where a T-cell response can be initiated. The migration will take place irrespective of whether the dendritic cells also have taken up antigen or not. This may induce unintended immune responses. On the other hand, local dendritic cells that have taken up the antigen, but not sufficient amounts of the adjuvant, may fail to activate T-cells or even induce anergy in antigen-specific T-cells.

Delivering antigen and adjuvant combined in a single molecule could potentially be a useful strategy to achieve strong adaptive immune responses, while limiting off-target effects. Indeed, Barbuto et al. has demonstrated that a DC-targeted anti-DEC205 antibody coupled to poly (dA:dT) and antigen led to an efficient immune response with low toxicity [10]. Using DNA vaccination and electroporation, Fredriksen et al. demonstrated that a strong immune response was generated in response to antigen delivered directly to APCs using a major histocompatibility complex (MHC) class II targeting antibody fusion protein [11]. We have previously shown that targeting CD11c on DCs with antibody-coupled DNA, lead to dendritic cell maturation [12]. Further, we have shown that targeting antigen to CD11c led to strong antibody production *in vivo* [13,14]. Antigen-adjuvant formulations may be created by conjugating the molecules chemically [12]. It is, however, time consuming and challenging to ensure structural uniformity of the vaccine from batch to batch using chemical methods.

Here, we present the production and characterization of two small recombinant fusion proteins including an antigen an oligonucleotide-binding domain. We demonstrate that the constructs does bind nucleotides, which leads to formation of fusion protein-nucleotide complex particles. The complexes are internalized by macrophages and dendritic cells, which become potently activated. Further, we demonstrate that the antigen can be expressed on MHC-I molecules on dendritic cells. *In vivo*, the fusion protein-nucleotide complexes induce a strong antibody response, with no observed side effects.

2. Materials and methods

2.1. Generation and evaluation of peptide construct

Two DNA constructs were made in the pET-30 Ek/Lic plasmid (Merck), one construct encoding amino acid region 341 to 443 (pmp8f) of polymorphic membrane protein 8 (pmp8) (accession number, PMP8_CHLPN), containing an T-helper cell epitope from *Chlamydia pneumoniae* (Fig. S1) [15], and one where the pmp8f sequence is

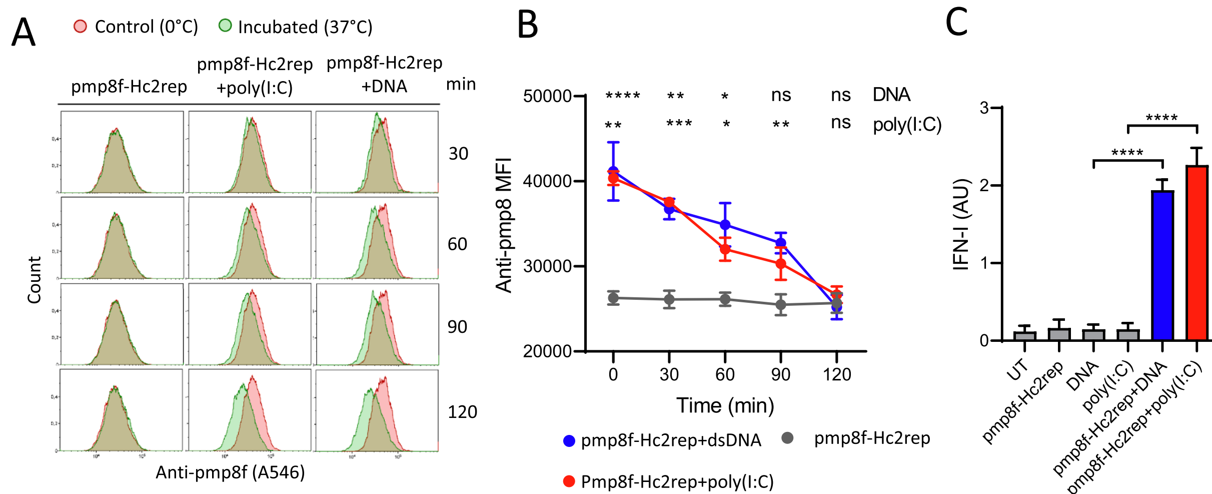


Fig. 2. Pmp8f-Hc2rep complexed with DNA or poly(I:C) binds to and become internalized by THP-1 cells. (A) Representative histograms of THP-1 cells treated with pmp8f, pmp8f-Hc2rep + poly(I:C) or pmp8f-Hc2rep + DNA, washed and kept on ice (control) (red), or incubated for 30, 60, 90 and 120 min at 37 °C (green). (B) Triplicate data from (A) showing the median fluorescence intensity (MFI). Statistically significant difference was measured using Two-way ANOVA, $*p \leq 0.05$ $**p \leq 0.01$ $***p \leq 0.001$ $****p \leq 0.0001$. (C) Type-I IFN production from THP-1-derived macrophages after stimulation with pmp8f-Hc2rep, nucleotides, pmp8f-Hc2rep + poly(I:C), or pmp8f-Hc2rep + DNA. Statistically significant difference was measured using One-way ANOVA, $****p \leq 0.0001$. Bars represent the mean ± SEM. Data are from three biological replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

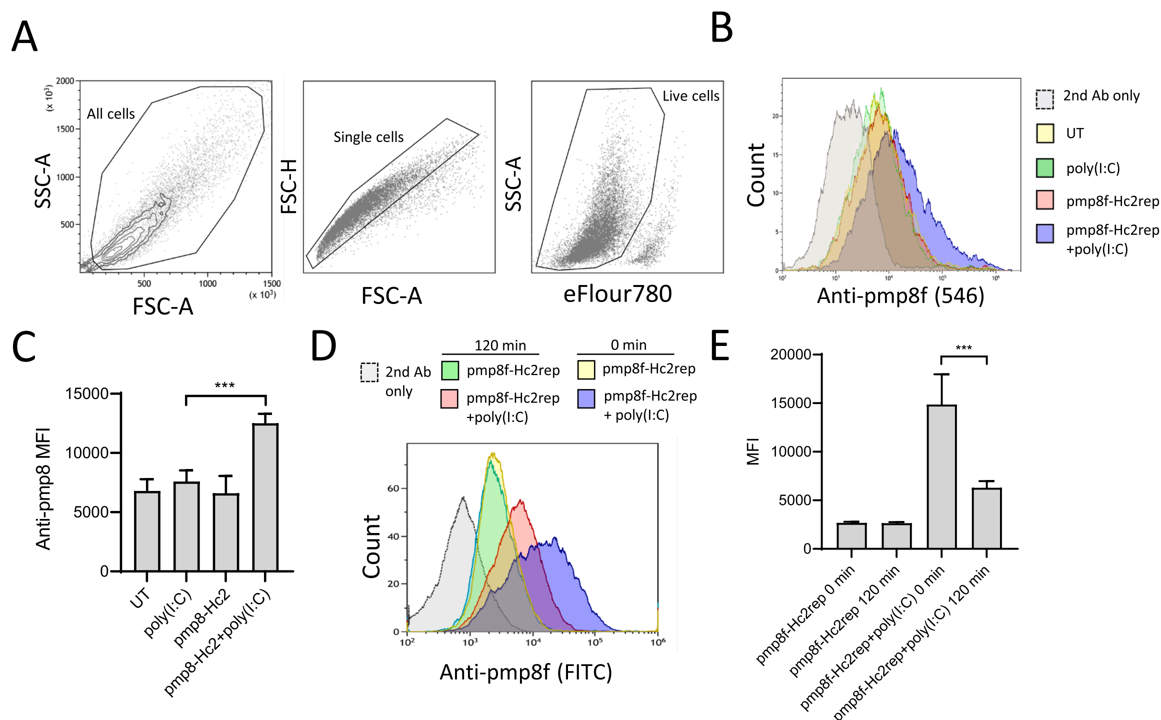


Fig. 3. Bone marrow-derived dendritic cells favor internalization of pmp8f-Hc2rep-poly(I:C) particles. (A) Flow cytometry gating strategy used in (B-E). (B) Binding of poly(I:C), pmp8f-Hc2rep, and pmp8f-Hc2rep- poly(I:C) to the surface of BMDCs after 30 min of incubation, 2nd Ab: background staining control from secondary antibody. (C) Analysis of triplicate data from (B), showing the median fluorescence intensity (MFI). (D) Internalization of pmp8f-Hc2rep and pmp8f-Hc2rep-poly(I:C) by BMDCs after 0 and 120 min of incubation. (E) Triplicate data from (D), showing the MFI. Bars represent the mean ± SEM. Data are from three biological replicates. Statistically significant difference was measured using One-way ANOVA, $***p \leq 0.001$.

followed by a sequence coding for 36 amino acids repeat (Hc2rep) from the histone H1-like *C. trachomatis* DNA-binding protein histone like protein 2 (Hc2) (Fig. S2 and suppl. Table 1) [16]. Plasmids was transformed into BL21 (DE3) *E. coli* (Merck) and protein expression was induced by 1 mM isopropyl-beta-D-thiogalactoside (IPTG) in Luria broth supplemented with 30 µg/ml kanamycin. The bacteria were lysed in 6 M Guanidinium, 0.1 M NaH_2PO_4, 10 mM Tris, 0.1 % β-mercaptoethanol (v/v), 0.5 % Noidet P-40 (v/v), pH 8.0 and sonicated. Proteins were collected by Ni^{++}-affinity chromatography (GE Healthcare, UK). Three washes with 60 % isopropanol were used to remove endotoxins [17]. Elution was performed by gradually lowering the pH of the elution buffer (8 M urea, 0.1 M sodium dihydrogen phosphate, 10 mM Tris, pH 5.9–4.5) with 20 % acetic acid in 6 M Guanidinium, 0.1 M NaH_2PO_4, 10 mM Tris. The protein containing fractions were subsequently dialyzed

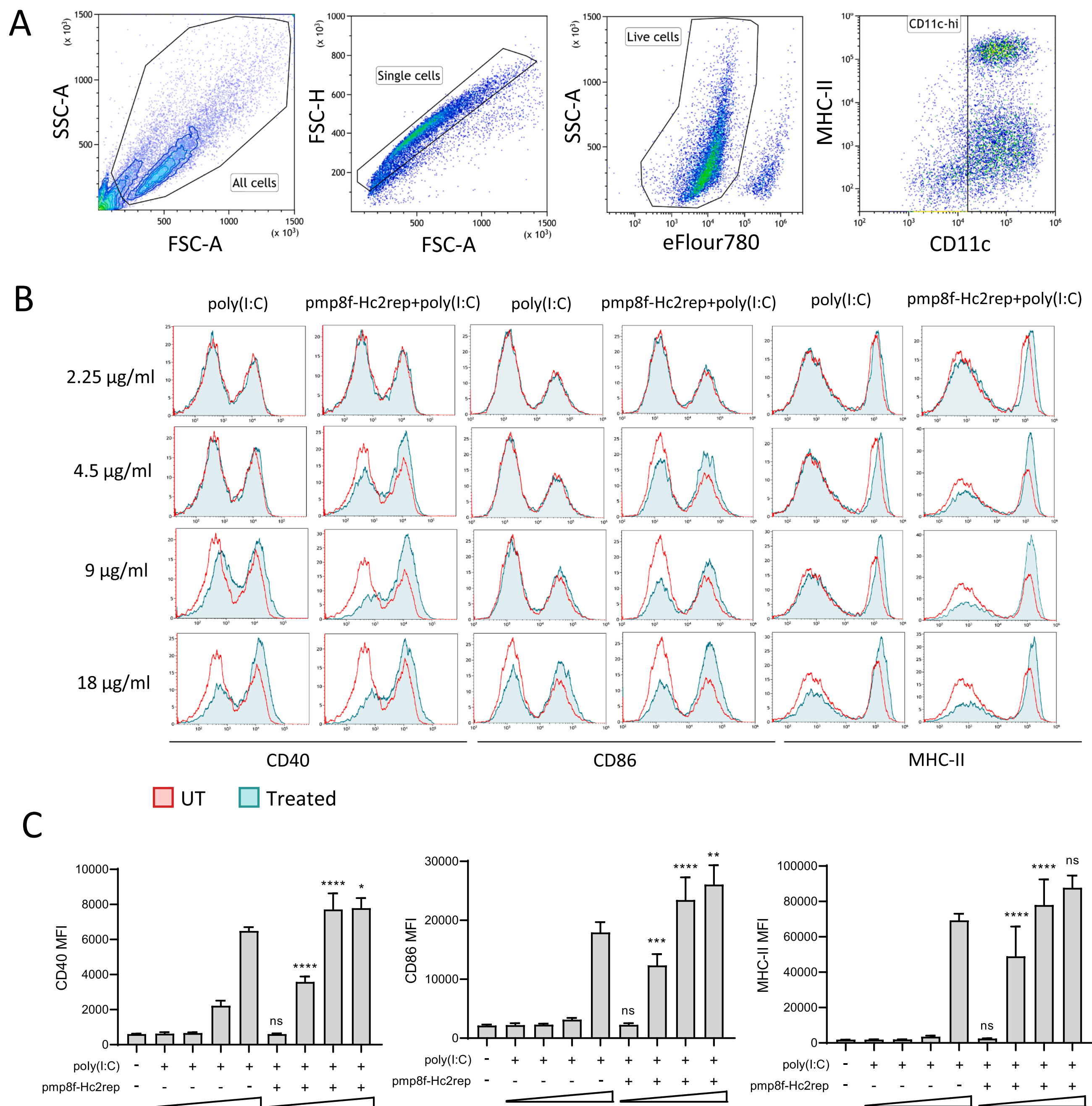


Fig. 4. Pmp8f-Hc2rep and poly(I:C)-induced maturation of murine BMDCs. (A) Flow cytometry gating strategy for gating out CD11c^{hi} BMDCs were used in (B-C). (B) Representative histograms showing the maturation induced by poly(I:C) and pmp8f-Hc2rep-poly(I:C) at various concentrations (0, 2.25, 5, 9, 18 µg/ml). Blue-green profiles show treated samples and untreated control is shown in red. (C) Triplicate data from (B) showing the median fluorescence intensity (MFI) of CD40, CD86, and MHC-II. Bars represent the mean ± SEM. Data are from three biological replicates. UT: untreated control (PBS). Statistically significant difference from UT measured using One-way ANOVA, $^{**}p \leq 0.01$ $^{***}p \leq 0.001$ $^{****}p \leq 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

against PBS over membranes with a molecular weight cut-off of 6–8 kDa (Spectra, USA). Endotoxin levels in purified proteins were determined using a Limulus amebocyte lysate (LAL) assay (Thermo Fisher Scientific) and was below 0.4 EU/mg. Recombinant Hc2rep was synthesized by Fmoc solid-phase-peptide synthesis on an automatic ABI 433 synthesizer (Applied Biosystems, Waltham, MA, USA) [16]. MHC-I and MHC-II epitopes of ovalbumin for the OVApep-Hc2rep construct were predicted using MHC Binding Prediction (IEDB Analysis Resource) and BepiPred-2.0 (DTU Health Tech, Denmark). The final construct was synthesized by Schafer-N (Denmark) (Fig. S3).

2.2. Cell growth and differentiation

Murine bone marrow was extracted from femur and tibia of BALB/c mice and cultured in RPMI 1640 (Gibco) supplemented with 10 % FCS (Thermo Fisher Scientific), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), 50 µM beta-mercaptoethanol (Sigma), 1 ng/ml IL-4 (Miltenyi Biotech Norden), and 40 ng/ml GM-CSF (Miltenyi Biotech Norden) for 10 days, with fresh IL-4 and GM-CSF added on day 3, 5 and 7. On day 10, non-adherent immature bone marrow-derived dendritic cells (BMDCs) were

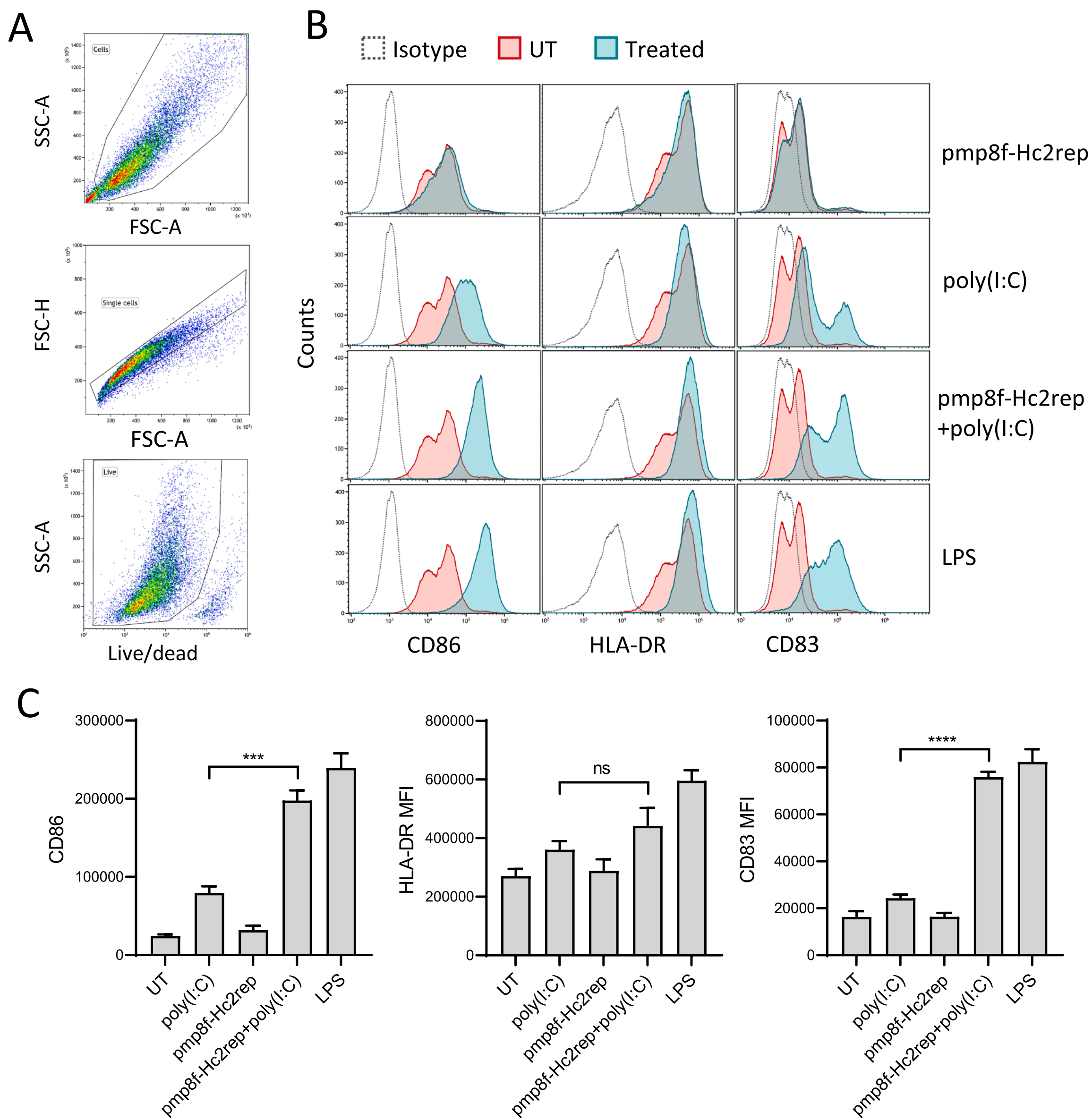


Fig. 5. Maturation response of moDCs to pmp8f-Hc2rep-poly(I:C) particles. (A) Flow cytometry gating strategy used in (B-C). (B) Representative histograms from (C), showing the maturation markers CD86, HLA-DR, and CD83 after stimulating moDCs with pmp8f-Hc2rep (5 μg/ml), poly(I:C) (5 μg/ml), pmp8f-Hc2rep + poly(I:C), and LPS as the positive control. (C) Median fluorescence intensity (MFI) of CD86, HLA-DR, and CD83. UT: untreated control (PBS). Bars represent the mean ± SEM. Data are from three biological replicates. Statistical difference was measured using One-way ANOVA, pmp8f-Hc2rep showed statistical difference from all other samples, ***$p \leq 0.001$ ****$p \leq 0.0001$.

collected. Human monocyte-derived dendritic cells (moDCs) were generated from monocytes, extracted from PBMCs from healthy donors, as previously described [12].

THP-1 cells (TIB-202, Leukemic monocyte, Tohoku Hospital Pediatrics-1 Cells) were grown in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Invitrogen) and 10 % FCS. THP-1 cells were induced to differentiate into macrophages by adding 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) for 24 h.

HEK-Blue IFN-α/β cells (kindly provided by Martin R Jakobsen, Aarhus University) were cultured in DMEM (Gibco) supplemented with 10 % FCS, 2 mM L-glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin, and 100 μg/ml normocin (InvivoGen) for 3 days, after which the medium was further supplemented with 30 μg/ml blasticidin (Life Technologies) and 100 μg/ml zeocin (InvivoGen). Cells were cultured for another 2–3 days, after which they were ready for following experiments.

2.3. *IFN- α/β detection assay*

HEK-Blue cells, possessing an active type I IFN signaling pathway and the reporter gene *secreted embryonic alkaline phosphatase* (*SEAP*), was used as a reporter cell to measure the levels of IFN-α/β in the culture medium of THP-1 cells, as per manufacturer's instructions (InvivoGen). Recombinant human IFN-αlpha (104 U/ml) were used as a positive control, and recombinant human IFN-γ (104 U/ml) as a negative control. Wavelengths of 620–655 nm were used to measure the color intensity, using a Tecan Sunrise microplate reader.

2.4. *Flow cytometry*

Mature moDCs were harvested by rinsing the wells with RPMI and PBS with 2 mM EDTA and washed with flow buffer (PBS, 0.1 % BSA, 0.01 % sodium azide, 1 mM EDTA). The cells were then stained with viability dye (eFluor® 780, eBioscience) followed by staining with

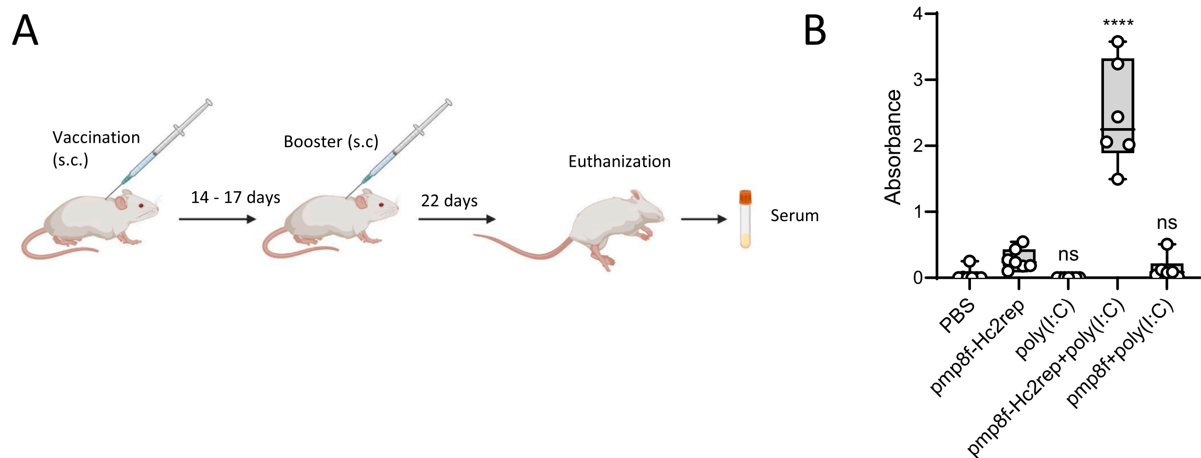


Fig. 6. Humoral response following *in vivo* immunization. (A) Schematic representation of experimental setup generated using BioRender. Mice were immunized twice after which serum was collected and analyzed. (B) Serum from mice immunized with PBS, pmp8f-Hc2rep, poly(I:C), pmp8f-Hc2rep-poly(I:C), or pmp8f with poly(I:C), was diluted 1:5000 and levels of pmp8f-specific antibodies were determined by ELISA (N = 6–7 mice per group). Bars represent the mean ± SEM and dots represent measurements from each individual mouse. Statistical difference was measured using One-way ANOVA, ***$p \leq 0.001$ ****$p \leq 0.0001$.

mouse anti-HLA-DR (PE, L203; RND systems), mouse anti-CD83 (PE-Cy7, HB15e; BD Biosciences), and mouse anti-CD86 (BV421, FUN-1; BD Biosciences) for 30 min at 4 °C. Finally, the cells were fixed with 1 % paraformaldehyde. Mature BMDCs were harvested and stained with rat monoclonal antibodies against murine MHC II (PE, M5/114.15.2; eBioscience), CD86 (eFluor450, GL1; eBioscience), and CD40 (APC, 1C10; Invitrogen) for 30 min at 4 °C before being fixed with 1 % paraformaldehyde. The cells were analyzed by flow cytometry using a CytoFLEX flow cytometer (Beckman Coulter) and using FlowJo v.10.8.1 software (BD Biosciences) for data analysis.

Internalization experiments were performed by treating the cells with Hc2rep, pmp8f or pmp8f-Hc2rep loaded with poly(I:C) or DNA and stained with mouse MAb11 anti-Lic-Tag (Loke Diagnostics) for 30 min at 4 °C and washed twice in PBS. Cells were incubated at 37 °C for various timepoints and moved to ice before analysis by flow cytometry using goat anti-mouse Alexa 488 (Jackson ImmunoResearch) or goat anti-mouse Alexa 546 (Molecular Probes). Control samples was kept on ice until analysis.

2.5. Electron microscopy

Electron microscopy of pmp8f-Hc2rep was performed as described earlier [18,19]. Complexes of 3 μg/ml protein and 30 μg/ml poly(I:C) (InvivoGen) or only 30 μg/ml poly(I:C) in 2 mM spermidine and 150 mM NaCl were applied to carbon-coated, glow discharged 400-mesh copper grids (Polaron) for 5 min. Afterwards, the grids were rinsed and dehydrated in 25, 50, 75, and 95 % ethanol and air dried. Specimens were rotary shadowed with tungsten.

2.6. Animals and immunizations

Experiments with Female BALB/cJRj mice (Janvier Labs, France) mice were approved by the Danish Animal Experiments Inspectorate (license #2021–15-0201–01004). Mice were injected subcutaneously in the scruff of the neck with 15 μg pmp8f-Hc2rep, 10 μg poly(I:C) (InvivoGen), 15 μg pmp8f-Hc2rep + 10 μg poly(I:C), 15 μg pmp8f + 10 μg poly(I:C), or PBS (Gibco). All substances were dissolved in PBS and the injected volume was 100 μl using a 22G needle. During injection, mice were kept under anesthesia with isoflurane. The mice received an identical booster injection after 14 or 17 days and were sacrificed by cervical dislocation after another 22 days. Blood from all mice were drawn from the facial vein and allowed to fully clot, whereafter serum was collected and stored at −80 °C before downstream analysis.

2.7. ELISA

96-well polystyrene plates (Nunc MaxiSorp, Thermo Scientific) were coated with 1 μg/ml recombinant pmp8f in coating buffer (10 mM carbonate/bicarbonate buffer, pH 9.6) overnight at 4 °C. Wells were washed three times in washing buffer (0.05 % Tween-20 in PBS) and blocked for 2 h at room temperature in blocking buffer (1 % BSA in PBS). Serum from immunized mice was diluted in blocking buffer and incubated for 1 h at room temperature. Wells were then washed three times and incubated with goat anti-mouse Ig-HRP (Dako) for 1 h at room temperature. Wells were washed three times, after which 100 μl Enhanced K Blue Substrate (Neogen) were added and incubated for 5 min. To stop the reaction, 100 μl stopping solution (1 M sulfuric acid in water) were used. Absorbance was measured at 450 nm with 620 nm reference on a MultiScan SkiHigh Microplate Reader (Thermo Scientific).

2.8. EMSA and SDS-PAGE

Protein production and purity was assessed by SDS-PAGE under denaturing conditions. Purified protein (3.5 μg) was denatured in LDS (lithium dodecyl sulfate) sample buffer and heated to 95 °C for 5 min before loaded and run on a 12 % polyacrylamide gel (Expedeon, USA). Gels were stained with Coomassie blue for protein band visualization. Electrophoretic mobility shift assay (EMSA) was performed by incubating various concentrations of purified Hc2rep, pmp8f or pmp8f-Hc2rep protein with 900 ng poly(I:C) (InvivoGen) or plasmid DNA (Agilent) for 10 min at 37 °C.

2.9. Statistical analysis

All data was analyzed using One-way ANOVA with multiple comparisons, with the exception of Fig. 2B, which was analyzed using Two-way ANOVA with multiple comparison and Turkeys correction. Statistics was performed using GraphPad Prism 9.

3. Results

3.1. Binding of dsDNA and dsRNA by Hc2rep leads to formation of small particles

We set out to generate an antigen-nucleotide-binding fusion protein, which could bind and form small particles when mixed with dsDNA or dsRNA. Initially, we generated a fusion protein consisting of an antigen

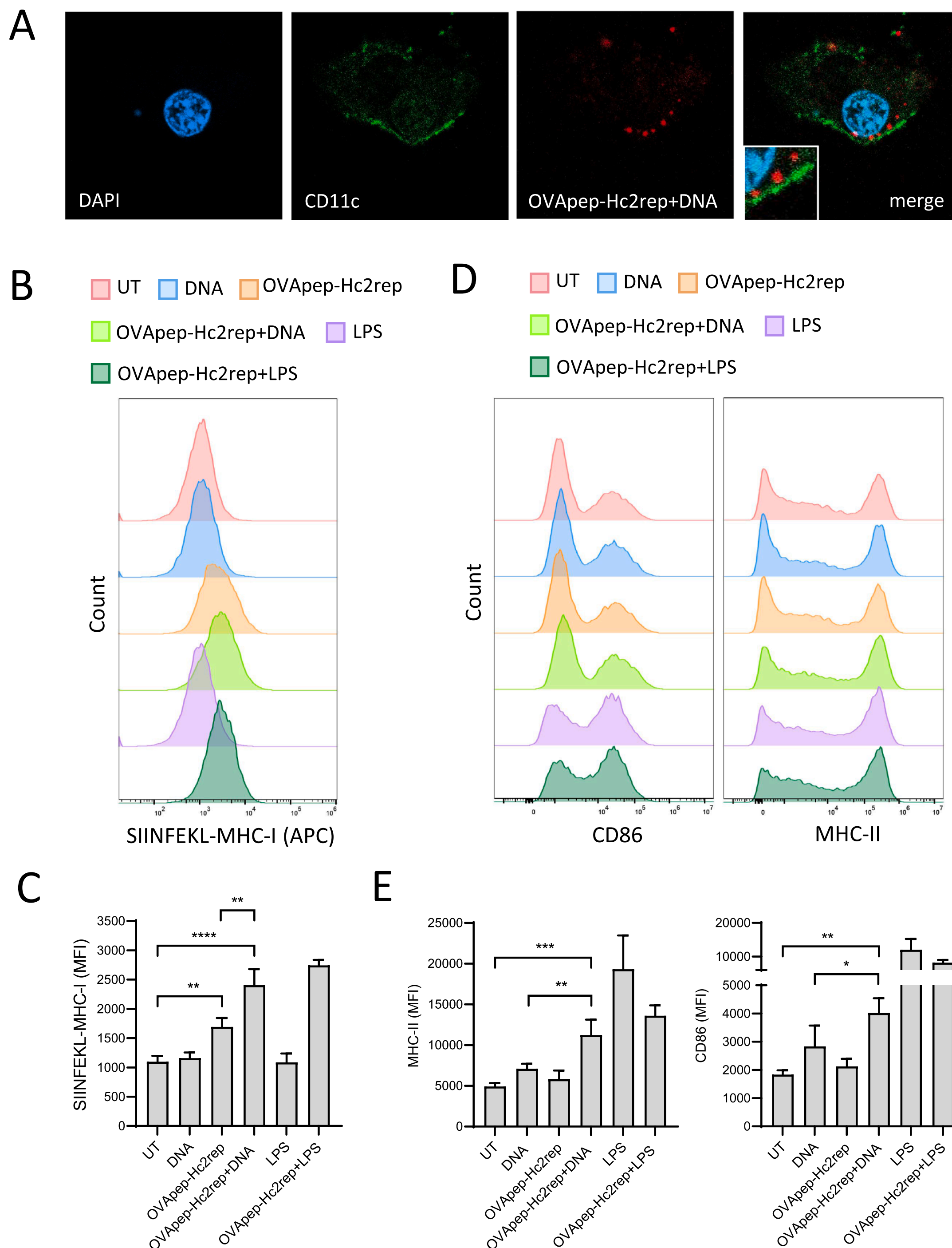


Fig. 7. SIINFEKL antigen is presented on mature BMDCs treated with OVApep-Hc2rep. (A) Confocal microscopy images of OVApep-Hc2rep internalization in BMDCs. The DC marker CD11c (FITC) is shown in green, the Cy5-DNA is shown in red and nucleus is shown in blue (DAPI). (B) Representative histograms from (C), showing expression of SIINFEKL-epitope-MHC-I on BMDCs treated with OVApep-Hc2rep with or without DNA. LPS was used as a positive control. (D) Median fluorescence intensity (MFI) of SIINFEKL-epitope-MHC-I expression. (D) Representative histograms from (E) showing the expression of maturation markers CD86 and MHC-II on the cells from the SIINFEKL-MHC-I-expression experiments (B & C). (E) Median fluorescence intensity (MFI) of CD86 and MHC-II expression on the SIINFEKL-expressing cells (B & C). Bars represent the mean ± SEM. Data are from three biological replicates. Statistically significant difference from UT measured using One-way ANOVA, *$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fragment (pmp8f) derived from the Polymorphic Membrane Protein 8 from *Chlamydia pneumoniae* and the nucleotide-binding domain (Hc2rep) from the Histone H1-like protein Hc2, from *Chlamydia trachomatis* (Fig. 1A). The Hc2rep domain is a small DNA/RNA binding domain [16], and pmp8f is known to be an antigen, which lead to a strong T-helper cell response in mice [15]. Hc2rep, pmp8f and pmp8f-Hc2rep fusion plasmids, were expressed in *E. coli*, purified, and analyzed on an SDS-PAGE gel. The fusion protein, and the Hc2rep was found to match the expected sizes of Hc2rep (3.8 kDa), pmp8f (15.6 kDa), and pmp8f-Hc2rep (19.4 kDa) (Fig. 1B).

Since Hc2rep has previously been found to bind dsDNA and ssRNA, leading to formation of small particles [20], an electrophoretic mobility

shift assay was performed to confirm that recombinant pmp8f-Hc2rep had the capacity to bind nucleotides. An increasing amount of Hc2rep, pmp8f, or pmp8f-Hc2rep was combined with a fixed amount of DNA and poly(I:C). Band-shifts was observed when Hc2rep or pmp8f-Hc2rep was mixed with DNA or poly(I:C), whereas pmp8f alone showed no band-shifts, thus confirming that pmp8f-Hc2rep formed complexes with the nucleotides (Fig. 1C). Interestingly, the pmp8f-Hc2rep fusion protein led to complex formation using lower amounts of DNA and poly(I:C) compared to Hc2rep alone.

To further examine the formation of small complexes, we conducted electron microscopy, which demonstrated that poly(I:C) was seen as strings and did not give rise to particle formation (Fig. 1D). In contrast, a mixture of pmp8f-Hc2rep and poly(I:C) resulted in particle formation due to binding between protein and poly(I:C) (Fig. 1D). This is in agreement with the mobility shift observed in gel electrophoresis (Fig. 1C).

3.2. *Vaccine particles binds to and activates THP-1 cells*

To investigate if the vaccine particles could bind, become internalized, and activate APCs, we initially used THP-1 cells. The cells were treated with pmp8f-Hc2rep alone or pmp8f-Hc2rep loaded with DNA or poly(I:C), incubated for various timepoints, stained with anti-pmp8f antibody and a Alexa546-conjugated secondary antibody and analyzed using flow cytometry (Fig. 2A-B). Our results demonstrated that cells treated with pmp8f-Hc2rep in the absence of DNA or poly(I:C), showed no discernible shifts in the median fluorescent intensity (MFI) at any of the timepoints (Fig. 2A-B). However, when pmp8f-Hc2rep was complexed with DNA or poly(I:C), a time-dependent decrease in MFI relative to pmp8f-Hc2rep alone could be observed (Fig. 2A-B). These results indicate that the construct is internalized steadily over time.

To address if vaccine particles taken up by THP-1 cells triggered their activation, the cells were subjected to treatment with DNA, poly(I:C), pmp8f-Hc2rep alone or pmp8f-Hc2rep loaded with DNA or poly(I:C), followed by measurements of type-I interferon (IFN) in the supernatant. DNA, poly(I:C) or pmp8f-Hc2rep alone did not induce measurable levels of type-I IFN production. On the contrary, when cells were treated with pmp8f-Hc2rep loaded with DNA or poly(I:C), equally high amounts of type-I IFN expression were observed (Fig. 2C). The complete lack of response in the THP-1 cells that were treated with DNA or poly(I:C) alone, is presumably due to the use of a relatively small amount of extracellularly added nucleotide, which are poorly internalized, and thus unable to activate the intracellular RNA and DNA sensors.

In summary, these findings show that pmp8f-Hc2rep complexed with DNA or RNA can be taken up by THP-1-derived macrophages, leading to activation of the cells.

3.3. *Bone marrow-derived DCs can internalize vaccine particles bound to their surface*

Since DCs are the most prominent APCs responsible for induction of adaptive immune responses, we wanted to investigate if DCs could also bind and internalize the vaccine particles. To make the experiments more compatible with mouse models, we used bone marrow-derived dendritic cells (BMDCs) and poly(I:C) for the investigations. BMDCs were treated with poly(I:C), pmp8f-Hc2rep, or pmp8f-Hc2rep-poly(I:C) and analyzed by flow cytometry. BMDCs treated with pmp8f-Hc2rep-poly(I:C) had an increased binding, compared to cells treated with pmp8f-Hc2rep or poly(I:C) alone (Fig. 3A-C).

To further investigate if the pmp8f-Hc2rep-poly(I:C) particles were also taken up, BMDCs were incubated with pmp8f-Hc2rep or pmp8f-Hc2rep-poly(I:C) for 0 or 120 min. Flow cytometric analysis revealed a significant decrease in MFI from 0 to 120 min of incubation with pmp8f-Hc2rep-poly(I:C) complexes (Fig. 3D-E). This finding did not apply for cells treated with pmp8f-Hc2rep alone (Fig. 3D-E), thus indicating that BMDCs take up the complex, as observed for the THP-1 cells.

3.4. *Dendritic cells mature upon treatment with vaccine particles in vitro*

Next, we wanted to assess whether the vaccine would induce DC maturation. Immature BMDCs were treated with different concentrations of poly(I:C) or pmp8f-Hc2rep-poly(I:C), and the expression level of maturation markers CD40, CD86 and MHC-II were analyzed by flow cytometry. Cells treated with a high dose of poly(I:C) showed increased expression of all three maturation markers CD40, CD86, and MHC-II, whereas lower doses did not (2.25 μg/ml to 9 μg/ml) (Fig. 4A-C). Treatment with pmp8f-Hc2rep-poly(I:C) led to an increased expression of all maturation markers at lower dosage level (4.5 μg/ml) than poly(I:C) alone (Fig. 4A-C).

To investigate if human DCs responded equally to the vaccine, immature monocyte-derived DCs (moDCs) were collected and stimulated with pmp8f-Hc2rep, poly(I:C), pmp8f-Hc2rep-poly(I:C), or LPS. Stimulated cells were analyzed for the expression of maturation markers CD86, HLA-DR, and CD83 (Fig. 5A-C). We found that moDCs treated with pmp8f-Hc2rep alone did not mature upon stimulation and moDCs treated only with poly(I:C) showed a minor upregulation of maturation markers. In contrast a high expression of maturation markers was observed in cells treated with pmp8f-Hc2rep-poly(I:C), similar to our findings using BMDCs, indicating that the vaccine works equally well on human DCs.

Collectively, this shows that DCs are able to take up pmp8f-Hc2rep-poly(I:C) particles, leading to a strong maturation of the cells.

3.5. *Potent antibody response in vivo with no visual toxicity*

Since pmp8f-Hc2rep-poly(I:C) particles led to activation and maturation of DCs *in vitro*, we wanted to evaluate the immune response *in vivo*. Mice received s.c. injections of vehicle, pmp8f-Hc2rep, poly(I:C), pmp8f-Hc2rep-poly(I:C), or pmp8f mixed with poly(I:C) and an identical booster injection 14 or 17 days later (Fig. 6A). An ELISA detecting anti-pmp8f antibodies was carried out on serum samples from the immunized mice and revealed that the combined delivery of pmp8f-Hc2rep and poly (I:C) provoked a strong antibody response (Fig. 6B). Injections of pmp8f-Hc2rep alone or pmp8f mixed with poly(I:C) did not give rise to increased antibody levels, although a small increase could be observed relative to control and poly(I:C) alone (Fig. 6B). Collectively, this indicate that formation of particles containing both antigen and poly(I:C) leads to a stronger response than un-coupled antigen and poly(I:C). Importantly, no side-effects, such as piloerection and squinched eyes was observed using this small amount of poly(I:C).

3.6. *Bone marrow-derived dendritic cells present vaccine-epitopes on MHC-I molecules*

In order to evaluate if an antigen-Hc2rep construct could lead to antigen-presentation on MHC-I, we generated a new construct comprised of an antigenic fragment from hen-egg ovalbumin (OVApep) (Fig. S3A). MHC-I presentation of the OVApep SIINFEKL-epitope can be analyzed with a SIINFEKL-MHC-I specific antibody. Initially, we analyzed the constructs ability to bind nucleotides and induce BMDC maturation. Similarly, to the pmp8f-Hc2rep construct, it was confirmed that the OVApep-Hc2rep construct also bound poly(I:C) and dsDNA (Fig. S3B). Analysis of BMDC maturation, showed that OVApep-Hc2rep, packaged with either poly(I:C) or dsDNA, could induce upregulation of the maturation markers CD40, CD86 and MHC-II (Fig. S3C – D), although OVApep-Hc2rep + poly(I:C) did not significantly increase the maturation, compared to poly(I:C) alone. To investigate internalization of the construct, we packaged OVApep-Hc2rep with Cy5-labled DNA, and performed confocal microscopy, which showed that BMDCs could take up the labeled particles (Fig. 7A). To confirm that epitopes from the internalized construct could be presented on MHC-I, we treated BMDCs with OVApep-Hc2rep or OVApep-Hc2rep + DNA for 24 h and stained the cells with antibody recognizing the SIINFEKL-epitope bound to

MHC-I (Fig. 7B & C). Both the construct with DNA and without DNA was presented on the BMDCs. Interestingly, the DNA-packaged construct led to a significant increase in presentation. Since this increase in presentation is also seen when OVA-pep-Hc2rep is co-delivered with LPS, it is likely due to the increased MHC-I expression in mature DCs. We further investigated the maturation status of the cells from the presentation experiments and found that only cells that received DNA or OVApep-Hc2 + DNA had upregulated maturation markers, and similarly to previous results, this was significantly higher in OVApep-Hc2 + DNA treated cells compared to OVApep-Hc2 alone (Fig. 7D & E).

4. Discussion

Vaccination is one of the greatest medical discoveries, saving millions of people every year from an untimely death caused by infectious diseases. Recent research indicate that vaccination may also become important in anti-cancer therapy [21]. Peptide vaccines have been employed for decades, but they have a relatively low efficacy compared to live and attenuated vaccines and require co-administration of adjuvant. A strategy to increase efficacy could be small peptide vaccines packaged with DAMP or PAMP adjuvants.

We have constructed two fusion-protein vaccines, which contains either a pmp8f antigen or an OVApep antigen and a DNA/RNA binding motif. The constructs are able to induce activation and antigen presentation in the same cell, thereby ensuring that adjuvant effect is restricted to the APCs that took up the antigen. This may reduce the amount of PRR agonist needed to get a beneficial immune activation.

DNA and RNA have been shown to be strong DAMPs, which lead to induction of type-I IFN, and effective activation of several immune cells, including DCs [22,23,24]. We and others have previously shown that DNA and poly(I:C) are potent DC activators when delivered directly to the cells [10,12,25]. Our results demonstrate that co-administration of the constructs and DNA or poly(I:C) leads to potent mouse and human DC maturation. We found that the SIINFEKL epitope from the OVApep-Hc2rep construct, was presented on MHC-I in treated DCs, and these DCs only matured when the construct was packaged with DNA. This confirms an uptake of the particles, followed by MHC-I presentation, which was enhanced in mature DCs.

Positively charged peptides have previously been used as carriers of DNA and RNA to cells, where transactivator of transcription (TAT) peptide derived from human immunodeficiency virus is one of the best described [26]. Park et al. used a TAT fusion protein to deliver antigen and poly(I:C) simultaneously and demonstrated an increased antigen-specific immune response evident by increased levels of type-I IFN producing T cells and reduced tumor volume in prophylactic and therapeutic models [27]. However, they observed no difference in the expression of maturation markers between TAT + poly(I:C) and poly(I:C)-treated DCs *in vitro*, which could indicate excess amounts of poly(I:C) were used. Although TAT has been used successfully in some fusion protein vaccines to deliver poly(I:C), it seems to bind poly(I:C) less efficiently than the vaccine in the present study, according to what was reported for Melan A epitope-TAT [28]. However, comparative studies have to be performed to determine the exact difference.

Since poly(I:C) is a powerful activator of TLR3, there may be a risk of severe side-effects *in vivo* and thus it is important to find an optimal delivery strategy to circumvent possible induced systemic toxicity [29]. It has been observed that poly(I:C) has a dose dependent toxicity [30,31,32]. We found that delivery of poly(I:C) packaged with pmp8f-Hc2rep or OVA-Hc2rep led to DC maturation at lower concentrations compared to poly(I:C) alone, which should enable using poly(I:C) as an effective adjuvant with very low levels, thus reducing toxicity. Indeed, no visual toxicity was found when either poly(I:C) or poly(I:C) complexed with pmp8f-Hc2rep were administered in mice.

In animal studies, poly(I:C) has been used for immune response studies of viral infections [33,34,35] and as an adjuvant for vaccines [27,36,37]. We found that immunization with pmp8f-Hc2rep packaged with a low dose of poly(I:C) led to a potent antibody response, with no response in mice vaccinated with pmp8f + poly(I:C), pmp8f or poly(I:C) alone. This indicate that delivering antigen coupled to a low dose of poly(I:C) to the same cells, greatly enhances the response and production of specific antibodies.

In conclusion, our results provide a new, easy and efficient way to deliver antigen and a low amount of nucleotide adjuvant to APCs *in vivo*. This could hold potential for future vaccine designs, as the need for high amounts of DNA or RNA may pose a risk for excessive activation of the immune system.

CRediT authorship contribution statement

Natasja Bruun: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **Marlene F. Laursen:** Conceptualization, Investigation, Methodology. **Rita Carmelo:** Investigation. **Esben Christensen:** Conceptualization, Investigation, Methodology. **Trine S. Jensen:** Formal analysis. **Gunna Christiansen:** Investigation, Methodology. **Svend Birkelund:** Conceptualization, Writing – review & editing. **Ralf Agger:** Conceptualization, Funding acquisition, Writing – review & editing. **Emil Kofod-Olsen:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

Funding: This work was supported by the Danish Cancer Research Foundation, Pedersen Charity Foundation, Familien Erichsens Mindefond, Fabrikant Einar Willumsens Mindelegat and Lions pris (Lions Denmark). All authors have declared that there are no financial conflicts of interest with regard to this work.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2024.03.058.

References

[1] World Health Organization. Immunization, 2019, https://www.who.int/news-room/facts-in-pictures/detail/immunization [accessed 16 October 2023].
[2] Bayani F, Hashkavaei NS, Arjmand S, Rezaei S, Usković V, Alijanianzadeh M, et al. An overview of the vaccine platforms to combat COVID-19 with a focus on the subunit vaccines. Prog Biophys Mol Biol 2023;178:32–49.
[3] Pulendran B, S. Arunachalam P, O'Hagan DT. Emerging concepts in the science of vaccine adjuvants. Nat Rev Drug Dis 2021;20(6):454-75.
[4] Hogenesch H. Mechanism of immunopotentiation and safety of aluminum adjuvants. Front Immunol 2013;3:406.
[5] Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. Immunity 2010;33(4):492–503.
[6] Luchner M, Reinke S, Milicic A. TLR agonists as vaccine adjuvants targeting cancer and infectious diseases. Pharmaceutics 2021;13(2).
[7] Medzhitov R, Flavell RA, Alexopoulou L, Holt AC. Recognition of double-stranded RNA and activation of NF-κB by toll-like receptor 3. Nature (London) 2001;413(6857):732–8.
[8] Scheiermann J, Klinman DM. Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer. Vaccine 2014;32(48):6377–89.
[9] Li J-K, Balic JJ, Yu L, Jenkins B. TLR agonists as adjuvants for cancer vaccines. Regul Inflamm Signal Health Disease 2017;1024:195–212.

[10] Barbuto S, Idoyaga J, Vila-Perello M, Longhi MP, Breton G, Steinman RM, et al. Induction of innate and adaptive immunity by delivery of poly dA:dT to dendritic cells. Nat Chem Biol 2013;9(4):250–6.
[11] Fredriksen AB, Sandlie I, Bogen B. DNA vaccines increase immunogenicity of idiotypic tumor antigen by targeting novel fusion proteins to antigen-presenting cells. Mol Ther 2006;13(4):776–85.
[12] Laursen MF, Christensen E, Degn LLT, Jønsson K, Jakobsen MR, Agger R, et al. CD11c-targeted Delivery of DNA to Dendritic Cells Leads to cGAS- and STING-dependent Maturation. Journal of immunotherapy (1997). 2018;41(1):9-18.
[13] Pugholm LH, Petersen LR, Sondergaard EK, Varming K, Agger R. Enhanced humoral responses induced by targeting of antigen to murine dendritic cells. Scand J Immunol 2015;82(6):515–22.
[14] Pugholm LH, Varming K, Agger R. In vitro assay for screening of optimal targets for antigen-delivery to murine dendritic cells. Scand J Immunol 2015;82(6):498–505.
[15] Mygind T, Vandahl B, Pedersen AS, Christiansen G, Höllsberg P, Birkelund S. Identification of an in vivo CD4 + T cell-mediated response to polymorphic membrane proteins of chlamydia pneumoniae during experimental infection. FEMS Immunol Med Microbiol 2004;40(2):129–37.
[16] Goncalves OSL, Christiansen G, Holm A, Herrmann B, Klintstedt M, Petersen SB, et al. The repeated 36 amino acid motif of chlamydia trachomatis Hc2 protein binds to the major groove of DNA. Res Microbiol 2019;170(6–7):256–62.
[17] Franken KL, Hiemstra HS, van Meijgaarden KE, Subronto Y, den Hartigh J, Ottenhoff TH, et al. Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. Protein Expr Purif 2000;18(1):95–9.
[18] Christiansen G, Pedersen LB, Koehler JE, Lundemose AG, Birkelund S. Interaction between the chlamydia trachomatis histone H1-like protein (Hc1) and DNA. Journal of Bacteriology: JB 1993;175(6):1785–95.
[19] Griffith JD, Christiansen G. Electron microscope visualization of chromatin and other DNA-protein complexes. Annu Rev Biophys Bioeng 1978;7:19–35.
[20] Pedersen LB, Birkelund S, Christiansen G. Purification of recombinant chlamydia trachomatis histone H1-like protein Hc2, and comparative functional analysis of Hc2 and Hc1. Mol Microbiol 1996;20(2):295–311.
[21] Liu J, Fu M, Wang M, Wan D, Wei Y, Wei X. Cancer vaccines as promising immuno-therapeutics: platforms and current progress. J Hematol Oncol 2022;15(1):28.
[22] Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature 2008;455(7219):674–8.
[23] Zhong B, Yang Y, Li S, Wang Y-Y, Li Y, Diao F, et al. The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. Immunity (Cambridge, Mass) 2008;29(4):538–50.
[24] Kis-Toth K, Szanto A, Thai T-H, Tsokos GC. Cytosolic DNA-activated human dendritic cells are potent activators of the adaptive immune response. J Immunol (1950) 2011;187(3):1222–34.
[25] Tacken PJ, Zeelenberg IS, Cruz LJ, van Hout-Kuijer MA, van de Glind G, Fokkink RG, et al. Targeted delivery of TLR ligands to human and mouse dendritic cells strongly enhances adjuvanticity. Blood 2011;118(26):6836–44.
[26] Zou L, Peng Q, Wang P, Zhou B. Progress in research and application of HIV-1 TAT-derived cell-penetrating peptide. J Membr Biol 2017;250(2):115–22.
[27] Park JS, Kim HS, Park HM, Kim CH, Kim TG. Efficient induction of anti-tumor immunity by a TAT-CEA fusion protein vaccine with poly(I:C) in a murine colorectal tumor model. Vaccine 2011;29(47):8642–8.
[28] Haenssle HA, Riedl P, Buhl T, Schardt A, Rosenberger A, Schon MP, et al. Intracellular delivery of major histocompatibility complex class I-binding epitopes: dendritic cells loaded and matured with cationic peptide/poly(I:C) complexes efficiently activate T cells. Exp Dermatol 2010;19(1):19–28.
[29] Hafner AM, Corthesy B, Merkle HP. Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant. Adv Drug Deliv Rev 2013;65(10):1386–99.
[30] Cornell JCJ, Smith KA, Cornwell rGG, Burke GP, McIntyre OR. Ssytemic effects of intravenous polyriboinosinic-polyribocytidylic acid in man. JNCI : Journal of the National Cancer Institute. 1976;57(6):1211-6.
[31] Fisher JC, Cooperband SR, Mannick JA. The effect of polyinosinic-polycytidylic acid on the immune response of mice to antigenically distinct tumors. Cancer research (Chicago, Ill) 1972;32(5):889–92.
[32] Homan ER, Zendzian RP, Schott LD, Levy HB, Adamson RH. Studies on poly I: C toxicity in experimental animals. Toxicol Appl Pharmacol 1972;23(4):579–88.
[33] Zhao J, Wohlford-Lenane C, Zhao J, Fleming E, Lane TE, McCray Jr PB, et al. Intranasal treatment with Poly(I·C) protects aged mice from lethal respiratory virus infections. J Virol 2012;86(21):11416–24.
[34] Zhou Z-X, Zhang B-C, Sun L. Poly(I:C) induces antiviral immune responses in japanese flounder (Paralichthys olivaceus) that require TLR3 and MDA5 and is negatively regulated by Myd88. PLoS One 2014;9(11).
[35] Gao X, Chan PKS, Lui GCY, Hui DSC, Chu I-M-T, Sun X, et al. Interleukin-38 ameliorates poly(I:C) induced lung inflammation: therapeutic implications in respiratory viral infections. Cell Death Dis 2021;12(1):53.
[36] Trumpfheller C, Caskey M, Nchinda G, Longhi MP, Mizenina O, Huang Y, et al. The microbial mimic poly IC induces durable and protective $CD4^{+}$ T cell immunity together with a dendritic cell targeted vaccine. Proc Nat Acad Sci - PNAS 2008;105 (7):2574–9.
[37] Apostólico JS, Lunardelli VAS, Yamamoto MM, Cunha-Neto E, Boscardin SB, Rosa DS. Poly(I:C) potentiates T cell immunity to a dendritic cell targeted HIV-multiepitope vaccine. Front Immunol 2019;10:843.