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A novel rabies virus lipopeptide provides a better protection by improving the magnitude of DCs activation and T cell responses

Rui Liu [1], Jingbo Wang [1], Yan Yang, Inamullah Khan, Yue Dong, Naishuo Zhu *

Laboratory of Molecular Immunology, State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, Shanghai 200438, China

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ABSTRACT

Besides rabies virus neutralizing antibody, non-neutralizing antibody to internal vital proteins, interferon, and possibly cell-mediated immunity also have a critical role in preventing the infection by rabies virus (RV). We identified novel CTL and Th epitopes which could induce lymphocyte proliferation and IFN-γ, IL-4 production, and designed linear and branched lipopeptides with these selected CTL and Th epitopes. Compared to linear construct, branched lipopeptides, especially Lipo C, stimulate stronger phenotypic and functional maturation of DCs, as well as more efficient $CD8^+$ T cell responses, evaluating by using FACS, $G_{333–341}$ tetramer staining and specific CTL assay. Lipo C could also assist rabies vaccine to induce an instant rabies virus neutralizing antibody production, and better protection against rabies virus challenge at early stage. These data reveal that Lipo C could be a promising component for developing novel rabies vaccines.

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1. Introduction

Rabies virus has the highest fatality rate of all known human viral pathogens, which causes over 31,000 annual death in Asia every year (Evans et al., 2012; Li et al., 2012). Up to now, the post exposure prophylaxis (PEP) with nonliving vaccine remains to be the only resistance to rabies (Li et al., 2012; Wen et al., 2011). Although serum RVNA titer is used as an indicator to evaluate the efficacy of rabies vaccine, there is compelling evidence showing that for effective clearance of virus from the CNS, non-neutralizing antibody to internal vital proteins, interferon, and possibly cell-mediated immunity such as Th1 cells and $CD8^+$ T cells also have a critical role in preventing the infection (Li et al., 2012; Schijns and Lavelle, 2011; Wirblich and Schnell, 2011). A study reported that 197 out of 725 human rabies cases were caused by vaccination failure, in which victims died before the 4th injection (Zhang et al., 2016). Thus, additional components which could activate protective immunity by stimulating earlier and higher level production of RVNAb and cell-mediated immune responses are under great demand.

Subunit vaccine which contains both CTL epitopes and Th epitopes could stimulate antivirus CTL activity and durable Ab (Schijns and Lavelle, 2011; Zaman and Toth, 2013). The use of Ags covalently coupled to palmitic acid, a biological ligand for TLR2 that acts as adjuvants, could activate transcription factors including NF-κB leading to DC maturation and secretion of proinflammatory cytokines(Chua et al., 2012; Dey et al., 2015; Li et al., 2013; Muller et al., 2015). These exogenous palmitic acid tailed peptide epitopes deliver both Ag and agonist to DCs simultaneously, leading to enhanced uptake, activation, and subsequent Ag presentation to T cells (Eriksson and Jackson, 2007; Renaudet et al., 2010; Zaman and Toth, 2013). This immune formulation has allowed us to design vaccine candidates that boost protective humoral and cellular immune responses against rabies virus infection.

In this study, we predicted CTL and Th epitopes from glycoprotein (G) and nucleocapsid protein (N) of RABV using bioinformatics software based on the model of BALB/c mice in order to construct lipopeptides. We designed different (lipo-)peptides by incorporating all the necessary components including CTL epitope, Th epitope and an internal immune-adjuvant, and studied how the position of the lipid moiety (i.e. either at N-terminal end or in the middle) affects the uptake of (lipo-)peptides, cytokines induction, and maturation of DCs, and the processing that lead its functional presentation to $CD8^+$ T cells. RVNAb titer was also examined to explore the immunogenicity of our lipopeptides adjuvanted vaccine. Overall the use of these RV specific lipopeptides will greatly facilitate the production of novel rabies vaccines, which are capable of inducing effector T cell function and humoral response.

* Corresponding author.
E-mail address: nzhu@fudan.edu.cn (N. Zhu).
[1] These authors contribute equally to this work.

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2. Materials and methods

2.1. Computational prediction of candidate RABV-specific CTL and Th epitopes

SYFPEITHI and BIMAS were used for the prediction of RABV-specific CTL epitopes, and Th epitopes was predicted by RANKPEP. The following criteria were used to select the candidate epitopes: (a) a higher ranking score; (b) the score analyzed by polynomial method exceeding the threshold; (c) occurrence in both prediction results.

2.2. Peptide and lipopeptide synthesis

Peptides and lipopeptides derived from the RABV glycoprotein and nucleoprotein were synthesized from Qiangyao Biochem (Shanghai, China). The purity of peptides was between 91 and 98% as determined by RP-HPLC (Vydac C18) and mass spectroscopy (Voyager MALDI-TOF System). For flow cytometry and confocal microscopy, peptides and lipopeptides with their free amino groups labeled with FITC were purchased from Qiangyao Biochem. Peptides were dissolved in 5% DMSO (Sigma–Aldrich, St. Louis, MO) in water at a concentration of 1 mg/ml and stored at −80 °C until used.

2.3. Immunization of mice

Female BALB/c (H-2d) mice, 5–7 weeks of age, were obtained from Shanghai Bikai Laboratory Animal Technology (Shanghai, China) and primed i.p. with 50 μg/mouse free peptide or lipopeptide three times at 7 day intervals. The rabies vaccine (vaccine efficacy >2.5 IU/dose) was purchased from Chengda Biotechnology Co. (Liaoning, China). Pre-immune and post-immune sera were collected weekly via the retro-orbital venous plexus. All mice were maintained under specific-pathogen-free conditions and treated according to the Guide for the Care and Use of Laboratory Animals of Fudan University.

2.4. BMDC culture

BM cells from BALB/c mice were cultured at a density of 2×10^6 cells/ml in dishes containing 10 ml complete RPMI-1640 medium (Gibco Life Technologies, UK) with 10 ng/ml mouse rGM-CSF and 1 ng/ml rIL-4 (PeproTech, Rocky Hill, USA). On Day 3, another 10 ml complete RPMI 1640 medium containing 10 ng/ml GM-CSF and 1 ng/ml rIL-4 was added. On Day 6, the cells were collected from each dish, washed and counted.

2.5. ELISPOT assay

Mouse IFN-γ and IL-4 ELISPOT kits (DAKEWE, China) were used for this assay. 14 days after the last injection, spleen cells were isolated and single cell suspensions were prepared. 10^6/well (100 μl) $CD8^+$ T cells were seeded into 96-wells plate and stimulated with 10 μl rabies vaccine, Pep A, or Lipo B, C, D (10 μM). After 48 h incubation in a humidified incubator at 37 °C and 5% CO_2, plates were washed and processed according to the manufacturer's protocol, spots were scanned and enumerated by SpotReader B1 (SLT Instruments, UK).

2.6. Internalization of peptides

BMDCs were incubated at 37 °C in complete RPMI-1640 medium with FITC-conjugated (Lipo-)peptides (10 μM). After 30 min, DCs were harvested and stained with PE-labeled anti-CD11c antibody (BioLegend, San Diego, USA) for 20 min after blocking the FcRs with an anti-mouse CD16/CD32 antibody (BD mouse Fc-block™). Surface-associated fluorescence was quenched by adding 0.1 M citrate buffer (pH 4.0) that contained 250 g/ml trypan blue (Sigma–Aldrich, St. Louis, MO). The samples were incubating for 1 min on ice before analysis (Song and Liu, 2015).The internalization of FITC-(lipo-) peptides by $CD11c^+$ cells was analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, USA).

Blocking experiments were performed by using anti-TLR2 mAb according to the study of Zhu et al. (Zhu et al., 2004). 10 μg/10^6 cells anti-TLR2 mAb (Abcam, Cambridge, UK) was added to DCs 45 min before the incubation of the FITC-(lipo-) peptides, the fluorescence was determined as described above.

2.7. Determination of DC maturation

1×10^6 DCs were treated with vaccine (0.1IU/10^6 cells) or (lipo-)peptides (1 μM) for 24 h and suspended in 100 μl PBS. Than incubated with 2 μg/ml anti-CD11c FITC, anti-CD40 PE, anti-CD80 PE or anti-CD86 PE Abs for 20 min on ice and washed twice with PBS. Flow cytometry was performed using FACSort (BD Biosciences, San Jose, CA), and data was analyzed using FlowJo software (Tree Star, San Caros, CA)

2.8. Confocal microscopy

To assess the internalization peptides/lipopeptides, BMDCs were seeded at a density of 1×10^5 cells/well into 12-well plates in 1.5 ml complete RPMI-1640 medium. Cells were incubated with FITC-labeled (lipo-)peptides (10 μM) for 0.5 h at 37 °C. After incubation, cells were fixed in 4% PFA (Sigma–Aldrich, St. Louis, MO). The cells were then visualized using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss AG, Germany).

Presentation and *in vitro* detection of peptide-specific $CD8^+$ T cells

Mice were vaccinated with two s.c. injections of rabies vaccine, Pep A, or Lipo B, C, D (30 μg) in IFA (Sigma–Aldrich, St. Louis, MO) one week apart. Spleens were harvested and the H–2 K^d-restricted CTL peptide ($G_{333-341}$, AYTIFNKTL) specific $CD8^+$ T cells were detected using a PE labeled $G_{333-341}$ tetramer (Kuangbo, China) and a FITC-labeled anti-CD8 mAb (BioLegend, San Diego, USA). The $G_{333-341}$-specific $CD8^+$ T cells were analyzed by flow cytometry.

2.9. Lymphocyte proliferation assay

Lymphocyte proliferation assay was performed as described previously (Hu et al., 2013). 2×10^5 cells were treated with 5 μg/ml ConA (Sigma–Aldrich, St. Louis, MO), PBS or 20 μg/ml (lipo-)peptides for nonspecific or specific stimulation. After 48 h incubation (5% CO_2, 37 °C), 10 μl CCK-8 solution (Beyotime Biotechnology, China) was added 4 h before the end of the incubation. The optical density was evaluated at 450 nm using SpectraMax M5 (Molecular Device, USA). Stimulation index = (Test OD- Blank OD)/(Negative OD-Blank OD). SI ≥ 2 indicated positive results.

2.10. Cytotoxicity assay

Specific cytotoxicity was determined based on CytoTox 96 Nonradioactive Cytotoxicity assay (Promega, Madison, USA). 2×10^4/well target cells (SP2/0) were treated with 2 μM (lipo-)peptides for 1 h and seeded into a 96 well plate. $CD8^+$ T were separated by microbead-labeled anti-CD8α and added at an effector: target cell ratio of 50:1 to 5:1. After 5 h at 37 °C, 50 μl of supernatants were assayed for LDH activity following the manufacturer's protocol. Target maximum release and spontaneous LDH release in effector and target cells were prepared as controls. The

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