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T cell responses to bluetongue virus are directed against multiple and identical CD4+ and CD8+ T cell epitopes from the VP7 core protein in mouse and sheep

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ABSTRACT

Bluetongue virus (BTV), an economically important *orbivirus* of the *Reoviridae* family, is a non-enveloped, dsRNA virus that causes a haemorrhagic disease mainly in sheep, but little is known of the cellular immunity elicited against BTV. We observed that vaccination of interferon type I-deficient mice (IFNAR$^{(-/-)}$), or inoculation of the wild type C57BL/6 strain with BTV-8, induced a strong T cell response. Therefore, we proceeded to identify some of the T cell epitopes targeted by the immune system. We selected, using H-2^{b}-binding predictive algorithms, 3 major histocompatibility complex (MHC)-class II-binding peptides and 7 MHC-class I binding peptides from the BTV-8 core protein VP7, as potential T cell epitopes. Peptide binding assays confirmed that all 7 MHC-class I predicted peptides bound MHC-class I molecules. Three MHC-class I and 2 MHC-class II binding peptide consistently elicited peptide-specific IFN-γ production (as measured by ELISPOT assays) in splenocytes from C57BL/6 BTV-8-inoculated mice and IFNAR$^{(-/-)}$-vaccinated mice. The functionality of these T cells was confirmed by proliferation and cytotoxicity assays. Flow cytometry analysis demonstrated that CD8+ T cells responded to MHC-class I binding peptides and CD4+ T cells to MHC-class II binding peptides. Importantly, these 5 epitopes were also able to induced IFN-γ production in sheep inoculated with BTV-8. Taken together, these data demonstrate the activation of BTV-specific T cells during infection and vaccination. The characterisation of these novel T cell epitopes may also provide an opportunity to develop DIVA-compliant vaccination approach to BTV encompassing a broad-spectrum of serotypes.

1. Introduction

Bluetongue virus (BTV) is a non-contagious, insect-transmitted viral pathogen of domestic and wild ruminants, considered one of the most economically important arbovirus [1]. The virus, for which 24 serotypes have been described so far, is transmitted to the vertebrate host by some species of biting midges from the *Culicoides* genus [2]. BTV is the prototype member of the *Orbivirus* genus of the *Reoviridae* family. This double-stranded RNA virus has a 10 segments genome that is enclosed by a complex capsid structure consisting in a nucleocapsid containing 5 proteins (major, VP3 and VP7; minor, VP1, VP4 and VP6). VP3 and VP7 are conserved proteins of hydrophobic nature, playing an important role in the structure integrity of the virus core [3]. VP1, VP4 and VP6 have RNA transcriptase- and RNA-modifying properties [4]. The outer capsid layer structure is composed of 2 additional major structural proteins (VP2 and VP5) [5–7]. VP2 is responsible for receptor binding, hemagglutination and eliciting serotype-specific neutralizing antibodies [8–10]. VP5 forms trimers in globular motifs of the outer layer of the BTV particle [11]. In addition to the structural proteins, 4 non-structural (NS) proteins NS1, NS2, NS3 and NS3A participate in the control of BTV replication, maturation and export from the infected cell [12,13].

BTV is capable of infecting cells of the immune system like monocytes [14], dendritic cells [15] and γδ T cells [16] and these are likely routes for dissemination of the virus within the host (and to the vector). Animals which recover from the disease develop a long-lasting immunity to the virus. Both neutralizing antibodies [17] and cytotoxic T lymphocytes (CTL) play a role in protective immunity to BTV [18], although cellular immunity is likely to be crucial as BTV protection can exist in the absence of neutralizing antibodies [19,20]. Moreover, adoptive transfer of lymphocytes could at least partially protect monozygotic sheep from subsequent BTV challenge [18]. Interestingly, BTV infection and vaccination induces CTLs in sheep capable of cross-reaction with different BTV serotypes [16,21–23]. Therefore vaccination designed to elicit CTL responses can potentially protect animals against a broad-spectrum of BTV serotypes.

Immunodominant serotype cross-reactive T-cell determinants have been located within the structural proteins of BTV-cores [24]. VP7 is a major BTV group reactive antigen [25] and sheep vaccinated with a capripox virus encoding VP7 showed clinical protection

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against heterotypic challenge, although the virus still replicated [26]. These data led us to define T cell epitopes in VP7. Thus, in the present report we identify novel CD4 and CD8 T cell epitopes recognized in mouse model from the VP7 core protein of BTV-8. Some of these epitopes are also recognized by T cells form infected sheep. This work underlines the necessity to further characterise anti-BTV immunity in order to develop more effective vaccinations.

2. Materials and methods

2.1. Virus

BTV stocks were prepared from infection of MC57 cells with multiplicity of infection (MOI) of 1. Virus titre was determined by standard plaque assays in Vero cell line. The virus (1×10^6 PFU prior to inactivation) was inactivated by incubation with freshly prepared 3 mM BEI for 24 h at 37 °C, and the reaction was stopped with 0.02 M sodium thiosulphate.

2.2. Mice, sheep and inoculations

C57BL/6 mice were purchased from Harlan Interfauna Ibérica SL and IFN-α/βR$^{0/0}$ (IFNAR$^{(-/-)}$) mice on a C57BL/6 genetic background were kindly provided by Professor R. Zinkernagel (Institute of Experimental Medicine, Zurich) and bred in our animal facility. C57BL/6 mice (8–12 week old) were infected with 100 PFU BTV-8 subcutaneously (s.c.) three times at 10–11 day intervals before sacrifice 3 days after the last infection. IFNAR$^{(-/-)}$ mice (8–16 week old) were inoculated s.c. twice with 100 μl of BEI-inactivated BTV-8 (equivalent to 1×10^6 PFU prior to inactivation) at a week interval and challenged s.c. with 10 PFU BTV-8 a week later. IFNAR$^{(-/-)}$ were sacrificed at day 6 post-challenge. Three month old female sheep (Mallorquina breed) were inoculated with 1×10^5 PFU BTV-8. All the procedures herein described were carried out under European Community guidelines and approved by the local ethical review committee.

2.3. Peptides

Peptides sequences from the VP7 core protein of BTV-8 (accession number: ACJ06230) were selected according to predictive algorithms and motifs for binding to H-2 D^b/K^b/A^b molecules available on the web [27–30]. VP7 peptides were purchased from Altabiosciences (Birmingham, UK). The gp(33–41) peptide (KAVYNFATC) from lymphocytic choriomeningitis virus (LCMV) known to bind D^b and K^b molecules was used as irrelevant peptide where mentioned.

2.4. Peptide binding assays

Peptide binding assay were performed following a modified methods from [31]. The transporter-associated protein-deficient RMA-S cell line was kindly provided by Dr McArdle (The Nottingham Trent University, UK). These cells were culture in complete RPMI + 10% FCS. For binding assays, $2–5 \times 10^5$ cells were incubated in serum-free RPMI for 1 h at 37 °C in the presence of varying concentration of peptides. Cells were stained with anti-D^b-FITC and anti-K^b antibodies or the appropriate isotype controls (eBiosciences). Cells were analysed on a BD FACSCalibur flow cytometer. The binding was calculated as the ratio of mean fluorescence intensity (MFI) for D^b or K^b molecules detected in the presence of peptide to the MFI detected in the absence of peptide. Data are presented as mean of 3 independent experiments.

2.5. Splenocyte cultures and IFN-γ ELISPOT assays

Splenocytes from infected C57BL/6 or vaccinated IFNAR$^{(-/-)}$ were obtained by mechanical disruption and cultured in T cell media (RPMI + 10%FCS + 4 mM L-glutamine + 10 mM HEPES + 1% 100X non-essential amino-acids + 1 mM sodium pyruvate + 100 U/ml penicillin/100 μg/ml streptomycin + 50 nM (-mercaptoethanol). IFN-γ ELISPOT assays were performed according to the manufacturer protocol (Diaclone, France). As negative control, cells were cultured either without stimuli, with DMSO (equivalent volume to that added with peptide), or with a lysate of the MC57 cell line (used in the virus preparation). All cultures were performed in triplicates and results are presented as average number of spots for each mouse. Assays were considered valid only when IFN-γ spot counts in control wells were below 20 for 2×10^5 cells, and standard deviations in positive wells below 10% of the average. A positive control of splenocytes activated with 0.5 μg/ml Concanavalin-A was always included to validate the ELISPOT assay.

2.6. Intracellular cytokine staining and flow cytometry

1×10^6 splenocytes were stimulated either with 20 μg/ml of peptide, BTV-8, BEI-BTV or the appropriate negative controls for 5 h (at 37 °C 5%CO_2) in U-bottom 96-well plates, in presence of 1 μg/ml brefeldin-A. Cells were harvested, washed twice with PBS + 1% FCS + 0.02% sodium azide and stained with anti-mouse CD4-FITC and anti-mouse CD8-PerCP antibodies (BD pharmingen). After washing cells were fixed and permeabilised with PBS containing 4% paraformaldehyde and 0.1% saponin. Cells were stained with anti-mouse IFN-γ-PE (BD pharmingen). The analyses was done on a BD FACSCalibur flow cytometer.

2.7. Proliferation assays

Splenocytes (2×10^5 per well) were plated in U-bottom 96-well plates in triplicate in presence of stimuli (either 20 μg/ml peptide, BEI-BTV or the appropriate negative controls). ^{3}H Thymidine (Hartmann Analytic, Germany) was added at a final concentration of 5 μCi/ml, and cells were cultured for 72–96 h at 37 °C 5%CO_2. Using a cell harvester, cells were transferred onto UniFilter-96 microplates (Perkin-Elmer) and counted on a 1450 MicroBeta Trilux counter (Perkin-Elmer).

2.8. Splenocyte restimulation, LPS blast generation and CTL assays

Splenocytes ($4–5 \times 10^6$ per well) were cultured for 5–6 days in 24-well plates in presence of 10 μg/ml of stimulating peptide in T cell media. These cells were used as effector cells in CTL assays. Depending on the experiment, RMA-S cells or syngeneic LPS blast were used as target cells. LPS blasts were generated from syngeneic splenocytes cultured at a density of 1.5×10^6/ml with 25 μg/ml LPS (Sigma) and 7 μg/ml Dextran Sulphate (Sigma) for 48 h [32]. Target cells (1×10^7) were harvested and labelled with an isotonic solution of $Na_2{}^{51}CrO_4$ (50 μCi) (Hartmann Analytic, Germany) for 1 h at 37 °C in serum-free RPMI. Target cells were then pulsed for 1 h at 37 °C, 5%CO_2 with 20 μg/ml VP7 peptide, irrelevant peptide gp(33–41) or BEI-BTV (equivalent to 1×10^5 PFU). Effector cells (E) and target cells (T) were plated in triplicates in U-bottom 96 well plates at different ratios (E:T) and incubated for 5 h at 37 °C, 5%CO_2. Maximum release was measured using the supernatants of target cells lysed with 0.1%SDS and spontaneous release by the