Vaccine 27 (2009) 5252–5258

Contents lists available at ScienceDirect

Vaccine

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

Effective CD8+ T cell priming and tumor protection by enterotoxin B subunit-conjugated peptides targeted to dendritic cells

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ARTICLE INFO

Article history:
Received 28 May 2009
Received in revised form 8 June 2009
Accepted 14 June 2009
Available online 2 July 2009

Keywords:
Dendritic cells
Cytotoxic T cells
Enterotoxins
MHC class I antigen presentation
Vaccination
Tumorimmunology

ABSTRACT

In our previous studies we have shown that bacterial enterotoxin B subunits are effective vehicles to deliver antigen into the MHC class I processing route. Here we have used the non-toxic *Escherichia coli* heat labile enterotoxin B subunit (EtxB) conjugated to OVA peptide (EtxB–peptide) to address the impact on induction of specific CD8+ T cells in vivo. Although incubation of DCs with these EtxB–peptide conjugates as such did not induce DC maturation in vitro MHC class I antigen presentation was much more efficient as compared to peptide alone. Antigen presentation was further enhanced upon DC maturation with the TLR-4 ligand LPS. Injection of matured DCs incubated with EtxB–peptide conjugates lead to strong induction of OVA-specific CD8+ T lymphocytes and fully prevented the outgrowth of lethal B16 melanoma in wild type mice. Our data demonstrate that bacterial non-toxic B subunit–peptide conjugates are potent vaccine vehicles for induction of protective CD8+ T cell responses.

1. Introduction

Dendritic cell (DC) based vaccines are increasingly used for immunotherapy [1,2]. Choice of antigen source, use of adjuvant (maturation of DC) and optimal route of antigen delivery are important parameters for designing effective cancer vaccines. In order to induce efficient CD8+ T lymphocyte responses, optimal antigen targeting into the cytosol for major histocompatibility complex (MHC) class I route is required.

CD8+ T lymphocytes recognize peptides derived from pathogens, pathogen infected cells or tumor cells in the context of MHC class I. Professional antigen presenting cells (APCs), such as DCs are specialized in the uptake and processing of such antigens (Ags). After internalization, these Ags are processed into peptide fragments in the cytosol by a protease complex, named proteasome. Hereafter, the peptides are translocated into the endoplasmic reticulum (ER) via the Transporter Associated with antigen Processing (TAP) where the peptides are loaded on newly synthesized MHC class I molecules. Subsequently, these peptide/MHC class I complexes are transported to the cell surface for recognition by cytotoxic T lymphocytes (CTLs).

Immature DCs are efficient in the uptake and processing of antigens for the MHC class I pathway. In order to induce an effective CTL response DCs need to be fully activated and thereby expressing high levels of MHC class I molecules and various costimulatory molecules [3] We and others have previous shown that coupling of peptides to bacterial toxins is an efficient strategy to target peptides to DCs for MHC class I presentation [4,5]. Non-toxic GM1-binding B subunits of *Escherichia coli* heat-labile toxin (EtxB) are highly efficient in delivering conjugated peptides into the class I processing pathway [4]. Epitope presentation by MHC class I molecules was found to require conjugation of peptides to EtxB and binding of these EtxB–peptide conjugates to GM1 ganglioside receptors. Furthermore, the efficiency of epitope presentation is significantly enhanced by the incorporation of a sequence derived from the DNA polymerase of herpes simplex virus type 1. This 10-amino acid stretch of non-polar and charged residues, termed the Pol-loop segment, has been proposed to have an intrinsic tendency to insert into lipid bilayers, thereby enhancing translocation [4,6]. In addition, we have performed an in vitro study on the trafficking and processing of the EtxB–peptide conjugate into the MHC class I pathway in a dendritic cell line [5].

In our the current study we have investigated whether efficient delivery of conjugated synthetic peptides (comprising the

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doi:10.1016/j.vaccine.2009.06.053

ovalbumin (OVA) CTL epitope sequence; SIINFEKL) into mature DC will lead to induction of $CD8^+$ T lymphocytes in vivo and whether this vaccine can protect against melanoma. Our results demonstrate that although EtxB-OVA peptide conjugates do not mature DCs the peptide conjugate is efficiently taken up and the OVA epitope is significantly better processed and presented in MHC class I than unconjugated peptide. Injection of mature DCs incubated with EtxB19mer conjugates lead to high induction of OVA-specific $CD8^+$ T lymphocytes. This vaccine is capable of protecting mice against a lethal tumor challenge with B16 melanoma. Thus, improved targeting of exogenous peptides via non-toxic bacterial B subunits into the MHC class I pathway of DCs elicits effective $CD8^+$ T lymphocyte responses in vivo.

2. Materials and methods

2.1. Mice

Female C57BL/6Kh (B6, H-2^b) mice were kept at the LUMC animal facility and used at 6–10 weeks of age in accordance with national legislation and under supervision of the animal experimental committee of the University of Leiden.

2.2. Cell line and, reagents

Freshly isolated DCs were cultured from bone marrow cells of C57BL/6 as described [7].

The D1 dendritic cell line, a long-term growth factor-dependent immature splenic DC cell line derived from C57BL/6 (H-2^b) mice, was cultured as described [8], except that glutamine addition to the medium is replaced by glutamax (Gibco, Invitrogen Life Technologies, ref. No. 35050-038). D1 cells behave in a similar way as freshly isolated bone marrow derived DCs [9].

DCs were matured as described [9] by incubation with 10 μg/ml LPS of *Escherichia coli* (Sigma–Aldrich, serotype 026:B6) for 48 h at 37 °C or left untreated. Cells were detached by 2 mM EDTA in PBS, collected and used for experiments.

B3Z is a T cell hybridoma specific for $OVA_{257–264}$ SIINFEKL in H-$2K^b$, which carries a β-galactosidase construct driven by NF-AT elements of the IL-2 promoter [10].

EG7 is an ovalbumin (OVA)-transfected subclone of EL-4, which is a C57BL/6 thymoma cell line.

B16-OVA, a murine melanoma cell line, which is transfected with ovalbumin.

Cell lines were cultured in IMDM (BioWhittaker, Verviers, Belgium) supplemented with 8% heat-inactivated FCS (Greiner, Alphen, The Netherlands), 100 IU/ml penicillin/streptavidin, 2 mM L-glutamine, and 20 μM 2-ME. The EtxB–peptide conjugates were prepared exactly as described [4]. Toxicity of the EtxB conjugate was evaluated by trypan blue or propidium iodide staining and the Nicoletti assay [11]. Briefly, DCs were incubated with LPS, or EtxB19mer for 24 h or exposed to UV for 90 s, followed by 24 h incubation. Cell death induced by the individual treatment was measured by flow cytometry.

2.3. MHC class I-restricted antigen presentation

DCs were either left immature or matured with 10 μg/ml LPS. After 48 h the cells were incubated with either 20 nM of the $OVA_{257–264}$ SIINFEKL (OVA8mer) or an OVA19mer peptide CAVGAGATAEESIINFEKL or EtxB conjugated to the OVA19mer peptide (EtxB19mer conjugate). After 2 h at 37 °C, the cells were extensively washed and plated in different densities for CTL recognition assays or injected into naïve C57BL/6 mice. For CTL recognition assays 5×10^4 B3Z T cells were added to each well and incubated for 24 h at 37 °C. Presentation of OVA8mer in H-$2K^b$ was measured by the activation of B3Z cells, measured by a colorimetric assay using chlorophenol red-β-D-galactopyranoside (CPRG) as substrate to detect *lacZ* activity in B3Z lysates.

2.4. Cell surface immunofluorescence

The following antibodies (Abs) were purchased from BD Pharmingen (San Diego, CA): FITC-coupled anti-MHC class I K^b Ab, FITC-coupled anti-CD86/B7.2 Ab, PE-coupled MHC class II I-A^b Ab, PE-coupled CD40 Ab and PercP-coupled anti-CD8α Ab. DCs were incubated with Abs in the presence of 30% 2.4G2 supernatant (rat anti-mouse FcγRIII/II) to block FcR binding. APC-conjugated $OVA_{257–264}$-loaded H-$2K^b$ tetramers were prepared as described [12]. Staining for $OVA_{257–264}$-loaded H-$2K^b$ tetramers and PercP-coupled anti-CD8α Ab was conducted on ice for 30 min. Propidium Iodide was added to exclude dead cells. Stained cells were analyzed using a FACScan flow cytometer equipped with CellQuest software (BD Biosciences, Mountain View, CA).

2.5. Induction of specific $CD8^+$ T cell response in vivo

To induce CTL responses in vivo, untreated DCs or DCs treated for 48 h with 10 μg/ml LPS were incubated with either OVA19mer peptide (20 nM) or EtxB19mer conjugate (20 nM) for 2 h at 37 °C. Subsequently, cells were washed five times and 1×10^6 DCs were injected intravenously into C57BL/6 mice in phosphate buffered saline (PBS) solution with 0.1% bovine serum albumin (BSA). After 14 days, spleen cells (5×10^6 per well) were restimulated with mitomycin-treated (1 h), irradiated (2500 rad/min) EG7 (5×10^5 per well) in 1 ml cultures in 24 wells plates in the absence of additional cytokines. After 7 days viable splenocytes were isolated over a ficoll gradient and stained for H-$2K^b$ Tetramer (TM)-$OVA_{257–264}$ and CD8b2 (clone 53-5.8), and propidium iodide to exclude dead cells.

2.6. ELISPOT

Briefly, 96-well nitrocellulose plates were coated overnight with 5 μg rat anti-mouse IFN-γ mab. The plates were then washed and unoccupied sites blocked with medium for 1 h at 37 °C. An aliquot of spleen cells after re-stimulation and ficoll purification (see above) were plated into the precoated 96-well plates and cells were subjected to stimulation in vitro with or without 1 μg/ml $OVA_{257–264}$ peptide (H2-Kb restricted SIINFEKL) overnight. After 24 h incubation at 37 °C in 5% CO_2 cells were removed from the ELISPOT plates by washing four times with PBS + 0.05% Tween 20. Wells were incubated for 60 min at RT with 0.1 μg rat anti mouse IFN-γ-biotinylated mAb. After washing four times with PBS + 0.05% Tween 20, 100 μl/well of extravidine–alkaline phosphatase (BioRad, München, Germany) was added for 60 min at room temperature. After another washing step with PBS + 0.05% Tween 20 BCIP/NBT substrate was added for 20–30 min. Color development was stopped by washing under running tap water. After drying overnight at room temperature colored spots were counted using an automated image analysis system ELISPOT reader.

2.7. Immunization with Ag loaded DC followed by tumor challenge

DCs treated for 48 h with 10 μg/ml LPS were incubated with either 5 or 20 nM of OVA8mer peptide, OVA19mer peptide or EtxB19mer conjugate for 2 h at 37 °C. Subsequently, cells were washed five times and 1×10^6 DCs were injected intravenously into C57BL/6 mice in PBS with 0.1% BSA. Fourteen days after immunization, mice were challenged subcutaneously with 8×10^4 B16-OVA