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Photosensitisation facilitates cross-priming of adjuvant-free protein vaccines and stimulation of tumour-suppressing CD8 T cells

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ABSTRACT

Cancer vaccines aim to induce CD8 T cells infiltrating the tumour. For protein-based vaccines, the main biological barrier to overcome is the default MHC class-II-pathway, with activation of CD4 T cells rather than CD8 T cells. The latter requires antigens to access the cytosol and MHC class I antigen presentation. We applied photosensitiser and light to trigger disruption of antigen-containing endosomes and thereby MHC class I cross-presentation of a model cancer vaccine. This "photochemical internalisation" resulted in activation, proliferation, and IFN-γ production of cytotoxic CD8 T cells, which suppressed tumour growth by infiltrating CD8 T cells and caspase-3-dependent apoptosis. The process was independent of MHC class II, MyD88, and TLR4 signalling, but dependent on trypsin- and caspase-like proteasome activity and partly also on chloroquine. This novel method of vaccination may find applications in cancer immunotherapy where the activation of CD8 T cells is important.

1. Introduction

Skin malignancies are candidate indications for tumour vaccines. In particular, the poor prognosis of metastatic melanoma patients [1,2] makes the search for new and improved therapeutic measures important for the patients, but challenging for researchers and clinicians. As melanoma can be resistant to chemotherapy and targeted drug therapies, adoptive immunotherapy and cancer vaccination may offer attractive and complementary treatment strategies [3,4]. Therapeutic vaccination is however challenging because tumours generate an immunosuppressive microenvironment for instance through down-regulation of p53 and other tumour-suppressors [5]. Nonetheless, the potential beneficial effect of cancer vaccines has been demonstrated in clinical trials [6], and the adoptive transfer of CD8-positive tumour infiltrating lymphocytes (TILs) in melanoma patients has showed promising treatment benefits [7–10]. Stimulation of tumour-specific CD8 T cells has also been the aim of peptide vaccines or autologous dendritic cells (DCs) treated ex vivo with melanoma antigens [11–14]. However, vaccination strategies often failed because of inappropriate antigen processing [15–17]. By a default mechanism, exogenous proteins are processed by the MHC class-II presentation pathway leading to activation of CD4 T cells. Stimulation of CD8 T cells requires antigen access to the MHC class-I pathway via cytosol. Hence, technologies that enable translocation of protein vaccines through the cell plasma membrane of APCs, or that enable antigen release from endosomes/phagosomes may permit or enhance stimulation of tumour-specific CD8 T-cell responses.

One popular method of targeting HIV antigens to cytosol has been to use adenoviruses, [18,19]. However, early enthusiasm for adenoviruses lessened after reported toxicity and even one death [20,21] as well as failures to meet expectations in clinical trials [22,23]. We recently demonstrated that properties of photosensitisers may be utilised to shuffle antigen into the cytosol of antigen-presenting cells (APCs) [24]. A photosensitiser was combined with a protein antigen to pulse DCs in vitro. The photosensitiser, due to its high affinity for cell membranes, was translocated to endosomes upon endocytosis of the antigen. After light excitation of the cells, photochemical disruption of the endosomal membrane leads to cytosolic release or "photochemical internalisation" (PCI) of the antigen and subsequently to MHC-class I presentation. In the current study, mice were directly immunised with protein antigen and a photosensitiser. While the previous study demonstrated that this novel technology of controlled vaccine delivery can cause stimulation of strong CD8 T cell responses (ref. [24]), the current study further investigate potential mechanisms by which such immune stimulation is mediated, namely, that light activation of the photosensitiser causes immediate and quantitative release of the antigen into cytosol, that the released antigen is processed by trypsin- and caspase-like proteasomes and presented in a TAP-dependent manner to CD8 T cells, independent on MHC class II, MyD88 and TLR4 signalling. Most importantly, we demonstrate that the PCI-based vaccination can therapeutically suppress existing melanoma tumour in mice by stimulating infiltration of specific CD8 T cell into the tumour parenchyma.

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2. Materials and methods

2.1. Animals

C57BL/6 mice were purchased from Harlan (Horst, The Netherlands). CD8 T-cell receptor transgenic OT-I mice (B6.129S6-*Rag2*^{tm1Fwa} Tg(TcraTcrb)1100Mjb) from Taconic Europe (Ry, Denmark) and MHC class II-deficient mice (B6.129S2-H2$^{dlAb1\text{-}Ea}$/J) from Jackson Laboratories (Bar Harbor, Maine) were bred in own specific pathogen-free (SPF) facilities at the University of Zurich; the OT-I CD8 T cells recognise the H-2K^{b}-restricted epitope SIINFEKL from ovalbumin (OVA, aa257-264). Myd88-deficient B6-MyD88^{tm1Aki} and TLR4-deficient B6-TRL4^{tm1Aki} mice were from The Swiss Immunological Mutant Mouse Repository (SwIMMR). All mice were kept under SPF conditions, and the procedures performed were approved by Swiss veterinary authorities (licence 69/2012).

2.2. Materials and cells

Chicken OVA was purchased from Sigma-Aldrich (Buchs, Switzerland) and the SIINFEKL peptide from EMC microcollections (Tuebingen, Germany). The photosensitiser tetraphenyl chlorine disulfonate (TPCS2a) was from PCI Biotech (Lysaker, Norway). OVA and TPCS2a were mixed in PBS, kept light protected, and administered to mice within 60 min of preparation. TPCS2a was activated by illumination at 435 nm with LumiSource™ (PCI Biotech) as described [24,25]. B16.F10 melanoma cells (ATCC® CRL-6322™), originally from C57BL/6 mice, were used to make a stable transfectant that expressed the whole OVA antigen (hereafter, B16-OVA).

2.3. Antigen presentation assay with dendritic cells in vitro

DCs were prepared from mouse bone marrow cells that were cultured in medium supplemented with 10% FCS, glutamine, sodium pyruvate, penicillin and streptomycin in the presence of 60 ng/ml GM-CSF. After six days, the loosely adherent DCs were harvested, washed and plated at 100,000 cell per well on flat-bottom 96-well culture plates. The DCs were then incubated 18 h with TPCS2a before they were added inhibitors of antigen presentation, 10 μM epoxomicin, 5 μM lactacystin, 2 μM brefeldin A, 10 μM leupeptin and 100 μM chloroquine (Sigma-Aldrich). After 2 h of incubation at 37 °C, the cells were washed and incubated with 0.5 μg/ml OVA for another 4 h. The DCs were then washed and illuminated with various light doses using the LumiSource®. The DC cultures were then added 100,000 OT-I cells per well and incubated for 24 h before analysis of IFN-γ into the supernatants.

2.4. Intradermal photosensitisation and immunisation of mice

C57BL/6 mice were immunised at 5–10 weeks of age as previously described [25]. One day prior to immunisation, 10,000 (for tumour experiments) or 2×10^6 (for immunological experiments) OT-I spleen and lymph node cells were administered by intravenous injection into recipient C57BL/6 mice. The next day, the fur was shaven off the abdominal region and 100 μl of the vaccine preparations was injected intradermally. The doses of OVA and TPCS2a were 10 μg and 100 μg, respectively. After 18 h, the mice were anaesthetised with intraperitoneal ketamine and xylazine and were placed on the light source for six minutes illumination (4.86 J/cm^2) to activate TPCS2a.

2.5. Analysis of immune responses by flow cytometry and ELISA

The frequency of antigen-specific CD8 T cells was monitored in blood and spleen by flow cytometry using H-2K^{b}/SIINFEKL Pro5 pentamer (Proimmune, Oxford, UK). Cell-surface expression of CD4, CD8, and CD44 and intracellular production of IFN-γ was analysed flow cytometry after Fc-receptor blocking with anti-CD16/32. The intracellular staining was after overnight incubation at 37 °C with 0.1 μg SIINFEKL. Brefeldin A (2.5 μg/ml) was added during the last 4 h. The cells were fixed with 4% formaldehyde for 10 min, permeabilised in 0.1% Nonidet P40 from Roche (Rotkreuz, Switzerland) for 3 min, and stained with anti-IFN-γ for 35 min. All stainings were performed at 4 °C and all steps intercepted by washing in PBS/FCS 2%. FACS antibodies were from eBioscience (Vienna, Austria) or BD Pharmingen (Basel, Switzerland). Acquisition was done on FACSCanto (BD Biosciences, San Jose, USA) and data were analysed with FlowJo 8.5.2 (Tree Star, Inc., Ashland, OR). For the analysis of cytokine secretion by ELISA, 2×10^5 splenocytes were re-stimulated in round-bottom 96-well plates with 0.1 μg SIINFEKL. Supernatants were collected after 24–72 h and analysed using cytokine ELISA kits for IL-2 and IFN-γ (eBioscience).

2.6. Analysis of in vivo cytotoxicity

Spleen cells were harvested from naïve C57BL/6 mice and pulsed with 10 μg/ml SIINFEKL (37 °C, 60 min) or left unpulsed. The SIINFEKL-pulsed cells were then labelled with a high concentration of 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; 37 °C, 15 min), while the non-pulsed cells were similarly labelled with a low concentration of CFSE (0.5 μM). Cells were washed with PBS and 5×10^6 cells from each population were injected i.v. in vaccinated and control mice. After 18 h, the mice were euthanized and the splenocytes harvested, stained with PE-labelled anti-CD4 and anti-CD8 antibodies and analysed by flow cytometry. Specific lysis or cytotoxicity was calculated for the T cell population and the non-T cell population using the following formula: percentage specific cytotoxicity = 100 − [100 × (CFSElow / CFSEhigh)].

2.7. Fluorescence microscopy of cytosolic antigen release

C57BL/6 bone-marrow cells were cultured in GM-CSF-containing medium for preparation of DCs as described [24]. The DCs were incubated with OVA-Alexa Fluor® 488 Conjugate (Life Technologies, Zug Switzerland) and TPCS2a as described below and washed with Ca^{2+}- and Mg^{2+}-containing PBS prior to epi-fluorescence microscopy using a 63× oil-immersion objective on an AxioImager™ Z1 Microscope (Carl Zeiss, Oberkochen, Germany). A 450–490 nm band pass excitation filter, a 495 nm dichroic mirror and a 500–550 nm band pass emission filter were used for the measurement of the OVA-Alexa Fluor® 488. TPCS2a fluorescence was recorded using a 395–440 nm excitation filter, with a 460 nm beam splitter and a 610 nm pass filter. All micrographs were processed with AxioVision™ software (Carl Zeiss).

2.8. Prophylactic vaccination against intradermal melanoma and monitoring of tumour growth and metastatic potential

C57BL/6 mice received an adoptive transfer of 10,000 OT-I cells (i.v.) one day before vaccination with OVA or OVA-PCI as described above. After 4–5 days, the mice were challenged with 500,000 B16-OVA-melanoma cells. The cells were injected intradermally into one of the flanks. The tumour growth was monitored by measuring the size of the B16 neoplasm with a calliper. The tumour volume was calculated using of the modified ellipsoid formula: (length × width^2)/2. For analysis of the metastatic potential, C57BL/6 mice were vaccinated as above with OVA or OVA-PCI, but challenged four days later with 500,000 B16-OVA cells given intravenously. On day 19, mice were euthanized and spleens and lungs were harvested. The lungs were analysed by counting melanoma spots as a measure for metastasis. Spleen cells were analysed by flow cytometry for CD8 T-cell activation as described above.