Vaccine (2008) **26**, 1626–1637

available at www.sciencedirect.com

ScienceDirect

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

TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses

Eva Schlosser[a], Marc Mueller[a], Stefan Fischer[b], Sameh Basta[c], Dirk H. Busch[d,e], Bruno Gander[b], Marcus Groettrup[a,f,*]

[a] *Division of Immunology, Department of Biology, D-78457 Konstanz, Germany*
[b] *Institute of Pharmaceutical Sciences, ETH Zürich, CH-8093 Zürich, Switzerland*
[c] *Department of Microbiology & Immunology, Queen's University, Kingston, K7L 3N6 Canada*
[d] *Institute for Medical Microbiology, Immunology, and Hygiene, Technical University Munich, 81675 Munich, Germany*
[e] *Clinical Cooperation Group Immune Monitoring GSF National, GSF Institute of Health and Environment/Neuherberg and Technical University Munich, Munich, Germany*
[f] *Biotechnology Institute Thurgau, CH-8274 Tägerwilen, Switzerland*

Received 14 March 2007; received in revised form 17 December 2007; accepted 17 January 2008
Available online 6 February 2008

KEYWORDS
Biodegradable microspheres;
Viral infection;
Cytotoxic T cells

Summary Dendritic cells phagocytose pathogens leading to maturation and cross-presentation on MHC class I. We found that the efficiency of cross-priming in mice after vaccination with biodegradable poly(D,L-lactide-*co*-glycolide) microspheres (MSs) was enhanced when ovalbumin was coencapsulated together with either a CpG oligonucleotide or polyI:C as compared to co-inoculation of ovalbumin-bearing MS with soluble or separately encapsulated adjuvants. A single immunization with MS containing coencaspsulated CpG and ovalbumin yielded 9% SIINFEKL/H-2K^b tetramer positive CTLs, production of IFN-γ, efficient cytolysis, and protection from vaccinia virus infection. Taken together, coencapsulation of adjuvant and antigen is an important paradigm for the generation of potent CTL responses.

Abbreviations: BMDC, bone marrow-derived dendritic cells; DCs, dendritic cells; MS, microspheres; OVA, ovalbumin; PAMP, pathogen-associated molecular pattern; PLGA, poly(D,L-lactide-*co*-glycolide).

* Corresponding author at: Lehrstuhl Immunologie, Universität Konstanz, Universitätsstrasse 10, D-78457 Konstanz, Germany. Tel.: +49 7531 882130; fax: +49 7531 883102.
E-mail address: Marcus.Groettrup@uni-konstanz.de (M. Groettrup).

0264-410X/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2008.01.030

Introduction

Dendritic cells (DCs) function as master switches that control whether the consequence of antigen encounter is tolerance induction or the stimulation of a cellular immune response [1]. In their immature state, DC reside in peripheral tissues to ''sample'' the environment by pinocytosis or phagocytic uptake of pathogens. The consequence of engulfing bacterial or viral particles and their disassembly in phagosomes are twofold. On the one hand antigenic peptides are generated and delivered to MHC class I and II molecules, on the other hand DC receive a maturation signal via TLRs which are stimulated by pathogen-associated molecular pattern (PAMP) molecules [2]. Mature DC then migrate to secondary lymphoid organs, where the acquired material is either presented directly on MHC class II molecules, thus triggering T cell help, or cross-presented to $CD8^+$ T cells on MHC-I [3–5]. In this way all systemically and peripherally expressed antigens can be presented to T cells. Nevertheless, this mechanism implies that self-antigens are presented on dendritic cells, which potentially leads to activation of silent autoreactive T cells finally leading to autoimmunity [6]. To prevent this, dendritic cells only get ''licensed'' to induce activation of T cells in the presence of TLR ligation.

Recently, Blander and Medzhitov [7,8] have addressed the question how it can be avoided that DC that simultaneously pick up self-antigens and pathogens activate self-reactive T cells. They found that only those phagosomes contributed effectively to presentation on MHC class II molecules *in vitro* which had endocytosed particulate cargo that contained both the TLR4 ligand LPS and the model antigen ovalbumin. Moreover, it was recently shown that the T helper response to the TLR11 ligand profilin from the parasite *Toxoplasma gondii* relied on TLR signal transduction by DCs suggesting that the physical association of TLR ligand and antigen is relevant *in vivo* [9]. These results have important consequences for the design of microparticulate vaccines especially if they can be extended to cross-priming of CTLs.

We and others have explored biodegradable microspheres (MSs) composed of poly(D,L-lactide-*co*-glycolide) (PLGA) as antigen delivery devices for macrophages and dendritic cells [10]. PLGA–MS of about 0.5–5 μm in diameter are actively phagocytosed by human and murine DC and can be used for the encapsulation of DNA, RNA, peptides or proteins. The PLGA polymer hydrolyzes slowly in aqueous environments [11], and releases encapsulated peptides and proteins into the processing pathways for presentation on MHC class I and II [12–14]. While pivotal biological properties of DC like cytokine secretion, migration, and T cell stimulation were not altered by the uptake of PLGA–MS, it became also evident that PLGA–MS by themselves do not trigger the maturation of DC [15]. However, the differentiation of immature human DC that had phagocytosed PLGA–MS occurred normally upon subsequent stimulation with TLR ligand or proinflammatory cytokines.

The overall goal of our work is the optimization of the parameters for subcutaneous vaccination with PLGA–MS for the generation of antigen-specific CTL responses *in vivo*. In this study, we first realized that the effect of polyI:C and CpG oligonucleotides was more prominent when these adjuvants were coinjected in microencapsulated rather than soluble form. Even better results, however, were consistently achieved when antigen and adjuvant were not separately microencapsulated in different MS and coinjected but when they were coencapsulated into the same microparticles. This procedure yielded unprecedented potent CTL responses upon a single injection and demonstrates that the principle described by Blander and Medzhitov for class II presentation *in vitro* can be extended to cross-priming of $CD8^+$ T cells *in vivo*.

Materials and methods

Preparation of microspheres (MS)

MS were prepared from 14 kDa PLGA 50:50 carrying hydroxyl- and carboxyl-end groups (Resomer RG502H, Boehringer Ingelheim, Ingelheim, Germany). The antigens and TLR ligands were microencapsulated by spray drying as described elsewhere [16]. Briefly, ovalbumin (Grade V, Sigma) and/or CpG oligonucleotide with a phosphothioate backbone (1826, Microsynth, Balgach, Switzerland) and/or polyI:C (Calbiochem, VWR, Dietikon, Switzerland) were dissolved in 0.5 ml aqueous medium (aqueous phase) and mixed with 1 g of PLGA dissolved in 20 ml of either dichloromethane or ethyl acetate (organic phase). For individual microencapsulation of OVA, CpG or polyI:C, 50 mg ovalbumin were dissolved in 0.5 ml H_2O and 1 g PLGA in 20 ml dichloromethane, or 5 mg of CpG or polyI:C were dissolved in 0.5 ml 0.1 M $NaHCO_3$ and 1 g PLGA in 20 ml ethyl formate. Coencapsulation of OVA with either of the two adjuvants was performed by co-dissolving 50 mg OVA and 5 mg adjuvant in 0.5 ml of 0.1 M $NaHCO_3$ and 1 g PLGA in 20 ml dichloromethane. The aqueous and organic phases were homogenized under ultrasonication (Hielscher, UP200 H, Ampl. 40%) for 10 s on ice. The obtained w/o-dispersion was spray-dried (Mini Spray-Dryer 191, Büchi, CH-Flawil) at a flow rate of 2 ml/min and inlet/outlet temperatures of 40/37 °C. The obtained MS were washed out of the spray-dryer's cyclone with 0.1% poloxamer 188 (Pluronic®F68, BASF), collected on a cellulose acetate membrane filter and dried under reduced pressure (20 mbar) for 18 h. Before use, indicated amounts of MS were dispersed in indicated aqueous media by ultrasonication for 1 min in order to obtain a homogenous MS suspension.

Release of antigen and TLR ligands from MS

Amounts of 10 mg of MS, accurately weighed, were suspended in 1 ml phosphate buffered saline (PBS; pH 7.4) and kept at 37 °C under mild rotational movement. After 3 days of incubation, the MS suspension was centrifuged, and the supernatant analysed for OVA and CpG. The OVA was assayed fluorimetrically ($\lambda_{ex}/\lambda_{em}$ = 280/334 nm; slits: 10 nm; Cary Eclipse, Varian Optical Spectroscopy Instruments, Mulgrave, Victoria, Australia), and the CpG with the Quant-iT™ Oligreen® ssDNA reagent (Invitrogen/Molecular Probes, Eugene, Oregon, USA) according to the manufacturer's protocol using a 96-well microplate fluorometer FluoroCount™ (Packard Instruments).

In order to determine the total amount of ovalbumin encapsulated into PLGA–MS, microspheres were incubated