Biomaterials 57 (2015) 153–160

Contents lists available at ScienceDirect

Biomaterials

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

Long-term persistence of immunity induced by OVA-coupled gas-filled microbubble vaccination partially protects mice against infection by OVA-expressing *Listeria*

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ARTICLE INFO

Article history:
Received 5 February 2015
Received in revised form
2 April 2015
Accepted 3 April 2015
Available online 28 April 2015

Keywords:
Vaccine
Memory
Antibody
T cells
Bacterial infection
Protection

ABSTRACT

Vaccination aims at generating memory immune responses able to protect individuals against pathogenic challenges over long periods of time. Subunit vaccine formulations based on safe, but poorly immunogenic, antigenic entities must be combined with adjuvant molecules to make them efficient against infections. We have previously shown that gas-filled microbubbles (MB) are potent antigen-delivery systems. This study compares the ability of various ovalbumin-associated MB (OVA-MB) formulations to induce antigen-specific memory immune responses and evaluates long-term protection toward bacterial infections. When initially testing dendritic cells reactivity to MB constituents, palmitic acid exhibited the highest degree of activation. Subcutaneous immunization of naïve wild-type mice with the OVA-MB formulation comprising the highest palmitic acid content and devoid of PEG2000 was found to trigger the more pronounced Th1-type response, as reflected by robust IFN-γ and IL-2 production. Both T cell and antibody responses persisted for at least 6 months after immunization. At that time, systemic infection with OVA-expressing *Listeria monocytgenes* was performed. Partial protection of vaccinated mice was demonstrated by reduction of the bacterial load in both the spleen and liver. We conclude that antigen-bound MB exhibit promising properties as a vaccine candidate ensuring prolonged maintenance of protective immunity.

1. Introduction

Vaccination is one of the most successful medical procedure to fight against pathogen infections and has allowed to drastically reduce several life-threatening conditions in humans [1]. It consists in the prophylactic induction and harnessing of pathogen-specific immunity that has to be ideally protective for decades. Depending on the target, vaccination relies on the generation of memory humoral and/or cellular immune responses with powerful neutralizing/effector functions. When available, vaccines based on the administration of live attenuated or killed/inactivated microorganisms have demonstrated the best efficacy to induce long-lived protective immunity, but their systematic use is questioned mainly due to safety issues.

Novel strategies to design safe and efficient vaccines have been explored and include the use of either RNA/DNA coding for pathogen-derived antigens (Ag) or purified/recombinant antigens (subunit vaccines). Because of their intrinsic lack of immunogenicity, subunit vaccines need to be combined with immunostimulatory adjuvants which stimulate both innate and adaptive immunity [2]. For example, administration of antigens with TLR ligands, such as MPLA, CpG or Imiquimod, has proven efficient in the induction of robust protective immunity in animal models [3] and is currently evaluated in human clinical trials. An alternative developing strategy is the formulation of antigens with nano-/microparticles, which mimic in size and/or structure microorganisms and serve as carriers to target antigen-presenting cells (APC), such

Abbreviations: Ab, antibody; Ag, antigen; DC, dendritic cell; DPPE-MPB, maleimidophenyl-butyryl-dipalmitoyl-phosphatidylethanolamine; DSPC, distearoyl-phosphatidyl-choline; DSPE, distearoyl-phosphatidyl-ethanolamine; ICS, intracellular cytokine staining; LN, lymph node; MB, gas-filled microbubbles; OVA-MB, OVA covalently linked to MB; OVA-*Lm*, OVA-expressing *Listeria monocytogenes*; PA, palmitic acid; PEG, polyethylene glycol; SN, supernatant.

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http://dx.doi.org/10.1016/j.biomaterials.2015.04.008
0142-9612/

as dendritic cells (DC) and macrophages [4]. Such structures may also expose repeated antigenic copies to B cells and mediate their efficient activation followed by the production of important amounts of Ab [5]. Particles can be immunogenic by themselves or are designed to combine adjuvant molecules for optimal efficacy, especially when aiming at inducing T cell responses [6–8]. In addition to canonical alum, biodegradable formulations including polymer-based nano-/microparticles, virosomes or lipid-based nano-/microparticles (e.g. liposomes or gas-filled microbubbles (MB)), are theoretically suitable for the design of vaccines for administration in humans [9,10].

MB are microparticles composed of a lipid monolayer entrapping a heavy gas, and routinely serve as ultrasound-responsive contrast agents in the clinic. Their use in the delivery of DNA/RNA/protein by ultrasound-mediated sonoporation has been reported in *in vitro* and *in vivo* studies [11]. MB composed of palmitic acid (PA), distearoyl-phosphatidyl-choline (DSPC) and distearoyl-phosphatidyl-ethanolamine (DSPE)-PEG2000-maleimide, serving as a linker for covalent attachment of the model Ag OVA, are able to induce protective immunity in mice against a systemic bacterial infection taking place shortly after prophylactic subcutaneous (s.c.) vaccination [10]. This occurred without the need for ultrasound application, did not require the addition of adjuvant molecules, and promoted robust specific Ab, CD4 and CD8 T cell responses after prime-boost parenteral administration [12,13].

Based on these initial results, we reasoned that tailored modifications in the structure of MB may lead to the design of more potent vaccine formulations. Comparison of the original preparation with formulations carrying increased percentage of PA and lacking PEG2000 was performed to assess establishment of long-lasting adaptive immunity in vaccinated mice. Efficiency of protection at 6 months was evaluated upon systemic challenge with lethal doses of OVA-expressing *Listeria monocytogenes*.

2. Materials and methods

2.1. Preparation of gas-filled microbubbles

OVA covalently linked to MB (OVA-MB) via a stable thio-ester bond were prepared as described [12] with minor modifications. The 4 formulations tested in the current study are described in Table 1. Phospholipids and derivatives were purchased from Avanti Polar Lipids (Alabaster, AL), except PA (Fluka, Buchs, Switzerland). Physical characteristics of MB were determined with a Multisizer 3 Coulter counter (Beckman Coulter Inc., Brea, CA) after dilution in 150 mM NaCl and zeta potential was measured using a Zetasizer 3000HSA (Malvern Instruments Ltd, Worcestershire, United Kingdom) after dilution of MB in 1 mM NaCl.

2.2. DC activation assay

Spleen-derived DC2.4 cells (provided by K. L. Rock, Department of Pathology, University of Massachusetts Medical School, Worcester, MA) were maintained in complete RPMI medium [12]. They were incubated for 24 h in the presence of plain medium, 100 ng/ml LPS (Sigma-Aldrich, St-Louis, MO), ethanol (vehicle for lipid solubilization) or different amounts of PA or DSPC. Alternatively, they were incubated with OVA-MB containing either 20% or 80% PA as described [13]. TNF-α in culture SN was quantified by ELISA (Max™ Set kit; BioLegend, San Diego, CA). Cells were blocked with mAb against CD16/32, stained with mAb against CD40 and analyzed with a Gallios flow cytometer (Beckman Coulter) and FlowJo software (Tree Star Inc, Ashland, OR).

2.3. Mice and cell suspensions

Four week-old female BALB/c mice were obtained from Charles River Laboratories (L'Arbresle, France) and used at the age of 8–10 weeks. They were housed in the animal facility of the Lausanne University State Hospital under standard conditions. All experiments were approved by the State Veterinary Office. Preparation of cell suspensions from spleens and inguinal lymph nodes (LN) was previously described [13].

2.4. MB administration and assessment of immune responses

Mice were immunized s.c. at the base of the tail with 4.5 μg of OVA in association with different MB formulations in a total volume of 100 μl. Endotoxin levels were measured by Limulus Amebocyte Lysate assay (Pierce, Rockford, IL, USA) and found to be below 1 endotoxin unit (EU) per μg of OVA. Three administrations at two-week intervals were performed and blood, spleen and inguinal LN were collected 8 days, 14 days, 2 months and 6 months after the last vaccine injection. Sera and cell suspensions were analyzed respectively for the presence of OVA-specific Ab by ELISA and T cells by CFSE-based proliferation assay as described elsewhere [13]. Cell culture SN were collected after 72 h of incubation and analyzed for the presence of IL-2, IFN-γ, IL-10, IL-4 and IL-5 (all ELISA Max™ Sets; BioLegend) and IL-13 (eBioscience, San Diego, CA). Flow cytometry analysis of splenic T cell proliferation was performed by gating on live (DAPI-negative) lymphocytes, followed by recording of $CD3^+CD4^+CD8^-CFSE^{low}$ cells on or $CD3^+CD4^-CD8^+CFSE^{low}$ cells. For intracellular cytokine staining (ICS), inguinal LN cell suspensions were incubated for 20 h with plain medium or OVA and brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the last 16 h. Cells were then stained with surface anti-CD4 and -CD8 mAb (BD Biosciences, San Jose, CA) and intracellular anti-IL-5 mAb (BD

Table 1
Characteristics of formulations.

	Structure				DV[b] (μm)	DN[c] (μm)	Conc. (MB/ml)[d]	Zeta potential (mV)[e]	OVA density (molec./μm²)[f]	OVA/MB (μg/mg)
	DSPC[a] (%)	PA[a] (%)	DSPE[a]- PEG2000-maleimide (%)	DPPE - MPB[a] (%)						
$OVA\text{-}MB_{20\%\ PA/PEG}$	78	19.5	2.5	0	3.19	1.24	$3.5x10^9$	−26.5	23743	428
$OVA\text{-}MB_{20\%\ PA}$	76.2	19	0	4.8	3.85	1.32	$3.4x10^9$	−24	19233	368
$OVA\text{-}MB_{80\%\ PA/PEG}$	19.5	78	2.5	0	4.07	1.37	$2.2x10^9$	−23.1	12708	350
$OVA\text{-}MB_{80\%\ PA}$	19	76.2	0	4.8	3.41	1.17	$2.4x10^9$	−28.1	20928	329

[a] DSPC, distearoyl-phosphatidyl-choline; PA, palmitic acid; DSPE, distearoyl-phosphatidyl-ethanolamine; DPPE-MPB, maleimidophenyl-butyryl-dipalmitoyl-phosphatidylethanolamine.
[b] DV, mean diameter from volume data.
[c] DN, mean diameter from number data.
[d] MB reconstituted in 1 ml of 150 mM NaCl.
[e] Measured using Zetasizer 3000HSA (Malvern) after dilution of MB in 1 mM saline.
[f] Determined by radioactivity.