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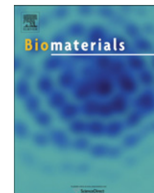

Gas-filled microbubble-mediated delivery of antigen and the induction of immune responses

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ABSTRACT

The use of well characterized recombinant or purified protein antigens (Ag) for vaccination is of interest for safety reasons and in the case where inactivated pathogens are not available (cancer, allergy). However it requires the addition of adjuvants such as Ag carrier or immune stimulators to potentiate their immunogenicity. In this study, we demonstrated that gas-filled microbubbles (MB) can serve as an efficient Ag delivery system to promote phagocytosis of the model Ag ovalbumin (OVA) without the need of ultrasound application. Once internalized by DC, OVA was processed and presented to both CD4 and CD8 T cells *in vitro*; such observations were coupled with the capacity of MB to activate DC. *In vivo* administration of MB-associated OVA in naïve wild-type Balb/c mice resulted in the induction of OVA-specific antibody and T cell responses. Detailed characterization of the generated immune response demonstrated the production of both IgG1 and IgG2a serum antibodies, as well as the secretion of IFN-γ and IL-10 by splenocytes. Interestingly, similar results were obtained with human DC in regards of Ag delivery and cell activation. Therefore, the data presented here settle the proof of principle for the further evaluation of MB-based immunomodulation studies.

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1. Introduction

Gas-filled microbubbles (MB) are micro-sized particles composed of a gas entrapped in a lipid- or polymer-based shell [1]. They have originally been developed as ultrasound (US) contrast agents for imaging of the bloodstream and are nowadays approved for clinical use; investigation of their potential for the targeted detection of diseases is under scrutiny [2,3]. Additionally, they have been widely studied for sonoporation of MB-associated, but not covalently linked, RNA/DNA, drugs or antigens (Ag) [4–7]. Such process relies on a close interaction between MB and the cell membrane with the generation of small disruptions in the target cell membrane induced by MB cavitation upon US application [8,9].

We have recently demonstrated that MB can be phagocytosed by human and mouse DC without the application of US [10]. Indeed, subcutaneous (s.c.) injection of fluorescent MB resulted in the recovery of skin-derived dendritic cells (DC) that have taken up the injected formulation, before having migrated to the draining lymph node (LN).

DC are professional antigen-presenting cells (APC) and therefore the most potent cells to prime specific CD8 and CD4 T cell responses through the presentation of ingested and processed Ag [11,12]. DC also participate in the generation of antibody (Ab) responses through their interactions with B cells. Engulfment of Ag encountered in the periphery promotes the migration of DC to afferent/draining LN, secondary lymphoid organs where the local initiation of immune responses occurs [13]. Alternatively, Ag that enter the body may have directly access to the draining LN where they are taken up by resident DC [14]. The environment in which the interaction between Ag and DC takes place delivers signals that instruct them to generate the appropriate profile of adaptive immune response [15,16]. Ag associated with harmful microorganisms are delivered together with so-called danger signals to ultimately trigger effector cells, whereas innocuous Ag usually activate tolerogenic cells.

Abbreviations: MB, gas-filled microbubbles; P-MB, plain MB; OVA-MB, OVA covalently linked to MB; Cy3:OVA, Cy3-labeled OVA; MFI, mean fluorescence intensity; APC, antigen-presenting cells; DC, dendritic cells; MoDC, monocyte-derived dendritic cells; mDC, myeloid DC; pDC, plasmacytoid DC; LN, lymph node; Ag, antigen; US, ultrasound; s.c., subcutaneous; DAPI, 4′,6′-diamidino-2-phenylindole; mAb, monoclonal antibodies; LB, latex microbeads; SN, supernatant.

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Safety concerns raised by the use of live attenuated pathogens for vaccination, paved the way for the administration of well characterized recombinant Ag [17], that intrinsically remained poorly immunogenic. Addition of immune modulators, generally derived from pathogen danger signals, proved efficient at ameliorating immunogenicity of vaccines [18]. Additionally, efforts have been made to improve specific delivery of Ag by: (1) their coupling to monoclonal antibodies (mAb) to target receptors or ligands expressed by different APC subsets [19,20]; (2) their concentration on nano- or microparticles, resulting in so-called particulated Ag, that will be taken up by APC [21]. Research on the use of carriers for vaccination was initiated decades ago with works on Ag adsorbed on alum [22], the most largely clinically accepted vaccine carrier/adjuvant. Currently, studies focus on the development of safe Ag delivery systems, such as liposomes, ISCOM matrix and polymeric nano- or micro-particles [23,24]. For induction of immune responses they allow efficient delivery of Ag, peptides, RNA or DNA to target cells. Particle charge, size and structure are important parameters that impact on uptake, *in vivo* behavior after administration and profile of induced immune responses [25,26], features that have to be considered when defining their area of application.

In the present study we sought to analyze the ability of MB to deliver the covalently linked model Ag OVA (OVA-MB) to DC *in vitro* and *in vivo* without application of US and subsequently to induce an immune response in naïve wild type mice after subcutaneous (s.c.) administration of OVA-MB.

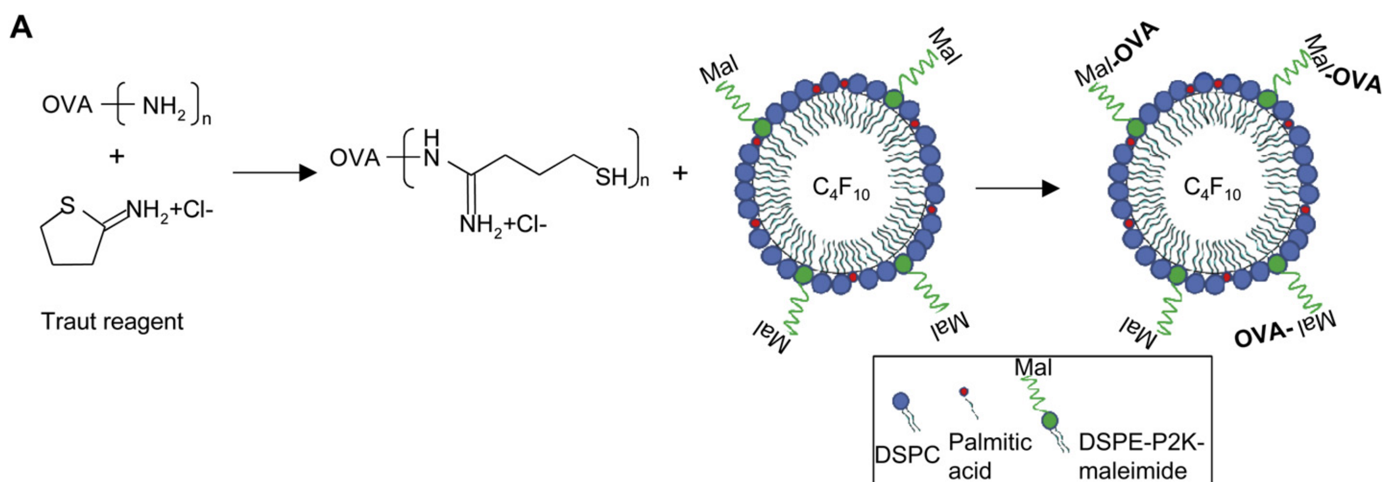
2. Materials and methods

2.1. Preparation of gas-filled OVA and plain microbubbles

Plain MB (P-MB) formulated as a powder for injection were produced by lyophilization of a PEG4000 solution containing small amounts (255 μg/vial) of neutral phospholipids, distearoylphosphatidylcholine (DSPC) and a pegylated phospholipid (DSPE-PEG2000-maleimide) (both from Avanti Polar Lipids, Alabaster, AL), and palmitic acid (Fluka, Buchs, Switzerland) at a molar ratio of 78/2.5/19.5. The headspace in the vial contains a mixture of perfluorobutane and nitrogen gases (35/65 vol/vol).

Thiolation of OVA (20 mg/ml) was performed using a 10-fold molar excess of Traut's reagent (Thermo Scientific-Pierce, Rockford, IL) in PBE buffer (25 mM phosphate, 150 mM saline, 1 mM EDTA, pH 8) for 1 h at RT on a rotating wheel. The excess of Traut's reagent was removed from the thiolated OVA solution by purification on a Zeba™ Desalt Spin Column (Thermo Scientific-Pierce) equilibrated with phosphate buffer 20 mM at pH 6. Thiolation of OVA was controlled with Ellman's reagent (Thermo Scientific - Pierce) according to the supplier instructions. The final solution of thiolated OVA was coupled to DSPE-PEG2000-maleimide by incubation at RT on a rotating wheel for 2.5 h (Fig. 1A).

Radiolabelled ^{125}I OVA was added to the native solution of OVA, enabling the quantification of coupled OVA on MB and calculation of the final OVA density on MB as molecules/μm^2. 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. In some cases, Cy3-labeled OVA (Cy3:OVA) or DQ:OVA (Molecular probes-Life Technologies Ltd., Paisley, United Kingdom) were linked to MB to generate fluorescent OVA-MB. The labeling of OVA with Cy3 (GE Healthcare Bio Sciences AB, Uppsala, Sweden) was performed by adding a 3 times molar excess of 10 mg/ml Cy3 to the solution of OVA at 20 mg/ml and let for 45 min under gentle stirring at RT in the dark. The labeled solution of OVA was then purified on a Zeba™ Desalt Spin Column (equilibrated with



B

	Structure	DV (μm)	DN (μm)	Conc. (MB/ml)[a]	Zeta potential (mV)	OVA density (molec./μm²)[b]	OVA/MB (μg/mg)
P-MB	DSPC/PA/DSPE-P2K-mal	3.05	1.36	2.80x10⁹	-12.8	0	0
Cy3:OVA-MB	DSPC/PA/DSPE-P2K-mal-OVA:Cy3	2.71	1.32	3.06x10⁹	-20.7	16'289	165.6
Cy3:OVA$_{low}$-MB	DSPC/PA/DSPE-P2K-mal-OVA:Cy3	2.94	1.33	2.69x10⁹	-14.2	8'256	69.8
Cy3:OVA-LB	LB-OVA:Cy3	1.86	1.85	1.17x10⁹	n.d.[c]	50'302	38.3
DQ:OVA-MB	DSPC/PA/DSPE-P2K-mal-OVA:DQ	2.90	1.35	3.24x10⁹	-22.8	9'611	131.9
OVA-LB	LB-OVA	1.92	1.94	1.03x10⁹	n.d.[c]	43'761	46.3
OVA-MB (A)	DSPC/PA/DSPE-P2K-mal-OVA	3.04	1.35	2.54x10⁹	-21.7	33'414	160.3
OVA-MB (B)	DSPC/PA/DSPE-P2K-mal-OVA	2.62	1.24	3.48x10⁹	-26.5	23'743	214.0

Fig. 1. *Generation of MB-associated OVA and characteristics of MB formulations*. (A) Schematic representation of thiolated OVA linkage to MB via maleimide. (B) Characteristics of MB formulations used in the study. DV, mean diameter from volume data; DN, mean diameter from number data; DSPC, distearoylphosphatidylcholine; PA, palmitic acid; DSPE-P2K-mal, distearoylphosphatidylethanolamine-poly(ethylene glycol)2000-maleimide; [a] MB reconstituted in 1 ml PBS; [b] determined by radioactivity; [c] not determined.

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