Gas-filled microbubbles: Novel mucosal antigen-delivery system for induction of anti-pathogen’s immune responses in the gut

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ABSTRACT
Despite important success in protecting individuals against many pathogenic infections, parenteral vaccination is not optimal to induce immunity at the site of pathogen entry, i.e. mucosal surfaces. Moreover, designing adequate delivery systems and safe adjuvants to overcome the inherent tolerogenic environment of the mucosal tissue is challenging, in particular in the gastrointestinal tract prone to antigen degradation. We recently demonstrated that intranasal administration of a Salmonella-derived antigen associated with gas-filled microbubbles induced specific Ab and T cell responses in the gut and was associated with a reduction in local and systemic bacterial load after oral Salmonella infection. Building on these promising data, the adequate choice of antigen(s) to be administered and how to make it suitable for possible human application are discussed. We additionally present novel data dealing with oral administration of microbubbles and describe research strategies to direct them to mucosal sampling/inductive sites.

KEYWORDS
mucosal vaccine; nasal administration; oral administration; microparticles; enteropathogen infections; gut immune responses

Mucosal surfaces are constantly exposed to, and challenged by, numerous innocuous or potentially harmful microorganisms and environmental antigens (Ags). Therefore, induction of appropriate local immune responses needs to be tightly controlled to maintain homeostasis on one hand and protect the integrity of the epithelium on the other hand.1 Mucosal responses to commensal microorganisms or food Ags are generally considered to be tolerogenic by nature. In contrast, danger signals generated by the sensing of pathogenic microorganisms promote an effector type of response relying on both humoral and cellular arms to eliminate the infection.2 Mechanisms that contribute to the protection of mucosal surfaces include: a) immune exclusion mediated by secretory IgA (SIgA), b) opsonization of pathogens by IgG, c) targeting of Ags to dendritic cells (DCs), d) production of IFN-γ and IL-17 that activate phagocytes, and e) production of IL-22 that preserves the integrity of the intestinal epithelium.3,4 Vaccination strategies to elicit mucosal immune responses may largely benefit from the knowledge acquired during the last decades on how the mucosal immune system works. An essential aspect is that only local DCs have the ability to promote the expression of specific homing receptors on B and T cells for trafficking back to mucosa.5,6 This implies that mucosal delivery of vaccine formulations should be preferred over parenteral application. Interestingly, immunization via the mucosal route also generates systemic immunity, which is crucial to contain the dissemination of invading pathogens. Additional advantages include the ease of administration, the reduced requirement for trained medical personal, the reduced costs for particular administration devices and the decreased risks of side-effects associated with injections.

Irrespective of the vaccination route, the magnitude and profile of induced immune responses are controlled by the formulation of the delivered Ags.7 In the context of mucosal application, parameters such as a) incorporation of adjuvants to overcome the inherent tolerance-prone environment of mucosae, b) association with delivery systems to improve the Ag stability/integrity, and c) targeting of the epithelium to ensure efficient sampling are of prime importance for vaccine
design and a matter of intense investigation. Apart from licensed live-attenuated vaccines which are relatively effective, but raise safety concerns for infants and immunocompromised people, several vaccination strategies have been evaluated. Recombinant or purified pathogen-derived antigenic entities have been associated with delivery systems (e.g., polymer-based nano- or microparticles, liposomes, virus-like particles) and/or with immunostimulatory components (adjuvants; e.g., Toll-like receptor ligands) to reinforce their otherwise poor immunogenicity. In addition, live lactic acid bacteria engineered to express vaccine Ag(s) or non-living lactic acid bacteria (Gram-positive enhancer matrix) have been tested with some success as delivery systems for mucosal vaccination.

We have evaluated the potential of gas-filled microbubbles (MBs) to serve as an alternative and biocompatible systemic and mucosal Ag delivery system for vaccine formulations. MBs used for vaccination purposes are lipidic microparticles composed of a monolayer of distearoyl-phosphatidylcholine and palmitic acid entrapping inert high molecular weight gases, which allows to stabilize the bubble-shape's structure. The presence of PEG grafted to the phospholipids ensures the resistance to pressure during administration. Similar reagents have been safely used for more than a decade for human application as intravenously delivered ultra-sonographic echo-contrast agents. We previously demonstrated that MB-associated Ags efficiently induce systemic IgG1/2a Abs, and IL-2-, IFN-γ-, TNF-α- and IL-10-producing Th1-type T cell responses in mice after subcutaneous injection. In direct comparative experiments, MBs performed quantitatively at least equally to liposomes, but qualitatively a more pronounced Th1-type profile of induced immune response was observed. Remarkably, the immune responses elicited by these vaccine formulations were still present 6 months after the last administration and were active in partially protecting against systemic bacterial infection. These promising results and the current needs for relevant mucosal vaccines to stem the wave of multidrug resistant pathogens paved the way to evaluate their efficacy for mucosal application.

**Mucosal application of MB-based vaccines for gut protection**

The mucosal immune system displays some degree of anatomic compartmentalization related to the migratory properties of lymphocytes primed in particular mucosal tissues. For example, nasal administration of vaccines preferentially induces immune responses in the upper respiratory tract, whereas the generation of gut immunity mostly requires oral application. However, in accordance with the concept of common mucosal immune system, the nasal route also allows to elicit specific immunity in the genital tract, rendering administration of potential vaccines against sexually transmitted infections more convenient. In addition, some studies demonstrated that a particular subset of lung DCs has the capacity to prime T cells with gut-homing properties, suggesting that intranasal vaccination may be used as a convenient route to protect against gastrointestinal infections. In anesthetized mice, administration of ≥ 20μl of particulated antigenic entities in the nose resulted in the delivery of the preparations not only to the nose-associated lymphoid tissues (NALT), but also to the dense network of DCs lining the trachea and the pulmonary mucosa. Similarly, intranasal application of fluorescent MBs resulted in their detection in lung CD11c+ DCs by flow cytometry. Similar administration of MB associated with the *Salmonella*-derived serodominant secreted effector protein B (SseB) demonstrated the ability of this formulation to induce not only NALT-related mucosal immunity, as shown by the presence of specific T cells in the cervical LNs which drain the NALT, but also lung and gut immune responses. Interestingly, specific Ab production and T cell activation were observed in both feces and mesenteric LNs. T cell responses were also found in Peyer’s patches (PPs; Fig. 1A), the primary inductive site of gut immunity and the portal of entry of many microbes. Similarly, SseB-specific SIgA were detected in intestinal washes, ready to prevent local pathogen entry. The sum of these data indicates that after priming in the lung, immune cells have the capacity to traffic from the activation site to reinitiate distant pathogen-specific immune responses.

Protection mediated by intranasal application of SseB-MBs was examined in the context of oral *Salmonella enterica* Typhimurium infection in mice. This model is known to mimic, for several pathological aspects, *Salmonella*-associated typhoid fever in humans, a disease that affects millions of individuals worldwide by causing symptoms such as fever, respiratory distress, hepatic, spleen and neurologic damages, and culminating in the host death in about 1% of the cases. Of note, besides safety concerns, the currently available oral live attenuated Ty21a and
Parenteral Vi capsular polysaccharide vaccines have only limited efficacy in the main at-risk populations and display lack of cross-reactivity to multiple serovars, which precludes their broad use in endemic areas. The use of a subunit vaccine applied mucosally and displaying an Ag (SseB) shared by many different Salmonella serovars may overcome several of the above-mentioned limitations. Characterization of naturally-induced protective immunity in both animal models and humans has demonstrated the importance of specific IgG Abs and IFN-γ production to limit Salmonella infection and dissemination, with an additional beneficial role played by SIgA Abs, IL-17 and IL-22 production.

Figure 1. Gut immune responses induced by intranasal administration of SseB-MBs. Female Balb/c mice (8–10 weeks of age) were intranasally immunized for 3 consecutive days a week for 4 weeks with the indicated SseB formulations, corresponding to a total of 3 μg SseB administered per week. The prototypic murine mucosal adjuvant cholera toxin (CT; 2 μg per administration) was used as control. (A) Cytokine production measured by ELISA in the culture supernatant of PP cell suspensions restimulated in vitro for 72 h with 10 μg/ml SseB. (B) SseB-specific Ab responses measured by ELISA in feces. n = 6 mice per group. The unpaired, nonparametric Kruskal-Wallis test, corrected with Dunn’s test for multiple comparisons, was applied to compare the indicated experimental groups. ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Strikingly, several of these essential molecular partners were induced after intranasal vaccination with SseB-MBs, as opposed to SseB alone that elicits poor immune responses. Detection of IL-17 and IFN-γ in PPs (Fig. 1A) and mesenteric LNs, together with specific IgG and SIgA Abs in feces and intestinal washes (Fig. 1B), underpin the onset of potential local protective immunity induced by SseB-MB. In addition, the presence of systemic specific IgG and IFN-γ production supposed to control Salmonella dissemination indeed translated into a significant reduction of bacterial load, in both the gut and the spleen, after intranasal administration of SseB-MBs. The underlying mechanism most probably relies on phagocytic-mediated elimination of the bacteria released by dying infected cells. IL-17 may also be involved locally by harnessing neutrophils known to control Salmonella infection. Of note, although strong systemic immunity was induced by subcutaneously injected SseB-MBs, this did not ensure protection against oral Salmonella infection. Thus, the adjuvant properties displayed by MBs suggest that they may be a valuable tool for mucosal vaccine’s development.

Possible development beyond SseB-MBs

When designing vaccines against infectious agents, the choice of the targeted Ag(s) to be associated with delivery systems and/or adjuvants is of outstanding importance. Ideally, its (their) expression by the pathogen must reach a sufficient level and occur with the adequate timing, so that it is (they are) accessible for recognition by vaccine-induced Abs and/or effector T cells. SseB is part of the TSS3–2 complex that is mainly expressed by Salmonella during infection of macrophages, i.e., when the bacterium has already entered mucosal tissues and starts to disseminate. Therefore, SseB-based vaccination may not be effective at preventing the entry of Salmonella within intestinal tissues. However, SseB-specific Ig- and T cell-mediated protection may occur at the interface between local and systemic compartments, as evidenced by the presence of such immune responses in mice and humans after recovery from Salmonella infection. It is therefore tempting to speculate that the association of a second Ag with MBs would be beneficial. In this context, FlIC that is expressed by the bacterium in the intestinal lumen or at the time it crosses the epithelial barrier sounds like an ideal candidate. Indeed, FlIC-specific immune responses induced by natural Salmonella infection are mainly composed of SIgA and IL-17, 2 local players important to prevent bacterium infection. Of importance, responses of the same nature are induced by MB vaccination (Fig. 1). More generally, in addition to the induction of immune responses in the gut, intranasal delivery of MBs may be an appropriate approach to elicit protective immunity in the context of pulmonary and urogenital infections such as Influenza, Streptococcus, Mycobacterium tuberculosis, HIV, HPV or Chlamydia.

Routes of administration for MB-based formulations: Pros and cons

Previous data raise important questions such as: can the results obtained in mice after intranasal delivery of SseB-MBs be translated for human application? Can MBs be appropriate to elicit protective immunity in the context of pulmonary and urogenital infections such as Influenza, Streptococcus, Mycobacterium tuberculosis, HIV, HPV or Chlamydia.

While valuable information can be provided by “preclinical” studies in animal models, one has to keep in mind that these latter do not fully recapitulate human anatomy and physiology. Indeed, despite some common features in terms of physiologic and immunological aspects, such as the presence of M cells allowing sampling of particulated Ags, the organization of NALT, and DC localization and phenotype in the nasal cavity and in the lung all differ between mice and humans. Mice have a concentrated aggregation of immune cells at the inductive site (NALT) in the nasal cavity. In humans, such structures are present early in infants, while upon aging they are replaced by alternative inductive sites, e.g., immune nodules in the upper nasal cavity, in the concha, and in Waldeyer’s rings (adenoids, tonsils) located in the pharynx. This suggests that the direct interspecies translation of the knowledge acquired on the uptake of vaccine formulations and their delivery to underlying/subepithelial DCs will need to be further evaluated.

As matter of fact, intranasal delivery of liquid drops (instillation) or spray (nebulization) of MB preparations would mostly be taken up through sampling sites within the nose and in the vicinity of the nasopharynx; however particulated Ags delivered via these protocols did not prove immunogenic. In
contrast, the use of aerosols would be the most potent way to reach down to the pulmonary tract and permit uptake by lung DCs. This approach has been found to be immunogenic, but this way of administration would imply that the stability of MBs is not impaired by transient overpressure for instance. An alternative to intranasal administration, sublingual delivery requires relatively low amounts of vaccine formulations to induce significant levels of systemic and lung immune responses. However, inconsistent results have been obtained regarding the generation of gut immune responses.

It is common knowledge that the most physiologic and potentially efficient administration route to vaccinate against gut infections remains the oral route, provided that the applied formulations survive the aggressive environment of the gastrointestinal tract. Indeed, the stomach’s acidity, the high protease content of the intestine, and the dynamic environment of the lumen, can all reduce the effectiveness of the administered formulations. This implies that, as compared with the delivery routes discussed above, larger amounts of vaccine preparations need to be applied to reach a sufficient degree of immunity. As a possible asset, the gut-associated immune system exhibits important similarities between mice and humans, suggesting that data obtained in animal models would be relevant for clinical approaches. Moreover, the reported similarities between NALT and PP organization and function suggest that oral administration, like intranasal application, of MB-based formulations may induce gut immunity. We therefore aimed at testing oral administration of SseB-MBs in mice and at analyzing immune responses induced in the gut. We first observed by flow cytometry that fluorescent MBs administered in a ligated intestinal loop were taken up by DCs present in PPs (Fig. 2). Moreover, preliminary data showed that oral administration of SseB-MBs induced specific T cell responses in both PPs and mesenteric LNs (Fig. 3A and B), whereas low Ab responses were found in intestinal washes (Fig. 3C). Even though such results need to be confirmed, they represent an interesting basis for the further evaluation of oral vaccination with MBs. Incorporation of vaccines in protective capsules and their administration within a mucoadhesive reagent such as chitosan solutions may guarantee vaccine stability. A further perspective would consist in testing existing strategies to potentiate vaccine delivery across the mucosal epithelium, as exemplified below.

**Improving the delivery and immunostimulatory properties of MBs**

The fact that SseB-MBs are able to induce gut immune responses suggests that MBs have the capacity to properly deliver Ag to intestinal inductive sites and/or activate antigen-presenting cells. The size of MBs, in the micrometer range, is consistent with the size of particles that are preferentially sampled from the lumen by M cells, and transported across the epithelial barrier to DCs in underlying PPs. However, the number of PPs along the intestine is relatively low indicating that specific delivery to these sites would improve vaccine sampling. Targeting of Ags to specific receptors expressed by DC subsets has shown some success for parenteral vaccination, a situation where administered formulations are directly contacting tissue-resident DCs or are passed to DCs in draining LNs. For mucosal administration, vaccine formulations have to be taken up by
intraepithelial or subepithelial DCs. Due to their dynamic structure, MBs can accommodate ligand motifs to enhance for example tissue- and cell-specific imaging procedures.\textsuperscript{41} Building on this approach, this opens the way to validation experiments in mice by achieving specific targeting of M cells through interactions with GlycoProtein-2 (GP-2) and Ulex Europeaus Lectin-1 (UEA-1).\textsuperscript{42-44} However, this approach is not completely suitable for humans, because expression of UEA-1 is not restricted to M cells, but occurs also on enterocytes.\textsuperscript{45} In contrast, the loading of SIgA Ab on MBs is an interesting option because (1) they can be retro-transported across the epithelium by M cells in both human and mouse via Dectin-1,\textsuperscript{46} (2) they are stable in the gastrointestinal environment and are trapped in the mucus to avoid elimination\textsuperscript{47} and (3) they are additionally targeted to PP DCs.\textsuperscript{48} Therefore, SIgA may serve as a cargo for the controlled delivery of associated particle payloads as this occurs naturally with microorganisms sampled from the lumen.\textsuperscript{49} Promising results have already been obtained by coupling SIgA with free Ags to improve mucosal delivery.\textsuperscript{50,51} Following the same approach with MBs known to elicit immune responses might further increase their effectiveness. Alternatively, targeting FcRn which is expressed on epithelial cells and hematopoietic cells, e.g., DCs, has also been demonstrated as an efficient mechanism to deliver IgG-based immune complexes across the epithelium and to underlying DCs.\textsuperscript{52} Such interesting strategies are currently being tested in the laboratory. The modularity ensured by tailoring the MB design and content, their stability as lyophilized preparation and their known safety as already established in medical imaging in humans make the appraisal of this vaccine candidate a promising development in the field of targeted mucosal immunomodulation.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Figure 3. Gut immune responses induced by oral administration of SseB-MBs. Female Balb/c mice (8–10 weeks of age) were immunized intragastrically once a week for 4 weeks with the indicated SseB formulations corresponding to a total of 6 μg SseB administered per week. The SseB + cholera toxin group (CT; 2 μg per administration) was used as control. (A) Cytokine production measured by ELISA in the culture supernatant of PP cell suspensions restimulated in vitro for 72 h with 10 μg/ml SseB. (B) T cell proliferative responses in mesenteric LNs (CFSE-based assay; restimulation of cells with 10 μg/ml SseB for 4 days) and cytokine production measured by ELISA in the culture supernatant of mesenteric LN cell suspensions restimulated in vitro for 72 h with 10 μg/ml SseB. (C) Ab responses measured by ELISA in feces. n = 3 mice per group.
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