

Review articles

Mucosal vaccination – an old but still vital strategy¹

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ABSTRACT. The basic premise of vaccinology is to achieve strong protective immunity against defined infectious agents by a vaccine mimicking the effects of natural primary exposure to a pathogen. Because an exposure of humans and animals to microbes occurs mostly through mucosal surfaces, targeting the mucosa seems a rational and efficient vaccination strategy. Many experimental and clinical data confirmed that mucosal immunization offers many advantages over widely used in human and veterinary medicine subcutaneous or intramuscular immunization. In the present article selected aspects regarding mucosal vaccination are discussed. The structure and function of mucosa-associated lymphoid tissue (MALT), comprised of four main mucosal compartments forming a structural and functional unity as well as pivotal cellular MALT components (dendritic and M cells) were briefly characterized. Particular attention was focused on the mode of simple but efficacious delivery of vaccine antigens to mucosal surfaces. A few trials to generate potential mucosal vaccines against toxoplasmosis introduced by nasal or oral routes to experimental animals are also presented.

Key words: MALT (mucosa-associated lymphoid tissue), immunity, vaccine, toxoplasmosis

Mucosa-associated lymphoid tissue

Mucosal immunity involves a complex network of cell types and soluble effector molecules that is responsible for protecting the host organism against infections at mucosal surfaces. The term MALT (mucosa-associated lymphoid tissue) refers to organized lymphoid tissues situated throughout the body, where GALT (gut-associated lymphoid tissue), NALT (nasopharynx- or nose-associated lymphoid tissue), and BALT (bronchus-associated lymphoid tissue) are its best-known anatomically subdivided representatives [1]. The genital tracts of females and males are also components of the common mucosal system (Fig.1). GENALT (genital-associated lymphoid tissue) is, to some degree, unique because of its special function – to maintain a delicate balance between tolerance to germinal center cells, spermatozoa and fetus and immune response to exogenous (e.g., microbial) antigens. The mucosal immune system in genital

tracts is characterized by a lack of typical inductive mucosal sites (like Peyer's patches) resulting in weak and localized immune responses, most likely without any significant involvement of other mucosal compartments. Besides, a considerable proportion of antibodies in genital tract secretions derives from the circulation [2]. Apart from four mentioned and well-known MALT representatives other compartments are also described, for instance LALT (larynx-associated lymphoid tissue) [3], EALT (eye-associated lymphoid tissue) etc. [4].

It is essential that the appearance of MALT, besides species differences [5], depends on the age and tissue state (i.e., normal/inflamed). Likewise, exogenous stimulation may affect the occurrence and size of some MALT structures which apparently do not develop prenatally (e.g., BALT is not regularly found in normal lungs of adults). The largest and the best defined is GALT, comprising Peyer's patches, an appendix and isolated lymphoid follicles. The inductive sites are formed by regional

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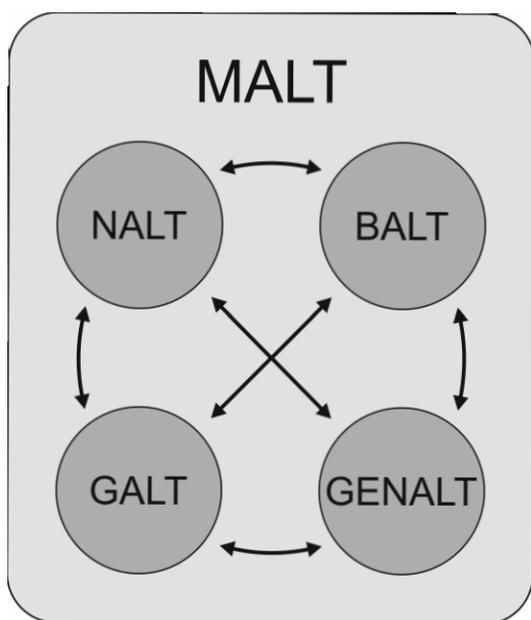


Fig. 1. Mucosal-associated lymphoid tissue (MALT) – an integrated mucosal immune system composed of NALT (nasopharynx-...), BALT (bronchus-....), GALT (gut -....), and GENALT (genital-associated lymphoid tissue) as main MALT parts

MALT with local mucosa-draining lymph nodes, where exogenous antigens (soluble or particulate) are actively transported from the gut lumen through a characteristic follicle-associated epithelium containing M cells to antigen presenting cells. Naive B and T lymphocytes, after being primed, migrate to peripheral blood to be subsequently extravasated at the mucosal epithelium. Effector sites are present in all mucosal tissues distributed throughout the *lamina propria* [6]. A significant feature of the mucosal immune response is the production and secretion of dimeric/multimeric immunoglobulin A antibodies by mucosal plasma cells. The structural analysis of secretory IgA (S-IgA) reveals an existence of the joining chain (J-chain) which enables binding to the pIgR receptor. The pIgR molecule mediates the transcytosis of S-IgA across the intraepithelial cell barrier – dimeric IgA are captured by pIgR at the basolateral surface, internalized by intraepithelial cells and transported to the apical surface. S-IgA antibodies are continuously delivered onto the intestinal mucosa in huge quantities (3-5 g of S-IgA per day), diffuse through the mucus and enhance the protection against specific pathogens by inhibition of the absorption of soluble and particulate antigens by forming intraluminal immune complexes. Due to a unique chain structure, S-IgA are resistant to

degradation by microbial proteases and play multiple roles in mucosal defense, e.g., by promoting the entrapment of pathogens in the mucus or by preventing direct contact of microorganisms with the mucosal surface (immune exclusion). Moreover, S-IgA might block microbial molecules that mediate attachment, as well as intercept an incoming pathogen during pIgR-mediated transport [7–10].

Noteworthy, in the MALT occur inductive or/and effector phases of delayed-type hypersensitivity (DTH, mediated by antigen-specific T lymphocytes), which plays an important role in the immunity to many infectious agents, oral tolerance, as well as immunopathology such as in the DTH reaction to gluten [11].

Key cellular components of MALT, particularly important for immune response induction and mucosal vaccination, are epithelial M cells characterized by the high transcytotic activity as well as dendritic cells (DC), which serve as potent antigen presenting cells (APC) and stimulate naive T cells.

Mucosal M cells

M cells („membranous” or „microvilli” cells), discovered in 1974, are specialized endothelial cells found in the epithelium overlaying mucosal lymphoid follicles called the follicle-associated epithelium (FAE). They are distinguished from neighbouring enterocytes and goblet cells in the villous epithelium by short and irregular microvilli. The apical surface, characterized by reduced surface glycocalyx, is flattened and basolateral cytoplasmic invagination creates a pocket containing lymphocytes and occasional macrophages (Fig. 2). Cytoplasm of M cells contains few lysosomes, more mitochondria, and on the cell membrane fucose epitopes binding a lectin UEA (UEA, *Ulex europaeus* agglutinin) are present [12,13]. The huge mucosal surface area is protected by a layer of tightly joined epithelial cells, preventing the mucosa from the penetration by environmental antigens including microbes but this natural physiological barrier is not completely tight. Villous M cells are principal antigen entry sites in the mucosal epithelium; they take up and transport numerous microbes and macromolecules from the lumen to the inside of Peyer’s patches follicles [14] where antigen-specific immune response is initiated.

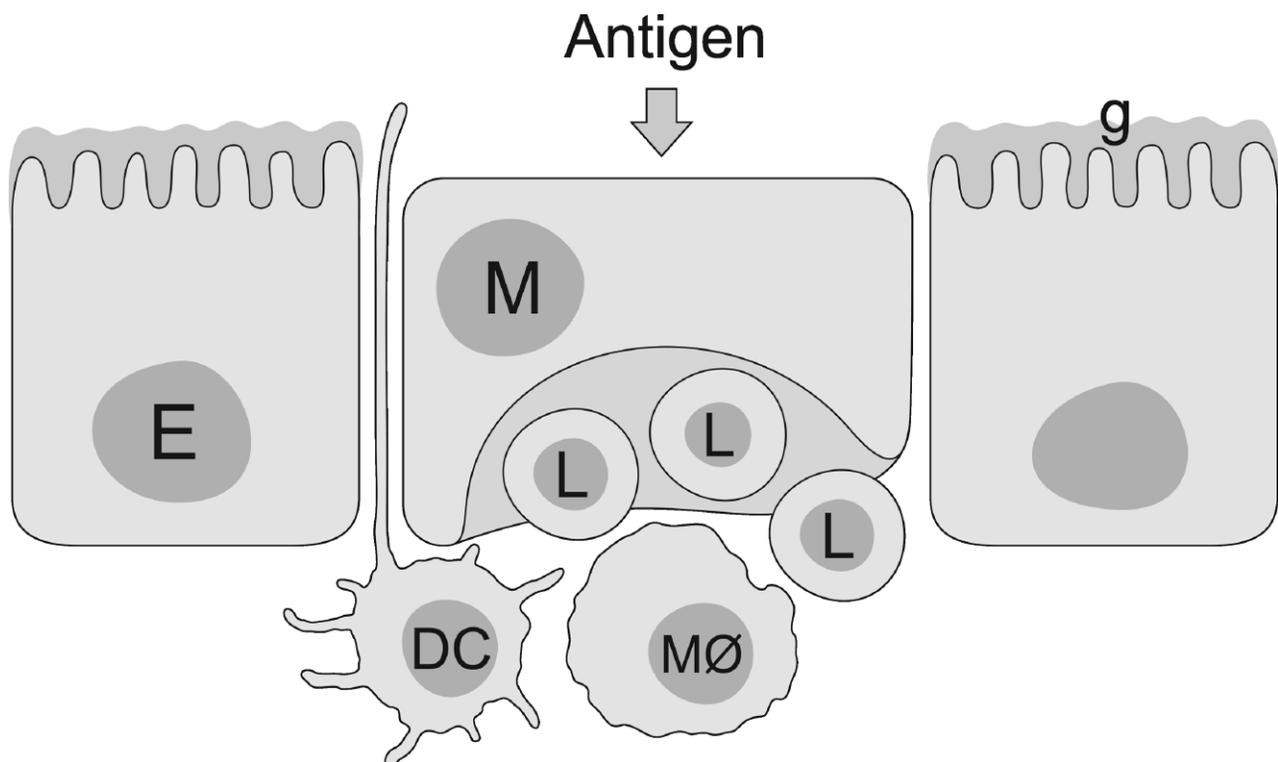


Fig. 2. Main cellular components of MALT: M – M cell, E – epithelial cell, Mφ – macrophage, DC – dendritic cell, L – lymphocyte, g – glycocalyx

Dendritic cells (DC)

The Nobel Assembly at Karolinska Institutet informed on 3rd October 2011 that a half of the Nobel Prize in Physiology or Medicine was given to Ralph M. Steinman „for his discovery of the dendritic cell and its role in adaptive immunity” [15]. In 1973 he identified a new cell type that he called the dendritic cell (déndron, being Greek for a tree) and subsequently demonstrated that dendritic cells stimulated T-cells to mount a strong immune response. Dendritic cells of both lymphoid and myeloid stem origin are present in many lymphoid and nonlymphoid organs, especially in these tissues that are in contact with the external environment, such as skin and the inner lining of the nose, lungs, stomach and intestines. Immature forms of those cells (with large cytoplasmic „veils”, veiled cells) are also present in the blood. Dendritic cells (DC) are not only potent professional antigen presenting cells but also crucial mediators of immune defense and tolerance. DC of mucosa (nasopharynx, bronchus, gut, and vagina) display different phenotypes and functions dependent on the tissue but their main role as peripheral sentinels is to recognize and respond to mucosal pathogens (i.e., uptake, processing and presentation of antigenic

material). Once activated, they migrate to the lymphoid tissues where they interact with T and B lymphocytes to initiate and shape the adaptive immune response [16]. Soloff and Barrat-Boyes [17] have clearly presented functional interplay between DC based on: a. their specialization (IFN- α production, antigen sampling etc.), b. their plasticity (determined by local cytokine and chemokine milieu), and c. cross-talk between DC subsets. This versatile interplay enables the induction of situation-specific and tailored immune responses to pathogens. The role of DC in vaccination is distinctly emphasized by the creation and activity of „International Society for Dendritic Cell and Vaccine Science”.

Mucosal vaccination

The contact with microorganisms occurs mostly by mucosa and thus the targeting of local immune components is crucial in the development of protective immunity at the infection gate and also in the design of effective mucosal vaccination strategies. The mucosal (nasal) immunization procedure was first reported in China in 10th century as a method of smallpox prevention (insufflation of powdered smallpox scabs called variolation) [18].

However, numerous well-documented and newer data on mucosal vaccination are connected mainly with the development of oral attenuated vaccines (OPVs) against poliomyelitis by several groups in the fifties of 20th century (Hilary Koprowski, Albert Sabin and Herald Rea Cox). Sabin's polio strains were chosen for a worldwide distribution and the Sabin vaccine, licensed in 1962, was introduced for immunoprevention. Individuals immunized with trivalent OPV develop long-lasting (frequently life-long) protective immunity against poliomyelitis. Currently, poliomyelitis has been eradicated from many regions and countries but the vaccine is still used because of many local disease focuses, and in the meantime only the vaccination scheme was modified [19]. Although the oral route of vaccination represents many advantages (such as high efficacy, low cost, no hazard) as compared to parenteral routes, only a small number of oral vaccines are routinely available today [20].

Mucosal vaccine targeting

To induce mucosal immune response, antigens must be transported across the epithelial barrier to organized lymphoid tissues such as Peyer's patches, in the process called transcytosis mediated by M cells. Targeting specific receptors on the apical surface of M cells seems a very promising approach in the development of an effective mucosal vaccine. The study on antigen uptake mechanisms showed several potential transcytotic molecules, among them: TLR4 (Toll-like receptor), PAF-R (platelet activating factor receptor), and $\alpha 5\beta 1$ integrin [21]. Hase et al. [22] found recently that glycoprotein 2 (GP2), specifically expressed on the apical membrane of human and mouse M cells, binds with Fim H, component of type I pili of enterobacteria (including *E. coli* and *S. typhimurium*) and serves as a transcytotic receptor. Shortly thereafter Kim et al. [23] reported the expression of C5a receptor on the apical surface of mouse Peyer's patch M cells, accompanied by co-expression of glycoprotein 2 (GP2). Oral immunization with an antigen complexed with *Yersinia enterocolitica* OmpH $\beta 1\alpha 1$ (Outer membrane protein H), a ligand of C5aR receptor, led to the uptake of the antigen by M cells. The presented results showed the diversity of potential molecules serving as antigen transcytotic components and provided new targets for the development of new mucosal vaccination strategies.

Numerous recent studies focus on the selection

of a proper carrier for antigen delivery to the mucosa. For example, to increase the uptake of the vaccine antigen (HBs, hepatitis B surface antigen) by M cells selectively, Gupta and Vyas [24] used liposomes modified by *Ulex europaeus* lectin 1, which binds specifically with α -L-fucose residues on mouse M cells. The modified liposomes induced enhanced immune response against HBs following oral administration in mice. Stano et al. [25] tested degradable polymer nanoparticles (50 nm) conjugated with a thiolated antigen by reversible disulfide bonds, and found that after intranasal administration the nanoparticles were efficiently transported *via* M cells, followed by the uptake by APC in NALT. What is more, co-conjugation with flagellin (FliC) as an adjuvant and ligand for TLR5 enhanced humoral responses, not only in airways but also in vaginal and rectal mucosa. Nanoparticles promoted antigen specific CD4⁺ T cells of Th1 profile.

Another approach in targeted antigen delivery is based on searching for individual short peptides capable of binding with mucosal cells to mimic the natural infection. The phage display technology was used to identify the peptides targeting human M cells, M-like cells, and enterocytes [26,27]. When polymeric nanoparticles were grafted with two peptide sequences (GCTGKSC and LRVG), their transport by the follicular-associated epithelium was significantly enhanced [26]. Co1, one of the selected by Kim et al. [27] ligands, fused with the antigen and administered orally, revealed adjuvant activity and could be used for targeted antigen providing to mucosal surfaces. Recently, the RDG peptide (a ligand for $\beta 1$ integrin) was conjugated with alginate coated chitosan particles and such a construct proved to be an efficient carrier for antigen transfer in oral vaccination [28].

The simple delivery of soluble antigens to mucosal membranes is ineffective as a rule and therefore the manner of vaccine antigen delivery to mucosa is a key factor defining the vaccination success. The destructive environment of the gastrointestinal tract is a challenge for the maintenance of transport vehicles (e.g., liposomes). Soudi et al. [29] found that rectal immunization with inactivated *Leishmania major*, co-administered with BCG, protected the susceptible BALB/c mice – no mortality and low parasite burden in the liver and the spleen were observed.

The injection of naked DNA encoding selected vaccine antigens is the simplest way to induce protective immunity but the major disadvantage of

the procedure is the inefficient entry of DNA into a cell. A very interesting approach to improve delivery efficacy seems to be an encapsulation of DNA vaccine material in virus-like particles (VLPs). The capsid of human papillomaviruses (HPV), responsible among others for cervical carcinoma, confers tropism for the basal epithelium. Produced *in vitro* HPV capsid major protein L1 is able to encapsidate plasmid bearing vaccine genes during self-assembly to form pseudovirions [30]. Graham et al. [31] used the method to transfer the M/M2 gene of the respiratory syncytial virus to mice and found that twofold intra-vaginal delivery elicited local and systemic CD8⁺ T lymphocytes and very intense antibody response as compared to non encapsulated DNA. Even a single immunization induced M/M2 specific IgA in nasal and vaginal secretions and the expression of vaccine antigen was restricted to the vaginal epithelium and lasted for a short time (<5 days). The encapsidation complements the high-quality DNA vaccines and seems very promising in mucosal immunization.

MALT as the integrated mucosal immune system; the choice of vaccination route

Many experimental data suggest that MALT is a unique structural and functional complex (Fig. 2) and immunity induced at one mucosal site disseminates to other (even distal) mucosal sites providing there also a highly-expressed immune response and protection. For example, oral delivery of *Helicobacter pylori* or *Campylobacter jejuni* (targeting intestinal M-cells by UEA-1 lectin agglutinated bacteria) induced a significant IgG and IgA antibody response in vaginal mucosa [32]. Similarly, using replication-defective adenovirus as a vector of herpes simplex gB (gB, viral envelope glycoprotein B) to colorectal mucosa, Zhu et al. [33] demonstrated that this immunization strategy provides strong immune response and protection against the challenge with the pathogenic HSV-2 virus, at both rectal and vaginal mucosa. The results obtained on a mouse experimental model are in agreement with the observations in humans. A live attenuated typhoid vaccine was administered twice to healthy female volunteers orally or/and rectally. Irrespective of the route of primary vaccine administration, specific IgA and IgG antibodies induced by primary vaccination in the genital tract were enhanced by subsequent rectal immunization [34].

Mucosal *Toxoplasma gondii* vaccination

Toxoplasma gondii, a cosmopolitan intracellular protozoan parasite, infects the host (humans and other mammals) through the oral route by ingestion of either tissue cysts (contaminated meat products) or oocysts (contaminated soil). The disease is of major medical (congenital toxoplasmosis, neurotoxoplasmosis in immunosuppressed individuals etc.) and veterinary (abortions and stillbirths in farming animals) importance [35]. The only commercially available vaccine (Toxovax) contains live attenuated *T. gondii* tachyzoites of S48 strain and is used in a limited range of countries for immunoprevention of congenital toxoplasmosis causing a decrease in the frequency of abortions in sheep and goats. Animals should be given intramuscularly a single dose at least 3 weeks prior to mating. The vaccine shows many disadvantages and is not suitable for humans. Many laboratories have been conducting intense research on the development of vaccines for medical and veterinary uses [34]. Because of the oral infection route, toxoplasmosis is one of the parasitoses which could be eradicated by efficient mucosal vaccination.

Till now, few studies have been carried out using the intranasal route to induce protective immunity in laboratory mice and *T. gondii* SAG1 antigen, a main surface antigen and marker of tachyzoites. For instance, Velge-Roussel et al. [37] found that twofold intranasal immunization of CBA/J mice with the natural *T. gondii* SAG1 antigen, adjuvanted with a cholera toxin, induced protective immunity in both NALT and GALT regions which was determined by proliferative T-cell responses, and reduction in brain cysts levels (50–60%) in mice challenged with low virulent and cyst forming 76 K *T. gondii*. The adoptive transfer of cervical and mesenteric lymphoid cells as well as intraepithelial lymphocytes of vaccinated mice confirmed additionally the obtained vaccination results.

For the first time in 2008 Igarashi et al. [38] tested a composition of recombinant instead of native *T. gondii* antigens (ROP2, GRA5, and GRA7, present in all development stages), supplemented with the cholera toxin adjuvant. Intranasal immunization with the trivalent vaccine (2 doses) followed by the oral challenge with VEG strain tissue cysts induced only a partial protection – the number of brain cysts was reduced by 58.3%.

A very interesting experimental vaccine trial was described by Cong et al. [39], who used attenuated

Salmonella typhimurium rods to deliver a recombinant plasmid encoding both *T. gondii* surface antigens (SAG1 and SAG2) and cholera toxin as an adjuvant. Intra-gastric (3 times) immunization of BALB/c mice resulted in the induction of Th1-biased humoral and cellular immune responses. After intraperitoneal challenge with the highly virulent RH strain the survival time of the mice was significantly prolonged as compared to all control groups and 40% survival rate was achieved. The efficacy of a similarly constructed vaccine, encoding the SAG1 antigen only, was lower and directly proportional to the number of *S. typhimurium* rods [40]. Approaches to immunization with nucleic acids are focused on DNA. The exception is the work of Dimier-Poisson et al. [41] in which the effectiveness of RNA vaccination (3 doses) by using the intranasal route was evaluated. Infection of C57BL/6 mice with a lethal dose of 76 K *T. gondii* cysts revealed a 50% survival rate, whereas a challenge with a sublethal parasite dose resulted in a 32% decrease in the brain cysts number.

Toll-like receptor ligands of parasites (e.g., profilin) are potentially attractive mucosal adjuvants. Heldhi et al. [42] demonstrated that *Eimeria* profilin-like protein would serve as an efficacious mucosal adjuvant through TLR11 activation. Although intranasal immunization elicited weak humoral and cellular response, the immunized mice were significantly protected against chronic toxoplasmosis (50% brain cyst reduction).

As mentioned above, *T. gondii* infections is an important cause of fetal mortality in sheep and therefore these farm animals should be one of the primary targets for vaccine development. *T. gondii* tachyzoites (RH strain) lysate as crude antigen preparation was encapsulated into PLG (poly(D,L-lactide-co-glycolide) microspheres, adjuvanted or not with a cholera toxin, and then used for intranasal immunization (3 times×200 µg) of young seronegative hogs. The vaccination elicited strong IgA nasal response, which persisted for several weeks, and induced also systemic cell-mediated immunity (lymphoproliferation and IFN-γ) but none of the immunized animals was protected against oral challenge with *T. gondii* oocysts [43].

Summarizing, so far few experimental approaches into mucosal vaccination showed admittedly promising results related to vaccine immunogenicity but the protection against highly

virulent and low virulent, cyst-forming *T. gondii* strains was not satisfactory.

Final remarks and comments

In general, mucosal vaccines represent many advantages as compared with the parenteral vaccines; they are safe, inexpensive, easy to administer, non-hazardous, and highly efficacious (induce both local and systemic protective immunity). Despite several advantages, only the limited number of mucosal vaccines was approved due to some challenges and unsolved problems. Recently Azizi et al. [44] summarized the advantages and disadvantages of each possible route of mucosal immunization (oral, nasal, rectal, sublingual, genital, and inhalation delivery). The most attractive and promising seems an oral vaccination although the route is also associated with the potential problems, as requiring a large antigen dose and harsh conditions in gastrointestinal tract which could damage vaccine preparation. Besides, several experimental data indicate that repeated oral administration of the antigen in high doses results in decreased T-cell mediated immune response to the parenteral immunization with the same antigen [45]. Thus, achieving effective antigen-specific immune response using the oral route, obstacles such as induction of oral tolerance and inefficient antigen delivery should be resolved. Another significant factor modulating immunological response to mucosal vaccines may also be natural microbiota, particularly abundant in the gastrointestinal tract and individually diversified. The influence of microorganisms is associated not only with their immunoregulatory activity but also with potential conversion of vaccine material driven by numerous fully metabolically active microorganisms at the mucosa [46]. Using BALT as an entry site for vaccine antigens is also quite difficult because BALT is not presenting obligatory in all species and age groups. A new concept would involve inducing BALT and/or increase its activity by local stimulation with MALP-2, an agonist of TLR2/6 [47]. Besides, several recent data showed that oral vaccination could prevent infections transmitted through non-mucosal routes (e.g. hepatitis B, malaria etc.) [48]. Thus, before new generation mucosal vaccines will be implemented for wide use, any particular problems should be first identified and then solved.

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