M Cell-Targeted Mucosal Vaccine Strategies

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Abstract Immune responses in the aerodigestive tract are characterized by production and transport of specific IgA antibodies across the epithelium to act as a first line of defense against pathogens in the external environment. To sample antigens on mucosal surfaces in the intestine and upper respiratory tract, the immune system relies on a close collaboration between specialized antigen-sampling epithelial M cells and lymphoid cells. Depending on various factors, local antigen presentation in the mucosal tissue leads to tolerance or initiation of an active immune response. Recently, molecules that could be used to target vaccine antigens to apical M cell surfaces have been identified. Here we review the M cell-targeted vaccine strategy, an approach that could be used to enhance uptake and efficacy of vaccines delivered in the nasal cavity or intestine.

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1 Introduction

Mucosal surfaces provide a first line of defense against the plethora of potential pathogens and nonself antigens that perturb the host. Since mucosal tissues normally harbor a vast variety of commensal microorganisms, the mucosa is a site of continuous stimulation requiring tolerance to the normal flora, but immune reactions to pathogens (Kiyono et al. 2008). These tissues are protected by secretory IgA (*S*IgA) antibodies which constitute greater than 80% of all antibodies produced in mucosa-associated tissues of humans. Mucosal IgA antibodies are induced, transported, and regulated by mechanisms that are completely different from those used to generate systemic antibody responses (Craig and Cebra 1971). The mucosal immune system can be subdivided into two primary components: the organized mucosa-associated lympho-reticular tissues (MALTs), where antigen-specific T and B cells are activated and imprinted with mucosal homing molecules for direct migration to specific effector sites, and the diffuse lamina propria regions or glandular tissues, which serve as the effector sites for synthesis of polymeric IgA and the execution of T cell responses (Kiyono and Fukuyama 2004; Kunisawa et al. 2005).

MALTs are situated along the surfaces of various mucosal tissues and include the nasopharynx-associated lymphoid tissue (NALT), bronchus-associated lymphoid tissue (BALT), and the gut-associated lymphoid tissue (GALT) such as Peyer's patches (PP), isolated lymphoid follicles, and colonic patches. The most extensively studied MALTs are PP and NALT in the digestive and airway tissues, respectively (Kiyono and Fukuyama 2004). Peyer's Patches usually number 8 to 10 in the small intestine of mice and hundreds in humans. In rodents, NALT is found on both sides of the nasopharyngeal duct dorsal to the cartilaginous soft palate. Humans generally do not share the same anatomical features of NALT, except possibly at an early age (Debertin et al. 2003), but they do possess oropharyngeal lymphoid tissues, including unpaired nasopharyngeal tonsils (adenoids), bilateral tubular palatines, and the lingual tonsil, which together are known as Waldeyer's ring and appear to be functionally equivalent to murine NALT (Kiyono and Fukuyama 2004).

The induction of SIgA responses is known to be generally dependent on cognate help provided by $CD4^+$ T helper (Th) cells in MALT. The antigen-specific lymphocytes in MALT are programmed to home primarily local effector tissues using a mucosal imprinting system which bridges mucosal inductive and effector sites, whereas lymphocytes activated in peripheral lymph nodes have a distinct systemic homing program (Kiyono and Fukuyama 2004; Kunisawa et al. 2008). The MALT contains antigen-presenting cells (APC) of all major types, including several dendritic cell (DC) subsets, macrophages, and MHC class II^+ B cells for initiation of mucosal immune responses. In addition, germinal centers are present with a high frequency of surface IgA^+ B cells and inter-nodular zones predominantly populated by $CD4^+$ and $CD8^+$ T cell subsets (Kiyono et al. 2008). The MALT is the site for the induction of regulatory T cells (T_{reg} cells) in addition to Th1 and Th2 cells (Izcue et al. 2009; Lloyd and Hawrylowicz 2009; Murai et al. 2009; Unutmaz and Pulendran 2009). Recently, it has been shown that some of these T_{reg} cells differentiate into follicular helper T cells (T_{FH} cells) which support IgA B cell responses in MALT (Tsuji et al. 2009). The $CD4^+$ T cell axis between T_{reg} and T_{FH} cells would account for the simultaneous induction of tolerance and IgA responses in mucosal compartments. Another important trait of MALTs is that these lymphoid tissues are covered by a specialized epithelium, termed follicle-associated epithelium (FAE), which contains micro-fold or membranous (M) cells, a professional antigen-sampling epithelial cell specialized for the uptake of antigens from the lumen of the aerodigestive tract (Bockman and Cooper 1973; Owen and Jones 1974). M cells have also been identified in the crypt epithelium of human tonsils and adenoids (Karchev and Kabakchiev 1984).

The development of effective mucosal vaccines will require the precise characterization and understanding of the molecular and cellular mechanisms involved in the antigen-sampling, -processing and -presentation initially executed by M cells and APC in MALT for the induction of protective immunity. In this article, we will summarize the recent progress made in the characterization of airway and digestive tract M cells. In particular, we will focus on the development of M cell-targeted vaccines for prevention of diseases in the aerodigestive tract.

2 Characterization of M Cells in the Aerodigestive Tract

2.1 Immunobiological Features of M Cells

Originally described as part of the FAE associated with GALT and NALT, M cells have shared unique structural features in comparison with other mucosal epithelial cells. M cells have tight junctions and desmosomes in contact with adjacent columnar epithelial cells and interdigitating lateral membranes (Fig. 1). The processes on their luminal surfaces are spaced more widely and are often shorter and more irregular in shape than the microvilli of absorptive cells (Kraehenbuhl and Neutra 2000; Neutra et al. 1996; Niedergang and Kraehenbuhl 2000).

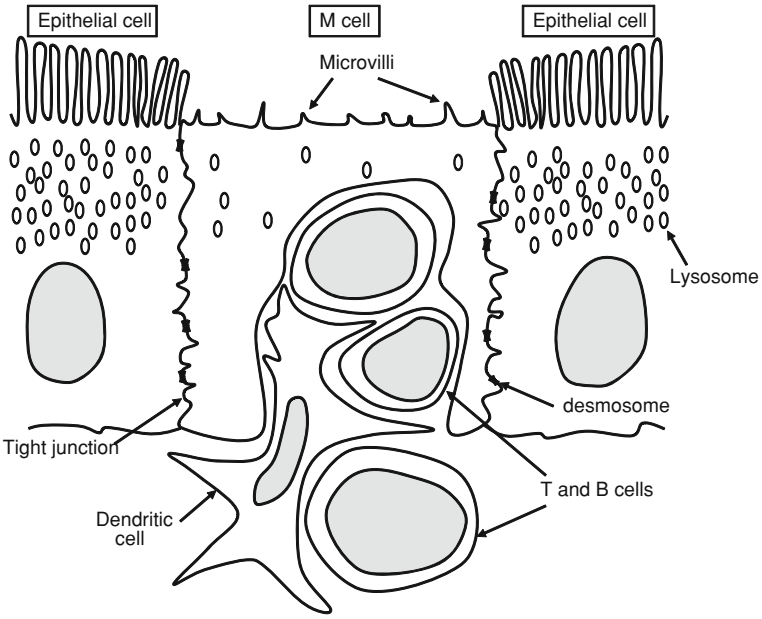

Fig. 1 Diagram of M cells in the follicle-associated epithelium. M cells have tight junctions and desmosomes in contact with adjacent columnar epithelial cells. Luminal surfaces of M cells are characterized by short microvilli, a thin mucus layer, small cytoplasmic vesicles, a reduced glycocalyx and sparse lysosomes. The basolateral surface of the M cells forms an intraepithelial pocket that contains DC, T cells or B cells

Furthermore, compared with adjacent columnar epithelial cells, they are characterized by short microvilli, a thin mucus layer, small cytoplasmic vesicles, a reduced glycocalyx, sparse lysosomes, and a deep invagination of their basolateral membrane which enfolds lymphoid cells (Frey et al. 1996; Kraehenbuhl and Neutra 2000; Neutra et al. 1996; Niedergang and Kraehenbuhl 2000). These unique features allow M cells to selectively and efficiently transfer inhaled or ingested luminal antigens to APC located in the M cell pocket or directly below in the FAE of MALT (Kraehenbuhl and Neutra 2000). M cells take up macromolecules, particles, and microorganisms by adsorptive endocytosis via clathrin-coated pits and vesicles (Neutra et al. 1987; Sicinski et al. 1990), fluid-phase endocytosis (Bockman and Cooper 1973; Owen 1977), and phagocytosis involving extension of cellular processes and reorganization of sub-membrane actin assemblies (Jones et al. 1994). All of these uptake mechanisms result in the transport of foreign material into endosomal tubules and vesicles and large multivesicular bodies that lie between the apical membrane and the intraepithelial pocket (Neutra et al. 1987). Immunocytochemical analysis has revealed the presence of an endosomal protease, cathepsin E, in rabbit M cells (Finzi et al. 1993), but the possible presence of other endosomal hydrolases in M cell transport vesicles has not yet been examined.

M cells are defined by a combination of the above described morphologic features and the presence of the fucose epitope recognized by the *Ulex europaeus* agglutinin-1 (UEA-1) lectin on mouse M cell membrane (Kraehenbuhl and Neutra 2000; Neutra et al. 1996). Recently, we generated a novel M cell-specific monoclonal antibody (NKM 16-2-4). This antibody reacts with murine M cells in FAE of PP, but not with epithelial cells or goblet cells (Nochi et al. 2007). M cells have been shown to develop in villous epithelium in addition to the FAE of organized lymphoid tissues in the intestine (Jang et al. 2004). These cells, termed villous M cells, take up bacteria, as well as bacterial antigens, for subsequent induction of antigen-specific immune responses (Jang et al. 2004), suggesting that villous M cells could be an alternative to the FAE-dependent antigen-sampling pathway. NKM 16-2-4 reacts with villous M cell. Thus, it is considered a pan-marker for murine PP and villous M cells (Nochi et al. 2007).

2.2 Origin of M Cells

The origin of M cells and the regulation of their development are still controversial. One study showed that intravenous injection of PP lymphocytes into severe combined immunodeficient mice resulted in the formation of new lymphoid follicles and FAE with typical M cells (Savidge and Smith 1995). A similar phenomenon was seen in vitro when co-culture of PP B cells with an enterocyte cell line triggered the conversion of enterocytes into M cell-like epithelial cells (Kerneis et al. 1997). Furthermore, B cells have been proposed to play a role in the organogenesis of the mucosal immune barrier system (Golovkina et al. 1999). Two different strains of B cell-null mice have exhibited drastic reductions in FAE size and M cell numbers (Golovkina et al. 1999). On the other hand, others have found that the absence of mature T and B cells does not prevent the formation of FAE and M cells, and instead suggest that signaling of lymphotoxin α/β from non-B and non-T cells plays a critical role in formation of M cells in FAE of PP (Debard et al. 2001).

2.3 Role of DC in Aerodigestive Tract

In addition to M cells, DC in the lamina propria extend their dendrites into the lumen and sample antigens (Chieppa et al. 2006; Niess et al. 2005; Rescigno et al. 2001). A recent study has suggested that these lamina propria DC are capable of initiating systemic IgG responses, whereas antigen transport by M cells into the PP is required for induction of intestinal IgA responses (Martinoli et al. 2007), a finding consistent with the report that DC in PP are responsible for intestinal IgA production (Fleeton et al. 2004). Villous M cells and intraepithelial DC have been reported in the respiratory tract (Jahnsen et al. 2006; Teitelbaum et al. 1999). Furthermore, we recently demonstrated the presence of M cells in the single layer of epithelium covering the nasal cavity turbinate in addition to the FAE in NALT

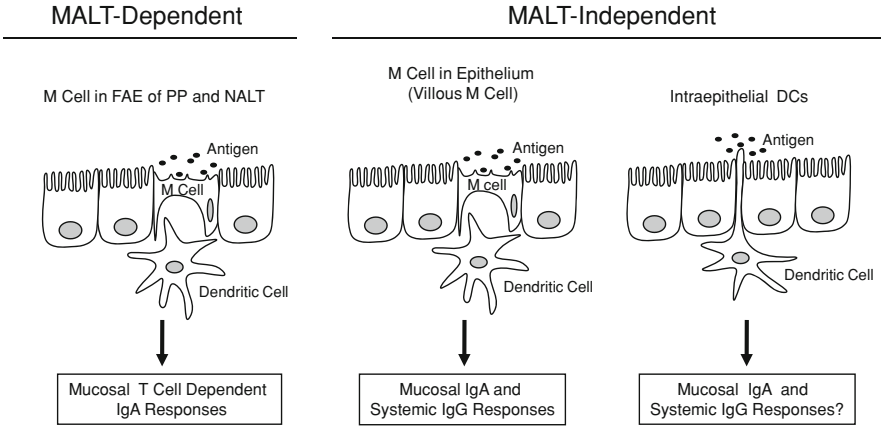

Fig. 2 MALT-dependent and -independent antigen-sampling system at aerodigestive surfaces. Antigens are captured by M cells located in follicle-associated epithelium (FAE) of lymphoid follicles, intestinal villi or the epithelial cell layer in the nasal cavity. The antigens are then transported to subepithelial DC for processing and presentation. Alternatively, lamina propia or intraepithelial DC extends their dendrites through the epithelial layer for direct capture of luminal antigens. Antigen uptake through M cells in FAE of MALT leads to the induction of mucosal IgA responses. On the other hand, M cells located in the intestinal villi or nasal epithelium as well as intraepithelial DC are thought to play a critical role in the induction of systemic IgG responses in addition to mucosal IgA

(submitted for publication). Taken together, these results suggest that tissue in the aerodigestive tracts is equipped with a diversified antigen-uptake and presenting system which consists of MALT M cells, villous M cells, lamina propria DC, and intraepithelial DC (Fig. 2).

3 Targeting Vaccines to Nasal M Cells for Induction of Specific IgA and CTL

Although most human pathogens infect the host by means of a mucosal surface, the majority of vaccines are administered intramuscularly, a route that poorly induces antigen-specific mucosal immune responses (Ogra et al. 2001). It is essential to select a mucosal route of vaccination to induce mucosal immune responses. Nasal administration of vaccines is preferred to oral administration because the former can be used to deliver vaccine without degradation by digestive enzymes and acids. Because M cells are extremely efficient in the uptake of luminal antigens, it is a logical strategy to target vaccines to these cells. In this regard, several approaches incorporating an M cell-specific lectin (Manocha et al. 2005; Wang et al. 2005), peptide (Higgins et al. 2004) or microbial invasion molecules of reovirus or Yersinia (Clark et al. 1998; Wang et al. 2003; Wu et al.

Table. 1 M cell-targeting molecules for development of mucosal vaccine

M cell ligands
Ulex europaeus agglutinin-1 (UEA-1)
Reovirus protein $\sigma 1$
Yersinia invasin
YQCSYTMPHPPV
M cell-specific molecules
NKM 16-2-4 (specific for $\alpha(1,2)$-fucose-containing carbohydrate moiety)
FimH of enterobacteria (specific for glycoprotein 2 expressed by M cells)

2001) have been tested as M cell-targeting delivery vehicles for nasal or oral vaccines (Table 1).

In our mucosal DNA vaccination studies, recombinant reovirus protein $\sigma 1$, which can bind NALT M cells, has proved effective as an antigen delivery molecule. It facilitated nasal immunization with luciferase and β-galactosidase plasmid reporter gene constructs (Wu et al. 2000, 2001). Furthermore, nasal delivery of a human immunodeficiency virus type 1 (HIV-1) envelope protein-encoding plasmid complexed to protein $\sigma 1$ via poly-L-lysine successfully induced potent and long-lived HIV-specific cytotoxic T lymphocytes (CTL) in both respiratory and systemic lymphoid tissues (Wang et al. 2003).

Alternative M cell ligands have also been tested, including UEA-1 (Table 1). When mice were nasally immunized with UEA-1-poly-L-lysine complexed to plasmid encoding HIV envelope, significant envelope-specific mucosal and systemic antibodies, as well as CTL, were induced (Wang et al. 2005). These findings suggest that targeted delivery of mucosal DNA vaccines to M cells could be highly effective for induction of cellular and humoral immunity against infectious diseases (Fig. 3).

4 Intestinal M Cell-Targeted Vaccine Strategies

Nasal immunization has proven to be an effective method for stimulating both mucosal and systemic immunity. However, nasal immunization can be problematic due to the possible migration of vaccine antigen, adjuvant, and/or delivery molecule into the central nervous system via the olfactory nerves. Nasally administered cholera toxin (CT) and adenovirus vectors have been shown to temporally accumulate in the olfactory nerves and epithelial regions of mice (Lemiale et al. 2003; van Ginkel et al. 2000). Clinical studies have also linked *Escherichia coli* heat-labile enterotoxin (LT)-based adjuvants with the development of Bell's palsy in nasal vaccine recipients (Lewis et al. 2009; Mutsch et al. 2004). This has raised concerns about potential health threats posed by nasal vaccines. It should be noted that the targeted delivery of vaccine to M cells in the respiratory tract could overcome this problem. Nevertheless, oral vaccine delivery may be safer in some cases, and oral immunization would be more effective for induction of immune responses in the intestine. A practical advantage of oral vaccination is the lack of necessity for

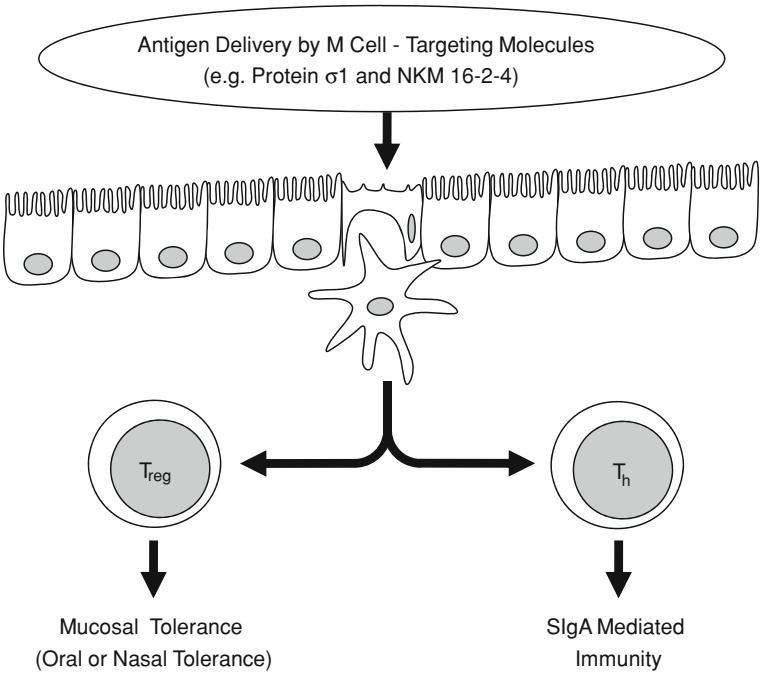

Fig. 3 FAE M cell-targeting antigen delivery system for induction of active or quiescent immunity. Different fusion molecules consisting of antigen and M cell-targeting molecules have been shown to effectively induce Th2 type cell-mediated protective immunity and/or T_{reg} cell-mediated mucosal tolerance

delivery devices (e.g. nebulizers, needles) but the obstacles are greater compared to other immunization routes because of the extremely large surface area and harsh degradative environment of the gastrointestinal tract.

Despite the hurdles, the success of oral poliovirus and rotavirus vaccines (Holmgren and Czerkinsky 2005) has encouraged many mucosal immunologists and vaccinologists to tackle the challenges associated with oral vaccine development. Oral administration of vaccine antigens in conjunction with enterotoxin adjuvants (CT, LT, or nontoxic mutant derivatives thereof) has been shown to effectively induce antigen-specific protective immune responses in both mucosal and systemic compartments (de Haan et al. 1996; Di Tommaso et al. 1996; Douce et al. 1997; Giuliani et al. 1998; Yamamoto et al. 1997, 1998). Moreover, the concept of M cell targeting has also been applied for the development of oral vaccines. For example, antigen expressed in attenuated *Salmonella* vectors, which can bind to M cells, has stimulated antigen-specific mucosal immune responses (Yamamoto et al. 2001). As described above, several M cell-specific molecules have been examined as M cell-targeted delivery vehicles for mucosal vaccines (Clark et al. 1998; Higgins et al. 2004; Manocha et al. 2005; Wang et al. 2003, 2005; Wu et al. 2001). However, some of these molecules are not solely

M cell-specific and bind to other neighboring cells. The murine M cell-specific UEA-1 lectin also reacts strongly with goblet cells and the mucus layer covering the intestinal epithelium (Kandori et al. 1996). Since the NKM 16-2-4 M cell-specific monoclonal antibody has been shown to be solely specific (Nochi et al. 2007), an obvious experiment was to test whether NKM 16-2-4 could be used as a carrier for M cell-targeted oral vaccines (Table 1). Indeed, oral administration of CT adjuvant with a chimeric vaccine consisting of botulinum toxoid (BT) and NKM 16-2-4 induced strong antigen-specific IgG and mucosal IgA responses, as well as protective immunity against lethal challenge with botulinum toxin (Nochi et al. 2007). Considering the anatomical and physiological conditions of the gastrointestinal tract, the selective targeting of vaccine to M cells should allow for lowering of vaccine dosage since the antigen can be specifically delivered to the inductive tissue. In this regard, it should be noted that because of the M cell targeting ability of NKM 16-2-4, as little as 50 μg BT was sufficient for the induction of protective immunity in this murine model. An epitope analysis indicated that NKM 16-2-4 distinguishes α(1,2)-fucosylated M cells from goblet cells containing abundant sialic acids neighboring the α(1,2) fucose moiety and from non-α(1,2)-fucosylated epithelial cells (Nochi et al. 2007). These results suggest that the use of monoclonal antibody NKM 16-2-4 to target vaccine antigens to the M cell-specific carbohydrate moiety could be highly effective for delivery of vaccines into the intestinal mucosa (Fig. 3).

In addition to NKM 16-2-4, glycoprotein 2 (GP2) has been found to be specifically expressed in the apical plasma membrane of PP M cells (Terahara et al. 2008). A recent study has shown that GP2 selectively binds a subset of commensal and pathogenic enterobacteria, including *E. coli* and *Salmonella typhimurium*, by recognizing FimH, a component of type I pili on the bacterial outer membrane (Hase et al. 2009). Interestingly, deficiency of bacterial FimH or host GP2 leads to defects in transcytosis of type-I-piliated bacteria through M cells, resulting in an attenuation of PP-mediated antigen-specific immune responses (Hase et al. 2009). These findings suggest that the GP2-dependent transcytotic pathway could provide another target for delivery of mucosal vaccines to M cells.

5 Targeting M Cells for Induction of Tolerance

Oral administration of a single high dose or repeated low doses of protein has been shown to induce mucosal tolerance (Mowat 2003; Weiner 2000; Xiao and Link 1997). The former mode induces tolerance by clonal anergy/deletion of effector cells, whereas the latter, based on repeated low-dose administration, causes active suppression of effector cells (Faria and Weiner 1999; Fujihashi et al. 2001a, b; Jun et al. 2005; Xiao and Link 1997). Previous studies have demonstrated that PP (Fujihashi et al. 2001a, b; Mowat 2003) and NALT (Kiyono and Fukuyama 2004; Wu et al. 2001) actively facilitate immunity or unresponsiveness by luminal antigen sampling (Fleeton et al. 2004; Weiner 2000; Wu et al. 2001) via M cells,

suggesting that M cells play a crucial role in the induction of mucosal tolerance. Previous studies have demonstrated that mucosal administration of ovalbumin fused to reovirus protein $\sigma 1$ (OVA-p$\sigma 1$) induces a state of unresponsiveness in both mucosal and systemic lymphoid tissues (Rynda et al. 2008; Suzuki et al. 2008). In fact, tolerance could be achieved even with a single low dose of OVA-p$\sigma 1$ delivered either nasally (Rynda et al. 2008) or orally (Suzuki et al. 2008). In contrast, parenteral delivery of OVA-p$\sigma 1$ failed to induce tolerance to OVA. Interestingly, the p$\sigma 1$-induced mucosal tolerance resisted co-treatment with the potent mucosal adjuvants, CT and CpG oligodeoxynucleotides, and tolerance was not broken after peripheral challenge with OVA (Rynda et al. 2008). In contrast, mucosal tolerance established by OVA without an M cell targeting vehicle has typically been abrogated by the presence of mucosal adjuvants (Lycke 2005).

It is now generally agreed that mucosal tolerance is established and maintained at the levels of T cells. Such suppression occurs via activation of specific regulatory cells, among which $CD25^+$ $CD4^+$ T regulatory (T_{reg}) cells have been best described (Fujihashi et al. 2001a, b; Weiner 2000). Specific T_{reg} cells are known to express the nuclear forkhead box P3 (FoxP3) transcription factor and suppress the immune response in an IL-10- and/or TGF-β-dependent fashion (Faria et al. 2003; Sakaguchi et al. 2006, 2008). Interestingly, $FoxP3^+$ $CD25^+$ $CD4^+$ T cells secreting IL-10 and TGF-β were significantly increased in the mucosal compartment after nasal or oral administration of OVA-p$\sigma 1$ (Rynda et al. 2008; Suzuki et al. 2008). Furthermore, adoptive transfer of OVA-p$\sigma 1$-primed $CD25^+$ $CD4^+$ or $CD25^-$ $CD4^+$ T cells significantly inhibited antigen-specific proliferation of OVA-transgenic $CD4^+$ T cells. This suppression was due to increased production of IL-10 by OVA-p$\sigma 1$-induced T_{reg} cells, as evidenced by the lack of OVA-specific tolerance in OVA-p$\sigma 1$-dosed IL-$10^{-/-}$ mice (Rynda et al. 2008). Mucosal administration of OVA-p$\sigma 1$ also induced clonal deletion of OVA-specific $CD4^+$ T cells (Rynda et al. 2008; Suzuki et al. 2008), offering an additional suppressive mechanism for protein $\sigma 1$ if it can survive delivery beyond the initial cell binding to mucosal epithelium or M cells. Taken together, these findings suggest that reovirus protein $\sigma 1$-mediated targeting of protein antigen to M cells could be an effective strategy for establishing tolerance (Fig. 3). Because the nasal and oral routes provide an easy way to administer antigens, vaccines, or drugs, the M cell-targeting protein antigen delivery system for induction of mucosal and systemic unresponsiveness could provide important advantages for the development of therapeutic approaches to treat diseases.

6 Conclusion and Future Directions

Recent progress in our understanding of the molecular and cellular characteristics of M cells in the aerodigestive tract has allowed testing the possibility of M cell-targeted vaccines. The mucosal immune system is a remarkable defense mechanism that provides the means to generate highly specific responses against a

myriad of potentially pathogenic microorganisms that invade via mucosal surfaces. The mucosal immune system consists of several distinct but harmonized antigen-sampling and presentation mechanisms for MALT-dependent and -independent induction of inhaled and/or ingested antigen-specific immune responses. Of course, one of the major portals of antigen entry is M cells. Despite the recent advances in our knowledge, we still lack a global view of how M cells develop and orchestrate mucosal immune responses after sampling antigens in the lumen of the aerodigestive tract. The orientation towards tolerance or active immune responses is also a critical issue to clarify. Finally, the development of vaccines that trigger mucosal as well as systemic immune responses is of global importance, and the M cell-targeted vaccine strategy offers the potential for safe and effective delivery of mucosal vaccines.

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