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Table I. Distinct molecular features for organogenesis of different MALTs

Mice	TALT	NALT	PP	CLN	MLN	ILF	Cryptopatch	References
Id2$^{-/-}$	+++	–	–	–	–	ND	ND	*1
Rorγt$^{-/-}$	+++	+++	–	–	–	–	+/–	*2
Ltα$^{-/-}$	+	+	–	–	+/–	–	+/–	*3
aly/aly	+	+	–	–	–	–	++	*4
Il-7rα$^{-/-}$	++	++	–	+/–	++	++	–	*5
Cxcl13$^{-/-}$	++	+	+/–	+/–	++	ND	ND	*6
plt/plt	+++	++	++	++	++	ND	ND	*7
Cxcl13$^{-/-}$*plt/plt*	+	+	+/–	–	++	ND	ND	*8

CLN, cervical LN; MLN, mesenteric LN. +++, developed well; ++, developed with decreased number of lymphocytes; +, developed with few number of lymphocytes; –, absent; +/–, present or absent, depends on individual. *1, Yokota et al., 1999; Fukuyama et al., 2002; Boos et al., 2007; *2, Sun et al., 2000; Harmsen et al., 2002; Eberl and Littman, 2004; Eberl et al., 2004; Naito et al., 2008; Tsuji et al., 2008; *3, De Togni et al., 1994; Banks et al., 1995; Suzuki et al., 2000; Fukuyama et al., 2002; Hamada et al., 2002; Harmsen et al., 2002; Taylor et al., 2004; *4, Kanamori et al., 1996; Shinkura et al., 1999; Fukuyama et al., 2002; Hamada et al., 2002; *5, Peschon et al., 1994; Kanamori et al., 1996; Adachi et al., 1998b; Fukuyama et al., 2002; Hamada et al., 2002; Luther et al., 2003; *6, Ansel et al., 2000; Rangel-Moreno et al., 2005; Fukuyama et al., 2006; *7, Nakano et al., 1997; Rangel-Moreno et al., 2005; Fukuyama et al., 2006; and *8, Luther et al., 2003; Rangel-Moreno et al., 2005; Fukuyama et al., 2006.

genesis mechanisms. The organogenesis of secondary lymphoid tissues has been shown to require several processes, including the trafficking/accumulation of LTi cells, the differentiation/activation of specialized stromal cells, and the trafficking/accumulation of conventional lymphocytes (Mebius, 2003). In this light, the genesis of these tissues can be separated into at least two phases, initiation and maturation; in other words, the migration of LTi cells and lymphocytes, respectively, to the tissue development site. Our results indicate that the initiation of TALT genesis operates independently of the requirement for the classical tissue genesis–associated signaling cascade of IL-7R/LTβR–NIK because leukocytes, including B lymphocyte, already migrated to TALT without this pathway. Further, the unique CD3$^-$CD4$^+$CD45$^+$ cells develop without a requirement for the LTi cell–associated transcriptional regulators Id2 and RORγt, and are identified as the first hematopoietic cell population that migrates to the TALT anlagen. To directly address the critical role of Id2- and RORγt-independent CD3$^-$CD4$^+$CD45$^+$ cells (or TALT inducer cells) in the initiation of TALT genesis, our efforts are now directed toward finding and/or developing TALT-deficient mice for the necessary adoptive transfer experiment.

TALT organogenesis occurs after birth, as does NALT genesis (Fukuyama et al., 2002). In contrast, PPs and pLNs are initially generated during the embryonic period (Mebius, 2003). These findings suggest that secondary lymphoid tissue genesis can be chronologically separated into two categories: a prenatal group (PPs and pLNs) and a postnatal group (TALT and NALT). However, initiation of genesis of all of these tissues, including TALT, occurs independently of microbial stimuli.

Ocular surface antigens are taken up by NALT, and NALT might function as an inductive site for tear IgA production (Ridley Lathers et al., 1998). However, our findings suggest that TALT is a key inductive tissue for immune responses, because TALT is a more important site for the generation of antigen-specific T cells than NALT and, thus, contributes to mucosal immune responses against ocularly encountered antigens. In support of this suggestion, our study showed the presence of a mucosal gateway population of M cells in TALT that is capable of taking up ocularly administered bacterial antigens (e.g., *Salmonella*). Ocular infection with *P. aeruginosa* causes corneal ulcers and sometimes loss of vision (Liesegang, 1998), and we found *P. aeruginosa* given by ocular challenge within TALT, leading to the subsequent formation of GCs. These findings indicate that TALT plays an important role in ocular immune surveillance and protection, providing the first line of defense of the host's eyesight; we can therefore expect it to be equivalent in its capacity for immunosurveillance to the other well-known mucosal inductive tissues in the aerodigestive tract, NALT and PPs.

The lacrimal glands are effector sites for IgA production because their tissue contains large numbers of IgA-producing cells (Sullivan and Allansmith, 1984; Peppard and Montgomery, 1987; Saitoh-Inagawa, 2000). We also found that a large number of IgA$^+$B220$^-$ plasma cells were distributed around the diffuse tissues of the NPs and in the tear duct in response to CT immunization via eye drops. Thus, TALT and various tissues of the tear duct are responsible for ocular immunity as inductive and effector sites, respectively.

In summary, our results demonstrated the presence of mouse TALT, providing the first definitive evidence for the existence of Id2-, RORγt-, and LTβR-independent lymphoid tissue genesis. In addition, TALT was shown to play an important role in the induction of antigen-specific immune responses and to function in immune surveillance in ocular immunity.

MATERIALS AND METHODS

Mice. C57BL/6 and BALB/c mice were purchased from Japan SLC; germ-free and *aly/aly* mice were purchased from CLEA Japan; and *Ltα*$^{-/-}$, *Igh6*$^{-/-}$, *Tcrβ*$^{-/-}$, and *Tcrδ*$^{-/-}$ mice were purchased from the Jackson Laboratory. *Il-7rα*$^{-/-}$ mice were provided by Immunex Corp., and were also purchased from the Jackson Laboratory. *Id2*$^{-/-}$, *Rorγt*$^{-/-}$, *Cxcl13*$^{-/-}$, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *MyD88*$^{-/-}$, *Cxcl13*$^{-/-}$*plt/plt*, *Trance*$^{-/-}$, and *Traf6*$^{-/-}$ mice were generated as previously described (Adachi et al., 1998a; Hoshino et al., 1999; Kong et al., 1999; Naito et al., 1999; Takeuchi et al., 1999;

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Yokota et al., 1999; Kurebayashi et al., 2000; Ebisuno et al., 2003; Fukuyama et al., 2006). Animal experiments were conducted in accordance with the guidelines of and with permission provided by the Animal Care and Use Committee of the University of Tokyo.

Histological analysis. Histological analysis was performed as previously described (Fukuyama et al., 2002). The antibodies and lectins used for confocal microscopy analysis were as follows: FITC- or PE-anti-CD11c (HL3; BD), FITC-anti-CD3ε (145-2C11; BD), FITC-anti-IgA (C10-3; BD), PE-anti-B220 (RA3-6B2; BD), PE-anti-CD45 (30-F11; BD), PE- or APC-anti-CD4 (RM4-5; BD), FITC-anti-TCRβ (H57-597; BD), PE-anti-CXCR5 (2G8; BD), rabbit polyclonal anti-AID (H-80; Santa Cruz Biotechnology, Inc.), purified anti-FDC (FDC-M1; BD), purified anti-PNAd (MECA 79; BD), biotinylated anti-MAdCAM-1 (MECA-89; BD), biotinylated anti-VCAM-1 (429; BD), biotinylated peanut agglutinin (PNA; Vector Laboratories), rhodamine-UEA-1 (Vector Laboratories), Alexa Fluor 633-wheat germ agglutinin (Invitrogen), and FITC-NKM16-2-4 (Nochi et al., 2007). To visualize AID, FDC, MAdCAM-1, VCAM-1, PNAd, and PNA, FITC-anti-rabbit IgG (Santa Cruz Biotechnology, Inc.), FITC-anti-rat Ig κ chain (MRK-1; BD), FITC-anti-rat IgM (G53-238; BD), streptavidin-FITC (BD), and streptavidin-PE (eBioscience) were used as secondary antibodies or reagents. In some experiments, tissues were counterstained with DAPI (Sigma-Aldrich) to visualize the nucleus. *P. aeruginosa* PAO1 was detected with a rabbit polyclonal antibody specific for the bacterium (Abcam), followed by staining with FITC-anti-rabbit IgG (Santa Cruz Biotechnology, Inc.).

Ocular administration of bacteria. GFP-*Salmonella* (Jang et al., 2004) and *P. aeruginosa* PAO1 (Parks and Hobden, 2005) were administered as ocular antigens. After a 30-min administration of GFP-*Salmonella*, the mouse's ocular surface was washed with 100 μg/ml gentamycin. *P. aeruginosa* PAO1 is characterized by motility, biofilm formation, acyl-homoserine lactone production, and virulence in a mouse infection model (Parks and Hobden, 2005). These bacteria were cultured in Luria broth medium at 37°C for 18 h and used for ocular administration, as previously described (Jang et al., 2004). PAO1 was given twice with an interval of 1 wk (Hazlett et al., 2001).

Immunization and analysis of antigen-specific immune responses. Mice were ocularly immunized with 1 μg CT per eye (Sigma-Aldrich) in 5 μl PBS by eye drops three times at weekly intervals. Tissues or cells were collected from the heads of the ocularly immunized mice 7 d after the final immunization. To characterize CT-specific antibody responses, a CT-B-specific ELISPOT assay was used. In brief, 96-well plates (MultiScreen; Millipore) were coated with 2 μg/ml CT-B in 100 μl PBS (pH 7.4) per well for 16 h. The plates were washed three times with PBS and blocked with 100 μl of RPMI 1640 supplemented with 10% FCS for 30 min. After the blocking solution was discarded, 100 μl of cell suspension was applied to the well (tear duct, 10^4 cells/well; spleen, 10^6 cells/well). After incubation for 4 h, the plates were washed three times with PBS, followed by three washes with 0.1% Tween-PBS. The plates were incubated with horseradish peroxidase-conjugated anti-mouse IgA or IgG (1:1,000 dilution [vol/vol] in 0.1% Tween-PBS) for 16 h. After the plate had been washed with PBS six times, antibody-producing cells were visualized with AECB-500 and AECM-100 conjugate solutions (Moss, Inc.). Plates were incubated for 30 min and washed with water. The plates were allowed to dry, and spot pictures were taken with a microscope. To detect CT-B-specific T cells, a CT-B/I-A^b tetramer was prepared and used for flow cytometric analysis, as previously described (Chang et al., 2008).

Cell preparation and RT-PCR. $CD3^-CD4^+CD45^+$ cells were isolated from mucosa-associated tissues as previously described (Fukuyama et al., 2006). In some experiments, mononuclear cells were isolated from the TALT, NALT, and NPs of immunized mice by mechanical dissociation (Fukuyama et al., 2002). Total RNA was extracted for RT-PCR as previously described (Shikina et al., 2004). The sequences of primers used were as follows: *Id2*, (sense) 5′-TCTGAGCTTATGTCGAATGATAGC-3′ and (anti-sense) 5′-CACAGCATTCAGTAGGCTCGTGTC-3′; *Rorγt*, (sense) 5′-ACCTCCACTGCCAGCTGTGTGCTGTC-3′ and (anti-sense) 5′-TTGTTTCTGCACTTCTGCATGTAGACTGTCCC-3′; *Gapdh*, (sense) 5′-TGAACGGGAAGCTCACTGG-3′ and (anti-sense) 5′-TCCACCACCCTGTTGCTGTA-3′; *Aid*, (sense) 5′-GGCTGAGGTTAGGGTTCCATCTCAG-3′ and (anti-sense) 5′-GAGGGAGTCAAGAAAGTCACGCTGGA-3′; and *β-actin*, (sense) 5′-TGGAATCCTGTGGCATCCATGAAA-3′ and (anti-sense) 5′-TAAAACGCAGCTCAGTAACAGTCC-3′.

Electron microscopy analysis. Electron microscopy was performed as previously described (Jang et al., 2004). Head tissue containing the tear ducts was prepared and fixed in a solution containing 0.5% glutaraldehyde, 4% paraformaldehyde, and 0.1 M of sodium phosphate buffer (pH 7.6) on ice for 1 h. After washes with 4% sucrose in 0.1 M of phosphate buffer, the tissue was decalcified with 2.5% EDTA solution for 5 d. After three washes, the samples were fixed with 2% osmium tetraoxide on ice for 1 h and dehydrated with a series of ethanol gradients. For scanning electron microscopy, dehydrated tissues were freeze embedded in *t*-butyl alcohol and freeze dried, and then coated with osmium and observed under a scanning electron microscope (S-4200; Hitachi). For transmission electron microscopy, the tissues were embedded in Epon 812 resin mixture, and ultrathin (70-nm) sections were cut with an ultramicrotome (Reichert Ultracut N; Leica). The ultrathin sections were stained with 2% uranyl acetate in 70% ethanol for 5 min at room temperature and then in Reynold's lead for 5 min at room temperature. Sections were analyzed with a transmission electron microscope (H-7500; Hitachi).

Online supplemental material. Fig. S1 shows postnatal organogenesis of TALT on BALB/c mice. Fig. S2 shows development of TALT in B cell- or T cell-null mice and in TLR signaling-null conditions. Fig. S3 shows the lymphoid structure of TALT in mice lacking molecules related to lymphoid tissue genesis. Paraffinized sections were prepared from 8-wk-old mice and stained with the indicated antibodies (PNAd and B220) for confocal microscopy analysis. Arrows indicate PNAd-expressing HEVs. Fig. S4 shows the absence of MAdCAM-1 in TALT and NALT. TALT (A) and NALT (B) of 8-wk-old C57BL/6 mice were stained with DAPI and an anti-MAdCAM-1 antibody, and confocal microscopy analysis was performed. PPs of 10-d-old mice were analyzed as a positive control for anti-MAdCAM-1 antibody (C). Fig. S5 shows the independence of TALT genesis in TRANCE-Traf6 signaling. TALT of 2–3-wk-old *Trance*-deficient and *Traf6*, *Tcrb* double-deficient mice was analyzed histologically with hematoxylin and eosin (HE) staining. $Traf6^{+/-}$, $Tcrb^{-/-}$ mice were examined as a control. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091436/DC1.

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Review

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Mucosal immunosenescence: new developments and vaccines to control infectious diseases

Kohtaro Fujihashi[1] and Hiroshi Kiyono[1,2]

[1] Departments of Pediatric Dentistry and Microbiology, The Immunobiology Vaccine Center, The Institute of Oral Health Research, The University of Alabama at Birmingham, Birmingham, AL 35294-0007, USA
[2] Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

Infection of the aero-digestive tract represents a major disease burden of the elderly, and despite recent advances in our understanding of the mucosal immune system, its immunosenescence remains poorly defined. Age-associated alterations of the intestinal and respiratory immune systems occur at distinct times and in a distinct manner. A reduction in gut-associated lymphoreticular tissues, intestinal antigen-specific IgA antibody responses and lack of oral tolerance induction are all associated with aging. By contrast, nasopharyngeal-associated lymphoreticular tissue function remains intact during aging with notable signs of immunosenescence seen only in the elderly. The distinct timing of mucosal immunosenescence seen between the gut and respiratory system suggests the nasal route of vaccination might be preferable for effective mucosal vaccines in the elderly.

Introduction

The mucosal immune system consists of an integrated network of tissues, lymphoid and mucous membrane-associated cells and innate effector (e.g. mucins and defensins) and acquired (e.g. antibody; Ab) molecules. Along with cytokines, chemokines and their receptors, these effector Ab molecules, which are primarily of the IgA isotype, are key players in mucosal immunity and seem to function in synergy with the innate immune system [1,2]. Mucosal inductive sites include the Peyer's patches (PPs), one of the well-characterized gut-associated lymphoreticular tissues (GALTs), and the Waldeyer's ring of tonsils and adenoids that form the nasopharyngeal-associated lymphoreticular tissues (NALTs). Collectively, these comprise a mucosa-associated lymphoreticular tissue (MALT) network that continuously supplies antigen (Ag)-specific memory B and T cells to diffuse mucosal effector sites [1,2]. The migration of lymphocytes from inductive to mucosal effector tissues is the basis for the concept of the mucosal immune system, where either nasal or oral vaccination induces mucosal immunity in multiple distal effector sites [1,2].

Despite considerable progress in characterizing the mucosal immune system (Box 1) [3–5], we still do not have a clear picture of the age-associated changes that occur to mucosal immunity. This is a crucial gap in our understanding because infections of the respiratory and gastrointestinal (GI) tracts represent the leading cause of morbidity in the elderly. In this review, we will attempt to shed light on the changes occurring to the mucosal immune system during aging, and this knowledge should eventually lead to the development of effective mucosal vaccines for the elderly where it is most desperately needed.

The intestinal microbiota shapes up mucosal immunity: its role in immunosenescence

The mammalian lower intestine contains up to 10^{12} bacteria per gram of intestine [6,7]. The normal microbiota is essential to maintain appropriate homeostatic conditions, providing energy in the form of short-chain fatty acids and nutrients (vitamins K and B_{12}) and protection against colonization by pathogenic bacteria [7–9]. In addition to these functions, the intestinal microbiota plays a major role in maturation of the host immune system including intestinal secretory IgA (SIgA) Ab production and intraepithelial lymphocyte (IEL) development [6,7,10,11]. For example, germ-free (GF) mice have an immature mucosal immune system that includes hypoplastic PPs and diminished numbers of IgA-producing cells and $CD4^+$ T cells [6,12,13]. Adapting GF mice to conventional housing or mono-association of GF mice with *Escherichia coli* results

Glossary

AID: activation-induced cytidine deaminase
FAE: follicle associated epithelium
GALT: gut-associated lymphoreticular tissue
GC: germinal center
GI: gastrointestinal
M cell: microfold cell
MALT: mucosa-associated lymphoreticular tissue
MLN: mesenteric lymph node
NALT: nasopharyngeal-associated lymphoreticular tissue
nCT: native cholera toxin
PP: Peyer's patch
SIgA: secretory immunoglobulin A
TT: tetanus toxoid

Corresponding author: Fujihashi, K. (kohtarof@uab.edu).

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Box 1. Organization of the mucosa-associated lymphoreticular tissue

The surface of the mucosa-associated lymphoreticular tissue (MALT) is covered by the specialized follicle associated epithelium (FAE), 10–20% of which is M cells that exhibit a unique topical morphology (i.e. microfold or membranous) and form pockets for the inclusion of lymphoid cells, including B and T cells, dendritic cells (DCs) and macrophages [1,2]. M cells, which have short microvilli, small cytoplasmic vesicles and few lysosomes, are adept at uptake and transport of luminal Ags, including soluble proteins and particulates such as viruses, bacteria, small parasites and microspheres [1,2].

In general, murine MALT [mainly gut-associated lymphoreticular tissue (GALT) and nasopharyngeal-associated lymphoreticular tissue (NALT)] contains follicles [B-cell zones with germinal centers (GCs)] and parafollicular regions enriched in T cells. Distinct follicles (B-cell zones) are located beneath the domed area of the Peyer's patch (PP) or main body of the NALT and contain GCs where significant B-cell division is seen. These GCs, which contain the majority of surface IgA-positive ($sIgA^+$) B cells [1,2], are considered to be sites where frequent B-cell isotype switching to IgA and affinity maturation occur. However, unlike peripheral lymph nodes and the spleen in the systemic compartment, the efficiency of plasma cell development is low. All major T-cell subsets are also found in the T cell–dependent areas adjacent to follicles. The parafollicular T cells are mature cells, and >97% of these T cells use the αβ heterodimeric form of the T-cell receptor (TCR). Approximately two thirds of αβ TCR^+ T cells are $CD4^+$ and exhibit properties of Th1 and Th2 cells, including support for IgA Ab responses [1,2]. Furthermore, recent studies showed the presence of different subsets of regulatory T cells (Treg). Approximately one third of the αβ T cells in MALT are $CD8^+$; this T-cell subset contains precursors of cytotoxic T lymphocytes (CTLs) [1,2]. The immunohistology of murine PPs has shown that $CD11c^+$, $CD11b^+$ and $CD8^-$ immature DCs, which show high endocytic activity and low levels of MHC and costimulatory molecule expression, form a dense layer of cells in the subepithelial area [1,2]. Mature interdigitating $CD11c^+$, $CD11b^-$ and $CD8^+$ DCs with low endocytic activity and high numbers of major histocompatibility complex (MHC) class I and class II as well as B7 molecules have been identified in the interfollicular T-cell regions [1,2]. It has been shown that MALT DCs play essential roles in the induction of mucosal immunity, tolerance and inflammation.

in normal maturation of the mucosal immune system [14,15]. Furthermore, it was reported that bacterial stimulation of human intestinal epithelial cells induced IgA2 subclass switching, the subclass associated with lower digestive tract immunity [16]. Conversely, aberrant expansion of segmented filamentous bacteria was noted in activation-induced cytidine deaminase (AID)-deficient mice that lack an appropriate molecular environment for IgA class switching [17]. Taken together, these results clearly demonstrate a complex interplay between the gut microbiota and the mucosal IgA Ab response and development.

In humans, gut lavages taken from either aged or young subjects were shown to contain comparable IgA levels [18]. A study in mice provided similar results, showing that fecal extract samples from aged mice contained essentially the same levels of IgA as young adult mice [19]. Similar results have also been reported for total IgA responses in the serum of aged mice, rats and humans [19–24]. These results demonstrate a lack of any gross age-associated impairment in total IgA synthesis. Thus, one could predict that a normal quality and number of microbiota is maintained in both aged animals and humans. However, during aging, significant alterations occur in the species composition of intestinal microflora, with proteolytic bacteria including *Fusobacteria*, *Propionibacteria* and *Clostridia* increasing in the elderly, yet the total number of anaerobes is essentially unchanged (Figure 1) [8]. Further, the numbers and species diversity of beneficial or protective anaerobes including *Bacteroides* and *Bifidobacteria* were diminished in aged individuals [8]. These changes in the quality of the microbiota might result in increased putrefaction, a greater susceptibility to gastroenteritis and infections. Further, it is possible that the quality of the SIgA response could be altered in the elderly even though the absolute quantity of these Abs can be unchanged (Figure 1).

As of now, we still do not understand the molecular and cellular relationship between the microbiota and intestinal mucosal immunity in the elderly. Although these black boxes are currently being investigated, especially in the normal adult condition, much work will be required in the future to elucidate the roles of commensal bacteria in the induction, regulation and maintenance of Ag-specific immunity and tolerance in mucosal senescence.

The mucosal immune system in aging

Effect of aging on intestinal immune responses

As mentioned above, the GI tract in the elderly is particularly susceptible to infectious diseases, in part due to changes in their intestinal microbiota but probably also because of dramatic changes to mucosal immunity itself [25,26]. For example, aged rats (>24 months of age) given native cholera toxin (nCT) orally show significantly reduced levels of anti-CT IgA responses than identically immunized young rats (3–6 months of age) [27,28]. Similar results were observed in rhesus macaques given oral nCT [29]. When aged mice (16–24 months of age) were orally immunized with *Haemophilus influenzae* type b oligosaccharide (Hib) conjugated to diphtheria toxoid and nCT as mucosal adjuvant, Hib-specific IgA Ab responses were also reduced compared with those responses seen in young, adult mice (6–8 weeks of age) [30]. These results clearly indicate that orally induced Ag-specific mucosal IgA responses are diminished in aged animals (Figure 2).

Age-associated immune dysregulation occurs in both the mucosal and the systemic immune compartments as early as 12–14 months of age (so-called 'aging' mice as opposed to 'aged' mice) (Figure 2). Groups of mice at 1 (i.e. aging) or 2 years (i.e. aged) of age show reduced levels of Ag-specific mucosal and systemic immune responses when given oral ovalbumin (OVA) as antigen plus nCT (Figure 2) [19]. When Ag-induced cytokine responses were examined at both protein and mRNA levels, $CD4^+$ T cells from the spleen and PPs of young adult mice (8–12 weeks old) revealed elevated levels of interleukin 4 (IL-4) production. However, these cytokine responses were already and significantly diminished in aging mice, because antigen-induced IL-4 synthesis is required for the generation of effector memory cells from naïve $CD4^+$ T cells, a pathway that seems to be defective in aged mice (Figure 2) [19]. By contrast, it has been reported that anti-CD3 mAb stimulation, which provides activation signals for existing memory $CD4^+$ T cells, elicited higher levels of IL-4 synthesis even in senescent animals [31–33]. These findings suggest that, although an age-related reduction of naïve $CD4^+$ T

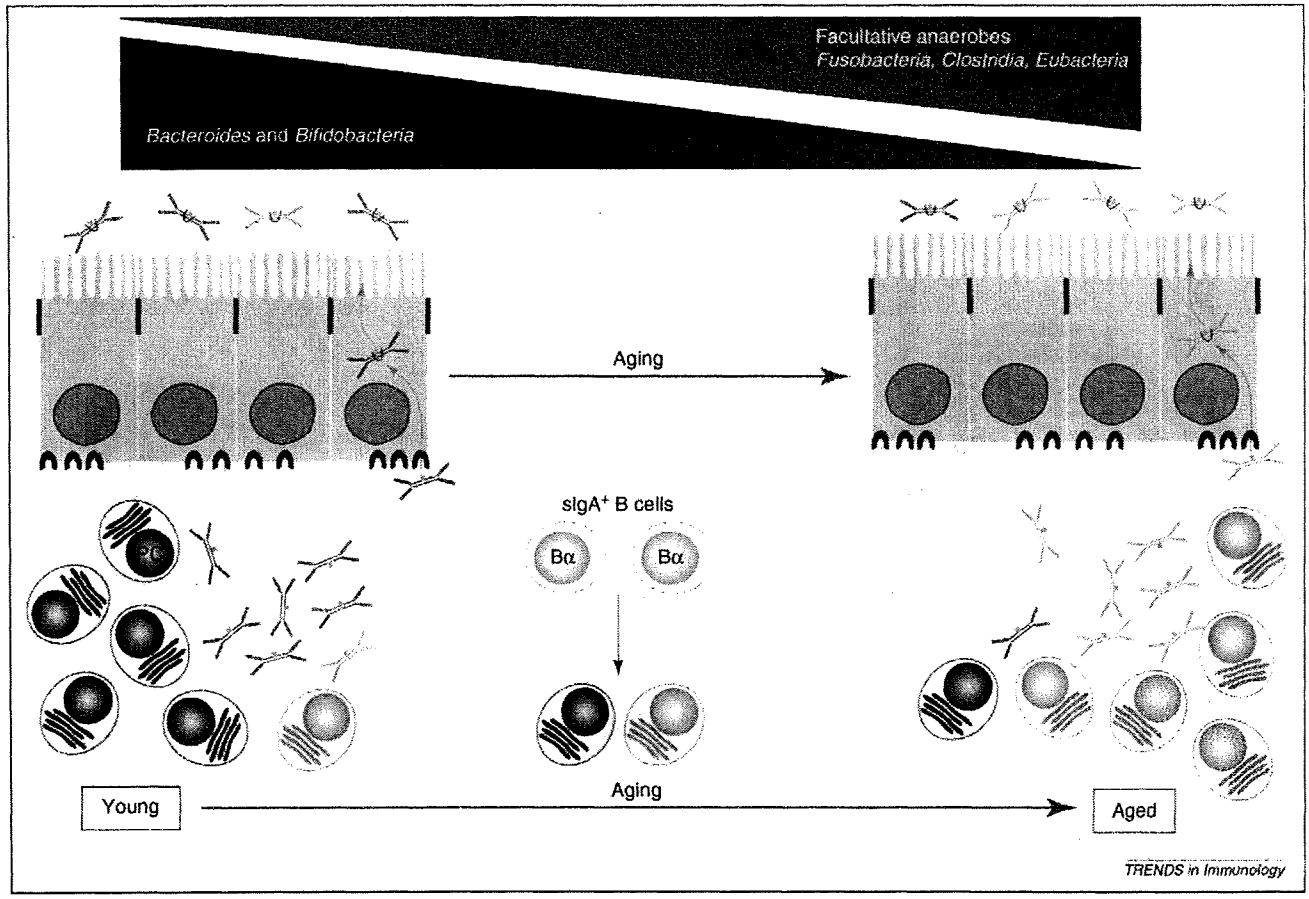

Figure 1. Age-associated changes in the intestinal microbiota of the elderly. The numbers and species diversity of beneficial or protective anaerobes including *Bacteroides* and *Bifidobacteria* diminish in the elderly. This age-related reduction is countered by a rise in *Fusobacteria, Clostridia* and *Eubacteria* species. As a direct result of these age-related changes in the microbiota, the quality of the secretory IgA (SIgA) response can be altered, although the absolute quantity of these Abs are generally unchanged. The alterations in the quality of SIgA Abs are indicated by the different colors of dimeric IgA (blue versus green) secreted by plasma cells. Bα cells indicate surface IgA^+ B cells that differentiate into plasma cells ($sIgA^+$).

cells is evident in the mucosa, a pool of memory T cells remain capable of responding to exogenous stimulation signals. Thus, one possible approach for the development of mucosal vaccines in the elderly might be accomplished by triggering of this memory $CD4^+$ T-cell pool.

When mice were immunized subcutaneously with OVA plus nCT as peripheral adjuvant, impaired OVA-specific, but intact CT-specific, immune responses were seen in the systemic immune compartment of 1-year-old mice [19]. Two-year-old mice showed the poorest priming, with responses to both OVA and CT being depressed. From these studies, one could suggest that the parenteral (i.e. nonoral) immune system in 1-year-old (or aging) mice might be in a transitional stage between a normal and age-associated deficiency (Figure 2). Thus, systemic Ab responses to the weak antigen OVA, which always requires an adjuvant for induction of specific immunity, was impaired in 1-year-old mice; however, nCT, a potent immunogen because of its innate cAMP-dependent adjuvant properties, induced normal plasma Ab responses in these mice. By contrast, mucosal immune responses including both OVA- and CT-specific Ab and cytokine responses, induced by oral OVA and nCT in 1-year-old mice (aging mice), were markedly reduced and were comparable to those seen in 2-year-old mice (aged mice) [19]. These results indicate that age-associated alterations might arise in the mucosal immune system of the GI tract earlier than in the parenteral immune compartment.

Oral tolerance in aging

The induction of mucosal and systemic immunity by oral Ag delivery is rather challenging and requires use of potent mucosal adjuvants, targeting vectors or other special delivery systems. The reason for this difficulty is that a major function of the mucosal immune system is in general to create a physiologically and immunologically quiescent state instead of an active condition [2,34]. Thus, tolerance (including both mucosal and systemic unresponsiveness) represents the most common response of the host to mucosally presented antigens. The continuous ingestion of several thousand different types of food protein is but one important example of oral tolerance, whereas tolerance to our indigenous microflora, which mostly colonizes the large intestine, represents another.

The nature, dose and frequency of Ag feeding, as well as host-related factors such as genetic background and the absence of a microflora in the GI tract, have been shown to influence the induction and maintenance of oral tolerance

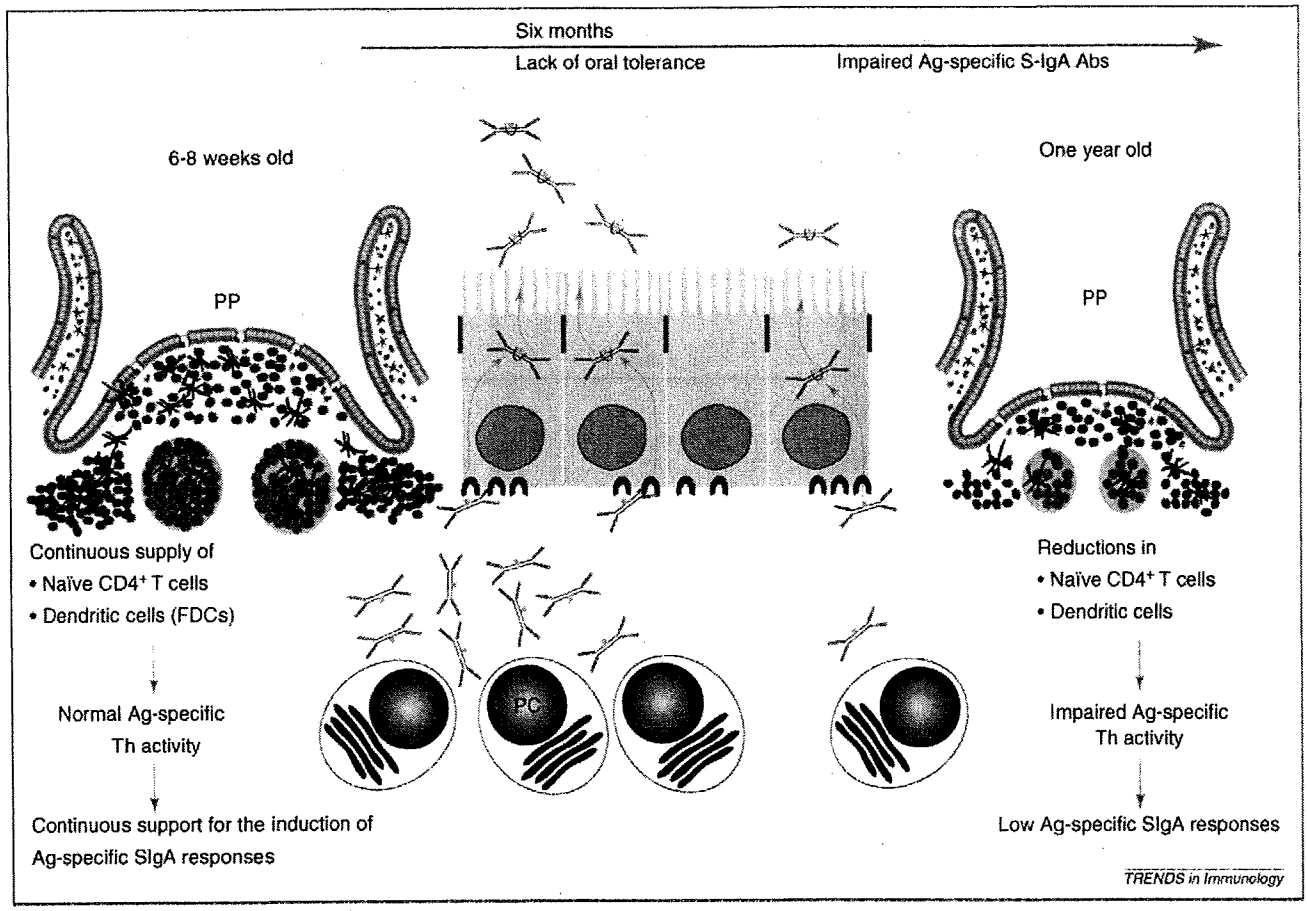

Figure 2. Evidence of early aging in the gastrointestinal (GI) tract. A reduction in Ag-specific intestinal secretory IgA (SIgA) responses can be seen in so-called 'aging' (1 year old) mice, and this is clearly manifested by a reduced Peyer's patch (PP). Reduced numbers of naïve CD4+ T cells and follicular dendritic cells (FDCs) are also seen in the PP of aging mice. Similarly, an impaired ability to induce oral tolerance also occurs as early as 6 months of age.

[2,35–37]. For instance, the induction of oral tolerance in GF mice is particularly difficult [38]. By this token, it is reasonable to predict that age-related changes in the intestinal microflora could adversely affect the ability to induce oral tolerance in the elderly. Indeed, oral tolerance to OVA could be induced in all strains of young mice and in C3H/HeJ and B6D2F$_1$ mice at 20 and 38 weeks of age, respectively [39]; however, oral tolerance induction was completely abolished in A/J mice by 38 weeks of age [39]. Similarly, it has been shown that induction of oral tolerance to sheep red blood cells (SRBCs) or OVA is impaired with aging, and this occurs as early as 6–8 months of age (Figure 2) [40,41]. These results were due to dysregulation of both T- and B-cell responses in the PPs of aging mice, which in turn could stem from the senescence of both subepithelial dendritic cells (DCs) and follicular DCs, key antigen-presenting and immune-instructing cells for the initiation of balanced Ag-specific immune responses in PPs [41]. Interestingly, although B6D2F$_1$ mice become less susceptible to oral tolerance induction at 25 weeks and are totally refractory at 70 weeks of age (aged mice), mice given oral OVA at 8 weeks remain tolerant to OVA at 70 weeks of age [42]. This highlights the important finding that aging affects the inductive but not the effector phase of oral tolerance. Thus, oral tolerance established in early age can be maintained despite aging, whereas the induction of oral tolerance to new antigens is impaired in aged mice.

Alternative Ag-specific T-cell responses in the gut of aging mice

A substantial age-associated decline in the absolute numbers of lymphoid cells was found in the MALT, specifically in PPs and mesenteric lymph nodes (MLNs) [43]. Further, it was suggested that mucosa-associated, T cell–dependent but not T cell–independent B-cell responses decline in aging. *In vitro* Ab production by B cells from aged PPs and MLNs were depressed when T cell–dependent, but minimally affected when T cell–independent, B-cell mitogens were used for stimulation [43]. This finding suggests that T cells are more susceptible than B cells to immunosenescence in the mucosal compartment. It has been shown that PPs play key roles in the initiation of mucosal IgA immunity and oral tolerance [44,45]. As indicated above, a significant size reduction in PPs was seen in 1-year-old mice along with reduced Ag-specific mucosal Ab responses [19]. When the frequencies of T-cell subsets were examined, the ratio of CD4$^+$ and CD8$^+$ T cells and B cells were unchanged [19,41]. However, actual numbers of lymphocyte counts in PPs of 1-year-old mice were

significantly lower than those seen in young adult mice (6–8 weeks old) [19,41]. Further, it was reported that Ag-specific T-cell helper and regulatory functions in PPs were diminished by aging [19,41,46]. Others have shown that cytotoxic T-lymphocyte (CTL) activity against viral infections was significantly reduced in aged mice and in the elderly [47]. These findings clearly suggest that the development of effector T cells is influenced by senescence. Indeed, it has been shown that age-associated alterations closely parallel increases in memory type and loss of the naïve T-cell phenotype during aging [48–51]. In this regard, when the actual cell numbers of naïve $CD4^+$ T cells between young adult (6–8 weeks old) and aging (1 year old) mice were compared, the PPs of aging mice showed significant reductions in $CD4^+CD45RB^+$ naïve T-cell frequencies in addition to total cell numbers compared with young, adult mice [52].

Impaired mucosal DC function in aging

The mucosal DC subsets and their unique functions in various mucosal immune compartments have been well characterized [2]. Recently, studies have also examined age-related changes in mucosal DC function. For instance, mucosal DCs from aged mice exhibit less ability to trigger T and B cells [53–55]. Similarly, plasmacytoid DCs from aged mice show reduced interferon α (IFN-α) production in response to herpes simplex virus type 2 infection due to an impairment in IFN regulatory factor 7 activation [56]. By contrast, in humans, the antigen-presenting cell (APC) functions of aged DCs seem to be intact [57–60]. DCs from old individuals (>65 years old) have been shown to be as effective as DCs from young individuals (<30 years old) for T-cell stimulation when highly immunogenic proteins (e.g. *Mycobacterium* PPD, inactivated influenza virus and tetanus toxoid) were used [57,59,60]. Thus, how the quality of DC antigen presenting function changes with age remains a controversial and incompletely defined issue, with discrepancies between the human and mouse systems. However, few studies have assessed the effects of aging on murine DCs in mucosal immune compartments and their responses to either tolerogens or immunogens [41,55]. Our previous study indicated that $CD11c^+$ DCs in the PP subepithelial dome decrease in number in mice >1 year of age [41]. Furthermore, fewer follicular DCs (FDCs) and germinal center B cells were noted in the B-cell zone of the PP during aging [41]. These results suggest that the impaired Ag-specific SIgA responses and the lack of oral tolerance induction seen in aging mice [19,41,55] might be associated

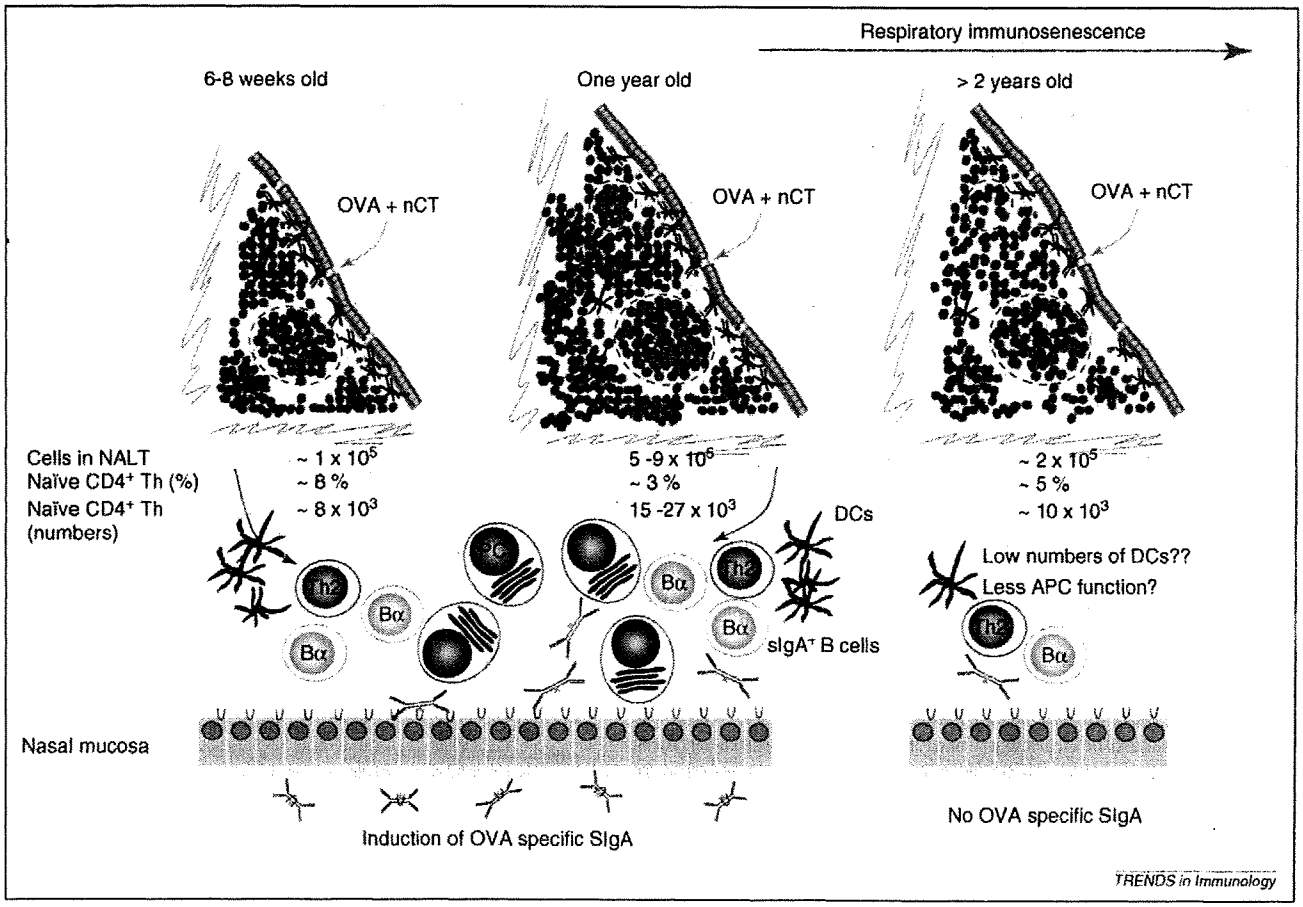

Figure 3. Nasal associated lymphoid tissue (NALT) exhibits a slower rate of immunosenescence. Nasal immunization with ovalbumin (OVA) plus native cholera toxin (nCT) induces OVA-specific secretory IgA (SIgA) and systemic IgG Ab responses in 1-year-old (i.e aging) mice which are identical to those in young adult (6–8 week-old) mice. By contrast, the signs of mucosal immunosenescence are already evident in the gut associated lymphoid tissue (GALT) of 1-year-old mice (see Figure 2). However, by 2 years of age, mice given OVA plus nCT nasally fail to induce OVA-specific SIgA but retain systemic IgG Ab responses. Therefore, NALT shows a relatively slower decline in immune responses with senescence becoming apparent only in aged mice. The absolute numbers of $CD4^+$ naïve T cells in the NALT are maintained in aging and aged mice.

with decreased DC and FDC functions in PPs in addition to impaired T-cell responses.

A recent study from another group indicated that DCs from the MLNs of aged mice showed less ability to stimulate Ag-specific T-cell immunity against infection by the protozoan *Encephalitozoon cuniculi* when compared with DCs from young adult mice [55]. Interestingly, IL-15 treatment restored this impaired DC function in aged mice [55] This finding could have important implications for intervention in the age-related decline of immune function.

Nasal immunity: a slower decline in function with age

Relatively little work has investigated the age-associated alterations in the upper respiratory tract. In this regard, Ag-specific mucosal immune responses were examined in aged mice nasally immunized with OVA plus nCT. We described above that oral administration of OVA along with nCT showed an impairment in mucosal and systemic responses in 1-year-old mice [19], the same age of mice have also been used to examine whether similar impairments could be seen when this antigen-adjuvant combination was administered nasally. In contrast to oral immunization, nasal immunization induced normal Ag-specific mucosal and systemic immune responses in 1-year-old (i.e. aging) mice [19,52]. Thus, equivalent levels of OVA-specific Ab responses in plasma and external secretions and Ag-specific Ab-forming cells (AFCs) in the nasal cavity were seen in both young and old mice, clearly showing nasal immunization could induce both mucosal and systemic immunity in aged mice (Figure 3) [52]. Furthermore, 1-year-old mice given nasal tetanus toxoid (TT) and nCT vaccine were protected from tetanus toxin challenge as were nasally vaccinated young adult mice [52]. These results suggest that the GALTs and NALTs are characterized by distinct rates of age-related functional decline.

To support this notion further, organogenesis of NALT has been shown to begin during the postnatal period, whereas GALT development initially occurs during gestation [61,62]. These studies clearly point to significant differences in the developmental timing of the two major mucosal inductive tissues. Considering these developmental differences, one would predict that age-associated alterations might also occur independently of each other. The findings that nasal but not oral immunization induced normal levels of Ag-specific immune responses in 1-year-old mice strongly supports the contention that aging occurs more slowly in NALT than GALT immune systems. Given these differences, the nasal route might therefore be the preferred and more effective means of administering vaccines to induce both Ag-specific mucosal and systemic immune responses. That said, one has to always take consideration of the safety issues related to nasal vaccines, especially the possibility of vaccine antigen deposition in the central nervous system (CNS). This is a genuine concern because it has been shown that nasal administration of mice with TT plus nCT resulted in the transient accumulation of these antigens in the CNS [63]. Although the results generated by the murine study must be carefully considered in any future development of nasal vaccines, we should also appreciate the fact that the anatomy of the nasal cavity is different between mice and humans.

It has been shown that increased numbers of memory-type and decreased numbers of naïve $CD4^+$ T cells are associated with aging [50,64–66]. When the frequencies of naïve $CD4^+$ T cells in NALT and GALT (i.e. the PPs) were compared in young adult and 1-year-old mice, reduced frequencies of $CD4^+$, $CD45RB^+$ T cells were seen in aging mice [52]. However, the size and the total lymphocyte count in the NALT increases approximately five- to ninefold during the aging process through the first year (Figure 3). Although the total lymphocyte count is ultimately reduced by 2 years of age, the NALT contains approximately twice the number of lymphocytes (Figure 3). These results suggest that the continuous generation of a naïve T-cell population in the NALT plays a pivotal role in maintaining young adult mouse levels for the induction of both systemic and mucosal immune responses to nasally administered antigens in aging (i.e. 1 year old) mice.

When examining immunosenescence, it is often suggested that experimental mice should be at least 2 years old before they can be considered equivalent to elderly humans [41,48,52]. When 2-year-old (or aged) mice were immunized nasally with OVA and nCT as adjuvant, they failed to undergo induction of Ag-specific SIgA responses in their external secretions (Figure 3) [52,67]. However, these same mice showed OVA-specific systemic immune responses (i.e. T-cell proliferation, Th1 and Th2

Box 2. Novel mucosal vaccine targeting strategies

The major mucosal inductive tissues like Peyer's patches (PPs) and nasopharyngeal-associated lymphoreticular tissue (NALT) are covered by follicle associated epithelium (FAE) containing M cells (see Box 1) [1,2]. Reoviruses initiate infection via M cells, an ability that has been associated with the protein sigma 1 ($p\sigma1$). Nasal M-cell targeting protocols have exploiting this phenomenon by using plasmid DNA (the vaccine) and the covalently attached reovirus $p\sigma1$ to poly-L-lysine (PL). This approach has effectively induced Ag-specific mucosal IgA Ab responses (Figure 4) [74]. Further, a novel M cell–specific mAb (NKM 16-2-4) has been used as a carrier for M-cell targeting with a mucosal vaccine. Oral administration of chimeric vaccine consisting of NKM 16-2-4 and tetanus toxoid or botulinum neurotoxin type A toxoid, together with native cholera toxin (nCT) as mucosal adjuvant, can induce high levels of Ag-specific plasma IgG and mucosal IgA Ab responses and protection against the toxin challenge (Figure 4) [75]. These studies clearly show that an M cell–targeting delivery system for the development of effective mucosal vaccines might also be effective in the elderly.

FluMist, a trivalent nasal vaccine consisting of type A (H1N1 and H3N2) and type B live attenuated influenza virus, is an example of a potent NALT immune system–targeting vaccine already in the current market. FluMist can successfully reduce the chances of influenza illness among children (age 15–85 months) by 92% compared with a placebo. Further, the effectiveness of FluMist was also demonstrated in adults 18–49 years of age; however, this efficacy was not seen in individuals 50–64 years of age. The safety studies resulted in a higher rate of sore throats in a group >65 years of age. Therefore, FluMist is approved for use in healthy non-pregnant people 2–49 years of age. Although nasal application of FluMist is not yet approved in the elderly, it would be possible that an adaptation of this strategy for specific mucosal targeting (M cells or mucosal dendritic cells) with adjuvant could be applied the elderly. Together, the evidence accumulated by the FluMist vaccine and current ongoing studies in the development of safe and potent mucosal targeting vehicles and adjuvants should lead to the generation of mucosal vaccines for the control of infectious diseases of the aged.

cytokines), which were essentially identical to the responses seen in young adult mice [52,67]. These results further reinforce the findings that immunosenescence occurs earlier in the mucosa than in the systemic immune system [19], even though the process of NALT immunosenescence is milder than that of GALT in 2-year-old mice.

Mucosal vaccines designed for the elderly

The elderly are in general much more susceptible to infections. In fact, the severity and mortality caused by the infectious pathogens invading mucosal surfaces such as influenza virus and the bacterial pathogen *Streptococcus pneumoniae* (pneumococcus) are sharply increased in humans of advanced age [68–70]. In the United States, influenza virus infection annually caused at least 36 000 deaths during 1990–1999 and 226 000 hospitalizations during 1979–2001 [71,72]. Pneumococcus is similarly a major human bacterial pathogen and a significant cause of morbidity and results in >40 000 deaths in the United States each year [73]. The highest incidence of influenza and pneumococcal diseases occurs among persons >65 years of age. The development of effective vaccines for the elderly remains a largely unmet need, so to provide effective protection against influenza and *S. pneumoniae* in the elderly, one should strongly consider developing a new generation of vaccines that could induce pathogen-specific immunity in the respiratory tract. Although it has been shown that effective protection can be provided by pathogen-specific systemic IgG without mucosal IgA responses [74], pathogen-specific SIgA responses are a necessary component for providing the first line of effective immunity against these respiratory pathogens at their entry site in the elderly. Pathogen-specific SIgA at the surface of the nasal mucosa and upper respiratory tract reduce invasion, dissemination and/or growth of pathogenic bacteria or viruses. Indeed, it was reported that influenza hemagglutinin (HA)-specific SIgA responses play a key role in protection against influenza in the upper respiratory tract and provide cross-protection against infection with a variant virus within the same subtype when compared with those of serum IgG Abs [75,76].

To explore new avenues for effective mucosal vaccine development, investigators have begun to target mucosal tissues and immune cells for vaccine delivery. To this end,

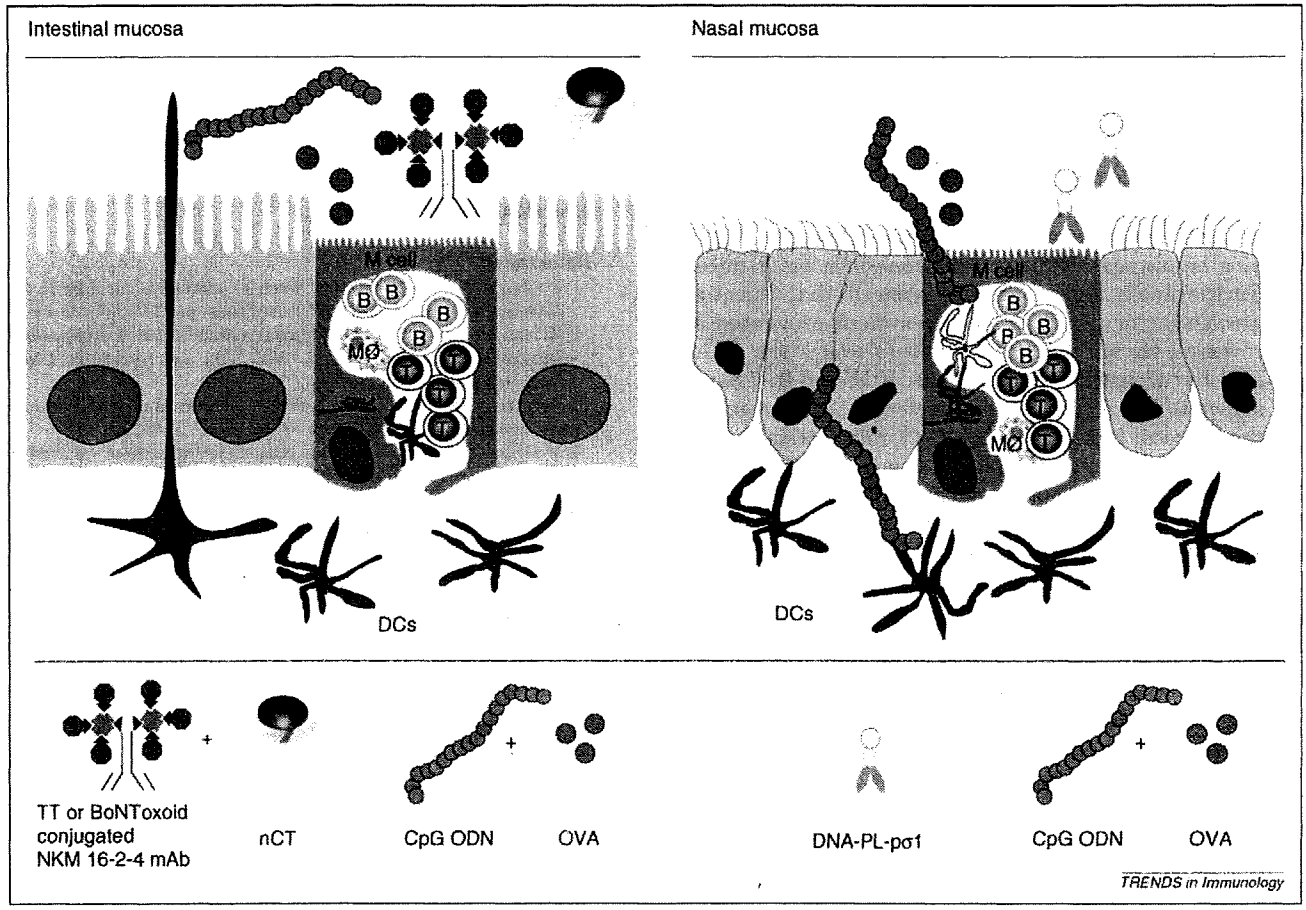

Figure 4. Mucosal targeting approaches for vaccination of the elderly. Mucosal M-cell and/or dendritic cell (DC) targeting can facilitate Ag-specific secretory IgA (SIgA) Ab responses to provide effective immunity at the entry site of pathogens. Specific M-cell targeting can be achieved by using protein σ1 of reovirus or the M cell–specific mAb NKM 16-2-4. Nasal immunization with the chimeric DNA vaccine (DNA-PL-pσ1) induces Ag-specific immune responses. Oral immunization with the chimeric vaccine (NKM 16-2-4 conjugated to BoNToxoid or TT) together with nCT results in the induction of protective immunity. The Toll-like receptor (TLR) 9 ligand CpG ODN is currently the most promising adjuvant used in vaccines for the elderly. In murine models, CpG ODN can be used as an effective mucosal (intestinal or respiratory) adjuvant to target mucosal plasmacytoid DCs for the induction of Ag-specific immune response. Thus, oral or nasal immunization with nominal antigen OVA plus CpG ODN results in the induction of Ag-specific immune responses in both the mucosal and systemic compartments of aged mice. BoNToxoid, botulinum neurotoxin type A; DC, dendritic cell; Mϕ, macrophage; nCT, native cholera toxin; OVA, ovalbumin; TT, tetanus toxoid.

mucosal M cell- or DC- targeting Ag delivery systems have been shown to induce Ag-specific SIgA responses (Box 2; Figure 4) [77–80]. CpG ODN as vaccine adjuvant has been shown to restore Ag-specific immune responses to OVA, diphtheria toxoid, hepatitis B, polysaccharide of *S. pnuemoniae*, amyloid β and tumor cells in aged mice and rats [81–87]. When 3-month-old (or young adult) and 18-month-old (or aged) mice were orally immunized with OVA plus CpG ODN as adjuvant, both groups of mice showed high and equivalent levels of OVA-specific systemic IgG and mucosal IgA Ab responses [88]. Furthermore, recent work showed that when a nasal adjuvant was constructed with a plasmid encoding the Flt3 ligand cDNA (pFL) coupled to CpG ODN and then given with OVA to 2-year-old mice, significant levels of OVA-specific IgA responses were induced in external secretions including nasal and vaginal washes and saliva [67]. By contrast, nCT as nasal adjuvant in 2-year-old (or aged) mice failed to induce mucosal immunity, although significant systemic immune responses were noted [52,67]. These findings demonstrate that mucosal delivery of CpG ODN as adjuvant offers an attractive possibility for the development of an effective mucosal vaccine for the elderly.

In addition to the above mucosal targeting system or adjuvants, direct mucosal application of cytokines, chemokines or costimulatory molecules possessing mucosal adjuvanticity [2] are also considered to be attractive candidates for the induction of protective immunity in the elderly. Indeed, it has been shown that the effect of aging on IL-2 production was abrogated by exogenous IL-2 delivery [48]. Thus, mucosal IL-2 treatment can overcome age-impaired mucosal immune responses by enhancing mucosal immunity or abrogating unresponsiveness in aged mice [89]. Furthermore, recent studies showed that keratinocyte growth factor (KGF) or IL-7 treatment prevented and/or reversed thymic atrophy [90,91] (also see the article by Lynch *et al.* in this issue). Because KGF and IL-7 have been shown to be involved in the development and regulation of the mucosal immune system [1,2], mucosal delivery of the growth factor cytokine family (e.g. IL-2, IL-7, IL-15 and KGF) could help improve mucosal vaccines for the elderly.

Are we there yet? Yes, we can...

Despite its relatively rapid immunosenescence, mucosal vaccination is still a particularly attractive route for inducing protective immunity against pathogens in the elderly. Because immunosenescence occurs earlier in the mucosa than in the systemic compartments, our efforts in the design of mucosal modulators including novel mucosal adjuvants that can overcome mucosal immunosenescence in the elderly are one of major priorities for aging research. Thus, one should continue to study not only CpG ODN but also seek other mucosal adjuvant candidates for supporting the induction of protective immunity in the elderly, especially against influenza and *S. pneumoniae* infections. The development of mucosal vaccines for the elderly has only just begun and offers both promise as well as significant hurdles. For instance, we must fill the gaps between mouse and human studies. A significant concern is that humans are exposed to environmental Ags, vaccines and antibiotic treatments, whereas mice stay in a relatively clean environment. Further, genetic and anatomic differences need to be considered. Especially, for nasal vaccine development, one must discover the human counterparts for mouse NALT, in addition to tonsils and adenoids. Translational research such as the use of nonhuman primates and *in vitro* human cell systems might be partial solutions. Furthermore, recent progress in the development of a new generation of humanized mice should offer a powerful new tool for understanding immunosenescence in humans. Differences in the immune responses of the elderly populations of various countries are influenced by commensal flora, nutritional uptake, genetic background, life style, and so forth. Thus, this complexity must also be taken into account when studying human mucosal immunosenescence, because the mucosal immune system is directly and continuously exposed to these micro- and macro-environments. The development of universal, safe and effective mucosal vaccines for both young and aged populations will require global enthusiasm and international cooperation at the bench-top and the clinic, as well as pharmaceutical and government support.

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Current Opinion in Immunology

New horizon of mucosal immunity and vaccines

Ichiro Takahashi[1,2], Tomonori Nochi[2], Yoshikazu Yuki[2] and Hiroshi Kiyono[2]

Progress in the past quarter-century on understanding the molecular, cellular, and *in vivo* components of the mucosal immune system have allowed us to develop a practical strategy for a novel mucosal vaccine. The mucosal immune system can induce secretory IgA (SIgA) and serum IgG responses to provide two layers of defense against mucosal pathogens. For SIgA-mediated immunity in the gastrointestinal tract, the gut-associated lymphoid tissue contains both the tissue-dependent and tissue-independent IgA components. Harnessing the mucosal immune system for vaccine development may help prevent the global health problems caused by enteric infectious diseases. We have therefore combined mucosal immunology and plant biology to create a rice-based mucosal vaccine that requires neither needles and syringes nor refrigeration.

Addresses
[1] Department of Mucosal Immunology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima 734-8553, Japan
[2] Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

Corresponding author: Kiyono, Hiroshi (kiyono@ims.u-tokyo.ac.jp)

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Introduction

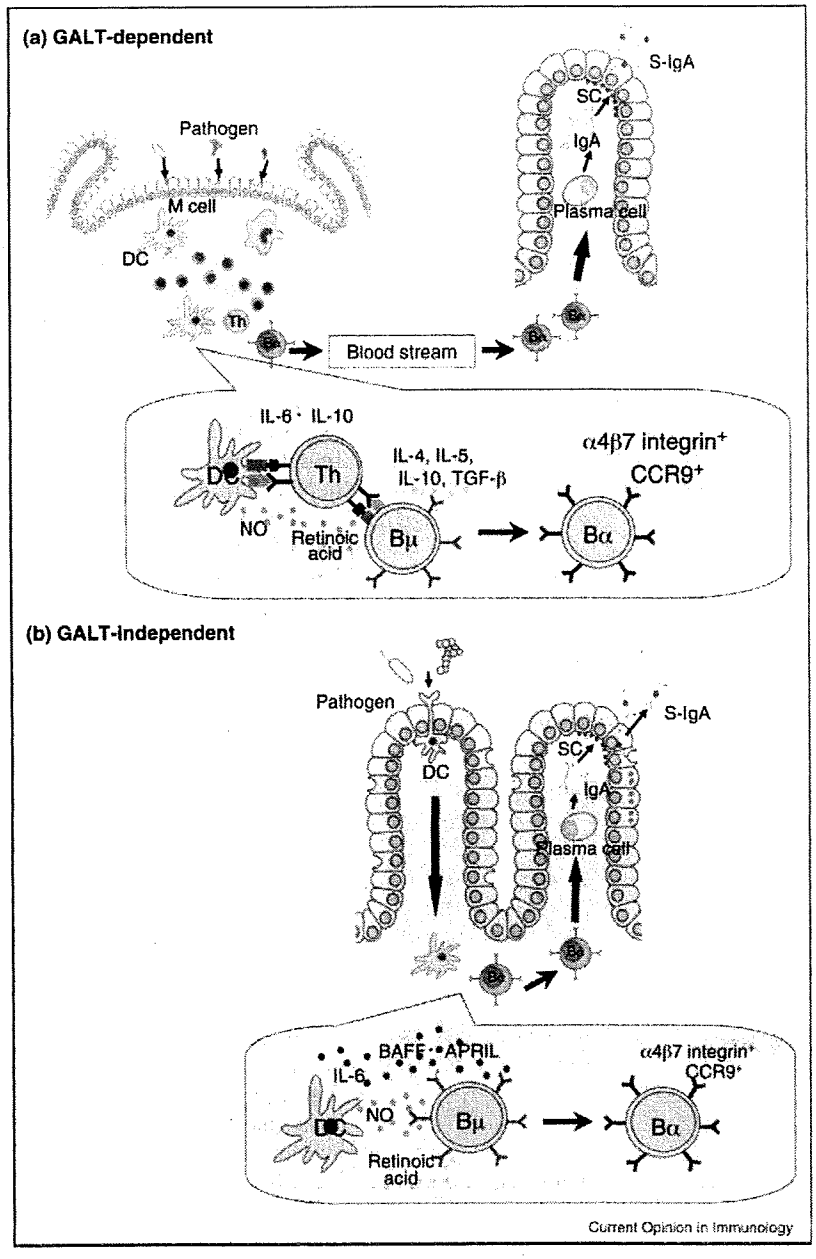
For decades, immunologists have been aiming at understanding the systemic immunity for the prevention of infectious diseases without much attention to one of the host major defensive weapons, the mucosal immune system. Thus, most widely used vaccines to date, such as those for measles, mumps, and rubella, have been aimed at stimulating the systemic immunity to produce serum antibodies against disease-causing organisms. Therefore, most vaccines have been given by injection for induction of effective systemic immunity. However, our present molecular and cellular understanding of the mucosal immune system allows us to consider the use of oral and nasal immunization for induction of antigen-specific immune responses at the mucosal surface as well as systemic compartment [1]. On the basis of the advanced knowledge of the mucosal immune system, it is now plausible to propose that the current injection-type vaccines should be advanced to needle/syringe-free and cold-chain-free forms of mucosal vaccine [2•].

The intestinal immune system for the development of oral vaccine: recent progress

The intestinal immune system consists of specialized local inductive sites, the organized gut-associated lymphoid tissue (GALT), and widely spread effector sites, the diffused intestinal epithelial and lamina propria (LP) regions, both of which are interconnected but separated from outside environment by mucus and epithelial barriers [3••,4]. The first step in the induction of an antigen-specific intestinal immune response is the uptake and transport of antigens across the epithelial barrier. The intestinal epithelium overlying GALT such as Peyer's patches (PP) is specialized to allow the transport of pathogens into the lymphoid tissue. This particular function is carried out by specialized antigen sampling epithelial cells termed M cells [5]. M cells have a folded luminal surface instead of the microvilli present on enterocytes. Unlike enterocytes, M cells do not secrete digestive enzymes or mucus and lack a thick surface glycocalyx. M cells are therefore easily accessible to microorganisms and particle antigens within the gut lumen and are the route by which antigens enter the PP from the lumen. The basolateral membrane of M cells forms pockets containing T and B lymphocytes and dendritic cells (DCs) [5]. Thus, antigens and microorganisms are transcytosed into the M cell pocket and subsequently transferred to underlying professional antigen processing and presenting cells such as DCs for the initiation of antigen-specific mucosal IgA responses. In this regard, our group showed that apical surface of M cells selectively express α(1, 2) fucose-containing carbohydrate moiety, and a monoclonal antibody recognizing the M-cell-specific carbohydrate moiety possessed the ability to efficiently deliver vaccine antigens into M cells and induce brisk antigen-specific mucosal IgA and systemic IgG responses [6•].

Following antigen processing and presentation in inductive sites (GALT), antigen-specific IgA-committed B cells proliferate locally and then migrate via the bloodstream to distant effector sites such as LP of the gut and exocrine tissues such as salivary glands (Figure 1a) [1,3••]. Recent several lines of evidence suggest that PP DCs play a pivotal role in the mucosa-tropism by the induction of gut homing molecules (e.g. α4β7 integrin and CCR9 chemokine receptor) on antigen-primed B and T lymphocytes through the production of retinoic acid from vitamin

Figure 1



The mucosal immune system. There are two distinctive pathways for the production of secretory IgA (SIgA). **(a)** In the conventional secondary lymphoid follicle such as Peyer's patch (PP)-dependent pathway, orally administered antigens are taken up by M cells in follicle-associated epithelium of PP and then processed and presented by dendritic cells (DCs) and macrophages for the generation of helper T (Th) cells and IgA-committed B cells. IL-4, IL-5, IL-10, and TGF-β produced by Th cells and nitric oxide (NO) and IL-6 produced by PP DCs allow B cells to undergo μ to α class switch recombination. Simultaneously, retinoic acid (RA) produced by DCs increases gut homing receptors (α4β7 integrin and CCR9) on antigen-primed Th cells as well as IgA-committed B cells. These antigen-primed Th cells and IgA committed B cells migrate to effector sites such as intestinal lamina propria (LP) for terminal differentiation to IgA producing plasma cells. **(b)** Another lineage of B cells, possibly originated from the peritoneal cavity, is considered as precursor for the generation of IgA, without help of Th cells. In the latter case, LP DCs directly sample antigen from the lumen and present it to the B cells, which under the influence of cytokines such as BAFF, APRIL, and TGF-β1, and NO derived from LP DCs trigger the process of isotype switching and differentiation to IgA producing plasma cells.

A [7], that is, effector memory lymphocytes that arise in response to antigens in the alimentary tract mostly express intestinal migration molecules, particularly the integrin $\alpha4\beta7$, the receptor for mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed by high endothelial venules in the gastrointestinal tracts, and CCR9, the receptor for TECK/CCL25, a chemokine expressed in the small intestine [8,9,10•]. *In vitro* activation of naïve lymphocytes with DCs from PP but not from peripheral lymph nodes (PLNs) or spleen instructs effector lymphocytes with high expression of $\alpha4\beta7$ and CCR9 and the capacity to migrate to the small intestine [8,9]. Importantly, many DCs from PP but few from PLNs or spleen expressed the prerequisite enzyme (retinal dehydrogenase) for oxidative conversion of vitamin A to retinoic acid, and inhibitors of the enzyme rendered intestinal DC incapable of inducing $\alpha4\beta7^+$ lymphocytes [11•]. These tissue-specific consequences of lymphocyte priming in PP explain why vaccination against intestinal infections requires immunization by a mucosal route, particularly oral immunization, because other routes, such as subcutaneous or intramuscular immunization, do not involve mucosal DC with the correct imprinting properties.

The production of IgA at mucosal surfaces is strictly regulated by the coordinated communication consisting of mucosal B cells, mucosal T cells, mucosal DCs, and epithelial cells. Although it has long been recognized that a major source of IgA plasma cells at mucosal surfaces comes from GALT such as PP [1,12], it was recently revealed that some IgA antibodies, particularly commensal microbiota-specific ones, occur even in the absence of GALT [13]. In this setting, DCs located in GALT (e.g. PP) as well as diffused effector tissues (e.g. LP) should be specialized in providing crucial molecular environment for generation of IgA committed B cells as well as their differentiation into IgA producing plasma cells. For example, a $CD11c^+CD11b^+CD8\alpha^-$DC subset isolated from PP, which preferentially produces IL-6 and polarizes antigen-specific T cells to produce Th2 cytokines, promotes IgA production by naïve B cells [14]. Furthermore, a novel PP DC subset for mucosal IgA responses has been identified; these PP DCs produce TNF-α and inducible nitric oxide synthase (iNOS), termed Tip-DCs, and function to induce the production of IgA-committed cells [15•]. Nitric oxide increases TGF-β receptor expression on naïve PP B cells, leading to the enhancement of class-switching recombination to IgA.

In addition to those located in GALT (e.g. PP), a unique population of mucosal DCs in the diffused effector sites (e.g. LP) has been identified and extensively characterized as an important DC subset involved in IgA responses (Figure 1b). One subset of $CD11c^{high}CD11b^{high}$ LP DCs expresses toll-like receptor 5 (TLR5) in the small intestine [16]. When stimulated by the TLR5 ligand bacterial flagellin, $TLR5^+$LP-DCs were able to induce differentiation of IgA committed B cells to IgA^+ plasma cells independent of T cells and GALT *in vivo*. Because $TLR5^+$LP-DCs can synthesize retinoic acid and IL-6 themselves, T cell-independent IgA^+ plasma cells can be generated.

Mucosal DCs located in the LP are likely to further activate B cells through B cell-activating factor of the TNF family (BAFF) and a proliferating-inducing ligand (APRIL), both of which are B-cell-stimulating factors structurally and functionally related to CD40L [17,18]. Growing evidence indicates that this BAFF–APRIL-mediated signaling pathway supports intestinal IgA production in a GALT-independent fashion. Recent data indicate that recognition of pathogen-associated molecular patterns (PAMPs) by TLRs at the intestinal epithelial barrier is essential for the production of BAFF and APRIL by LP DCs [19]. TLR signaling not only stimulates DC production of BAFF and APRIL but also elicits DC expression of iNOS, an enzyme that augments BAFF and APRIL synthesis through the generation of nitric oxide [15•]. BAFF and APRIL induce IgA class switching by activating B cells in cooperation with IgA inducing cytokines released by DCs or other mucosa-associated cells (e.g. macrophages and epithelial cells), including IL-10 and TGF-$\beta1$ [13].

The antigen-specific IgA-committed B cells generated by GALT-dependent and GALT-independent pathways finally differentiate into dimeric IgA producing plasma cells under the influence of another group of IgA inducing cytokines, IL-5 and IL-6 in diffused LP. Dimeric IgA antibodies are transported across epithelial cells into glandular and mucosal secretions via polymeric Ig-receptor-mediated transcytosis and thus released as a secretory form of IgA (SIgA). Because SIgA antibody appears to be the most important defensive molecule in non-invasive enteric infections such as those caused by *Vibrio cholerae* and enterotoxigenic *Escherichia coli* [2•,20], oral vaccines should effectively stimulate the mucosal immune system to induce antigen-specific IgA responses in the gut.

Cholera is still a global health problem

Despite our recent progress in the molecular and cellular understanding of the mucosal immunity, infectious diseases are responsible for a third of all deaths worldwide, killing at least 15 million people a year [21]. Of these, more than five million are children under five years of age.

The most effective way to reduce morbidity and mortality from infectious diseases is to vaccinate the susceptible population [21]. The impact of vaccination on global health in both developing and developed countries cannot be overemphasized. With the exception of water sanitation, no other modality has had such a major effect on mortality reduction. Nevertheless, at least two million

children die every year from diseases preventable by available, low-cost vaccines [21].

Cholera is an acutely dehydrating, watery diarrheal disease caused by an intestinal infection after ingestion of food or water contaminated with *V. cholerae* [20,22•]. *V. cholerae* is a non-invasive, Gram-negative, motile bacterium that colonizes the epithelial lining of the gut after penetrating the mucous layer. Cholera affects the small intestine through its secreted exotoxin (cholera toxin; CT), which is composed of five receptor-binding B subunits surrounding a catalytic A subunit. CT binds to a specific receptor, monosialosyl ganglioside GM1, on the surface of all nucleated cells, including the brush border of polarized epithelial cells. Once bound, CT is endocytosed, and the A subunit then activates the stimulatory G protein for adenylate cyclase and increases intracellular cyclic AMP, resulting in secretion of chloride and bicarbonate into the small intestine. This secretion causes water to be drawn from the intravascular and extracellular spaces of the body and to be rapidly lost into the gut lumen.

Cholera no longer poses a threat in countries with minimum standards of hygiene but remains a challenge in countries where access to safe drinking water and adequate sanitation cannot be guaranteed [22•,23•]. Many developing countries still face cholera outbreaks or the threat of cholera epidemics.

Protective immune responses against cholera

Protective immunity to cholera is mediated mainly by IgA antibodies produced locally in the intestinal mucosa and secreted onto the mucosal surface [2•,24•]. These antibodies are directed against bacterial adhesins, CT, and lipopolysaccharide, inhibit bacterial colonization and multiplication, and block CT toxicity. IgA, IgG, and IgM antibodies to *V. cholerae* antigens occur in the intestinal lumen of cholera patients, but intestinal IgA antibodies to CT provide the best protective immunity [24•]. Protective antitoxic antibodies in the gut are specific to the B subunit of CT (CT-B), and prevent clinical manifestation by blocking toxin binding to epithelial GM1 ganglioside receptors [24•]. Because of its potent immunogenicity in mucosal tissues, CT has become the model mucosal immunogen and adjuvant. Oral administration of microgram amounts of CT induces SIgA and serum IgG antibodies [1,24•]. Furthermore, CT does not induce oral tolerance and can abrogate oral tolerance to unrelated proteins when administered simultaneously [1].

Currently available cholera vaccines

Currently, two types of oral cholera vaccines have been shown to be safe, immunogenic, and efficacious [22•,23•,24•]. Although these approved vaccines are mainly used by travelers, they are now under consideration for public health use in pandemic areas. Several countries have already attempted to vaccinate populations at high risk of cholera outbreaks [22•,23•,24•].

Dukoral: a killed whole-cell (WC)/rCT-B cholera vaccine

Dukoral consists of killed whole-cell *V. cholerae* O1 with purified recombinant CT-B (WC/rCT-B) [24•]. Dukoral vaccine evokes antitoxic as well as antibacterial mucosal immunity, to provide synergistic cooperative protection against cholera [25]. Clinical trials have been performed in Bangladesh and Peru and in US volunteers. Efficacy trials in Bangladesh have shown that this vaccine is safe and confers 85–90% protection for six months in all age groups (>two years) after administration of two oral doses, 10 to 14 days apart [24•,26]. However, a large volume of liquid (75–150 mL) is needed for individual administration, meaning that the vaccine cannot be given to children under two years of age. The initial protection declined rapidly after six months in young children, but was still about 60% protection of older children (>five years) and adults after one year.

Orochol: a live, attenuated WC cholera vaccine

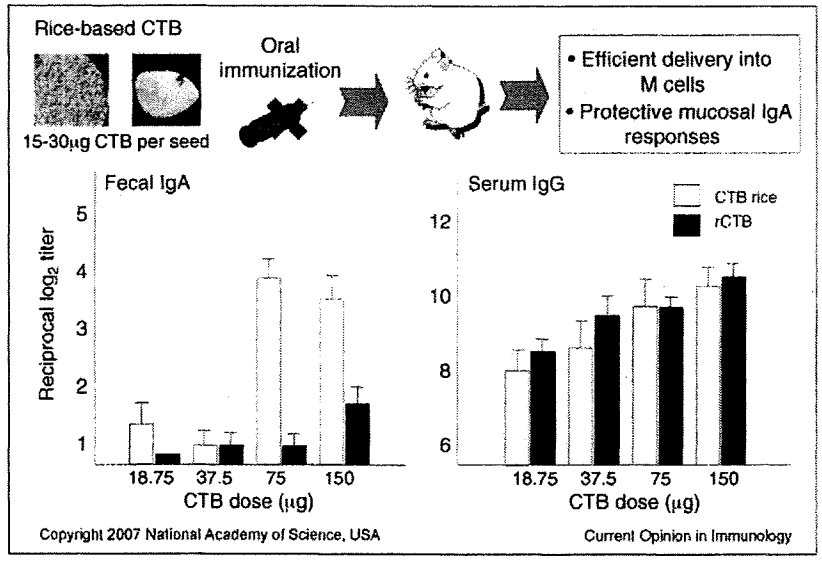
Given that the principal virulence factor of *V. cholerae* is CT itself, the development of a live vaccine deleted for a portion of CT is an obvious first approach. Orochol consists of a live, attenuated, genetically modified *V. cholerae* O1 strain (CVD 103-HgR) that has been engineered to produce CT-B but not CT-A [22•,23•,27]. Placebo-controlled trials in several countries have demonstrated the safety and immunogenicity of Orochol [27,28]. A single dose of this live vaccine conferred good protection (60–100%) in adult volunteers in the USA challenged three months after vaccination. A large field trial performed in Indonesia has not shown convincing protection in a population exposed to cholera long after immunization [22•,29].

Limitation of currently available cholera vaccines and an innovative strategy for a plant-based vaccine

The limitations of available vaccines are apparent in large-scale interventions. The buffer solution requires sterile water for Dukoral, and two doses at separate times require access to the same population twice. In addition, these vaccines require continuous cold storage. These constraints limit the usefulness in pandemic regions or in emergencies because the at-risk populations are mobile and hard to reach.

One innovative approach to improving storage and distribution, as well as to reducing the high production costs associated with antigen purification from traditional expression systems (e.g. microbial and mammalian expression), is to construct plants with transgenic grains that express vaccine antigens. Ideally, these vaccines could be administered to children under two years in a

Figure 2



Mucosal and serum anti-CT-B-specific antibody responses induced by rice-based oral vaccine administration in mice. Orally administered rice-based CT-B (or MucoRice-CTB) was effectively taken-up by PP M cells and induced brisk CT-B-specific serum IgG as well as fecal IgA response in a dose-dependent manner. It was noteworthy that the level of fecal IgA response elicited by oral administration of rice-based CT-B was much higher than that of the equivalent amount of purified recombinant CT-B (rCT-B).

single dose, without water, and would confer long-term protection [2•].

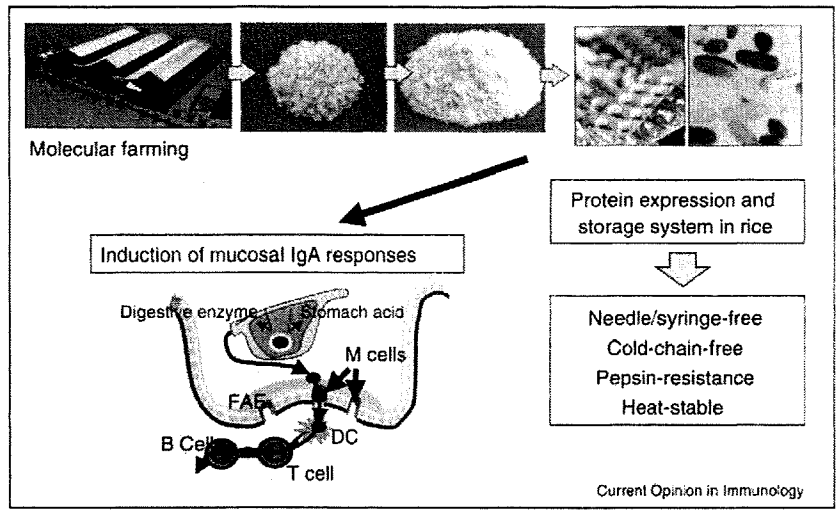
Oral feeding of mice with CT-B and the related, heat-labile enterotoxin B subunit from enterotoxigenic *E. coli* (LT-B) expressed in raw potato induced potent serum IgG as well as mucosal IgA responses against CT-B/LT-B, and protected animals from toxin challenge [30,31]. Furthermore, ingestion of uncooked potato synthesizing LT-B elicited both serum IgG and fecal IgA responses in human volunteers [32]. Antibody-secreting cells derived from GALT were observed in blood of vaccinated volunteers a week after oral vaccination [32]. LT-B antigen expressed in maize could also induce responses in human volunteers [33]. These results demonstrated the attractiveness of plant-based vaccine as a new prototype for needle/syringe free one. However, these potato and maize systems showed rapid antigen degradation in the digestive tract, and consistent dosing was difficult to achieve [34].

MucoRice CT-B: a novel unrefrigerated, needle-free vaccine

Our group recently developed a rice-based oral vaccine expressing CT-B (MucoRice CT-B) as a new possible form of oral cholera vaccine [35•]. Transgenic rice is stabile in the harsh environment of the gastrointestinal tract. Rice seeds possess two kinds of protein storage organelles, protein body I (PB-I) and PB-II. PB-I is alcohol-soluble but water-insoluble, making it resistant to the gastrointestinal environment. To examine the ability of CT-B in rice PB to withstand protease digestion in the stomach, total seed proteins were subjected to pepsin treatment *in vitro*. Prolamins in PB-I were absolutely resistant to the treatment, and ~75% of the CT-B accumulated in rice seed remained intact after pepsin treatment, whereas 90% of the glutelins accumulated in PB-II were digested by pepsin under the same conditions. M cells take up particles smaller than 10 μm [5]. Because PB-I is 1–2 μm in diameter, MucoRice CT-B is taken up by M cells and induces CT-B–specific serum IgG as well as intestinal IgA responses to protect against oral challenges with cholera toxin (Figure 2) [35•]. Furthermore, MucoRice CT-B does not stimulate serum IgG or fecal IgA responses to rice storage proteins [35•], putatively because of its much higher antigenicity. In addition, rice preserved at room temperature for 24 months shows equivalent CT-B–specific mucosal immunogenicity to that of freshly harvested rice, obviating the need for cold storage.

Achieving high levels of recombinant protein in transgenic plants is challenging, with most transgenic proteins of medical interest expressed at 0.01–0.40% of total protein [36]. Our group addressed this challenge by expressing CT-B under the control of the rice-derived promoter, and recombinant CT-B in rice reached 2.1% of the total seed protein [35•].

Figure 3



Overview of rice-based mucosal vaccine development; departure from edible vaccine to plant-based oral vaccine. Rice-based oral vaccine system possesses several advantages of circumventing limitations inherent to the currently available injection-type vaccines, including requirement of professional skill and syringe/needle for administration, cold-chain (or refrigeration storage), and physicochemical-stability. These properties are suitable for the prevalence of global vaccine development for many emerging and re-emerging infectious diseases. However, highly sophisticated and closed soilless farming facility with artificial sunlight should be suitable technical advancement for the rice-based transgenic vaccine system.

Although, ingestion of the tablet (or powder) form of plant-based vaccines, even transgenic rice, has to overcome several social and practical concerns, including public confusion and hesitation about genetically modified plants in general, unease about segregating transgenic vaccine plants from food plants, and questions about the consistency of antigen concentration in different lots of transgenic plants. These issues need to be addressed by scientific evidence and improved technology. For example, the development of a soilless molecular farming facility with artificial sunlight will address segregation issues. The term 'edible vaccine' has expanded the development of novel oral vaccines [37,38], but has also created unnecessary confusion that the vaccine could be a part of other food products and not a separate medical preparation. Thus, we propose to use the precise term 'plant-based oral vaccine' (Figure 3). The scientific community in vaccinology, immunology, and plant biology must provide new experimental results and technological advancements to address these public concerns.

Conclusion

The MucoRice system circumvents limitations of other forms of oral vaccine, including physicochemical stability and the requirement for continuous refrigeration. MucoRice CT-B is stable at room temperature for 24 months and does not require purification. Rice plants do not scatter pollen as widely as other crops modified to produce vaccines, for example, maize and wheat, and so have less risk of contaminating non-transgenic crops. Nevertheless, a closed facility would be required for production of the MucoRice vaccine. A rice-based oral vaccine offers a practical global strategy for unrefrigerated, needle-free vaccination against important infectious diseases not only for cholera but also for other re-emerging and emerging infectious diseases, including influenza, botulism, and anthrax.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
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