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Protective Mucosal Immunity in Aging Is Associated with Functional CD4\(^+\) T Cells in Nasopharyngeal-Associated Lymphoreticular Tissue¹

Yukari Hagiwara,* Jerry R. McGhee,* Keiko Fujihashi,* Ryoki Kobayashi,* Naoto Yoshino,* Kosuke Kataoka,* Yuri Etani,* Mi-Na Kweon,† Shinichi Tamura,‡ Takeshi Kurata,§ Yoshifumi Takeda,∥ Hiroshi Kiyono,*†∥ and Kohtaro Fujihashi²*

Our previous studies showed that mucosal immunity was impaired in 1-year-old mice that had been orally immunized with OVA and native cholera toxin (nCT) as mucosal adjuvant. In this study, we queried whether similar immune dysregulation was also present in mucosal compartments of mice immunized by the nasal route. Both 1-year-old and young adult mice were immunized weekly with three nasal doses of OVA and nCT or with a nontoxic chimeric enterotoxin (mutant cholera toxin-A E112K/B subunit of native labile toxin) from Brevibacillus choshinensis. Elevated levels of OVA-specific IgG Abs in plasma and secretory IgA Abs in mucosal secretions (nasal washes, saliva, and fecal extracts) were noted in both young adult and 1-year-old mice given nCT or chimeric enterotoxin as mucosal adjuvants. Significant levels of OVA-specific CD4\(^+\) T cell proliferative and OVA-induced Th1- and Th2-type cytokine responses were noted in cervical lymph nodes and spleen of 1-year-old mice. In this regard, CD4\(^+\), CD45RB\(^+\) T cells were detected in greater numbers in the nasopharyngeal-associated lymphoreticular tissues of 1-year-old mice than of young adult mice, but the same did not hold true for Peyer’s patches or spleen. One-year-old mice given nasal tetanus toxoid plus the chimeric toxin as adjuvant were protected from lethal challenge with tetanus toxin. This result reinforced our findings that age-associated immune alterations occur first in gut-associated lymphoreticular tissues, and thus nasal delivery of vaccines for nasopharyngeal-associated lymphoreticular tissue-based mucosal immunity offers an attractive possibility to protect the elderly. The Journal of Immunology, 2003, 170: 1754–1762.

The cellular and molecular mechanisms of immunosenescence have been extensively investigated in systemic lymphoid compartments. For example, it has been suggested that altered T cell functions are major factors in dysregulated immune responses that commonly occur in the elderly (1–7). These include alterations in T cell phenotypes, reduced IL-2 production and IL-2R expression, aberrant signal transduction, and enhanced programmed cell death of naïve T cells (1–4). Furthermore, reduced responses to mitogens and impaired cytokine production have also been reported in the elderly (5–7). It has been shown that these alterations closely parallel increases in memory type and loss of the naïve T cell phenotype during aging (1, 4, 8). Dysfunctions in B cells and Ab responses also occur in aging (7, 8). For example, recent studies showed that pre-B cells develop poorly in the bone marrow of aged BALB/c mice (8).

Although age-associated changes in the mucosal immune system are less well understood when compared with immunosenescence in systemic immunity, it has been shown that the mucosal immune system is also altered by aging because the elderly are much more susceptible to infections of the gastrointestinal (GI) tract (9, 10). Furthermore, marked increases in the severity and mortality caused by respiratory pathogens such as influenza virus and the bacterial pathogen Streplococcus pneumoniae are seen in the elderly (11–13). Additionally, it was reported that the number of lymphocytes in Peyer’s patches and mesenteric lymph nodes (LN) decreased in aged rats (14). Our recent study showed that Ag-specific mucosal and systemic immune responses were diminished in 1-year-old mice immunized orally with OVA and native cholera toxin (nCT), whereas significant immune responses were seen in orally immunized, young adult mice in both mucosal and systemic lymphoid compartments (15). Thus, these studies clearly indicate that the development of age-associated alterations occur earlier in the GI tract mucosa than in systemic immune compartments (15).

Recent studies support the notion that nasopharyngeal-associated lymphoreticular tissue (NALT) is a major mucosal inductive site. To understand the precise contribution of NALT in the induction of IgA responses to inhaled Ags, previous studies have

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³ Abbreviations used in this paper: GI, gastrointestinal; AFC, Ab-forming cell; CLN, cervical lymph node; GALT, gut-associated lymphoreticular tissue; LT, lymphotoxin; LT-B, subunit of native labile toxin; mCT, mutant cholera toxin; NALT, nasopharyngeal-associated lymphoreticular tissue; nCT, native cholera toxin; NP, nasal passage; S-IgA, secretory IgA; sIg, surface Ig; SMG, submandibular gland; TT, tetanus toxoid.
isolated and characterized lymphoid cells from NALT of mice and rats (16–19). In these species, NALT consists of bilateral strips of nonencapsulated lymphoid tissues underlying the epithelium on the ventral aspect of the posterior nasal tract and exhibits a bell-type shape in cross sections (20). Like gut-associated lymphoepithelial tissues (GALT), M cells are present in NALT for Ag uptake (21, 22). Thus, M cell-targeted immunization with a reovirus sigma-1 protein coupled to Ag effectively induced Ag-specific immune responses (23). Although dense aggregates of lymphocytes have been observed in the NALT of normal mice, germinal centers are absent, but could be induced by nasal application of Ag (18). Thus, uncommitted B cells (slgM+?) have been found in high proportions (80–85%), while low numbers of slgA− and slgG− B cells (3–4% and 0–1%, respectively) have been noted in mononuclear cells isolated from NALT (17, 19). Approximately 30–40% of NALT are CD3+ T cells with a CD4:CD8 ratio of 3–0 (17, 19). The majority of NALT CD3+ T cells coexpress CD45RB, and thus are naive, resting T cells (17, 19). Because transcriptional single cell analysis revealed the expression of mRNA for both Th1 and Th2 cytokines, the majority of CD4+ T cells are considered to be of the undifferentiated Th0 type (2). Furthermore, stimulation via the TCR-CD3 complex resulted in development of both Th1- and Th2-type cells in NALT.

Most current nasal immunization studies instill vaccine into each nostril (usually 5–10 μl/nostril), and normal inhalation subsequently results in effective delivery of vaccine, presumably into NALT. nCT and mutants of CT (mCTs) are effective mucosal adjuvants and have been widely used for nasal immunization with protein Ags, bacterial components, viruses, or virus-related peptides for the induction of secretory IgA (S-IgA) Ab responses and/or protection (24–28). Nasal immunization with OVA and mCT as adjuvant resulted in S-IgA anti-OVA Ab responses in various mucosal tissues (24). Furthermore, mice nasally immunized with pneumococcal surface protein A Ag plus mCT revealed pneumococcal surface protein A-specific IgA Ab responses associated with effective protection against capsular serotype 3 S. pneumoniae A66 (25). These Ag-specific S-IgA Ab responses were associated with polarized Th2-type responses in cervical LNs (CLN) (24, 25).

In this study, we have examined whether nasal vaccines are effective in aged mice. Our results indicate that nasal immunization with the weak Ag OVA or with the vaccine tetanus toxoid (TT) plus nCT or mCT-A E112K/B subunit of native labile toxin (LT-B) is an effective vaccine approach to induce protective immune responses in aged mice. Furthermore, significant numbers of naive CD4+ T cells were noted in NALT, but not in GALT of 1-year-old mice, and this subset was associated with the induction of protective immunity.

Materials and Methods

Mice

Young adult (6- to 8-wk-old) C57BL/6 and BALB/c mice were purchased from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). The retired C57BL/6 and BALB/c male breeders (8 mo old) were obtained from The Jackson Laboratory (Bar Harbor, ME). Upon arrival, all mice were immediately transferred to microisolators, maintained in horizontal laminar flow cabinets, and provided sterile food and water ad libitum. Experiments were performed using young adult C57BL/6 and BALB/c mice between 6 and 8 wk of age or aged mice either between 12 and 14 mo (~1 year), or those over 2 years of age. The health of the mice was tested semianually, and mice of all ages used in these experiments were free of bacterial and viral pathogens.

Chimeric mCT-A E112K/LT-B

A plasmid containing both the mCT-A E112K and LT-B genes was constructed, as previously described (29). Briefly, the mCT-A E112K gene was amplified by PCR using pUC119-E112K as a template, and the PCR product was inserted into the Ncol/HindIII site of pNCmPO2 (pNCmPO2-mCT-A E112K). The LT-B gene was also amplified by PCR using the chromosome of enterotoxigenic Escherichia coli WT-1. The PCR product was cloned into the Ncol/BamHI site of pNCmPO2 (pNCmPO2-LT-B). Next, the mCT-A E112K gene together with the preceding ribosome binding site (SD2) of Brevibacillus choshinensis cell wall protein gene was amplified using the BAMSD primer; 5′-AAA GGA TCG TAG AGG AGG AGA ACA CAA GG-3′ and mCT-A-R primer from pNCmPO2-mCT-A E112K. The amplified SD2-mCT-A E112K gene was digested and then cloned into the BamHI/HindIII site of pNCmPO2-LT-B, resulting in pNCmPO2-LT-B-mCT-A E112K (29). The expression plasmid, pNCmPO2-LT-B-mCT-A E112K, was introduced into B. choshinensis HPD31 by electroporation. The B. choshinensis HPD31 vector containing the plasmid pNCmPO2 gene was cultured in 2SLN medium at 30°C for 3 days (29). After cultivation, the mCT-A E112K/LT-B in the culture supernatant was purified using a t-galactoside immobilized column (Pierce, Rockford, IL), as described previously (29, 30).

Nasal immunization and sample collection

Aged and young adult mice were immunized three times at weekly intervals with nasal doses of 100 μg of OVA (fraction V; Sigma-Aldrich, St. Louis, MO) and 0.5 μg of nCT (List Biological Laboratories, Campbell, CA) or 5 μg of mCT-A E112K/LT-B in PBS (24, 25, 29). To compare mucosal immune responses in aged mice immunized by different routes, animals were given oral doses of 1 mg of OVA and 10 μg of nCT (15). In tetanus toxoid challenge experiments, groups of mice were immunized with TT (25 μg) and mCT-A E112K/LT-B or nCT. TT was kindly provided by Y. Higashi from The Biken Foundation, Osaka University (Osaka, Japan). Plasma and mucosal secretions (nasal washes, saliva, and fecal extracts) were collected on day 21. Saliva was obtained from mice following i.p. injection with 100 μg of sterile pilocarpine (31). Fecal pellets (100 μg) were suspended into 1 ml of PBS containing 0.1% sodium azide and were then extracted by vortexing for 5 min. The samples were spun at 10,000 × g for 1 min, and the supernatants were collected and stored at −20°C (24, 25, 31). The mice were sacrificed 7 days after the last immunization. The nasal washes were obtained by injecting 1 ml of PBS on three occasions into the posterior opening of the nasopharynx with a hypodermic needle (32).

Ab assays

Ab titers in plasma and mucosal secretions were determined by an ELISA (15, 24, 25, 30). Falcon microtiter assay plates (BD Biosciences, Oxnard, CA) were coated with an optimal concentration of OVA (100 μl of 1 mg/ml) in PBS overnight at 4°C. Two-fold serial dilutions of samples were added after blocking the wells with 5% BSA. To detect Ag-specific Abs, biotinylated mAbs specific for IgG1 and IgG2a (BD Pharmingen, San Diego, CA) and peroxidase-conjugated goat anti-mouse μ, γ, or α. H chain-specific Abs were used (Southern Biotechnology Associates, Birmingham, AL). For IgG Ab subclass determinations, biotinylated mAbs specific for IgG1 and IgG2a (BD Pharmingen, San Diego, CA) and peroxidase-conjugated goat anti-biotin Ab were used. End point titers were expressed as the last dilution yielding an OD414 of >0.1 U above negative control values after a 15-min incubation.

Enumeration of Ab-forming cells

The spleens and CLNs were removed aseptically, and single cell suspensions were prepared, as described elsewhere (15, 24, 25, 29). For isolation of mononuclear cells from NALT and nasal passages (NPs), a modified dissociation method was used based upon a previously described protocol (17–19). Individual NALT were carefully removed using microsurgical tweezers under a stereoscopic microscope. Following the removal of NALT, the NP tissues were also removed from the nasal cavity. Cells from individual tissues were prepared by gently teasing them through sterile stainless steel screens, followed by enzymatic dissociation using collagenase type IV (0.5 mg/ml; Sigma) to obtain single cell preparations (17–19). Mononuclear cells were purified on discontinuous Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden). Mononuclear cells in the interface between the 40 and 75% layers were removed, washed, and resuspended in RPMI 1640 (Cellgro Mediatech, Washington, DC) supplemented with HEPES buffer (15 mM), L-glutamine (2 mM), D-glucoses immobilized column (Pierce, Rockford, IL), as described previously (29, 30).
CD4+ T cells were purified by the magnetic-activated cell sorter system (Miltenyi Biotec, Auburn, CA), as described previously (15, 24). Briefly, isolated cells were incubated in a nylon wool column (Polysciences, Warrington, PA) to remove B cells and macrophages. Enriched T cell fractions were then incubated with biotinylated anti-CD4 (5 μg/ml) mAb, followed by streptavidin-conjugated microbeads, and passed through a magnetized column. The purified T cell fractions were >97% CD4+ and were >99% viable. Cells were resuspended in complete medium, and purified CD4+ T cells (4 × 10⁵ cells/ml) were cultured with or without 1 mg/ml of OVA in the presence of T cell-depleted, irradiated (3000 rad) splenic APCs. These APCs were derived from naive mice and were placed in 96- or 24-well tissue culture plates (Corning Glass Works, Corning, NY) for 5 days at 37°C in a moist atmosphere of 5% CO₂ in air. To assess OVA-specific T cell responses, 0.5 μCi of tritiated [³H]TdR (Amersham, Arlington Heights, IL) was added for the final 18 h of incubation. The cells were harvested, and the degree of [³H]TdR incorporation was determined by scintillation counting. In some experiments, culture supernatants were harvested after 2 or 5 days of incubation, and were then subjected to cytokine-specific ELISA.

Cytokine-specific ELISA

Levels of cytokines in culture supernatants were measured by ELISA. The details of the ELISA for IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10 have been described previously (9, 10, 28, 30). For coating and detection, the following mAbs were used: for anti-IFN-γ, R4-6A2 and XMG 1.2 mAbs; for anti-IL-2, JES6-1A12 and JES6-5H4 mAbs; for anti-IL-4, BVD4-1D11 and BVD6-24G2 mAbs; for anti-IL-5, TRFK-5 and TRFK-4 mAbs; for anti-IL-6, MP-20F3 and MP5-32C11 mAbs; and for anti-IL-10, JES5-2A5 and JES-16E3 mAbs. The levels of Ag-specific cytokine production were calculated by subtracting the results of control cultures (e.g., without OVA stimulation) from those of OVA-stimulated T cell cultures. This ELISA was capable of detecting 0.78 ng/ml IFN-γ; 8 pg/ml IL-2; 23.4 pg/ml IL-4; 0.78 pg/ml IL-5; 200 pg/ml IL-6; and 0.4 ng/ml IL-10. CD4+ T cells stimulated with a combination of anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) mAb produced 20 ± 5 ng/ml IFN-γ; 40 ± 8 ng/ml IL-2; 120 ± 12 pg/ml IL-4; 1850 ± 150 pg IL-5; 1850 ± 150 pg IL-6; and 20 ± 4 ng/ml IL-10.

Flow cytometry analysis

To assess the frequencies of naive and memory cells in various tissues from young adult and aged mice, aliquots of mononuclear cells (0.2 × 10⁹) from NALT, spleen, and Peyer’s patches were isolated from young adult and 1-year-old mice. To obtain single cell preparations, Peyer’s patches were dissociated using collagenase type IV (0.5 mg/ml; Sigma-Aldrich) after being carefully excised from the small intestinal wall (31). Mononuclear cells (0.2 × 10⁹) from individual tissues were washed with PBS containing 1% BSA (PBS-BSA). Cells were stained with FITC-conjugated anti-mouse CD45RB (23G1; BD PharMingen), and biotinylated anti-mouse CD44 (Pgp-1; BD Pharmingen) mAb at 4°C for 30 min. Cells were washed with PBS-BSA and incubated with CyChrome-streptavidin at 4°C for 30 min. These samples were subjected to flow cytometry (FACS Calibur; BD Biosciences) for cell subset analysis.

Tetanus toxin challenge

Tetanus toxin for the challenge experiment was kindly provided by Y. Higashi (The Biken Foundation). The toxin was diluted in 0.5% gelatin/PBS, and an appropriate minimum lethal dose (130 LD₅₀) was given s.c. to each mouse. Normal OVA-specific IgA Ab responses occur in 1-year-old mice (Fig. 1, Table I). Thus, OVA-specific Ab levels in 1-year-old mice were comparable to those seen in young adult mice (Fig. 1, Table I). Similarly, high levels of plasma IgG and mucosal S-IgA Ab responses were induced in BALB/c mice given nasal OVA and nCT (Table I). In contrast, 1-year-old mice given oral OVA and nCT showed significantly reduced Ab responses in plasma and fecal extracts (Table I). These results show that mucosal immune responses after nasal immunization are distinct from those that occur following oral immunization.

OVA-specific plasma Ab and AFC responses in 1-year-old mice

Because it has been shown that both oral and nasal immunization induce Ag-specific immune responses in systemic as well as mucosal sites, OVA-specific plasma Ab levels were assessed. Significant and comparable OVA-specific plasma IgA Ab responses were seen in 1-year-old mice following nasal immunization when compared with young adult mice (Fig. 2). Levels of OVA-specific IgG Abs were elevated in the plasma of young adult mice (Fig. 2). Similarly, 1-year-old mice given nasal OVA plus nCT exhibited OVA-specific, systemic IgG Ab responses that were slightly lower than those seen in young adult mice (Fig. 2). Furthermore, 1-year-old mice given nasal OVA plus nCT showed reduced, but levels still comparable to plasma IgG1 Ab responses of young adult mice (Fig. 2). As expected, low OVA-specific IgG2a Ab responses were

### Table 1. Comparison of OVA-specific Ab responses in C57BL/6 and BALB/c mice induced by different immunization routes

<table>
<thead>
<tr>
<th>Anti-OVA Ab</th>
<th>Age of Mice</th>
<th>Reciprocal Log₂ Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nasal</td>
<td>Oral</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>BALB/c</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>IgA* (Fecal extracts)</td>
<td>6–8 wk</td>
<td>12–14 mo</td>
</tr>
<tr>
<td></td>
<td>4.3 ± 0.5</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>4.0 ± 0.5</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>IgG* (Plasma)</td>
<td>6–8 wk</td>
<td>12–14 mo</td>
</tr>
<tr>
<td></td>
<td>19.3 ± 0.2</td>
<td>19.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>17.2 ± 0.3</td>
<td>17.2 ± 0.1</td>
</tr>
</tbody>
</table>

* C57BL/6 or BALB/c mice were immunized with OVA (nasoal, 100 μg; oral, 1 mg) with nCT (nasoal, 0.5 μg; oral, 10 μg) three times at weekly intervals.

* Plasma and fecal extract samples were collected at day 21 and subjected to OVA-specific ELISA.

* Values represent the mean end point titer ± SEM for 10–15 mice in each experimental group.
seen in young adult as well as in 1-year-old mice, and this was due to the propensity of nCT to induce Th2-type responses when used as mucosal adjuvant (24, 25). These results indicate that 1-year-old mice nasally immunized with protein Ag plus adjuvant possess comparable levels of Ag-specific IgG and IgA Ab responses in plasma and in mucosal secretions as those seen in young adult mice. In addition, similar anti-CT-B Ab responses were seen in 1-year-old and young adult mice (data not shown).

Mononuclear cells from NP, submandibular glands (SMG), CLN, and spleen taken from nasally immunized young adult or from 1-year-old mice were subjected to an OVA-specific ELISPOT assay to determine the numbers and isotypes of AFCs present. One-year-old mice exhibit comparable numbers of OVA-specific AFCs in IgA effector sites such as NPs and SMG (Fig. 3). Similarly, 1-year-old mice given OVA and nCT as nasal adjuvant showed intact AFC responses in both spleen and CLNs. Furthermore, 1-year-old mice showed increased numbers of OVA-specific IgM AFCs in spleen, confirming results that this isotype increases in mice as they age (Fig. 3). These results confirm that mucosal immunity mediated through the NALT immune system is maintained in 1-year-old mice. The findings are remarkably different from our previous studies that showed impaired mucosal Ab responses in 1-year-old mice given oral OVA plus nCT (15).

T cell proliferative and cytokine responses in 1-year-old mice

Our findings to this point suggest that early, age-associated changes do not occur in the NALT immune system. To examine the roles of nasally induced CD4⁺ T cells in the induction of mucosal immunity, we next assessed OVA-specific T cell proliferative responses in nasally immunized, young adult and aged mice. The CD4⁺ T cells from spleen or CLN were cultured with OVA in the presence of irradiated, T cell-depleted spleen cells. Similar level of splenic CD4⁺ T cell proliferative responses were seen in young adult and 1-year-old mice (Fig. 4). The CD4⁺ T cells from CLNs of 1-year-old mice immunized nasally with OVA plus nCT showed lower (but significant (stimulation index = 15)) proliferative responses than did those of young adult mice (Fig. 4). These results clearly support our observations that the NALT-mediated immune responses in 1-year-old mice are intact and suggest that mucosal age-associated alterations have not taken place in the NALT immune system.

We next examined Ag-specific Th1 and Th2 cytokine responses in young adult and 1-year-old mice. The culture supernatants harvested from OVA-stimulated CD4⁺ T cells isolated from spleen and CLNs of 1-year-old mice given nasal OVA plus nCT exhibited high levels of OVA-induced IL-4, IL-5, IL-6, and IL-10 responses (Fig. 5). The levels of these Th2-type cytokine responses were almost identical with those of young adult mice and ~20–50% of the cytokine synthesis induced by anti-CD3 and anti-CD28 mAb-triggered, splenic CD4⁺ T cells. In contrast, only low levels of OVA-induced IFN-γ and IL-2 production were detected in the CD4⁺ T cell cultures from spleen and CLNs of both young adult and aged mice when compared with anti-CD3 and anti-CD28 mAb-stimulated cultures (Fig. 5). These results indicate that Th2-type cytokines, especially IL-4, mediate the adjuvant effects of nCT and were maintained in 1-year-old mice when this adjuvant was given by the nasal route, although mice of this age failed to respond to oral OVA plus nCT as adjuvant (15). Taken together, our results indicate that when mice are immunized via the NALT immune system, OVA-specific, CD4⁺ T cell responses escape the influence of age-associated dysfunction that occurs in 1-year-old mice immunized via GALT.

Nasal immunization induces plasma, but not mucosal Abs in 2-year-old mice

To this point, our findings indicate that nasal immunization effectively induces both mucosal and systemic immune responses in 1-year-old mice. Thus, it was important to examine whether these
responses also occur in severely aged mice. Two-year-old mice were immunized nasally with OVA plus nCT three times at weekly intervals, and mucosal secretions and plasma were collected 1 wk after the final immunization. Significant reductions in OVA-specific, S-IgA Ab responses were noted in all mucosal secretions (nasal washes, saliva, and fecal extracts) of 2-year-old mice given nCT nasally when compared with identically immunized, young adult or 1-year-old mice (Fig. 6, p < 0.05). Conversely, plasma IgG and IgG subclass anti-OVA Ab levels in 2-year-old mice that received nasal nCT as adjuvant were significant and were comparable to those of young adult and 1-year-old mice (Fig. 6).

Specific OVA-induced Th2-type cytokine responses supported the presence of these OVA-specific Ab responses in the systemic compartment of 2-year-old mice because essentially the same levels of IL-4, IL-5, and IL-6 synthesis by OVA-stimulated splenic CD4+ T cells were noted (Fig. 5). These results suggest that although mucosal immunosenescence is delayed in the NALT immune system when compared with GALT, age-associated alterations initially take place in mucosal compartments before they occur in systemic lymphoid tissues.

**Analysis of NALT T cell subsets in aging**

To explore the mechanism for mucosal immune responses in 1-year-old mice given nasal vaccine, the frequencies of naive CD4+ T cells, which are responsible for initiation of immune responses, were compared with those in young adult mice. Mononuclear cells from NALT, Peyer’s patches, and spleen of nonimmunized mice were stained with FITC-conjugated anti-CD4, PE-labeled anti-CD45RB, and biotinylated anti-CD44 mAbs, followed by CyChrome-streptavidin, and were then subjected to flow cytometry analysis. The naive CD4+ T cell subset in spleen, Peyer’s patches, and NALT of 1-year-old mice was significantly reduced when compared with those cells in young adult mice. In addition, the actual number of mononuclear cells in spleen and Peyer’s patches was also reduced; however, the total number of cells in NALT doubled in 1-year-old mice (Table II). Thus, no overall reductions in the actual numbers of CD4+, CD45RB+ T cells were noted in NALT of 1-year-old mice (Table II). These results indicate that...
naive CD4⁺, CD45RB⁺ T cells in NALT are key players for the maintenance of the respiratory immune system in the induction of mucosal and systemic immune responses in 1-year-old mice.

**Chimeric mCT-A E112K/LT-B induces OVA-specific Ab responses in 1-year-old mice**

Our results to date indicate that nasal application of nCT induces mucosal and systemic Ab and T cell responses in 1-year-old mice. Our recent studies showed that newly constructed chimeric mCT-A E112K/LT-B elicits adjuvant activity without IgE Ab responses and potential toxicity in the CNS of young adult mice (30). Thus, it was important to examine whether similar adjuvant effects would be induced in 1-year-old mice when mCT-A E112K/LT-B was administered via the nasal route. In this regard, 1-year-old and young adult mice were nasally immunized with OVA plus mCT-A E112K/LT-B. When OVA-specific Ab responses in nasal washes, saliva, and fecal extracts were examined, significant S-IgA Ab responses occurred in mucosal secretions of both young adult and 1-year-old mice (Fig. 7). In addition, significant and comparable OVA-specific plasma IgA Ab responses were also seen in aged mice when compared with young adult mice (Fig. 7). Furthermore, both 1-year-old and young adult mice elicited similar levels of plasma IgG anti-OVA Ab responses.

Interestingly, both young adult and 1-year-old mice immunized nasally with OVA plus chimeric mCT-A E112K/LT-B showed a similar pattern of IgG subclass response. Thus, increased IgG1 and IgG2b, but essentially no IgG2a, anti-OVA Ab responses were detected (Fig. 7). Comparable levels of CD4⁺ T cell proliferative responses were seen in CLNs of 1-year-old mice given nasal OVA plus chimeric mCT-A E112K/LT-B (stimulation index = ~10). Although stimulation indices in spleen of aged mice given nasal OVA plus chimeric mCT-A E112K/LT-B resulted in lower proliferative responses than occurred in young adult mice, the proliferative responses were nevertheless maintained (stimulation index = ~5–10). Taken together, these findings show that nasal administration of a nontoxic, safe mucosal adjuvant, mCT-A E112K/LT-B, effectively induced OVA-specific Ab and T cell responses in 1-year-old mice.

**Induction of protective immunity**

Because nasal immunization with OVA and chimeric enterotoxin as mucosal adjuvant induced both mucosal and systemic Ab responses in 1-year-old mice, it was important to determine whether

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**Table II. Comparison of naive and memory CD4⁺ T cells and cell yields from lymphoid tissues of young adult and aged mice**

<table>
<thead>
<tr>
<th>Source of Tissue</th>
<th>Age of Mice</th>
<th>CD45RB⁺/CD4⁺T Cells (% of Total CD4⁺ T cells)</th>
<th>Lymphocyte Counts/Mouse</th>
<th>Actual Numbers of CD4⁺ Naive T Cells/Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>NALT</td>
<td>8–12 wk</td>
<td>76.5 ± 4.2</td>
<td>~1.0 × 10⁵</td>
<td>~8.1 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>12–14 mo</td>
<td>29.1 ± 4.0</td>
<td>~0.5–9.5 × 10⁵</td>
<td>16.1–30.5 × 10⁵</td>
</tr>
<tr>
<td>Peyer’s patches</td>
<td>8–12 wk</td>
<td>43.6 ± 8.8</td>
<td>~15–20 × 10⁶</td>
<td>11.2–14.9 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>12–14 mo</td>
<td>24.1 ± 6.6</td>
<td>~10–16 × 10⁶</td>
<td>2.9 × 10⁵</td>
</tr>
<tr>
<td>Spleen</td>
<td>8–12 wk</td>
<td>67.0 ± 2.0</td>
<td>~60–75 × 10⁶</td>
<td>10.5–13.1 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>12–14 mo</td>
<td>50.0 ± 0.1</td>
<td>~20–26 × 10⁶</td>
<td>1.9–2.5 × 10⁶</td>
</tr>
</tbody>
</table>

* Mononuclear cells from NALT, Peyer’s patches, and spleen of immunized mice were stained with FITC-conjugated anti-CD4, PE-labeled anti-CD45RB, and biotinylated anti-CD44 mAbs, followed by CyChrome-streptavidin, and then subjected to flow cytometry analysis by FACSCalibur.

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**FIGURE 7.** Comparison of OVA-specific IgA Ab responses in nasal washes, fecal extracts, saliva, and plasma of 1-year-old (filled bars) and young adult (open bars) mice given nasal chimeric mutant toxin. Each mouse group was nasally immunized weekly for 3 consecutive wk with 100 μg of OVA plus 5 μg of chimeric mCT-A E112K/LT-B as mucosal adjuvants. Seven days later, Ab levels in nasal washes, fecal extracts, saliva, and plasma were determined by OVA-specific ELISA. The values shown are the mean ± SEM of 15 mice in each experimental group.

**FIGURE 8.** Nasal immunization with TT and chimera or nCT adjuvants elicited protective immunity in 1- and 2-year-old mice. Both groups of 1-year-old (• or ●) and young adult (○ or △) mice were immunized nasally with 10 μg of TT and 5 μg of chimeric mCT-A E112K/LT-B (○ or ●) or 10 μg of TT alone (▲ or △) three times at weekly intervals. Two-year-old mice (□) were given nasal TT (10 μg) and nCT (0.5 μg) three times at weekly intervals. Mice were s.c. challenged with a lethal dose (130 LD₅₀) of tetanus toxin in 0.5 ml of PBS including 0.2% gelatin. Each group was comprised of five mice, and the data are representative of two separate experiments.
these Ag-specific Ab responses could protect mice from infection or intoxication. In this regard, both groups of 1-year-old and young adult mice were immunized nasally with TT and chimeric mCT-A E112K/LT-B or TT alone three times at weekly intervals. The mice were challenged s.c. with a lethal dose (130 LD$_{50}$) of tetanus toxin injected 1 wk after the last immunization. As expected, young adult mice given nasal TT plus chimeric enterotoxin as mucosal adjuvant were completely protected (Fig. 8). Importantly, 1-year-old mice given TT and mCT-A E112K/LT-B showed a degree of protection comparable to that seen in young adult mice, even though 1 of 15 mice died 4 days after challenge. Furthermore, 2-year-old mice were given nasal TT, and nCT showed complete protection from challenge (Fig. 8). In contrast, both 1-year-old and young adult mice given TT alone failed to protect from the paralysis and death that normally occurs within 2 days after tetanus toxin administration (Fig. 8). These findings clearly show that TT-specific, plasma IgG Abs in both 1- and 2-year-old mice induced by the chimeric mCT-A E112K/LT-B or nCT mucosal adjuvants were protective.

**Discussion**

This study has revealed that nasal administration of nCT or chimeric mCT-A E112K/LT-B, in contrast to oral immunization (15), effectively induced mucosal and systemic immune responses in 1-year-old mice. Thus, Ag-specific Ab responses were seen in plasma and mucosal secretions, and Ag-specific AFCs were detected in NPs, SMG, and the spleen, clearly showing that both mucosal and systemic immunity occurred in 1-year-old mice following nasal immunization. Furthermore, elevated levels of CD4$^+$ T cell proliferative and OVA-induced Th2-type cytokine responses were seen in CLN and spleen of 1-year-old mice given nasal OVA plus nCT or mCT-A E112K/LT-B chimera. In 2-year-old mice, Ag-specific plasma Ab responses were seen; however, mucosal S-IgA Ab responses were not induced when nCT was used as nasal adjuvant. When the frequencies of naive CD4$^+$ T cells in NALT, Peyer’s patches, and spleen were compared in young adult and 1-year-old mice, reduced frequencies of CD4$^+$, CD45RB$^+$ T cells were seen in aged mice. Importantly, the actual cell counts of naive CD4$^+$ T cells in NALT of 1-year-old mice were higher than those seen in young adult mice. Finally, 1-year-old mice given a nasal TT vaccine and chimeric mCT-A E112K/LT-B as adjuvant were protected from systemic challenge with tetanus toxin.

It has been suggested that the mucosal immune system is compartmentalized and differs remarkably from the systemic one (34). In this regard, either nasal or oral immunization induces Ag-specific immune responses at mucosal surfaces as well as in systemic lymphoid tissues via the common mucosal immune system. In contrast, parenteral immunization does not effectively elicit mucosal immunity (34). It is evident that different types of mucosal immune responses occur when Ag is given either by the oral or nasal routes. Oral immunization effectively induces Ag-specific S-IgA Ab responses in the GI tract and saliva, but is less effective for S-IgA responses in the respiratory and reproductive tracts (31, 35, 36). In contrast, previous studies clearly showed that a nasal immunization regimen elicited significant immunity not only in the respiratory tract, but also in saliva, as well as in the GI and reproductive tracts (24, 25). These results suggest that distinct immune regulatory mechanisms in GALT vs NALT account for the induction of mucosal S-IgA and parenteral IgG Ab responses. Indeed, our current findings in 1-year-old mice given nasal vaccine revealed significant Ag-specific immune responses that provided effective protection, whereas our previous study had clearly shown that oral immunization with OVA plus nCT failed to elicit either OVA- or CT-B-specific Ab responses (28). Taken together, these two separate studies suggest that the regulation of the NALT-mediated mucosal immune system is distinct from the GALT-directed system. Furthermore, the nasal route may be the preferred method for administering vaccines to induce both Ag-specific mucosal and systemic immune responses in the elderly.

Additional evidence for differences between NALT and GALT has been provided by the finding that organogenesis of NALT occurs independently of the lymphotoxin $\beta$ (LT$\beta$) and LT$\beta$ receptor signaling pathways (37, 38). Thus, LT signaling pathways are essential for the genesis of Peyer’s patches or GALT as well as for lymph nodes (LN) and spleen (39). For example, both LT$\alpha$ and LT$\beta$ gene knockout mice lack Peyer’s patches and LNs (40–44). Furthermore, administration of LT$\beta$R-Ig during gestation disrupted the development of LNs and Peyer’s patches (45, 46). It was also shown that IL-7 and the IL-7R pathway are essential for the development of GALT (47–50); however, an intact NALT system was present, but was reduced in size in IL-7R$^+$ knockout mice (37, 38). Furthermore, organogenesis of NALT was shown to begin after birth, while GALT development began during gestation (38). These studies clearly point to significant differences in the development of the two major mucosal inductive tissues, e.g., GALT and NALT. Because of these differences, one would predict that age-associated alterations may independently occur in these mucosal inductive tissues. Our findings that nasal, but not oral immunization induced Ag-specific immune responses in 1-year-old mice strongly support the notion that the aging develops more slowly in NALT than GALT.

It has been shown that increased numbers of memory-type cells are associated with aging (51–54). Thus, aged mice showed decreased numbers of naive CD4$^+$ T cells that express CD45RB molecules. In this regard, overexpression of the Fas gene under the CD2 promoter resulted in reduced numbers of memory-type T cells in aged mice, and rejuvenated immune responses were seen in aged Fas-CD2 transgenic mice that resembled those of young adult mice (55). Furthermore, it was suggested that effector T cell production from naive T cells is also impaired in aged mice (56, 57). IL-2, but not other common $\gamma$-chain cytokine receptor-related ILs, e.g., IL-4, IL-7, or IL-15, effectively restored development of effector cells from naive precursors in aged mice (57). In this regard, it is important to investigate the frequencies of naive T cell populations in various mucosal tissues of aged mice. Our findings clearly show that the actual numbers of CD4$^+$, CD45RB$^+$ T cells in the NALT of aged mice were essentially the same as those of young adult mice. Thus, although the frequency of CD4$^+$, CD45RB$^+$ T cells was reduced, total numbers of lymphocytes in NALT were twice as high in 1-year-old mice as in young adult mice. In contrast, the total numbers of CD4$^+$, CD45RB$^+$ T cells were reduced, and CD4$^+$, CD44$^+$ T cell populations were increased in the spleen of aged mice. Furthermore, Peyer’s patches of aged mice showed significant reductions in CD4$^+$, CD45RB$^+$ T cell frequencies in addition to total cell numbers when compared with young adult mice. These results suggest that the naive T cell population in NALT plays a pivotal role in the induction of both systemic and mucosal immune responses in 1-year-old mice.

Our results also suggest that naive T cell functions in NALT are maintained and these T cells are capable of developing into effector T cells. Further support for the presence of intact NALT T cell function in senescent mice, our results showed that high levels of Ag-specific CD4$^+$ T cell proliferative responses occur in CLN and spleen of 1-year-old mice given OVA and nCT. Furthermore, these CD4$^+$ T cells were capable of producing Th2-type cytokines when T cells were restimulated with OVA in vitro. Interestingly, when 2-year-old mice were given nasal OVA plus nCT, OVA-specific CD4$^+$ T cell immune responses were also maintained in the CLN.
and splenic compartments. These results further confirm our prediction that naive CD4+ T cells in the NALT of 1-year-old mice become effector CD4+ T cells and migrate into both systemic and mucosal lymphoid compartments.

It is often suggested that experimental mice should be at least 2 years old to be suitable models for vaccine evaluation in elderly humans. In this regard, 2-year-old mice were immunized nasally with OVA and nCT as adjuvant. In terms of Ag-specific Ab responses, mice given nCT failed to undergo mucosal S-IgA Ab responses; however, and interestingly, mice that received nCT as mucosal adjuvant showed Ag-specific, systemic immune responses that were essentially identical with the responses seen in young adult mice. Similarly, CD4+ T cell proliferative as well as Th1 and Th2 cytokine responses in the spleen of 2-year-old mice were comparable to those of young adult mice when nCT was used as nasal adjuvant. These results further confirm our previous findings that mucosal immunosenescence takes place before systemic immune dysregulation (15), even though nasal, but not oral immunization is still effective in 2-year-old mice. To overcome this immune dysregulation (15), even though nasal, but not oral immunization using nCT as mucosal adjuvant may induce sufficient CTL responses in 2-year-old mice. Supporting this notion, our findings showed that intact Ag-specific T cell immune responses occur in 2-year-old mice given nasal OVA and nCT. Furthermore, nCT is known to elicit CTL activity when coadministered with viral protein Ag given by the nasal route (60). We are currently investigating Ag-specific CTL responses in NALT of 2-year-old mice given nCT as mucosal adjuvant.

Although nCT-A E112K/LT-B failed to elicit Ag-specific T and B cell and Ab responses in 2-year-old mice when given with OVA, this mucosal adjuvant successfully induced Ag-specific immune responses in 1-year-old mice. Importantly, these immune responses provided immune protection. Thus, 1-year-old mice given TT and mutant chimera toxin showed significant survival rates (>80%) when s.c. challenged with tetanus toxin. Based upon these results, mCT-A E112K/LT-B shows promise as a practical mucosal adjuvant to accompany vaccines for the elderly. Early vaccination in the elderly would be expected to be more effective, and vaccination in a middle-aged population may provide sufficient immunity later in life.

An additional reason for use of mCT-A E112K/LT-B chimera in a mucosal vaccine is its safety. Our separate studies showed that mCT-A E112K/LT-B chimera was essentially nontoxic when compared with nCT (29). In this regard, reduced levels of Ag-specific IgE Ab responses were noted in young adult mice given nasal TT and mCT-A E112K/LT-B chimera. Furthermore, this chimera toxin showed essentially no Ag redirection into CNS tissues in contrast to mice given nCT (29). Because safety is an important issue for nasal vaccines, the features of mCT-A E112K/LT-B as a nasal adjuvant offer significant advantages in the development of vaccines for the elderly.

Our current study has clearly shown the effectiveness of nasal immunization in senescent mice that is mediated through a naive CD4+ T cell subset in NALT. Nasal immunization with protein Ag plus nCT or mCT-A E112K/LT-B as mucosal adjuvants was shown to induce protective Ag-specific Ab responses in 1-year-old mice. In contrast, our previous study showed that impaired Ag-specific Ab responses occur in 1-year-old mice given OVA plus nCT by the oral route (15). Taken together, these studies clearly show that the route of immunization is a critical factor for effective induction of protective mucosal immunity in the elderly. In addition, the age of initial mucosal immunization is another important factor, because our study showed that nCT failed to induce mucosal Ab responses in 2-year-old mice, but not in 1-year-old mice. Because the efficacy and safety of mucosal adjuvants are essential elements in mucosal vaccine development, it will be necessary to continue optimizing mucosal adjuvants that are suitable for use in the elderly.

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References


