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Upper Respiratory Tract Resistance to Influenza Infection Is Not Prevented by the Absence of Either Nasal-Associated Lymphoid Tissue or Cervical Lymph Nodes[1]

James A. Wiley,[2]* Michael P. Tighe,[†] and Allen G. Harmsen*

The murine nasal-associated lymphoid tissue (NALT) and cervical lymph nodes (CLN) are involved in the generation of local immune responses within the upper respiratory tract (URT). However, their involvement in these responses does not imply the necessity for resistance to URT infections. We surgically removed NALT or CLN to address the necessity of these lymphatic tissues for the development of a local protective immune response after a URT influenza infection. No histological evidence of the re-establishment of either tissue was detected after surgery and the subsequent infection. Removal of NALT did not elicit changes in serum or nasal mucosa-associated influenza-specific Ig levels. However, increases in PR8-specific serum IgG and nasal mucosa-associated IgA were detected after removal of CLN. Recruitment of influenza-specific CD4 T cells into the nasal mucosa was not altered by removal of NALT. The removal of NALT or CLN did not alter the recruitment of influenza-specific CD8 T cells into the URT. However, increased levels of influenza-specific CD8 T cells were observed in the tracheal-bronchial lymph nodes after CLN surgery. The rate of viral clearance from nasal mucosa and lungs was not altered by removal of NALT or CLN. These studies demonstrate that despite the participation of NALT and CLN in the generation of local immunity to influenza infections, neither tissue is essential for the development of protective immunity and viral clearance in URT. *The Journal of Immunology,* **2005, 175: 3186–3196.**

Evidence from murine infectious disease models has demonstrated the participation of nasal-associated lymphatic tissues (NALT)[3] and cervical lymph nodes (CLN) in the generation of local humoral and cellular immune responses within the upper respiratory tract (URT) after intranasal infection (1, 2). Both of these lymphatic tissues are also known to be involved in the induction of distal mucosal as well as systemic immunity after the administration of intranasally targeted vaccines (3–5). Nevertheless, the participation of these local lymphatic tissues in the generation of pathogen- or vaccine-derived immunity does not imply that their presence is necessary to successfully resist infections within URT. In developing surgical models to remove NALT or CLN, we were able to determine whether these URT lymphatic tissues play an essential role in resistance to infections in the URT.

In the human URT, local immune induction sites include the mucosal lymphatic tissues of Waldeyer's ring and the various systemic lymph nodes located within the neck. The major lymphatic tissues of Waldeyer's ring consist of the nasopharyngeal or adenoid tonsil, the paired tubal tonsils at the Eustachian tube openings, the paired palatine tonsils on either side of the oral pharynx, and the lingual tonsil in the posterior third of the tongue (6). Surgical removal of the palatine tonsils has long been a common procedure in patients with recurrent throat infections. This tonsillectomy procedure is not associated with any loss in the ability to generate protective immunity within the human URT. However, because the palatine tonsils comprise only a portion of Waldeyer's ring, it is likely that the remaining mucosal lymphatic tissues could compensate for this loss, thus maintaining the involvement of local mucosal lymphatic tissues in the generation of URT immunity. Therefore, the necessity of having local mucosal lymphatic tissues participating in the generation of protective immunity in response to URT infections remains to be determined.

In addition to the involvement of CLN in the generation of local immunity, this systemic lymph node is critical for the induction of immunological tolerance to intranasally administered Ag (7). Removal of the CLN resulted in the loss of localized tolerance induction. Tolerance to nasally administered Ag could be restored by transplant of another CLN, but not by transplant of other peripheral lymph nodes. Although this evidence defined the CLN as a unique site for the induction of local tolerance, it remained to be determined how critical this systemic lymphatic tissue was in the generation of local protective immunity after pathogen exposure within the URT.

In determining the necessity for a particular component of an immune response, it has become a common practice to make use of knockout ($-/-$) mice lacking the component of interest. The use of lymphotoxin $\alpha^{-/-}$ ($LT\alpha^{-/-}$) mice has demonstrated that this cytokine is required for the structural organization of the constituent immune cells within the NALT in order for this mucosal lymphatic tissue to properly function as a local immune induction site (8, 9). The defects in cellular organization observed in the NALT of $LT\alpha^{-/-}$ mice also extend to the absence of and/or organizational defects in other secondary lymph nodes of these mice (10). Despite the lack of $LT\alpha$, these mice are able to mount a

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[3] Abbreviations used in this paper: NALT, nasal-associated lymphoid tissue; BAL, bronchoalveolar lavage; CLN, cervical lymph node; LTα, lymphotoxin α; NP, nucleoprotein peptide; PA, polymerase peptide; TBLN, tracheal-bronchial lymph node; URT, upper respiratory tract.

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delayed, yet effective, response to intranasal influenza infections. Other −/− models involving TNF/TNFR family members also demonstrated lymphoid tissue defects that extended to their secondary lymphoid tissues. Within lymphatic tissues, networks of immunological mediators exert their influence on the development of constituent immune cell subsets to regulate the development of local immunity. A consequence of these immunological networks is that the functional necessity of any given lymphatic tissue is unlikely to be determined through the use of animal models that are genetically deficient in single or even multiple gene products. To determine how dependent the development of protective immunity within the URT is upon specific local lymphatic tissues, we developed surgical techniques to selectively remove or disrupt the structural and thus the functional integrity of the lymphatic tissue in question.

In the following series of experiments we describe how the surgical destruction of the organizational structure of NALT or the removal of CLN affects the subsequent development of Ag-specific protective immunity within the URT after an intranasal influenza virus infection. No re-establishment of NALT or CLN was detected before or after the infection of those animals that had undergone surgery. The loss of NALT did not result in any consistent difference in the level of nasal-associated or serum influenza-specific Ig detected in the NALT vs sham surgery groups. In contrast, the loss of CLN did result in some enhancement of the PR8-specific, nasal-associated IgA and serum PR8-specific IgG levels. The loss of NALT did not significantly alter the recruitment of influenza-specific CD4 or CD8 T cells to effector sites within the nasal mucosa. Removal of CLN did not alter the ability of the host to recruit influenza-specific CD8 T cells into the nasal mucosa. However, CLN surgery elicited increased accumulations of influenza-specific CD8 T cells in the tracheal-bronchial lymph nodes. Removal of CLN or NALT did not compromise the ability of those animals to clear virus from their nasal mucosa or the lungs. These studies demonstrated that although CLN and NALT participate in URT immune responses, they are not essential lymphatic tissues in the development of effective resistance to URT influenza infections.

Materials and Methods

Mice

Male and female BALB/c Thy 1.1 and C57BL/6 mice, 4–6 wk of age, were used in these experiments. These animals were purchased from the National Cancer Institute or bred at Montana State University. These animals were housed at the Animal Resource Center of Montana State University for the duration of the experimental procedures. The BALB/c Thy1.2 CD4/HNT TCR transgenic mice used in these experiments were obtained from the Trudeau Institute breeding facility and bred at the Animal Resource Center of Montana State University. The CD4/HNT T cells of these mice express the Vβ8.3 TCR derived from clone T2.5-5 and are specific for the PR8 influenza hemagglutinin peptide 126–138 (HNT NGVTAACSHE) presented in the context of I-A^d. The animals and procedures used throughout these experiments complied with the approved institutional animal care and use committee protocols established at Montana State University.

NALT surgical procedure

This surgery was performed on 5- to 6-wk-old mice. The animals were anesthetized by i.p. administration of a sublethal dose of sodium pentobarbital (60 μg/g). When the sedated animals reached the point at which no pedal reflex was detected, they were laid in a supine position with their mouths held open to reveal access to the hard palate. The topical analgesic, maracine (0.5% solution), was applied to the hard palate. A small 2- to 3-mm incision was made along the midline of the hard palate, and a 0.5-mm microdissection curette (Roboz) was inserted through the incision. The area containing each NALT strip was scrapped to disrupt the structural integrity of the tissue. The retraction on the mouth was then released, and the incision was sutured shut using a single absorbable surgical suture (Roboz). The mice were allowed to recover from the anesthesia under a constant flow of 100% O_2. After this, the animals were returned to their cages and allowed to continue their recovery from surgery for the next 2 wk before their continued use in the experiments. In these experiments a sham surgical control group and a NALT surgery experimental group were used. The sham surgical control group was subjected to the surgical procedure only to the point at which a midline incision into the hard palate was made. These mice did not undergo any disruption of the NALT. The incision was then sutured shut, and the animals were allowed to recover as described.

CLN surgical procedure

This surgery was also performed on 5- to 6-wk-old mice. The animals were anesthetized by i.p. administration of a sublethal dose of sodium pentobarbital (60 μg/g). When the sedated animals reached the point at which no pedal reflex was detected, they were laid in a supine position, and the hair covering the throat region was gently shaved to reveal the skin. A single-line, 0.5-cm incision was made along the center of the neck, and the skin was gently teased back to reveal the superficial or mandibular CLN on each side of the neck lying on the surface of the masseter muscle (11). Within the facia adjacent to each jugular vein lies the internal jugular CLN on each side of the neck (7). These four CLN were then excised, and the incision was closed with a liquid suture material. The mice were allowed to recover from the anesthesia under a constant flow of 100% O_2. After this, the animals were returned to their cages and allowed to continue their recovery from surgery over the next 2 wk before their continued use in the experiments. In this series of experiments, a sham surgical control group was included in which the animals were subjected to the same surgical procedure, except that the CLN was not excised. This group was included to control for the effects of the surgical manipulations. Animals taken at each time point were initially examined for any re-establishment of CLN or to determine whether any CLN remained after surgery.

Viral infection of mice

For the purposes of these experiments, the influenza viruses A/PR8/8/34 (PR8; H1N1) and A/HKx31(X31; H2N3) were used. These two virus strains share the same internal proteins, whereas their surface hemagglutinin and neuraminidase proteins are derived from different serotypes (12). Both influenza virus strains were grown in the allantoic fluid of 10-day-old embryonated chicken eggs. The infected eggs were grown for 48 h at 35°C before the allantoic fluid was harvested and then stored at −80°C. The virus preparation was made at the Trudeau Institute. To address how the absence of a functional NALT or CLN affected the development of the URT immune response, sham control or surgically manipulated mice were inoculated intranasally with 5 μl/nare of allantoic fluid containing 1.5×10^4 PFU of PR8 or 5×10^6 PFU of X31influenza virus while they were under light isoflurane anesthetization. Primary infections with the PR8 influenza virus in these experiments took place 2 wk after surgery. This was considered day 0 in these experiments. Secondary PR8 intranasal infections were given 34 days later in the same manner. Secondary X31 infections, used to test heterosubtypic T cell immunity, were administered 14 days after NALT surgery had been performed on animals that were infected with the PR8 influenza strain 25 days before surgery.

Tissue sampling

At designated time points after the PR8 influenza infections, selected tissues were extracted from both surgery groups. The mice were placed under deep anesthesia using a lethal i.p. injection of sodium pentobarbital (100 μg/g). At the absence of any pedal reflex, the abdominal cavity was opened, and the mice were exsanguinated by perforation of their abdominal aorta. Bronchoalveolar lavage (BAL) fluids were obtained by fully inflating the lungs three times with the same 1-ml aliquot of 3 mM EDTA in HBSS. Nasal wash fluids were obtained by slowly flushing the nasal cavity once with 0.7 ml of 3 mM EDTA in HBSS. To perform this nasal wash without incurring any damage to the interior of the nasal cavity, the trachea was perforated, and a small tube was inserted into the opening. A total of 0.7 ml of HBSS was flushed through the nasal cavity and collected from the nares. After the nasal wash, the hard palate region was carefully dissected away from the mouth and nasal cavity to ensure that the NALT region was included in the excised tissue. Each tissue was gently passed through a mesh screen to produce a single-cell suspension. The total number of cells obtained was determined, and a differential cell count analysis was performed on Diff-Quik (Dade Behring)-stained cytospins of each NALT preparation. The total number of lymphocytes was calculated by multiplying the percentage of lymphocytes obtained in the differential cell analysis by the total number of cells in the single-cell suspension. The CLN, tracheal-bronchial lymph node (TBLN), and nasal mucosa were extracted as previously described

(2) and processed for flow cytometry. A sample of each nasal mucosa tissue was immediately processed or snap-frozen in liquid nitrogen for assessment of viral recovery. If the lungs were removed, they were placed in 2 ml of 2% BSA-supplemented DMEM (Invitrogen Life Technologies) and immediately snap-frozen in liquid nitrogen. Assessment of virus present in the nasal mucosa or lungs was conducted by plaque assay using Madin-Darby canine kidney cells as previously described (13).

Analysis of total and PR8-specific Ab levels

The determination of total and PR8-specific Ig levels in the nasal washes and serum was accomplished using 96-well, high binding, polystyrene ELISA plates coated with either 100 μl of a goat anti-mouse IgA or IgG capture Ab (Southern Biotechnology Associates) at 2 μg/ml for total Ig levels or coated with 100 μl/well of a 2 μg/ml solution of a PR8 influenza membrane preparation in the case of the Ag-specific Ig levels, in a 0.05 M carbonate buffer (0.159 g of Na_2CO_3 and 0.293 g of $NaHCO_3$, pH 9.6). The plates were coated overnight at 4°C, then washed in PBS/Tween 20. Nonspecific binding sites were blocked with 5% nonfat skim milk in PBS for 1 h at 37°C. Aliquots of nasal wash and serum samples (diluted 1/100 in PBS) were added to the wells and incubated for 1 h at 37°C. After three PBS/Tween 20 washes, the secondary alkaline phosphatase-conjugated, anti-mouse, isotype-specific Ab (Southern Biotechnology Associates) was added and allowed to incubate for 2 h at 37°C. The presence of bound Ab was detected by the addition of *p*-nitrophenyl phosphate in diethanolamine buffer and was visualized at 405 nm. The Ig contents of the nasal wash samples were compared with standard curves generated from the 405 nm absorbance levels of known amounts of IgG or IgA.

The PR8 influenza membrane preparation was derived from the outer membrane of the PR8 influenza virus, which contains the hemagglutinin and neuraminidase proteins. A purified virus preparation was made by pelleting the virus from the allantoic fluid at 145,000 $\times$ *g* for 45 min (38,000 rpm; Ti60 rotor; Beckman Coulter) and then banding the pelleted virus on a 30–45-60% sucrose step gradient for 45 min (25,000 rpm; SW28 rotor; Beckman Coulter). The fractions from the gradient containing the highest hemagglutination titers were extracted and pooled, and the virus was pelleted at 145,000 $\times$ *g* for 45 min (38,000 rpm; Ti60 rotor; Beckman Coulter). The purified virus preparation was resuspended in a sodium acetate buffer (0.05 M sodium acetate, 2 mM $CaCl_2$, and 0.2 mM EDTA, pH 7.0), then lysed by adding an equal volume of the sodium acetate buffer containing 15% octyl-β-D-thioglucoside (Calbiochem) to the virus suspension. The preparation was centrifuged for 1 h (16,500 rpm; Ti60 rotor; Beckman Coulter). The supernatant was retained, dialyzed against HBSS, and concentrated, then assessed for its hemagglutination activity and protein content and stored at −80°C until used.

Adoptive transfer of CD4 T cells

Naive CD4/HNT T cells used in adoptive transfer experiments were prepared from male and female CD4/HNT transgenic BALB/c Thy1.2 mice. Single-cell suspensions made from the donor spleens after lysis of the RBC were labeled with CFSE (Molecular Probes). The CD4 T cell population was enriched by passage of the cell preparation through a CD4 T cell subset column (R&D Systems) according to the manufacturer's instructions. Each recipient BALB/c Thy1.1 mouse received 5×10^5 Thy1.2 cells by i.v. tail injection 2 days before intranasal influenza infection.

Flow cytometric analysis

Flow cytometric analysis was performed on single-cell suspensions of selected tissues in each experiment. Each tissue was passed through a mesh screen to obtain a single-cell suspension. The RBC content of the nasal mucosa sample was lysed using a standard lysis buffer (150 mM NH_4Cl, 1.0 mM $KHCO_3$, and 0.1 mM Na_2-EDTA). Live gating during FACS analysis on the recovered lymphocyte populations was used in the analysis of CD4 and CD8 T cells in these experiments. Staining for the adoptively transferred CD4 T cell populations was accomplished using CyChrome-conjugated anti-mouse CD4, biotin-conjugated anti-mouse CD90.2 (Thy1.2), and allophycocyanin-conjugated avidin (BD Pharmingen). Analysis of the proliferation of the adoptively transferred CD4/HNT T cell population was accomplished by selective live gating on the recovered adoptively transferred T cells according to their $CD4^+/Thy1.2^+$ expression and their CSFE florescence level. Ag-specific CD8 T cells in C57BL/6 mice were detected within the live lymphocyte-gated population using CyChrome-conjugated anti-mouse CD8 (BD Pharmingen) and H-$2D^b$-restricted allophycocyanin-conjugated tetramer-binding complexes. These complexes recognize CD8 T cells specific for the nucleoprotein peptide $NP_{366–374}$ (ASNENMETH) or the polymerase peptide $PA_{224–233}$ (SSLEN FRAYV; Trudeau Institute). The FACS data were acquired on a FACSCalibur, then analyzed using CellQuest software (BD Pharmingen). The total number of each T cell subpopulation of interest in a given tissue was determined by multiplying the total cell count from the recovered tissue by the percentage of the total number of cells found by FACS analysis to be expressing the phenotype of interest.

Results

Surgical disruption of the NALT or removal of the CLN was not followed by re-establishment of that lymphatic tissue

Surgical ablation of NALT or CLN was conducted 2 wk before the administration of an intranasal influenza infection. To confirm that we had successfully ablated the NALT and that the NALT did not re-establish itself before the influenza infection, we examined histological sections from the nasal cavities of mice that had undergone NALT surgery up to 3 wk previously (Fig. 1, *A* and *B*). No evidence of a structurally intact NALT was detected in the mice

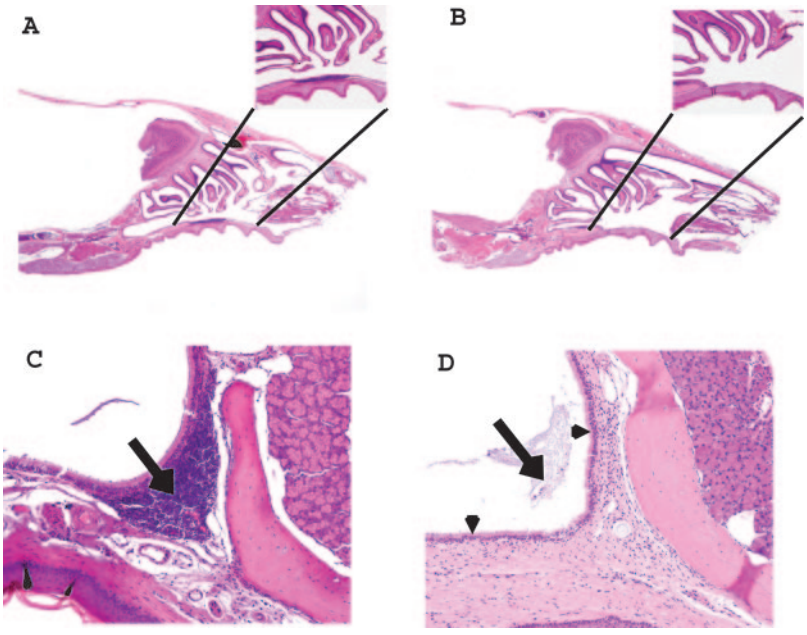

FIGURE 1. Histological sections cut from NALT and sham surgery mice. Midsagittal sections were cut from the nasal cavity of animals in the sham surgery (*A*) and NALT surgery (*B*) groups 3 wk after surgery. The *insets* in *A* and *B* are enlargements of the NALT-containing region. Frontal cross-sections were cut from the sham surgery (*C*) and NALT surgery (*D*) groups 3 wk after surgery. The arrow in *C* indicates the location of the NALT 3 wk after the sham surgery procedure. The arrow in *D* indicates the location previously occupied by the NALT 3 wk after NALT surgery. After a 3-wk recovery period from NALT surgery, the NALT is not re-established, and the location formerly occupied by the NALT remains devoid of any organized lymphatic tissue. Surgical debris, scar tissue, and the reconstituted nasal epithelium (arrowheads) now occupy this space. Sections were cut from formalin-fixed decalcified nasal cavities and H&E stained.

after the NALT surgery. By 3 wk after the surgery, only the respiratory epithelium had re-established itself in the region previously occupied by NALT (Fig. 1, *C* and *D*). Lymphocyte recruitment into the lamina propria area formerly occupied by NALT remained at significantly low levels even in mice that had been intranasally infected with influenza virus before NALT was removed (Fig. 2). In the absence of a presurgery intranasal influenza infection, similar significant reductions in lymphocyte recoveries were observed in the NALT region 2 wk after surgery (data not shown). NALT region lymphocytes detected in the NALT surgery group were present in the reconstituted respiratory epithelium and lamina propria where the organized NALT had been. The lymphocytes within the former NALT region were found at densities equivalent to those of lymphocytes in other areas of the nasal mucosa and were never found as organized lymphoid tissues.

In those experiments in which CLN were removed, no evidence of any redevelopment of this systemic lymphatic tissue site could be seen when the animals were assessed for local immune response (data not shown).

Total and PR8-specific Ab levels after NALT surgery

The loss of NALT did not consistently alter the levels of total or PR8-specific IgA (Fig. 3, *A* and *B*) in the nasal mucosa after a primary or a secondary intranasal infection. In both surgery groups, the levels of nasal mucosa-associated PR8-specific IgA displayed equivalent increases in titer that are typically associated with secondary responses. Total IgG was detectable in the nasal mucosa of most animals in both surgery groups throughout the experiment (Fig. 3*C*). In contrast, detectable levels of PR8-specific IgG in the nasal mucosa were absent from both groups at some time points or, when detected, were present in only half or fewer of the animals in each group (Fig. 3*D*). The levels of total and PR8-specific IgG in the nasal wash remained typically lower than the corresponding detected IgA levels in response to the intranasal influenza infection as reported by others (14). PR8-specific serum IgG levels (Fig. 3*E*) and PR8-specific serum IgA levels (data not shown) were not consistently different between the NALT surgery and sham surgery groups during the responses to infection. Although the PR8-specific serum IgG level was significantly higher in the sham surgery group on day 14, the two groups tended toward more equivalent levels as the primary response waned. This equivalence briefly carried into the secondary response until the NALT surgery group displayed a significantly greater PR8-specific serum IgG level by day 46. The loss of NALT did not result in any consistent differences in the PR8-specific IgG or IgA Ab levels recovered in the BAL (data not shown).

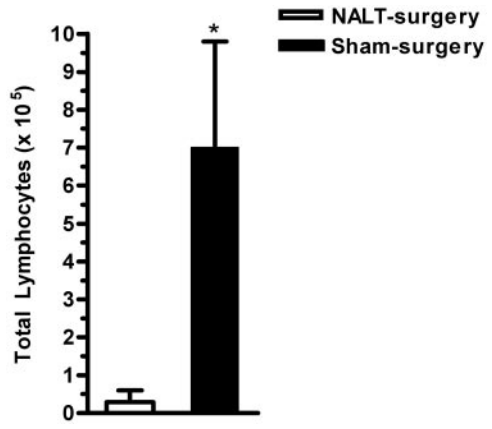

FIGURE 2. Total lymphocytes recovered from the NALT region of sham and NALT surgery animals. Animals were infected with PR8 influenza virus and allowed to recover for 25 days. NALT and sham surgeries were then performed, and the animals were allowed to recover for 14 days. At this time the animals were killed, and the NALT region was carefully dissected away from the nasal mucosa. The total number of lymphocytes was calculated by multiplying the total number of cells obtained by the percentage of lymphocytes detected in the differential cell count. Values are the mean ± SD ($n = 9$ animals/group). *, $p < 0.0001$.

Total and PR8-specific Ab levels after CLN surgery

In the absence of CLN, total and PR8-specific IgA levels in the nasal washes became equivalent or significantly exceeded those in the sham surgery group after the first week of infection (Fig. 4, *A* and *B*). Before this, total IgA levels in the nasal washes were higher in the sham surgery group, and PR8-specific IgA nasal wash levels were at trace or low levels in both groups. Although the total and PR8-specific IgG levels of the nasal washes displayed extensive fluctuations at some time points in both surgery groups, no consistent differences in these IgG levels attributable to the presence vs the absence of CLN were observed (Fig. 4, *C* and *D*). Serum levels of PR8-specific IgG were equivalent or greater in the CLN surgery group throughout the primary and secondary responses (Fig. 4*E*). The absence of CLN did not cause alterations in the PR8-specific serum IgA levels (data not shown). PR8-specific serum IgA Ab levels remained low throughout the primary and secondary responses in both surgery groups.

Influenza-specific CD4 and CD8 T cells recovered from nasal mucosa after NALT surgery

To determine whether the NALT has a role in the recruitment of virus-specific CD4 T cells to effector sites within the adjacent nasal mucosal tissues, we adoptively transferred CSFE-labeled BALB/c Thy1.2 CD4 T cells specific for the PR8 hemagglutinin peptide 126–138 (HNTNGVTAACSHE) into sham and NALT surgery groups 2 days before intranasal influenza virus infection. Detectable levels of adoptively transferred CD4/HNT T cells recruited into the nasal mucosa of both surgery groups were detectable by day 4 after infection and were equivalent in both groups at this time (Fig. 5*A*). Before this, only inconsistent trace levels were observed in both groups. By day 5 after infection, recruitment of the adoptively transferred, influenza-specific CD4 T cells to the nasal mucosa had substantially increased in both groups and was somewhat, but not significantly, greater in the NALT surgery animals. In both groups, essentially all the adoptively transferred, influenza-specific CD4 T cells recruited into the nasal mucosa had already proliferated several times, as indicated by the low CSFE levels within all detectable cells.

We used tetramer analysis of the CD8 T cell response in NALT and sham surgery animals to similarly address the requirement for the NALT in the recruitment of host-derived, influenza-specific CD8 T cells to the effector sites within the surrounding nasal mucosal tissue. We have previously reported that the peak recruitment levels of host-derived, influenza-specific CD8 T cells to the nasal mucosa occurred at 1 wk after infection (2). In our sham and NALT surgery groups, we were able to detect similar trace levels of influenza-specific NP and PA CD8 T cells in the nasal mucosa on day 5 after infection (Fig. 5*B*). On day 8 after infection, the recruitment of influenza-specific CD8 T cells to the nasal mucosa had significantly increased and remained equivalent in both surgery groups.

Recovery of host-derived, influenza-specific CD8 T cells after infection of mice lacking CLN

To determine whether the CLN was critical to the expansion of the local influenza-specific CD8 T cell response, we examined the host-derived NP and PA-specific CD8 T cells recovered from nasal

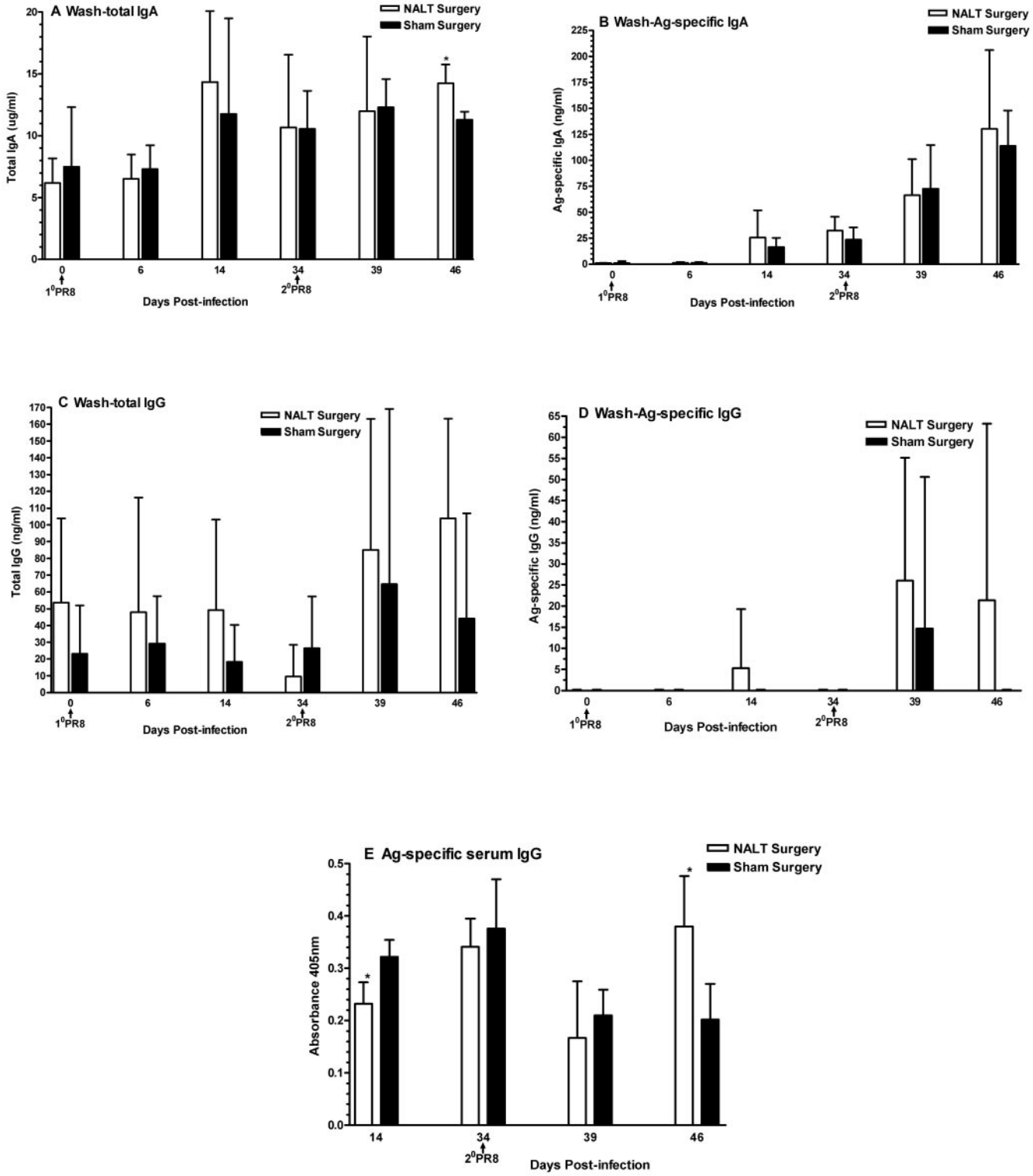

FIGURE 3. Total and PR8-specific Ab levels detected in nasal washes and serum in animals after NALT surgery. Animals underwent sham or NALT surgical procedures 2 wk before a primary intranasal influenza infection on day 0. Total and PR8-specific IgA (*A* and *B*) and total and PR8-specific IgG (*C* and *D*) levels in the nasal washes were examined at designated time points. Secondary PR8 challenge was administered 34 days after the primary infection. Ab titers were determined using a standard curve derived from the 405 nm absorbance of known amounts of IgA or IgG. The levels of PR8-specific serum IgG (*E*) were expressed according to the direct absorbance values from the ELISA plate readings. No serum samples were tested on days 0 and 6. Values are the mean ± SD ($n = 6$ or 7 mice/group). *, $p < 0.05$).

mucosa and from the remaining lymphatic tissues associated with the respiratory tract after primary and secondary intranasal influenza infections. In the CLN surgery group, accumulations of influenza NP- and PA-specific CD8 T cells in the TBLN were consistently and at times significantly greater during the primary response (Fig. 6*A*). After the secondary influenza challenge, the

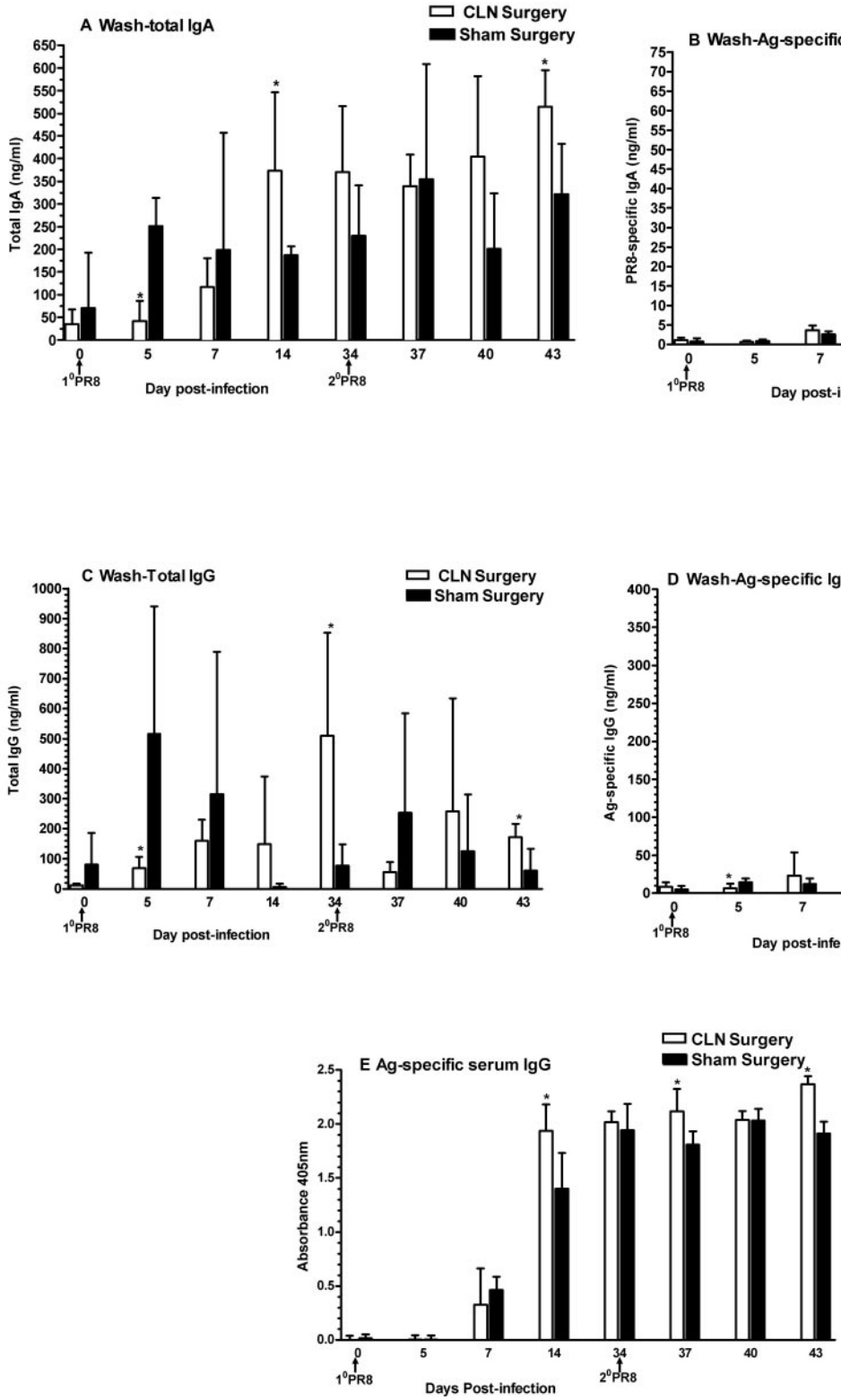

FIGURE 4. Total and PR8-specific Ab levels detected in nasal washes and serum in animals after CLN surgery. Animals underwent sham or CLN surgical procedures 2 wk before a primary intranasal influenza infection on day 0. Total IgA (*A*), PR8-specific IgA (*B*), total IgG (*C*), and IgG PR8-specific IgG (*D*) levels in the nasal washes were examined by ELISA at designated time points. Secondary PR8 infection was administered 34 days after the primary infection. Ab titers were determined using a standard curve derived from the 405 nm absorbance of known amounts of IgA or IgG. The levels of PR8-specific serum IgG (*E*) were expressed according to the direct absorbance values from the ELISA plate readings. Values are the mean ± SD (n = 5 animals/group). ∗, $p < 0.05$.

levels of influenza-specific CD8 T cells detected in the TBLN of each surgery group tended to became more equivalent. The accumulation of these CD8 T cells in the NALT during the primary and secondary responses were not affected by the loss of CLN (Fig. 6*B*). In the nasal mucosa, detectable levels of NP- and PA-specific CD8 T cells were not observed until day 7 after infection in both

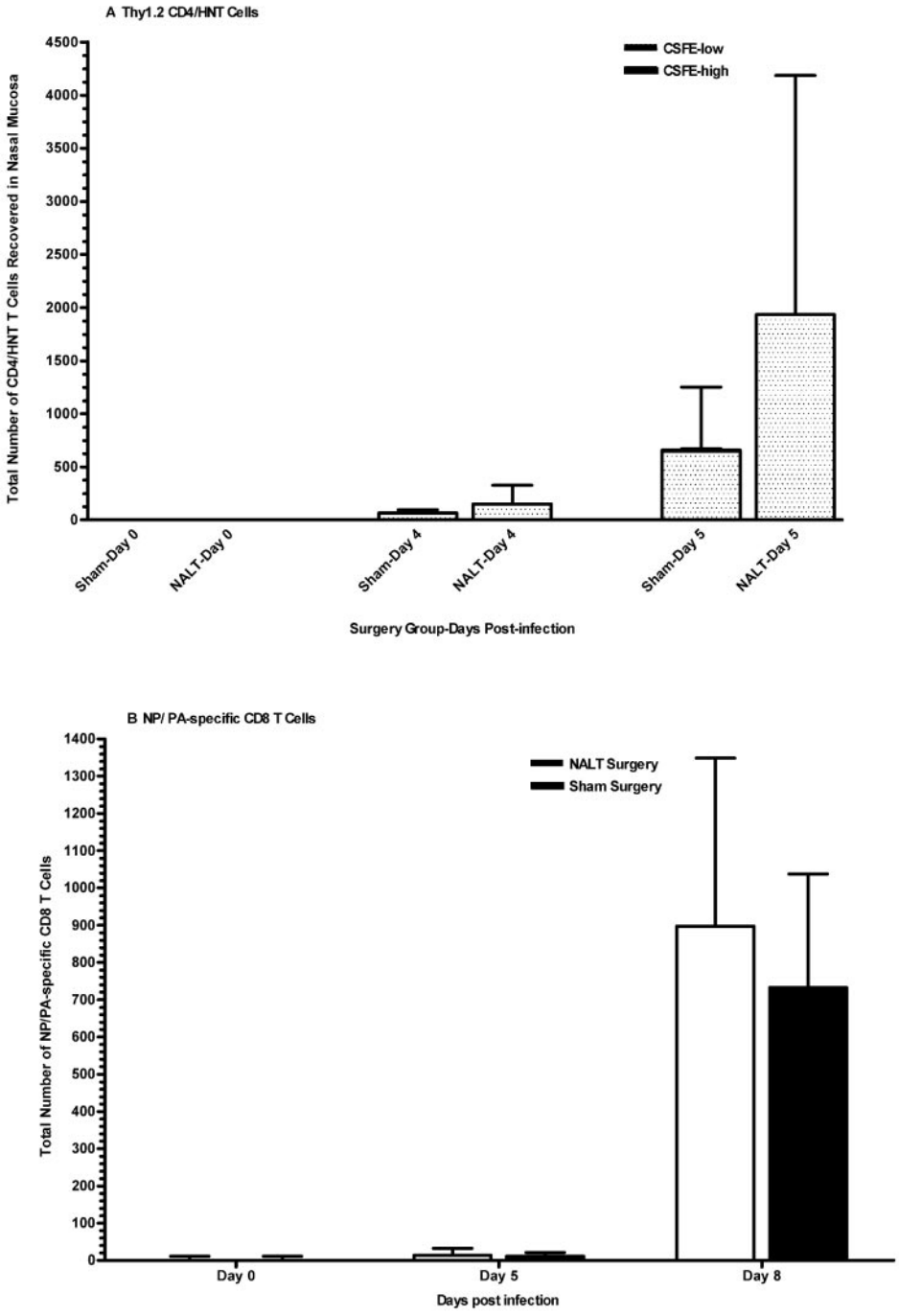

FIGURE 5. Recovery of influenza-specific T cells recruited to the nasal mucosa of sham and NALT surgery animals after primary intranasal influenza infection. *A*, CSFE-labeled Thy1.2 CD4/HNT T cells (5×10^5 cells) were adoptively transferred into Thy1.1 BALB/c recipient mice 2 days before infection. Recruitment of these cells to the nasal mucosa was detected on days 4 and 5 after infection by FACS analysis. *B*, Host-generated CD8 T cells specific for the NP and PA epitopes of PR8 were recovered from the nasal mucosa on days 5 and 8 after infection in animals that had undergone sham or CLN surgery 2 wk before infection. Values are the mean ± SD (n = 3–7 mice/group).

groups (Fig. 6*C*). At this time, the accumulation of these influenza-specific CD8 T cells was slightly greater in the sham surgery animals. However, this difference did not persist into the remainder of the primary response and throughout the secondary response. Each surgery group accumulated similar numbers of influenza-specific CD8 T cells in the nasal mucosa.

Viral recoveries from the nasal mucosa and/or lungs after removal of NALT or CLN

The rate of viral proliferation and clearance within the nasal mucosa was not altered by removal of NALT (Fig. 7*A*). Peak viral recoveries from the nasal mucosa still occurred between days 3 and 4 after infection in the NALT surgery group, and viral clearance was observed by day 10 after infection in both surgery groups. Despite the addition of adoptively transferred, influenza-specific CD4/HNT T cells into the local immune response, no differences in viral proliferation or clearance from the nasal mucosa or lungs could be detected between the sham and NALT surgery groups (Fig. 7, *B* and *C*). Removal of the CLN did not result in any change in viral proliferation or clearance within the nasal mucosa or lungs (Fig. 8).

To determine the necessity of NALT for the generation of protective heterosubtypic T cell immunity, animals were infected with the PR8 strain of influenza virus and then allowed to recover before undergoing NALT surgery. Fourteen days after NALT surgery, these animals were infected with a heterologous strain of influenza virus, the X31 strain. The rate of clearance of the X31 influenza virus from the nasal mucosa of the NALT and sham

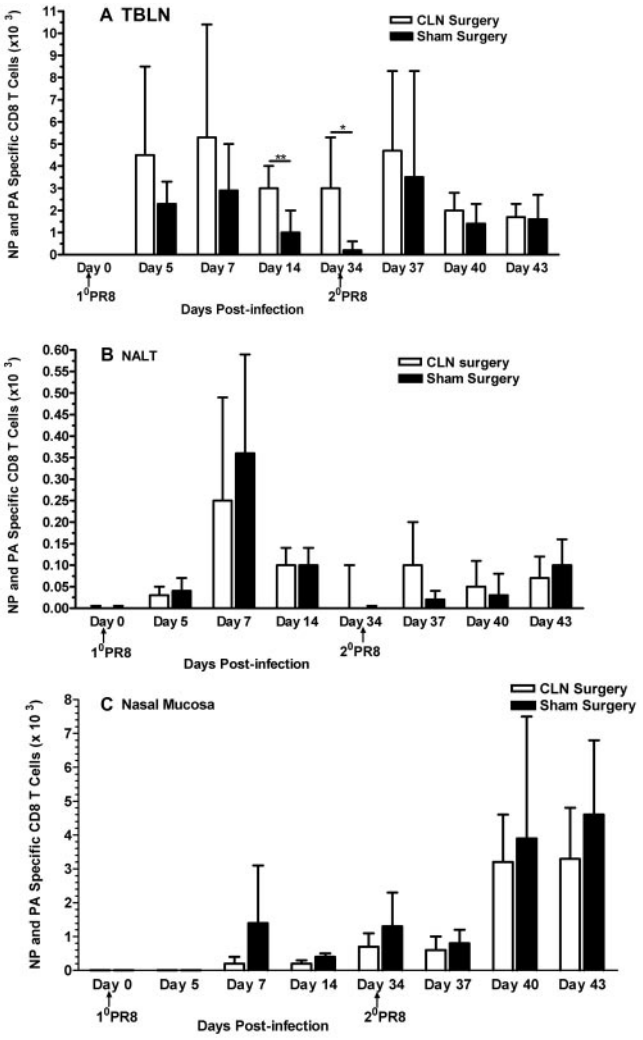

FIGURE 6. Recovery of host-derived NP- and PA-specific CD8 T cells in selected tissues from sham or CLN surgery animals that received primary and secondary intranasal influenza infections. Primary intranasal influenza infection was given 2 wk after sham or CLN surgery. Recovery of the Ag-specific CD8 T cells during the primary response was assessed on days 5, 7, 14, and 34 after infection. A secondary challenge was given to the remaining animals on day 34. The recruitment into selected tissues during the secondary response was assessed 3, 6, and 9 days after the secondary challenge. Values are the mean ± SD ($n = 5$ animals/group). *, $p < 0.05$; **, $p < 0.005$.

surgery groups was not different (Table I). In fact, the amount of X31 virus recovered from the nasal mucosa 4 and 7 days after the secondary X31 challenge was significantly less than the amount of PR8 virus recovered 4 and 7 days after the primary PR8 challenge despite administering >100 times more X31 influenza virus in the secondary challenge than PR8 influenza virus in the primary challenge.

Discussion

Host mucosal surfaces provide vast and easily accessible tissue sites for pathogen entry. Exposure of these surfaces within the URT to pathogens or Ags elicits the participation of URT systemic and mucosal lymphatic tissues in the development of local immune responses (1, 2, 15). The CLN is essential in the induction of local tolerance to intranasally administered Ags (7). As a local mucosal lymphatic tissue within the URT, the NALT is unique in its ability to acquire and respond to local Ags. Although these disparate lymphatic tissues participate in the generation of URT immunity, the necessity for their presence for the development of resistance to URT infections remains to be established. To determine whether these two URT lymphatic tissues are required for the successful generation of resistance to influenza infections within the URT, we developed surgical models to remove either the NALT or CLN.

Our results demonstrated that after ablation of NALT or CLN, there is no re-establishment of either tissue, nor was there evidence of any significant aggregation of immune cells into the location that they previously occupied. Even the generation of a localized immune response environment within adjacent URT tissues before NALT surgery failed to encourage any re-establishment of the NALT in those animals that had undergone NALT surgery. This suggested that the NALT is not capable of re-establishing itself under normal homeostatic conditions or under conditions in which the local environment had been altered by the development of a recent local immune response. This contrasts with the rescue of a previously existing, but disorganized, NALT in LT$\alpha^{-/-}$ mice by the reconstitution of these mice with bone marrow from wild-type donors (8). Interestingly, although a previously established, yet defectively organized, NALT can be rescued by wild-type bone marrow reconstitution, the NALT cannot re-establish itself after its removal from a wild-type animal even though this wild-type host retains the full complement of bone marrow used to reconstitute the defective LT$\alpha^{-/-}$ recipient.

The surgical removal of the NALT effectively eliminated efficient M cell acquisition of Ag from the nasal mucosa. However, the appearance and levels of total and PR8-specific IgA detected in the nasal washes of both surgery groups during the course of the primary and secondary responses were not consistently different. In addition, both surgery groups displayed equivalent increases in Ab titers that are typical of secondary response kinetics. These observations support the conclusion that the production of total and PR8-specific mucosal-associated IgA in the nasal cavity during primary and secondary responses is not dependent upon the presence of NALT.

Over the course of the primary and secondary responses, levels of total IgG in the nasal washes were slightly elevated at most time points in the NALT surgery group. Under normal circumstances, the presence of IgG at the surface of the nasal mucosa is associated with transudation of serum IgG across the respiratory epithelium (14). Viral damage to the nasal respiratory epithelium will increase the permeability of this tissue to allow an increase in transudation of serum Ig into the nasal cavity (14, 16). The levels of total IgG detected in the nasal washes of both groups coincide with increases in Ag-specific Ab titers after primary infections (17) as well as with the ongoing repair of viral and surgical-induced damage to the nasal epithelium. In our model, this viral-induced damage, in combination with the degree of surgery-related damage from which the NALT surgery group were in the process of recovering at the time of infection, would permit more leakage of serum Ig into the nasal cavity of this group. Both sources of damage and the extent of epithelial repair from these injuries will influence the IgG levels recovered in the nasal washes. Although the level of total IgG in the nasal wash of the NALT surgery group is slightly greater, it is, in fact, very low relative to the corresponding IgA levels.

The serum PR8-specific IgG levels did show small, yet significant, differences on days 14 and 46. However, no biological impact on the recovery of virus from the lungs was detected despite the role of serum IgG in the protection of the lung (14). The lack of any consistent differences in the PR8-specific serum and nasal wash Ig levels between the surgery groups suggests that despite loss of NALT and its M cells, the delivery of Ag to downstream

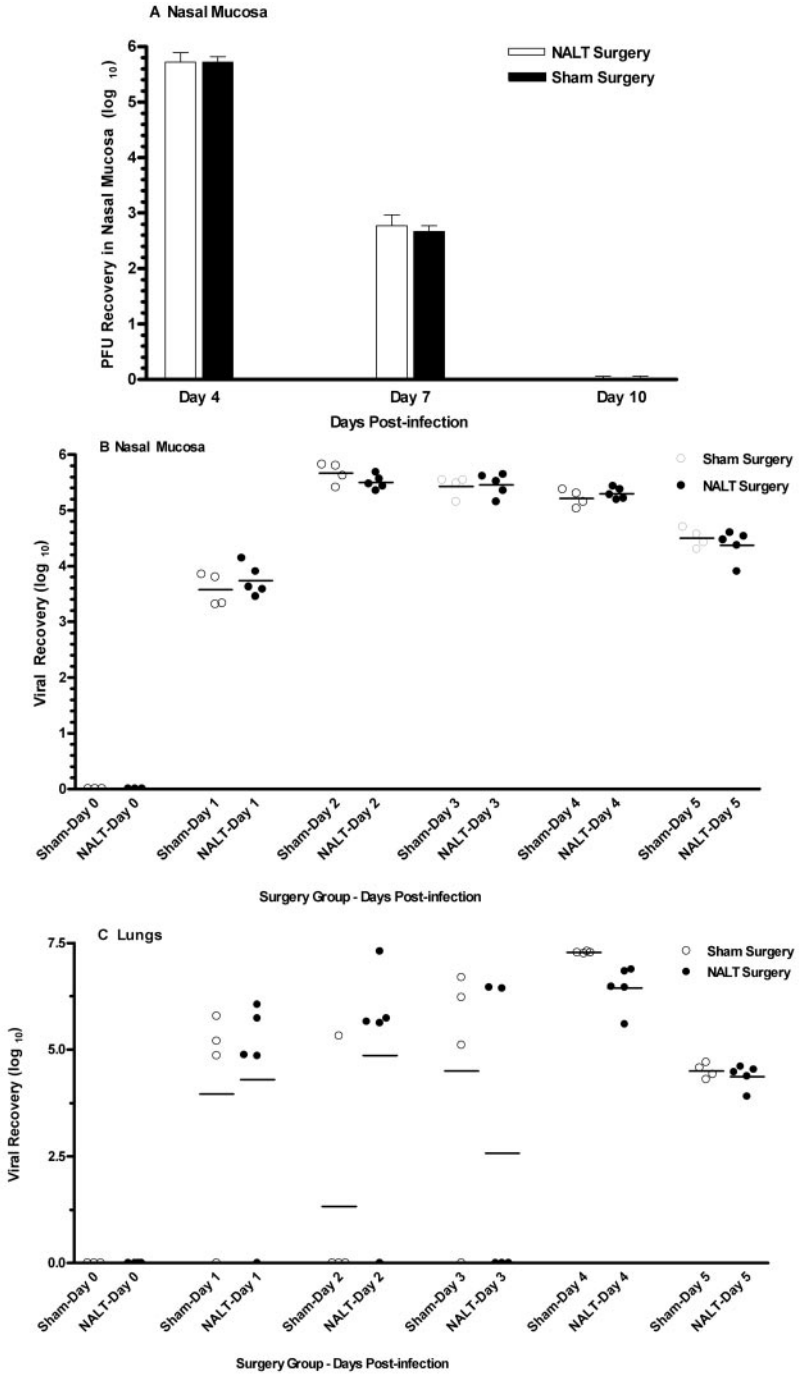

FIGURE 7. Recovery of influenza virus from the nasal mucosa and lungs of sham surgery or NALT surgery mice. *A*, Two weeks after sham or NALT surgery, animals were given an intranasal influenza virus infection. Viral recovery was assessed on days 4, 7, and 10 after infection. Values are the mean ± SD (n = 5–6 animals/group). *B* and *C*, Two weeks after sham or NALT surgery, mice were given adoptively transferred CSFE-labeled Thy1.2 CD4/HNT T cells specific for a PR8 hemagglutinin epitope. Two days later, both surgery groups were given an intranasal influenza infection. Viral recovery in the nasal mucosa and lungs was assessed by plaque assay on days 1–5 after infection. The bar in each group is the mean viral recovery (n = 3–5 animals/group).

lymphatic tissues was not affected, and the absence of NALT did not affect the production of PR8-specific Ab by the Ab-secreting B cells that were recruited into the respiratory epithelium of the nasal mucosa.

Different experimental approaches were used to examine how the absence of NALT might affect the recruitment of PR8-specific CD4 and CD8 T cells into the surrounding nasal mucosa in response to intranasal infection. No evidence was found that would support the necessity of the NALT for the recruitment of PR8-specific CD4 or CD8 T cells to the nasal mucosa. The lack of NALT did not alter the recruitment pattern of proliferating vs nonproliferating CD4 T cells. The adoptively transferred CD4 T cells recovered in the nasal mucosa of both surgery groups had already undergone numerous cell divisions before their arrival in the nasal mucosa, as indicated by their low CSFE content. This also suggested that the proliferative response by the adoptively transferred CD4 T cells was not dependent upon the presence of NALT. The generation and recruitment of sufficient levels of host-derived, influenza-specific CD8 T cells in response to a primary PR8 infection were not compromised by the lack of NALT. The equivalent rates of viral clearance in response to the X31 viral challenge support the conclusion that the NALT is not required for virus-specific CD8 T cell responses to heterosubtypic infections.

Removal of the CLN was associated with total and Ag-specific IgA nasal wash levels that were equivalent or significantly greater than those of the sham surgery group after the first week of the primary response and throughout the secondary response. In this model, as in the NALT surgery model, the extent of viral damage and the progression of epithelial repair in each animal after intranasal influenza infection will alter any regulated transudation of

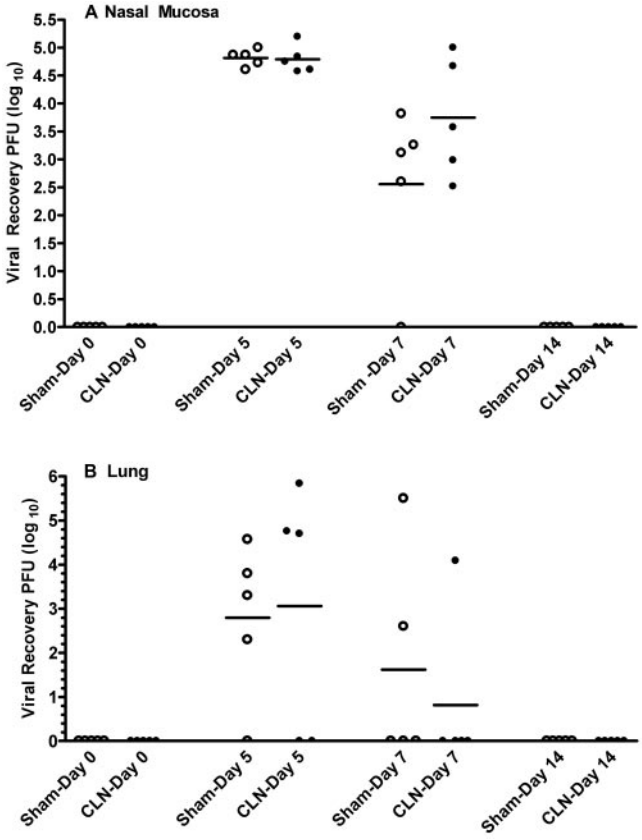

FIGURE 8. Recovery of influenza virus from the nasal mucosa and lungs of sham surgery and CLN surgery mice. Two weeks after sham or CLN surgery, animals were given an intranasal influenza infection. Viral recovery from the nasal mucosa (*A*) and lungs (*B*) was determined by plaque assay on days 5, 7, and 14 after infection. The bar in each group is the mean viral recovery ($n = 5$ animals/group).

Table I. *Recovery of X31 influenza virus*[a]

Surgery Group[b]	Day 4 Postinfection	Day 7 Postinfection
Nalt surgery	2.35 ± 0.29[c]	0.45 ± 0.85
Sham surgery	2.80 ± 0.52	1.05 ± 1.00

[a] Recovery of X31 influenza virus from the nasal mucosa of NALT and sham-surgery mice that had previously been immunized with the PR8 influenza virus. Mice were given the PR8 strain of influenza virus intranasally (5 μl, 1.5×10^4 PFU) 25 days before undergoing NALT surgery. Fourteen days after the NALT surgery these mice were given the X31 influenza virus intranasally (5 μl, 5.0×10^6 PFU). Nasal mucosa tissues were assessed by plaque assay for the clearance of the X31 virus.

[b] $n = 10$ mice per group.

[c] Log_{10} of the PFU recovery from the nasal mucosa.

serum IgG into the nasal cavity. The fluctuations in nasal wash IgG levels within each surgery group confound conclusions that may link the presence or the absence of CLN to the levels of IgG within the nasal cavity. The more significant observation related to PR8-specific IgG was that levels of these Abs in serum were equivalent or significantly greater than those detected in the sham surgery animals throughout the primary and secondary responses. This evidence suggests that the loss of CLN does not diminish the ability of the host to generate normal levels of PR8-specific IgG. In fact, the increased levels of PR8-specific serum IgG and nasal-associated IgA could be the result of a compensatory generation of Ag-specific Ab-secreting B cells by other lymphatic tissues and/or an increase in Ab production by the elicited Ag-specific Ab-secreting B cells. The PR8-specific IgA levels detected in the nasal wash also suggest that the recruitment of PR8-specific Ab-secreting B cells into the respiratory epithelium is not diminished by the absence of CLN.

The levels of Ag-specific CD8 T cells in selected induction and effector tissues within the respiratory tract were examined in the CLN and sham surgery groups. Although the loss of CLN did not elicit significant changes in the accumulation of these cells in NALT and nasal mucosa, levels of Ag-specific CD8 T cells in the TBLN were greater in the absence of CLN during the course of the primary response. Although the TBLN is of greater significance to the lymphatic drainage of the lung, it is reasonable that the generation of local systemic immunity within the respiratory tract is compartmentalized to the CLN and TBLN depending upon the distribution of infection foci within the URT and the lung (18). However, in the absence of either draining lymph node, it is plausible that the remaining lymph node may be capable of providing some level of compensatory response. Additional experiments involving the surgical removal of CLN in a heterosubtypic virus challenge model need to be completed to determine how critical the CLN is in the generation and maintenance of the local virus-specific CD8 T cell memory response and to determine whether the TBLN would be involved in any sort of compensatory protective heterosubtypic immune response that would have involved the CLN.

The surgical models used in these studies effectively eliminated the participation of the mucosal (NALT) or the systemic (CLN) lymphatic tissues from the generation of a local protective immune response in the URT. The evidence suggests that the absence of NALT was not detected by the host. No compensation or diminution in any of the response parameters measured was consistently observed, and the NALT surgery animals were able to clear the virus from their nasal mucosa and lungs as effectively as the sham surgery controls. Despite its specialized Ag acquisition features, the cellular contribution of the NALT to the local immune response was probably insignificant and thus not missed by the host. The NALT would seem to be a nonessential lymphatic tissue that participates in the generation of protective immunity in the URT. In contrast, the loss of CLN was detected by the host. Our evidence suggests that removal of CLN merely transfers the responsibility of generating sufficient levels of Ag-specific effector immune cells to other systemic lymphatic tissues, rather than being reliant on the NALT as a possible compensatory mucosal lymphatic tissue. The increased recoveries of influenza-specific CD8 T cells in TBLN of the CLN surgery group suggest that this lymphatic tissue might be involved in this responsibility. Additional studies to investigate how other lymphatic tissues might compensate for the loss of CLN in URT infection models will be required to confirm their involvement in such a role. A compensatory response by other local systemic lymphatic tissues implies an element of redundancy in the generation of local protective immunity. In our model, the CLN is a participating, but nonessential, systemic lymphatic tissue in the generation of local protective immunity in the URT. A possible caveat is that its nonessential status is not because its immune cell contribution would be missed by the host, as might be the case for NALT, but, rather, the CLN′s contribution to the URT response might be compensated for by increased immune proliferation within other systemic lymphatic tissues. This nonessential status in resistance to URT infection contrasts with the required presence of CLN for the induction of local tolerance within the URT (7).

Previous investigators have discussed the distinctions and compartmentalization of mucosal and systemic immunity based upon routes of vaccination and the response to systemic viral infections (19, 20). The targeting of mucosal vaccines to the NALT allows

for access to the common mucosal immune system and has resulted in the induction of protective immunity at distal mucosal sites (3, 21). In our surgery models, the nonessential nature of CLN and NALT is confined to the generation of local protective immunity within the URT. In this regard, the immunity induced at distal sites after administration of NALT-targeted vaccines may be more dependent upon the existence of NALT than is the generation of local immunity in the URT. Additional investigations concerning the role of NALT and CLN in the generation of local tolerance, local protective immunity, and distal protective immunity will be required to fully understand what makes a lymphatic tissue essential for a successful immune response.

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Disclosures

The authors have no financial conflict of interest.

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