Reduced Frequencies and Heightened CD103 Expression among Virus-Induced CD8$^+$ T Cells in the Respiratory Tract Airways of Vitamin A-Deficient Mice

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Vitamin A deficiency (VAD) has profound effects on immune responses in the gut, but its effect on other mucosal responses is less well understood. Sendai virus (SeV) is a candidate human parainfluenza virus type 1 (hPIV-1) vaccine and a candidate vaccine vector for other respiratory viruses. A single intranasal dose of SeV elicits a protective immune response against hPIV-1 within days after vaccination. To define the effect of VAD on acute responses toward SeV, we monitored both antibodies and CD8$^+$ T cells in mice. On day 10 following SeV infection, there was a trend toward lower antibody activities in the nasal washes of VAD mice than in those of controls, while bronchoalveolar lavage (BAL) fluid and serum antibodies were not reduced. In contrast, there was a dramatic reduction of immunodominant CD8$^+$ T cell frequencies in the lower respiratory tract (LRT) airways of VAD animals. These T cells also showed unusually high CD103 (the $\alpha E$ subunit of $\alpha E\beta 7$) expression patterns. In both VAD and control mice, E-cadherin (the ligand for $\alpha E\beta 7$) was better expressed among epithelial cells lining the upper respiratory tract (URT) than in LRT airways. The results support a working hypothesis that the high CD103 expression among T cell populations in VAD mice alters mechanisms of T cell cross talk with URT and LRT epithelial cells, thereby inhibiting T cell migration and egress into the lower airway. Our data emphasize that the consequences of VAD are not limited to gut-resident cells and characterize VAD influences on an immune response to a respiratory virus vaccine.

Vitamin A deficiency (VAD) is a common dietary concern, particularly in developing countries. The deficiency is associated with increased susceptibility to disease, poor responses to vaccination, and increased mortality (28, 35, 37, 38). Vitamin A supplements can improve health in some circumstances, but there are also risks associated with excessive vitamin A supplementation (4, 6, 11, 30, 43, 48).

Vitamin A is usually acquired from the diet as all-trans-retinol, retinyl ester, or beta-carotene. All-trans-retinol and beta-carotene can be oxidized to all-trans-retinal by ubiquitous alcohol dehydrogenases, after which all-trans-retinal can be oxidized to all-trans-retinoic acid (RA) by retinal dehydrogenases (RALDHs), which are expressed primarily by intestinal epithelial cells and gut-associated dendritic cells (DCs) (6, 9, 20, 21, 23–25). RA is capable of activating nuclear receptors of the retinoic acid receptor (RAR) family and altering the transcription of many genes. Among its many functions, RA can increase the expression of metalloproteinasomes in DCs and migration to lymph nodes. RA can also enhance DC maturation and antigen presentation in the presence of inflammatory stimuli such as tumor necrosis factor (TNF) (3, 22, 31, 32).

Immune responses of the gut are particularly susceptible to VAD, in part due to RA influences on intestinal B and T cell activities (9, 20, 21, 23–25, 36, 46). For example, vitamin A and RA can support B cell activation, inhibit B cell apoptosis, augment IgA antibody-forming cells (AFCs) in the gut mucosa, and increase levels of total IgA in the intestinal lumen. Improvements to immune function in the gut are greatly influenced by the effects of vitamin A on lymphocyte homing. RA induces retinol-metabolizing activity in gut DCs, which can, in turn, “imprint” T cells to adopt specific tissue homing properties. As examples, T cells marked with high $\alpha 4\beta 7$-integrin and CC-chemokine receptor 9 (CCR9) molecules exhibit enhanced T cell infiltration into the small intestine (6, 13, 23, 40), while cells marked with $\alpha E\beta 7$-integrin and low CCR9 levels may more readily traffic to other sites. Imprinting is most pronounced on Peyer’s patch-induced effector and memory lymphocytes, and many T lymphocyte subsets can be influenced (e.g., CD8$^+$, Th17, and CD4$^+$ T-regulatory cells [16, 20, 21, 23–25, 45]). Blocking RAR can cause a significant decrease in T cell homing. Similarly, B cells can be educated to acquire or lose gut-homing potential when they are stimulated in the presence or absence, respectively, of RA (24, 25). There is some debate concerning the relative influence of vitamin A on nongut tissues, because cells in locations outside the intestine are relatively weak in vitamin A metabolizing capacity (17, 23, 35, 38).

Sendai virus (SeV; a murine parainfluenza virus type 1 [PIV-1]) is currently being studied as a candidate respiratory viral vaccine for pediatric group caused by its cousin, human parainfluenza type 1 (hPIV-1). Due to its close sequence and structural similarities with hPIV-1, SeV stimulates cross-reactive responses and protection against hPIV-1 in cotton rats and in nonhuman primates (12, 34). SeV can also be used in a recombinant form to protect against other serious human pediatric viruses.
such as respiratory syncytial virus and human PIV-3 in animal models (14, 41, 42, 49, 50).

SeV is particularly attractive as a human pediatric vaccine and vaccine backbone because of the rapidity with which immune response can be generated. Both antibodies and CD8⁺ T cell effectors are induced within 10 days following a single intranasal (i.n.) inoculation with SeV (33, 34). When SeV is tested in a cotton rat model, a model susceptible to human respiratory virus infections, protection against human challenge viruses can also be detected within days after vaccination (34). This rapid induction of protective immunity may be essential for a respiratory virus vaccine that targets the human pediatric arena, because young infants require immediate protection against viral disease.

We sought to determine whether the acute murine response to an i.n. inoculation with SeV is influenced by VAD. We show that in the context of VAD, (i) there is a trend toward reduction of SeV-specific antibody in the upper respiratory tract (URT), (ii) there is a dramatic reduction of immunodominant SeV-specific CD8⁺ T cells in the lower respiratory tract (LRT) airway, (iii) this population exhibits an unusually high percentage of CD103⁺ positive cells, and (iv) E-cadherin, the ligand for αEβ7, exhibits normal expression levels in VAD mice; there is much higher E-cadherin expression in URT than in LRT airway epithelial cells, a pattern that may influence the migration of SeV-specific CD103-positive T cells. Data emphasize that the impact of VAD is not exclusive to the gut. Rather, this dietary deficiency can significantly alter an acute immune response to a respiratory viral vaccine.

MATERIALS AND METHODS

Animals and housing. Pregnant female C57BL/6 (H-2b) mice were purchased from Charles River (Wilmington, MA) for the VAD studies. SeV-seronegative animals were housed in filter-top cages in a biosafety level 2 containment area with sentinel caged mice, following guidelines outlined by the Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC). Studies were approved by the Institutional Animal Care and Use Committee (IACUC).

To establish VAD mice, day 4 to 5 estrus C57BL/6 females were placed purchased from Charles River (Wilmington, MA) for the VAD studies. SeV-seronegative animals were housed in filter-top cages in a biosafety level 2 containment area with sentinel caged mice, following guidelines outlined by the Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC). Studies were approved by the Institutional Animal Care and Use Committee (IACUC).

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staining of SeV-specific immunodominant CD8+ T cells, major histo-
compatibility complex (MHC) class I (Kb) tetramers were generated by
folding Sendai virus NP324–332 (FAPGNYPAL) with heavy and light
chains as described previously (7, 26). Tetramers were stored as aliquots at
4°C. BAL fluid lymphocytes were stained with phycoerythrin (PE)-con-
jugated tetrameric reagent for 1 h at RT, followed by staining with labeled
anti-CD62L, anti-CD44, anti-CD11a, anti-CD69, anti-CD103, or anti-
LPAM-1 (BD Pharmingen, San Diego, CA) on ice for 20 min. Samples
were analyzed on a FACSCalibur. Lymphocytes were gated for analyses
based on forward- and side-scatter parameters.

To examine digested URT and LRT tissues before or after culture, cells
were stained with anti-CD11b (BD Pharmingen) and 7AAD (1 µg/ml;
Invitrogen). Additional stains were anti-E-cadherin (R&D Systems)
and anti-ICAM-1 (Biolegend). Dead cells, platelets, red blood cells
(RBCs), small lymphocytes, and macrophages were excluded from
analyses based on forward scatter and positivity for 7AAD and CD11b.

FIG 1 Robust virus-specific acute-phase serum antibody responses in VAD mice. Ten days after infection with 250 PFU of SeV, sera were serially diluted and
tested for the presence of SeV-specific antibodies. ELISA results are shown for a representative of 4 individual experiments. Each group included 5 mice (x axis),
and each dilution for each serum sample was tested in replicate. Means and standard deviations are shown. Naïve mouse serum samples served as negative
controls. Antibody levels in mice deficient in vitamin A (B) were not reduced compared with antibody levels in mice on the control diet (A).

FIG 2 Virus-specific acute-phase antibody responses in nasal wash and BAL fluid of VAD mice. Ten days after infection with 250 PFU of SeV, nasal wash (A and
B) and BAL fluid (C and D) samples were collected from 3 individual control (A and C) and VAD mice (B and D) for assessment of antibody activity. ELISA
results are shown for a representative of 2 independent experiments. Each dilution for each nasal or BAL sample was tested in replicate. Means and standard
deviations are shown. The pound sign identifies one bar in which an unexpectedly high outlier value was associated with the 1:50 sample dilution. It was higher
than all values associated with the 1:10 sample dilution and was therefore excluded from the calculation.
Data were analyzed using FlowJo version 7.6.4 software (Tree Star Inc., Ashland, OR).

Virus measurements in infected animals. The lungs and nasal turbinates were removed aseptically and washed in PBS. Suspensions were centrifuged at 2,000 \( \times g \) for 10 min to clear cellular debris. Virus titers were measured as 50% tissue culture infectious doses (TCID50). The TCID50 measurements were performed by plating serial 10-fold dilutions of lung and nasal turbinate suspensions on LLC-MK2 cells with minimal essential medium containing 0.1% bovine serum albumin in the presence of 5 \( \mu g/ml \) of acetylated trypsin and 50 \( \mu g/ml \) of gentamicin. Cell supernatants were collected after 4 to 5 days of incubation and mixed 1:1 with chicken red blood cells (0.5%) in PBS for hemagglutination detection. TCID50 values were calculated using the Reed-Muench formula.

RESULTS

Robust virus-specific serum antibody responses in VAD animals on day 10 postinfection. Previous work has shown that the antibody responses to SeV are rapidly induced and are long-lasting (14, 33, 34). We therefore questioned whether serum antibody responses to SeV might be reduced in the context of VAD. Sera from VAD and control animals were sampled on day 10 after infection for SeV-specific antibody levels. As demonstrated in Fig. 1, the serum antibody levels were not reduced in VAD compared to control animals.

Virus-specific antibody responses in nasal washes and BAL fluid of VAD animals on day 10 postinfection. We also questioned whether SeV-specific immune responses were altered in the nasal washes or BAL fluid of vaccinated VAD animals compared to controls. As shown in Fig. 2A and B, there was a trend toward lower antibody levels in nasal washes of VAD animals (right) in each of four separate experiments. Values from 4 individually tested control and VAD mice from one experiment were tabulated and compared using an unpaired t test, demonstrating a significant difference (GraphPad Prism software).

VAD animals exhibit reduced frequencies of SeV-specific \( CD8^+ \) T cells in the BAL fluid. To examine \( CD8^+ \) T cell responses in VAD animals, we used a tetramer that marks immunodominant SeV-specific \( CD8^+ \) T cells in C57BL/6 mice (7). In normal animals, the \( CD8^+ \) T cell response can typically be measured by sampling the BAL fluid 10 days after infection (very few cells can be harvested from the nasal wash). As expected, we found that TET\(^4\) T cells were present at high frequency among lymphocytes in the BAL fluid of control animals by day 10 (Fig. 3A). However, in VAD animals, there was a reproducible and significant reduction of TET\(^4\) T cell frequencies. The results from 4 individual mice in an independent experiment are shown in Fig. 3B, demonstrating statistical significance. Total lymphocyte counts in the BAL fluid were often reduced by as much as 50% in VAD mice compared to controls, indicating that TET\(^4\) T cells in the LRT airways of VAD mice were reduced both by percentages among lymphocytes and by absolute numbers.

![FIG 3](https://example.com/figure3.png)

**FIG 3** Reduced frequencies of immunodominant virus specific (TET\(^4\)) T cells in the BAL fluid of infected VAD mice. Ten days after inoculation of C57BL/6 mice with 250 PFU of SeV, BAL fluid cells were collected for T cell phenotype analyses. (A) Cells pooled from 5 mice per group were gated on lymphocytes, and markers were set to discriminate between the TET-positive (circled values) and TET-negative phenotypes (naive BAL fluid cells do not stain with the TET reagent). There was a high frequency of TET\(^4\) T cells in control animals (left) but not in VAD animals (right) in each of four separate experiments. (B) Values from 4 individually tested control and VAD mice from one experiment were tabulated and compared using an unpaired t test, demonstrating a significant difference (GraphPad Prism software).

| Samples | % TET+ among lymphocytes |
|---------|--------------------------|
|         | 1 | 2 | 3 | 4 | Average |
| Control | 36.4 | 12.6 | 24.0 | 34.0 | 28.6 |
| VAD     | 9.2 | 10.3 | 10.8 | 4.5 | 8.7 |

**Significance** \( P < .05 \)

![FIG 4](https://example.com/figure4.png)

**FIG 4** Levels of homing and activation antigens on TET\(^4\) T cells in VAD animals. Markers CD62L, CD11a, CD69, and CD44 were examined among TET\(^4\) cells in control (left) and VAD (right) mice 10 days after infection with 250 PFU of SeV. Cells from 5 mice per group were pooled. Representative histograms are shown. Results were similar for each of 4 separate experiments. Histogram markers were set to define differences between VAD and control mice, either in terms of positive or negative membrane protein expression (as for CD62L) or in terms of high or low protein expression (as for CD11a).
Heightened frequencies of CD103-positive cells among Tet⁺ T cells in VAD animals. To help explain the reduced appearance of vaccine-induced Tet⁺ T cells in the BAL fluid of VAD animals, we examined the phenotype of these cells in test and control mice. Of particular interest were the patterns of membrane homing and activation antigens. As shown in Fig. 4, an array of markers were tested, including CD62L (L-selectin, a cell adhesion protein that binds the high endothelial venules of peripheral lymphoid tissues), CD11a (a subunit of integrin LFA-1, a receptor for ICAMs), CD69 (a marker of early activation, indicative of recent antigen encounter), and CD44 (an activation and adhesion membrane protein), but significant differences were not observed between VAD and control animals. In Fig. 5A and B are shown the results with antibodies specific for LPAM-1 (α4β7, an integrin prevalent on gut-associated T cells) and CD103 (the αE subunit of a second β7-integrin, αEβ7). We found that CD103 was upregulated in VAD animals compared to controls in multiple experiments. The results from the testing of individual mice in a representative experiment are shown in Fig. 5C, illustrating statistical significance.

E-cadherin, the ligand for αEβ7, is expressed similarly in the respiratory tracts of control and VAD mice, with increased levels in URT compared to LRT tissues. CD103 is the αE subunit of the αEβ7-integrin protein with affinity for a membrane ligand, E-cadherin, that marks respiratory tract epithelial cells. We questioned whether E-cadherin levels were altered in the context of VAD. In Fig. 6 are shown fixed tissue sections from the URT and LRT airways of uninfected control and VAD animals. As shown, there was relatively low expression in the bronchoalveolar epithelial tissue sections (LRT) compared to nasal epithelial tissue sections (URT), but there was no discernible difference between VAD and control animals. To better quantify E-cadherin levels in both control and VAD animals, we digested URT and LRT tissues with collagenase, dispase, and DNase to release matrix cells for analyses. Cells were placed in overnight culture followed by a 1-day culture with or without SeV. Cells were then costained with antibodies for E-cadherin and ICAM-1, the ligand for CD11a. Results in Fig. 7 support those in Fig. 6, in that cells from control and VAD mice were similar and there were higher levels of E-cadherin in URT than in LRT cells. The E-cadherinHIGH cells in the URT were generally ICAM-1LOW in cultures with or without SeV (circled values indicate percentages of E-cadherinHIGH ICAM-1LOW cells). E-cadherinLOW ICAM-1HIGH cells were more prevalent in the LRT cultures, particularly after SeV infection.

URT and LRT cells from control and VAD animals were also examined after a 10-day in vivo infection with SeV (Fig. 8). In this case, cells were not cultured prior to analyses. Again as expected,
the percentages of total E-cadherinHIGH cells (as indicated by circled values) were similar between control and VAD mice and were significantly higher in URT than in LRT tissues. Figure 8A and B show representative profiles, while Fig. 8C shows results from individually tested mice. It is likely that the ultimate positioning of T cells in LRT versus URT tissues following an i.n. infection is dependent on these differential displays of matrix membrane molecules.

Weight loss and virus load in VAD mice. To determine if VAD impacted SeV infectivity, virus loads were compared between VAD and test mice on day 7 after SeV exposure. As demonstrated in Fig. 9A, the lung and nasal turbinate SeV titers in both VAD and control mice averaged within the same order of magnitude. In a separate set of experiments, animal weights were measured every day for 10 days after SeV infection. As demonstrated in Fig. 9B, both control and VAD mice lost weight approximately 1 week after SeV infection and then reached a plateau or showed signs of improvement by day 10. Data encourage future experimentation to determine if the aberrant immune responses elicited by this primary infection in VAD mice will protect against infection and weight loss upon secondary challenge with SeV.

**DISCUSSION**

VAD alters the SeV-specific immune response. The work described here was conducted to examine the effect of VAD on vaccine-induced immune responses in the respiratory tract. SeV is well known for its rapid induction of protective immunity. Both antibodies and CD8+ T cell responses are induced within 10 days of a single i.n. inoculation (33). To define the acute effect of VAD on SeV-specific immune activities, we first examined serum antibody levels and found that these were not reduced compared to those in control mice. BAL fluid antibody levels were similar among groups, but there was a trend toward lower antibody levels in nasal washes of VAD animals. When the immunodominant SeV-specific CD8+ T cell population was analyzed, significant changes were evident. These cells were diminished in the lower airway of VAD animals, and they exhibited unusually high CD103 membrane expression patterns.

When E-cadherin, the ligand for eß7, was examined, no differences were noted between VAD and control animals. However, differences between URT and LRT expression patterns were noted in both VAD and control animals, infected or uninfected. Whereas E-cadherin was relatively weakly expressed in the LRT, URT cells were strongly positive. E-cadherinHIGH cells in the URT were usually found to be ICAM-1LOW, as demonstrated by in vitro culture of dispase-, collagenase-, and DNase-digested cells with or without SeV.

We have previously shown that T cells capable of migration to URT or LRT tissues are clonally related (39), and others have shown that adherence of T cells to epithelial cell membrane markers (e.g., ICAM-1 and ICAM-2) affect cell migration and airway egress (27, 29). The increases in ICAM-1, a ligand for CD11a, among LRT cells following SeV infection (Fig. 7) may promote, at least in part, T cell residence in the LRT. It is also likely that the differing E-cadherin and ICAM-1 phenotypes between LRT and URT epithelial cells influence patterns of T cell migration, particularly when CD103 is overexpressed on responding T cells, as in VAD animals. Results support a working hypothesis that the high CD103 expression among T cell populations in VAD mice alters T cell interactions with URT and LRT epithelial cells, thus inhibiting T cell migration and egress into the lower airway. A further analysis of kinetics and migration patterns of Tet− T cells in VAD mice.
is now warranted to test our hypothesis. Intravital imaging experiments may be conducted in concert with in vitro migration and adherence studies to facilitate a comprehensive examination of CD8+/H11001 T cell migration potentials in vaccinated VAD and control animals. Studies are also warranted to define the mechanism for upregulation of CD103 on membranes of respiratory tract Tet+/H11001 T cells, and the specific influences of various DC populations and cytokines (e.g., transforming growth factor β [TGF-β]) on this process. Expectations are that respiratory tract DCs, like gut DCs, are altered by VAD in terms of CD103 expression, migration, and functional capacities. The VAD influence on DCs may then be relayed to T cells during the processes of antigen presentation and imprinting.

**VAD and altered T cell imprinting in gut mucosal tissues.**

Our work supplements the more extensive studies of VAD effects on gut immunity. Respiratory and intestinal tracts share numerous features in that each is lined by layers of epithelial cells which are constantly bombarded by foreign antigens. Associated with the epithelial cells are DCs which sample foreign antigens, migrate to lymph nodes, and activate naïve B and T cell populations. The activated lymphocytes may then leave lymph nodes, enter the bloodstream, and traffic back to the site of original antigen exposure.

There have already been detailed analyses of mechanisms by which intestinal DCs imprint gut B and T cell populations. As described above, DC-mediated imprinting of cells with CCR9 and α4β7-integrin directs homing of lymphocytes to the small intestine (9, 20). Obstruction of vitamin A functions (e.g., by inhibition of RAR with drug [LE540] [21, 23, 25]) alters lymphocyte marking and migration patterns, similar to the situation defined in our studies of the respiratory tract.

CD103 has previously been described as influencing lymphocyte homing potential in the gut. However, the influences on T cell migration and residence are complex. For example, early studies suggested that lymphocyte homing to the gut was more dependent on αEβ7 than on α4β7-integrin. The conclusion was based on a finding that β7-integrin-deficient mice, but not α4-integrin-defi-
from the direct study of knockout mice, one may consider that the lymphocyte markers (16). It is perhaps expected that the two student experiments.

after infection with 500 PFU of SeV. Results were similar in each of 2 independent experiments. (B) In a separate experiment, mice were weighed every day for 10 days after infection with 500 PFU of SeV. Sample titers were determined, and each dot indicates a result from a different animal (4 or 5 mice per group). Horizontal bars indicate averages per group.

FIG 9 Weight loss and virus growth in SeV-infected VAD animals. (A) Lungs and nasal turbinates were removed on day 7 after infection with 500 PFU of SeV. Sample titers were determined, and each dot indicates a result from a different animal (4 or 5 mice per group). Horizontal bars indicate averages per group. (B) In a separate experiment, mice were weighed every day for 10 days after infection with 500 PFU of SeV. Results were similar in each of 2 independent experiments.

cient mice, exhibited reduced lymphocyte numbers in the gut’s epithelial cell lining (1, 16, 44). A different conclusion was reached when mixed ovalbumin-specific transgenic CD8⁺ T cells (OT-1) from αE⁺ and αE⁻ sources, or from β7⁺ and β7⁻ sources, were transferred to wild-type host animals subsequently infected with a recombinant vesicular stomatitis virus (VSV) encoding ovalbumin. In this case, the transfer of mixed αE⁺ and αE⁻ donor T cell populations resulted in the appearance of both cell types among intraepithelial lymphocytes (IEL) and in the lamina propria, but the transfer of mixed β7⁺ and β7⁻ donor cells resulted in the preferential migration of β7⁺ cells. Researchers then proposed that T cell homing was more dependent on αEβ7 than on αEβ7 lymphocyte markers (16). It is perhaps expected that the two studies support different conclusions. When considering the results from the direct study of knockout mice, one may consider that the lack of gene expression can affect multiple cell types in the animals and that there may be compensatory events during animal development. In the case of mixed, transferred T cell populations, one might also consider that one T cell population may exert positive or negative influences on the migration patterns of another. Our results demonstrate additional complexity in that the Tet⁺ T cells in LRT airways were not homogeneous with regard to membrane marker expression in either VAD or control animals. Clearly, cell-cell interactions facilitated by αEβ7-integrins are not autonomous but integrate with a variety of additional adherence mechanisms (e.g., CD11a interactions with ICAM-1) to define the overall outcome of T cell migration.

VAD, infectious disease, and vaccination. VAD continues to pose a health risk to children and adults, as multiple organ systems depend upon dietary vitamin A. Immune defects associated with VAD include increased susceptibility to infectious disease and weakened responses to vaccination. VAD deficiencies can in some cases be corrected by vitamin A supplements, but in other cases vitamin A supplements cause additional harm (4, 11, 18, 19). A fine vitamin balance must be achieved to avoid a spiral of events that may upset cell differentiation and homing marker expression, upset T cell migration, and thereby impair the natural course of an adaptive immune response.

This report begins to dissect the influences of VAD on vaccine-induced immune responses in the respiratory tract. There are multiple additional respiratory tract-resident cells other than CD8⁺ T cells that may be influenced by vitamin A (e.g., Th2, Th17, and CD4⁺ Foxp3⁺ T-regulatory cells [5, 45]) and there are multiple cytokines (e.g., interleukin-2 [IL-2], IL-5, IL-6, and TGF-β [20, 21, 23–25, 36]) which may alter vitamin A effects. As stated above, the changes described here may be secondary to VAD-induced modifications of the innate immune system and/or the stromal cells of the respiratory tract. A comprehensive investigation of lymphocytes, dendritic cells, matrix cells, and soluble factors may be necessary to define the full influence of VAD on vaccines that are administered by the i.n. route.

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