Effect of Vitamin A Deficiency in Dysregulating Immune Responses to Influenza Virus and Increasing Mortality Rates After Bacterial Coinfections

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Background: Secondary bacterial coinfections are ranked as a leading cause of hospitalization and morbid conditions associated with influenza. Because vitamin A deficiency (VAD) and insufficiency are frequent in both developed and developing countries, we asked how VAD influences coinfection severity.

Methods: VAD and control mice were infected with influenza virus for evaluation of inflammatory cytokines, cellular immune responses, and viral clearance. Influenza-infected mice were coinfecting with *Streptococcus pneumoniae* to study weight loss and survival.

Results: Naive VAD mouse lungs exhibited dysregulated immune function. Neutrophils were enhanced in frequency and there was a significant reduction in RANTES (regulated on activation of normal T cells expressed and secreted), a chemokine instrumental in T-cell homing and recruitment. After influenza virus infection, VAD mice experienced failures in CD4⁺ T-cell recruitment and B-cell organization into lymphoid structures in the lung. VAD mice exhibited higher viral titers than controls and slow viral clearance. There were elevated levels of inflammatory cytokines and innate cell subsets in the lungs. However, arginase, a marker of alternatively activated M2 macrophages, was rare. When influenza-infected VAD animals were exposed to bacteria, they experienced a 100% mortality rate.

Conclusion: Data showed that VAD dysregulated the immune response. Consequently, secondary bacterial infections were 100% lethal in influenza-infected VAD mice.

Key words. respiratory virus; coinfections; host response.

Globally, influenza virus kills approximately 290 000–650 000 individuals per year, with dramatic increases in lethality during pandemic events. Bacterial infections frequently accompany influenza infections and are a major cause of hospitalizations and morbid conditions [1–3]. Common bacterial coinfections with influenza involve *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus* [1, 2]. During the 1918 influenza pandemic, as many as 95% of all deaths involved bacterial coinfections and pneumonia [4, 5]. Studies in mice have shown that nonlethal influenza and bacterial infections become fatal when pathogens are administered sequentially (influenza followed by bacterial coinfection) [6–8]. Coinfections are not restricted to patients infected with influenza virus. Many respiratory viruses, including rhinoviruses and coronaviruses are

associated with bacterial coinfections [9]. In fact, the current severe acute respiratory syndrome coronavirus 2/coronavirus disease 19 pandemic is characterized by frequent coinfections and an alarming mortality rate [10].

Influenza-associated secondary bacterial infections are well characterized among respiratory viruses. Features of influenza disease that can enhance the severe consequences of subsequent bacterial coinfections include high levels of select cytokines, reduced or impaired resident macrophages, impairment of innate functions, depletion of adaptive immune cells/dysfunction, and destruction of barrier integrity. However, our understanding of the host parameters that render individuals susceptible to viral and bacterial coinfections is far from complete [1, 11–14].

Vitamin A deficiency (VAD) was once considered a problem in developing countries only, but more recently, VAD and vitamin A insufficiencies have become a relatively common feature of the developed world [15–17]. We and others have demonstrated the role of vitamin A in maintaining a healthy immune response and control of respiratory pathogens [18–20]. Vitamin A supports epithelial cell integrity [21] and influences both adaptive and innate immune cell activity [22–24]. VAD mice exhibit poor immune responses to respiratory viruses and bacteria [25, 26]. Antibodies, antibody-forming cells, and T-cell

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responses are weak, in the contexts of both infection and vaccination [23, 24].

Because individuals infected with a respiratory virus often succumb to bacterial coinfections, we focus our attention on immune parameters before and after viral infection that render individuals with VAD vulnerable to bacteria. Our results demonstrate that both innate and adaptive immune responses are altered in the context of VAD, resulting in increased inflammation after influenza infection and heightened susceptibility to disease caused by secondary bacterial infections.

MATERIALS AND METHODS

VAD and Control Mice

Mice were housed as specified by the Association for Assessment and Accreditation for Laboratory Animal Care guidelines and all protocols were approved by the Institutional Animal Care and Use Committee. Pregnant C57BL/6 mice (days 4–5 gestation) were purchased from Jackson Laboratories and placed on either a control (catalog no. 5WA2; TestDiets) or VAD (catalog no. 5W9M; TestDiets) diet on arrival in the animal facility at St Jude Children's Research Hospital, as described elsewhere [23]. Three weeks after delivery, pups were weaned and continued on the appropriate diet. Same-sex pups from multiple mothers were commonly combined in a single cage. Experiments used male and female 7-week-old mice. Mice were euthanized by anesthetization with tribromoethanol followed by exsanguination or by carbon dioxide asphyxiation followed by cervical dislocation.

Infection Experiments

Experiments were done using the mouse-adapted influenza A/Puerto Rico/8/34 (H1N1) (PR8) and type 3 pneumococcal strain A66.1. Virus and bacteria stocks were diluted in phosphate-buffered saline (PBS) to 100 plaque-forming units (PFUs) in 50 μ L and 75–100 colony-forming units (CFUs) in 100 μ L, respectively, and administered intranasally to mice anesthetized with 2.5% isoflurane. Mice were weighed daily and euthanized if they became moribund or lost $\geq 30\%$ of their body weight.

Harvesting/Processing of Blood and Lung

Blood was collected by cardiac puncture into heparin and incubated in red cell lysis buffer (catalog no. 07850; Stem Cell Technologies). Lungs were aseptically harvested, washed in PBS, digested with collagenase (1 mg/mL; Sigma C0130), and physically homogenized against a 40- μ m cell strainer. Cell suspensions were centrifuged and supernatants were used to determine viral titers and cytokine/chemokine levels.

Lung Titers

Viral titers were obtained using serial dilutions on Madin-Darby canine kidney monolayers, as described elsewhere [27]. On rare occasions, influenza-inoculated mice exhibited no signs of infection (no weight loss or detectable viral titers in the lungs on

days 3 and 7). These animals were excluded from cytokine and cellular analyses.

Flow Cytometric Analysis

Flow cytometry (LSRII Fortessa; Becton Dickinson) was performed on single-cell suspensions after 30 minutes in 1:2 dilution of Fc block (human γ -globulin) and 30 minutes surface staining. Viable cells and singlets were gated using forward/side scatter plots. For lung cell analyses, after neutrophil (Ly6C^{MID}Ly6G^{HI}) exclusion, T cells were gated as CD3⁺ and further defined as CD8⁺ or CD4⁺. Among CD3-negative cells, natural killer (NK) cells (F4/80^{neg}NK1.1⁺) and F4/80⁺ CD11c^{MID/}^{HI} cells (termed "macrophages" for simplicity) were quantified. Macrophages were further characterized as CD11b^{LO} or CD11b^{HI}. The former includes alveolar macrophages, while the latter includes interstitial and recruited exudate cells [28, 29]. For blood cell analyses, neutrophil and T-cell gating was done as described above. All cell frequencies were calculated as the percentage of viable singlets.

Cytokines

Cytokines and chemokines were measured in lung supernatants by means of an enzyme-linked immunosorbent assay (interferon [IFN] α and β) or using the Luminex platform (granulocyte-macrophage colony-stimulating factor, IFN- γ , interleukin 1 α , 1 β , 2, 6, 10, 12[p40], and 12[p70], keratinocyte-derived cytokine, CXCL-1 (KC), monocyte chemoattractant protein 1, macrophage inflammatory protein 1 α and 1 β , RANTES [regulated on activation of normal T cells expressed and secreted], and tumor necrosis factor α). See the Supplementary Materials for additional details.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software version 8. Influenza titer, cytokine data and weight loss data were evaluated individually each day, for diet- and sex-specific differences. Data were evaluated for normality and equality of variance. Unpaired *t* tests (with or without Welch correction) and Mann-Whitney *U* tests were performed as appropriate. Cellular data were evaluated using 2-way analysis of variance (ANOVA) followed by a Sidak multiple comparisons test to determine the contribution of diet to significant differences in cellular populations. Sex-specific differences in cellular populations were evaluated individually each day using unpaired *t* or Mann-Whitney *U* tests, as appropriate.

Pathology

Lungs were inflated in situ via tracheal infusion with 10% neutral-buffered formalin solution (ThermoFisher Scientific), followed by continued fixation in neutral-buffered formalin for ≥ 2 weeks. Lungs were then embedded in paraffin, sectioned at 4 μ m, mounted on positively charged glass slides (Superfrost Plus; ThermoFisher Scientific), and dried at 60°C for 20 minutes

before dewaxing and staining with hematoxylin-eosin using standard methods. For immunohistochemical staining, antibodies were as follows: for macrophages, anti-Iba1 (1:300 dilution; catalog no. CP290A; Biocare Medical) and anti-arginase 1 (1:40 dilution; catalog no. sc-20150; Biocare Medical); for B cells, anti-B220 (1:1000 dilution; catalog no. 553084; BD PharMingen); and for eosinophils, anti-mouse eosinophil major basic protein (1:3000 dilution; clone MT-14.7; Mayo Clinic). The presence of lymphocyte aggregates, indicative of organized tertiary lymphoid follicles, was evaluated using light microscopy, and semiquantitative scores ranging from 1–100 were assigned as grades. See the Supplementary Materials for additional details. All sections were examined by a pathologist blinded to the experimental group assignments.

RESULTS

Delayed Viral Clearance and Increased Inflammatory Cytokine Production in VAD Mice

To investigate how vitamin A influences the immune response to influenza infection, VAD and control mice were intranasally inoculated with 100 PFUs of PR8 ($n = 8$ –10 per group), a nonlethal dose in control mice. Lungs were harvested from naive mice (day 0) and infected mice (days 3, 7, and 10 after infection), and viral burden was determined. No significant difference in viral burden was observed on day 3. However, by day 7, when control mice had begun to clear infection, titers remained significantly elevated in VAD mice (Figure 1) ($P = .009$). By day 10 most control mice had cleared virus, whereas many VAD mice were still infected ($P = .05$). Among VAD mice, males exhibited higher viral titers than females ($P < .05$).

Anticipating that elevated viral titers and poor viral clearance may be associated with increased inflammation, we examined a select set of inflammatory cytokines and chemokines. At day 10 after infection, IFN- β , IFN- γ , and interleukin 1 β (Figure 2) were significantly elevated in VAD mice, and multiple, additional cytokines and chemokines trended higher (Supplementary Figure 1). Cytokines significantly elevated at day 10, were also frequently elevated earlier during infection. Interestingly, only 1 cytokine or chemokine (RANTES) was significantly reduced on day 10 in VAD mice. This critical cytokine for T-cell homing and recruitment was largely absent before infection and was significantly reduced in VAD mice, on days 7 and 10 (Figure 2). Although VAD male mice exhibited higher viral titers, no significant, sex-specific differences in cytokine expression were observed.

VAD Alteration of Innate Immune Cell Composition in the Lung

Using flow cytometry, we examined innate and adaptive immune cell populations, including T cells, macrophages, NK cells, and neutrophils in the lungs of naive and infected VAD and control mice. In general, there was an elevated presence of innate immune cells in the lungs of VAD mice. Specifically, VAD

Figure 1. Vitamin A–deficient (VAD) mice experienced higher viral burden at late times during influenza infection. VAD and control mice ($n = 8$ –10 per group) were intranasally infected with 100 plaque-forming units of influenza virus (PR8). Viral burden was determined in the lungs of infected mice on days 3, 7, and 10 after infection. Mice exhibiting no detectable virus when lung supernatants were assayed neat were assigned a titer value of 1. Significant differences were determined using Mann-Whitney U tests. Each symbol represents an individual mouse, with male and female mice represented by circles and diamonds, respectively. Hash tag (#) denotes a significant difference ($P < .05$) between male and female mice within a group, determined by the Mann-Whitney U test. Data shown represent 1 of 2 replicate experiments. Abbreviation: TCID₅₀, median tissue culture infective dose.

mice showed significantly increased percentages of neutrophils (Ly6C^{MID}Ly6G^{HI}) both before and after infection compared with controls ($P < .001$ by 2-way ANOVA including all time points; Figure 3A). In addition, Ly6G^{neg}CD3^{neg}F4/80⁺CD11c^{MID/HI} cells (termed macrophages for simplicity [28, 29]) were elevated in VAD mice ($P < .001$ by 2-way ANOVA including all time points), with differences being most pronounced at day 7 (Figure 3B).

No significant sex-specific differences were observed in the macrophage response to infection, though female and male mice exhibited significantly different frequencies before infection among both VAD and control mice ($P < .05$; Figure 3B). No significant differences were observed in NK cell frequencies (Ly6G^{neg}CD3^{neg}F4/80^{neg}NK1.1⁺; data not shown). However, there was intra-alveolar eosinophilia on day 7 in VAD mouse lung sections (Figure 3C and 3D, pink stain; additional images are included in Supplementary Figure 2). Altogether, results identified dysregulation of immunomodulating factors and innate cells in VAD animals both before and during virus infection.

Presence of Alveolar Macrophages that Fail to Polarize Toward the M2 Pathway in VAD Mice

Given the important role of macrophages in the immune response to primary influenza and secondary bacterial infections [27, 28, 30, 31], we investigated whether VAD influenced the number of CD11b^{LO} macrophages (inclusive of alveolar macrophages) during influenza infection, but no significant differences were detected between VAD and control mice (Figure 4A). CD11b^{HI} macrophages (inclusive of interstitial and recruited exudate macrophages) were more frequent in the

Figure 2. Dysregulation of cytokine expression in vitamin A-deficient (VAD) mice throughout the time course of influenza virus infection. VAD and control mice ($n = 8$ – 10 per group) were intranasally infected with 100 plaque-forming units of influenza virus (PR8). Lungs were harvested from naive and infected mice on days 3, 7, and 10. Cytokine levels were evaluated using the Luminex platform (interferon [IFN] γ , interleukin 1 β [IL-1 β], and RANTES [regulated on activation of normal T cells expressed and secreted]) or with enzyme-linked immunosorbent assay (IFN- β). Each symbol represents an individual mouse, with male and female mice represented by circles and diamonds, respectively. Data shown are from 1 of 2 replicate experiments. Significant differences were determined using Mann-Whitney U tests.

lungs of VAD mice before and after infection ($P < .001$ by 2-way ANOVA; [Figure 4B](#)).

To investigate further, we stained cells for arginase, often used to define an alternative activation pathway for macrophages indicative of an anti-inflammatory (M2) phenotype associated with tissue repair [32, 33]. Strikingly, though arginase-positive cells (pink stain in [Figure 4](#); B220 $^{+}$ B cells stained brown) were widely dispersed and abundant in the lungs of control mice on day 7, they were largely absent in VAD mice ([Figure 4C and 4D](#); additional images are in [Supplementary Figure 3](#)). Additional sections (not shown) were stained for Iba1, a macrophage marker, to confirm that macrophages were abundant in control and VAD mice. Results showed that in VAD mice, cells were not polarized toward a phenotype known to resolve inflammation.

VAD Impairment of CD4 $^{+}$ T-Cell Recruitment and Formation of Organized Lymphoid Structures

We next evaluated the effects of VAD on adaptive immune cells during influenza infection by quantifying T-cell frequencies in the lungs of naive and infected mice. CD8 $^{+}$ T cells were recruited into lungs in both VAD and control mice ([Figure 5A](#)), though VAD mice showed lower frequencies ($P = .01$ by 2-way ANOVA including all time points), with the difference most

pronounced at day 10 ($P = .006$). There was a striking reduction in total CD4 $^{+}$ T-cell frequencies in the lungs of VAD mice compared with controls at day 10 ($P < .001$; [Figure 5B](#)). In a preliminary experiment we observed no significant difference between CD4 $^{+}$ and CD8 $^{+}$ T-cell frequencies in the blood of VAD and control mice at day 10 after infection ([Supplementary Figure 4](#)), though T-cell frequencies were lower in VAD mice at earlier times during infection, suggesting that there was a defect in the recruitment of T cells into the lungs of VAD mice following influenza infection. In both the lung and blood, we observed sex-specific significant differences ($P < .05$) in T-cell frequencies in VAD and control mice, with a trend toward higher frequencies of T cells in female mice regardless of diet.

To investigate the B-cell response to influenza infection, we evaluated lung sections stained for B220 ([Figure 5C and 5D](#), brown stain; additional images are in [Supplementary Figure 3](#)). On day 7, VAD mice had small numbers of B220 $^{+}$ B cells dispersed throughout the lung interstitium, but no detectable tertiary structures had formed ([Figure 5D](#)). Control mice, in contrast, had tertiary lymphoid tissues distributed throughout the lung ([Figure 5C](#)). Separately, when lymphoid nodule aggregates, which include proliferating germinal center B cells, were scored by a pathologist from hematoxylin-eosin-stained

Figure 3. Elevated numbers of innate immune cells are present in the lungs of naive and influenza-infected vitamin A-deficient (VAD) mice. *A, B*, VAD and control mice ($n = 8$ – 10 per group) were intranasally infected with 100 plaque-forming units of influenza virus (PR8). Lungs were harvested from naive mice and infected mice on days 3, 7, and 10 after infection and cellular composition was evaluated by flow cytometry. Neutrophils were defined as $\text{Ly6G}^{\text{H}}\text{Ly6C}^{\text{MID}}$. $\text{Ly6G}^{\text{neg}}\text{CD3}^{\text{neg}}\text{F4/80}^{\text{+}}\text{CD11c}^{\text{MID/HI}}$ cells were termed “macrophages” for simplicity. See Materials and Methods for gating and staining details. Significant differences were determined using 2-way analysis of variance, followed by a Sidak multiple comparisons test. Each symbol represents an individual mouse, with male and female mice represented by circles and diamonds, respectively. Significant differences ($P < .05$) between male and female mice within a group were identified by Mann-Whitney U tests and are denoted by hash tags (#). Data shown are from 1 of 2 replicate experiments. *C, D*, On day 7 after infection, lungs collected from control (*C*) and VAD (*D*) mice were fixed and stained immunohistochemically for major basic protein (pink) as a marker for eosinophil infiltration. Experiments were performed in replicate, with 4–6 mice per group. Representative images are shown.

sections, VAD mice had significantly reduced numbers on day 7 compared with controls (Figure 5E). Organized peribronchiolar/perivascular lymphoid structures are not present in normal mouse lungs, but their formation can be triggered by infections, including influenza, and may play an important role in protection in mice [34, 35]. Lymphoid structures function primarily to facilitate lymphocyte activation, expansion, and differentiation [36]. Our findings suggest that the B-cell response is poorly coordinated in VAD mice.

Increased Susceptibility to Secondary Bacterial Infections in VAD Mice

The immune dysregulation we observed during influenza infection led us to hypothesize that secondary bacterial infections would be more severe in VAD mice. To test this hypothesis, we infected VAD and control mice intranasally with 100 PFUs of PR8, the same dose used in the experiments described above. Mice were then infected with a low dose of *Streptococcus pneumoniae* (strain A66.1, 75 colony-forming units) on day 7 after influenza infection. A second set of mice received PBS instead of bacteria (“influenza only”) and a third set of mice

received PBS instead of influenza (“bacteria only”). Mice were euthanized if they became moribund or lost 30% of their starting body weight.

Among the control mice in the influenza-only group, animals usually began to regain weight by day 10 (Figure 6A) and all animals recovered from the infection (Figure 6B). VAD mice in the influenza-only group continued to lose weight and most (10 of 12) succumbed to infection. Although male VAD mice lost significantly more weight than female VAD mice early during infection, no significant difference was observed in survival (Supplementary Figures 5 and 6A). Among control mice in the bacteria-only group, there were no morbid effects (Figure 6C). However, several VAD mice (2 of 10, both male) succumbed to infection after receiving bacteria (Figure 6D and Supplementary Figure 6B).

The lethality rate was significantly higher among coinfecting mice than among those that received either pathogen alone ($P < .001$ for coinfection compared to bacteria alone and $P < .05$ for coinfection compared to influenza alone, for all mice). Across 3 replicate experiments (see Figure 6E and 6F

Figure 4. Vitamin A-deficient (VAD) mice exhibit significantly elevated numbers of CD11b^{HI} macrophages but lack arginase-positive cells. *A, B*, VAD and control mice ($n = 8-10$ per group) were intranasally infected with 100 plaque-forming units of influenza virus (PR8). Lungs were harvested from naive and infected mice on days 3, 7, and 10 after infection. Cellular composition was evaluated by flow cytometry as Ly6G^{neg}CD3^{neg}F4/80⁺CD11c^{MID/Hi} CD11b^{LO} (inclusive of alveolar macrophages) and Ly6G^{neg}CD3^{neg}F4/80⁺CD11c^{MID/Hi} CD11b^{HI} (inclusive of interstitial and recruited exudate macrophages). Significant differences were determined using 2-way analysis of variance, followed by a Sidak multiple comparisons test. Each symbol represents an individual mouse, with male and female mice represented by circles and diamonds, respectively. Significant differences ($P < .05$) between male and female mice within a group were identified by Mann-Whitney U tests and are denoted by hash tags (#). Data shown are from 1 of 2 replicate experiments. *C, D*, On day 7 after infection, lungs collected from control (*C*) and VAD (*D*) mice were fixed and stained by immunohistochemistry for arginase (pink) as a marker of M2 macrophages. B220⁺ B cells were stained in brown. Experiments were performed in replicate with 4–6 mice per group. Representative images are shown.

and [Supplementary Figure 6C](#) for a representative experiment), 30%–50% of control mice survived, while 100% of VAD mice succumbed to coinfection and did so more rapidly than control mice (median survival, 11 and 12.5 days, respectively; $P = .008$).

DISCUSSION

Altered Immune Parameters in VAD Mice

Here, we describe how VAD contributes to dysregulated immune responses during influenza virus infection in mice, leading to increased influenza disease severity and an extreme susceptibility to disease caused by secondary bacterial infection. Naive VAD animals exhibited high neutrophil and CD11b^{HI} macrophage numbers and low levels of RANTES in the lung. After influenza virus infection, VAD animals showed poor T-cell frequencies and failed B-cell organization in the lung. Collectively, this resulted in higher viral loads in VAD animals compared with controls and slow viral clearance. High influenza virus titers were accompanied by high levels of IFN- β ,

IFN- γ , and interleukin 1 β and enhanced recruitment of neutrophils, eosinophils, and CD11b^{HI} macrophages into the lung. When VAD animals were exposed to bacteria 7 days after influenza, they experienced a worse outcome than controls and a 100% mortality rate.

Notably, RANTES was the only tested cytokine or chemokine that was reduced in VAD mice compared with controls. Poor RANTES production can mark both a cause and effect of T-cell malfunction, because RANTES can be produced by and can regulate T cells [37, 38]. Hence, the low expression of RANTES observed in naive VAD mice may have contributed to the poor recruitment of T cells during influenza infection, which may explain the reduced expression of RANTES late in infection. Coinciding with the changes in cytokine/chemokine production and poor T-cell recruitment, VAD mice exhibited abnormal B-cell responses. Although B cells were present in the lungs of VAD mice, there was poor B-cell organization ([Figure 5](#)), which may explain the commonly observed phenomenon of poor

Figure 5. Vitamin A-deficiency (VAD) impairs the CD4⁺ T-cell response to infection and leads to a disorganized adaptive immune response. *A, B*, VAD and control mice ($n = 8-10$ per group) were intranasally infected with 100 plaque-forming units of influenza virus (PR8). Lungs were harvested from naive and infected mice on days 3, 7, and 10 after infection. CD4⁺ and CD8⁺ T-cell numbers were evaluated by flow cytometry (Ly6G^{neg}CD3⁺CD4⁺CD8^{neg} and Ly6G^{neg}CD3⁺CD4^{neg}CD8⁺, respectively). Significant differences were determined using 2-way analysis of variance (ANOVA) followed by a Sidak multiple comparisons test. Each symbol represents an individual mouse, with male and female mice represented by circles and diamonds, respectively. Significant differences ($P < .05$) between male and female mice within a group were identified by Mann-Whitney *U* tests and are denoted by hash tags (#). *A, B*, Data shown are from 1 of 2 replicate experiments. *C, D*, Lungs collected from control (*C*) and VAD (*D*) mice were fixed and stained by immunohistochemistry for B220 (in brown), a marker for B cells. Arrows indicate organized B-cell structures. Representative images from day 7 are shown. *E*, The presence of lymphoid aggregates/nodules was determined by a pathologist using hematoxylin-eosin-stained lung sections taken before and after infection. Results of lymphoid scoring from 2 experiments (4–6 mice per group) were combined and showed significant differences using 2-way ANOVA followed by a Sidak multiple comparisons test.

antibody production in the context of VAD [23]. The low CD4⁺ T-cell frequencies may explain the poor B-cell organization observed in our study, although vitamin A can also act directly on B-cell development and function [39–45].

A prominent feature of VAD is the lack of normal homing receptors on lymphocytes, resulting in aberrant cell trafficking to mucosal tissues. Retinoic acid has been shown to regulate the

expression of gut-homing receptors on activated T cells [42]. This abnormal T-cell phenotype in VAD mice suggests that aberrant homing leads to reduced frequencies of T cells in the lung [24]. Altogether, our results point to impaired homing and effectors (including B cells, CD4⁺ and CD8⁺ T cells, antibodies, and alternatively activated M2 cells) as contributing factors to poor outcomes after viral-bacterial coinfections [2, 18, 23, 30].

Figure 6. Influenza-infected vitamin A deficient (VAD) mice are highly susceptible to secondary bacterial infections. VAD mice (grey dotted lines) and control mice (black solid lines) ($n = 9-12$ per group) were intranasally infected with 100 plaque-forming units of influenza virus (PR8) or phosphate-buffered saline (PBS). On day 7 after infection, mice were intranasally infected with 75 colony-forming units of *Streptococcus pneumoniae* (strain A66.1) or PBS. Mice were monitored for weight loss (A, C, E) and survival (B, D, F). Each group received treatments as follows: influenza only, PR8 followed by PBS (A, B); bacteria only, PBS followed by A66.1 (C, D); coinfection, PR8 followed by A66.1 (E, F). Arrows indicate the administration of PBS or A66.1 bacteria (day 7). Significant differences in weight loss were evaluated for each individual day using an unpaired t test with Welch correction (owing to significant differences in variance between groups). $*P < .05$; significant differences in survival were evaluated using a log-rank (Mantel-Cox) test. Data shown are from 1 of 3 replicate experiments. For analysis of weight loss and survival by sex, see Supplementary Figures 5 and 6, respectively.

Vitamin A, a Nuclear Receptor Ligand

Differences between male and female mice were noted throughout the study. This may be explained by the fact that both vitamin A and estrogen are nuclear receptor ligands. Vitamin A binds the retinoic acid receptor (RAR), and estrogen binds the estrogen receptor (ER). These transcription factors bind DNA response elements that often overlap, forcing an interplay of ligand-regulated ER and RAR. Our laboratory has identified a response element hot spot within the immunoglobulin heavy chain locus within a key

switch site necessary for immunoglobulin G, E, and A production [40]. The interplay of ER and RAR at this and other locations in the genome may explain significant differences in immune responses between male and female VAD mice [45].

Effects of a Loss of Balance Between Immune Function and Immunopathology

The causes and effects of immune responses and virus control are circular and complex. Innate and adaptive immune cells, if

rapid in function, can remove a respiratory pathogen so quickly that the infection occurs without symptoms. However, if effectors are not properly recruited (as for T cells, [Figure 5A](#) and [5B](#)) or lack appropriate organization/function (as for B cells, [Figure 5D](#) and [5E](#)), and if viral infection is not controlled, a once-beneficial innate and adaptive immune response can cause damage. An overrecruitment of cells into the airway (as observed for neutrophils, CD11b^{HI} macrophages, and eosinophils; [Figure 3](#)), may exacerbate disease and render individuals vulnerable to further insults.

Vitamin A has been viewed as an anti-inflammatory agent *in vitro* [43], but its effects *in vivo* are often beneficial rather than inhibitory to adaptive immunity [46]. When vitamin A promotes functional CD4⁺ T cells, CD8⁺ T cells, and B cells in the respiratory tract, it may quell pathogen spread and thereby prevent cell death and immunopathology. In the absence of vitamin A, viral clearance is compromised and inflammation does not resolve properly. Interestingly, our data suggest that some VAD mice may still succumb to infection despite clearing infectious virus from the lungs, which may be the direct result of immunopathology driven by the dysregulated immune response.

Correcting Low Dietary Intake of Vitamin A

Our results demonstrate the importance of maintaining sufficient levels of vitamin A to prevent severe morbid effects of infectious diseases. In VAD mice, vitamin supplementation corrects immune deficiencies [47] and in mice with diet-induced obesity (mice that exhibit unusually low retinol levels in lung tissues), vitamin A supplements (VASs) are again corrective [48]. In the developing world, supplementation programs have been designed to prevent VAD and have proved beneficial [49]. In the United States, however, because VAD is assumed to be rare, it may go unnoticed [15] and there is little emphasis on ensuring adequate vitamin A intake. Should high-dose VASs be provided to children in at-risk communities? The answer is unclear, as vitamin A overdosing may pose a risk. Our recent pediatric study of influenza virus vaccination demonstrated a correlation between baseline vitamin A levels and vaccine-induced serum antibodies. Vitamin A and D supplementation was beneficial, but benefit was recognized only among children who were vitamin A and D deficient or insufficient at baseline [17].

The use of high-dose VASs in patients who are hospitalized after respiratory infections is another topic of debate. A benefit was demonstrated among patients hospitalized with measles in the developing world [49], but similar studies have not been performed in the United States. One study of VASs among respiratory syncytial virus-infected, hospitalized children in the United States showed that supplements caused longer hospital stays compared with placebos [50]. Controlled clinical studies are needed to determine precisely if or when VASs are beneficial

in the developed world. Before acquisition of new clinical data, a cautious approach may be to encourage adequate dietary intake of vitamin A and to reserve VASs for individuals who cannot access vitamin-rich foods.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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