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Vitamin A corrects tissue deficits in diet-induced obese mice and reduces influenza infection after vaccination and challenge

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Abstract

OBJECTIVE—Individuals with obesity suffer from an increased susceptibility to severe respiratory viral infections and respond poorly to vaccinations, making it imperative to identify interventions. Recent evidence suggesting that obesity leads to tissue-specific vitamin A deficiency (VAD) led us to investigate whether high-dose oral vitamin A, a strategy used for remediating VAD in developing countries, could correct obesity-associated tissue deficits.

METHODS—Adult, C57BL/6, diet-induced obese (DIO) mice were supplemented with vitamin A for four weeks. A subset of mice were then vaccinated with inactivated influenza and challenged. Following supplementation, tissue vitamin A levels, lung immune cell composition, blood inflammatory cytokines, antibody responses and viral clearance were evaluated.

RESULTS—Supplementation significantly improved vitamin A levels in lung and adipose tissues in DIO mice. Additionally, supplementation decreased inflammatory cytokines in the blood and altered the lung immune environment. Importantly, vaccinated, vitamin A-treated DIO mice exhibited improved antibody responses and significantly reduced viral loads post-challenge compared to PBS-treated mice.

CONCLUSIONS—Results demonstrate a low-cost intervention that may correct vitamin A tissue deficits and help control respiratory viral infections in individuals with obesity.

Keywords

Immunology; infections; inflammation; vitamins; vaccines

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INTRODUCTION

Today, obesity affects approximately 13% of individuals worldwide and more than 1 in 3 adults in the United States. Obesity is associated with poor immune responses toward vaccines and pathogens, including influenza virus and tetanus (1), and a heightened risk for complications due to infectious disease (2, 3). In particular, following influenza virus infection individuals with obesity exhibit high virus loads and prolonged virus shedding, and are more vulnerable to hospitalization, ICU admission, and mortality (4, 5, 6).

The diets of individuals with obesity tend to favor high calorie, nutrient-poor foods, and thus vitamin deficiencies are common in this population. Recently, Trasino et. al. (7) demonstrated that obese mice fail to properly store vitamin A, resulting in tissue deficiency, despite elevated levels of vitamins in the blood. It is likely that these deficiencies are also present in humans, as non-alcoholic fatty acid disease, which alters vitamin A storage and metabolism (8), is common in individuals with obesity. The extent of tissue deficiency in individuals with obesity is unknown, because vitamin A status is routinely only measured using serum assays or is not assessed at all.

The interplay between malnutrition and immunity has been well established in the literature, particularly in the context of undernutrition. Notably, vitamin A is an essential micronutrient that regulates antibody production, immune cell (T, B and innate cell) residence, and the integrity of mucosal epithelial cells (9, 10, 11). Weaknesses in immune responses and natural barriers result in an increased vulnerability of vitamin A deficient (VAD) populations to infectious diseases (12, 13, 14). We and others have shown that vitamin A supplements benefit individuals with VAD or vitamin A insufficiencies to improve immune responses and protect against infectious diseases (14, 15, 16). Based on these findings, we asked if high-dose oral vitamin A supplementation could be beneficial in the context of obesity. Here we show that supplementation corrected vitamin A tissue deficits in obese mice, improved responses to influenza vaccination and protected against infection following challenge in vaccinated mice.

MATERIALS AND METHODS

Mice

Animal experiments followed AAALAC guidelines and were in accordance with Institutional Animal Care and Use Committee (IACUC) protocols. Three-week-old male C57BL/6 (B6) mice (Jackson Laboratories, Bar harbor, ME) were fed *ad libitum* a 60% high fat diet (cat #58Y1, LabDiets) for 15–19 weeks before experimentation, at which time mice weighed ~45–55 g. Age-matched B6 mice fed a standard rodent diet (cat #5001, LabDiets) were lean controls. Euthanasia was by CO_2 inhalation and cervical dislocation.

Vaccination and Vitamin A Supplementation

Mice received vitamin A supplements on days (d.) 0,3,7,21,24, and 28 by oral gavage with 100 μL containing 600 IU vitamin A (retinyl palmitate; Nutrisorb A, Interplexus Inc.). Mice were vaccinated intramuscularly on d.0 and d.21 with a sucrose purified, betapropiolactone

(BPL)-inactivated A/California/04/2009 pdmH1N1(CA09) H1N1 virus (1.2 μg hemagglutinin [HA], 50 μL PBS). Control mice received PBS, vitamin A, or vaccine only.

Vitamin A measurements in tissues

DIO and lean mice were euthanized on d.35 for blood collection by cardiac puncture. Liver, lung and white adipose tissues were frozen on dry ice. Serum retinol was quantified as described previously (16).

Virus challenge and lung titers

Two weeks following the second dose of vaccine, mice were anesthetized with isoflurane followed by intranasal inoculations (30 μL) with egg-grown A/California/04/2009 pdmH1N1 (10^6 $TCID_{50}$ on MDCK cells). Mice were sacrificed on d.3. Lungs were homogenized in 2 ml PBS and serially diluted in DMEM/0.1% BSA + 1 μg/ml acetylated trypsin. Dilutions were plated on MDCK cells in duplicate. Plates were incubated at 37°C for 4 days. 50 μL from each well were mixed with 50 μL 5% turkey red blood cells for 30 min at room temperature (RT) to test hemagglutination. $TCID_{50}$ values were calculated using the Reed-Muench formula.

Enzyme linked immunosorbent assays (ELISAs)

Sera were collected 10–14 days after the second dose of vaccine and tested for influenza-specific IgG and IgM. Plates were coated with 5 μg/mL of sucrose-purified pdmH1N1virus (overnight, 4°C), washed 3x with PBS and blocked with 1% BSA in PBS overnight at 4°C. Sera were diluted 1:100, 1:500 and 1:2500 in dilution buffer (PBS +1%BSA +0.05% Tween), and added to plates (1 hour, RT). Plates were washed 4x with PBS +0.05% Tween. Anti-IgG or anti-IgM (cat #1030-04 and 1020-04, Southern Biotech, 1:1000) were added (1 hour, RT). Plates were washed 4x and developed with pNPP (1mg/mL in diethanolamine buffer; cat #20-106, Sigma Aldrich), and read at 405 nm (VersaMax microplate reader).

Cytokine assays

Unvaccinated animals were euthanized and blood was collected by cardiac puncture one week following administration of the last dose of vitamin A. Thirty-two cytokines/chemokines were measured using a Milliplex MAP Kit (cat #: MCYTOMA-70K-PX32, Millipore). Samples (diluted 1:2 in PBS) were evaluated using a Luminex 200 Multiplexing Instrument and xPonent software.

Flow Cytometry

Lungs were collected (d.35) into gentleMACS C Tubes (cat #130-093-237, Miltenyi Biotech) and were processed with a Mouse Lung Dissociation Kit (cat #130-095-927, Miltenyi). Cells were suspended (70 μM strainer) and pelleted and red blood cells were lysed (red cell lysis buffer, cat #07850, Stem Cell Technologies). Cells were counted (Biorad TC20 automated counter), stained, and analyzed using an LSRFortessa X-20 (BD Biosciences) and FCS Express Software. All cells were stained with 7AAD (cat #A1310, Invitrogen) for live gating. Additional stains from Biolegend included: anti-CD3 (cat #100349), anti-CD4 (cat #100443), anti-CD8 (cat #100730), anti-CD45 (cat #103112), anti-

F4/80 (cat #123133), anti-CD11b (cat #101206), anti-CD11c (cat #117301), anti-SiglecF (cat #155506).

Statistics

Analyses were performed with GraphPad Prism. Data were evaluated for normality and equality of variance. Unpaired T or Mann Whitney U tests and two-way ANOVA were performed as appropriate.

RESULTS AND DISCUSSION

Vitamin A supplements correct deficits in lung, liver and adipose tissues in animals with obesity

To test the corrective potential of vitamin A supplements, we administered 600 IU vitamin A or PBS orally to obese and control mice on d.0, 3, 7, 21, 24, and 28. Vitamin A levels, measured in the form of retinol, were evaluated on d.35 (Figure 1). Similar to previous reports (7), we observed elevated levels of retinol in the sera and reduced levels in the tissues of obese mice compared to lean controls. Vitamin A supplementation significantly improved vitamin A levels in the lung and adipose tissues, but not in the sera or liver, of obese mice. Importantly, vitamin A levels in the lungs of supplemented DIO mice were closely matched to those of lean controls.

Supportive of our previous findings with non-obese mice (17), we found that vitamin A supplementation associated with marginally higher CD4+ and CD8+ T cell percentages among CD45+ cells in the respiratory tract (Figures 2A–B). Additionally, the percentages of $CD3^{NEG}B220^{NEG}F4/80^{+}CD11c^{HI/MED}CD11b^{LOW}SiglecF^{+}$ cells (inclusive of alveolar macrophages) were marginally improved and the percentages of $CD3^{NEG}B220^{NEG}F4/80^{+}CD11c^{HI/MED}CD11b^{HI}SiglecF^{NEG}$ cells (inclusive of interstitial macrophages and termed interstitial macrophages for simplicity (18)) were significantly reduced among $CD45^{+}$ cells in the lung (Figures 2C–D).

Vitamin A supplements improve virus control in vaccinated mice following challenge

Mice were vaccinated via a prime-boost regimen with an inactivated influenza virus (pdmH1N1) and vitamin A or PBS was administered orally on d.0, 3 and 7 post-vaccination. Two weeks post-boost serum was evaluated for virus-specific antibodies. Overall, obese mice demonstrated a poor antibody response to vaccination compared to control mice (Figure 3). Only when vaccine was co-delivered with vitamin A were influenza-specific IgG antibody responses significantly above background (Figure 3B). Supplementation also increased IgG/IgM ratios, indicating enhanced antibody class switching (Figure 3C).

Two weeks after the second dose of vaccine, animals were challenged with a non-lethal dose (10^6 $TCID_{50}$) of homologous influenza virus. There was transient, mild, weight loss after infection that was more pronounced in vitamin A-treated versus PBS-treated animals (Supplementary Figure 1), although absolute weights were similar between test and control groups throughout the time course. Weight loss may have reflected virus-specific immune responses (19), but significant correlations between weight loss and virus-specific antibodies

were not observed (data not shown). Vaccination alone provided some protection against infection. Importantly, on d.3 post-infection vaccinated mice that received vitamin A exhibited a profound reduction in viral lung titers compared to vaccinated, unsupplemented animals (Figure 3D, $p < 0.001$).

CONCLUSION

Vitamin A tissue deficits may be common in individuals with obesity, but masked and under-reported due to normal or above-normal serum retinol levels. Vitamin A is pleiotropic in function and influences virtually every mammalian cell, including cells involved in adaptive and innate immune responses. We show that vitamin A supplements provide benefit to individuals with obesity by correcting tissue vitamin deficits, altering lung immune cell composition, improving vaccine responses and assisting in respiratory virus clearance. These findings are particularly relevant as the world scrambles to deal with the SARS-CoV-2 pandemic. Individuals with underlying health conditions, including obesity (20), are particularly vulnerable to serious disease (COVID-19) caused by SARS-CoV-2. Our demonstration that vitamin A supplements provide protection against a respiratory virus infection may inform healthcare recommendations for prophylaxis against severe disease from respiratory viral infection in individuals with obesity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

$TCID_{50}$	tissue culture infectious doses-50
d	day
VAD	vitamin A deficient

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What is already known about this subject?

- Individuals with obesity are highly susceptible to increased morbidity and mortality to respiratory viral infections
- Tissue-specific vitamin A deficiencies have been demonstrated in mouse models of obesity

What are the new findings in your manuscript?

- Vitamin A supplementation corrects tissue-specific deficiencies and improves immune responses in obese mice
- Following challenge, vitamin A supplementation accelerates viral clearance in vaccinated obese mice

How might your results change the direction of research or the focus of clinical practice?

- Results identify a low-cost intervention that may correct vitamin A tissue deficits, improve vaccine-induced immune responses, and control respiratory viral infections in individuals with obesity

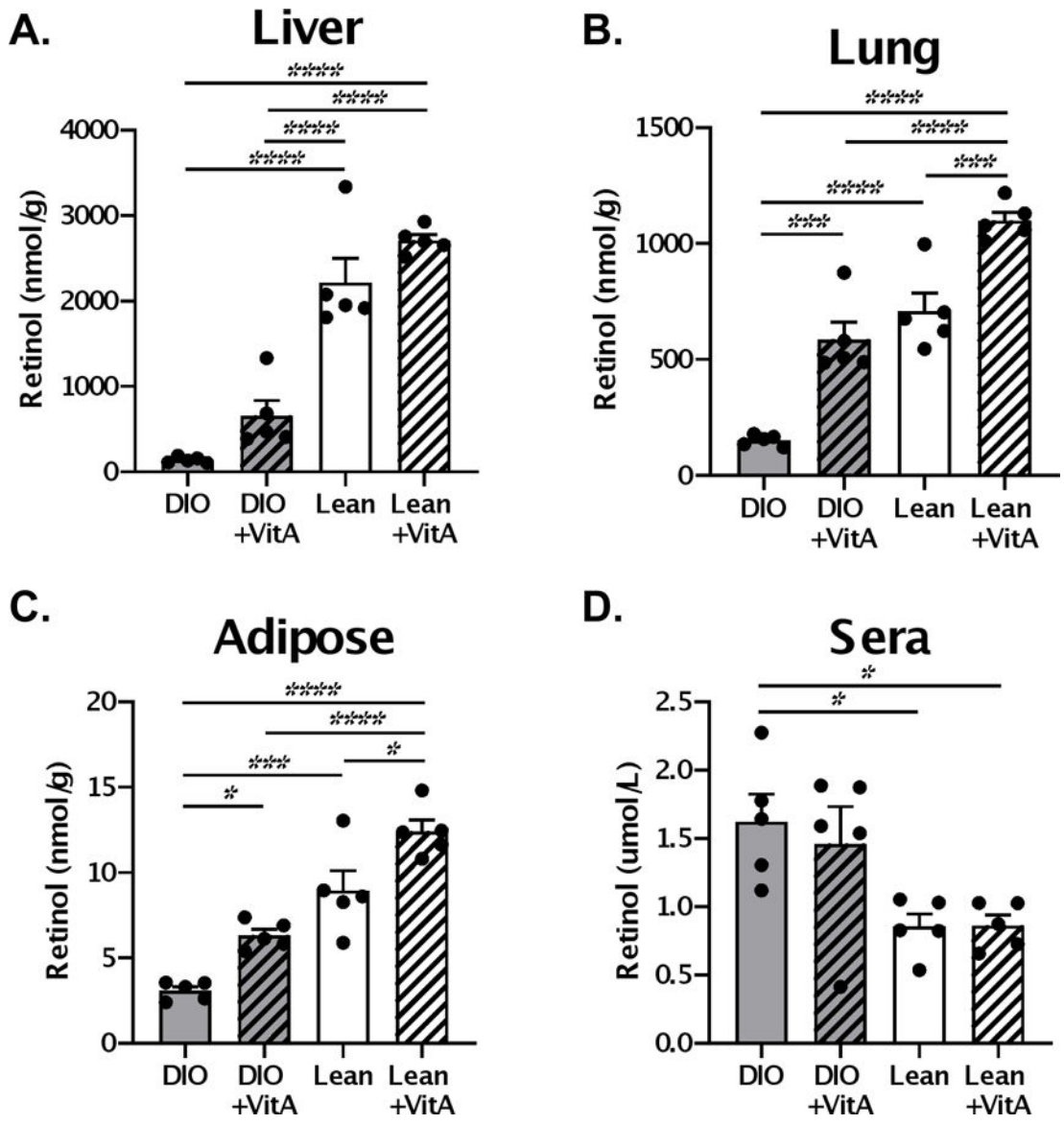

Figure 1. High-dose vitamin A supplementation improves retinol levels in tissues of obese mice.

Mice received 600 IU vitamin A or PBS orally on d.0,3,7,21, 24 and 28. Retinol levels were evaluated in the (A) liver, (B) lung, (C) adipose tissue and (D) of lean and diet-induced obese (DIO) mice one week after administration of last vitamin A dose (d.35). Each dot represents a single mouse with 5 mice per group. A representative experiment is shown for two independent experiments, each with 5 mice per group. Significant differences were determined by two-way ANOVA followed by Tukey's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

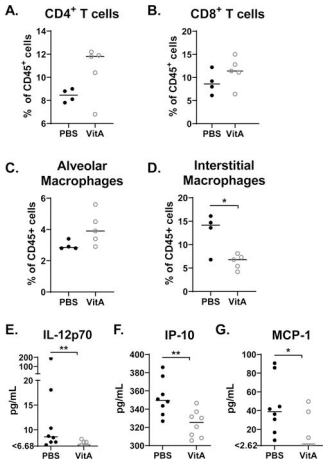

Figure 2. Vitamin A supplementation improves lung cell composition and blood cytokines in obese mice.

Lungs were analyzed by flow cytometry for percentages of (**A**) CD8+ and CD4+ T cells, and (**B**) cell populations inclusive of alveolar macrophages and interstitial macrophages. (**C**) Blood was analyzed for IL-12p70, IP-10, and MCP-1. For the cellular analyses, a representative experiment is shown for two independent experiments, each with 3–5 mice per group. Each dot represents a single mouse. For cytokine analyses, combined results are shown from two independent experiments, which included 3 mice per group and 5 mice per group, respectively. Significant differences were determined by Mann Whitney U tests. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Cell percentages were determined as percent of live $CD45^+$ cells. Populations were defined as follows: $CD8^+$ T cells: $CD3^+CD4^{NEG}CD8^+$; $CD4^+$ T cells: $CD3^+CD4^+CD8^{NEG}$; population inclusive of alveolar macrophages: $CD3^{NEG}B220^{NEG}F4/80^+CD11c^{HI/MED}CD11b^{LOW}SiglecF^+$; population inclusive of interstitial macrophages: $CD3^{NEG}B220^{NEG}F4/80^+CD11c^{HI/MED}CD11b^{HI}SiglecF^{NEG}$ (termed interstitial macrophages for simplicity).

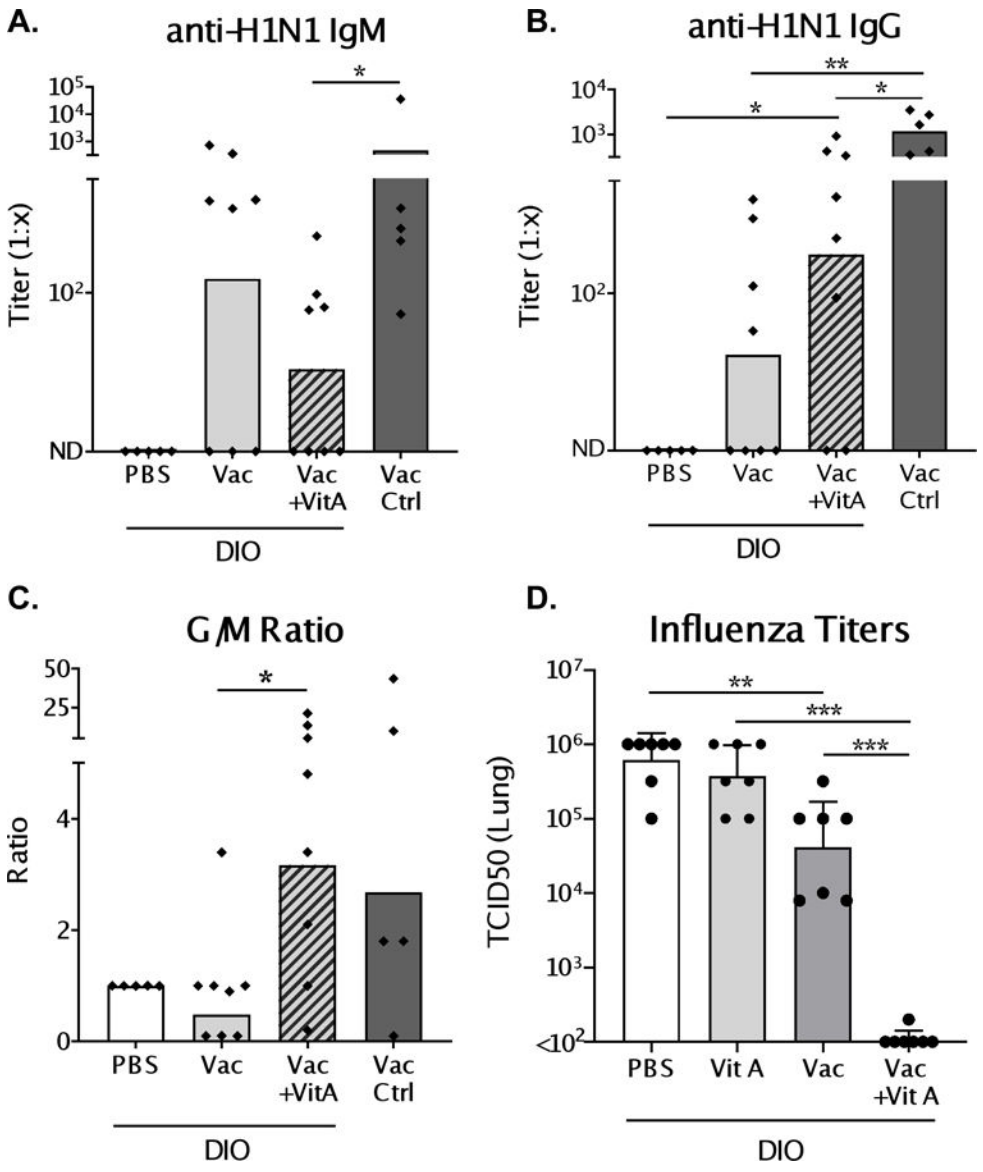

Figure 3. Virus-specific antibodies post-vaccination and virus load post-challenge in diet-induced obese (DIO) mice.

Mice were vaccinated with inactivated pdmH1N1 virus in a prime-boost scheme. At the time of vaccination, and d.3 and d.7 post-vaccination, mice received either 600 IU vitamin A or PBS. One week after boosting, sera were collected and tested by ELISA for IgM (**A**) and IgG (**B**) virus-specific antibody responses. (**C**) Ratio of IgG to IgM (G/M) titers were determined to evaluate the amount of antibody isotype switching. Mice were challenged intranasally with homologous virus (10^6 $TCID_{50}$) two weeks after the last dose of vaccine. Viral titers in the lungs (**D**) were measured in both vaccinated and unvaccinated DIO mice treated with vitamin A or PBS on d.3 post-infection. Each dot represents a single mouse. For antibody results, a representative experiment is shown for 4 independent experiments with 5–8 mice per group [PBS/C57BL/6 control mice: n=5; vaccinated/vaccinated+vitamin A test groups: n=8]. For influenza virus titers, a representative experiment is shown for two

experiments, each with 7 mice per group. Significant differences were determined by Mann Whitney U tests. *p<0.05, **p<0.01, ***p<0.001.