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Prenatally Acquired Vitamin A Deficiency Alters Innate Immune Responses to Human Rotavirus in a Gnotobiotic Pig Model

Anastasia N. Vlasova,* Kuldeep S. Chattha,* Sukumar Kandasamy,* Christine S. Siegismund,† and Linda J. Saif*†

We examined how prenatally acquired vitamin A deficiency (VAD) modulates innate immune responses and human rotavirus (HRV) vaccine efficacy in a gnotobiotic (Gn) piglet model of HRV diarrhea. The VAD and vitamin A–sufficient (VAS) Gn pigs were vaccinated with attenuated HRV (AttHRV) with or without concurrent oral vitamin A supplementation (100,000 IU) and challenged with virulent HRV (VirHRV). Regardless of vaccination status, the numbers of conventional and plasmacytoid dendritic cells (cDCs and pDCs) were higher in VAD piglets prechallenge, but decreased substantially postchallenge as compared with VAS pigs. We observed significantly higher frequency of CD103 (integrin αEβ7) expressing DCs in VAD versus VAS piglets postchallenge, indicating that VAD may interfere with homing (including intestinal) phenotype acquisition. Post-VirHRV challenge, we observed longer and more pronounced diarrhea and higher VirHRV fecal titers in nonvaccinated VAD piglets. Consistent with higher VirHRV shedding titers, higher IFN-α levels were induced in control VAD versus VAS piglet sera at postchallenge day 2. Ex vivo HRV-stimulated mononuclear cells (MNCs) isolated from spleen and blood of VAD pigs prechallenge also produced more IFN-α. In contrast, at postchallenge day 10, we observed reduced IFN-α levels in VAD pigs that coincided with decreased TLR3+ MNC frequencies. Numbers of necrotic MNCs were higher in VAD pigs in spleen (coincident with splenomegaly in other VAD animals) prechallenge and intestinal tissues (coincident with higher VirHRV induced intestinal damage) postchallenge. Thus, prenatal VAD caused an imbalance in innate immune responses and exacerbated VirHRV infection, whereas vitamin A supplementation failed to compensate for these VAD effects. The Journal of Immunology, 2013, 190: 4742–4753.

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Abbreviations used in this article: ATRA, all-trans retinoic acid; AttHRV, attenuated human rotavirus; CCIF, cell-culture immunofluorescence; cDC, conventional dendritic cell; CPE, cytopathic effects; FFU, fluorescent-forming unit; Gn, gnotobiotic; HRV, human rotavirus; MNC, mononuclear cell; PCD, postchallenge day; pDC, plasmacytoid dendritic cell; PID, postinoculation day; RA, retinoic acid; RARα, retinoic acid receptor-α; RBPF, retinol-binding protein 4; RS, rectal swab; RV, rotavirus; Treg, regulatory T; VAD, vitamin A deficiency; VAS, vitamin A–sufficient; VirHRV, virulent human rotavirus; VSV, vesicular stomatitis virus; WHO, World Health Organization.

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hereditary keratitis was more rapid, and the clinical disease, inflammatory response, incidence of epithelial ulceration, and necrosis were more severe in VAD rats than control rats (22). VAD in rats has been associated with decreased IFN production by spleen cells and diminished NK cell function in spleen (17, 23, 24), both of which were restored after oral vitamin A supplementation (23). Vitamin A–deficient mice showed a moderate reduction in the T cell area of the spleen, a significant reduction in thymus mass, and a reduced number of goblet cells per duodenal villus (20). There was a marked destruction of the villus tips in VAD mice infected with RV, but neither VAD nor RV infection alone produced such a marked effect. Recent studies of mice demonstrated that VAD alters splenic dendritic cell (DC) subpopulations, possibly contributing to skewed immune responses (25). However, in most of these studies that demonstrate vitamin A repletion effects, VAD was induced by dietary manipulations after birth, whereas the actual situation in humans may be more complex, including both prenatal (maternal) and dietary VAD.

The adjuvant properties of retinol were first reported in 1968 by Dresser (26), who showed that retinol-treated mice produced Abs to soluble bovine γ-globulin, which is not immunogenic in the mouse. Cytokine production, lymphocyte transformation, and resistance to tumor cells have all been reported to be greater in normal animals supplemented with high doses of vitamin A, possibly by recruiting lymphocytes and monocytes to the circulation and altering membrane structure (27, 28). Phagocytosis by peritoneal macrophages in mice and Salmonella typhimurium clearance in rats was greater in groups fed diets high in retinyl palmitate, probably through macrophage and T lymphocyte activation (29, 30). More recent studies on vitamin A adjuvant characteristics are somewhat contradictory and suggest that vitamin A adjuvancy can be pathogen/Ag and sex specific (31–35).

The immune function of retinoic acid (RA; a metabolite of vitamin A) includes enhancement of B and T cell mucosal homing and vitamin A concentrations were measured by HPLC.

**Materials and Methods**

**Virus**

The VirHRV Wa strain was used for pig challenge at a dose of 1 × 10^5 fluorescent-forming units (FFU). The ID_{50} of VirHRV in pigs was determined as ∼1 FFU (39, 42). Cell culture–adapted AntHRV Wa strain was maintained in MA 104 monkey kidney cells and used as a vaccine (three doses of 5 × 10^5 FFU/dose) and positive control in the cell-culture immunofluorescence (CCIF) assay (39, 42).

**Experimental design**

This study was approved by the Institutional Animal Care and Use Committee of The Ohio State University and conducted in compliance with local and federal guidelines. Pregnant sows were assigned to VAD or VAS diet at approximately gestational day 34 (when pregnancy was confirmed) or ∼80 d prior to derivation of Gn piglets. The sows fed a VAS diet were given an additional 500,000 IU injectable vitamin A (NDC50989-178-12; Vedco) to compensate for the naturally declining vitamin A levels during gestation. The Gn pigs were derived by hysterectomy and given a vitamin A–containing formula, vitamin A supplemented milk powder (Parmaflor), and vitamin A–containing formula, vitamin A–supplemented milk powder (Palmate). Vitamin A supplementation from the milk diet was estimated as 350 IU daily (∼3 times less than recommended by the National Research Council, 1998, for pigs), allowing us to maintain low vitamin A levels in VAD pigs, but not depleting vitamin A liver storage in the VAS pigs. Additionally, supplemental vitamin A (50,000 IU dose) was given i.m. to VAS piglets at birth to stably maintain adequate vitamin A levels. Retinyl palmitate (R3375, Sigma-Aldrich) at a 100,000 IU dose was used for oral supplementation with each vaccine dose or mock inoculation. Pigs were randomly assigned to each of four treatment groups as follows: 1) vaccinated, non–vitamin A–supplemented (3×AttHRV) (VAD, n = 12; VAS, n = 11); 2) vaccinated, vitamin A–supplemented with each vaccination (3×AttHRV, vitamin A 100,000 IU) (VAD, n = 9; VAS, n = 10); 3) nonvaccinated, non–vitamin A–supplemented (control) (VAD, n = 9; VAS, n = 9); and 4) nonvaccinated, vitamin A–supplemented (control, VitA 100,000 IU) (VAD, n = 9; VAS, n = 8). Nonvaccinated control pigs were given an equal volume of diluent in place of vaccine. All pigs were given 5 ml 100 mM sodium bicarbonate immediately to reduce the pain and to reduce any potential adverse effects of a mock or mock inoculation. After three sequential vaccinations with AttHRV (postinoculation day [PID]10, PID 10, and PID 20), a subset of Gn piglets in each group was euthanized at PID 26 to assess prechallenge immune responses, and the others were challenged with VirHRV Wa strain and euthanized 10 d later (PID 36) to assess postchallenge immune responses and vaccination efficacy. Rectal swabs (RSs) were collected daily to assay VirHRV shedding (postchallenge day [PCD]0–10). Pigs were examined daily for diarrhea postchallenge as described previously (38, 40, 41). Serum samples were collected at PID 10, -2, -4, PID 26/PCD 0, and PID 36/PCD 10 to assess vitamin A and IFN-α levels. Pigs were euthanized at PID 26/PCD 0 and PID 36/PCD 10 to isolate the mononuclear cells (MNCs) from ileum, duodenum, spleen, and peripheral blood for determining frequencies of DCs and apoptotic/necrotic MNCs. Liver samples were collected at euthanasia for assessment of vitamin A levels. Spleen and body weights were measured for VAS and VAD pigs, and spleen/body weight ratios were calculated to estimate the relative spleen weight increases.

**Detection of rectal HRV shedding by CCIF**

RS samples were used to quantitate infectious HRV postchallenge by CCIF assay as previously described (42, 43). Briefly, RS samples were initially diluted 1:25 in serum-free MEM, then diluted 1:4, and serially diluted 10-fold thereafter. RS fluids from mock-inoculated pigs were used as negative controls. The HRV-positive RS fluids from reference HRV-inoculated pigs were used as positive controls. The final CCIF titers were calculated and expressed as the reciprocal of the highest dilution showing positive fluorescent cells.

**Serum and liver vitamin A levels**

Serum and liver samples were submitted to the Diagnostic Center for Population and Animal Health (Michigan State University, Lansing, MI), and vitamin A concentrations were measured by HPLC.
**Isolation of MNCs**

Spleen, blood, duodenum, and ileum were collected the day of euthanasia and processed for isolation of MNC as previously described (38, 41). After isolation, the cells were diluted in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Atlas Biological, Fort Collins, CO), 1% l-glutamine, sodium pyruvate, nonessential amino acids, and ampicillin/gentamicin (200 μg/ml) (Invitrogen; DC media) and kept at room temperature until testing. Flow cytometry stainings were performed the same day immediately after the isolation of all tissue-derived cells. MNCs (400,000) for quantitative RT-PCR (retinol-binding protein 4 [RBP4]/RA receptor-α [RARα] gene expression) were frozen at −70°C in RNA Later Buffer (Qiagen, Venica, CA).

**Flow cytometry to assess plasmacytoid and conventional dendritic cell frequencies and distribution**

Procedures for flow cytometry staining (including buffers used) were performed as described previously (44–47) with minor modifications. The 96-well plates (0030 521.102; Eppendorf, Hamburg, Germany) were used to perform all flow cytometry stainings. To assess DC subset frequencies and distribution, cells were stained with specific and cross-reactive mAbs to porcine and human cell-surface markers, respectively, isotype control and secondary Abs (Table I). Swine plasmacytoid DCs (pDCs) were defined as SWC3α+/CD4+/CD11R1+ and conventional DCs (cDCs) were defined as SWC3α+/CD4+CD11R1+. Expression of CD103 marker (Table I) was assessed for both DC subsets and the total MNC population. After the cell-surface marker stainings were completed, the cells were permeabilized using Cytofix/Cytoplperm (554714; BD Biosciences), washed in Perm/Wash Buffer (554723; BD Biosciences), and resuspended in Perm/Wash Buffer. Intracellular staining for TLR3+ (Table I) was performed as recommended by the manufacturer, and acquisition of 50,000 events was done using Accuri6 flow cytometer (Accuri cytometers; BD Biosciences). Analyses were performed using CFlow software (Accuri cytometers; BD Biosciences).

**Frequencies and tissue distribution of apoptotic and necrotic MNCs by flow cytometry**

Annexin V Apoptosis Detection Kit APC (88-8007-74; eBioscience,) and Propidium Iodide Staining Solution (006990-50; eBioscience) were used according to the manufacturer’s protocols to detect and discriminate apoptotic and necrotic MNCs. Within 4 h after the staining, acquisition of 50,000 events was performed using Accuri6 flow cytometer (BD Biosciences), and analyses were performed using CFlow software (BD Biosciences).

**RNA isolation and real-time RT-PCR for the RBP4 and RARα mRNA quantification**

Total RNA was extracted from the purified MNCs using an RNaseasy mini kit (Qiagen) according to the manufacturer’s protocols. RNA was digested with DNase I (RNase-Free DNase set; Qiagen). RT-PCR was conducted using an equal amount of total RNA (150 ng) with QuantiTect SYBR Green RT-PCR Kit (Qiagen). The real-time quantitative RT-PCR was done in a final volume of 20 μl, which contained 10 μl 2× SYBR Mix, 0.2 μl RT Mix, and 25 pmol each primer for the detection of RBP4, RARα, and β-actin that was used as a housekeeping gene. All reactions were done in duplicate in PCR eight-tube strips using the Eppendorf MasterCycler EP Realplex (Eppendorf). The following primers were designed in this study and used for RBP4 and RARα mRNA quantification, respectively: pRBP4-NF, 5′-GCAAGATGGAATGGTTTGT-3′ (sense) and pRBP4-NR, 5′-GTTCCTTGTGACTCGAAGTGGT-3′ (antisense); and pRARα-forward, 5′-ACGTGGCAATACATACCTACGAA-3′ (sense) and pRARα-reverse, 5′-GGCGACGCGTTGCTGCGAATC-3′ (antisense).

**All-trans RA treatment of splenic MNCs and proliferation assay**

A stock solution of 1 mM retinoic acid (R2625; Sigma-Aldrich) was prepared in 95% ethanol. Splenic MNCs were resuspended in enriched RPMI 1640 medium (Invitrogen-Life Technologies, Grand Island, NY) supplemented with 10% FBS, 2 mM glutamine, 100 U ampicillin, and 100 mg gentamicin/ml and stimulated ex vivo with different final concentrations (0.1, 1, and 10 μM) of all-trans RA (ATRA) for 24 h. To assess ex vivo proliferation, we used the Click-it Edu Alexa Fluor 488 Flow Cytometry Assay Kit (Invitrogen-Life Technologies) according to the manufacturer’s instructions.

**IFN-α production by splenic MNCs after ex vivo restimulation with HRV**

MNCs/DCs (400,000 cells/well, each sample in duplicate) were cultured for 24 h at 37°C, with 5% CO₂, in 24-well plates in enriched RPMI 1640 in the presence of HRV Ag or PMA (Sigma-Aldrich) as a control added at final concentrations of 12 and 25 μg/ml, respectively. After 24 h, cells were removed by centrifugation, and supernatants were stored at −80°C.

**IFN-α bioassay**

The cytopathic effect (CPE) inhibition bioassay for IFN-α has been described elsewhere (49, 50). Briefly, monolayer cultures of 1-d-old Madin Darby bovine kidney cells in 96-well microtiter plates were rinsed twice and incubated with 4-fold serial dilutions of samples (sera or MNC supernatants) or standard (recombinant porcine IFN-α) for 16 h and then rinsed with fresh medium and challenged with vesicular stomatitis virus (VSV, Indiana strain). Forty-eight hours later, when complete CPE developed in VSV control wells (without IFN-α), the medium was discarded from all wells, the plates were rinsed, and fresh medium was added, and then 10 μl AlamarBlue (Invitrogen) was added to each well. Five hours later, the fluorescence was read at 530–560 and 590 nm using Fluoroskan Ascent FL and Ascent software (Thermo Electron). A measurement of 1 IU/ml is defined in this paper as that concentration that in the bioassay protects 50% of the cells in a culture against VSV-induced CPE (reciprocal of the dilution producing 50% CPE × 20).

**Statistical analyses**

The Fisher exact test was used to compare proportions of pigs with diarrhea and virus shedding among groups. One-way ANOVA (ANOVA general linear model), followed by Duncan’s multiple range test, was used to compare mean duration of virus shedding and diarrhea, vitamin A, and

<table>
<thead>
<tr>
<th>Table I. Abs used for flow cytometry analyses</th>
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<td><strong>Marker</strong></td>
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<tr>
<td>SWC3a (porcine)</td>
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<tr>
<td>CD4 (porcine)</td>
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<tr>
<td>CD11R1 (porcine)</td>
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<tr>
<td>CD103 (human)</td>
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<tr>
<td>TLR3 (human)</td>
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<tr>
<td>Isotype control, IgG1-PE</td>
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<tr>
<td>Isotype control, IgG2b-SPRD</td>
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<tr>
<td>Isotype control, IgG1-FITC</td>
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<td>Secondary Ab, anti-mouse IgG1, APC</td>
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PE: R-phycocerythrin; SPRD: SpectralRed.
IFN-α levels. The frequencies of cell populations measured by flow cytometry were compared among or within groups using the Kruskal-Wallis rank-sum (nonparametric) test. Statistical significance was assessed at $p \leq 0.05$ for all comparisons. All statistical analyses were performed using the Minitab 16 program (Minitab).

Results

Hepatic vitamin A levels were significantly higher in VAS and vitamin A–supplemented piglets

Hepatic vitamin A levels for VAD piglets were significantly lower than for VAS piglets in all treatment groups (Fig. 1A). Hepatic vitamin A levels of nonsupplemented piglets were significantly lower than those of supplemented piglets, respectively, on PID26/PCD0 and PID36/PCD10. Serum vitamin A levels of VAS piglets were within the normal range defined for nursing piglets (0.3–1 IU/ml) and stayed at similar levels throughout the experiment, reflecting that liver and serum vitamin A levels are under homeostatic regulation (Fig. 1B). In contrast, in VAD piglets, during the course of experiment, serum vitamin A levels increased ~2-fold (Fig. 1B), suggesting a positive 30-d dose response that indicates the presence of subclinical VAD as observed in human clinical studies (12). The three-dose vitamin A supplementation (100,000 IU) did not affect serum vitamin A levels in VAD or VAS piglets (data not shown).

Interestingly, VirHRV challenge of VAS piglets resulted in significantly reduced hepatic vitamin A postchallenge (PCD10) (Fig. 1A) and a transient decrease in serum vitamin A early after the challenge (PCD2) that was restored by PCD10, likely by means of hepatic vitamin A release into serum (Fig. 1C). Thus, VirHRV or VirHRV-induced diarrhea may affect vitamin A metabolism or absorption.

RBP4 and RARα mRNA levels were higher in VAS piglets

RBP4 and RARα mRNA levels were measured by quantitative RT-PCR in MNCs isolated from ileum, duodenum, spleen, and blood at pre- (PID26/PCD0) and post-VirHRV (PID36/PCD10) challenge. However, detectable levels of RBP4 and/or RARα were found only in VAS piglets postchallenge.
mRNA and differences between the treatment groups were only observed for splenic MNCs. Consistent with vitamin A levels, RBP4 and RARα mRNA levels were affected by vitamin A status, vitamin A supplementation, and VirHRV challenge, but not by the vaccination status. Overall, they were higher in splenic MNCs of VAS pigs as compared with VAD pigs (Fig. 2A, 2B). Interestingly, RBP4 and RARα mRNA expression was increased postchallenge in both VAS and VAD animals (Fig. 2C, 2D). RARα mRNA levels were increased in vitamin A–supplemented VAS and VAD pigs postchallenge (Fig. 2D), whereas RBP4 mRNA levels were decreased in vitamin A–supplemented VAS and increased in vitamin A–supplemented VAD pigs postchallenge (Fig. 2C).

**HRV shedding and diarrhea were higher in VAD piglets**

VAD control piglets had significantly more severe and longer duration of diarrhea compared with VAS piglets (Table II). Noteworthy, in control VAS piglets, fecal scores peaked by PCD3 and declined thereafter, whereas in control VAD piglets, fecal scores were significantly higher at PCD3–5 and peaked at PCD4 (data not shown). VAD control piglets had longer fecal HRV shedding and higher HRV titers and diarrhea postchallenge as compared with VAS control piglets (Table II). All vaccinated VAS (with or without vitamin A supplementation), but only vaccinated and vitamin A–supplemented VAD piglets were completely protected against RV shedding (Table II). Only 75% (three out of four) of VAD-vaccinated nonsupplemented piglets were protected against VirHRV fecal shedding. These results indicate that the oral AttHRV vaccine–induced protective efficacy and adaptive immune responses (K.S. Chattha, S. Kandasamy, A.N. Vlasova, C.S. Siegismund, and L.J. Saif, unpublished observations) were not as severely compromised as innate immunity, suggesting that even low-level postnatal vitamin A supplementation (in the milk diet) may have had beneficial effects. Mean cumulative fecal score and average peak fecal HRV titers were significantly higher in control VAD versus VAS pigs (Table II). Mean HRV shedding duration differed numerically, but did not differ significantly between these two groups. Thus, VAD increases HRV-induced diarrhea severity and fecal HRV shedding titers and duration, whereas the three-dose vitamin A supplementation to VAD pigs did not provide consistent compensatory effects in the Gn pig model.

**Circulating IFN-α levels were higher in sera from VAD piglets postchallenge**

At PID2, vaccinated and, surprisingly, some nonvaccinated control VAD piglets had very low but detectable IFN-α levels in sera, whereas in VAS piglets, IFN-α was detectable only in vaccinated animals (data not shown). This suggests that in VAD pigs, IFN-α production may be induced via other than a TLR3 (viral dsRNA)-dependent pathway.

At PCD2 (peak of HRV-induced IFN-α in serum), we observed four times higher levels of IFN-α in serum samples of control VAD versus VAS piglets (Fig. 3B), likely due to higher VirHRV shedding levels (Table II) and/or the imbalanced immune responses. At PCD4, IFN-α was still detectable in challenged control VAD piglets, but not in VAS piglets (Fig. 3B). Notably, serum IFN-α levels appeared to coincide with the extent of HRV replication, occurring in all vaccinated pigs after the primary AttHRV vaccine dose (Fig. 3A). Postchallenge, high IFN-α levels in control pigs compared with vaccinated pigs (Fig. 3B) coincided with the control pigs having higher HRV shedding titers postchallenge, but also

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**FIGURE 2.** Relative RBP4 and RARα mRNA levels in splenic MNCs of VAD/VAS pigs measured by real-time RT-PCR and expressed as fold change relative to VAS group (A, B) or to untreated (no vitamin A supplementation, vaccination, or challenge) VAS group (C, D). (A and B) Fold change is shown regardless of treatment (vitamin A supplementation, vaccination, or challenge) for RBP4 and RARα mRNA levels, respectively. (C and D) Fold change is shown pre- and postchallenge for each group for RBP4 and RARα mRNA levels, respectively. Data are the mean ± SEM from one experiment. VitA, Vitamin A.
corresponding to the 25% shedding rate in VAD-vaccinated pigs only (Table II).

Similarly to IFN-α levels in vivo, after ex vivo stimulation (HRV Ag), MNCs from spleen and blood of VAD piglets pre-challenge also produced 10- and 100-fold, respectively, higher amounts of IFN-α than MNCs from VAS piglets (Fig. 3C, 3D). In contrast, after VirHRV challenge, the situation was the opposite: IFN-α production by MNCs isolated from VAD piglets decreased

FIGURE 3. Circulating IFN-α in VAD- and VAS-vaccinated (A) and control (B) piglets’ sera pre- and postchallenge. Different capital letters (A, B) indicate that IFN-α level was significantly lower ($p < 0.05$) in VAS piglet sera at PCD2. Pooled data are the mean ± SEM from three independent experiments IFN-α production by splenic (C) and blood (D) MNCs (post-HRV Ag stimulation) of control VAD and VAS piglets pre- and postchallenge. Pooled data are the mean ± SEM from two independent experiments. Asterisks indicate that IFN-α production by splenic and blood MNCs was significantly decreased (arrow down) for VAD piglets and significantly increased (arrow up) for VAS piglets postchallenge.
4- and 8-fold (for blood and spleen MNCs), and IFN-α production by MNCs isolated from VAS piglets increased 2-fold for spleen and 430-fold for blood MNCs (Fig. 3C, 3D).

**Total DC frequencies were increased in VAD piglets prechallenge, whereas frequencies of CD103-expressing DC were decreased in VAD piglets postchallenge**

In the Gn pigs, in steady state or upon VirHRV challenge, regardless of treatments (vaccination or vitamin A status), the major residential tissue for pDCs was duodenum, whereas the highest frequencies of cDCs were observed in spleen (Fig. 4A, 4B). Total pDC and cDC frequencies were lower post-VirHRV challenge. Total frequencies of pDCs were significantly higher in gut tissues of VAD piglets, but in systemic tissues of VAS piglets prechallenge, with lower and similar (between VAD and VAS) levels postchallenge (Fig. 4A). cDC frequencies were higher in all tissues of VAD piglets prechallenge and were also decreased and similar to those ofVAS piglets postchallenge (Fig. 4B). These enhanced frequencies of DCs in VAD piglets in the absence of pathogen prechallenge may subsequently lead to immune system imbalance and debilitation, with inability to control VirHRV postchallenge. The observed trends were not affected by vaccination status and vitamin A supplementation, so the data were collapsed for analysis.

Frequencies of specialized CD103+ (αEβ7) DCs (pDCs and cDCs) were consistently (pre- and postchallenge) and significantly (postchallenge) higher in VAS versus VAD piglets (Fig. 4C, 4D), suggesting a potential for subsequent less inflammatory and more regulatory immune profiles in VAS piglets. Notably, whereas in VAS piglets, frequencies of CD103+ DCs increased 2–4-fold postchallenge, in VAD piglets, those frequencies were unchanged, providing a possible explanation for the less-efficient resolution of VirHRV postchallenge in VAD piglets (Fig. 4C, 4D). Additionally, no effect of vitamin A supplementation on DC subsets was observed pre- or postchallenge in VAD and VAS control and vaccinated pigs (data not shown).

**TLR3+ MNC frequencies were significantly higher in intestinal and systemic tissues of VAS piglets**

TLR3+ MNCs were detectable only postchallenge, and their frequencies were significantly higher in all tissues of VAS as compared with VAD piglets (Fig. 5). This is consistent with decreased IFN-α production by splenic and blood MNCs of VAD piglets at PCD10, suggesting that postchallenge IFN-α production is activated via a TLR3 dependent pathway.

Decreased TLR3 expression by MNCs from vaccinated piglets compared with control piglets likely reflects their partial protection against VirHRV replication. A similar situation is probable in the case of marginally lower TLR3+ MNC frequencies for vaccinated and vitamin A–supplemented piglets (as compared with vaccinated, nonsupplemented): the vitamin A–supplemented piglets also had improved protection against VirHRV replication.

**Necrotic MNC frequencies were increased and apoptotic MNC frequencies were decreased in local and systemic tissues of VAD piglets**

We observed significantly higher (~2.5-fold) frequencies of necrotic MNCs in spleens of VAD piglets prechallenge (Fig. 6A). There was a slight moderation in the frequencies of necrotic
MNCs due to vitamin A supplementation as evident prechallenge (Fig. 6A). Higher cDC (Fig. 4B) and necrotic MNC (Fig. 6A) frequencies, potentially accompanied by inflammatory cell (including macrophages and neutrophils) infiltration and cell debris accumulation in spleens of VAD piglets prechallenge, could contribute to the relative increase in spleen/body weight ratio observed in this study (Fig. 7).

Additionally, necrotic MNC numbers were increased (1.5–2 times) in ileum and duodenum of VAD piglets compared with VAS piglets postchallenge, likely due to a combined effect of VAD and HRV infection (Fig. 6B). Apoptotic MNC frequencies postchallenge were significantly higher in spleen of VAS pigs as compared with VAD pigs, suggesting insufficiency of immunoregulatory function of the latter pigs (Fig. 6C) with no effect of vitamin A supplementation evident (data not shown).

Splenic MNCs from VAS piglets were more responsive to ATRA treatment ex vivo

In our study, we did not observe a consistent effect (or minor if observed) of three doses vitamin A supplementation on innate immune responses in either VAD or VAS neonatal piglets. In VAS piglets with adequate circulating and hepatic vitamin A levels, all supplemented vitamin A is likely being stored in the liver, whereas in VAD piglets (with positive 30-d dose response indicating subclinical VAD), we expected that some supplemental vitamin A would be converted into ATRA and compensate for negative VAD effects. To address the observed lack of such consistent compensatory effects of vitamin A supplementation in vivo, we analyzed the effects of ATRA (at concentrations of 0.1, 1, and 10 μM) ex vivo on splenic MNCs derived from VAD and VAS control pigs. The ATRA effect on proliferation, apoptosis, and CD103 expression was dose dependent (data not shown). Interestingly, ex vivo stimulation with ATRA (1 μM) resulted in increased proliferation and increased apoptosis of MNCs from VAS, but not from VAD pigs (Table III). ATRA stimulation improved viability of VAD MNCs as reflected by decreased frequency of necrosis, whereas in VAS MNCs, it was lower initially and did not change (Table III). Additionally, ATRA treatment significantly increased CD103 expression by VAS MNCs, whereas no effect on VAD MNCs was apparent (Table III). These findings demonstrate that VAD MNCs were less responsive to ATRA stimulation (no effect of ATRA except for necrosis frequencies) than VAS MNCs and suggest that vitamin A absorption efficiency and/or metabolism may be compromised in VAD neonatal piglets. Alternatively, it may require a longer time period to improve the immune function and alter the life cycle of immune cells in vivo by means of vitamin A supplementation.

Discussion

Reduced efficacy of oral vaccines and increased mortality rates from infectious diseases are prevalent in children in impoverished countries and often are associated with their poor micronutrient status. In particular, VAD is strongly associated with an increased risk of respiratory and alimentary tract diseases. Rotavirus causes life-threatening gastroenteritis with high fatality rates in developing countries, where VAD is prevalent in preschool children (1, 51). We have successfully established a VAD and VAS Gn pig model and studied the impact of VAD and vitamin A supple-
VITAMIN A DEFICIENCY AND INNATE IMMUNITY

4750

FIGURE 7. Spleen to body-weight ratio of VAD/VAS pigs pre-challenge. Asterisk indicates that this ratio was significantly higher for VAD pigs, which reflects an increase in relative spleen weight. Pooled data are the mean ± SEM from two independent experiments.

plementation (following the WHO recommendations) among AttHRV-vaccinated and VirHRV-challenged groups. In our Gn pig model, we recreated subclinical VAD, with significantly decreased liver vitamin A levels and positive serum 30-d dose-response test (12). VirHRV challenge depressed vitamin A levels in serum early postchallenge (PCD2), as it was previously shown for measles virus (52). This finding suggests that VirHRV, which causes intestinal villous atrophy and malabsorption (41), may affect vitamin A metabolism or its absorption from the milk diet. Nonvaccinated VAD pigs had more severe diarrhea and higher HRV shedding compared with VAS pigs, suggesting that vitamin A mediates its effect by maintaining the epithelial barrier and/or by regulating immune functions. Consistent with this, higher frequencies of necrotic MNCs in ileum and duodenum are reflective of more profound intestinal damage in VAD pigs. Vitamin A supplementation had no consistent effect on diarrhea or HRV shedding. Our neonatal Gn pig model with VAD induced in mothers may represent severe effects of VAD development in the prenatal period. We speculate that earlier (shortly prior to vaccination or virulent HRV exposure) or longer vitamin A supplementation or vitamin A supplementation of both mothers and their offspring may be needed to improve intestinal health and normalize immune responses. Also, the effect of vitamin A supplementation may vary for different pathogens or even strains as was shown previously (53, 54).

According to the previously published results, RBP4 and RARα mRNA expression is affected by VAD, and they are reflective of RBP4 and RAR protein levels, respectively (55–59). RBP4 is secreted by adipocytes and carries retinol through the plasma to extrahepatic tissues (60). Although liver is considered to be the major source of RBP4, other RBP4 expression sites are suggested, including human macrophages that are closely related genetically to adipocytes and are capable of RBP4 expression (61). We showed for the first time, to our knowledge, that porcine RBP4 mRNA is expressed by splenic MNCs, and its levels are affected by VAD, vitamin A supplementation, and VirHRV challenge, similar to the latter effects on vitamin A levels. Notably, vitamin A supplementation increased RBP4 mRNA levels in VAD pigs postchallenge and decreased RBP4 mRNA levels in VAS pigs, possibly indicating vitamin A saturation in the latter pigs.

In our study, RARα mRNA expression by splenic MNCs was positively correlated with vitamin A status, supplementation, and VirHRV challenge. We observed decreased RARα mRNA levels and lower relative responses to supplemental vitamin A in VAD versus VAS pigs postchallenge. This suggests that in VAD pigs, supplemental vitamin A may not be metabolized to ATRA efficiently, or insufficient RARα expression results in aberrant cell signaling and the observed lack of consistent compensatory effects of such supplementation. Whether postchallenge increases of RBP4 and RARα mRNA expression were a compensatory response to the transient drop in serum vitamin A at PCD2 and decreased hepatic vitamin A storage postchallenge remains to be determined.

Numerous studies have demonstrated that VAD decreases resistance to and aggravates respiratory and enteric infections by depressing immune function; thus, we expected to see reduced innate immune responses (IFN-α, pDCs, and cDCs) in our study. In our experiments, however, we observed not just a decrease, but VAD-induced dysregulation of IFN-α production in response to AttHRV or VirHRV. Significantly higher amounts of circulating IFN-α in VAD piglets at PCD2 were consistent with higher VirHRV replication titers and more prominent intestinal inflammation (as reflected by higher necrotic MNC frequencies). However, at PCD10, the capacity of IFN-α production by MNCs from VAD piglets ex vivo was significantly decreased as compared with prechallenge and to MNCs from VAS pigs postchallenge. This, together with higher amounts of IFN-α in VAD piglets’ sera, suggests that imbalanced IFN-α release by immune cells may contribute to increased inflammation and does not efficiently control HRV infection. Additionally, although RVs are known to be potent IFN-α inducers, the role of IFN-α in RV clearance is uncertain and varies between homologous and heterologous infections (62–64). Moreover, type I and II IFNs were not demonstrated to be major inhibitors of RV replication in mice (63, 64). In this study, we did not observe a negative correlation between IFN-α levels and HRV fecal shedding in either VAS or VAD pigs, which could be attributable to HRV replication in the porcine (heterologous) host or reflect the failure of IFNs to inhibit RV replication (63, 64).

Consistent with IFN-α response dysregulation, we observed higher total frequencies of pDCs (gut tissues) and cDCs (all tis-

Table III. The effect of ATRA treatment on the frequencies of proliferating, necrotic, apoptotic, and CD103+ MNCs in the spleen of control VAD/VAS Gn pigs

<table>
<thead>
<tr>
<th></th>
<th>VAD (n = 4 of 9)</th>
<th>No ATRA (n = 4 of 10)</th>
<th>VAS (n = 2 of 16)</th>
<th>No ATRA (n = 2 of 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frequency of (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferation</td>
<td>1.99</td>
<td>2.02</td>
<td>1.08</td>
<td>2.25</td>
</tr>
<tr>
<td>Necrosis</td>
<td>41.2</td>
<td>31.0</td>
<td>2.10</td>
<td>2.02</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>7.85</td>
<td>8.00</td>
<td>11.85</td>
<td>9.75</td>
</tr>
<tr>
<td>CD103 expression</td>
<td>3.58</td>
<td>3.43</td>
<td>9.52*</td>
<td>6.38*</td>
</tr>
</tbody>
</table>

Numbers in boldface indicate that an ATRA effect was observed. Means in the same row with asterisks differed significantly: p ≤ 0.05 (one-way ANOVA; Minitab). Necrosis, apoptosis, and CD103 expression: VAD, ATRA, n = 9; VAD, no ATRA, n = 10; VAS, ATRA, n = 16; VAS, no ATRA, n = 17. Proliferation: VAD, ATRA, n = 4; VAD, no ATRA, n = 4; VAS, ATRA, n = 2; VAS, no ATRA, n = 2.

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sues) in VAD piglets prechallenge compared with VAS piglets regardless of vaccination or vitamin A supplementation status. This result is in agreement with previous findings by others that VAD causes a systemic expansion of myeloid cells in SENCAR mice and an increase in polymorphonuclear neutrophils, lymphoid DCs, and memory CD8^+ T cells in C57BL/6J mice (25, 65). Vitamin D deficiency was previously shown to be prevalent in patients with systemic lupus erythematosus and associated with overactive DCs and overexpression of IFN-regulated genes (66). This, together with our findings, suggests that some universal immunoregulatory mechanisms involve DC overactivation and are affected by various vitamin deficiencies.

Declines in pDC and cDC frequencies in both VAD and VAS piglets postchallenge may have been due to VirHRV infection or VirHRV-induced apoptosis. A marked reduction in splenic pDCs in mice was reported following HSV 1, VSV, and murine CMV infections, with IFN-I contributing to pDC death through induction of the expression of proapoptotic molecules and caspase activation (67). Decreased numbers of circulating pDCs have also been observed in patients infected with HIV, hepatitis B or C, and measles viruses (68–72). Our data provide the first in vivo evidence, to our knowledge, that an acute gastrointestinal infection may cause at least a transient decrease in cDC and pDC frequencies in local and systemic tissues.

Small intestinal CD103⁺ DCs are imprinted with an ability to metabolize vitamin A (retinol) and generate gut-tropic T cells (expressing CCR-9 and α4β7). It was recently shown that RA regulates generation of gut-tropic migratory DC precursors that give rise to CD103⁺ intestinal pDCs and cDCs (73). Lamina propria DCs expressing CD103 were shown to convert naive CD4⁺ T cells into Foxp3⁺ Treg cells (74). Interestingly, we observed slightly lower CD103⁺ pDC and cDC frequencies in ileum, duodenum, and spleen of VAD piglets prechallenge, and they were decreased significantly postchallenge as compared with VAS piglets. The loss of CD103⁺ DCs could be due to the experimental induced colitis was described in mice (75), suggesting that VAD-induced MNC necrosis, possibly reflecting intestinal inflammation in our Gn pig model, could contribute to or be the result of the significantly decreased CD103⁺ DC frequencies. CD103⁺ DCs express the tight junction proteins (zonula, occluding, and claudin I) preserving the integrity of the epithelial barrier and preventing the over inflammatory reactions to intestinal pathogens (76, 77). Additionally, α6β1 (CD103) integrin was demonstrated to influence cellular intraepithelial morphogenesis and motility (78), which are critical for the proper communication among pathogens, DCs, and T and B lymphocytes. Therefore, the VAD-induced loss of CD103 expression by DCs that we observed in our experiments could have resulted in aberrant innate immune responses against VirHRV and compromised resolution of the infection. Ultimately, disruption of signaling between DCs and T/B lymphocytes may be due to lack of proper Ag presentation to the latter cells, resulting in lowered HRV specific IgA Ab titers that we observed (K.S. Chattha, A.N. Vlasova, C.S. Siegismund, and L.J. Saif, unpublished observations) and diminished NK cell function (L.J. Saif and Gourapura, unpublished observations) in VAD piglets.

Interestingly, in this study, frequencies of apoptotic MNCs were slightly higher in all treatment groups of VAS piglets prechallenge (G.J. Renukaradhya, A.N. Vlasova, K.S. Chattha, S. Kandasamy, C.S. Siegismund, and L.J. Saif, data not shown), confirming that adequate vitamin A levels improve immunoregulatory function and programmed cell death, a mechanism involved in regulation of autoimmunity and T cell tolerance (79). Additionally, we observed an increase in apoptotic MNC frequencies postchallenge in VAD and VAS pigs (data not shown), supporting previous findings regarding RV-induced apoptosis in various cell lines of human, simian, and rat origin (80–84).

Mice with VAD (65) and mice treated with an RAR antagonist showed accumulation of immature myeloid cells similar to the immature myeloid-suppressive cells found in cancer patients (85). Additionally, a recent study demonstrated that RA suppresses immune cell proliferation and induces tolerogenic DC development, which indicates its therapeutic potential for the treatment of numerous inflammatory or autoimmune conditions (86). In our present study, despite the higher total frequencies of cDCs, we observed significantly lower numbers of specialized CD103⁺ cDCs in VAD piglets regardless of vaccination/challenge status. This confirms previous findings on VAD effects on the immune system including development of autoimmune conditions and tumorogenesis. Interestingly, we also observed significantly higher frequencies of apoptotic MNCs in VAS piglets in all groups, indicating that a mechanism of programmed cell death is affected by VAD, and therefore, untreated VAD may result in an overactive immune system and subsequently contribute to the development of autoimmune conditions.

Overexpansion of myeloid cells due to VAD in mice was reported previously and also coincided with increased spleen size (65). Also, it was suggested that inflammatory responses and accumulation of cell debris may have contributed to increased spleen size (splenomegaly) observed in VAD mice and lambs (65, 87, 88). Intriguingly, our observations of RBP4/RARα mRNA expression in spleen, increased relative spleen weight, and necrotic MNC frequencies in VAD pigs indicate that spleen, a systemic lymphoid organ, may play an important role in vitamin A metabolism and retinoid signaling and is affected by VAD, thereby altering immune responses at the systemic level. Additionally, we observed a decreased ability of splenic MNCs from VAD pigs to respond to vitamin A supplementation in vivo (as reflected by RBP4/RARα mRNA levels) or to ATRA stimulation ex vivo, indicating that prenatal VAD has a profound effect on intrinsic properties and life cycle of the immune cells.

In conclusion, our results indicate that under steady state, VAD in Gn pigs results in innate immune system overactivity and possibly disrupted cell signaling. This suggests that one of the major vitamin A functions is to maintain the immunoregulatory profile in the gut (mucosal) and at systemic immune sites. The VAD-induced loss of this immunoregulatory function may lead to imbalanced innate immune responses and subsequent development of autoimmune conditions, as reported by others. CD103⁺ DCs appeared to be the only subset examined that was significantly increased in VAS piglets, suggesting their critical role in efficient immune responses to HRV. Finally, a 3× high-dose vitamin A supplementation concurrent with the AttHRV vaccine did not compensate for the VAD effects or act as an adjuvant for AttHRV vaccine, possibly indicating a need for maternal, longer, or higher levels of postnatal vitamin A supplementation.

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