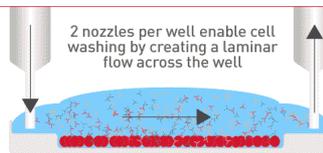


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# Vitamin A Deficiency Impairs Vaccine-Elicited Gastrointestinal Immunity

David R. Kaufman,\* Jaime De Calisto,<sup>†</sup> Nathaniel L. Simmons,\* Ashley N. Cruz,\*  
Eduardo J. Villablanca,<sup>†</sup> J. Rodrigo Mora,<sup>†</sup> and Dan H. Barouch\*<sup>‡</sup>

Vitamin A deficiency is highly prevalent in much of the developing world, where vaccination programs are of paramount importance to public health. However, the impact of vitamin A deficiency on the immunogenicity and protective efficacy of vaccines has not been defined previously. In this article, we show that the vitamin A metabolite retinoic acid is critical for trafficking of vaccine-elicited T lymphocytes to the gastrointestinal mucosa and for vaccine protective efficacy in mice. Moderate vitamin A deficiency abrogated Ag-specific T lymphocyte trafficking to the gastrointestinal tract, gastrointestinal cellular immune responses, and protection against a mucosal challenge following immunization with a recombinant adenovirus vaccine vector. Oral vitamin A supplementation as well as retinoic acid administration fully restored the mucosal immune responses and vaccine protective efficacy. These data suggest that oral vitamin A supplementation may be important for optimizing the success of vaccines against HIV-1 and other mucosal pathogens in the developing world, highlighting a critical relationship between host nutritional status and vaccine efficacy. *The Journal of Immunology*, 2011, 187: 1877–1883.

The World Health Organization estimates that vitamin A deficiency affects >20% of preschool-age children and >10% of pregnant women in >50 countries, including much of Africa and southern and central Asia (1). These are regions where the burden of infectious diseases is high and where vaccination programs may bring enormous public-health benefits. However, the impact of vitamin A deficiency on vaccine-elicited cellular immunity has not been defined previously. Although some clinical and animal studies have shown reduced vaccine-elicited peripheral Ab production and seroconversion in the setting of vitamin A deficiency (2–5), the majority of studies have failed to demonstrate an impact of either vitamin A deficiency or supplementation on vaccine-elicited Ab responses (6). Comparable studies of vaccine-elicited cellular immunity and immunity at mucosal surfaces have not been performed previously.

We explored whether vitamin A may be specifically required for the induction of vaccine-elicited cellular immunity in the gastrointestinal tract, as the vitamin A metabolite all-*trans* retinoic acid (RA) has been shown to be critical for programming B and T lymphocytes to upregulate mucosal homing molecules and traffic to the gastrointestinal mucosa (7, 8). Although vitamin A deficiency has been shown to adversely impact the steady-state

accumulation of T and B lymphocytes in the gastrointestinal mucosa (7), the impact of vitamin A deficiency on Ag-specific mucosal T cell responses has not been defined previously.

Gastrointestinal cellular immunity may be critical for vaccine-elicited protection against HIV-1 and other pathogens. HIV-1 preferentially targets CD4<sup>+</sup> T lymphocytes within the gastrointestinal mucosa (9–12), resulting in chronic intestinal inflammation, breakdown of mucosal epithelial integrity, and translocation of gut microflora into the intestinal lamina propria (13). These processes drive systemic inflammation that contributes significantly to HIV-1 pathogenesis (13). Recombinant adenovirus (rAd)-based vectors are currently being investigated as HIV-1 vaccine candidates and have been shown to induce potent gastrointestinal cellular immunity in mice and rhesus monkeys (14) and to abrogate the mucosal destruction of CD4<sup>+</sup> T lymphocytes following SIV infection (15). In this study, we demonstrate that vitamin A deficiency selectively impairs vaccine-elicited gastrointestinal immunity by interfering with RA-dependent upregulation of mucosal homing integrins, blocking the trafficking of Ag-specific T lymphocytes to the gastrointestinal tract, and abrogating protection from gastrointestinal infectious challenge.

## Materials and Methods

### Animals, vectors, treatments, and immunizations

rAd5, rAd26, and rAd5HVR48 vectors and pCMV plasmids expressing SIVmac239 Gag and Ova have been described previously (16–19). All immunizations were performed i.m. in 100  $\mu$ l PBS divided equally between both quadriceps. rAd vectors were administered at a dose of 10<sup>9</sup> viral particles (VP). C57BL/6 (B6), B6.SJL, B6.PL,  $\beta$  integrin<sup>-/-</sup>, and Ova TCR-I and II (OT-I and OT-II) mice were obtained from The Jackson Laboratory. Vitamin A-deficient mice were generated at The Jackson Laboratory. Pregnant B6 and B6.SJL mice were maintained on a vitamin A-sufficient (4000 IU/kg; Harlan TD96007) diet through day 10 of gestation and then switched to a vitamin A-deficient diet (Harlan TD88407) through weaning. Pups were maintained on vitamin A-replete (20,000 IU/kg; Harlan TD88406), vitamin A-deficient (TD88407), or low vitamin A (400 IU/kg; Harlan TD09775) diets. Serum retinol levels were determined in serum by HPLC at AniLytics Experiments were initiated when animals were 8 wk of age. FTY720 (Cayman Chemical) was administered daily at 1 mg/kg in DMSO by i.p. injection beginning at 48 h prior to immunization. All-*trans* RA (Sigma-Aldrich) was administered at 250  $\mu$ g/mouse

\*Division of Vaccine Research, Beth Israel Deaconess Medical Center, Boston, MA 02215; <sup>†</sup>Gastrointestinal Unit, Massachusetts General Hospital, Boston, MA 02114; and <sup>‡</sup>Ragon Institute of MGH, MIT and Harvard University, Boston, MA 02114

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Address correspondence and reprint requests to Dr. Dan H. Barouch, Division of Vaccine Research, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215. E-mail address: dbarouch@bidmc.harvard.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; LN, lymph node; OT-I, Ova TCR-I; OT-II, Ova TCR-II; RA, retinoic acid; rAd, recombinant adenovirus; rAd5-Ova, recombinant adenovirus serotype 5 vector expressing Ova; RALDH, retinaldehyde dehydrogenase; RP, retinyl palmitate; VP, viral particle; WT, wild-type.

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in DMSO by i.p. injection on days  $-1$  and  $+1$  relative to immunization. Retinyl palmitate (RP) (Sigma-Aldrich) was administered at 600 IU/mouse in vegetable oil by gavage. Mice were immunized i.m. in the quadriceps with  $10^9$  VP of each rAd vector. All animals used in this study were maintained in accordance with institutional guidelines, and all studies were approved by the institutional animal care and use committee of Beth Israel Deaconess Medical Center.

#### Lymphocyte adoptive transfer studies

Lymphocytes were isolated from the spleens and cervical, axillary, and inguinal lymph nodes (LN) of naive OT-I or OT-II mice.  $CD4^+$  or  $CD8^+$  T lymphocytes were purified from pooled lymphocyte populations by negative selection using immunomagnetic beads ( $CD4^+$  and  $CD8^+$  T cell isolation kits; Miltenyi Biotec). OT-I  $CD8^+$  T lymphocytes were  $>95\%$  pure by OT-I tetramer-binding assays.  $CD4^+$  T lymphocytes were  $>90\%$  pure by immunophenotyping. Purified OT-I and OT-II lymphocytes were labeled with CFSE (Invitrogen), and  $2.5 \times 10^6$  cells were transferred to recipient  $CD45$ -congenic (B6.SJL) mice by tail vein injection. Mice were immunized i.m. at 48 h following adoptive transfer with  $10^9$  VP rAd-Ova. For competitive adoptive transfer studies, B6.PL ( $Thy1.1^+$ ,  $CD45.1^-$ ) and  $\beta_7$  integrin $^{-/-}$  ( $Thy1.1^-$ ,  $CD45.1^-$ ) mice were primed i.m. at week 0 with  $10^9$  VP rAd26-Gag and boosted at week 6 with  $10^9$  VP rAd5HVR48-Gag. At day 10 following the boost immunization,  $CD8^+$  T lymphocytes were purified from splenocytes by negative selection using immunomagnetic beads as above. The percentage of Gag-specific  $CD8^+$  T lymphocytes was determined in each population using D<sup>9</sup>/AL11 tetramer-binding assays. Gag-specific  $CD8^+$  T lymphocytes from wild-type (WT) and  $\beta_7$  integrin $^{-/-}$  mice were pooled at a 1:1 ratio, and  $2.5 \times 10^6$  total Gag-specific  $CD8^+$  T lymphocytes were transferred to each naive B6.SJL ( $Thy1.1^-$ ,  $CD45.1^+$ ) recipient by tail vein injection.

#### Multiparameter flow cytometry and tetramer-binding assays

H-2D<sup>b</sup> tetramers labeled with PE and folded around the immunodominant SIV Gag epitope AL11 (AAVKNWMTQTLL) were used to stain lymphocytes as described previously (20). mAbs used for flow cytometry were from BD

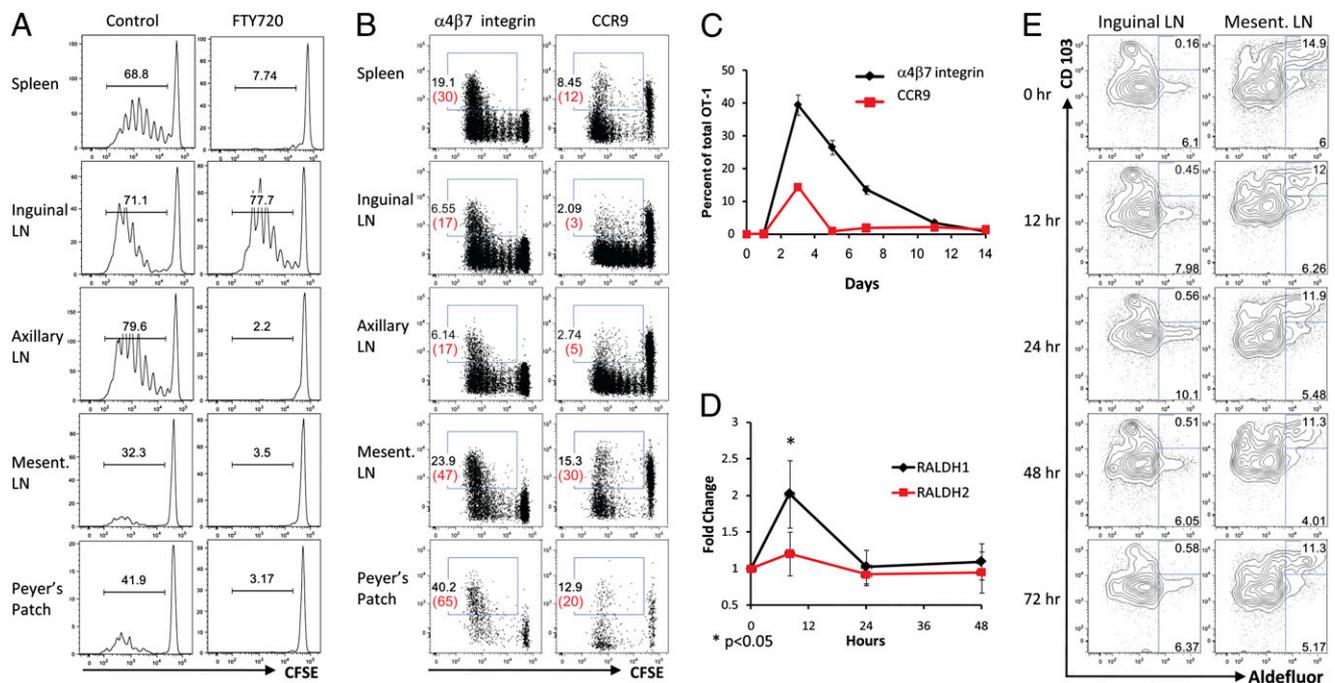
Biosciences ( $CD8\alpha$ -PerCP-Cy5.5 [53-6.7],  $CD3$ -allophycocyanin [145-2C11],  $CD3$ -PE [145-2C11], [D11c-PerCP-Cy5.5 [HL.3],  $CD19$ -PE [1D3], NK1.1-PE [PK136], and  $CD11b$ -PE [M1/70]) and eBioscience ( $CD45.1$ -PE-Cy7 [A20],  $CD45.2$  allophycocyanin [104],  $Thy1.2$ -allophycocyanin [53-2.1]  $\alpha_4\beta_7$  integrin-PE [DATK32],  $CCR9$ -PE [CW1.2], and  $CD103$ -allophycocyanin [2e7]). LIVE/DEAD Fixable Violet (Invitrogen) and Aldefluor (StemCell Technologies) were used according to the manufacturer's instructions. Samples were analyzed using an LSRII flow cytometer (BD Biosciences) and FloJo software (Tree Star).

#### Mucosal lymphocyte isolation

Mucosal lymphocyte populations were isolated as described previously (14). Bowel specimens were cleaned, minced, and incubated with HBSS supplemented with 0.2 mM EDTA and 10% FBS at 37°C for 30 min with shaking to release the intraepithelial lymphocytes. Cells were resuspended in 40% Percoll (Sigma-Aldrich), layered over 67% Percoll and centrifuged at  $1000 \times g$  for 20 min. Lymphocytes were isolated from the interface. Bowel specimens were then digested with two serial 30-min incubations at 37°C in RPMI 1640 medium containing 5% FBS supplemented with type II collagenase (Sigma-Aldrich) at 300 U/ml with vigorous shaking to release the lamina propria lymphocytes, which were purified on a Percoll gradient as described above.

#### Dendritic cell isolation, Aldefluor assays, and real-time PCR

Total RNA was extracted from inguinal LN lysates using the RNeasy kit (Qiagen). Total RNA (2  $\mu$ g) was used to synthesize cDNA using iSCRIPT cDNA Synthesis kit (Bio-Rad). Gene expression was quantified using TaqMan PCR master mix (Applied Biosystems) with primer probes for *Aldh1a1* and *Aldh1a2* (Mm00657317\_m1 and Mm00501306\_m1; Applied Biosystems). Relative expression was calculated using the  $\Delta\Delta CT$  method, and samples were normalized to  $\beta$ -actin. For Aldefluor assays, LN were digested with Liberase TL (0.15 mg/ml; Roche) and DNase I (325 U/ml; Sigma-Aldrich) in HBSS for 45 min at 37°C. Remaining tissue was disrupted mechanically. Cells were incubated with anti- $CD11c$  microbeads (Miltenyi Biotec) for 20 min and purified by positive magnetic selection. A purity of



**FIGURE 1.** Vaccine-elicited  $CD8^+$  T lymphocyte priming and mucosal homing marker upregulation. **A**, Localization of vaccine-elicited  $CD8^+$  T lymphocyte priming. A total of  $2.5 \times 10^6$  CFSE-labeled OT-I  $CD8^+$  T lymphocytes were adoptively transferred to naive,  $CD45$ -congenic recipients (B6.SJL;  $n = 8$ /group) that were untreated or treated i.p. from 48 h prior to immunization with FTY720. Mice were immunized i.m. in the quadriceps with  $10^9$  VP rAd5-Ova, and proliferation was assessed by CFSE dilution on day 3 following immunization. **B**, Vaccine-elicited mucosal homing marker upregulation. Following adoptive transfer and immunization as in **A**,  $CCR9$  and  $\alpha_4\beta_7$  integrin upregulation was evaluated on proliferating  $CD8^+$  T lymphocytes at day 3 following immunization. Numbers in black and red represent percentages of total and proliferating OT-I cells, respectively. **C**, Kinetics of  $\alpha_4\beta_7$  integrin upregulation in blood following adoptive transfer and immunization as in **B**. **D**, Induction of *Aldh1a1* and *Aldh1a2* mRNA, encoding RALDH1 and RALDH2, following rAd immunization. C57BL/6 mice ( $n = 4$ /group) were immunized IM with  $10^9$  VP rAd5-Gag. RALDH expression was determined in inguinal LN by real-time PCR. **E**, DC aldehyde dehydrogenase activity following rAd immunization. B6 mice ( $n = 4$ /group) were immunized as in **D**. DC were isolated from inguinal and mesenteric LN, and aldehyde dehydrogenase activity in DC subsets was determined by Aldefluor assay. Error bars are  $\pm$  SE. \* $p < 0.05$ .

98% CD11c<sup>+</sup> cells was routinely attained. Cell suspensions ( $1 \times 10^6$  cells/ml) were incubated for 45 min at 37°C in Aldefluor assay buffer containing activated Aldefluor (StemCell Technologies). Cells were stained with specific mAbs and analyzed on an LSR-II flow cytometer (BD Biosciences). Background Aldefluor staining was <0.5% in the presence of the retinaldehyde dehydrogenase (RALDH) inhibitor diethylaminobenzaldehyde.

### Oral *Listeria* challenge

*Listeria monocytogenes*-expressing Ova was a gift from F. Frankel (University of Pennsylvania). Mice were fasted overnight and challenged orally with  $10^{10}$  CFU Lm-Ova in PBS by gavage. At day 3 following oral challenge, livers were harvested and homogenized in 1.5 ml PBS/1% saponin. Serial dilutions were plated on brain heart infusion agar plates, and quantitative colony counts were performed following a 36-h incubation at 30°C. The identity of bacterial colonies was confirmed by PCR for the Ova insert.

### Statistical analyses

Statistical analyses were performed with GraphPad Prism version 4.01 (GraphPad Software). Immune responses among groups of mice are presented as means with SEs. Comparisons of mean immune responses were performed using two-sided *t* tests or Fisher's exact test. In all cases,  $p < 0.05$  was considered significant.

## Results

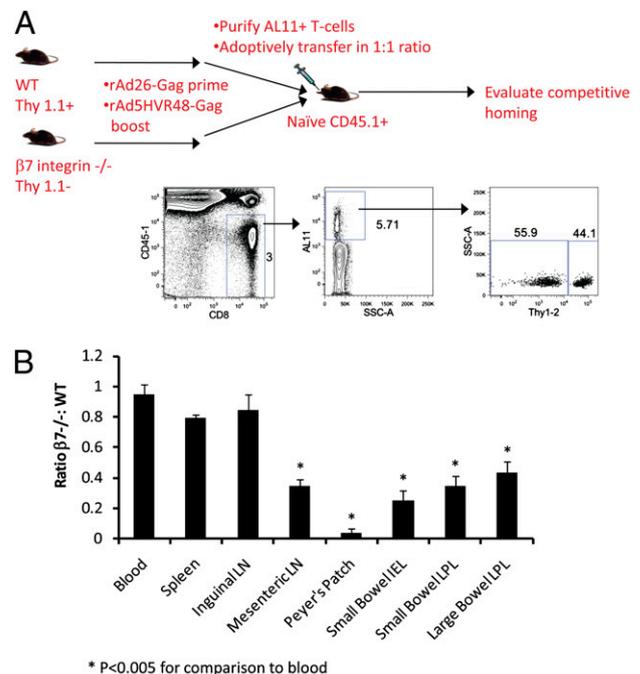
We first evaluated the mechanism underlying the capacity of i.m. immunization of rAd vectors to induce mucosal immunity in mice. Naive peripheral CD8<sup>+</sup> T lymphocytes from OT-I mice, which express a transgenic TCR specific for a MHC class I-restricted epitope from chicken Ova (21), were labeled with CFSE and i.v. transferred to naive CD45-congenic recipients (B6.SJL). Recipient mice were immunized i.m. in the quadriceps with  $10^9$  VP of a rAd serotype 5 vector expressing Ova (rAd5-Ova). Ova-specific T cell proliferation was evaluated at 60 h following immunization, which represented the earliest time point at which vaccine-elicited T-cell responses could be detected. Vigorous Ag-specific CD8<sup>+</sup> T lymphocyte proliferation, as assessed by CFSE dilution, was observed at multiple systemic and mucosal lymphoid inductive sites in immunized animals (Fig. 1A). However, in mice treated with FTY720, a sphingosine-1 phosphate receptor agonist that impedes the egress of T lymphocytes from lymph nodes (LN) (22), vaccine-elicited CD8<sup>+</sup> T lymphocyte proliferation was restricted to the inguinal LN draining the inoculation site (Fig. 1A). These data demonstrate that vaccine-elicited CD8<sup>+</sup> T lymphocytes were primed locally in peripheral LN but then rapidly acquired the capacity to traffic to distant systemic and mucosal lymphoid inductive sites.

We next assessed the induction of  $\alpha_4\beta_7$  integrin and CCR9, cell surface molecules critical for lymphocyte homing to the small intestine (23–25), on vaccine-elicited CD8<sup>+</sup> T lymphocytes. At 60 h following immunization,  $\alpha_4\beta_7$  integrin was upregulated on 30–65% of responding cells in the spleen, mesenteric LN, and Peyer's patches (Fig. 1B). CCR9 was initially downregulated during the first three to four rounds of cell division but was subsequently upregulated on 20–30% of responding cells that had trafficked to mesenteric LN and Peyer's patches (Fig. 1B). Peripheral  $\alpha_4\beta_7$  integrin and CCR9 upregulation peaked on day 3 following immunization and then rapidly declined (Fig. 1C), consistent with previous observations (26). Thus, peripheral vaccine-elicited CD8<sup>+</sup> T lymphocytes exhibited a marked induction of gastrointestinal homing markers within the first few days following immunization.

RA regulates the expression of  $\alpha_4\beta_7$  integrin and CCR9 on T and B lymphocytes (7, 8). Although a subset of mucosal dendritic cells (DC) constitutively produces RA (7, 27, 28), peripheral DC can produce RA inducibly when activated through TLR (29).

Therefore, we evaluated RA production at the anatomic sites of vaccine-elicited CD8<sup>+</sup> T lymphocyte priming and early trafficking. In the inguinal LN draining the inoculation site, i.m. immunization with  $10^9$  VP rAd5 induced a transient 3-fold induction of the retinaldehyde dehydrogenase isoform RALDH1 and a marginal induction of RALDH2, as measured by real-time PCR at 8 h following immunization (Fig. 1D;  $p < 0.05$  using a two-tailed *t* test for RALDH1), consistent with previous in vitro observations (26). This was accompanied by a comparable and transient 1.5-fold increase in aldehyde dehydrogenase activity in inguinal LN CD11c<sup>+</sup>CD103<sup>-</sup> DC at 24 h following immunization (Fig. 1E). In contrast, mesenteric LN DC exhibited a high basal level of aldehyde dehydrogenase activity that was restricted primarily to the CD11c<sup>+</sup>CD103<sup>+</sup> subset and was not affected by rAd immunization, as expected (Fig. 1E). These data suggest that vaccine-elicited CD8<sup>+</sup> T lymphocytes encountered RA first during local priming in inguinal LN and subsequently following trafficking to mucosal lymphoid inductive sites.

We determined the functional significance of vaccine-induced  $\alpha_4\beta_7$  integrin upregulation by comparing the anatomic trafficking patterns of vaccine-elicited CD8<sup>+</sup> T lymphocytes from WT and  $\beta_7$  integrin-deficient mice. Mice were immunized with a heterologous rAd26/rAd5HVR48 prime-boost regimen using vectors expressing SIV Gag (14) to generate high frequencies of circulating Gag-specific CD8<sup>+</sup> T lymphocytes (15–20% of total CD8<sup>+</sup> T lymphocytes; data not shown). Peripheral WT and  $\beta_7$  integrin-deficient Gag-specific CD8<sup>+</sup> T lymphocytes were then purified and transferred i.v. in a 1:1 ratio to naive recipients in competitive



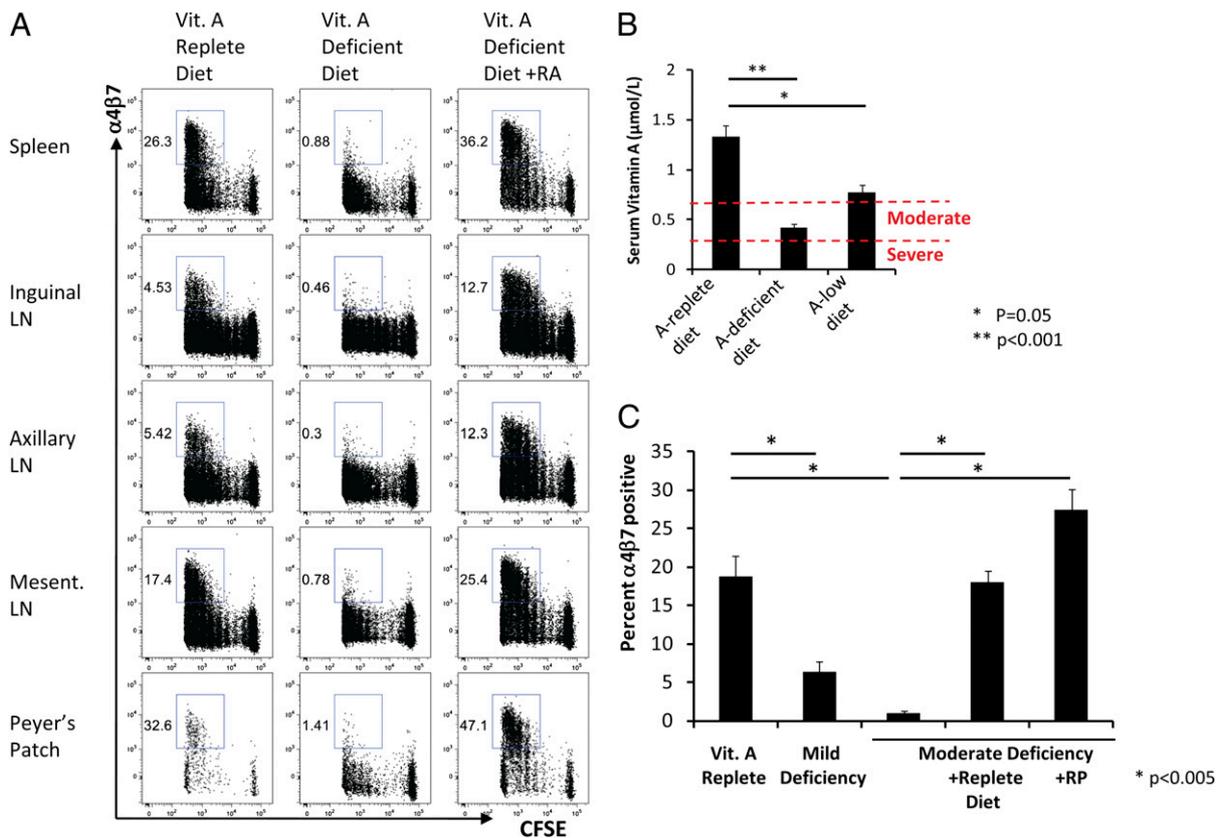
**FIGURE 2.** Vaccine-elicited CD8<sup>+</sup> T lymphocyte trafficking to the gastrointestinal mucosa is  $\beta_7$  integrin dependent. **A**, Experimental design for competitive adoptive transfers. Naive B6.PL (Thy 1.1<sup>+</sup>CD45.2<sup>+</sup>) and  $\beta_7$  integrin<sup>-/-</sup> (Thy 1.2<sup>+</sup>CD45.2<sup>+</sup>) mice were primed at week 0 with  $10^9$  VP rAd26-Gag and boosted at week 6 with  $10^9$  VP rAd5HVR48-Gag. At day 10 following the boost immunization, equal numbers of peripheral WT and  $\beta_7$  integrin<sup>-/-</sup> Gag-specific CD8<sup>+</sup> T lymphocytes were competitively adoptively transferred to naive B6.SJL (Thy 1.2<sup>+</sup>CD45.1<sup>+</sup>) recipients ( $n = 4$ /group). Trafficking of WT and  $\beta_7$  integrin<sup>-/-</sup> CD8<sup>+</sup> T lymphocytes was evaluated at day 10 following transfer. **B**, The ratio of WT to  $\beta_7$  integrin<sup>-/-</sup> Gag-specific CD8<sup>+</sup> T lymphocytes was determined at each anatomic site. Error bars are  $\pm$ SE. \* $p < 0.005$ .

adoptive transfer studies. WT and  $\beta_7$  integrin-deficient CD8<sup>+</sup> T lymphocytes expressed distinct congenic markers, allowing their trafficking efficiency to be compared at multiple anatomic sites (Fig. 2A). Gag-specific CD8<sup>+</sup> T lymphocytes from WT and  $\beta_7$  integrin-deficient mice trafficked with comparable efficiency to the spleen and inguinal LN, as expected. However,  $\beta_7$  integrin-deficient lymphocytes were selectively impaired in their capacity to traffic to mesenteric LN, Peyer's patches, and mucosa of the small and large intestine (Fig. 2B;  $p < 0.005$  for each comparison using two-tailed  $t$  tests), showing the importance of  $\beta_7$  integrin-dependent mechanisms for vaccine-elicited CD8<sup>+</sup> T lymphocyte trafficking to the gastrointestinal tract.

We next assessed the impact of vitamin A deficiency on mucosal homing marker upregulation on vaccine-elicited CD8<sup>+</sup> T lymphocytes. Naive CFSE-labeled OT-I lymphocytes from healthy mice were adoptively transferred to recipient mice that were bred from gestation on either a normal diet or a vitamin A-deficient diet. Following i.m. immunization with  $10^9$  VP rAd5-Ova, total CD8<sup>+</sup> T lymphocyte proliferation was comparable in both groups of mice at 60 h following immunization (Fig. 3A). However,  $\alpha_4\beta_7$  integrin upregulation on proliferating CD8<sup>+</sup> T lymphocytes was markedly abrogated in animals that received the vitamin A-deficient diet (Fig. 3A).  $\alpha_4\beta_7$  integrin upregulation on proliferating CD4<sup>+</sup> T lymphocytes was also abrogated in mice fed the vitamin A-deficient diet (Supplemental Fig. 1). Intraperitoneal administration of RA to animals fed the vitamin A-deficient diet completely re-

stored  $\alpha_4\beta_7$  integrin upregulation (Fig. 3A), indicating that RA can fully compensate for the defective induction of mucosal homing markers induced by vitamin A deficiency.

Mice bred on a vitamin A-deficient diet had a mean serum retinol level of 0.42  $\mu\text{mol/l}$ , whereas mice bred on a diet containing 400 IU/kg vitamin A had a mean serum retinol level of 0.77  $\mu\text{mol/l}$  (Fig. 3B). On the basis of World Health Organization-defined levels for severe ( $<0.35 \mu\text{mol/l}$ ) or moderate ( $<0.7 \mu\text{mol/l}$ ) vitamin A deficiency in humans (1), these data suggest that mice that received the vitamin A-deficient diet and the low vitamin A diet exhibited physiologically relevant moderate and mild levels of vitamin A deficiency, respectively. In contrast, mice bred on the control diet had a mean serum retinol level of 1.33  $\mu\text{mol/l}$ , within the normal range for humans (30).  $\alpha_4\beta_7$  integrin upregulation on vaccine-elicited CD8<sup>+</sup> T lymphocytes was dramatically abrogated in moderately vitamin A-deficient mice but was also partially decreased in mice with only mild levels of vitamin A deficiency (Fig. 3C;  $p < 0.05$  for each comparison with control animals). Vaccine-elicited  $\alpha_4\beta_7$  integrin induction was completely restored in moderately vitamin A-deficient mice that received either a normal vitamin A-containing diet for 1 wk prior to immunization or two oral doses of RP, a vitamin A formulation commonly used for dietary supplementation in humans (Fig. 3C). These data demonstrate that clinically relevant levels of vitamin A deficiency and typical dietary supplementation regimens markedly impacted vaccine-elicited mucosal homing marker upregulation.



**FIGURE 3.** Vaccine-induced mucosal homing marker upregulation is abrogated by vitamin A deficiency but restored by RA or oral vitamin A. **A**, Mucosal homing marker upregulation in vitamin A-deficient hosts. A total of  $2.5 \times 10^6$  CFSE-labeled OT-I CD8<sup>+</sup> T lymphocytes were adoptively transferred to vitamin A-sufficient or vitamin A-deficient, CD45-congenic recipients (B6.SJL;  $n = 4/\text{group}$ ). RA supplementation was given as indicated on days  $-1$  and  $+1$  relative to immunization. Recipient mice were immunized i.m. with  $10^9$  VP rAd5-Ova.  $\alpha_4\beta_7$  integrin upregulation was evaluated at day 3 following immunization. **B**, Serum retinol levels were determined by HPLC in mice ( $n = 8/\text{group}$ ) bred on vitamin A-sufficient, vitamin A-deficient, or 400 IU/kg vitamin A diets. \* $p = 0.05$ , \*\* $p < 0.001$ . **C**, Adoptive transfer and immunization were performed as in **A** using mice ( $n = 4/\text{group}$ ) bred on the indicated diets. Oral vitamin A supplementation was provided either by gavage with 600 IU RP on days  $-1$  and  $-3$  relative to immunization or with a vitamin A-sufficient diet for 1 wk prior to immunization. Error bars are  $\pm$ SE. \* $p < 0.005$ .

We next evaluated the impact of vitamin A deficiency on vaccine-elicited, Ag-specific cellular immune responses. Control mice and mice with moderate vitamin A deficiency were immunized i.m. with  $10^9$  VP of a rAd vector expressing SIV Gag, and Gag-specific CD8<sup>+</sup> T lymphocyte responses were evaluated by D<sup>b</sup>/AL11 tetramer-binding assays at day 14 following immunization (20). Comparable Gag-specific CD8<sup>+</sup> T lymphocyte responses were observed in the peripheral blood of vitamin A-replete and moderately vitamin A-deficient animals (Fig. 4A, Supplemental Fig. 2). In contrast, Gag-specific CD8<sup>+</sup> T lymphocyte responses were dramatically abrogated in the small intestine and were moderately reduced in the large intestine of the mice with moderate vitamin A deficiency (Fig. 4A, Supplemental Fig. 2;  $p < 0.005$  in by two-tailed *t* tests). The less dramatic effect of vitamin A deficiency in the large intestine likely reflects compensatory  $\alpha_4\beta_7$  integrin- and CCR9-independent mechanisms of T lymphocyte trafficking (31, 32). Importantly, administration of either i.p. RA or oral RP prior to immunization fully restored Gag-specific CD8<sup>+</sup> T lymphocyte responses in the gastrointestinal mucosa (Fig. 4A).

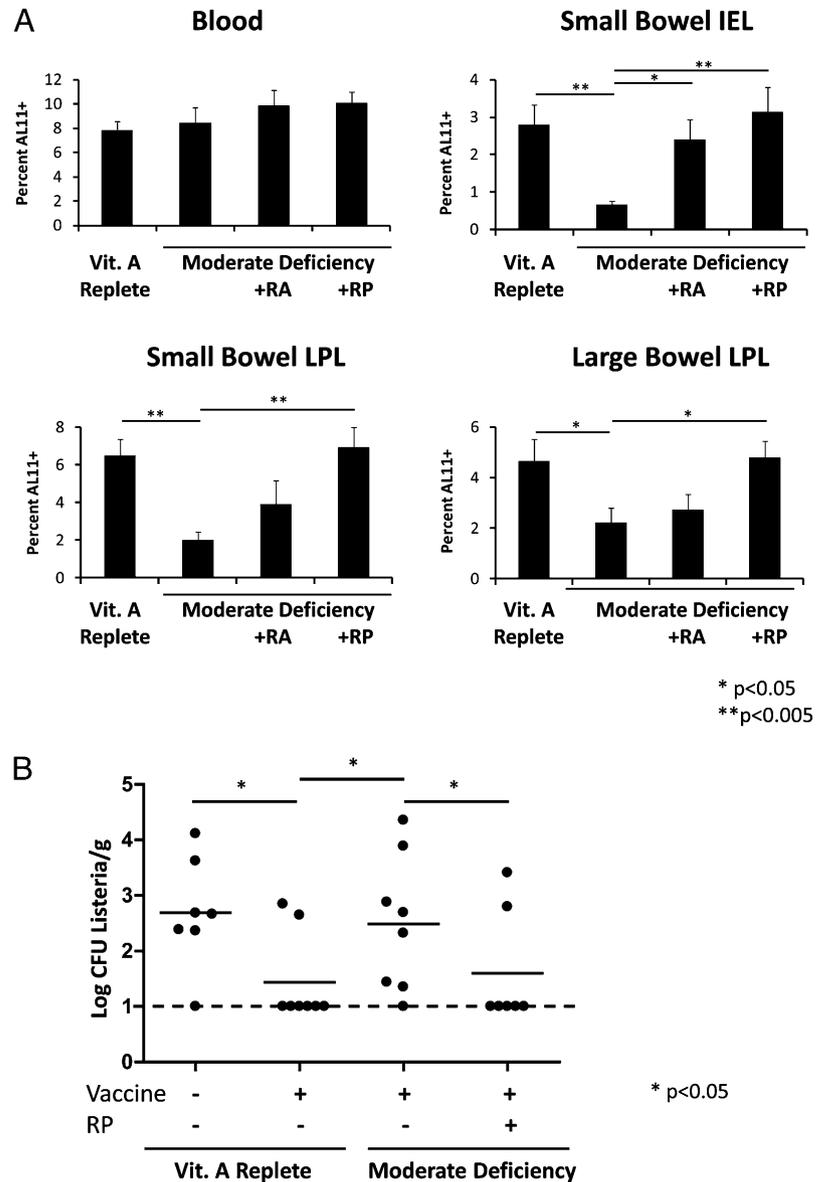
To investigate the impact of vitamin A deficiency on vaccine protective efficacy against a mucosal challenge, we infected control

and moderately vitamin A-deficient mice orally with the gastrointestinal pathogen *L. monocytogenes* expressing Ova (rLm-Ova) (33). Protective efficacy was assessed by determining bacterial loads in the liver on day 3 following challenge to quantitate bacterial translocation from the intestine (34). To evaluate vaccine-induced cellular immune protection, mice were immunized i.m. with  $10^9$  VP of a rAd vector that expressed the immunodominant CD8<sup>+</sup> T cell epitope from Ova (rAd5-SIINFEKL). Immunization with rAd5-SIINFEKL afforded significant protective efficacy against oral Lm-Ova challenge, as evidenced by a substantial reduction of bacterial loads following challenge of vaccinated mice as compared with unvaccinated mice (Fig. 4B;  $p < 0.05$  by Fisher's exact test). Importantly, this vaccine-elicited protection was dramatically abrogated in vaccinated mice with moderate vitamin A deficiency (Fig. 4B;  $p < 0.05$ ) but could be fully restored by oral RP administration immediately prior to vaccination (Fig. 4B;  $p < 0.05$ ).

### Discussion

Vitamin A deficiency remains an important public-health problem in the developing world. Recent meta-analyses suggest that vitamin A supplementation can significantly reduce all-cause and diarrheal

**FIGURE 4.** Vitamin A deficiency abrogates vaccine-elicited protection from gastrointestinal mucosal challenge. **A**, Control and vitamin A-deficient B6 mice ( $n = 8$ /group) were immunized i.m. with  $10^9$  VP rAd5HVR48-Gag. At week 2 following immunization, Gag-specific CD8<sup>+</sup> T lymphocyte responses were assessed in the blood and small bowel mucosa by D<sup>b</sup>/AL11 tetramer-binding assays. Mice were supplemented as indicated with RA at days -1 and +1 relative to immunization or orally with RP at days -3 and -1 relative to immunization. Error bars are  $\pm$ SE. **B**, Vitamin A-replete or -deficient CB6 mice were immunized i.m. with  $10^9$  VP rAd5-luc-SIINFEKL. Oral vitamin A supplementation was provided by gavage with 600 IU RP on days -1 and -3 relative to immunization. At day 21 following immunization, mice were challenged orally with Lm-Ova by gavage. At day 3 following challenge, liver homogenates were prepared, and quantitative colony counts were performed by plating serial dilutions of the homogenates. \* $p < 0.05$ , \*\* $p < 0.005$ . IEL, intraepithelial lymphocyte; LPL, lamina propria lymphocyte.



disease-specific mortality in infants and children in this setting (35, 36), whereas a benefit of vitamin A supplementation in preventing mortality from other causes such as measles, respiratory diseases, or HIV is not evident (35–37). Moreover, vitamin A supplementation has specifically been shown to reduce the prevalence and clinical severity of gastrointestinal norovirus infections while prolonging viral shedding (38). It is unknown whether specific immunologic mechanisms underlie these effects. In particular, no studies have yet explored whether specific defects in RA-mediated T and B lymphocyte trafficking to mucosal surfaces underlie the clinical outcomes observed in these studies.

Clinical studies that have evaluated the impact of vitamin A deficiency or supplementation on vaccine-elicited immunity have primarily focused on seroconversion or peripheral immune responses as immunologic end points and have not evaluated responses at mucosal surfaces (6). These studies have typically been performed using vaccines that have a high rate of peripheral seroconversion such as tetanus, measles, and polio (4, 26, 34, 39–44). A preponderance of these studies has failed to demonstrate a significant effect of vitamin A deficiency or supplementation on peripheral vaccine-elicited immunity. Studies in animal models have shown that severe vitamin A deficiency can impact peripheral B cell development, germinal center formation, and Ag-specific Ab responses (33). However, the impact of vitamin A deficiency on vaccine-induced gastrointestinal immunity has not been previously described in either clinical studies or animal models, despite the critical importance of gastrointestinal immunity for protection against HIV-1 and other mucosal pathogens.

Our data define key molecular mechanisms that account for gastrointestinal cellular immunity elicited by rAd vaccine vectors and, to our knowledge, demonstrate for the first time the impact of vitamin A deficiency on vaccine-induced gastrointestinal cellular immune protection. Vitamin A and its metabolite RA potentiate vaccine-elicited gastrointestinal cellular immune responses by upregulating mucosal homing receptors on Ag-specific CD8<sup>+</sup> T lymphocytes, likely first in draining LN and subsequently in mucosal inductive sites. These responses are selectively abrogated in mice with moderate vitamin A deficiency but can be fully restored by the administration of oral RP at the time of vaccination. Similar results were seen following i.m. immunization of plasmid DNA vaccines (Supplemental Fig. 3), suggesting the generalizability of our findings for other vaccine platforms.

Previous studies have shown that T lymphocytes transiently upregulate gastrointestinal homing markers following activation by a wide variety of antigenic stimuli, including systemic infection or infection localized outside of the gastrointestinal tract (23, 26, 35). We demonstrate that transient gastrointestinal homing marker induction also occurs when T cell priming is restricted to a peripheral lymphoid inductive site following i.m. immunization. Moreover, our data demonstrate that this phenomenon is not part of the intrinsic T lymphocyte activation program but rather is dependent on exogenous vitamin A and RA and therefore adversely impacted in the setting of vitamin A deficiency. Although the RA dependence of T lymphocyte mucosal homing marker upregulation has been well documented *in vitro* (7, 45, 46), our data demonstrate that vitamin A-derived RA is also strictly required for mucosal homing marker induction *in vivo*.

Our data demonstrate that oral vitamin A supplementation at the time of vaccination is critical for mucosal immune responses and protective efficacy in mice. Although our current studies are restricted to a murine model, the dramatic abrogation of gastrointestinal cellular immunity at clinically relevant levels of vitamin A deficiency and the complete restoration of these responses by oral vitamin A supplementation suggests potential clinical implications

of these results. Given the high prevalence of vitamin A deficiency, our data suggest that oral vitamin A supplementation may be critical for optimizing the success of vaccines against mucosal pathogens in the developing world. Moreover, our findings highlight a critical mechanism that links nutritional status with vaccine protective efficacy.

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## Disclosures

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