Vitamin A deficient hosts become non-symptomatic reservoirs of *Escherichia coli*-like enteric infections.

Kaitlin L. McDaniel, Katherine H. Restori, Jeffery W. Dodds, Mary J. Kennett, A. Catharine Ross, and Margherita T. Cantorna

Running title: Vitamin A and enteric infection.

1Department of Veterinary and Biomedical Science, The Pennsylvania State University, University Park, PA 16802, USA

2Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA 16802, USA

3Integrative Biosciences Graduate Program

4Research Institute of the McGill University Health Centre, Montreal General Hospital, Montreal, QC, H3G 14A

5Center for Molecular Immunology and Infectious Disease, The Pennsylvania State University, University Park, PA 16802, USA

6Pathobiology Graduate Program, The Pennsylvania State University, University Park, PA 16802, USA

Correspondence to: Dr. Margherita T. Cantorna, Department of Veterinary and Biomedical Sciences, The Center for Molecular Immunology and Infectious Disease, 115 Henning Bldg., University Park, PA 16802, USA.

Phone: 814-863-2819

Fax: 814-863-6140

E-mail address: mxc69@psu.edu
Vitamin A deficiency (A-) remains a public health concern in developing countries and is associated with increased susceptibility to infection. Citrobacter rodentium was used to model human *Escherichia coli* infections. A- mice developed a severe and lethal (40%) infection. Vitamin A sufficient (A+) mice survived and cleared the infection by d25. Retinoic acid treatment of A- mice at the peak of the infection eliminated *C. rodentium* within 16d. Inflammation was not different in A+ and A- colons although the A- mice were still infected at d37. Increased mortality of A- mice was not due to systemic cytokine production, an inability to clear systemic *C. rodentium* or increased pathogenicity. Instead A- mice developed a severe gut infection with most of the A- mice surviving and resolving inflammation but not eliminating the infection. Improvements in vitamin A status might decrease susceptibility to enteric pathogens and eliminate potential carriers from spreading infection to susceptible populations.
INTRODUCTION

Vitamin A deficiency is a significant problem in developing countries where inadequate micronutrient intake remains a public health concern (1). The World Health Organization estimates that over 20% of preschool aged children are clinically vitamin A deficient (1, 2). Vitamin A deficiency contributes to the higher prevalence of respiratory and diarrheal diseases as well as increased childhood mortality. Conversely, vitamin A supplementation is practiced as a means to reduce mortality in preschool-aged children by reducing severity of infectious diseases (3).

Vitamin A and its active metabolite, retinoic acid (RA) are important regulators of T cell responses. RA inhibited IFN-γ production from T cells in vitro (4). In addition, T cells from vitamin A deficient (A-) mice overproduced IFN-γ (5). RA also inhibited Th17 cells in vitro and in vivo (6). RA increased the expression of the gut homing receptors, α4β7 and CCR9, on T cells (7), which recruits T cells to the gut mucosa. In vitro, RA inhibited IL-17 and induced expression of the transcription factor FoxP3, associated with regulatory T cells, and IL-10 production (8). Vitamin A and RA are key regulators of T cell cytokine production and gut homing.

Several lines of experimental evidence support a beneficial effect of vitamin A and RA on the host response to infection (9, 10). In the gut, the mechanisms that account for the anti-infective effects of vitamin A include support of B cell function and T cell-dependent B-cell antibody responses (11). Gut infection of A- mice with Trichinella spiralis resulted in T cells that produced IFN-γ but not IL-4, and as a result, reduced the rate of parasite clearance (9). Furthermore, RA treatment reduced colonic inflammation caused by dextran sodium sulfate and infection (12). The reduction in gastrointestinal
(GI) inflammation with RA treatment was attributed to the inhibition of IL-17 and IFN-γ (12, 13). These data suggest that vitamin A and RA regulate T cell function to limit inflammation following chemical and infectious injury in the gut.

*Citrobacter rodentium* is a mouse pathogen that models human infections with enteropathogenic *Escherichia coli* and causes attaching and effacing lesions of the cecum and colon in mice (14). The natural route of *C. rodentium* transmission is fecal-oral. Resistant mouse strains including C57BL/6 mice are infected transiently with *C. rodentium* and clear the infection within 2-3 weeks (14). The acquired immune system was required for early protection from *C. rodentium*, as demonstrated in recombination-activating gene (Rag) knockout (KO) mice lacking T and B cells that were unable to clear the infection (15). Robust IL-22 production from innate lymphoid cells and macrophages has been shown to induce protective Th17 responses that resulted in clearance (16, 17). T cells and B cells were essential for resolution of *C. rodentium* infection since mice without T cells (CD4 KO) or B cells (Igμ KO) developed fatal infections (17). Host resistance to *C. rodentium* depends on IL-22 production from innate cells, T cells, B cells and Th17 cells.

Here, we determined the effect of vitamin A deficiency on host resistance to *C. rodentium* infection in C57BL/6 mice; a normally resistant mouse strain. Because of the well-demonstrated inhibitory effects of RA on the differentiation of Th1 and Th17 cells, we predicted that a bacterial infection that required Th17 cell responses for resistance might be less severe in A- mice, and exacerbated in RA-treated mice. Interestingly and contrary to expectations, A- mice developed a chronic infection with *C. rodentium*. The
C. rodentium infection was lethal for 40% of A- mice while none of the vitamin A sufficient (A+) or RA treated A- mice died prematurely from infection.
MATERIALS AND METHODS

Mice. C57BL6 mice were originally from Jackson Laboratories (Bar Harbor, MN) and bred at the Pennsylvania State University (University Park, PA) for experiments. Vitamin A deficient (A-) and vitamin A sufficient (A+) mice generated as previously described (5, 18). Briefly, mice were fed a purified diet that did not contain any vitamin A (A-) or that contained 25 µg of retinyl acetate (vitamin A) per day (A+). At weaning, mice were continuously fed the A- or A+ diet until the end of the study. Serum retinol status was determined by ultra pressure liquid chromatography at 6-7 weeks of age in pooled samples. For some experiments, A- mice were treated with 37.5 µg of all-trans RA (Sigma Aldrich, St Louis, MO) administered orally in 10 µl corn oil three times per week (19). For some experiments, mice were injected i.p. with E. coli O111:B4 LPS (6 mg/kg, Sigma-Aldrich). Experimental procedures were approved by the Office of Research Protection Institutional Animal Care and Use Committee of the Pennsylvania State University, University Park, PA.

C. rodentium infection. C. rodentium strain ICC169 (nalidixic acid resistant) and bioluminescent strain ICC180 (kanamycin resistant) were kind gifts of Gad Frankel (London School of Medicine and Dentistry, London UK). C. rodentium ICC169 was cultured in Luria-Bertani (LB, EMD Chemicals, Inc., Gibbstown, NJ) broth containing 50 µg/ml nalidixic acid (Sigma-Aldrich) while, C. rodentium ICC180 was cultured in LB broth containing 100 µg/ml kanamycin (Sigma-Aldrich). Mice 8-10 weeks of age were infected by oral gavage with 5×10⁹ CFU in 200 µl of C. rodentium strain unless otherwise noted. For studies looking at in vivo infection kinetic, 5×10⁹ CFU C. rodentium ICC180 was used. Animals were imaged every other day using the IVIS50.
small animal imaging system (Xenogen Corp., Alameda, CA, USA). Images were analyzed by Living Image software (PerkinElmer, Waltham, MA). Additional groups of mice were injected i.v. with 10⁴-10⁸ CFU of *C. rodentium* strain ICC169. Feces and other tissues were collected, homogenized and plated in serial dilutions on LB agar plates containing naladixic acid.

For most experiments mice were housed 1 per cage from the time of infection to prevent transmission from mouse to mouse. Natural transmission experiments were performed as previously described (20). Briefly, A-, RA d0, and A+ mice were infected via oral gavage with 5×10⁹ CFU. Three days post-infection, each infected “seed” mouse was co-housed with two naive A+ mice.

**Histology:** Distal colon sections were fixed in 10% formalin, sectioned and stained with hematoxylin and eosin (Pennsylvania State University Animal Diagnostic Laboratory, University Park, PA). Specimens were coded and evaluated in a blinded fashion by a board certified laboratory animal veterinarian with training in pathology. Crypt measurements were taken at 100X using the cellSens software (Olympus Corp., Center Valley, PA, USA). Sections were scored on a scale from 0 to 4 as follows: severity of inflammation (0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = extensive); epithelial sloughing (0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = extensive); distention of muscularis (0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = extensive); edema (0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = extensive). Total histology scores were generated by adding the scores for each category together, generating a value from 0-16 for each sample.

**Flow cytometry:** Colonic IELs were isolated as previously described and stained for
flow cytometry (21). Cells were counted and stained with PE-Cy5 TCR β (BD Pharmingen, San Jose, CA USA), FITC CD8 β (eBioscience San Diego, CA, USA), PE-Cy7 CD8 α (BioLegend, San Diego, CA, USA) or PE Texas Red CD4 (Invitrogen, Carlsbad, CA, USA). Cells were analyzed on a FC500 Bench top cytometer (Beckman Coulter, Brea, CA, USA) and data was analyzed using FlowJo 7.6.1 software (Tree Star, Ashland, OR, USA).

ELISA: Cytokine production in the serum was measured for TNF-α, IL-1β and IFN-γ levels by ELISA and following the manufacturer’s instructions (BD Biosciences, Minneapolis, MN).

Statistical Analysis: Statistical analyses were performed using GraphPad Prism software (Graphpad, La Jolla, CA, USA). Two-tailed Student’s t-tests were used for serum retinol analysis. One-way ANOVA with Tukey’s post-hoc test were used to compare the systemic C. rodentium loads, bioluminescence quantification, and cell population analysis. Two-way ANOVA with Bonferonni’s post-hoc test were used to compare CFU, histology scores and crypt lengths. Log-rank tests were used for the survival curves and ratios of serum cytokine producers. For all analyses, $P<0.05$ was used as the limit for significance.

RESULTS

A- mice are more susceptible to C. rodentium infection than A+ mice. A+ and A- mice were generated as previously described (5, 18). As expected (22), 6-7 week old A- mice had significantly lower serum retinol than A+ mice (Fig. 1A). Bacterial fecal shedding in A+ mice peaked around d10 and cleared within 25 days post-infection (Fig. 1B). The infection in A- mice followed the same kinetics as A+ mice until d14. A+ and
A- mice had similar numbers of *C. rodentium* in the cecum and feces at d10 post-infection (cecum data not shown and Fig. 1A). After d14 the A- mice continued to shed high numbers of *C. rodentium* in their feces, whereas the A+ mice began to clear the infection (Fig. 1B). All of the infected mice showed a small amount of weight loss (5% of starting weight) within the first 2-4 days of infection but all of the A+ mice recovered and no A+ mice died following *C. rodentium* infection (Fig. 1C). Conversely, some of the A- mice failed to recover and instead lost significantly more of their initial body weight (10-20%), which resulted in the premature lethality of 40% of the A- mice (Fig. 1C). The A- mice that survived did not lose any more weight than the A+ mice, had normal exploratory behaviors (data not shown) and otherwise were undistinguishable from their A+ infected counterparts. A subset of A- mice died following weight loss while, the remaining A- mice resume normal behaviors but don’t clear *C. rodentium*.

To visualize the intestinal passage of *C. rodentium* in vivo, we made use of a bioluminescent *C. rodentium* strain, and live animal imaging. Between d2 and d6 post-infection, low levels of *C. rodentium* bioluminescence were detected in the upper parts of the gastrointestinal (GI) tract of A+ mice (Fig. 1D). Just before the peak in fecal shedding (Fig. 1B) the intensity of bioluminescence increased and moved into the lower GI tract of A+ mice (Fig. 1D). After d10 of infection, the intensity decreased in A+ mice and by d14, when fecal shedding of *C. rodentium* was on the decline in A+ mice, less bioluminescence was detected in the intact A+ animals (Fig. 1D). The kinetics of *C. rodentium* transit in the A+ mice was similar to that reported previously in wild-type mice (23). The bioluminescence in the A- mice matched that of the A+ mice at d2 (Fig. 1D). However, as early as d4, the A- mice showed bioluminescence throughout the GI tract.
tract, which persisted in intensity through d14 (Fig. 1D). Unlike the A+ mice, the intensity of bioluminescence in A- mice between d4 and d14 was high throughout the GI tract and significantly higher than in A+ mice (Fig. 1D and Supplementary (S)Fig. 1). Thus, the transit of *C. rodentium* through the GI tract occurred more rapidly and persisted for longer in A- than A+ mice.

**T cell frequencies are lower in the vitamin A deficient gut.** To determine if vitamin A status causes differences in gut mucosal immune cell populations, the intraepithelial lymphocyte (IEL) populations of the colon were characterized. IELs are in direct contact with the intestinal epithelium and a source of T cells from the colon (24). In addition, the T cells in the IEL are in close contact with the microbiota and enteric pathogens in the colon. Fewer total IEL cells were isolated from the colons of uninfected A- versus A+ mice (Fig. 2A). *C. rodentium* infection resulted in a significant increase in the number of cells in the colon of both A- and A+ mice (Fig. 2A). Interestingly, although the A+ mice resolved the infection by d37, as shown in Fig. 1B, the numbers of T cells (TCRβ+) did not return to baseline by d37 pi (Fig. 2A and B). The total number of IELs and TCRβ+ T cells in the colon of A- mice increased following infection but never reached the levels present in infected A+ mice (Fig. 2A). The numbers of TCRβ+, TCRβ+/CD8α+, and TCRβ+/CD8αβ+ T cells in the colon IEL compartment were lower in A- than in A+ mice, both before and after infection (Fig. 2A-E). Therefore, although the increases in total cell numbers and T cell subpopulations were similar in A+ and A- mice, the A- mice consistently had fewer T cells in the colonic IEL compartment compared to A+ mice.

Differences in T cell numbers and populations between A+ and A- mice were not
associated with clearance of *C. rodentium* in A+ mice and persistence of *C. rodentium* in A- mice.

**Exacerbated inflammation and epithelial hyperplasia in A- mice following *C. rodentium* infection.** Histopathology sections of the colon were evaluated before and after infection for signs of inflammation, tissue damage and hyperplasia, including measurements of crypt length. Before infection, A+ and A- mice had low histopathology scores that did not differ with vitamin A status (Fig. 2F and SFig. 2). Crypt lengths also did not differ between uninfected A+ and A- mice (Fig. 2G). After infection, the histopathology scores of A+ colons did not change significantly, either at peak infection (d10) or after resolution of infection (d37) (Fig. 2F, SFig. 2). In A- mice, histopathology scores were significantly higher at d10 post-infection, both compared with baseline A- scores and scores for A+ mice at d10 (Fig. 2F, SFig. 2). By d37, the histopathology scores of A+ and A- mice were the same in spite of the fact that A+ mice had cleared the infection, while A- mice had not (Fig. 2F, SFig. 2). Crypt length in the A+ mice was not affected by infection (Fig. 2G). Crypt length in the A- mice increased significantly after infection and were significantly longer on both d10 and d37 than at baseline and as compared to the A+ mice at all time points (Fig. 2G). Overall, although A- mice exhibited more inflammation than A+ mice at d10 post-infection the effect was not present at d37 even though A- mice continued to harbor *C. rodentium* and A+ mice did not.

**RA treatment of A- mice results in clearance of *C. rodentium*.** To determine if RA could rescue the severe infection in A- mice, A- mice were orally dosed with RA. RA dosing began either 2 wks before infection (RA -d14), or on the day of infection (RA d0).
(Fig. 3). Once started, RA dosing continued 3 times weekly throughout the remainder of the experiment. Treatment of A- mice with RA, either 2 wks before (RA -d14) or on the day of infection (RA d0), resulted in fecal shedding of *C. rodentium* similar to that of A+ mice (Fig. 1B and Fig. 3). In a separate experiment, the RA d0 mice were also infected with bioluminescent *C. rodentium*. The bioluminescence in the RA d0 mice was of a high intensity and resembled that in A- rather than A+ mice (Fig. 1D and SFig. 1). Interestingly, by d14 the intensity of bioluminescence in the RA d0 group was lower and resembled the A+ rather than the A- mice (Fig. 1D and SFig. 1).

To determine whether RA could be used therapeutically in A- mice with an established *C. rodentium* infection, the RA treatment of A- mice was started on d14 post-infection (RA +d14). Day 14 of infection occurs just after the peak of fecal shedding and before A+ mice show a rapid decline in *C. rodentium* shedding (Fig. 1B). Treatment of A- mice with RA, three times weekly, beginning on d14 resulted in a gradual decline in fecal shedding of *C. rodentium* (Fig. 3), which was significant as early as 4 days following the start of RA treatment, as compared to untreated A- mice (d18 post-infection, Fig. 3). As observed in earlier experiments, untreated A- mice maintained a persistent infection while the RA +d14 treated mice cleared the infection 18d after the start of the RA treatment (d32 post-infection, Fig. 3). Thus, RA treatment of A- mice cleared the *C. rodentium* infection, which otherwise was persistent.

**Increased *C. rodentium* CFU in the liver and spleen of A- mice.** At d14 post-infection mice that had been infected with the bioluminescence strain of *C. rodentium* were euthanized to determine which organs harbored *C. rodentium*. In the A+ group, bioluminescence was detected in the colon but not in other parts of the GI tract (SFig.
A+ mice did not have visible bioluminescence in their spleen or liver at d14 post-infection (SFig. 3B). A- mice had high levels of bioluminescence in the upper portions of the GI tract that were significantly higher than in A+ mice (SFig. 3C). A- mice also had visible bioluminescence at d14 in both the spleen and liver (SFig. 3B). The bioluminescence in the spleen and liver of RA d0 mice was more similar to A+ mice than to A- mice at d14 post-infection (Fig. 4A and SFig. 3B). Quantification of the bioluminescence showed that A- mice had higher bioluminescence in the spleen, liver and SI than either the A+ or the RA d0 mice (Fig. 4A).

Next we determined whether viable *C. rodentium* was detectable in the spleen and liver of our mice following gastric infection with *C. rodentium*. Cultures of the spleen and liver at d10 and d14 post-infection showed that A+ mice had detectable *C. rodentium* in their internal organs (Fig. 4B, and d14 data not shown). A- mice had significantly more *C. rodentium* in the spleen and liver than A+ mice (Fig. 4B), while the spleen and liver of RA d0 mice did not differ from those of A+ mice but were significantly different than those of A- mice (Fig. 4B). At d37 of infection the A- mice no longer had *C. rodentium* present in the SI, spleen and liver even though they continued to shed *C. rodentium* in the feces (data not shown).

**Mortality of A- mice following infection with *C. rodentium* is not due to over-production of systemic cytokines.** To determine whether A- mice died due to cytokine over-production, serum cytokine levels were determined at before (d0) and at the peak (d10) of infection in A+ and A- mice (Table 1). None of the A+ mice had detectable levels of any cytokines before infection. At d0, several of the A- mice had detectable levels of TNF-α, IL-1β or IFN-γ in their serum (Table 1). Infection resulted in detectable
TNF-α, IL-1β and IFN-γ in the serum of both A+ and A- mice. There was significantly more A- mice with IL-1β in the serum (10 of 19) than A+ (1 of 13) mice at d10 post-infection (Table 1). Serum IFN-γ was higher but not significantly different in A- mice compared to A+ mice at d10 post-infection ($P=0.0817$). To measure the capacity for cytokine production, LPS was injected ip into A- and A+ mice to measure serum cytokine response. A- mice produced significantly more TNF-α than A+ mice following LPS injection, while the IL-1β response was the same in A+ and A- mice (Fig. 5A and IL-1β data not shown). Overall A- mice were more likely to have IL-1β detectable in the serum after *C. rodentium* infection and produced more TNF-α after LPS than A+ mice. We hypothesized that A- mice were dying following oral challenge with *C. rodentium* because of systemic spread. We attempted to determine the LD$_{50}$ of i.v. injected *C. rodentium* in A+ mice. All of the A+ mice survived an i.v. dose of $10^8$ *C. rodentium* and readily cleared the bacteria. A- mice also survived an i.v. injection of $10^8$ *C. rodentium*. A+ mice had the highest number of organisms in the spleen and liver 1d post-i.v. infection with $10^5$ CFU, which declined at d3 and again at d5 and was completely cleared by d10 post-infection (Fig. 5B and 5C). The group x time interaction showed no differences in the clearance of i.v. injected *C. rodentium* between A+ and A- mice (Fig. 5B and 5C). In addition, i.v. injection was not lethal and did not induce a systemic cytokine response in either A+ or A- mice (data not shown).

**Host vitamin A status does not alter *C. rodentium* infectivity.** Previous reports have demonstrated that natural infection of *C. rodentium* to co-housed uninfected mice occurs with several fewer log units of bacteria than with laboratory grown *C. rodentium* (20). We hypothesized that vitamin A and RA were attenuating the infectivity (virulence) of
C. rodentium and that C. rodentium that has been passaged through A- mice might be more virulent. A+, RA d0, and A- seed mice were infected by gavage using laboratory grown C. rodentium and 3d later each was co-housed with naïve A+ mice, housed in groups of 2 with the seed mice. As described previously (20), all seed mice, despite their vitamin A status, infected all co-housed naïve A+ mice within 3 days of exposure (data not shown). Culturing of the feces, spleen and liver from the cage mates of A+, A- and RA d0 seed mice showed that there were no differences in the CFU of C. rodentium recovered in the spleen, liver and feces via natural transmission from either A+, A- or RA d0 seed mice (Fig. 6). Thus the data do not show an effect of vitamin A status and or RA treatment on C. rodentium infectivity.
A- mice were found to be significantly more susceptible to *C. rodentium* infection compared to A+ mice. The increased susceptibility of A- mice included lethality of 40% of the A- mice by d14. The kinetics of shedding of *C. rodentium* in A+ and A- mice were not different before d14, after which the A+ mice proceeded to clear the infection while the A- mice either died or developed a persistent infection. The data suggest that early innate immune responses are adequate in A- mice during the initial phase of the infection, when fecal bacterial counts are increasing, and instead suggest that A- mice failed to generate an acquired immune response necessary to clear the infection. Our results unexpectedly showed that A- mice became chronic carriers of *C. rodentium*, while no longer showing symptoms (inflammation of the colon, diarrhea etc.). Correcting vitamin A status or treating A- mice with RA effectively eliminated the infection and improved survival of A- mice, suggesting an added benefit not previously recognized for vitamin A and/or RA interventions.

The increased mortality of A- mice following GI infection was not due to the systemic spread of *C. rodentium*, increased systemic cytokine production or the virulence of *C. rodentium*. Others have reported mortality following gavage of *C. rodentium* in mice and have attributed that mortality to systemic spread and poly-microbial sepsis (15, 25, 26). Susceptible strains of mice (C3H/HeJ, C3H/HeOuJ and C3H/HeN) develop a severe infection with 100% lethality due to bacterial translocation from the gut, cytokines in the serum and crypt cell apoptosis (25). By comparison, C57BL/6 mice are relatively resistant to *C. rodentium* (15, 17, 25). For the first time, our data clearly show that *C. rodentium* does not grow following i.v. injection of large numbers of bacteria in either
A+ or A- mice, indicating that vitamin A deficiency does not affect the ability to survive a systemic infection with *C. rodentium*. However, A- mice were more susceptible to oral infection with *C. rodentium*. We necropsied one recently deceased and one moribund A-mouse. Very little food was found in the stomach of either mouse, suggesting that the mice had stopped eating. The necropsy of A- mice failed to identify evidence of systemic infection (data not shown). It has been shown that LPS-induced proinflammatory cytokines such as TNF-α and IL-1β can result in anorexia (27). Our data are consistent with TNF-α and IL-1β induced anorexia, since the A- mice that died had lost significant amounts of weight (10-20% of original body weight). In addition, A- mice had higher TNF-α levels after LPS challenge and were more likely to have detectable IL-1β in their serum after infection than A+ mice. Surviving A- mice may have been just below the threshold of the response and therefore survived the infection-induced anorexia. Based on the inability of *C. rodentium* to grow following an iv injection and the necropsies of moribund and dead A- mice, we concluded that the mortality in our A- mice was not due to sepsis and/or the systemic spread of the infection.

A- mice had reduced numbers of total colonic IELs and all IEL T cell subsets in the colon before infection. Infection induced homing and expansion of T cells in the gut of both A+ and A- mice. Although the A- mice were persistently infected with *C. rodentium*, colonic inflammation as determined by histological score resolved and was not different at d37 from A+ mice. This same phenomenon occurred in germfree mice mono-associated with *C. rodentium* (28). In germfree mice *C. rodentium* infection was not cleared but the inflammatory response in the colon was nevertheless resolved (28). It therefore seems that vitamin A is not required to resolve inflammation in the colon and
that in the absence of vitamin A, T cells are able to arrive and respond to the infection.

However, the acquired immune response in the A- mice was ineffective at eliminating the infection. The data suggest a requirement for vitamin A/RA to mount effective protective immunity after the initial infection. Since RA treatments effectively reduced *C. rodentium* numbers in already infected A- mice within 4 days of treatment, it will be of interest to identify the targets of RA in the GI tract for *C. rodentium* clearance.

In our study, A- mice developed persistent and sometimes fatal enteric infections. The cause of the premature lethality of A- mice was not due to sepsis and/or systemic spread of *C. rodentium*. Instead it seems that a high load of *C. rodentium* in the gut of A- mice results in infection-induced anorexia in 40% of the mice. When given early, vitamin A and/or RA interventions protected A- mice from the lethality following *C. rodentium* infection and when administered later RA cleared the persistent infections that occurred in surviving A- mice. Our work has important implications for the developing world where vitamin A deficiency is prevalent. In particular, vitamin A-deficient humans and animals could be reservoirs for *E. coli*-like enteric pathogens. Vitamin A and RA treatments might be useful interventions to decrease morbidity and mortality from enteric infections. We show here a novel and unappreciated role for vitamin A/RA for eliminating persistent enteric infections and show added benefits to improving vitamin A status in the developing world.
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COMPETING FINANCIAL INTERESTS: The authors have nothing to declare.
FIGURE LEGENDS

Figure 1. A- mice are more susceptible to C. rodentium infection than A+ mice. A) Serum retinol analysis from groups of 6-7 week old A+ and A- mice. Each data point represents the serum retinol values in pooled samples from a different litter of mice (n=10-13 liters). B) C. rodentium CFU in the feces. Data is shown as mean ± SEM and 1 representative of 5 independent experiments with n=3-5/group. C) Survival of A- and A+ mice during C. rodentium infection with data combined from 5 experiments n=9-15/group. D) Whole body imaging of A+, A-, and RA d0 treated mice infected with bioluminescent C. rodentium and imaged. The image shows 1 mouse from a total of 3-4 mice/treatment group. Means of the bioluminescence from all the mice are shown in SFig.1. Two-tailed Student t-test (A), two way ANOVA with Bonferroni post-hoc tests (B), log-rank test (C), *P<0.05, **P<0.01, ***P<0.001

Figure 2. T cells in the IEL of the colon from A+ and A- mice before and after infection. A) Immune cell numbers in the colon IEL. B) TCRβ+ T cell numbers in the colon IEL. C) CD8α+ T cell numbers in the colon IEL. D) CD8αβ+ T cell numbers in the colon IEL. E) CD4+ T cells numbers in the colon IEL. Values are the mean ± SEM combined data from 2-3 independent experiments with n=6-15/group. Two way ANOVA with Bonferroni post-hoc tests. Asterisks indicate significant differences between groups at specific days by post test, *P<0.05, **P<0.01, ***P<0.001. F) Histology scores and G) crypt length of colons during infection. Values with different letters are significantly different from each other, P<0.05. A) Two way ANOVA with Bonferroni post-hoc tests, *P<0.05, ***P<0.001.
Figure 3. RA treatment of A- mice eliminates *C. rodentium* infection. A) CFU in the feces of A+ mice A- mice, A- mice treated with RA for 2wks before infection (RA -d14), A- mice treated with RA on the day of infection (RA d0) and A- mice treated with RA starting on d14 of infection (RA d+14). Values are the mean ± SEM of one representative of 2 independent experiments and n=3-5/group. Two-way ANOVA with Bonferroni post-hoc tests. Values with different letters are significantly different from each other, P<0.01-0.0001.

Figure 4. Systemic spread of *C. rodentium* following GI infection. Mice were sacrificed at d14 post-infection with the bioluminescent strain of *C. rodentium* and the A) GI tract and B) spleen and liver were imaged and quantitated. An example image is shown in SFig. 3. Values are from the images of n=3-4 mice/treatment group. CFU of *C. rodentium* recovered in the B) spleen and liver of d10 infected mice. Values in B) are the mean ± SEM combined from three independent experiments with n=9-15/group. One-way ANOVA with Tukey post-tests.

Figure 5. The effect of vitamin A status on LPS response and i.v challenge with *C. rodentium*. A) Serum TNF-α in A- and A+ mice treated with LPS. CFU in the B) spleen and C) liver of mice following i.v. injection of *C. rodentium*. Values are the mean ± SEM combined from two-three independent experiments with n=8-9/group/time-point. Two-tailed Student t-test (A), Two-way ANOVA with Bonferroni post-hoc tests (B and C). Asterisks indicate significant differences between groups at specific days by post test.; *P<0.05.

Figure 6. Natural transmission of *C. rodentium* is not affected by the host vitamin A status. CFU in the feces A), spleen B), and liver C) of A+ cage mates of A-, RA d0 and
A+ laboratory inoculated mice. N=4-6/group. Values are the mean ± SEM combined for two independent experiments. One-way ANOVA with Tukey post-tests.
### Table 1. Serum cytokines in A+ and A- mice

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<th>Day</th>
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<th>IL-1β</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>A+</td>
<td>0/</td>
<td>1/8</td>
<td>0/</td>
</tr>
<tr>
<td></td>
<td>A-</td>
<td>4/8</td>
<td>0/</td>
<td>3/6</td>
</tr>
<tr>
<td>10</td>
<td>A+</td>
<td>2/13</td>
<td>4/19</td>
<td>1/13</td>
</tr>
<tr>
<td></td>
<td>A-</td>
<td>10/19*</td>
<td>0/13</td>
<td>4/19</td>
</tr>
</tbody>
</table>

* A- value is significantly different than A+, P<0.05.

<sup>1</sup> n with detectable levels of cytokine/total n
REFERENCES


Figure 1

A. Serum Retinol (mmol/L) vs. A+ and A-

B. Log10 CFU/g feces vs. Group x Time: P<0.0001

C. Survival % vs. days post-infection

D. Imaging data for A+, A-, and RA d0 at various days post-infection.
Figure 2

Panel A: Cell number (x 10^6)
- Group effect: P<0.0001
- Time effect: P<0.0001
- Group x Time: P=0.7352

Panel B: TCRβ+ cell (x 10^5)
- Group effect: P<0.0001
- Time effect: P<0.0001
- Group x Time: P=0.0302

Panel C: CD8α.TCRβ+ cell (x 10^4)
- Group effect: P=0.0064
- Time effect: P<0.0001
- Group x Time: P=0.3190

Panel D: CD8α.TCRβ+ cell (x 10^4)
- Group effect: P<0.0001
- Time effect: P<0.0001
- Group x Time: P=0.0920

Panel E: CD4.TCRβ+ cell (x 10^4)
- Group effect: P=0.0925
- Time effect: P=0.0231
- Group x Time: P=0.3734

Panel F: Histology Score
- Group effect: P=0.0535
- Time effect: P=0.0305
- Group x Time: P=0.5705

Panel G: Crypt Length (um)
- Group effect: P=0.0925
- Time effect: P=0.0231
- Group x Time: P=0.3734
Figure 4

A

Spleen

Liver

B

log_{10} \text{photons/sec}

log_{10} \text{CFU/g}

...
Figure 5

A

B

C

Days post-injection

Group effect:    P=0.1706
Time effect:      P<0.0001
Group xTime:   P=0.2962

Group effect:    P=0.1920
Time effect:      P<0.0001
Group xTime:   P=0.6752
Figure 6

A

Spleen

Liver

log_{10} (CFU/g feces)

log_{10} CFU/g tissue

log_{10} CFU/g tissue

log_{10} CFU/g tissue