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Retinoid Levels Influence Enterohemorrhagic *Escherichia coli* Infection and Shiga Toxin 2 Susceptibility in Mice

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Enterohemorrhagic *Escherichia coli* (EHEC) is a food-borne pathogen that produces Shiga toxin (Stx) and causes hemorrhagic colitis. Under some circumstances, Stx produced within the intestinal tract enters the bloodstream, leading to systemic complications that may cause the potentially fatal hemolytic-uremic syndrome. Although retinoids like vitamin A (VA) and retinoic acid (RA) are beneficial to gut integrity and the immune system, the effect of VA supplementation on gastrointestinal infections of different etiologies has been controversial. Thus, the aim of this work was to study the influence of different VA status on the outcome of an EHEC intestinal infection in mice. We report that VA deficiency worsened the intestinal damage during EHEC infection but simultaneously improved survival. Since death is associated mainly with Stx toxicity, Stx was intravenously inoculated to analyze whether retinoid levels affect Stx susceptibility. Interestingly, while VA-deficient (VA-D) mice were resistant to a lethal dose of Stx2, RA-supplemented mice were more susceptible to it. Given that peripheral blood polymorphonuclear cells (PMNs) are known to potentiate Stx2 toxicity, we studied the influence of retinoid levels on the absolute number and function of PMNs. We found that VA-D mice had decreased PMN numbers and a diminished capacity to produce reactive oxygen species, while RA supplementation had the opposite effect. These results are in line with the well-known function of retinoids in maintaining the homeostasis of the gut but support the idea that they have a proinflammatory effect by acting, in part, on the PMN population.

Since its first documented outbreak in 1982 (1), enterohemorrhagic *Escherichia coli* (EHEC) has been recognized as an emerging food-borne pathogen. In Argentina, there is a high prevalence of EHEC O157:H7 infections and the highest incidence of hemolytic-uremic syndrome (HUS) in the world (17.5/100,000 children <5 years old). Therefore, EHEC-associated pathologies, with high morbidity and mortality rates, presume a serious public health problem (2, 3). EHEC has the capacity to intimately attach to and efface intestinal epithelial cells, a pathology called the attaching-and-effacing (A/E) lesion (4, 5). Most of the genes involved in A/E lesion formation are carried on the locus of enterocyte effacement pathogenicity island (6). Additionally, EHEC produces Shiga toxin (Stx), which is responsible for the severe complications associated with EHEC, including hemorrhagic colitis and HUS (7, 8). Under some circumstances, Stx produced within the intestinal tract reaches the systemic circulation (9) and targets its receptor (globotriaosylceramide [Gb3]) in the endothelium and other susceptible tissues. It results in intestinal, as well as systemic, dysfunction, with the main organs affected being the kidneys and the central nervous system (10, 11). EHEC O157:H7 has been the serotype most frequently associated with large outbreaks and sporadic cases of hemorrhagic colitis and HUS in many countries (12). In addition, clinical and experimental evidence strongly suggests that certain components of the inflammatory response, particularly peripheral blood polymorphonuclear cells (PMNs), play a central pathogenic role, through several mechanisms (10, 11). Initially, EHEC colonization induces acute colonic inflammation. In this regard, the infiltration of the gut and the presence of leukocytes in feces are seen in many EHEC-infected patients. PMN recruitment in the intestine may induce the Stx2 prophage mainly through the production of H_2O_2, thus augmenting Stx2 production (13). Additionally, PMNs may facilitate the systemic absorption of Stx2, which in turn increases the risk of developing HUS (3, 14). A high peripheral blood PMN count at presentation is the poor-prognosis factor most consistently reported, and the degree of renal impairment has been correlated with the level of activation of circulating PMNs (15). Recently, it has been demonstrated that Stx2 induces an oxidative imbalance *in vivo*, and ROS production by neutrophils may be one of the major sources of oxidative stress during Stx intoxication (16), contributing to the amplification of microvascular injury in the kidneys (17).

Retinoids like vitamin A (VA) and its main metabolite all-*trans* retinoic acid (RA) influence a wide variety of mammalian cellular processes, including immune regulation, embryonic development, survival, apoptosis, and cell growth (18–20). VA is known to be necessary for the growth and differentiation of epithelial

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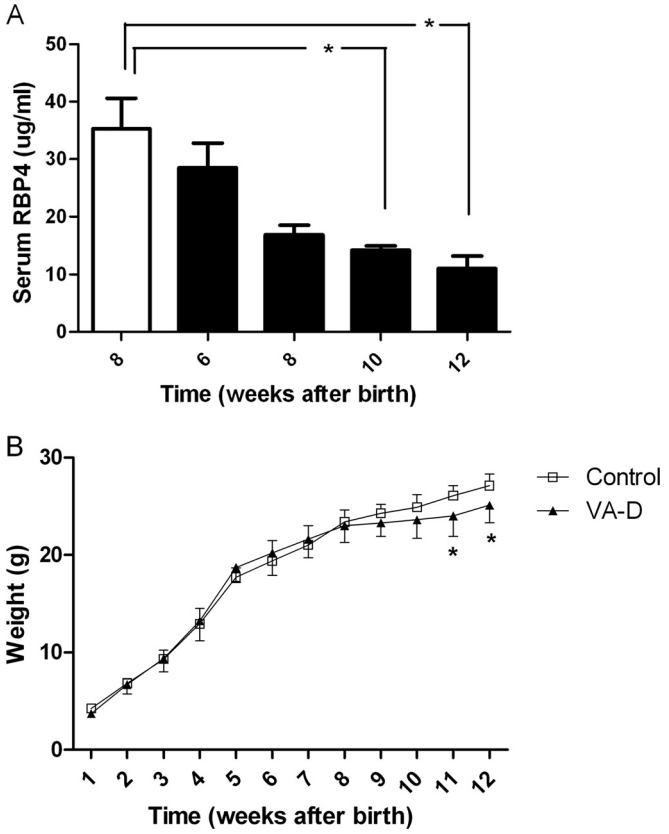

FIG 1 Establishment of the VA-D mouse model. (A) RBP4 concentrations (μg/ml) in the serum of control (white bars) and VA-D mice (black bars) were measured at different times after birth. The values shown are means ± standard errors (three mice per experimental group were used). All data were analyzed by ANOVA and *a posteriori* Tukey test. *, $P < 0.05$. The experiment was performed three times with similar results. (B) Body weights of male mice fed a VA-D diet (black triangles) or pair fed a control diet (white squares) were measured weekly after birth. The values shown are the mean body weights of 8 control mice and 10 VA-D mice ± the standard errors. All data were analyzed by a two-tailed Student *t* test comparing control and VA-D mice of similar ages. *, $P < 0.05$. The experiment was performed three times with similar results.

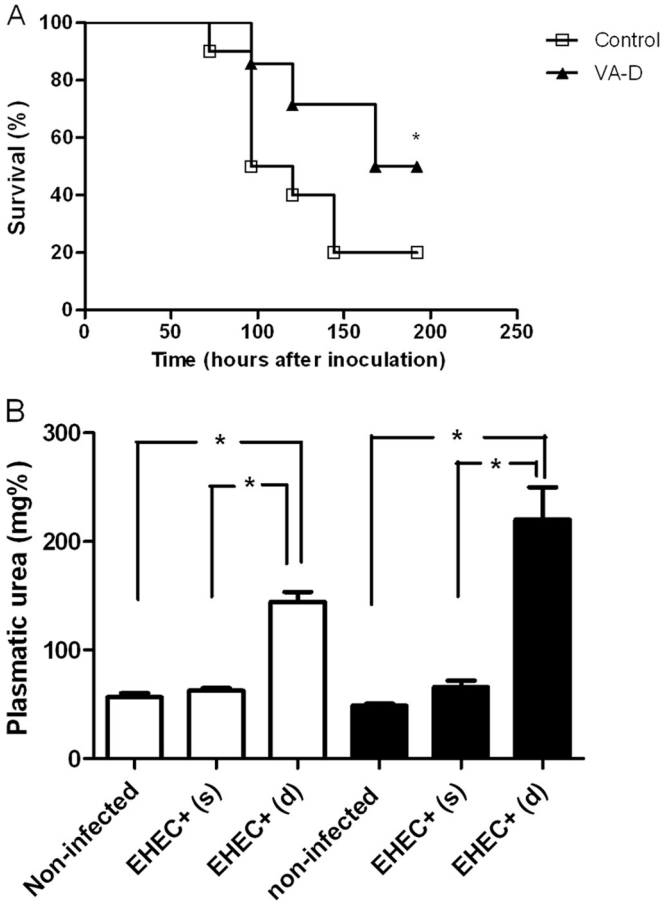

FIG 2 Survival and serum urea concentrations after EHEC infection. (A) A dose of 7×10^{11} CFU of EHEC/kg of body weight was orally administered to 11-week-old control (white squares) or VA-D (black triangles) mice. Survival of EHEC-infected mice was monitored daily after bacterial inoculation. Nine control and 14 VA-D mice were used. *, $P < 0.05$ (log rank test). The experiment was performed three times with similar results using doses in the range of 6×10^{11} to 9×10^{11} CFU of EHEC/kg of body weight. (B) Mice were bled 96 h after oral EHEC inoculation, and urea concentrations in plasma were evaluated. Infected mice were classified retrospectively according to their evaluation as survivors (s) or dead (d). The values shown are mean urea concentrations (mg%) ± the standard errors. Control (white bars) or VA-D (black bars) mice were classified as noninfected, EHEC+ (s), or EHEC+ (d). Numbers of control mice per experimental group: noninfected, 6; EHEC+ (s), 2; EHEC+ (d), 3. Numbers of VA-D mice per experimental group: noninfected, 4; EHEC+ (s), 7; EHEC+ (d), 2. All data were analyzed by ANOVA and *a posteriori* Tukey test. *, $P < 0.05$. The experiment was performed three times with similar results.

tissues (21–23), and RA plays a central role in the homeostasis of the gut-associated lymphoid tissue (24–26).

Although VA supplementation is beneficial in most cases of severe malnutrition, results concerning VA treatment of diarrheas of different etiologies have been controversial (27, 28). These inconsistencies support the notion that retinoid supplementation could be positive, negative, or neutral, depending on the pathogenic mechanisms involved (2, 28). The conflicting results concerning VA supplementation are not restricted to its effect on diarrhea. It has been reported that retinoid supplementation can be deleterious through exacerbation of the inflammatory response by affecting the maturation and function of neutrophils (29–32). This situation could be of particular importance in the context of HUS pathogenesis, where the inflammatory response and neutrophils have been demonstrated to potentiate Stx2 toxicity.

Since clinical and experimental evidence indicates that retinoids affect gut integrity and the inflammatory response, factors that are critical in HUS pathogenesis, the aim of this work was to analyze the influence of retinoids on EHEC infection.

MATERIALS AND METHODS

Mice. BALB/c mice were maintained under specific-pathogen-free conditions in the animal facility of the Instituto de Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas, Academia Nacional de Medicina, Buenos Aires, Argentina. The experiments described here were approved by the Instituto de Medicina Experimental Care Committee (protocol 1013) in accordance with the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (33). Mice were housed in standard transparent polypropylene cages under environmentally controlled conditions (temperature, 24 ± 2°C; humidity, 50% ± 10%) with a 12-h light-dark cycle.

Throughout these studies, the health and behavior of the mice were assessed three times a day. Any mice that became moribund were hu-

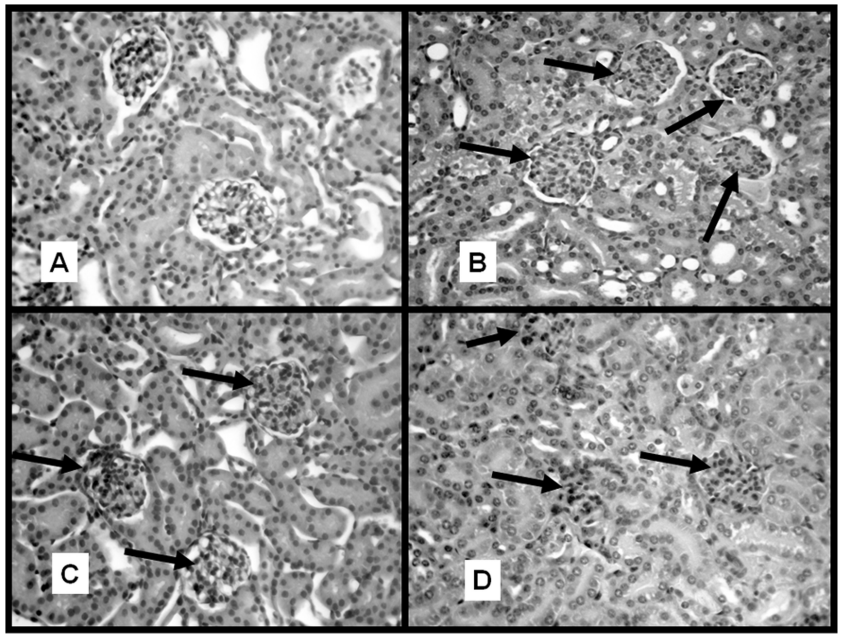

FIG 3 Histological studies of kidney samples. Kidney samples from control and VA-D mice inoculated with PBS or EHEC were excised at 96 hpi, fixed, and stained with hematoxylin and eosin. Images were acquired with a Carl Zeiss III photomicroscope (Carl Zeiss AG, Oberkochen, Germany). Original magnification, ×250. (A) Kidney tissue of a noninfected control mouse with normal-aspect glomeruli and tubular epithelia preserved. (B) Kidney tissue of an EHEC-infected control mouse showing glomeruli (arrows) with hypercellularity, with some of the glomeruli retracted and the proximal and distal tubular epithelial cytoplasm showing a frosted-glass appearance and frayed luminal edges. (C) Kidney tissue of a noninfected VA-D mouse with normal-aspect glomeruli (arrows) and preserved tubular epithelia. (D) Kidney tissue of an EHEC-infected VA-D mouse showing glomeruli with marked shrinkage (arrow), mesangial hypercellularity, absence of Bowman's space (arrows), proximal and distal tubular epithelia with scant pale cytoplasm, and images of bare nuclei.

manely euthanized. Institutional Animal Care and Use Committee (IACUC) guidelines were used to define humane endpoints.

Establishment of the VA-D mouse model. To generate VA-deficient (VA-D) mice, pregnant females were fed a VA-D diet (AIN-93G Growing Rodent Diet without added VA; Research Diets) since the second week of gestation and pups were given *ad libitum* access to the same diet after weaning (20 to 21 days of age) (34). Pups of pregnant females were pair fed a VA-sufficient diet (AIN-93G Growing Rodent Diet; Research Diets) and used as controls.

Retinol binding protein 4 (RBP4) was chosen as a surrogate marker for retinol because of the close association between retinol and RBP4 (35, 36). RBP4 concentrations in serum were measured monthly by enzyme-linked immunosorbent assay (Adipogen) to check the time course of the development of VA deficiency. In addition, body weights were measured weekly.

Unless indicated otherwise, 11-week-old mice were used in all of the experiments. The weights of the mice used ranged between the following values: male control mice, 25 to 27 g; male VA-D mice, 24 to 26 g; female control mice, 22 to 24 g; female VA-D mice, 21 to 23 g. The experiments were performed with either male or female mice.

Refeeding group. To generate VA-recovered mice, 3-month-old VA-D mice were given free access to the VA-sufficient diet for 1 month, after which their serum RBP4 concentrations returned to the normal basal level.

RA supplementation. RA (Roche, Kaiseraugst, Switzerland) was dissolved in the vehicle (liquid paraffin) at a final concentration of 10 mg/ml. RA-supplemented (RA-S) mice received daily intraperitoneal injections of RA (10 mg/kg body weight) for 5 consecutive days in a manner similar to that previously described (37). Control mice received an equal volume of the vehicle. Both control and RA-S mice were inoculated intravenously (i.v.) with 1 30% lethal dose (LD_{30}) of Stx2 on day 5 of RA treatment.

Bacterial strain. The EHEC O157:H7 bacterial strain used in this study was isolated from fecal specimens from a patient with HUS. The strain belonged to seropathotype A, according to the classification of Karmali et al. (38). The origin, toxin type, *eae* variant, EHEC Hly production, and 50% cytotoxic dose in a Vero cell assay of this EHEC strain were previously characterized by Fernández-Brando et al. (39).

EHEC infection. Bacterial suspensions of EHEC O157:H7 were diluted to an appropriate concentration and delivered directly into the stomachs of mice after 3 h of food starvation. A stainless steel cannula (model 7.7.1, 0.38-mm outside diameter, 22 gauge; Harvard Apparatus) was used. Animals received a total inoculum of 6×10^{11} to 9×10^{11} CFU of EHEC/kg of body weight. Control animals received the same volume of sterile phosphate-buffered saline (PBS). After 4 h of bacterial ingestion, both food and water were provided *ad libitum*.

Stx2 i.v. inoculation. Stx2 was prepared as described previously (40). In order to analyze specific Stx2-dependent effects, different doses of Stx2 were injected i.v. into the retro-orbital plexus of isoflurane-anesthetized control mice to establish a dose-response curve. We selected a 100% lethal dose (LD_{100}) of 160 ng/kg and a 30% lethal dose (LD_{30}) of 40 ng/kg, which induced 100% and 30% mortality, respectively, in control mice between 72 and 96 h after injection. These doses were selected on the basis of the necessity to highlight the protective or deleterious effect of VA deficiency and RA supplementation, respectively. The same batch and dose of Stx2 was used for all experiments.

Urea and PMNs. At 96 h after EHEC infection or i.v. inoculation of Stx2, blood samples were obtained by puncture of the retro-orbital plexus under isoflurane anesthesia. PMNs were counted in Neubauer chambers. To determine urea concentrations, blood samples were centrifuged and the plasma was separated and stored at –20°C. Urea concentrations were measured with the urea color kit in accordance with the manufacturer's instructions (Wiener Lab, Rosario, Argentina).

Survival analysis. EHEC- or Stx2-inoculated control VA-D or RA-S mice were used in a survival analysis. All moribund mice were humanely euthanized. IACUC guidelines were used to define humane endpoints.

Histology. Kidneys and cross-sectional intestinal samples obtained at different heights were fixed in a 10% Formol–PBS solution at 96 or 120 h postinfection. At least three mice per experimental group were used. Sections were stained with hematoxylin and eosin and examined by light microscopy. Histological colon sample scoring was performed in a blinded fashion by a pathologist to produce a combined score of mucosal thinning (on a scale of 0 to 3), goblet cell depletion (on a scale of 0 to 3), mucosal inflammatory cell infiltration (on a scale of 0 to 3), and submucosal inflammatory cell infiltration (on a scale of 0 to 3) in a manner similar to that previously reported (41).

Isolation of PMNs and ROS generation. Blood samples were obtained from control, VA-D, or RA-S mice by puncture of the retro-orbital plexus. PMNs were isolated from a pool of heparinized blood obtained from two mice. The cells were harvested by Ficoll-Hypaque separation, followed by dextran sedimentation, as described by Gomez et al. (16).

Dihydrorhodamine 123 (DHR-123), a derivative of rhodamine 123, was used to determine the production of ROS by flow cytometry (16). Briefly, 2×10^5 isolated PMNs were incubated for 15 min at 37°C with 1 μM DHR-123. The cells were then incubated with or without phorbol myristate acetate (PMA) at 50 ng/ml for 15 min at 37°C in a 5% CO_2 atmosphere. Finally, the cells were washed and suspended in 200 μl of Isoflow (BD, Buenos Aires, Argentina). Green fluorescence was measured in 100,000 events with a FACScan cytometer (BD, Argentina). Data were analyzed by using CELLQUEST software (Becton, Dickinson Immunocytometry Systems). PMNs were identified and gated by using forward- and side-scatter dot plot profiles, and the mean fluorescence intensity (MFI) of cells within the neutrophil gate was determined.

Statistical analysis. Levels of significance were determined by analysis of variance (ANOVA), a two-tailed Student *t* test, or a log rank test with a confidence level of >95% ($P < 0.05$).

RESULTS

Establishment of the VA-D mouse model. VA deficiency was induced as previously reported (34), and serum RBP4 concentrations were measured as a surrogate marker of VA status (35). As shown in Fig. 1A, the RBP4 concentration in the serum of VA-D mice decreased progressively after 6 weeks of age and decreased significantly after 10 weeks. By week 12, the serum RBP4 concentration had decreased 3-fold in VA-D mice (nearly 10 μg/ml) compared to normal values of 30 to 40 μg/ml.

Figure 1B shows that the body weights of VA-D and pair-fed control mice increased similarly at least until 10 weeks of age.

On the basis of these results, in the experiments we used mice at 11 weeks of age, when the RBP4 concentration showed a significant difference between VA-D and pair-fed control mice and their body weights just started to differ significantly.

VA deficiency attenuates the susceptibility to EHEC infection. On the basis of the concentration of bacteria previously reported to be necessary to cause pathogenesis in different mouse models of EHEC infection (42), a broad range of concentrations was evaluated to select the optimal dose to be used. The survival of control and VA-D mice was not affected by doses lower than 5×10^{11} CFU of EHEC/kg of body weight. In addition, bacterial doses greater than 1×10^{12} CFU of EHEC/kg of body weight caused 100% mortality in both groups of mice (data not shown). Thus, an average dose of 7×10^{11} CFU of EHEC/kg of body weight, which caused 80% mortality of control mice, was used from then on.

Interestingly, VA-D mice showed a survival rate higher than that of control mice after infection (Fig. 2A), suggesting that VA deficiency attenuates the susceptibility to EHEC infection.

VA-D mice that died after EHEC infection show alterations related to Stx2 toxicity. We have previously shown that EHEC infection of weaned BALB/c mice can be lethal, as a consequence of systemic damage related to Stx2 toxicity (39). In addition, we have also demonstrated that renal dysfunction secondary to EHEC infection is blocked when anti-Stx2-polyclonal serum is simultaneously inoculated, confirming that Stx is responsible for renal damage and an increased plasma urea concentration (43).

To assess renal dysfunction, the urea concentration was measured at 96 h postinoculation (hpi) and data were analyzed retrospectively according to the final outcomes of the inoculated mice. Our results showed that control and VA-D mice that died after EHEC infection displayed similar high plasma urea concentrations at 96 hpi (Fig. 2B). In contrast, control or VA-D mice that survived infection did not show a pathological plasma urea concentration increase (Fig. 2B).

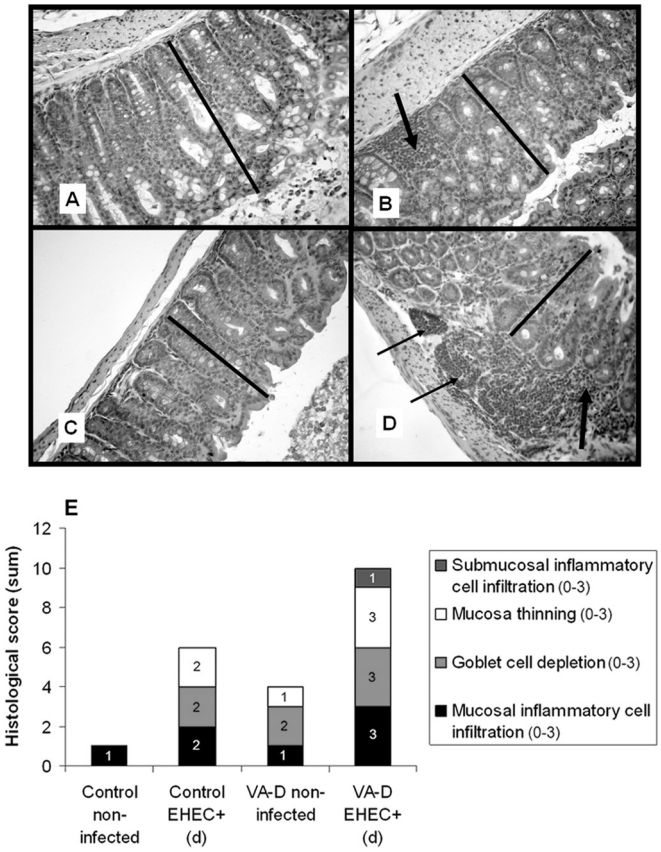

FIG 4 Histological studies of colon samples. Colon samples from control and VA-D mice inoculated with PBS or EHEC were excised at 96 hpi, fixed, and stained with hematoxylin and eosin. Images were acquired with a Carl Zeiss III photomicroscope (Carl Zeiss AG, Oberkochen, Germany). Original magnification, ×250. (A) Noninfected control mice show colonic mucosa with normal thickness (bar), preserved architecture, and abundant goblet cells. (B) EHEC-infected control mice show moderately thinned colonic mucosa (bar), a moderately decreased number of goblet cells, and lymphocyte inflammatory infiltrate in the lamina propria (arrow). (C) Noninfected VA-D mice show colonic mucosa with slightly reduced thickness (bar) and a decreased number of goblet cells with a mildly altered glandular architecture. (D) EHEC-infected VA-D mice show colonic mucosa with greatly reduced thickness (bar), architectural alterations, a sharp reduction in the number of goblet cells, and the presence of a chronic inflammatory infiltrate in the lamina propria (thick arrow) and submucosa (thin arrows). (E) Histological scoring of colon samples was performed in a blinded fashion by a pathologist. The *y* axis shows the sum of the histological scores for the following parameters: mucosal thinning (on a scale 0 to 3), goblet cell depletion (on a scale 0 to 3), mucosal inflammatory-cell infiltration (on a scale 0 to 3), and submucosal inflammatory-cell infiltration (on a scale 0 to 3). Three mice per experimental group were used.

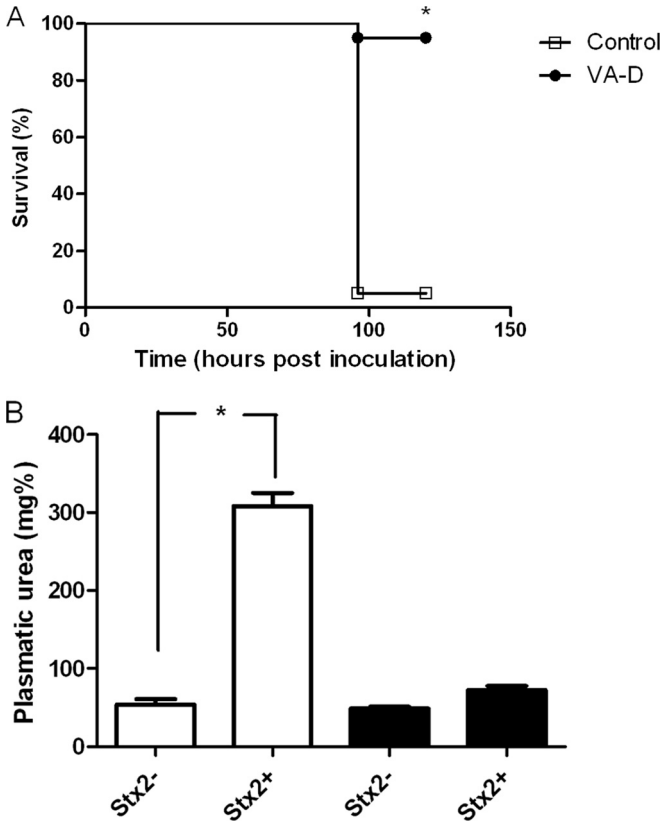

FIG 5 VA deficiency and Stx2 inoculation. (A) The survival of control (white squares) and VA-D (black circles) mice was monitored daily after the i.v. administration of 1 LD_{100} of Stx2. Ten mice per experimental group were used. *, $P < 0.05$ (log rank test). The experiment was performed twice with similar results. (B) Control (white bars) and VA-D (black bars) mice were bled 96 h after i.v. inoculation with Stx2, and plasma urea concentrations were evaluated. The values shown are mean urea concentrations (mg%) ± the standard errors. All data were analyzed by ANOVA and *a posteriori* Tukey test. *, $P < 0.05$. Five mice per experimental group were used. The experiment was performed twice with similar results.

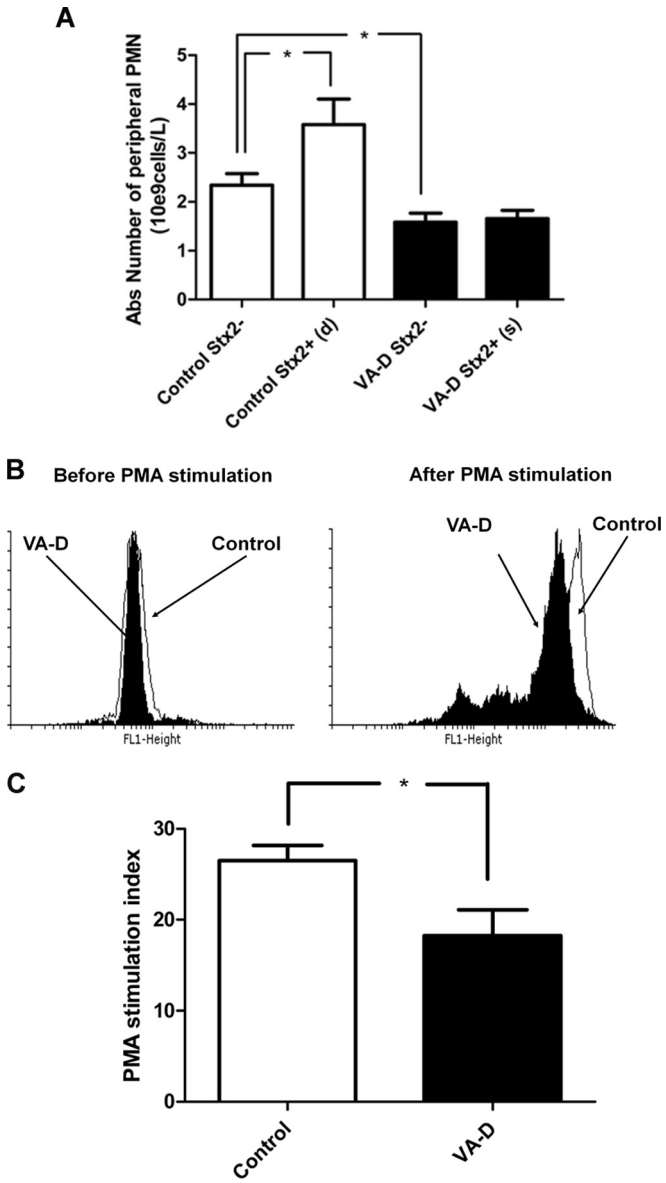

FIG 6 VA deficiency and PMNs. (A) Control and VA-D mice were bled 96 h after i.v. inoculation with 1 LD_{100} of Stx2, and the number of PMNs in their blood was determined. Control (white bars) or VA-D (black bars) mice were classified as $Stx2^-$ (four per group) or $Stx2^+$ (six per group). Mice were classified retrospectively according to their evaluation as survivors (s) or dead (d). The values shown are mean numbers of PMNs (10^9 cells/liter) ± the standard errors. All data were analyzed by ANOVA and *a posteriori* Tukey test. *, $P < 0.05$. The experiment was performed three times with similar results. Abs, absolute. (B, C) Control and VA-D mice were bled, PMNs were isolated, and ROS production was measured by flow cytometry by using 2×10^5 PMNs per experimental group, as detailed in Materials and Methods. (B) Representative histograms showing MFI before and after PMA stimulation of previously DHR-123-treated PMNs. (C) The PMA stimulation index (MFI after PMA stimulation/MFI before PMA stimulation) of VA-D mice was significantly decreased. *, $P < 0.05$. Data were analyzed by Student *t* test. Samples from eight mice were pooled in pairs into four pools per experimental group. The experiment was performed twice with similar results.

Histological examination of the kidneys showed that control and VA-D noninfected mice had similar and normal histology (Fig. 3A and C). On the contrary, the kidneys of control and VA-D mice that died after EHEC infection showed severe alterations, including mesangial hypercellularity and altered tubular epithelia (Fig. 3B and D).

Since all of the control and VA-D mice that died after infection displayed high plasma urea concentrations and renal histology compatible with renal failure associated with Stx2 toxicity, these results suggest that Stx2-related complications were the cause of death in the experiments performed, even in VA-D mice.

The guts of VA-D mice that died after EHEC infection were severely affected. It is very well known that VA is involved in the homeostasis of the gut epithelial mucosa (21, 44). Since EHEC affects the colons of both humans and mice (45), colon tissue samples were excised to analyze pathological changes in VA-D mice before and after EHEC infection.

Histological examination showed that the colons of noninfected VA-D mice had a decreased number of goblet cells (Fig. 4C), as previously reported (21).

In addition, although colon samples from both control and VA-D mice that died after infection were altered (Fig. 4B and D), colon samples from VA-D mice showed more severe damage than did those from control mice, as evidenced by higher mucosal thinning scores, depletion of goblet cells, and infiltration of both mu-

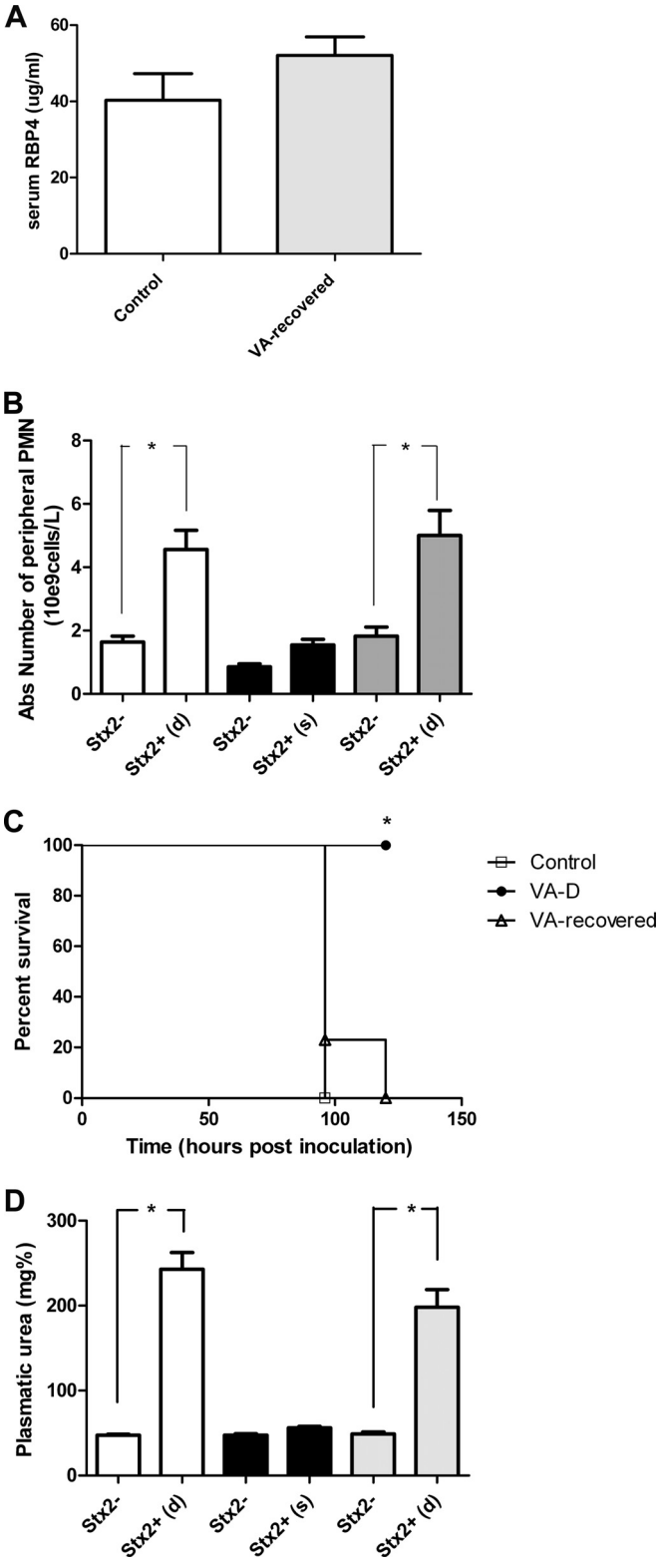

FIG 7 Reversibility of VA deficiency. (A) RBP4 concentrations in the serum (μg/ml) of control (white bar) and VA-recovered (gray bar) mice were measured. The values shown are means ± the standard errors (five mice per experimental group were used). No significant differences in RBP4 concentrations were detected. The experiment was performed twice with similar results. (B) Control (white bars), VA-D (black bars), and VA-recovered (gray) mice were bled 96 h after i.v. inoculation with 1 LD_{100} of Stx2 (six mice per experimental group), and the absolute (Abs) number of PMNs in blood was evaluated. Mice were classified retrospectively according to their evaluation as survivors (s) or dead (d). All data were analyzed by ANOVA and *a posteriori* Tukey test. *, $P < 0.05$. Four non-Stx2-inoculated mice per control, VA-D, or VA-recovered group were used. The experiment was performed three times with similar results. (C) The survival of control (white squares), VA-D (black circles), and VA-recovered (black triangles) mice was monitored daily after the i.v. administration of 1 LD_{100} of Stx2 (six mice per experimental group were used). *, $P < 0.05$ (log rank test comparing VA-D and VA-recovered mice). The experiment was performed twice with similar results. (D) Control (white bars), VA-D (black bars), and VA-recovered (gray bars) mice were bled 96 h after i.v. inoculation with Stx2, and the urea concentrations in their plasma were evaluated. The values shown are mean concentrations (mg%) ± the standard errors. All data were analyzed by ANOVA and *a posteriori* Tukey test. *, $P < 0.05$. Six Stx2-inoculated and five non-Stx2-inoculated mice per control, VA-D, or VA-recovered group were used. The experiment was performed twice with similar results.

cosal and submucosal cells. Figure 4E depicts the histological scores of different parameters of tissue damage for each experimental group of mice. The damage and the score increased in the following order: noninfected control mice, noninfected VA-D mice, EHEC-infected control mice that died, and EHEC-infected VA-D mice that died. Control or VA-D mice that did not die after EHEC infection showed histology similar to that of the corresponding noninfected mice.

Thus, these results show that VA deficiency not only affects the basal state of the epithelium but also potentiates the damage induced by EHEC infection.

VA-D mice are less susceptible to i.v. inoculation with Stx2. In order to evaluate the influence of VA deficiency directly on Stx2 susceptibility, control and VA-D mice were i.v. inoculated with 1 LD_{100} of Stx2.

Our results showed that VA deficiency made mice resistant to Stx2, at least to the Stx2 dose evaluated. Only 5% (1/20) of the VA-D mice died 96 h after Stx2 inoculation, while 95% (19/20) of the control mice did so (Fig. 5A).

In concordance with the survival rate, only control mice inoculated with Stx2 showed increased plasma urea concentrations at 96 hpi (Fig. 5B).

The fact that VA deficiency made mice resistant to 1 LD_{100} of Stx2 strongly suggests that VA deficiency attenuates susceptibility to EHEC infection by decreasing susceptibility to Stx2.

VA-D mice show alterations in the number of PMNs before and after Stx2 inoculation. We have previously reported that neutrophilia plays an important pathogenic role in Stx2 toxicity (17, 39, 46, 47). To gain insight into the mechanisms involved in Stx2 resistance, the influence of VA deficiency on the number of PMNs was analyzed. Interestingly, VA-D mice had a lower basal number of PMNs than control mice (Fig. 6A). In addition, the inoculation of 1 LD_{100} of Stx2 increased the absolute number of PMNs only in control mice (Fig. 6A).

These results indicate that VA deficiency decreases the basal number of PMNs and abrogates the increase in those cells after Stx2 inoculation.

ROS production by PMNs is decreased in VA-D mice. Since the oxidative stress induced by Stx2 plays an important role in the pathogenicity of HUS (16), the production of ROS by PMNs from VA-D mice was assessed.

Figure 6B depicts the production of ROS by DHR-123-treated PMNs from control and VA-D mice before and after PMA stim-

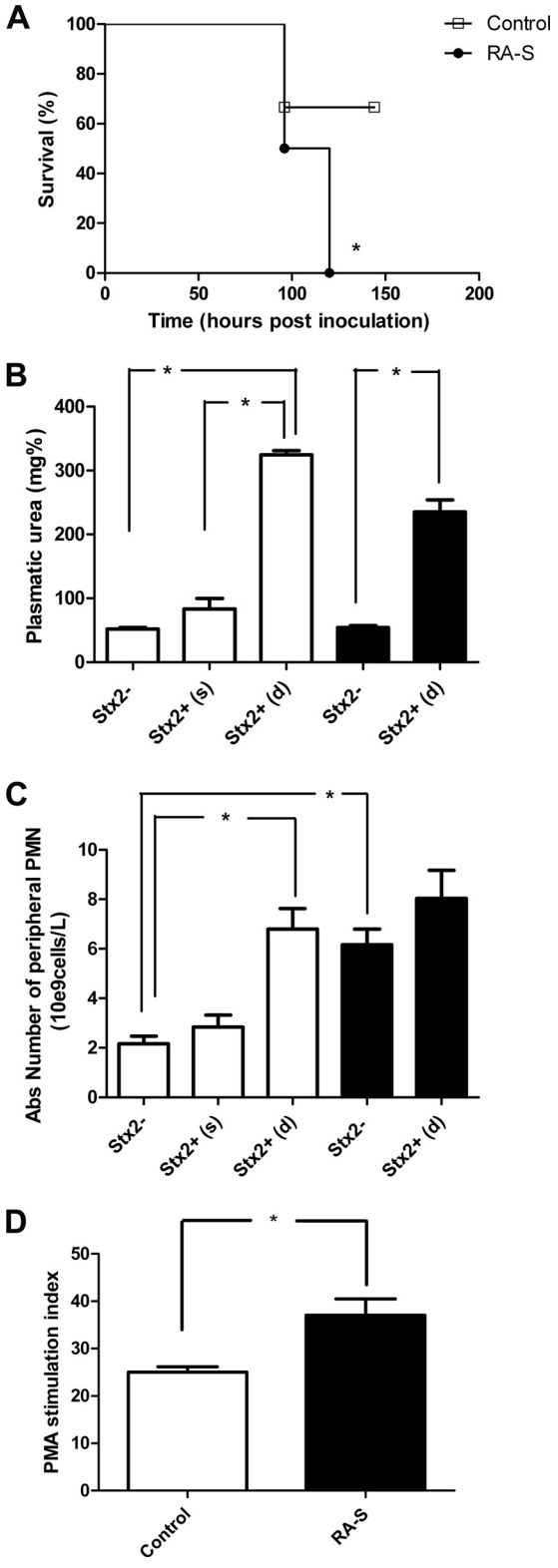

FIG 8 RA supplementation influences Stx2 toxicity, PMN numbers, and ROS production. (A) The survival of control (white squares) and RA-S (black circles) mice was monitored daily after the i.v. administration of 1 LD_{30} of Stx2. Twelve mice per experimental group were used. *, $P < 0.05$ (log rank test). The experiment was performed twice with similar results. (B, C) Control (white bars) and RA-S (black bars) mice were bled 96 h after i.v. inoculation with Stx2. Mice were classified retrospectively according to their evaluation as survivors (s) or dead (d). Numbers of control mice per experimental group: noninfected ($Stx2^-$), 4; $Stx2^+$(s), 8; $Stx2^+$(d), 4. Numbers of RA-S mice per experimental group: noninfected ($Stx2^-$), 4; $Stx2^+$(d), 12. (B) The values shown are mean urea concentrations (mg%) ± the standard errors. All of the data were analyzed by ANOVA and *a posteriori* Tukey test. *, $P < 0.05$. (C) The values shown are mean percentages of PMNs ± the standard errors. All of the data were analyzed by ANOVA and *a posteriori* Tukey test. *, $P < 0.05$. The experiment was performed three times with similar results. Abs, absolute. (D) Control and RA-S mice were bled, PMNs were isolated, and ROS production was measured by flow cytometry as detailed in Materials and Methods. The PMA stimulation index (MFI after PMA stimulation/MFI before PMA stimulation) of RA-S mice was significantly increased (*, $P < 0.05$). Data were analyzed by Student *t* test; samples from eight mice were combined in pairs into four pools per experimental group. The experiment was performed twice with similar results.

ulation. Figure 6C shows that the PMA stimulation index (MFI after PMA stimulation/MFI before PMA stimulation) in VA-D mice is significantly lower than that of control mice. Thus, our results indicate that not only the number but also the oxidative response of PMNs is decreased in VA-D mice.

The effect of VA deficiency on Stx2 toxicity is reversible. To confirm that VA deficiency accounted for the high Stx2 resistance observed, 3-month-old VA-D mice were switched to a normal diet for 1 month to reverse their VA deficiency (VA-recovered mice). VA-recovered mice had normal levels of RBP4 in serum and normal numbers of PMNs in blood and became as susceptible as control mice to 1 LD_{100} of Stx2 (Fig. 7A to C). Moreover, an Stx2 challenge increased the plasma urea concentrations at 96 hpi in both control and VA-recovered mice, confirming that low retinol levels account for Stx2 resistance (Fig. 7D).

RA administration potentiates Stx2 toxicity. Since VA deficiency made mice resistant to Stx2 toxicity, we analyzed whether RA supplementation increases susceptibility to Stx2. For these experiments, control mice were inoculated with a small dose of Stx2 that induces a mortality rate of around 30% (1 LD_{30}). Strikingly, RA-S mice inoculated with 1 LD_{30} had a 100% mortality rate (Fig. 8A) and all of them had high plasma urea concentrations (Fig. 8B). The mortality rate of noninoculated control and RA-S mice was 0%. Interestingly, RA-S mice had an increased number of PMNs before Stx2 intoxication, similar to the number of PMNs in control mice after Stx2 inoculation (Fig. 8C). Finally, DHR-123-treated PMNs from RA-S mice showed a higher ROS production level after PMA stimulation (Fig. 8D). Taken together, these results demonstrate that RA supplementation potentiates Stx2 toxicity, at least in part, by increasing the number and oxidative response of PMNs.

DISCUSSION

This is the first report showing that retinoid levels influence the outcome of EHEC infection in mice. Interestingly, results reported here showed that VA deficiency did not increase the EHEC-associated mortality rate of mice, as expected *a priori*, but instead conferred an overall resistance to EHEC pathogenicity.

Since Stx2 is the most important pathogenic factor that influences survival after EHEC infection, biochemical and histological parameters associated with Stx2 toxicity were evaluated. All control or VA-D mice that died after infection displayed signs of renal damage, compatible with Stx2-related toxicity, as we have previously shown (43), confirming that Stx2-related complications were the cause of death in the experiments performed, even in VA-D mice.

It is well known that retinoids play an important role in gut homeostasis and intestinal epithelial integrity (21). In line with this, our results showed that VA deficiency worsened the intestinal phase of the disease. Indeed, the colon histology of VA-D mice showed severe alterations under both basal and postinfective conditions. Noninfected VA-D mice showed mucosa thinning and depletion of goblet cells compared with noninfected control mice, indicating that the VA-D mouse gut shows changes corresponding to VA deficiency. Moreover, although both control and VA-D mice showed relevant tissue damage after EHEC-infection, VA-D mice displayed a more severe score of damage that included mucosal and submucosal inflammatory cell infiltration. In spite of having an altered gut epithelium, VA-D mice had a better outcome of EHEC infection, suggesting that VA deficiency may attenuate the systemic complications due to Stx2.

To directly analyze the influence of VA deficiency on the systemic effects of Stx2, a large dose of Stx2 (1 LD_{100}) was i.v. inoculated into control and VA-D mice.

Of interest, VA-D mice were much less susceptible than control mice to Stx2. In fact, the difference in survival between VA-D and control mice was greater after i.v. inoculation with Stx2 than after EHEC infection.

It has been reported that VA deficiency also decreases the levels of intestinal IgA antibodies, defensins, and mucus, allowing easier translocation of bacteria or bacterial factors (48–50). Thus, it is reasonable to speculate that larger quantities of pathogenic bacterial factors, including Stx2 and lipopolysaccharide (LPS), would gain access to the bloodstream in VA-D mice during EHEC infection, suggesting that gut alterations in VA-D mice tend to decrease the huge difference in survival observed after the i.v. inoculation of Stx2. In other words, these results indicate that VA deficiency induces intestinal damage but at the same time greatly decreases Stx2 susceptibility, leading to a final attenuation of EHEC pathogenicity.

After ingesting a control diet, VA-recovered mice lost Stx2 resistance and became as susceptible as control mice to i.v. inoculation with Stx2, further demonstrating that the effect of VA deficiency on Stx2 toxicity is reversible and supporting a close correlation between VA levels and susceptibility to Stx2 toxicity.

Clinical and experimental evidence has shown not only that PMNs directly participate in the pathogenic mechanism of Stx2 (14, 16) but also that retinoids modulate PMN maturation (31) and phagocytic function (30, 51). Thus, the number of circulating PMNs and their ROS-producing capacity were analyzed in order to gain insight into the mechanisms behind the Stx2 resistance of VA-D mice. Interestingly, VA-D mice had a reduced basal number of PMNs, which did not increase after Stx2 intoxication, and PMNs showed a decreased capacity to produce ROS upon *in vitro* activation, indicating that the functionality of those cells is affected.

Since VA deficiency attenuated Stx2 toxicity, we directly addressed whether RA supplementation would potentiate it. Notably, RA-S mice were more susceptible than control mice to a small dose of Stx2 (1 LD_{30}), as evidenced by increased mortality rates and Stx2-associated renal dysfunction. Moreover, RA treatment *per se* increased the absolute number of circulating PMNs and the ROS-producing capacity of those cells. These results suggest that a higher number and a greater oxidative response of PMNs contribute to the potentiation of Stx2 toxicity in RA-S mice. Taken together, these results strongly suggest that alterations in PMNs account, at least in part, for the direct relationship between VA levels and susceptibility to Stx2 toxicity.

Further investigations are necessary to delineate which functions mediated by neutrophils are critical for defining EHEC evolution. Gut infiltration, phage induction, delivery of Stx, the oxidative response, bystander endothelial damage, and activation of the thrombotic cascade are among the multiple PMN-pathogenic activities described during Stx intoxication.

Interestingly, some studies have reported that VA or RA supplementation may, under some circumstances, cause a detrimental effect influencing the inflammatory response. For instance, mice fed high dietary levels of VA showed severe lung hyperactivity and inflammation in a mouse model of asthma, while VA deficiency impaired these processes (21); RA supplementation of rats injected with LPS led to increased nitric oxide synthase 2 pathway activation and decreased host survival (37); oral treatment with an agonist of RA receptor α/β during *Trichuris muris* infection had a proinflammatory effect on mice (52); and patients with acute promyelocytic leukemia undergoing RA-based therapy may develop the RA syndrome, which in some cases includes pulmonary neutrophilic infiltrates and other effects resembling molecular events that occur during inflammatory responses (32, 53).

Thus, although it is well established that VA supplementation is generally beneficial, in particular, in cases of children with malnutrition (54), results presented herein suggest that VA or RA supplementation may have a detrimental effect when it exceeds normal values, acting in a proinflammatory manner. In addition, this draws attention to the implications that VA supplementation might have for patients with EHEC-associated diarrhea. In line with this conclusion, an updated meta-analysis has shown that VA supplementation has no consistent overall protective effect on the incidence of diarrhea, suggesting that further research is required to understand the mechanisms involved in each pathogenic infection (28).

Although we did not detect alterations in the Stx2 cellular receptor (Gb3) in renal samples from VA-D or RA-S mice (data not shown), further research is required to completely define the mechanisms that underlie the interaction between VA levels and Stx2 susceptibility.

This is the first report showing that retinoid levels may significantly modulate the outcome of EHEC infection in mice by modulating the absolute numbers and function of PMNs. Moreover, the close correlation between PMN status and Stx2 susceptibility highlights the key role of PMNs in EHEC infection.

Taken together, these results are in line with the very well-known function of retinoids in maintaining the homeostasis of the gut but support the idea that they can act in a proinflammatory manner by affecting at least the PMN population.

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