Retinoic acid induces homing of protective T and B cells to the gut after subcutaneous immunization in mice

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Diarrheal diseases represent a major health burden in developing countries. Parenteral immunization typically does not induce efficient protection against enteropathogens because it does not stimulate migration of immune cells to the gut. Retinoic acid (RA) is critical for gut immunity, inducing upregulation of gut-homing receptors on activated T cells. In this study, we have demonstrated that RA can redirect immune responses elicited by s.c. vaccination of mice from skin-draining inguinal LNs (ingLNs) to the gut. When present during priming, RA induced robust upregulation of gut-homing receptors in ingLNs, imprinting gut-homing capacity on T cells. Concurrently, RA triggered the generation of gut-tropic IgA$^+$ plasma cells in ingLNs and raised the levels of antigen-specific IgA in the intestinal lumen and blood. RA applied s.c. in vivo induced autonomous RA production in ingLN DCs, further driving efficient induction of gut-homing molecules on effector cells. Importantly, RA-supplemented s.c. immunization elicited a potent immune response in the small intestine that protected mice from cholera toxin–induced diarrhea and diminished bacterial loads in Peyer patches after oral infection with *Salmonella*. Thus, the use of RA as a gut-homing navigator represents a powerful tool to induce protective immunity in the intestine after s.c. immunization, offering what we believe to be a novel approach for vaccination against enteropathogens.

Introduction

Diarrheal diseases are among the major causes of death in developing countries, particularly in young children. Although many parenterally applied vaccines are highly efficient in preventing systemic infectious diseases, this route of vaccination frequently results in rather weak protection against enteropathogens. This is mainly caused by the fact that both effector T cells and antibody-producing plasma cells induced by antigen (applied s.c., intramuscularly, or intravenously) fail to express gut-homing molecules; consequently, a protective shield in the gut mucosa is not installed properly. On the other hand, it is well documented that oral application of appropriate vaccines allows for the induction of protective mucosal immunity in the intestine. Indeed, several studies have shown that such a vaccination strategy generates far more potent mucosal protection than does systemic immunization (1–4). Unfortunately, in developing countries, several oral vaccines have performed rather poorly compared with their effect in industrialized states. Factors responsible for inadequate protection conferred by oral vaccines in developing countries include poor sanitation, digestive and absorptive malfunction, intestinal flora overgrowth, and vitamin A deficiency of the vaccines (5). Moreover, oral vaccination can result in immune tolerance when low-virulent or dead material is used as antigen. Together, these findings underline the urgent need for vaccines that generate effective and long-lasting immunity in the digestive tract.

Effective pathogen clearance requires rapid migration of effector T cells to the site of infection. The homing capacity of effector T cells to distinct nonlymphoid tissues depends on the lymphoid organ in which lymphocyte activation occurs (reviewed in ref. 6). This represents a key feature of the locally restricted immune subsystems. Migration of antigen-experienced T cells into the small intestine requires expression of α4β7-integrin and the chemokine receptor CCR9. The CCR9/CCL25 interaction also guides plasma cells as well as plasmacytoid DCs into the small intestine (7, 8). In contrast, effector T cells expressing E-selectin ligand (ESL), P-selectin ligand (PSL), CCR4, and very late antigen–4 (VLA-4) preferentially home into the inflamed skin.

Several studies indicated that DCs play a decisive role in the imprinting of tissue tropism of effector T cells. In vitro stimulation by gut-associated lymphoid tissue–derived (GALT-derived) DCs is sufficient to induce expression of α4β7-integrin and CCR9 on T cells (9, 10), whereas DCs isolated from skin-draining LNs confer expression of CCR4, PSL, and ESL (11, 12). LN transplantation studies demonstrated that, aside from GALT-derived DCs, mesenteric LN (mLN) stromal cells are essential for the in vivo generation of gut-homing T cells (13, 14). A key component inducing the expression of α4β7-integrin and CCR9 is all-*trans* retinoic acid (RA) (15). RA is generated from dietary vitamin A involving retinal dehydrogenases (RALDHs). The ability of GALT-derived DCs and mLN stromal cells to induce gut-tropic T cells critically rests on their selective expression of RALDH (13–15). In addition to imprinting activated T cells, GALT-derived RA confers gut-tropism to B cells and synergizes with IL-6 and IL-5 to induce IgA secretion (16). IgA antibodies represent the predominant Ig class in mucosal secretions and provide specific protection of mucosal surfaces by blocking attachment of pathogens.

Given that the vitamin A metabolite RA confers gut-tropism to effector lymphocytes and acts as cofactor for IgA class switch, we hypothesized that mucosal protection can be induced by s.c. antigen application in the presence of RA. Indeed, we found that such an immunization approach resulted in the induction of gut-

Conflict of interest: The authors have declared that no conflict of interest exists.
Citation for this article: *J Clin Invest* doi:10.1172/JCI44262.

homing molecules and allowed for robust homing of effector T cells and plasma cells to the small intestine. Moreover, s.c. immunization combined with RA treatment established a pronounced IgA response in serum as well as intestinal washes. As a result, RA supplementation after s.c. immunization conferred mucosal protection against cholera toxin–induced (CT-induced) diarrhea and reduced bacterial burden in Peyer patches after oral *Salmonella* infection. Mechanistically, we found that RA applied s.c. induced increased RALDH activity in ingLN DCs in vivo. These DCs subsequently triggered expression of gut-homing molecules on activated T and B cells. Together, these results suggested that RA-supplemented s.c. vaccination efficiently induces intestinal protection by redirecting immune responses initiated in skin-draining LNs toward the small intestine. We believe these findings offer new alternatives for the development of vaccination strategies against enteric pathogens.

Results

s.c. RA imprints gut-homing specificity on T cells primed in skin-draining LNs. RA has previously been shown to efficiently instruct activated lymphocytes to upregulate the gut-homing molecules CCR9 and α4β7-integrin in vitro (15). We therefore sought to determine whether local abundance of RA, provided by s.c. delivery, allows for induction of gut-homing molecules on activated T cells in the skin-draining inguinal LN (ingLN). To test whether RA provokes conversion of the homing receptor pattern commonly induced in skin-draining LNs, TCR transgenic OT-I or DO11.10 cells (see Methods) were fluorescently labeled with CFSE and adoptively transferred into C57BL/6 or BALB/c wild-type recipients, respectively; 2 hours later, mice received a single s.c. injection of 50 μg ovalbumin (Ova) into the flanks or were fed 50 mg Ova by gavage. We used Ova of greater than 90% purity, which is known to contain immunologically relevant amounts of endotoxins that served as adjuvant in this experimental setup. A group of s.c. immunized mice additionally received s.c. injections of 150 μg all-*trans* RA on days 0, 1, and 2 after antigen delivery. On day 3, cells were isolated from gut-draining mLNs as well as skin-draining ingLNs and analyzed by flow cytometry. After standard s.c. immunization, $CD8^+$ OT-I T cells proliferating in the ingLN acquired a skin-homing phenotype, characterized by upregulation of PSL and ESL and no apparent expression of α4β7-integrin or CCR9 (Figure 1, A and B). In contrast, T cells primed in ingLNs of RA-treated mice readily upregulated α4β7-integrin and CCR9, albeit to a lesser extent than did T cells activated in the mLNs after oral application of Ova. Notably, $CD8^+$ OT-I cells of RA-treated mice no longer showed strong and characteristic induction of the skin-homing molecules PSL and ESL, but were comparable to OT-I cells primed in mLNs with regard to the expression of these molecules (Figure 1, A and B). Similarly, $CD4^+$ DO11.10 T cells upregulated α4β7-integrin and CCR9 after s.c. antigen plus RA application, even though expression levels were lower than in DO11.10 cells activated in the mLNs after oral antigen delivery (Figure 1, C and D). Similar results were obtained with $CD4^+$ OT-II cells (data not shown). Given the role of RA in inducing Tregs, we sought to determine whether addition of RA affects Treg generation. s.c. RA treatment did not enhance the induction of $FoxP3^+$ Tregs (Figure 1, E and F), in contrast to a previous report showing generation of $FoxP3^+$ T cells in the skin-draining LN after s.c. RA application (17). Of note, the considerably higher amounts of RA used in the prior study might account for the observed differences.

In contrast to all-*trans* RA, the isomeric 13-*cis* RA did not affect the regulation of CCR9 and α4β7-integrin and induced only a mild reduction of ESL and PSL expression (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI44262DS1). To determine whether the effect of RA is restricted to the draining LN, we analyzed the pattern of induced homing receptors in popliteal LNs of mice receiving s.c. Ova into the footpad and s.c. RA into the neck region. Of interest, the popliteal LN supported induction of gut-homing receptors and concomitant suppression of skin-homing receptors, albeit to a lesser extent compared with draining axial LNs of mice that received both Ova and RA s.c. into the neck region (Supplemental Figure 2). Thus, the effect was strongest in — but not restricted to — the LN draining the site of RA injection.

Together, these observations show that all-*trans* RA delivered exogenously via the skin allowed for efficient induction of gut-homing molecules on responding T cells in ingLNs. Our findings are consistent with previous in vitro studies showing that RA-mediated induction of gut-homing molecules goes along with reduced expression of skin-homing molecules (12), which would be the default pathway for T cells activated in skin-draining LNs.

We next addressed whether RA-induced upregulation of gut-homing molecules in vivo actually allows for efficient homing of activated $CD8^+$ and $CD4^+$ T cells to the small intestine. We compared the frequencies of adoptively transferred OT-I or DO11.10 cells in the small intestine of RA-treated and nontreated wild-type recipients on day 5 after s.c. immunization with Ova. As expected, after standard s.c. immunization, very few $CD8^+$ or $CD4^+$ T cells migrated to the small intestine, as determined by the frequency of $Ly5.1^+V\beta5^+$ cells within the $CD8\alpha\beta^+$ population and the by frequency of DO11.10 cells (as determined by the clonotypic mAb KJ1-26) within the $CD4^+$ population (Figure 2). Compared with mice immunized according to the standard s.c. protocol, both s.c. RA-treated and Ova-gavaged animals showed increased frequencies of OT-I cells recruited to the intestinal epithelial lymphocyte (IEL) and lamina propria lymphocyte (LPL) compartments of the small intestine (Figure 2). It should be noted that only the s.c. Ova plus RA route reached statistical significance in recruiting OT-I cells to the IEL compartment; Ova gavage did not cause a statistically significant increase of OT-I cells in the IEL or LPL compartment (Figure 2B). Given that the intestinal epithelium rather than the lamina propria is the major site where $CD8^+$ T cells reside, our findings demonstrated that s.c. application of RA together with antigen is most effective in recruiting antigen-specific $CD8^+$ T cells to the intestinal epithelium. Along the same lines, s.c. application of antigen together with RA was efficient in recruiting $CD4^+$ T cells to the LPL as well as the IEL compartment, whereas oral application by gavage had no effect on recruiting these cells to either compartment (Figure 2, C and D). Collectively, these data demonstrate that the s.c. application of RA allows not only the induction of gut-homing molecules on activated $CD4^+$ and $CD8^+$ T cells, but also their efficient homing to the small intestine.

Skin-draining LNs are the major site for the generation of gut-tropic effector T cells after s.c. antigen and RA application. After s.c. RA injection, gut-homing molecules are induced in the skin-draining LNs. However, it remains possible that a large fraction of the gut-tropic effector cells did not acquire gut-homing capacity in the skin-draining LNs but instead disseminated after priming into distant LNs (18), such as the mLN, to acquire gut-tropism there. To assess whether ingLN imprinting in RA-treated mice is sufficient to generate sub-

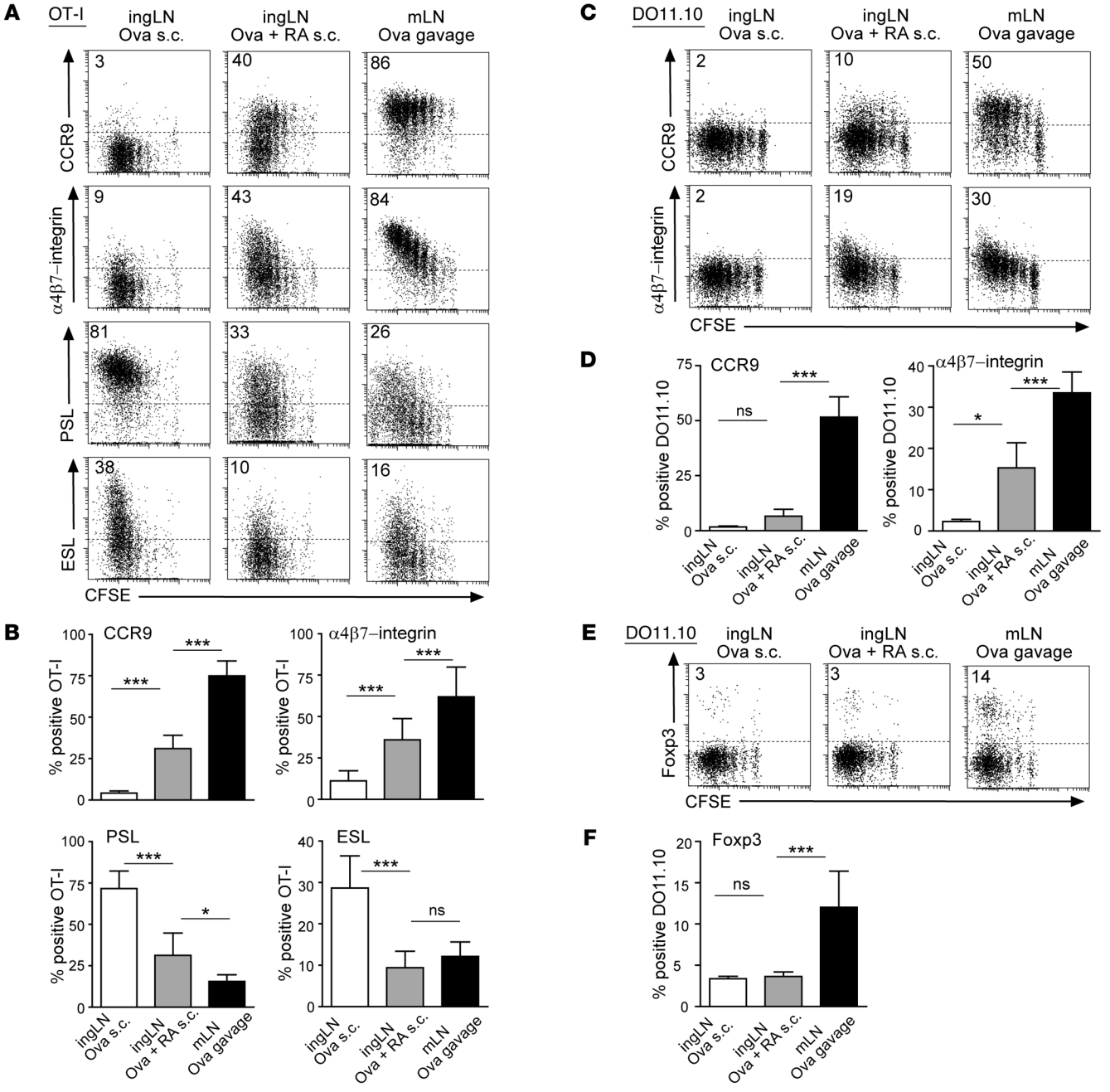


Figure 1

s.c. Ova immunization with RA as additive leads to upregulation of gut-homing molecules on antigen-specific T cells in skin-draining ingLNs. CFSE-labeled OT-I or DO11.10 cells were adoptively transferred into C57BL/6 or BALB/c mice on day 0. 2 hours later, a single dose of Ova was applied orally or injected s.c. A group of s.c. immunized mice additionally received s.c. injections of 150 μg all-*trans* RA on days 0, 1, and 2. (**A**, **C**, and **E**) Representative flow cytometry plots obtained on day 3 for α4β7-integrin, CCR9, Foxp3, ESL, and PSL expression on OT-I ($DAPI^-Ly5.1^+V\beta5^+CD8\beta^+$; **A**) or DO11.10 ($DAPI^-CD4^+DO11.10^+$; **C** and **E**) T cells activated in the mLN after oral antigen application or in the ingLN after s.c. injection of antigen. Numbers indicate percent positive OT-I or DO11.10 cells. (**B**, **D**, and **F**) Percent $\alpha4\beta7\text{-integrin}^+$, $CCR9^+$, $Foxp3^+$, ESL^+, and PSL^+ cells among transferred OT-I T cells (**B**) or DO11.10 T cells (**D** and **F**) from the indicated organs. Data were derived from 6–12 mice per group analyzed in 3 independent experiments (OT-I) or 6–10 mice per group analyzed in 2 independent experiments (DO11.10). $^*P < 0.05$; $^{***}P < 0.001$.

stantial amounts of gut-tropic effector cells, we compared the generation of gut-homing T cells in mice whose mLN was surgically removed with mice that underwent sham surgery. At 5 days after s.c. RA-supplemented Ova immunization, mLN-explanted OT-I recipients showed the same frequency of antigen-specific T cells in the small intestine as did sham-manipulated OT-I recipients (Figure 3). These data show that the mLN does not contribute to the generation of gut-tropic T cells after s.c. immunization and RA

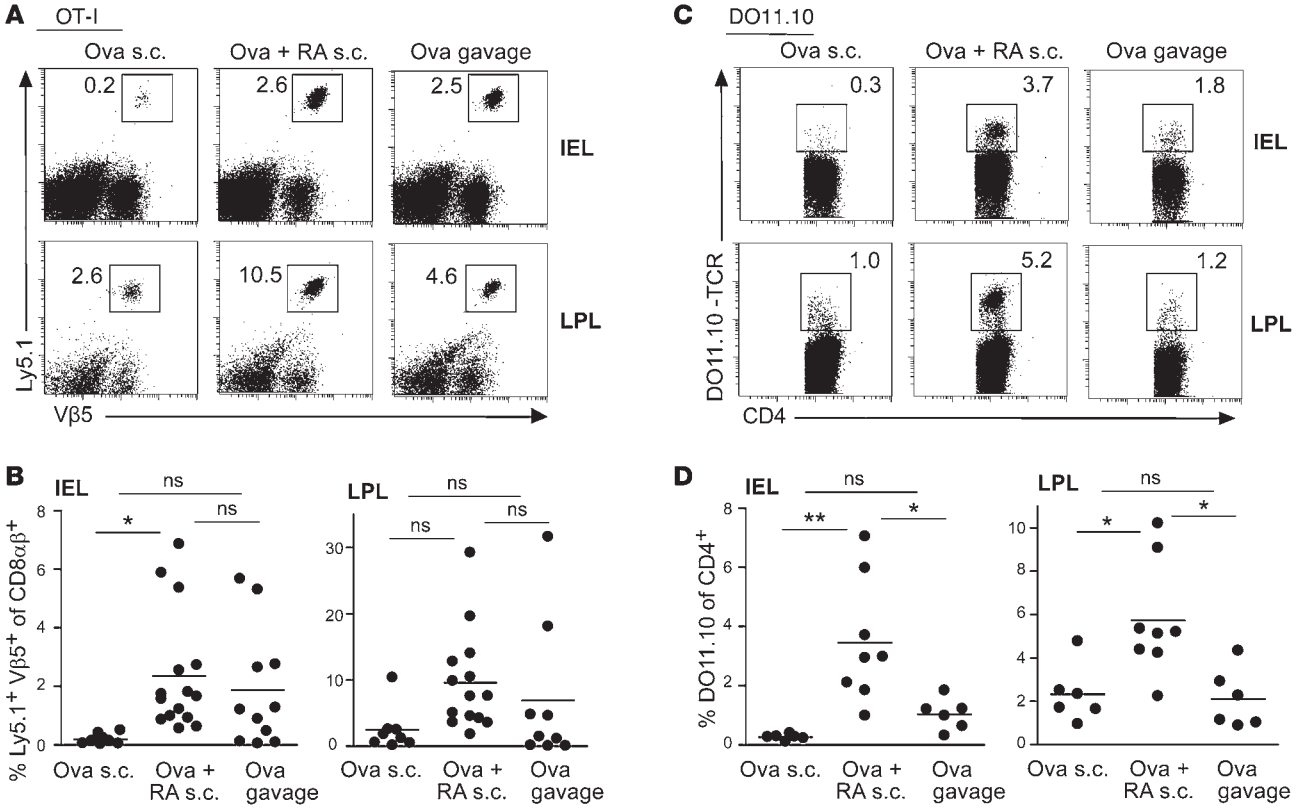


Figure 2

Exogenous RA imprints gut-homing specificity on T cells primed in skin-draining ingLN. CFSE-labeled OT-I or DO11.10 cells were adoptively transferred into C57BL/6 or Balb/c mice on day 0. 2 hours later, a single dose of Ova was applied orally or injected s.c. A group of s.c. immunized mice received additional s.c. RA injections on days 0, 1, and 2. T cell homing to the small intestine was determined on day 5. (**A** and **C**) Representative flow cytometry plots for OT-I (**A**) and DO11.10 (**C**) T cell migration to the small intestinal IEL and LPL compartments. (**B**) Percent Ly5.1$^+$Vβ5$^+$ cells within the gated CD8αβ$^+$ population. (**D**) Percent DO11.10-TCR$^+$ cells within the gated CD4$^+$ population. Bars represent mean values; symbols represent individual mice pooled from 3 (OT-I) or 2 (DO11.10) independent experiments. *P < 0.05; **P < 0.01.

treatment. Instead, local priming and imprinting in skin-draining LN is responsible and sufficient for the induction of gut-homing lymphocytes upon s.c. administration of RA.

s.c. RA application induces upregulation of gut-homing molecules on plasma cells and class switch to IgA in skin-draining ingLNs. RA not only confers gut-tropism to T and B cells, but also acts as a cofactor to promote IgA secretion (16). The finding that s.c. RA injection modifies the T cell response in skin-draining LN prompted us to investigate whether s.c. RA treatment similarly modifies the outcome of the humoral immune response. Mice were immunized on days 0 and 10 either s.c. with 1 μg CT or orally with 10 μg CT. A group of s.c. immunized mice additionally received 150 μg RA s.c. on days 0, 1, 2, 3, 6, 10, and 13 after antigen delivery. On day 14, the levels of CT-specific Igs in serum and intestinal wash were determined by ELISA. Although it provoked a strong IgG and IgM response in the serum, s.c. immunization with CT failed to elicit detectable anti-CT IgA in serum or intestinal wash (Figure 4A). In contrast, RA-treated mice showed increased levels of antigen-specific IgA levels in serum and intestinal wash, albeit to a lesser extent than orally immunized mice. Notably, antigen-specific IgM and IgG titers of RA-treated mice were as high as those of mice receiving only CT s.c. (Figure 4A). To extend these studies, we assessed the abundance of anti-CT IgA–secreting plasma cells in the small intestinal lamina propria by ELISPOT. Few anti-CT IgA-secreting plasma cells localized to the lamina propria of s.c. immunized mice (Figure 4B). Consistent with the increased antigen-specific IgA titers in the intestinal washes, RA-treated mice also showed increased numbers of anti-CT IgA-secreting plasma cells in the small intestine, and their numbers were comparable to those isolated from orally immunized mice. These observations demonstrated that RA-assisted s.c. immunization results in pronounced localization of antigen-specific plasma cells in the small intestine and a robust IgA response in serum and intestinal wash.

After oral challenge, induction of mucosal IgA secretion largely occurs in the organized GALT, such as mLN or Peyer patches. We therefore sought to determine the origin of IgA$^+$ plasma cells giving rise to the antigen-specific IgA detected in s.c. immunized RA-treated mice. Immunohistology of ingLN of RA-treated mice revealed the abundant presence of IgA$^+$ cells, which were absent in ingLN of s.c. immunized mice without RA treatment (Figure 4, C and D). IgA-expressing cells localized near Lyve-1$^+$ lymphatic endothelium, indicative of their imminent egress from the LN. Interestingly, a major fraction of the IgA$^+$ plasmablasts also expressed CCR9 in RA-treated mice, but not in nontreated mice (Figure 4, E and F), which explains their increased frequency in the intestinal lamina propria. Importantly, CT-binding plasma cells were detect-

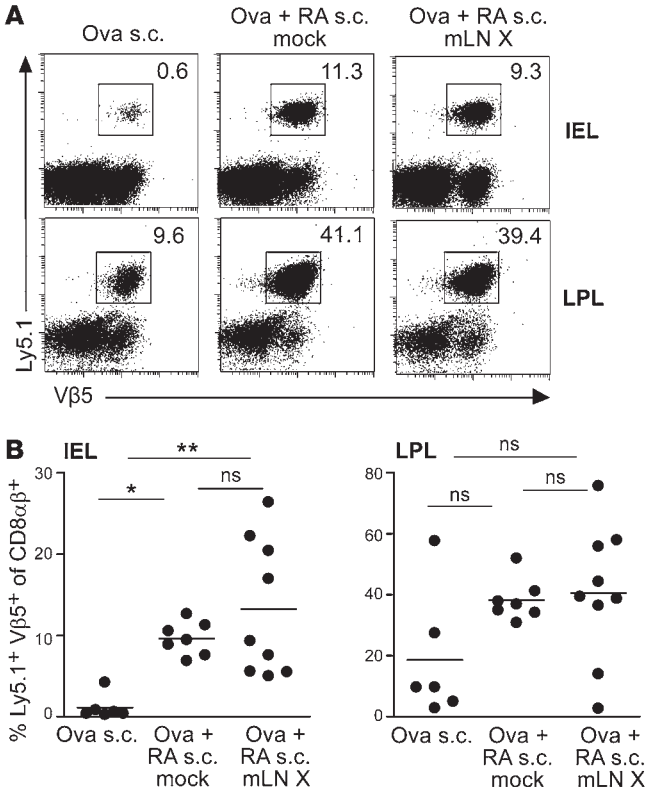


Figure 3
mLN does not contribute to the generation of gut-tropic T cells after s.c. immunization and RA treatment. mLN-explanted (mLN X) and mLN-sufficient mice were adoptively transferred with CFSE-labeled OT-I cells. Mice were s.c. immunized with Ova on day 0 and received additional s.c. RA injections on days 0, 1, and 2. T cell migration to the small intestine was determined on day 5. (**A**) Representative flow cytometry plots for OT-I T cell migration to the small intestinal IEL and LPL compartments. (**B**) Percent Ly5.1$^+$Vβ5$^+$ cells within the gated CD8αβ$^+$ population. Bars represent mean values; symbols represent individual mice pooled from 2 independent experiments. $^*P < 0.05$; $^{**}P < 0.01$.

ed in ingLN, but not mLN, of RA-treated mice (Figure 4, G and H), although IgA$^+$ plasma cells were frequently present in mLN. Since IgA$^+$ plasma cells were also found in mLN of nonimmunized mice (data not shown), these cells probably form a constitutively present population. Together, these results identify the skin-draining ingLN of RA-treated mice as a major site for the induction of gut-homing receptors and IgA expression of activated plasmablasts after s.c. RA injection.

RA-supplemented s.c. immunization protects mice from CT-induced diarrhea and diminishes bacterial loads in Peyer patches after oral Salmonella infection. To address whether the intestinal IgA antibodies induced by s.c. application of RA mediate protective immunity, mice were subjected to CT-induced diarrhea (19, 20). When applied orally, CT induces elevated levels of cAMP in intestinal epithelial cells, resulting in secretion of water and chloride ions into the small intestine. A previous study demonstrated that CT-specific secretory IgA in the intestinal lumen was responsible for protective immunity against CT-induced diarrhea (20). Therefore, groups of mice were immunized with CT s.c., orally, or s.c. plus RA treatment, or were left untreated. On day 14, mice were challenged orally by applying 10 μg CT by gavage. At 2 hours after CT challenge, mice were sacrificed, and the weight of the small intestine together with its fluid content was determined. The weight provides information about the degree of fluid influx and gives a rough estimate of antitoxic protection. Standard s.c. immunization did not establish prominent protection, and no significant difference was found in the fluid influx compared with that in nonimmunized animals (Figure 5A). In contrast, the concomitant s.c. RA treatment generated a protective immunity which was comparable in degree to that induced by oral CT immunization. Importantly, the protection provided by s.c. application of the antigen plus RA treatment prevented any detectable fluid influx, since there was no significant increase in weight compared with nonimmunized, nonchallenged mice (Figure 5A). In addition to antitoxic protection, immunity against viable mucosal pathogens is also of high importance; therefore, we assessed protection against *Salmonella* infection. Mice were s.c. immunized with dead *Salmonella* Typhimurium SL1344 lacking the *aroA* gene on days 0 and 2. A group of s.c. immunized mice additionally received s.c. injections of RA on days 0, 1, 2, 3, 4, 7, 8, and 9. On day 14, mice were orally infected with wild-type *Salmonella* Typhimurium SL1344, and 2 days after infection, bacterial loads were determined in single Peyer patches. RA-treated mice showed significantly less bacteria in Peyer patches than did mice lacking RA treatment (Figure 5B). This difference was more prominent when 7×10^7 bacteria were inoculated compared with high-dose infection with 4×10^9 bacteria. Collectively, these results demonstrated that s.c. application of antigen in RA-treated mice allowed for induction of protective immunity against CT-induced diarrhea and reduced bacterial burden in Peyer patches after oral *Salmonella* infection.

RA treatment facilitates induction of gut-tropism on T and B cells by increasing RALDH activity of skin-draining LN DCs. As described above, s.c. delivery of exogenous RA supported the generation of gut-homing effector T cells and plasma cells in the ingLN. RA has been reported to directly act on activated lymphocytes (15), but there is ample evidence that RA can also affect DC function (21–23). In particular, RA has been shown to induce RALDH expression of bone marrow–derived DCs (24–27). Inversely, mLN DCs of mice raised with a vitamin A–deficient diet show markedly reduced RALDH activity (24–27). Therefore, we assumed that s.c. applied RA might not only act directly on activated lymphocytes in the ingLN, but also exert imprinting activity on resident DC to efficiently synthesize RA. Cells isolated from the ingLN of untreated or s.c. RA-treated mice were incubated with the fluorescent RALDH substrate ALDEFLUOR, and RALDH activity was assessed by flow cytometry. Consistent with a recent study (28), we found that skin-draining LNs of untreated mice contained a small population of RA-producing DCs (Figure 6, A and B). However, ingLN DCs of RA-treated mice showed significantly higher fractions of RA-producing cells than did ingLN DCs of untreated mice, although the proportion of ALDEFLUOR$^+$ ingLN DCs derived from RA-treated animals did not reach that of DCs isolated from the mLN. Under all conditions tested, RALDH activity was blocked by the RALDH inhibitor diethylaminobenzaldehyde (DEAB) (Figure 6, A and B), demonstrating the specificity of the assay.

Finally, we assessed whether these differences in RALDH activity correlate with the ability of RA-imprinted ingLN DCs to induce gut-homing molecules on responding T cells. CFSE-labeled OT-I cells were cocultured with antigen-loaded ingLN

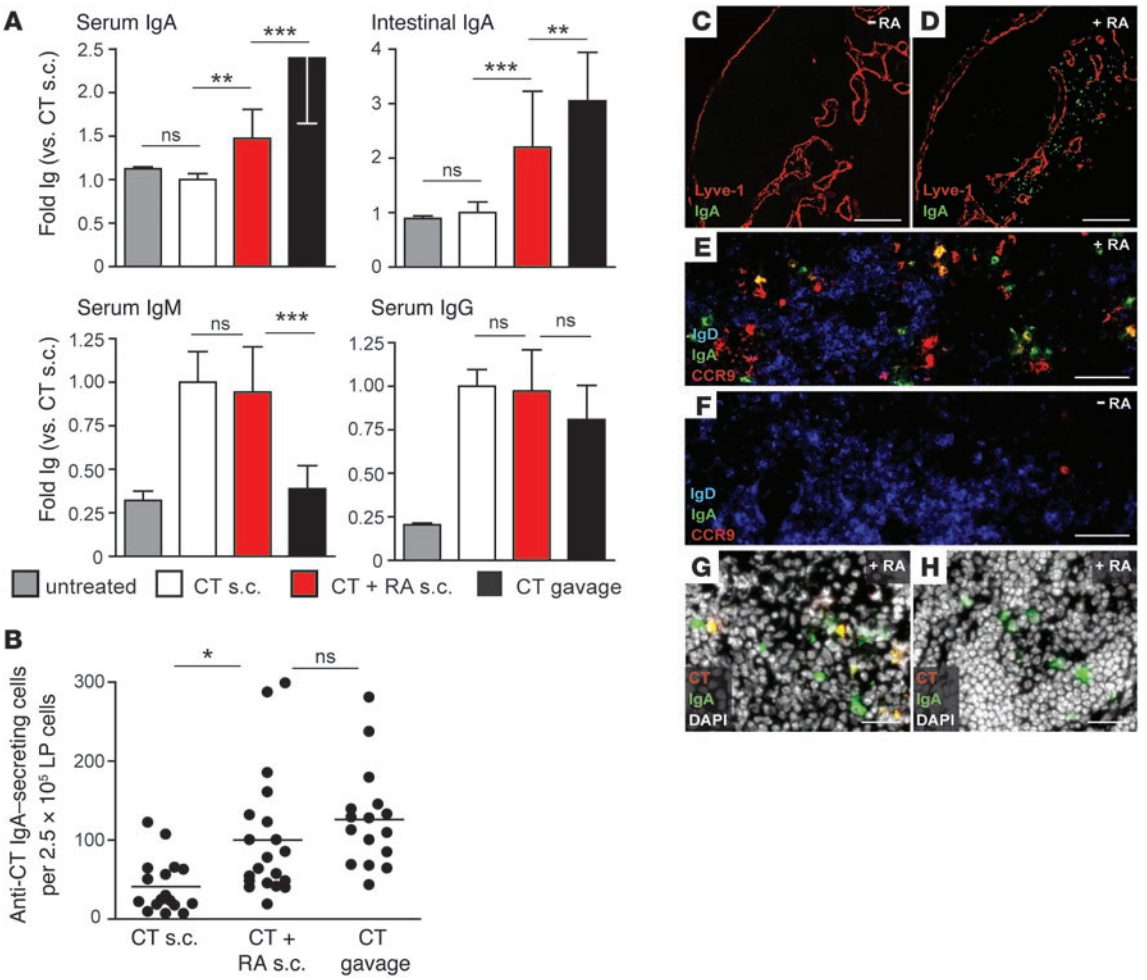


Figure 4

Skin-draining ingLNs of RA-treated mice support induction of gut-homing receptor and IgA expression on B cells. Mice were immunized on days 0 and 10 either s.c. with 1 μg CT or orally with 10 μg CT. A group of s.c. immunized mice additionally received s.c. RA injections on days 0, 1, 2, 3, 6, 10, and 13 after antigen delivery. On day 14, mice were sacrificed. (**A**) Levels of CT-specific Ig in serum and intestinal wash were determined by ELISA. Results are pooled from 4 independent experiments with at least 9 mice total per group. (**B**) Number of anti-CT IgA-secreting cells in the small intestinal lamina propria (LP), assessed by ELISPOT. Bars denote mean values; symbols denote individual mice pooled from 4 independent experiments. (**C**–**H**) Sections from ingLNs of s.c. immunized mice with (**D** and **E**) or without (**C** and **F**) RA treatment were either analyzed at day 14 by fluorescence microscopy for IgA and Lyve-1 (**C** and **D**) or stained for IgA, CCR9, and IgD (**E** and **F**). Sections from ingLNs (**G**) and mLNs (**H**) from RA-treated s.c. immunized mice were stained for IgA and CT-binding cells. Images are representative for 6 mice analyzed per group in 2 independent experiments. Scale bars: 200 μm (**C** and **D**); 50 μm (**E** and **F**); 25 μm (**G** and **H**). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

DCs isolated from untreated or RA-treated mice, and their expression of CCR9 and α4β7-integrin was analyzed by flow cytometry. ingLN DCs from RA-treated mice induced significantly more CCR9 and α4β7-integrin on responding T cells than did ingLN DCs from untreated mice (Figure 6, C and D), but were less potent than mLN DCs in upregulating CCR9 (56.4% ± 27.0%) or α4β7-integrin (36.7% ± 28.0%). Upregulation of CCR9 and α4β7-integrin could be blocked by addition of DEAB. These observations indicate that in vitro induction of gut-homing receptors relies on active RA production by ingLN DCs from RA-treated mice, rather than on passive RA carry-over. Apart from T cells, B cells activated in the presence of ingLN DCs from RA-treated mice gained expression of α4β7-integrin (Figure 6E). Interestingly, ingLN DCs of RA-treated mice lost the ability to strongly induce PSL and ESL on activated T cells (Figure 6, C and D). Notably, upregulation of ESL and PSL was also suppressed when RALDH activity was blocked by DEAB. In consideration of a potential link between RA abundance and Langerin-expressing DC generation (29), we next determined Langerin expression by ingLN DCs. Intriguingly, s.c. injection of RA decreased the frequency of Langerin^{+} DCs within the MHCII^{hi}CD11c^{+} ingLN population (Figure 6, F–H). Together, these results showed that RA modifies DC phenotype and function in terms of RALDH and Langerin expression.

Discussion

Given the fact that diarrheal diseases are among the major causes of morbidity and mortality among young children in developing countries, there is an urgent need for vaccination regimens that generate effective and long-lasting intestinal immunity. In view

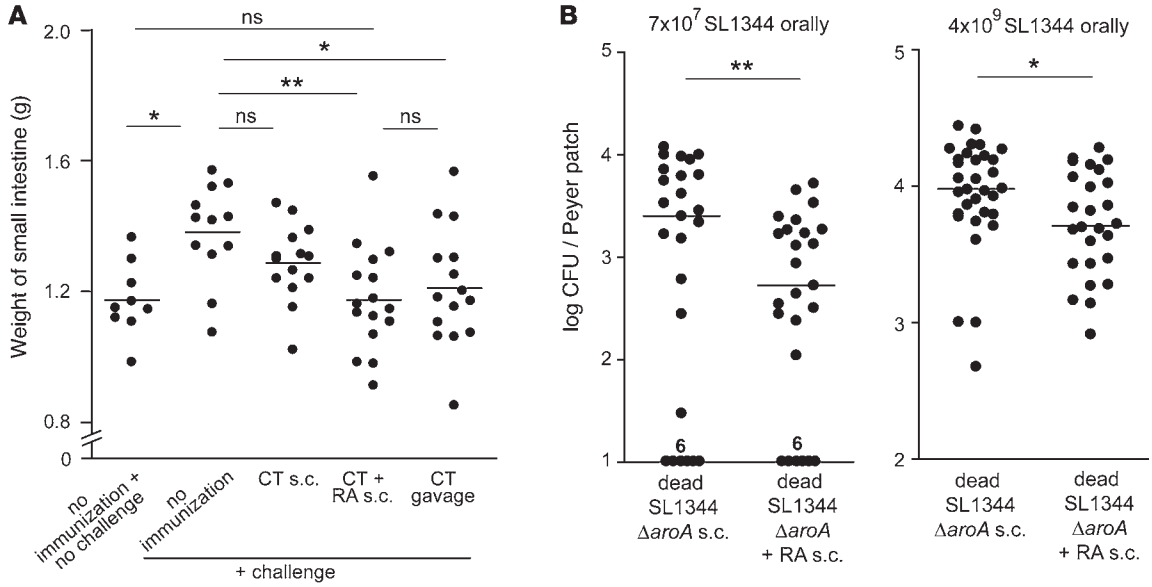


Figure 5

RA-supplemented s.c. immunization confers mucosal protection against CT-induced diarrhea and diminishes bacterial loads in Peyer patches after oral *Salmonella* infection. (**A**) Mice were treated with CT as in Figure 4 or were left untreated (no immunization). At day 14, mice were challenged orally by gavage with 10 μg CT. 2 hours after challenge, mice were sacrificed, and the weight of the small intestine together with its fluid content was determined. Small intestines of mice receiving neither immunization nor challenge served as controls. Bars represent mean values; symbols represent individual mice pooled from 3 independent experiments. Note the discontinuous *y* axis. (**B**) Mice were s.c. immunized with 10^6 PFA-fixed *Salmonella* Typhimurium SL1344 lacking the *aroA* gene (SL1344 Δ*aroA*) on days 0 and 2. A group of s.c. immunized mice was additionally s.c. injected with RA on days 0, 1, 2, 3, 4, 7, 8, and 9. On day 14, mice were orally inoculated with 7×10^7 or 4×10^9 wild-type *Salmonella* Typhimurium SL1344. 2 days after infection, bacterial loads were determined in single Peyer patches. Bars represent median values; symbols represent individual Peyer patches from 3 (7×10^7) or 4 (4×10^9) mice per group. Numerals within the graph indicate the number of sterile Peyer patches. **P* < 0.05; ***P* < 0.01.

of the poor performance of several oral vaccines in developing regions compared with industrialized regions (5), we aimed to develop an immunization strategy based on the targeted homing of immune effector cells to the intestine. Here, we report that mucosal protection was induced by s.c. applied antigens in the presence of RA. RA delivered via s.c. injection induced the generation of gut-homing T cells and gut-tropic IgA-expressing plasma cells in skin-draining LNs. Mechanistically, we showed that s.c. injected RA imprinted ingLN DCs with the ability to generate RA endogenously, which resulted in the capacity to induce gut-homing receptors on activated lymphocytes.

It is conceivable that, in contrast to the rather diluted RA arriving via lymph, the RA produced on-site can be delivered in a highly concentrated fashion to those cells directly contacting the DCs. Thus, exogenously applied RA might act not only directly on lymphocytes, but also indirectly via DC-based enhancement of RA signaling. In vivo, this may endow the DC with the capacity to potently manipulate homing, which permits an immune response to be redirected to the intestine even though it originated in the "wrong" compartment, the skin-draining LNs. It is currently unclear whether exogenously applied RA also has some imprinting capacity on stromal cells residing in skin-draining LNs. Since we previously failed to induce gut-homing molecules on T cells in skin-draining LNs transplanted into the mesenterium (13), these results suggest that stromal cells might be less reactive to RA than DCs are, but a role for RA in controlling RALDH expression in stromal cells has also been recently reported (26). Our findings regarding DC imprinting are in line with reports from others suggesting that RA regulates its own synthesis by a positive feedback loop through RA receptor signaling and retinal production (30–32). Furthermore, numerous recent studies provide direct evidence that RA induces RALDH activity in DCs (24–27). In addition to promoting RALDH activity, s.c. applied RA decreased the frequency of Langerin$^+$ DCs in skin-draining LNs. Similarly, Chang and colleagues suggest a link between availability of RA and expression of Langerin by showing that vitamin A–deficient mice display increased numbers of Langerin$^+$ DCs in GALT (29). It remains to be determined whether the reduction of Langerin$^+$ ingLN DCs is responsible for induction of a mucosal immune response after RA-supplemented s.c. immunization. Interestingly, RA has recently been studied as an additive for transcutaneous immunization with inactivated influenza virus (33). This immunization regimen induced a pronounced IgA response in the lung and conferred protection against intranasal influenza virus challenge. The authors attributed this effect primarily to enhanced mobilization of DCs to the skin-draining LNs (33). However, enhanced, RA-driven targeting of lymphocytes to mucosal tissues including the lung might also be a relevant mechanism leading to improved protection. Furthermore, it has been reported that exogenously applied RA leads to increased germinal centers and augmented antibody production (34), which indicates that RA has effects on the immune response other than changing the profile of homing molecules. It also should be mentioned that RA treatment might cause unwanted side-effects, such as allergic responses (35) and weight loss (36).

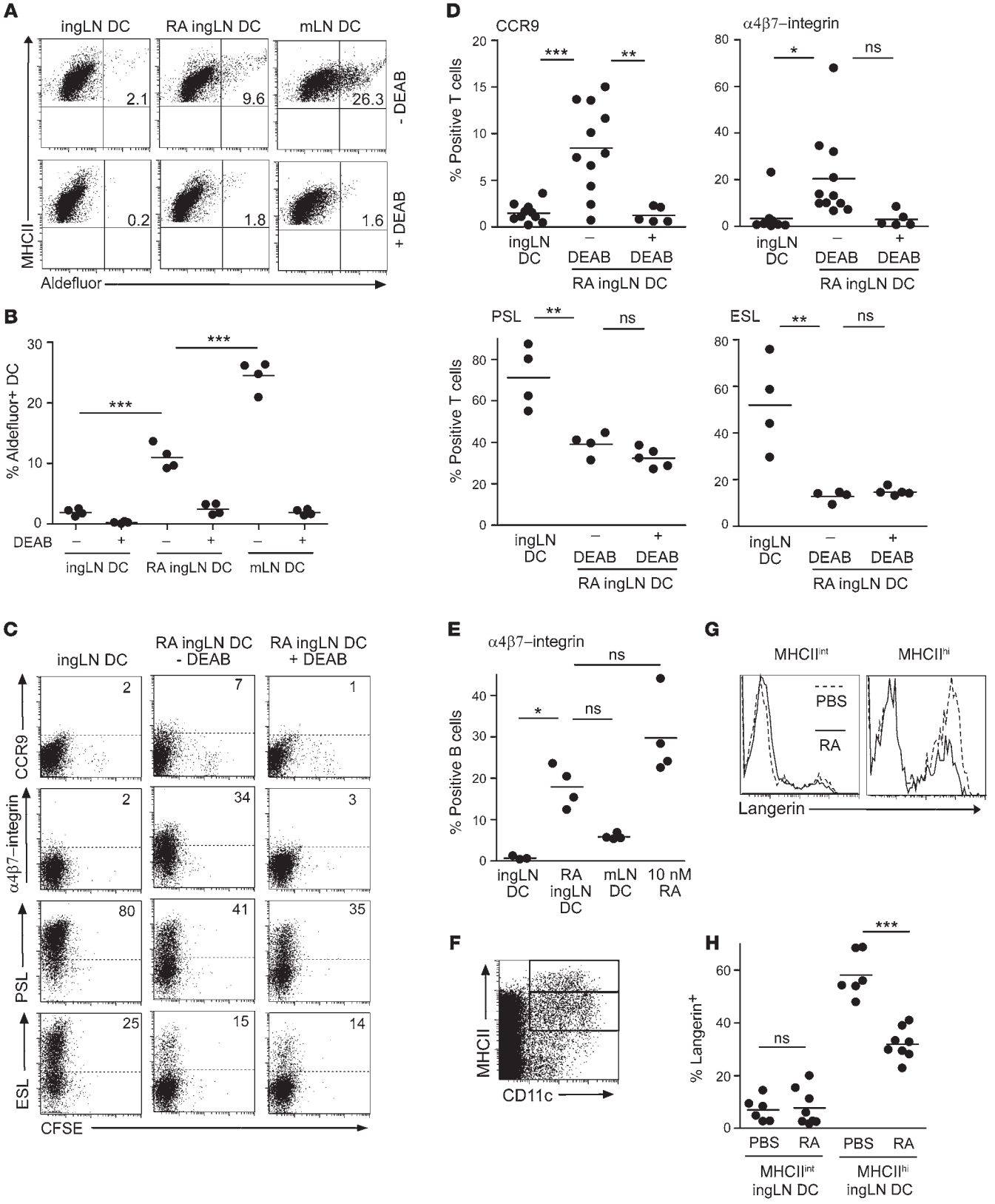

A
ingLN DC
RA ingLN DC
mLN DC
2.1
9.6
26.3
- DEAB
0.2
1.8
1.6
+ DEAB
MHCII
Aldefluor
B
% Aldefluor+ DC
DEAB
ingLN DC
RA ingLN DC
mLN DC
C
ingLN DC
RA ingLN DC - DEAB
RA ingLN DC + DEAB
CCR9
α4β7–integrin
PSL
ESL
CFSE
D
CCR9
α4β7–integrin
% Positive T cells
ingLN DC
DEAB
RA ingLN DC
PSL
ESL
E
α4β7–integrin
% Positive B cells
ingLN DC
RA ingLN DC
mLN DC
10 nM RA
F
MHCII
CD11c
G
MHCIIint
MHCIIhi
PBS
RA
Langerin
H
% Langerin+
PBS
RA
MHCIIint ingLN DC
MHCIIhi ingLN DC

Figure 6
Exogenous RA promotes in vivo the ability of ingLN DCs to induce gut-tropism by increasing their RALDH activity. (**A** and **B**) Mice were s.c. immunized with 50 μg Ova; 1 group additionally received RA s.c. at days 0, 1, and 2. At day 3, cells were isolated from mLNs and ingLNs and analyzed for RALDH activity. (**A**) Flow cytometry plots of gated MHCII$^+$CD11c$^+$ cells with or without DEAB. (**B**) Percent ALDEFLUOR$^+$ DCs within gated MHCII$^+$CD11c$^+$ cells. (**C** and **D**) CFSE-labeled OT-I cells were cocultured with Ova-loaded ingLN DCs, mLN DCs from untreated mice, or ingLN DCs from RA-treated mice (RA ingLN DC) with or without DEAB. (**C**) Flow cytometry plots for α4β7-integrin, CCR9, PSL, and ESL expression on OT-I cells on day 5 of coculture. (**D**) Percent α4β7-integrin$^+$, CCR9$^+$, ESL$^+$, and PSL$^+$ cells among OT-I T cells. (**E**) Percent α4β7-integrin$^+$ cells among B cells. CFSE-labeled splenic B cells were activated in the presence of ingLN DCs, mLN DCs, or ingLN DCs from RA-treated mice. (**F**–**H**) Langerin$^+$ cells were found among CD11c$^+$MHCII^{hi} cells (**F**, top gate) of skin-draining LNs, whereas MHCII^{int} DCs (bottom gate) contained few Langerin$^+$ cells; Langerin$^+$MHCII^{hi} DC frequency was reduced by RA treatment (**G** and **H**). (**B**, **D**, **E**, and **H**) Bars represent mean values, symbols denote individual mice. Pooled from 2 (**E** and **H**) or 3 (**D**) independent experiments, or (**B**) similar results were obtained in 2 additional independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

There is now ample evidence that the induction of skin-homing and gut-homing potential of effector cells of the adaptive immune system is intimately linked to the site of lymphocyte activation. Recent work suggests that DCs and LN stromal cells cooperate in shaping a LN environment that favors induction of the respective homing receptors. Here we report that exogenously applied RA overrides the LN-specific factors driving induction of skin-homing molecules in skin-draining LNs. In doing so, RA redirects the induced plasma cell and T cell response to the intestine. Conversion of the induced type of tissue tropism was triggered by s.c. injections of 150 μg RA, while 20 μg did not show any effect (data not shown). Thus, it appears that distinct threshold levels of RA are required to efficiently affect the microenvironment of the ingLN, investigated in the present study. Importantly, mLN-explanted mice directed T cells to the small intestine as efficiently as did mLN-proficient mice. Furthermore, CT-specific plasma cells were only detectable in ingLN, not mLN, of RA-treated mice. Thus, the mLN does not seem to contribute to the generation of gut-tropic T and B cells after s.c. immunization and RA treatment. Instead, local priming and imprinting in skin-draining LNs is responsible and sufficient for induction of gut-homing lymphocytes upon s.c. administration of RA. In contrast, the mLN was reported to be the site of IgA class switching after topical application of an antigen plus adjuvant to the intact skin surface (37). As additional examples of redirecting immune responses, 2 studies reported that parenteral, adenovirus-based immunizations enhance expansion of gut-homing T cells (38, 39). In mice, the pronounced CD8$^+$ T cell response in the gut was attributed to adenovirus-driven induction of RALDH activity in DC (38).

We demonstrated in this study that RA-assisted s.c. immunization generated intestinal immunity and conferred the benefits of mucosal protection otherwise generated only when antigen was applied orally. s.c. RA imprinted gut-homing specificity on T cells and plasma cells activated in skin-draining LNs. Furthermore, RA treatment initiated class switch to IgA in local skin-draining LNs and generated a pronounced IgA response in serum and intestinal lumen. Consequently, RA-assisted s.c. immunization protected mice from CT-induced diarrhea and reduced bacterial loads in Peyer patches after oral *Salmonella* infection. Notably, RA-supplemented s.c. immunization had the advantage of simultaneously inducing both mucosal and systemic humoral immune responses. As a mode of action of exogenously applied RA in redirecting immune responses, we found that RA treatment imprinted ingLN DCs in vivo with the ability to generate gut-tropic B and T cells by increasing their RA-producing capacities.

Thus, we propose that RA or its precursors, such as vitamin A, might be used as gut-homing navigators for vaccinations given s.c. or intramuscularly in order to generate protective immunity against enteric pathogens. Since vitamin A is already routinely delivered in vaccine clinics to young children in countries in which vitamin A deficiency is a problem, RA-assisted immunization might offer a feasible approach to efficiently combat enteric infections.

Methods

Mice. C57BL/6-Tg(Tcra Tcrb)1100MjbJ mice and BALB/c-Tg(DO11.10) mice (referred to herein as OT-I and DO11.10 mice, respectively) were bred under specific pathogen–free conditions at the central animal facility of Hannover Medical School. C57BL/6 and BALB/c mice were purchased from Charles River. All experiments were approved by the institutional review board and the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit.

Antibodies. The following antibodies, fusion proteins, and conjugates were used in this study: PE-conjugated anti-CD45.1 (A20), biotin-conjugated anti-MHCII(1A^b) (AF6-120.1), anti-α4β7 (DATK32), PerCP-conjugated anti-CD4 (RM4-5), PE-conjugated anti-MHCII (I-A/I-E) (M5/114.1), and PE-conjugated anti-α4β7 (DATK32), all from BioLegend; biotin-conjugated anti–Vβ5.1,5.2 TCR (MR9-4), PE-conjugated anti-Vα2 (B20.1), allophycocyanin-Cy7–conjugated anti-CD8α (53-6.7), biotin-conjugated anti-IgM (R6-60.2), biotin-conjugated anti-IgG1 (A85-1), biotin-conjugated anti-IgG2a (R19-15), biotin-conjugated anti-IgG3 (R40-82), and allophycocyanin-Cy7–conjugated anti-CD19 (1D3), all from BD Biosciences; PE-Cy7–conjugated anti-CD11c (N418), allophycocyanin-conjugated anti-CD11c (N418), PE-Cy7–conjugated anti-CD19 (1D3), PE-conjugated rat IgG2a isotype control (eBR2a), PerCP-Cy5.5–conjugated anti-CD45.2 (104), and PE-conjugated anti-Foxp3 (FJK-16s), all from eBioscience; poly(oxadiazole)-conjugated anti-IgA (Jackson), FITC-conjugated anti-IgA, biotin-conjugated anti–DO11.10-TCR (KJ1-26) from Caltag; anti–Lyve-1 from Acris; Alexa Fluor 488–conjugated anti-Langerin (929F3.01) from Dendritics; and recombinant mouse E-selectin/Fc chimera and recombinant mouse P-selectin/Fc chimera from R&D Systems. Anti-CD8β (Rm CD8-2), anti-CCR9 (7E7-1-1), and anti-IgD (HB250) were produced in our laboratory. Cy3 and Cy5 conjugates (Amersham) as well as Pacific Orange conjugates (Invitrogen) of antibodies were prepared as recommended by the manufacturers. Biotinylated antibodies were recognized by streptavidin coupled to PerCP (BD Biosciences) or Pacific Orange (Invitrogen). Anti-CCR9 and anti-α4β7 were detected using Cy5-conjugated mouse anti-rat (Jackson). E-selectin/Fc chimera and P-selectin/Fc chimera were detected with Cy5-conjugated goat anti-human (Jackson). For intracellular staining, cells were fixed with Fix/Perm working solution (eBioscience) for Foxp3 staining or with Cytofix/Cytoperm (BD Biosciences) for Langerin staining.

Mesenteric lymphadenectomy. Surgery was performed as previously described (40). Briefly, the small intestine and cecum, together with MLN, were exteriorized through a 1-cm-wide incision along the abdomen and kept humid with PBS. Mesenteric lymphadenectomy was performed by microdissection along the length of the superior mesenteric artery to the aortic root. After surgery, the small intestine and cecum were reintroduced into the abdomen, the lesion of the abdominal wall was stitched with degradable thread, and the outer skin

was sealed with wound clips. 6 weeks after surgery, animals were adoptively transferred with OT-I cells and s.c. immunized with Ova plus RA.

Adoptive cell transfer. OT-I or DO11.10 mice were used as cell donors for adoptive transfer into syngeneic recipient animals. Lymphocytes were isolated from ingLNs, brachial LNs, axillary LNs, mLNs, and spleen and labeled with 5 μM CFSE (Invitrogen) for 10 minutes at 37°C. After washing twice with PBS containing 3% FCS, 5×10^6 cells per mouse were injected into the tail vein.

Immunization. Recipients of OT-I or DO11.10 cells were immunized by oral gavage of 50 mg Ova (grade III; Sigma-Aldrich) in 200 μl PBS or by s.c. injection of 50 μg Ova in 2 × 50 μl PBS into the dorsal flanks. In other experiments, mice received 10 μg CT (Sigma-Aldrich) orally by gavage in 200 μl PBS or 1 μg CT in 2 × 50 μl PBS s.c. Some s.c. immunized mice additionally received 150 μg all-*trans* RA (Sigma-Aldrich) or, in one experimental setup, 150 μg 13-*cis* RA (Enzo Life Sciences) in 2 × 50 μl Polyethylenglycol 400 (Sigma-Aldrich) s.c. into the dorsal flanks. For the CT-induced diarrhea, 10 μg CT in 200 μl PBS was orally administered by gavage. 2 hours after application, mice were sacrificed, and the weight of the small intestine was determined.

Bacterial challenge. Mice were s.c. immunized on days 0 and 2 with 10^6 PFA-fixed *Salmonella* Typhimurium SL1344 lacking the *aroA* gene. A group of s.c. immunized mice additionally received s.c. injections of RA on days 0, 1, 2, 3, 4, 7, 8, and 9. On day 14, mice were inoculated orally by gavage with wild-type *Salmonella* Typhimurium SL1344. 2 days after infection, bacterial loads were determined in single Peyer patches by plating.

Cell isolation. For isolation of IELs and LPLs, Peyer patches were excised, and the intestine was opened longitudinally and incubated 3 times for 15 minutes in HBSS with 10% FCS and 2 mM EDTA at 37°C to remove epithelial cells. After each incubation step, tubes were shaken for 10 seconds, and the supernatant containing epithelial cells was collected. The remaining tissue was incubated for 45 minutes with RPMI 1640 containing 10% FCS, 0.24 mg/ml collagenase A, and 40 U/ml DNase I (both from Roche); tubes were shaken for 10 seconds; and lamina propria cell suspension was collected. The resulting cell suspensions were filtered and purified by density gradient centrifugation with 40%–70% Percoll (GE Healthcare). To isolate LN DCs, LNs were cut into pieces, incubated in RPMI with 0.1 mg/ml Liberase Blendzyme2 and 50 U/ml DNase I (both from Roche) for 30 minutes at 37°C, and meshed through nylon gauze. $CD11c^+$ cells were enriched by CD11c Microbeads using AutoMACS (Miltenyi Biotec), yielding an average purity of greater than 90% $MHCII^+CD11^+$ cells.

ALDEFLUOR assay. ALDH activity was determined using the ALDEFLUOR staining kit (STEMCELL Technologies) according to the manufacturer's instructions, with some modifications. Briefly, LN cells were suspended at 10^6 cells/ml in ALDEFLUOR assay buffer containing activated ALDEFLUOR substrate (final concentration, 1.5 μM) with or without DEAB (final concentration, 45 μM) and incubated for 30 minutes at 37°C. For immunophenotyping of ALDEFLUOR-reactive cells, the cells were subsequently stained with PE-, PE-Cy7–, and Pacific Orange–conjugated mAbs in ice-cold ALDEFLUOR assay buffer. ALDEFLUOR-reactive cells were detected in the FITC channel.

Elispot. 96-well multiscreen plates (Millipore) were coated with 5 μg/ml CT (Sigma-Aldrich), followed by blocking with RPMI containing 10% FCS. 2.5×10^5 and 1×10^5 LPLs/well in duplicates were incubated overnight at 37°C, 5% CO_2. After incubation, cells were lysed and washed away. Plates were incubated with biotin-conjugated anti-IgA (AbD Serotec) followed by streptavidin peroxidase (Jackson). For the detection of peroxidase activity, 3-amino-9-ethylcarbazole substrate solution (Sigma-Aldrich) was added. Spots were counted with Eli.Analyse version 4.1 software (A.EL.VIS GmbH).

ELISA. Intestinal washes were collected by flushing the distal 5 cm of the small intestine with 400 μl PBS containing 0.1 mg/ml trypsin inhibitor, 50 mM EDTA, and 0.1% BSA. ELISAs were performed by coating 96-well plates (Nunc) with 2.5 μg/ml CT, followed by blocking with PBS containing 2% BSA. After incubation of sera and intestinal washes, IgG, IgM, and IgA were detected by using biotinylated antibodies followed by streptavidin peroxidase (Jackson). For detection of peroxidase activity, o-phenylenediamine dihydrochloride (Sigma-Aldrich) was added, and absorbance was measured at 450 nm.

Immunohistochemistry. After freezing the tissue in OCT compound, cryosections (8 μm) were prepared and fixed for 10 minutes in ice-cold acetone. Immunohistochemistry was performed according to standard protocols. Briefly, sections were rehydrated in TBST (0.1 M Tris, pH 7.5; 0.15 M NaCl; 0.1% Tween-20), preincubated with TBST containing 5% mouse serum, and stained with unconjugated rat antibodies in 2.5% mouse serum in TBST. Unconjugated rat antibodies were detected by Cy3- or Cy5-conjugated mouse anti-rat (both Jackson). After blocking with 5% rat serum in TBST, sections were incubated with a mixture of fluorescent dye–coupled antibodies in 2.5% rat serum in TBST. To detect antigen-specific plasma cells, sections were incubated with biotinylated CT B subunit (Sigma-Aldrich) and then stained with Cy3-coupled streptavidin (Jackson). Nuclei were visualized by DAPI staining (1 μg/ml DAPI in TBST), and sections were mounted with MOWIOL. Pictures were taken by fluorescence microscopy (Axiovert) using AxioVision 4.6 software (both from Carl Zeiss Inc.).

In vitro cocultures. 10^5 CFSE-labeled purified $CD8^+$ OT-I cells ($CD8^+$ T Cell Isolation kit; Miltenyi Biotec) were activated by adding 5×10^4 LN DCs that were previously loaded with antigen by incubation with 1 mg/ml Ova for 2 hours at 37°C. Alternatively, 10^5 CFSE-labeled, sorted $CD19^+$ splenic B cells were stimulated in the presence of LN DCs by adding 20 μg/ml F(ab′)2 goat anti-mouse IgM (Jackson) and 50 μg/ml anti-CD40 (FGK45; ref. 41). FGK45 was provided by S. Weiss (Helmholtz Centre for Infection Research, Braunschweig, Germany).

Statistics. Statistical analysis was performed with GraphPadPrism software. All significant values were determined using 1-way ANOVA with Bonferroni post-hoc test, for comparing more than 2 groups, or unpaired 2-tailed *t* test, for comparing 2 groups. Data are mean ± SD. A *P* value less than 0.05 was considered significant.

Acknowledgments

We thank Siegfried Weiss for providing FGK45 and G. Bernhardt, I. Prinz, and A. Krueger for valuable suggestions on the manuscript. This work was supported by Deutsche Forschungsgemeinschaft grant SFB621-A1 to R. Förster.

Received for publication July 6, 2010, and accepted in revised form May 11, 2011.

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