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Retinoic Acid Determines the Precise Tissue Tropism of Inflammatory Th17 Cells in the Intestine

Chuanwu Wang,* Seung G. Kang,* Harm HogenEsch,* Paul E. Love,† and Chang H. Kim*

T helper cells expressing IL-17 (Th17 cells) are major effector T cells in the intestine, but the regulation of their tissue tropism within the gut is poorly understood. We investigated the roles of vitamin A and retinoic acid in generation of inflammatory Th17 cells with distinct tissue tropisms within the intestine. We found that Th17 cells with distinct tissue tropisms and pathogenic activities are generated depending on the available concentration of retinoic acid (RA). In contrast to the widespread perception that RA would suppress the generation of Th17 cells, we provide evidence that RA is actually required for generation of Th17 cells with specific tissue tropisms within the gut. Th17 cells induced at suboptimal serum concentrations of RA migrated and induced moderate inflammation mainly in the large intestine, whereas the Th17 cells induced with optimal levels of exogenous RA (∼10 nM) migrated to the small intestine and induced more severe inflammation. The Th17 cells, induced in the presence or absence of RA, differentially expressed the trafficking receptors CCR9 and α4β7. CCR9 is required for Th17 cell migration to the small intestine, whereas α4β7 is required for the migration of Th17 cells throughout the whole intestine. Our results identified RA as a major signal that regulates the generation of gut Th17 cells with distinct capacities in migration and inflammatory activities. The results indicate also that specific gut tropism of Th17 cells is determined by the combination of trafficking receptors regulated by the RA signal. The Journal of Immunology, 2010, 184: 000–000.

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Abbreviations used in this paper: Itg, integrin; MLN, mesenteric lymph node; PP, Peyer’s patch; RA, retinoic acid; Th17, retinoid-induced Th17; Th17 cells, T helper cells expressing IL-17; TRITC, tetramethylrhodamine isothiocyanate.

Materials and Methods

Cell isolation and culture

Naive CD4+ T cells were isolated from pooled mononuclear cells of peripheral lymph nodes, mesenteric lymph nodes (MLNs), and spleens as described before (2). Naive CD4+ T cells were cultured in complete RPMI 1640 medium (10% FBS) supplemented with Con A (2.5 μg/ml; PeproTech), hTGF-β1 (5 ng/ml; PeproTech, Rocky Hill, NJ), mIL-6 (20 ng/ml; PeproTech), mIL-21 (10 ng/ml; PeproTech), mIL-23 (10 ng/ml; R&D Systems, Minneapolis, MN), mIL-18 (10 ng/ml; PeproTech), mTNF-α (20 ng/ml; PeproTech), anti-mIL-4 (11B11, 10 μg/ml; BioLegend, San Diego, CA), anti-mIFN-γ (XMG2.4, 10 μg/ml; BioLegend) and anti-mIL-2 (S4B6, 2.5 μg/ml; BD Biosciences, San Jose, CA) for 7 d to generate control Th17 cells that were induced without exogenous All-trans-RA (hereafter called “RA”). RA Th17 cells were prepared using the same condition with RA at physiologic concentrations (10–20 nM). In some experiments, naive T cells were CFSE-labeled and cultured in the Th17 cell induction condition to determine the relationship between cell division and expression of the gut-trafficking receptors (Supplemental Fig. 1). The Th17 cells were also recultured for an additional 7 d to determine the stability of trafficking receptor expression (Supplemental Fig. 2).
Animals and generation of vitamin A-deficient or -sufficient mice

All the experiments with animals in this study were approved by the Purdue University Animal Care and Use Committee. CCR9-deficient mice were described previously (26). Integrin (Itg) β7-deficient mice (C57BL/6-Itgb7tm1Cgn/J) and Rag1-deficient (B6.129s7-Rag1tm1Mom/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). AKR/J mice (The Jackson Laboratory) were kept on custom research diets based on AIN-93G containing 25,000 IU/kg or 0 IU/kg levels of retinyl acetate (Harlan Teklad TD-06528 and 07267) immediately following birth. The pups were weaned at 4 wk old and maintained on the same diets for an additional 9 wk. Vitamin A deficiency was verified by determining defective CCR9 expression by small intestinal T cells, as described previously (2).

FIGURE 1. Gut homing CCR9+α4β7+ Th17 cells are induced in the presence of physiological concentrations of RA. A, Naive T cells were cultured in a Th17 cell induction condition for 7 d at the indicated concentrations of RA. B, Expression of gut homing receptors (CCR9 and α4β7) by Th17 cells was examined. C, The chemotactic ability of RA Th17 cells and control Th17 cells to CCL25 and CCL19. D, Twenty-hour, short-term homing capacity of RA Th17 cells and control Th17 cells was compared in C57BL/6 mice. The Th17 cells were injected i.v. Homing index of RA Th17 cells is shown as percent of control Th17 cells. *A significant increase in migration of RA Th17 cells over control Th17 cells. E, Migration of RA Th17 cells is shown as percent distribution among the indicated organs. *Significant differences from control Th17 cells. F, In situ visualization of the RA Th17 cells and control Th17 cells migrated into indicated organs (original magnification ×200). All experiments were performed at least three times and combined (A–E) or representative data (F) are shown. Averages and SEM are shown in the combined data.
Flow cytometry to identify Th17 cells and to determine their expression of trafficking receptors

Intracellular cytokine staining for IL-17 (TC11-18H10.1; BioLegend) expression was performed as described previously after activation with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (1 μM) in the presence of monensin (10 μg/ml) (2). Before activation, the cells were stained for surface expression of CD4 (clone RM4-5; BioLegend), CD44 (clone IM7; BioLegend), CCR9 (clone 242504; R&D Systems), α4β7 (clone DATK32; BioLegend), and/or αEβ7 (Clone 2E7; BioLegend). Stained cells were analyzed using a BD Canto II (BD Biosciences).

Homing experiment

Th17 cells generated in vitro (1 × 10⁷ cells per mouse) were labeled with CFSE or tetramethylrhodamine isothiocyanate (TRITC) and coinjected into wild type C57BL/6 mice via a tail vein; ∼20 h later, the mice were sacrificed and single cell suspension was prepared from selected organs after collagenase digestion (2). The numbers of injected CFSE⁺ or TRITC⁺ Th17 cells migrated into each organ were determined with flow cytometry. The relative homing index to an organ or tissue was determined according to the formula

\[
\% \text{homing index for organ } A = \frac{\text{No. of CFSE}^+ \text{ cells in organ } A}{\text{No. of TRITC}^+ \text{ cells in organ } A} / \frac{\text{No. of CFSE}^+ \text{ cells in input}}{\text{No. of TRITC}^+ \text{ cells in input}} \times 100
\]

The percent distribution of injected Th17 cells (Fig. 1E) was calculated based on the numbers of migrated cells into the indicated organs.

Chemotaxis

Chemotaxis was performed using Transwells (Corning Glass, Corning, NY) with 5-μm pores as described previously (27). Optimal concentrations of CCL25 (2.5 μg/ml) and CCL19 (1 μg/ml), determined by a preliminary titration experiment, were used.

In vivo intestinal inflammation study to assess the effector function of Th17 cells

B6.129S7-Rag1tm1Mom/J mice were injected i.p. with 1 × 10⁶ control or RA-induced Th17 cells. Weight change was monitored, and the mice were sacrificed on day 30–32 postinjection when some mice became moribund.

FIGURE 2. Th17 cells are decreased specifically in the small intestine of vitamin A-deficient animals. The frequencies of Th17 cells in various organs of vitamin A-sufficient and -deficient AKR/J mice were determined. Representative (A) and combined (B) data of three independent experiments are shown. *Significant differences between RA and control Th17 cells.

FIGURE 3. RA Th17 cells use αEβ7 to migrate to the whole intestine including the lamina propria and GALTs. A, Expression of CCR9 and α4β7 by wild type and αEβ7 (−/-) RA Th17 cells. B, The Th17 cells were injected i.v., and the migration of Th17 cells to the indicated organs were determined 20 h after cell injection. C, The localization of the injected Th17 cells in the indicated tissues was determined with a confocal microscope (original magnification ×200). The experiments were performed three times, and combined data (B) or representative data (C) are shown. *Significant changes.

FIGURE 4. RA Th17 cells use CCR9 to migrate to the small intestinal lamina propria. A, Expression of CCR9 and α4β7 by wild type and CCR9 (−/-) RA Th17 cells. B, The Th17 cells were injected i.v. and the short-term (20 h) migration of Th17 cells was determined. C, The localization of the Th17 cells was determined with confocal microscopy (original magnification ×200). Combined data (B) or representative data (C) of three independent experiments are shown. *Significant changes.
Intestinal inflammation in Rag1-deficient mice was scored as previously described with some modification (27). We scored the degree of inflammation and the degree of mucosal hyperplasia and loss of villi on a scale of 0–4. The two scores were combined to obtain the final inflammation index. The histologic images were obtained with a bright-field Leica (Leica Microsystems, Deerfield, IL) microscope equipped with a color camera at 3200 magnification.

Confocal analysis to determine localization of injected Th17 cells

Indicated tissues (spleen, peripheral lymph nodes, mesenteric lymph nodes, and Peyer’s patches [PPs]) were harvested from euthanized mice that were previously injected with cells stained with TRITIC and CFSE, and frozen in Tissue-Tek (Sakura, Torrance, CA). Six-micrometer frozen tissue sections were fixed in acetone and stained with anti–CD3-APC. A Zeiss LSM 710 confocal microscope system (Zeiss, Thornwood, NY) was used to image the cells within the tissues.

Statistical analyses

Student paired and unpaired two-tailed t tests were used to compare the significance of differences in means of two groups of related or unrelated data. p ≤ 0.05 was considered significant.

Results

**Naive T cells differentiate into CCR9+α4β7+ Th17 cells at physiologic concentrations of RA**

RA (i.e., All-trans-RA or At-RA) is the major biologically active metabolite of vitamin A. To determine the role of RA in induction of gut homing Th17 cells, we activated naive T cells in a Th17 cell-inducing condition in the presence of increasing concentrations of RA. The induction of Th17 cells was reduced but still occurred at significant levels in the presence of RA lower than 30 nM (Fig. 1A). Severe reduction occurred only at high concentrations of RA (≥100 nM). An interesting feature of these RA Th17 cells was the expression of CCR9 and α4β7 (Fig. 1B); however, they poorly expressed CD103 (or αEβ7), another mucosal homing receptor (Supplemental Fig. 3). Compared with CCR9, α4β7 was more readily expressed by the Th17 cells at low (∼1 nM) RA concentrations (Fig. 1B). α4β7 was induced at a medium level, even in the absence of exogenous RA, perhaps in response to the basal level of RA present in the serum. The induction of CCR9 and α4β7 on Th17 cells by RA was prompt, occurring after one to two cell divisions following T cell activation (Supplemental Fig. 1). Only RA Th17 cells were able to migrate to the CCR9 ligand CCL25 (Fig. 1C). Expression of α4β7 induced by RA was relatively more stable than that of CCR9 on Th17 cells upon subsequent T cell activation in the absence of RA (Supplemental Fig. 2). In contrast, CCR9, once induced on Th17 cells by RA, quickly disappeared upon restimulation of Th17 cells in the absence of exogenous RA.

The RA-induced Th17 cells preferentially migrate to the small intestine

We performed a short-term (20 h) homing assay to determine whether RA Th17 cells, induced in the presence of exogenous RA,
Th17 cells are decreased specifically in the small intestine of vitamin A-deficient mice. The results shown in Fig. 1 that RA induced gut homing Th17 cells is surprising, because it has been reported that RA suppresses the induction of Th17 cells from naive T cells in vitro. To determine whether vitamin A is required for the presence of Th17 cells in vivo, we induced vitamin A deficiency in mice and examined the frequency of Th17 cells in different organs, including the intestine. We found that the frequencies of Th17 cells were decreased in the small intestine and PP of vitamin A-deficient mice (Fig. 2A, 2B). However, the numbers in spleen and MLNs were unchanged. The data suggest that vitamin A has a tissue-specific role in population of Th17 cells, and it supports the role of RA in generating small intestine homing Th17 cells.

**Differential roles of α4β7 and CCR9 in migration of gut-homing Th17 cells**

Next, we investigated the function of α4β7 and CCR9 in the migration of Th17 cells. First, we compared Th17 cells derived from wild type and integrin β7 (Itgβ7)-deficient mice (Fig. 3A). Itgβ7 is a subunit for both αEβ7 and α4β7. Because RA Th17 cells have low expression of ItgαE (Supplemental Fig. 3), most of the Itgβ7 subunit would be paired with the α4 subunit. We performed a 20 h short-term homing assay to compare wild type and Itgβ7-deficient RA Th17 cells in migration to various organs. The migration of Itgβ7-deficient Th17 cells to the intestine including MLN, PP, small intestine, colon, and cecum was defective (Fig. 3B). Instead, the migration of these Th17 cells to other non-intestinal sites, such as peripheral lymph nodes (313% ± 52), spleen (235% ± 9.5), liver (166% ± 48), and marrow (206% ± 78), was generally enhanced. The Itgβ7-deficient Th17 cells failed to enter MLNs, PP, and the lamina propria of the intestine (Fig. 3C).

When CCR9-deficient RA Th17 cells were compared with wild type Th17 cells (Fig. 4A), they were defective in migration only to
the small intestine lamina propria (Fig. 4B, 4C). Although not statistically significant, the migration of CCR9-deficient RA-Th17 cells to other mucosal tissues, such as MLNs (163% ± 34), the colon (180% ± 56), PPs (155% ± 30), and the peritoneal cavity (148% ± 16), was increased (Fig. 4B). Overall, compared with α4β7, CCR9 had a more specific role in the migration of Th17 cells to the small intestine lamina propria.

RA regulates the gut tissue tropism of Th17 cells in pathogenesis

We hypothesized that RA-Th17 cells and control Th17 cells would induce inflammation in different locations of the intestine because these two T cell populations have different tissue tropisms. To test this hypothesis, the RA-Th17 cells and control Th17 cells were separately injected i.p. into Rag1-deficient mice. We found that RA-Th17 cells induced greater weight loss compared with control Th17 cells that were induced in the absence of RA (Fig. 5A). Mice injected with control Th17 cells had more severe inflammation in the distal colon than in the small intestine. In contrast, mice injected with RA-Th17 cells had the most severe inflammation in the small intestine (Fig. 5B, 5C). The inflammation in the small intestine was accompanied by mucosa/crypt hyperplasia and loss of villi (Fig. 5B, 5C). The inflammation consisted primarily of infiltration by lymphocytes and macrophages with fewer neutrophils and occasional eosinophils. In support of the tissue tropism of RA-Th17 cells, many more Th17 cells were found in the Rag1-deficient mice injected with RA-Th17 cells compared with control Th17 cells, even ∼30 d after the injection of the Th17 cells (Supplemental Fig. 4). In contrast, there was no statistically significant difference in numbers of Th1 and T regulatory cells in the small intestine. Overall, the RA-Th17 cells and control Th17 cells induce inflammation largely in distinct segments of the intestine.

The higher inflammatory activity of RA-Th17 cells could be due to their differences in expression of trafficking receptors and migration ability. Alternatively, this is due to other nonmigratory features of the cells. We compared the inflammatory activities of wild type and CCR9 or Itgβ7-deficient RA-Th17 cells (Fig. 6A). We found that both CCR9 (−/−) RA-Th17 cells and Itgβ7 (−/−) RA-Th17 cells had decreased inflammatory activities compared with wild type RA-Th17 cells. Particularly, the Itgβ7 deficiency completely blocked the inflammatory activity of RA-Th17 cells in all segments of the intestine (Fig. 6B). In contrast, CCR9 deficiency led to blockade of inflammation largely in the small intestine (Fig. 6C).

The decreased inflammation by CCR9 (−/−) RA-Th17 cells and Itgβ7 (−/−) RA-Th17 cells was consistent with decreased numbers of Th17 and T regulatory cells in the small intestine (Fig. 7). Moreover, the number of Th1 cells was decreased in the large intestine of the mice injected with Itgβ7 (−/−) RA-Th17 cells. FoxP3+ T cells were increased in the spleen and large intestine of the mice injected with CCR9 (−/−) or Itgβ7 (−/−) Th17 cells. These results show that the two trafficking receptors that are induced by RA—CCR9 and α4β7—have essential functions in induction of the intestinal inflammation initiated by RA-Th17 cells.

Discussion

We report the presence of Th17 cell subpopulations with distinct in vivo tissue tropisms that is regulated by the RA signal. The tissue tropism of these Th17 cells is determined by differential expression of gut homing receptors. Strikingly, the Th17 cell subpopulations with different tissue tropism induced inflammation in different parts of the intestine. We believe that this is the first demonstration of the presence of Th17 cell subsets with inflammatory activities in distinct compartments of the intestine. What concentration of RA is really available for T cells undergoing Ag activation in vivo? The RA concentration in normal human blood plasma is ∼4.9 ng/ml (28). Comparably, ∼2 ng/ml of RA was detected in rat serum. It is estimated that 50–75% of the RA in serum or plasma is At-RA. Therefore, the concentration of At-RA in normal blood plasma is estimated to be ∼5 nM. The balance between RA-synthesizing retinaldehyde dehydrogenases and RA-inactivating cytochrome P450RAI (CYP26) determines the availability of RA in a given tissue microenvironment. The expression patterns of CYP26 and RA-synthesizing retinaldehyde dehydrogenase type 2 are largely complementary in developing embryos (29–31), and it is expected that a similar pattern of complementary expression of the two enzymes would occur in adults. Based on the sensitivity of the expression of CCR9 and α4β7 in response to RA, it is estimated that the intestinal Ag-priming environment would have at least 2–3 nM and potentially higher levels of active RA (<30 nM). The concentration of RA in peripheral lymph nodes, where CCR9 is not induced, is expected to be <1 nM. We estimate that RA levels between 0.1 and 30 nM are within the physiologic range. RA concentrations higher than this range could be found in vivo but the need for that high RA signal is unclear.

A most characteristic feature of the Th17 cells induced at physiologic levels (10–20 nM) of RA is the expression of both CCR9 and α4β7. These two trafficking receptors are somewhat different from each other in requirement for RA for persistent expression. α4β7 initially induced by RA persists, even after subsequent Ag priming in the absence of exogenous RA. In contrast, expression of CCR9 on Th17 cells is transient requiring continued presence of RA. Moreover, CCR9 is not induced well in response to RA on the Th17 cells that were previously activated in the absence of suboptimal concentrations of RA. We found that α4β7 is a general homing receptor of Th17 cells for the intestine and associated lymphoid tissues, whereas CCR9 is a receptor more specific for the small intestine. This finding suggests that at least several populations of Th17 cells (CCR9− α4β7+, CCR9+ α4β7+, and CCR9− α4β7−) with different homing behaviors can
be generated in different sites of the intestine, depending on the RA availability during repetitive priming processes.

The potent activity of retinoid-induced Th17 cells in inducing small intestine inflammation is notable and is associated with the expression of α4β7 and CCR9. α4β7 is widely expressed by intestinal lymphocytes and serves as the major homing receptor (32, 33). α4β7 is an adhesion molecule for MadCAM-1 (32, 34) and can guide the Th17 cells to the entire gut system. It appears that even basal levels of α4β7, induced in the presence of low (serum) levels of RA, are required for migration of Th17 cells into the intestine and associated lymphoid tissues. Thus, higher expression of α4β7, induced at optimal levels of RA over the physiologic range, confers Th17 cells with the enhanced ability to stay within the intestine and to induce inflammation. CCR9 is expressed more specifically by the T cells in the small intestine and acts as a homing receptor that is specific for this tissue (35–38). CCR9 can guide the Th17 cells to the small intestine lamina propria compartment close to epithelial cells where CCL25 is expressed (35, 39, 40), and therefore would cause the more focused migration and inflammation in the small intestine. Consistently in our study, Itgβ7 deficiency had a broader effect on Th17 cell migration to the intestine, whereas CCR9 deficiency had a specific effect on their homing to the small intestine. In line with the differential roles of these trafficking receptors in determining the tissue tropism of Th17 cells, Itgβ7 deficiency had greater impact than CCR9 deficiency on the inflammatory activity of the retinoid-induced Th17 cells. This finding is supported by the fact that, without proper expression of Itgβ7 or α4β7, Th17 cells fail to migrate to any site of the entire intestinal system, including the small intestine, despite their normal expression of CCR9. This finding is in line with the established roles of these trafficking receptors for other T cells (32, 33, 35, 39, 40), and it suggests that Th17 cells do not deviate from the general trafficking behavior of gut T cells. Some studies have reported that IL-22 has a protective role in intestinal inflammation (12, 41), whereas others reported proinflammatory roles of IL-22 in intestinal inflammation (42, 43).

We would like to point out that there was no significant difference in expression of IL-22 by control and RA Th17 cells (Supplemental Fig. 5).

Because of the previously reported in vitro phenomenon that induction of Th17 cells is suppressed by RA (19, 22, 23), it has been assumed that vitamin A antagonized the population of Th17 cells in the intestine. Our finding with vitamin A-deficient mice indicates that the RA signal is actually important for the presence of Th17 cells in the small intestine. A similar decrease of small intestine Th17 cells in vitamin A deficiency was observed in a mouse model of chronic intestinal inflammation (44), suggesting that it occurs broadly in both normal and inflammatory conditions. We do not rule out the possibility that induction of Th17 cells could be somewhat suppressed by the RA in the small intestine in vivo. However, the increase of gut homing receptor-expressing Th17 cells would compensate for this small decrease caused by the presence of RA. Thus, there would be an overall increase in the number of gut Th17 cells in the intestine in response to RA.

Our results provide useful targets of intervention in regulation of the inflammatory activities of Th17 cells in the intestine. It is possible to regulate the inflammatory activity of Th17 cells in the small versus other compartments of the intestine by selective blocking of CCR9 or Itgβ7. This projection is well supported by therapies approved by the U.S. Food and Drug Administration and other proposed therapies targeting α4 or αβ7 integrin. For example, blocking of the Itgα4 using mAbs to integrin α4 is a treatment for Crohn’s disease approved by the U.S. Food and Drug Administration (45). It was also shown to be effective in the amelioration of colitis in animal models (45–47). Whereas this therapy targets all α4 integrins including α4β7 and αβ1, mAbs more specifically blocking Itgβ7 or MadCAM-1 were also effective in inhibiting colitis in animal models (48, 49). We propose that these reagents can act on the α4 integrins of Th17 cells and can therefore block their migration and inflammatory activities in the intestine. If the inflammatory activity of Th17 cells is localized to the small intestine, blocking of CCR9 would be more specific in treating the localized inflammation without the potential side effects of broad immune-suppression in the body.

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Disclosures
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