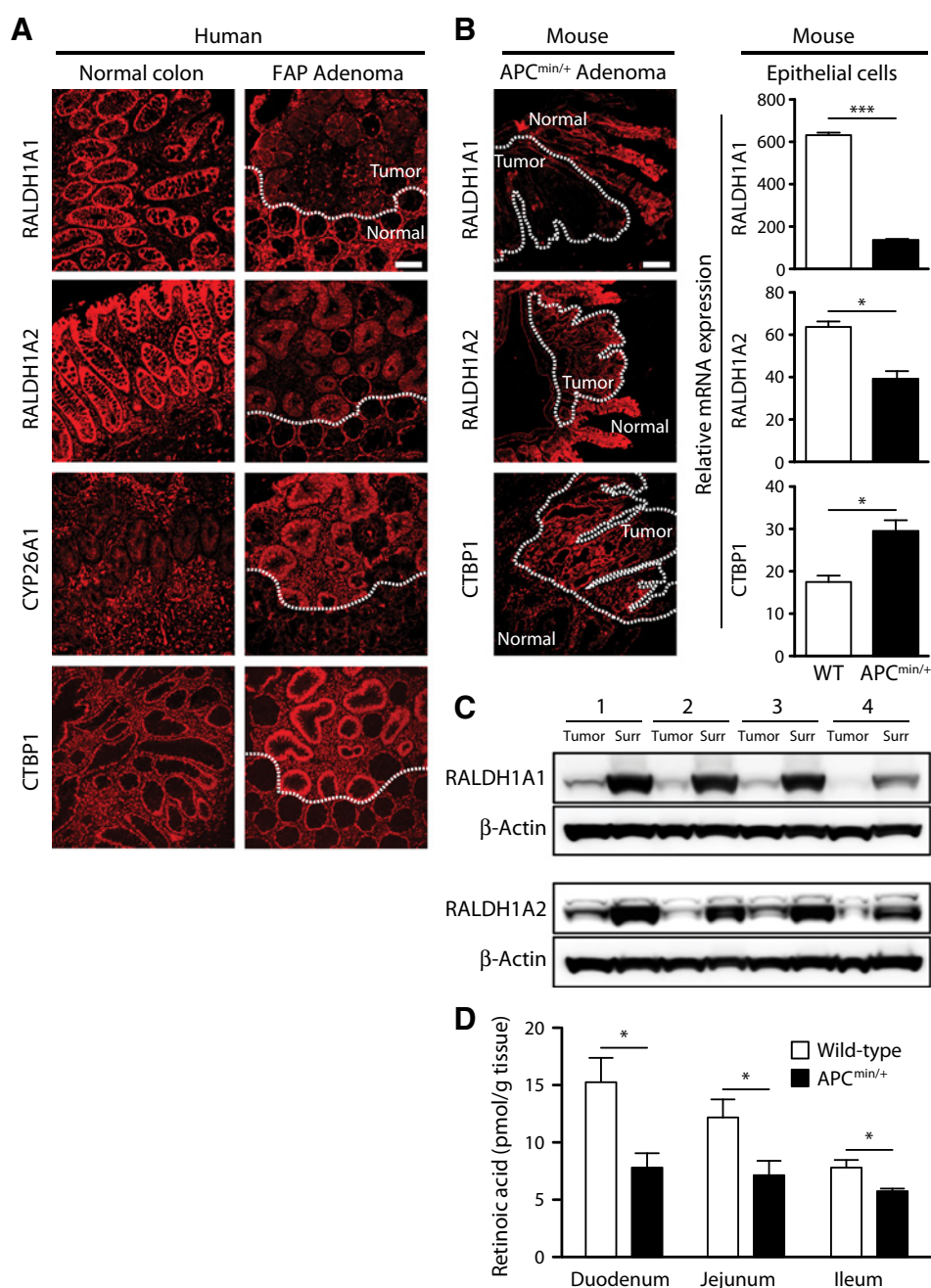








Penny et al.



other disease indicators such as body weight and hematocrit (data not shown).

Given the tight physiological control of RA levels, a potential explanation for the failure of RA i.p. to increase RA was that administered RA is rapidly catabolized by the upregulated CYP26A1 in APC<sup>Min/+</sup> tissue (20). On this basis, we evaluated an alternative strategy to reconstitute intestinal RA by targeting the upregulated CYP26A1 with liarozole, an inhibitor of this enzyme (23). When 8-week-old APC<sup>Min/+</sup> mice were fed chow containing liarozole at 40 ppm for 6 weeks, RA was restored in the ileum of liarozole-treated mice (Fig. 2A). Moreover, compared with base diet-treated controls, liarozole-fed APC<sup>Min/+</sup> mice exhibited a reduction in overall tumor burden (Fig. 2B), as well as a substan-

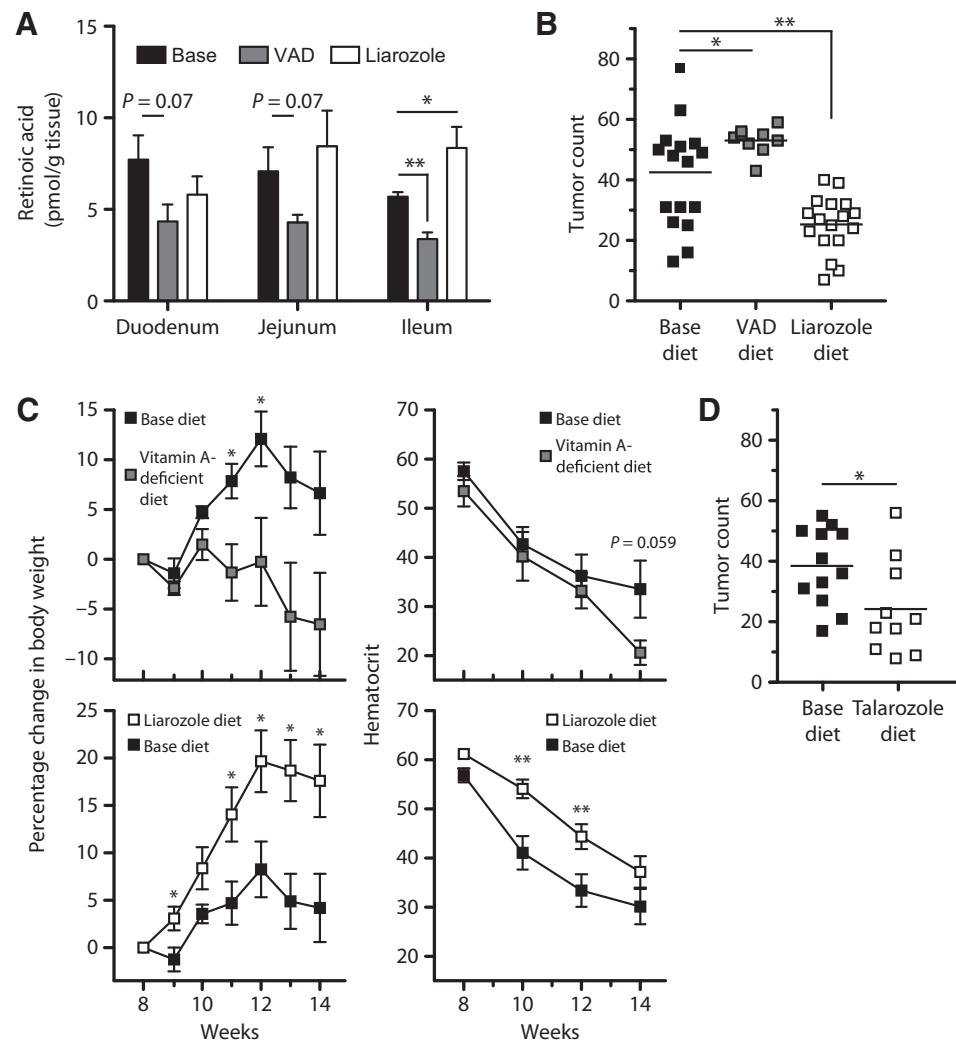
tial increase in body weight and a lesser decrease in hematocrit (Fig. 2C). To confirm the detrimental effects of upregulated CYP26A1 and validate our results with liarozole, we treated APC<sup>Min/+</sup> mice with talarozole, a highly selective inhibitor of CYP26 that is structurally distinct from liarozole (24). Consistent with the results from liarozole treatment, talarozole improved disease outcome (Fig. 2D).

#### APC<sup>Min/+</sup> LPDCs preferentially induce Th17 cells

Given the importance of DCs in maintaining immune tolerance in the intestine, we hypothesized that low RA in the intestine of APC<sup>Min/+</sup> mice might affect local DC function. At steady state, DCs in the gut consist of three phenotypically

**Figure 2.**

VAD reduces intestinal RA and exacerbates disease, while CYP26A1 inhibition restores intestinal RA levels and ameliorates disease. Base ■; VAD ■; liarozole □. Groups of 8 week-old  $APC^{Min/+}$  mice were placed on base diet, VAD diet, base diet containing liarozole 40 ppm for 6 consecutive weeks. **A**, as in Fig. 1D, RA was quantified by LC/MS, with 5 mice per dietary treatment. Mean number of tumors (**B**) and mean change in percentage body weight and hematocrit (**C**) at 14 weeks are shown for  $APC^{Min/+}$  mice given these treatments. **D**, additional groups of 8 week-old  $APC^{Min/+}$  mice were fed chow containing talarozole 8 ppm for 6 weeks, and intestinal tumors were enumerated. For **A-D**, data for VAD-fed mice are aggregated from 2 independent experiments, with 4 mice per experiment; data for liarozole-treated mice are aggregated from 4 independent experiments, with at least 4 mice per experiment. For **D**, data for talarozole-treated mice are aggregated from 2 independent experiments, with 5 mice per experiment. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .



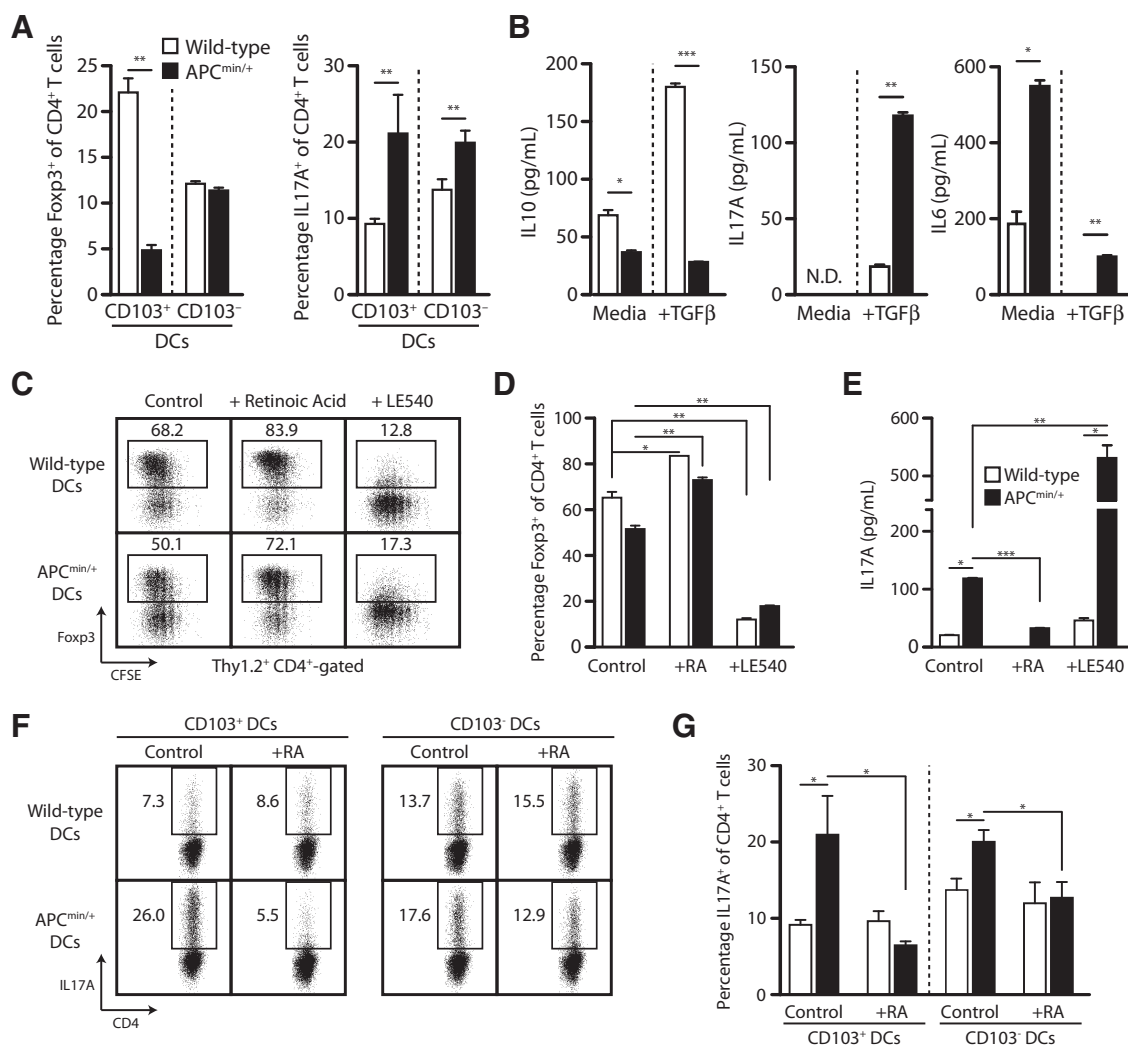
distinct populations:  $CD103^+CD11b^-$ ,  $CD103^+CD11b^+$ , and  $CD103^-CD11b^+$  DCs. No significant differences in the frequencies of the three main DC subsets were observed in the small intestine LP (SI-LP) of  $APC^{Min/+}$  mice compared with WT mice (Supplementary Fig. S2C). Further studies of SI-LPDCs from  $APC^{Min/+}$  mice revealed that, although their expression of costimulatory molecules was similar to that of WT SI-LPDCs (Supplementary Fig. S2D), they secreted much more proinflammatory cytokines such as  $TNF\alpha$ , IL6, and IL12p40 under basal conditions and in response to a panel of Toll-like receptor agonists (Supplementary Fig. S2E). As  $CD103^+$  LPDCs are the main cells responsible for generating Tregs in the intestinal environment (12), we sorted LPDCs into  $CD103^+$  and  $CD103^-$  subsets (sorting strategy shown in Supplementary Fig. S3) and assessed the role of each subset in a conventional TGF $\beta$ -dependent Treg induction assay (12, 14, 15). This assay utilizes a relatively low concentration of peptide as this is important for Treg differentiation (25–27). Although we attempted to sort the highest CD11c-expressing cells from the lamina propria, it is possible that the  $CD103^-$  cell population had some contaminating macrophages.  $APC^{Min/+}$   $CD103^+$  LPDCs only induced 25% of the  $Foxp3^+$  T cells compared with their WT counterparts

(Fig. 3A). In contrast,  $CD103^-$  LPDCs from WT and  $APC^{Min/+}$  mice induced  $Foxp3^+$  T cells equally (Fig. 3A). Both  $CD103^-$  and  $CD103^+$   $APC^{Min/+}$  LPDCs induced more Th17 cells compared with the WT LPDC subsets (Fig. 3A). Consistent with these results, supernatants obtained from whole  $APC^{Min/+}$  LPDC-T-cell cocultures contained only one-sixth of the IL10 compared with WT LPDC cocultures (Fig. 3B). Moreover, there was a concomitant and similarly dramatic increase in IL17A (Fig. 3B), an inflammatory cytokine that plays an important role in adenoma development (9). Because Th17 differentiation requires IL6 in addition to TGF $\beta$  (28) and no exogenous IL6 was added to our cultures, we measured IL6 in coculture supernatants and, as expected, found more in  $APC^{Min/+}$  LPDC cocultures compared with WT cocultures (Fig. 3B).

#### RA reverses the inflammatory phenotype of $APC^{Min/+}$ LPDCs *in vitro*

$APC^{Min/+}$  LPDCs expressed less RALDH1A2 compared with WT LPDCs (Supplementary Fig. S4), possibly reducing their RA synthesis capacity and exacerbating their proinflammatory phenotype. Therefore, we hypothesized that the proinflammatory phenotype of  $APC^{Min/+}$  LPDCs could be modulated by RA

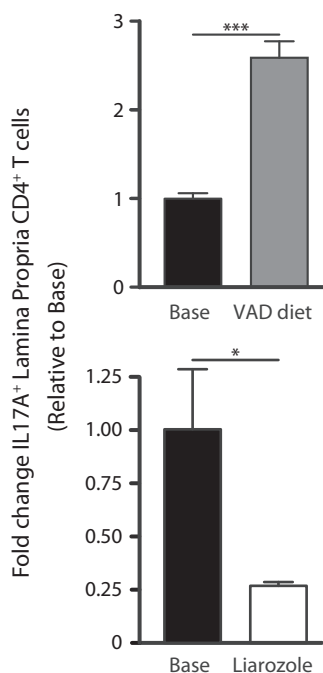
Penny et al.

**Figure 3.**

Exposure of APC<sup>Min/+</sup> LPDCs to RA, *in vitro*, reverses their inflammatory phenotype. **A**, to assess Treg induction capacity, LPDCs, defined as PI<sup>-</sup> EpCAM<sup>-</sup> CD45<sup>+</sup> Lin<sup>-</sup> CD11c<sup>hi</sup> MHCII<sup>+</sup> (Supplementary Fig. S3), were further sorted into CD103<sup>+</sup> and CD103<sup>-</sup> subsets and cocultured with CD4<sup>+</sup>CD62L<sup>+</sup>Foxp3<sup>-</sup> naive T cells from OT-II mice, along with OVA<sub>323-339</sub> peptide and TGFβ, and the frequency of Foxp3<sup>+</sup> and IL17A<sup>+</sup> T cells was determined after 4 days. Shown are the mean frequencies of Foxp3<sup>+</sup> T cells and IL17A<sup>+</sup> T cells induced in these cultures, representative of 3 independent experiments. **B**, whole LPDC-T-cell cocultures were stimulated with plate-bound anti-CD3 and anti-CD28 for the last 6 hours of coculture and IL10, IL17A, and IL6 were assayed in the supernatants. The experiment shown is representative of 4 independent experiments. **C-E**, all-trans RA or the RAR antagonist LE540 was added to whole CD11c<sup>+</sup> LPDC-T-cell coculture wells at the initiation of coculture, and induced Foxp3<sup>+</sup> T cells are shown in representative dot plots and bar graphs (**C** and **D**). **E**, cells were stimulated with plate-bound anti-CD3 and anti-CD28 for the last 6 hours of coculture. Bar graphs show mean production of IL17A in culture supernatants harvested at the end of coculture. For **C-E**, results shown are representative of 4 independent experiments. **F** and **G**, CD103<sup>+</sup> and CD103<sup>-</sup> LPDCs were incubated with all-trans RA (+RA) or in media alone (control) for 18 hours, washed extensively and cocultured with naive T cells as before. Representative dot plots and bar graphs show the mean frequency of the induced IL17A<sup>+</sup>CD4<sup>+</sup> T cells. For **F** and **G**, results shown are representative of 3 independent experiments. For **A-G**, DCs are pooled from 5 WT and 3 APC<sup>Min/+</sup> mice per experiment, all at 18 weeks of age. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

exposure. To assess this, we added RA or the RA receptor antagonist LE540 to LPDC-T-cell cocultures. The addition of RA to APC<sup>Min/+</sup> LPDC-T-cell cocultures restored the induction of Foxp3<sup>+</sup> CD4<sup>+</sup> T cells to WT coculture numbers (Fig. 3C and D) and also strongly inhibited the production of IL17A (Fig. 3E). In contrast, LE540 abrogated Foxp3 induction in both WT and APC<sup>Min/+</sup> LPDC cocultures (Fig. 3C and D) and greatly enhanced IL17A production in APC<sup>Min/+</sup> cocultures (Fig. 3E). To determine whether the normalizing effects of RA were due to direct action on

DCs, CD103<sup>+</sup> and CD103<sup>-</sup> LPDCs from WT and APC<sup>Min/+</sup> mice were incubated with 10 nmol/L RA for 18 hours, extensively washed, and then cocultured with OT-II T cells to assess their effects on T-cell differentiation. RA treatment completely reversed the proinflammatory phenotype of both APC<sup>Min/+</sup> LPDC subsets, reducing their induction of Th17 cells to WT levels (Fig. 3F and G). Although induction of Foxp3<sup>+</sup>CD4<sup>+</sup> T cells by RA-treated APC<sup>Min/+</sup> LPDCs trended toward WT levels, this increase did not reach statistical significance (data not shown). Taken together,

**Figure 4.**

Restoration of RA *in vivo* reverses the proinflammatory phenotype of LPDCs of APC<sup>Min/+</sup> mice. Fold change in the mean frequency of IL17A<sup>+</sup> CD4<sup>+</sup> T cells in LP from APC<sup>Min/+</sup> mice treated with liarozole, VAD or base diet, as described in Fig. 2. The experiment shown is representative of 2 independent experiments, with 4 mice per diet. DCs obtained were pooled from all mice on the same diet in each experiment. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ .

these results indicate that RA can act directly on APC<sup>Min/+</sup> LPDCs to suppress their Th17-polarizing capacity.

#### Th17 inflammation is exacerbated by VAD diet and alleviated by RA restoration

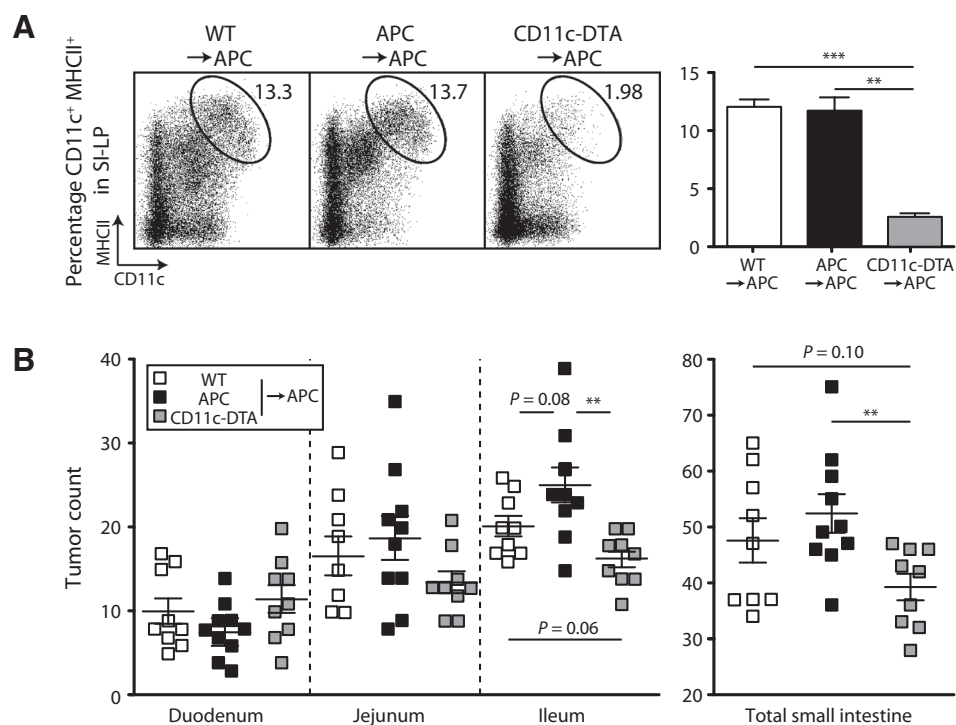
Because altered RA concentrations in the intestine have been linked to mucosal inflammation, we sought to determine whether modulation of RA *in vivo* via VAD diet or blockade of RA catabolism via liarozole treatment affected Th17 frequency. Mice maintained on a VAD diet had an almost 3-fold increase in Th17 cells relative to untreated control mice, whereas liarozole-treated APC<sup>Min/+</sup> mice had a nearly 75% reduction in the number of Th17 cells (Fig. 4). These findings show that repletion of RA reverses the proinflammatory phenotype of LPDCs, attenuates Th17-driven inflammation, and ameliorates disease, whereas depletion of RA exacerbates inflammation and disease.

#### Ablation of DCs reduces tumor burden in APC<sup>Min/+</sup> mice

To directly assess the role of DCs in tumor progression, we generated bone marrow (BM) chimeras using donor cells in which diphtheria toxin A (DTA) is activated in CD11c-expressing cells (CD11c:DTA mice; ref. 29). Chimeras generated with CD11c:DTA BM exhibited greatly reduced numbers of DCs in the SI-LP compared with chimeras with WT and APC<sup>Min/+</sup> BM (Fig. 5A). As shown in Fig. 5B, DC depletion in APC<sup>Min/+</sup> mice resulted in a decrease in total tumor frequency relative to mice reconstituted with APC<sup>Min/+</sup> BM. Reconstitution with WT BM also led to a reduction in tumor number, although the reduction did not reach statistical significance. These results directly demonstrate that DCs in APC<sup>Min/+</sup> mice exacerbate tumor progression, and are

**Figure 5.**

Ablation of CD11c<sup>+</sup> DCs in APC<sup>Min/+</sup> mice reduces adenoma formation. WT □; CD11c:DTA ■; APC<sup>Min/+</sup> ■. Six-week-old APC<sup>Min/+</sup> mice were lethally irradiated and reconstituted with WT, APC<sup>Min/+</sup>, or CD11c:DTA BM. **A**, representative dot plots and bar graphs depict DC populations in the SI-LP of CD11c:DTA BM-reconstituted APC<sup>Min/+</sup> mice compared with those reconstituted with WT or APC<sup>Min/+</sup> BM, using 3 mice per group. **B**, scatter plots of tumors in the small intestine enumerated at 24 weeks, as aggregated from 2 independent experiments, with at least 4 mice per group per experiment. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .



consistent with our finding that a factor in the gut microenvironment, which we believe to be RA, regulates these cells.

## Discussion

Our study quantitatively demonstrates RA deficiency in the intestinal tissue of a murine model of spontaneous familial intestinal polyposis. In addition, we report that restoration of intestinal RA attenuates inflammation with a concomitant reduction in polyp formation. Although RA inhibits the growth of a variety of tumor cell lines, including osteosarcoma (30), breast (31, 32), thyroid (33), and even head and neck (34) cancer, such *in vitro* findings lack the clinical relevance of the APC<sup>Min/+</sup> model. In an azoxymethane-induced rat model of colon cancer, 9-*cis*-RA reduces the multiplicity of aberrant crypt foci in the colon (35). This finding is consistent with our findings, although the model of colon cancer utilized was chemically induced, and may not recapitulate the physiologic condition as well as the APC<sup>Min/+</sup> model. Whereas these previous studies focused on the antiproliferative and differentiating effects of RA, we highlight its potent capacity to modulate the tumor immune profile. RA repletion alone can attenuate inflammation as well as impede disease progression in the APC<sup>Min/+</sup> model.

The APC<sup>Min/+</sup> model differs from FAP in one major respect, the principal location of tumors (ileum vs. colon). Nonetheless, the model is considered highly relevant to FAP due to its similarities in virtually all other aspects of the disease. Moreover, anti-inflammatory drugs and other agents that reduce inflammation and tumor growth in APC<sup>Min/+</sup> mice also reduce tumor growth in the colon of FAP patients (36). Our data show that affected colons from FAP patients express inflammatory markers and abnormalities in RA metabolism consistent with those seen in the APC<sup>Min/+</sup> small intestine. Although we saw substantial decreases in RA concentration in all sections of the APC<sup>Min/+</sup> small intestine, it remains to be understood why the majority of polyps develop in the ileum, with few (<3) polyps ever observed in the colon.

We found that APC<sup>Min/+</sup> mice have an RA deficit in their intestines. The reduction was due to both diminished synthesis and excessive breakdown of the molecule. Expression of the RALDH enzymes that promote RA production was reduced in APC<sup>Min/+</sup> intestinal epithelial cells (IEC), along with a marked accumulation of CTBP1, which normally suppresses RDH (21). Constitutive expression of  $\beta$ -catenin downstream of the APC mutation upregulates the major RA catabolic enzyme, CYP26A1 (20). Indeed, we observed upregulated CYP26A1 expression in APC<sup>Min/+</sup> and FAP adenomas, consistent with previous findings in sporadic colorectal carcinomas and the intestine of APC-mutant zebrafish embryos (20). Taken together, these results point to several mechanisms that cooperate to decrease RA concentration in the tumor milieu.

The role of intestinal inflammation in the development of adenomas in the APC<sup>Min/+</sup> model of spontaneous neoplasia is well documented. However, the few immunological studies performed on APC<sup>Min/+</sup> mice and related APC mutation models have focused almost exclusively on altered T-cell responses, with little attention directed at the underlying cause of these changes (7, 9, 37, 38). Our findings suggest that proinflammatory LPDCs in these mice may play an important role in inducing and maintaining Th17 cells. This contrasts with the LPDCs of healthy mice, which do not promote inflammation but instead maintain immune tolerance through the generation of Tregs (12, 14,

15, 39). Stimulation with various TLR ligands causes LPDCs to produce more IL6 and TNF when compared with LPDCs from wild-type mice. It is well known that efficient induction of Th17 cells requires TGF $\beta$  and IL6 (40). The addition of TGF $\beta$  to cultures simultaneously reduced IL6 release from LPDCs as well as stimulated the generation of IL17-producing Th17 cells. Given the widely reported pleiotropism of TGF $\beta$  proteins, this seemingly contradictory effect is not surprising and potentially represents a control mechanism for restricting the induction of Th17 cells. When proinflammatory LPDCs from APC<sup>Min/+</sup> mice are cultured in the presence of RA, *in vitro*, or exposed to increased concentrations of RA *in vivo*, they revert to tolerogenic cells. Although these findings do not prove that LPDCs are the only immune cells affected by RA deficiency, they highlight their importance in promoting the intestinal inflammation that characterizes this disorder.

A subset of intestinal macrophages also expresses CD11c and MHC II (41). As such, these cells may have been included in our DC-enriched LP preparations, although their contribution to intestinal inflammation in APC<sup>Min/+</sup> mice is unknown. Regardless, the induction of Th17 cells by LP antigen-presenting cells might explain the dependence of tumor growth on IL17 in the APC<sup>Min/+</sup> intestine (9) and potentially represents a critical control point in shaping the inflammatory milieu that drives tumor growth.

Our studies also examined the impact of RA metabolism on intestinal DC subsets. The data indicate that both CD103<sup>+</sup> and CD103<sup>-</sup> LPDC subsets are responsive to local fluctuations in RA concentration and adopt a proinflammatory phenotype in the presence of reduced RA. The CD103<sup>+</sup> LPDCs isolated from APC<sup>Min/+</sup> mice were more phenotypically altered than the CD103<sup>-</sup> LPDCs, following *in vitro* and *in vivo* modulation of RA concentrations. Although our studies indicate that RA can act directly on APC<sup>Min/+</sup> LPDCs to reverse their inflammatory phenotype, RA may affect intestinal inflammation through additional mechanisms. One potential mechanism involves IL22, which is a potent inducer of pathologic inflammation (42) that promotes intestinal tumorigenesis in APC<sup>Min/+</sup> mice (43). The secreted receptor that inhibits IL22, IL22BP, is constitutively produced by mouse intestinal CD11b<sup>+</sup>CD103<sup>+</sup> DCs (44). RA can elicit the expression of IL22BP in human immature monocyte-derived DCs (44), suggesting that RA may regulate intestinal tolerance *in vivo* via expression of IL22BP by the mouse CD11b<sup>+</sup>CD103<sup>+</sup> DC subset. In another study, mice given a pan-RAR antagonist or subjected to radiation-induced mucosal injury to obtain an acute VAD state selectively lost the CD11b<sup>+</sup>CD103<sup>+</sup> DC subset (45). These findings suggest that CD11b<sup>+</sup>CD103<sup>+</sup> DCs are particularly dependent on RA for their function. Whether the effect of RA on APC<sup>Min/+</sup> LPDCs depends on IL22 remains to be determined.

Large i.p. doses of RA did not reduce tumor burden or affect GI inflammation, which initially puzzled us, but was explained by the lack of increased RA concentration in the intestine which, in turn, is likely due to rapid catabolism of the injected RA by upregulated CYP26A1. This result is consistent with a prior report of orally administered RA not benefiting and, in fact worsening, disease in APC<sup>Min/+</sup> mice (46). In contrast to systemically administered RA, our study showed that the CYP26A1 inhibitor liarozole increased intestinal RA, reduced Th17 inflammation, and ameliorated disease. Because liarozole can affect other CYP enzymes in addition to CYP26A1, we studied talarozole, a third-generation RA metabolism-blocking agent. Like liarozole,



talarozole inhibits CYP26A1 from hydroxylating carbon 4 of RA (23), thus preventing the catabolism of RA. However, in contrast to liarozole, talarozole has virtually no effect on other CYP-dependent activities (47) and is specific to certain isoforms of CYP26A1. Moreover, it is structurally unrelated to liarozole and generates different metabolites (23). As shown in our study, talarozole, like liarozole, ameliorated disease, confirming that tumor development induced by intestinal inflammation can be reversed by inhibiting RA breakdown.

Conversely, a VAD diet resulted in more pronounced DC-mediated inflammation and exacerbation of all disease parameters. A standard protocol for achieving vitamin A deficiency in mice is to place pregnant dams on a 0 IU/g vitamin A diet beginning days 7 to 10 of gestation (48). However, we observed that RA concentrations were further reduced from baseline in APC<sup>Min/+</sup> mice fed a VAD diet for 6 weeks starting at 8 weeks of age, suggesting that the existing RA deficiency in the APC<sup>Min/+</sup> intestine may make these animals more susceptible to the effects of VAD. In contrast, WT mice subjected to the same VAD diet and protocol did not exhibit any signs of inanition and appeared to tolerate this diet well (data not shown).

In studies of a mouse model of Crohn's disease in which TNF $\alpha$  is constitutively produced, Collins and colleagues showed that RA concentrations are reduced in this model and RA supplementation attenuates ileitis (22). Like APC<sup>Min/+</sup> mice, regulatory LPDCs were reduced in frequency in this model and recovered with RA treatment, but proinflammatory LPDCs were not found. Also, whereas oral RA proved effective in the Crohn's model, it was ineffective in APC<sup>Min/+</sup> mice, suggesting that the mechanism responsible for the RA deficit in these two models is distinct. Despite these differences, the combined results suggest that RA deficiency may underlie several inflammatory disorders of the intestine.

Although we did not measure RA in FAP tissues, an RA deficit likely occurs in this disease, based on the severe vitamin A metabolism defects seen in FAP intestine. Given their many shared features, it is reasonable to hypothesize that the beneficial impact of RA reconstitution on APC<sup>Min/+</sup> disease might also be seen in FAP. Attempts to increase RA in the intestine of patients with inflammatory bowel disorders must be undertaken with caution, because RA in combination with IL15 has been reported to induce proinflammatory DCs in a mouse model of celiac

disease and pharmacologic retinoid treatment may be a risk factor for inflammatory bowel disease (49, 50). Nonetheless, our results provide a strong rationale for studying CYP26A1 inhibitors in appropriate patients. Liarozole and talarozole have been evaluated in clinical trials for diseases unrelated to FAP or colorectal cancer and are apparently well tolerated (51, 52). These or other agents that safely increase intestinal RA have the potential to reverse inflammation and reduce tumor burden as observed here in APC<sup>Min/+</sup> mice.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

**Conception and design:** H.L. Penny, T.R. Prestwood, N. Bhattacharya, E.G. Engleman

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** H.L. Penny, T.R. Prestwood, N. Bhattacharya, E.G. Engleman, J. Wang, J.L. Napoli

**Writing, review and/or revision of manuscript:** H.L. Penny, T.R. Prestwood, N. Bhattacharya, E.G. Engleman, J.A. Kenkel, M.G. Davidson, J.L. Napoli

**Study supervision:** E.G. Engleman

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Penny et al.

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