Retinoic Acid Is Essential for Th1 Cell Lineage Stability and Prevents Transition to a Th17 Cell Program

Highlights
- Retinoic acid (RA) stabilizes Th1 fate commitment
- Signaling through RA receptor α (RARα) activates enhancers of Th1-cell-lineage-specifying genes
- RA-RARα represses Th17-cell genes in Th1 cells and constrains Th1-cell plasticity
- RA-RARα prevents development of pathogenic Th17 cells in vivo

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In Brief
Maintenance of T helper (Th)-cell identity is critical for appropriate immune responses; however, the factors that regulate Th-cell plasticity are unresolved. Brown et al. show that retinoic-acid signaling confers Th1 cell stability and restrains their conversion to Th17 cells.
Retinoic Acid Is Essential for Th1 Cell Lineage Stability and Prevents Transition to a Th17 Cell Program


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SUMMARY

CD4+ T cells differentiate into phenotypically distinct T helper cells upon antigenic stimulation. Regulation of plasticity between these CD4+ T-cell lineages is critical for immune homeostasis and prevention of autoimmune disease. However, the factors that regulate lineage stability are largely unknown. Here we investigate a role for retinoic acid (RA) in the regulation of lineage stability using T helper 1 (Th1) cells, traditionally considered the most phenomenally stable Th subset. We found that RA, through its receptor RARα, sustains stable expression of Th1 lineage specifying genes, as well as repressing genes that instruct Th17-cell fate. RA signaling is essential for limiting Th1-cell conversion into Th17 effectors and for preventing pathogenic Th17 responses in vivo. Our study identifies RA-RARα as a key component of the regulatory network governing maintenance and plasticity of Th1-cell fate and defines an additional pathway for the development of Th17 cells.

INTRODUCTION

Functional plasticity within cells of the innate and adaptive immune system increases the breadth of response to pathogens while also limiting responses detrimental to the host. CD4+ T cells diversify into distinct effector subsets upon antigenic stimulation. Cytokines and other microenvironmental factors present during T-cell priming direct differentiation via induction of lineage specifying transcription factors (TFs): T-bet is the “master” regulator for T helper 1 (Th1) cells, RORγt for Th17 cells, and GATA3 directs the Th2 program. In vivo, the presence of cells that express TFs and cytokines from opposing Th lineages indicates flexibility between those subsets. Late-stage developmental plasticity is potentially perilous: interferon-γ (IFN-γ) Th17 cells have been implicated in several human autoimmune diseases including inflammatory bowel disease (Annunziato et al., 2007), juvenile idiopathic arthritis (Nistala et al., 2010), and multiple sclerosis (Kebir et al., 2009); ex-Foxp3+ Th17 cells play a pathogenic role in rheumatoid arthritis (Komatsu et al., 2014); and interleukin-17 (IL-17)+ Th2 cells have been positively linked to the severity of asthma (Irvin et al., 2014). Elucidating the developmental pathways for these hybrid cells and identifying the factors that regulate Th-cell stability are therefore of critical importance.

Initial lineage specification is driven by cytokines, which activate signal transducer and activator and transcription (STAT) proteins: expression of T-bet is driven by IFN-γ-STAT1 and IL-12-STAT4 (Schulz et al., 2009); RORγt by STAT3 downstream of IL-6, IL-21, and IL-23 (Zhou et al., 2007). Less is known about the molecular mechanisms that sustain lineage identity. Epigenetic modifications stabilize gene expression and as such, are thought to play a key role in the maintenance of cell-fate commitment. However, the factors that co-ordinate chromatin changes with evolving TF networks in differentiating Th cells are not fully defined. One candidate is the vitamin A metabolite, retinoic acid (RA). RA is known to play a key role in directing the lineage fate of hematopoietic stem cells (Chanda et al., 2013), dendritic cells (DCs) (Klebanoff et al., 2013), innate lymphoid cells (ILCs) (Spencer et al., 2014), and CD4+ T cells (Reis et al., 2013) through activation of the nuclear RA receptor (RAR). In addition to its classical role as a transcriptional regulator, recent studies in embryonic stem cells have identified RA-RAR as an epigenetic regulator (Kashyap et al., 2013; Urvalek and Gudas, 2014). RA synthesis is dynamically controlled at sites of T-cell priming during inflammation, where RA signaling on T cells has been demonstrated (Aoyama et al., 2013; Pino-Lagos et al., 2011). These studies suggest a potential role for RA in Th-cell plasticity. Indeed, RA is critical for Th1-cell immunity (Hall et al., 2011; Pino-Lagos et al., 2011) and RA has also been implicated in Th17-cell differentiation where its impact appears to be...
of the transcriptional targets of RARα in CD4+ T cells (Hall et al., 2011), to date a comprehensive analysis has been identified as the critical mediator of RA actions in Immunity 2007; Takahashi et al., 2012; Xiao et al., 2008). Although RARα in vivo negatively regulates Th17-cell responses (Mucida et al., 2007), administration of higher concentrations of RA both in vitro and in Th17-cell differentiation in vitro (Takahashi et al., 2012), yet dose dependent: physiological concentrations of RA enhance Th17-cell differentiation in vitro and oral antigen induced inflammation resulted in impaired Th17-cell fate. In the absence of RA signaling, infectious lineage-defining genes while repressing genes that regulate Tbx21 and Gata3 expression of Th17 cells (Mucida et al., 2007). Therefore it is likely that under steady-state conditions RA is critical for differentiation of Th1 cells, while also limiting the differentiation of Th17 cells.

RESULTS

RA-RARα Regulates the Balance between Th1 and Th17 Cells

To directly assess the role of RA in Th-cell differentiation in vivo we used mice carrying a sequence encoding a dominant-negative form of the RA receptor RARα (RARαx403) targeted to ROSA26 downstream of a loxP-flanked “stop” (Isi) cassette. As shown previously (Pino-Lagos et al., 2011), interbreeding with mice expressing Cre recombinase from the Cd4 promoter generates Cd4cre/dnRaralsl/lsl progeny (dnRARα mice) in which RA signaling is abrogated within the T-cell compartment. In contrast to Rara−/− mice, expression of this dnRARα disrupts the RA dependent activity of RARα while retaining the ligand independent effects, allowing the specific analysis of RA-dependent functions.

To investigate the role of RA in the generation of Th-cell subsets under steady-state conditions, we determined the expression of cytokines within CD4+ T cells with an activated CD44 hi phenotype. Examination of the peripheral CD4+ T-cell compartment revealed equivalent frequencies and absolute numbers of CD44hiCD62loCD4+ memory cells in 8-week-old dnRARα and WT mice (Figures 1A–1C). dnRARα effector cells displayed reduced production of IFN-γ compared to their WT counterparts with a >5-fold increase in the frequency of IL-17+ cells (Figures 1D and 1E). Examination of transscripts for the signature lineage-determining TFs showed reduced mRNA expression of Tbx21 and significantly higher expression of Rorc in dnRARα effector CD4+ T cells (Figure 1F). Loss of RA signaling had no impact on Th2 effectors with equivalent levels of Gata3 expression between dnRARα and WT mice (Figure 1F) and similar frequencies of IL-4 producing CD4+ T cells (data not shown).

The frequency and numbers of Foxp3+ T cells in the periphery and thymus of dnRARα mice were similar to control mice (Figures S1A and S1B), indicating that the increase in Th17 cells was not a consequence of reciprocal regulation by RA of Foxp3+CD4+ T cells and Th17 cells (Mucida et al., 2007). Therefore it is likely that under steady-state conditions RA is critical for differentiation of Th1 cells, while also limiting the differentiation of Th17 cells.
RA Promotes Th1-Cell Differentiation and Inhibits Development of Th17 Cells from Th1 Cell Precursors

We considered two alternative explanations of why dnRara mice exhibit reduced memory effector Th1 cells, in parallel with enhanced Th17 cells. The first possibility was that RA is required for the development of Th1 cells while independently suppressing the primary differentiation of Th17 cells. The alternative possibility was that RA is critical in restraining conversion of Th1 cells to Th17 cells. In order to resolve these two possibilities, naive CD4+ T cells were differentiated in the presence of Th1 or Th17 polarizing cytokines. dnRara expressing CD4+ T cells differentiated under Th1 cell conditions showed a markedly reduced capacity for IFN-γ production (Figure 2A). Diminished intracellular production was not a consequence of impaired proliferative responses as naive CD4+ T cells differentiated under Th1-cell conditions showed robust proliferation, equivalent to WT cells (Figure S2A). In addition, upregulation of the activation markers CD25 and CD44 indicated that dnRara T cells were not impaired in their ability to differentiate into effector cells (Figure S2B).

Analysis of TF expression showed that ablating RA signaling resulted in a dramatic reduction in the expression of T-bet in CD4+ T cells differentiated under Th1-cell conditions (Figure 2B). Strikingly, a substantial proportion of dnRara Th1 cells expressed RORyt and co-expression of T-bet and RORyt was observed at the single-cell level. Although we did not observe intracellular IL-17A in cells following brief stimulation with phorbol myristate (PMA) and ionomycin, analysis of supernatants from Th1 polarized cells revealed a substantial proportion of dnRara Th1 cells. Exogenous IFN-γ was not observed with comparable amounts of T-bet expression between WT and dnRara T cells at day 3 of culture, indicating that RA-RARα signaling is not required for early Th1 lineage commitment (Figure 3A). However, T-bet expression was not sustained in dnRara Th1 cells, with substantially diminished expression of T-bet by day 5 of culture. Given that IFN-γ promotes T-bet expression, the expression of T-bet was examined in the presence of recombinant IFN-γ, in order to avoid potential indirect effects caused by reduced IFN-γ production in dnRara Th1 cells. Exogenous IFN-γ enhanced early T-bet expression in both dnRara and WT Th1 cells but did not rescue the late (>72 hr) impairment in T-bet expression (Figure 3A). IFN-γ signaling, as measured by STAT1 phosphorylation, was not impaired at either time point (data not shown).

The late IL-12-dependent peak of T-bet expression observed in the presence of blocking IFN-γ antibodies was abrogated in dnRara Th1-cell polarized cells (Figure 3A) suggesting impaired STAT4 activity. At day 3 of culture, comparable amounts of phosphorylated STAT4 (pSTAT4) were observed between dnRara and WT mice. By contrast, at day 6 of culture, IL-12 induced pSTAT4 was markedly impaired in dnRara T cells (Figure 3B) despite comparable expression of IL-12Rβ2 mRNA and protein expression and increased expression of Il12rb1 mRNA (Figure 3C and 3D). Analysis of Stat4 expression, demonstrated impaired induction of Stat4 in the absence of RA signaling (Figure 3E) with reduced amounts of total STAT4 protein (Figure 3F). These findings suggest that the observed reduction in pSTAT4 in dnRara Th1 cells is a consequence of diminished STAT4 expression. Consistent with deviation toward the Th17-cell lineage, we observed enhanced pSTAT3 activity in Th1-cell polarized dnRara cells with an increased ratio of pSTAT3/pSTAT4 (Figures S3A and S3B).

To evaluate whether the impairment in T-bet and STAT4 expression correlated with changes in IFN-γ, the time course
Figure 2. RA Signaling Required for Th1-Cell Differentiation and Repression of Th17-cell Fate in Th1-Cell Precursors

Sorted naive CD4+ T cells from dnRara or WT mice were cultured under Th1 conditions for 6 days.

(A) Intracellular expression of IFN-γ and IL-17A following stimulation with PMA and ionomycin.

(B) T-bet and RORγt expression. Grey histograms indicate staining for Tbx21−/− (left panel) or isotype control antibody (right panel). Numbers show MFI. Numbers in quadrants represent percent cells in each.

(C) Amount of IL-17A, IL-21, IL-22, and IL-10 in supernatants following restimulation of cells as in (A) with α-CD3 and α-CD28 for 24 hr as measured by multiplex bead array. Triplicate culture wells.

(D) Quantitative real time PCR analysis of Th1 and Th17-cell signature cytokine and TF genes following stimulation with PMA and ionomycin.

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of IFN-γ expression following initiation of Th1-cell polarization was analyzed in naive dnRara-Ifng<sup>eYFP</sup> expressing CD4<sup>+</sup> T cells. The kinetics of IFN-γ induction, as measured by frequency of eYFP<sup>+</sup> cells, closely mirrored WT cells during the first 72 hr of culture but expression was not sustained in the absence of RA signaling (Figure 3G). Collectively, these data show that RA plays a temporal role in Th1 differentiation, maintaining Th1-cell commitment through regulation of T-bet and STAT4.

RA-RAR<sub>a</sub> Regulates Th1-Cell Plasticity

Alterations in the stable expression of lineage-determining TFs are thought to underlie Th-cell stability or plasticity. The emergence of Th1-Th17 cells together with the loss of T-bet expression, suggested a role for RA in the regulation of Th1-cell plasticity. However, diminished T-bet and STAT4 activity from day 3 of primary Th1-cell differentiation prevented assessment of lineage stability in fully differentiated Th1 cells. To determine whether RA-RAR<sub>a</sub> was required for long-term Th1-cell fate, we differentiated naive CD4<sup>+</sup> T cells from dnRara<sup>lsl/lsl</sup> mice under Th1-cell conditions, treated them with TAT-Cre (Wadia et al., 2004) on days 5 and 7, and restimulated them under Th1-cell conditions for a further 5 days. The temporal loss of RA signaling in Th1 cells resulted in decreased T-bet expression with a reciprocal increase in ROR<sub>γt</sub> expression (Figure 4A). ~50% of cells expressed ROR<sub>γt</sub>, which suggests that ongoing RA-RAR<sub>a</sub> activity is critical for sustaining T-bet and suppressing Th17-cell fate. Alterations in the lineage determining TFs did not impact on the cytokine phenotype (Figure S4A). This might in part reflect T-bet independent regulation of the Ifng locus at late stages in Th1-cell development.

To further examine the role of RA in Th1-cell stability, naive CD4<sup>+</sup> T cells from Ifng<sup>eYFP</sup> mice were differentiated under
To better understand the molecular mechanism by which RARα determines Th1-cell genes, RA-RARα signaling in fully committed Th1 cells leads to transdifferentiation of progeny with features of the Th17 lineage and support a model of transcriptional repression within Th1 cells. A recent study identified a role for RA-RARα in regulating enhancer activity. To test this, we mapped genome-wide binding of RA-RARα, combining with transcriptional profiling of dnRara-expressing Th1 cells in order to identify functional targets of RA-RARα. Selected loci were validated by ChIP-qPCR. RA-RARα binding was identified at 1,766 sites in 1,567 genes. RA-RARα binding was detected at 10.3% (76 of 740 regions) of genes downregulated in the absence of RA signaling (Table S2) (hereafter referred to as positively regulated) and 4.8% (56 of 1,169) of the upregulated genes (Table S3). In keeping with its classical role as a positive regulator of transcriptional activation there was significant enrichment of RA-RARα binding at genes positively regulated by RA (Fisher’s exact test, p < 0.0001). However, the presence of RA-RARα at a subset of the negatively regulated genes indicates that RA-RARα also plays a role in transcriptional repression within Th1 cells.

RA-RARα-dependent loci included Th1-cell lineage-defining genes (Tbx21 and Stat1). In addition to targeting the Tbx21 promoter (Figures 5A and 5B), modest RA-RARα binding was observed at the conserved T-bet enhancer element, 12kb upstream of the transcriptional start site (TSS) (Yang et al., 2007). This was confirmed by ChIP-qPCR (Figure 5B). Intergenic regions with increased intensity of H3K4me1, p300, and H3K27ac histone modifications in dnRara and WT Th1-cells, validating selected regions by ChIP-q-PCR. Active enhancers were operationally defined as regions with increased H3K4me3 and p300 binding (Figure S5D), arguing for a direct contribution of RA-RARα to transcriptional repression within Th1 cells.

RA-RARα Regulates Enhancer Activity at Lineage Determining Th1-Cell Genes

To better understand the molecular mechanism by which RA-RARα regulates Th-cell fate, we performed genome-wide analysis of RA-RARα binding in WT Th1 cells by ChIP-Seq, combined with transcriptional profiling of dnRara-expressing Th1 cells in order to identify functional targets of RA-RARα. Selected loci were validated by ChIP-qPCR. RA-RARα binding was identified at 1,766 sites in 1,567 genes. RA-RARα binding was detected at 10.3% (76 of 740 genes) of genes downregulated in the absence of RA signaling (Table S2) (hereafter referred to as positively regulated) and 4.8% (56 of 1,169) of the upregulated genes (Table S3). In keeping with its classical role as a positive regulator of transcriptional activation there was significant enrichment of RA-RARα binding at genes positively regulated by RA (Fisher’s exact test, p < 0.0001). However, the presence of RA-RARα at a subset of the negatively regulated genes indicates that RA-RARα also plays a role in transcriptional repression within Th1 cells.

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RA-RARα was also detected at the Stat4-Stat1 locus and an Ifng enhancer element (Figures S5A and S5B). RA binding to nuclear RA-RARα results in recruitment of co-activator complexes containing the histone acetyl-transferases p300 and CBP (Kamei et al., 1996), p300 is highly enriched at enhancer regions where it acetylates H3K27, a marker of active enhancers (Rada-Iglesias et al., 2011), suggesting a possible role for RA-RARα in regulating enhancer activity. To test this, we mapped genome-wide binding of p300, H3K4me1, H3K4me3, and H3K27ac histone modifications in dnRara and WT Th1-cells, validating selected regions by ChIP-q-PCR. Active enhancers were operationally defined as regions with increased intensity of H3K4me3, p300, and H3K27ac with low or absent H3K4me3 (Rada-Iglesias et al., 2011).

RA-RARα binding at the Tbx21, Stat4, and Ifng loci co-localized with p300 binding at enhancer regions (Figures S5A and S5B). dnRARα lacks the activation function 2 (AF2) domain which is required for RA-dependent recruitment of coactivators. Consistent with this, dnRara expressing T cells exhibited a significant reduction in p300 occupancy and H3K27ac deposition at the Tbx21 enhancer, supporting the direct regulation of enhancer activity by RA-RARα (Figures 5A and 5C). p300 binding at the Ifng and putative Stat4 intergenic enhancers was also dependent on RA-RARα (Figures S5A and S5C). Loss of p300 binding at the Stat4-Stat1 intergenic enhancer in dnRara Th1 cells correlated with reduced Stat4 transcripts, whereas Stat1 expression was actually increased, suggesting that this enhancer element regulated Stat4 transcription. A recent study identified a role for STAT4 in the regulation of Th1 enhancers (Vahedi et al., 2012). Given that Stat4 expression was reduced in dnRara Th1 cells, it was possible that the loss of p300 was in part due to reduced expression of Stat4. To address this issue, we assessed the binding of STAT4 in WT Th1 cells and compared p300 occupancy in WT and Stat4−/− Th1 cells using publicly available ChIP-seq data (Table S1) (Vahedi et al., 2012; Wei et al., 2010). Although STAT4 binding was observed at the Tbx21 enhancer, loss of STAT4 was not associated with obvious differences in p300 binding (Figure S5D), arguing for a direct contribution of RA-RARα to p300 recruitment and enhancer activity. Collectively, these data show that RA regulates expression of key Th1-cell lineage genes through remodeling of enhancer regions.

Figure 4. Loss of RA Signaling in Fully Committed Th1 Cells Leads to Th1 Plasticity and Divergence Toward the Th17 Lineage

(a) Naïve CD4+ T cells from dnRara/lsl/lsl mice were differentiated under Th1 conditions. Th1 cells were transduced with TAT-Cre on days 5 and 7 and re-polarized under Th1 conditions for a further 5 days. Intracellular expression of T-bet and RORγt was determined by flow cytometry. (b) Naïve CD4+ T cells from Ifng−/− eYFP mice were differentiated under Th1 conditions. IFN-γ (eYFP) cells were sorted on day 7 and restimulated under Th1 conditions for 5 days in the presence of Veh or RAi. Intracellular expression of T-bet and RORγt was determined by flow cytometry. Data representative of two independent experiments.
RA-RARα Represses Th17-Cell Fate in Th1 Cells through Direct Regulation of Th17-Cell Genes

The earlier finding that Th1 cells acquired features of Th17 cells in the absence of RA signaling led us to evaluate direct regulation of Th17-cell-instructing genes by RA-RARα. We first investigated effects of RA on the Th17-cell pioneer factors BATF and IRF4. As previously reported (Basu et al., 2013), these genes were expressed in WT Th1 cells. Strikingly, kinetic analysis of BATF and IRF4 expression in naive cells stimulated under Th1-cell conditions revealed dramatic upregulation of IRF4 (40- to 60-fold) during the initial phase of Th1-cell polarization with comparable expression between dnRara and WT cells (Figure 5D). Loss of RA signaling resulted in derepression of BATF-IRF4 target genes, Rorc, Il23r, Il22, Il21, and Il12rb1 (Figure 5E). This suggested that “balancing” factors must be induced in an RA-dependent manner to restrict the actions of BATF-IRF4 complexes at Th17-cell genes. IRF8, an alternative binding partner for BATF, previously shown to suppress Th17 differentiation (Ouyang et al., 2011), was one of the RA-RARα target genes most suppressed in dnRara Th1 cells. In WT Th1 cells, induction of Irf8 expression paralleled Irf4 expression. However, in dnRara cells Irf8 expression was not sustained past 24 hr (Figure 5D). RA-RARα bound at a putative upstream enhancer (Figures 5F and 5G) and in the absence of RA signaling, reduced p300 and H3K27ac were observed at this locus (Figure 5H and 5I). Together, these data show that RA directly regulates expression of IRF8 in Th1 differentiating cells and suggests a potential mechanism by which BATF-IRF4 activity is constrained within early Th1 cells.

Transcriptional activation of BATF-IRF4 target genes is dependent on STAT3 and RORγt (Ciofani et al., 2012). Various genes for cytokines and cytokine receptors associated with STAT3 activation (Il21, Il1r1, Il6ra, and Il23r) were derepressed in dnRara Th1 cells (Figure 5E). RARα targeted the promoter and an upstream enhancer in the Il6ra locus (Figure 5G) with increased H3K27ac observed at the enhancer element in dnRara Th1 cells (Figure 5J). Consistent with this, dnRara Th1 cells failed to downregulate mRNA and cell-surface IL-6-Rx expression during Th1 polarization (Figures S5E and S5F). These findings suggest that RA regulates Th1-cell plasticity in part by inhibiting responsiveness to IL-6.

RORγt was not a direct target of RARα. However, disruption of RA signaling resulted in increased expression of Runx1, a TF associated with transactivation of Rorc (Figure S5E) (Zhang et al., 2008). ChIP analysis confirmed direct regulation of short and long Runx1 isoform promoters by RA-RARα (Figure 5G). In Th1 cells, the Rorc locus is epigenetically silenced by T-bet (Mukasa et al., 2010). However, in dnRara cells, the repressive H3K27me3 mark was reduced at RORγt isoform-specific exon (Figure 5K), consistent with loss of T-bet. These findings suggest that increased RORγt expression in the absence of RARα signaling is in part due to increased accessibility of the Rorc locus, with unrestrained activation by Runx1. Collectively these data indicate that RA-RARα antagonises the activity of the core Th17-cell instructing TFs (IRF4, BATF, STAT3, and RORγt), both directly and indirectly, to suppress the Th17-cell gene program. Notably, Th2-cell-associated genes were not identified as targets of RARα (Tables S2 and S3) suggesting that direct repression of alternative cell fates by RA-RARα is specific to the Th17-cell program.

Th1-like Th17 Cells Emerge during Infection with L. monocytogenes in the Absence of RA Signaling

To assess the significance of these findings for immune responses in vivo, we intravenously infected WT and dnRara mice with an attenuated strain of L. monocytogenes (ΔActA), Lm-2W, which allows tracking of CD4+ T cells specific for listeriolysin O peptide LLO190–201 (LLOp). At the peak of the response, CD4+ T cells were isolated from the spleen and LLOp antigen-specific T cells were assayed for expression of cytokines and the TFs, T-bet, and RORγt. dnRara mice mounted an effectort-cell response of similar magnitude to WT mice with comparable frequencies and total numbers of CD44hiLLOp:I-ΔA-specific CD4+ T cells (Figures 6A and 6B). In WT mice, Lm-2W induced a Th1-cell restricted response, as evidenced by high T-bet expression within the LLOp-specific T-cell fraction (Figure 6C). LLOp:I-ΔA hi CD4+ T cells from dnRara mice expressed lower amounts of T-bet and a substantial proportion expressed RORγt, with co-expression of these TFs observed in a subset of cells (Figure 6C). At day 7 post-infection, a significant proportion of CD4+ T cells isolated from the spleen of dnRara mice were IL-17+ or dual IL-17A+IFN-γ+ with a trend toward reduced frequency of IFN-γ+ cells (Figure 6D). Measurement of cytokine protein concentrations from splenocytes restimulated with LLOp confirmed reduced amounts of IFN-γ and concomitant increase in IL-17A (Figure S6A). We did not detect IL-4 production by intracellular staining or protein secretion (Figure S6A and S6B). Consistent with our in vitro data showing downregulation of IL6-Rx on WT Th1 cells, cell-surface IL-6-Rx was not detectable on WT LLOp:I-ΔA hi CD4+ T cells. However, dnRara LLOp:I-ΔA hi CD4+ T cells retained expression of IL-6-Rx (Figure S6C), supporting a potential role for IL-6 signaling in the regulation of Th1-cell plasticity. These findings establish that RA-RARα signaling in T cells constrains the emergence of Th17 cells in a Th1-cell-instructing microenvironment in vivo.

RA Regulates the Th1-Th17-Cell Axis in the Gut and Prevents the Development of Intestinal Inflammation

RA is constitutively synthesized by a subset of DCS in the gut. To address the physiological importance of RA signaling in the regulation of pathogenic intestinal CD4+ T cells, we interbred dnRara mice with OTII mice that transgenically express an ovalbumin (OVA)-specific TCR and transferred naive CD4+ T cells into Rag1−/− hosts. Recipients were maintained on an OVA-containing diet for 7 days to induce differentiation within the transferred cells and migration to the intestinal tissue. Consistent with the infection experiments, feeding OTII(dnRara)–recipient mice OVA resulted in a shift in the Th1-Th17-cell balance with a deficiency in IFN-γ-producing cells and increased frequency of IL-17+ and dual IFN-γ+IL-17+ cells in the mesenteric lymph node (MLN), lamina propria lymphocytes (LPL), and spleen (Sp), 7 days after transfer (Figures 7B and 7C). To address the functional significance of the dysregulated cytokine response in dnRara T cells, we orally challenged mice with OVA and evaluated them for development of intestinal inflammation and diarrhea (Figure 7A). Recipients of OTII(dnRara) cells developed accelerated wasting disease relative to mice that received WT OTII cells (Figure 7D). Whereas all of the recipients of OTII(dnRara) cells developed severe diarrhea
by day 12 (Figure 7E), recipients of WT cells remained diarrhea free. Cytokine production was also assessed after the first gavage and confirmed an increased frequency of IL-17 + cells with concomitant reduction in IFN-γ + cells. Notably, enhanced IL-17 responses were not a consequence of impaired Foxp3 + conversion (Figure 7F). Homing of transferred cells to the gut was not affected in this model with similar frequencies of CD4+ T cells detected in the gut tissues (Figure S7A). We conclude that loss of RA signaling leads to deviation from Th1 to Th17 phenotype both in the periphery and the gut where these Th17 cells are associated with significant intestinal inflammation.

Figure 5. RA-RARα Regulates Enhancer Activity at Th1 Lineage Associated Loci and Represses Th17 Genes
Naive CD4+ T cells from WT and dnRara mice were cultured for 6 days under Th1 conditions prior to chromatin precipitation and transcriptional profiling.
(A) ChIP-seq binding tracks at Tbx21 locus for RARα in WT Th1 cells and p300 binding, H3K27ac, H3K4me1, and H3K4me3 modifications in WT and dnRara Th1 cells.
(B) Validation of the RARα-binding regions in WT Th1 cells by ChIP-qPCR. Untr6 region serves as a negative control. Binding events per 1,000 cells displayed as “Enrichment.”
(C) The effects of dnRara expression on p300 and H3k27ac abundance at the Tbx21 locus were validated by ChIP-qPCR.
(D) Quantitative real-time PCR analysis of Batf, Irf4, and Irf8 mRNA in naive CD4+ T cells from dnRara or WT cells differentiated under Th1-cell conditions for 0, 24, 48, 72 hr. Mean ± SEM, replicate wells.
(E) Log2 values of fold changes in gene expression as measured by microarray analyses. Average fold change depicted.
(F) ChIP-seq binding tracks at Irf8 locus for cells as in (A).
(G) Validation of RARα ChIP-seq regions by ChIP-qPCR.
(H–J) ChIP analysis of p300 and H3K27ac at selected loci.
(K) ChIP analysis of H3K27me3 at the RORc locus. Actb locus serves as a negative control. Data from three independent experiments (E) or representative of two independent experiments (B–D, G–K); Mean ± SD unless noted otherwise. Abbreviation: pro, promoter.
See also Figure S5.

DISCUSSION
Dysregulated Th-cell responses underlie the pathogenesis of autoimmune and allergic disease. In contrast to T regulatory (Treg) cells and Th17 cells, the Th1-cell lineage is thought to be relatively stable. However, the factors that control maintenance of the Th1-cell lineage were not previously known. This study identifies RA-RARα as a central regulatory node in the transcriptional network governing Th1-cell stability. We found that RA-RARα directly sustained the expression of lineage determining Th1-cell-associated genes during naive T-cell differentiation while also repressing signature Th17-cell-associated genes. Ablation of RA signaling in Th1-committed cells resulted in enhanced Th1-cell plasticity with deviation towards a Th17-cell phenotype. Using ChIP-seq to identify regulatory elements, we found that RARα bound at enhancers and recruitment of p300 to these regions was dependent on RA signaling. In vivo, both Th17 and Th1-Th17 cells emerged during infection with L. monocytogenes and in a model...
Figure 7. Loss of RA Signaling Causes Dysregulated Th1 and Th17 Response and Increased Pathogenicity in a Model of Gut Inflammation

(A) Schematic illustration of the adoptive transfer experiment.

(B) Intracellular expression of IL-17A and IFN-γ among CD4+ cells from the spleen (Sp), mesenteric lymph nodes (MLN), and lymphocytes from the lamina propria (LPL) of mice as in (A) 7 days after transfer.

(C) Statistical data for frequency of IFN-γ+, IL-17+, and IFN-γ+IL-17+ cells as in (B) in MLN and Sp.

(D) Percentile change of original body weight in Rag1−/− recipients treated as in (A) (n = 5–7 per group). Mean ± SD.

(E) Frequency of diarrhea-free mice among Rag1−/− recipients as in (A) (OTII recipients n = 3, OT-II(dnRara) recipients n = 5).

(F) Frequencies of IL-17, IFN-γ, and Foxp3 in CD4+ cells isolated from Sp, MLN, LPL, and IELs of mice as in (A), 9 days after transfer (n = 5 or 6 per group).

Data from one experiment (B and C), pooled from two independent experiments (D and F), or representative of two independent experiments (E). Mean ± SEM unless otherwise noted.
of oral tolerance. In the latter, their presence was associated with significant pathology.

Enhancers play a key role in directing cell fate through the regulation of lineage specifying genes. Enhancer profiling in WT and dnRara T cells revealed RA-dependent activation of enhancers at genes critical for Th1 identity (Tbx21, Stat4, Ifng, and Irf8). RA-dependent changes in p300 and H3K27ac were reflected at the transcriptional level suggesting that, in addition to its classical role as a transcriptional regulator, RA regulates gene expression in an enhancer-dependent manner. Although the ability of RA-RARα to target p300-CBP complexes to nucleosomes is well established, regulation of enhancers by RA has not been widely studied. We propose that unliganded RARα at enhancer elements acts as a gatekeeper, enabling initiation of enhancer activation once T cells sense RA in the microenvironment. A similar role has been demonstrated for STAT proteins (Vahedi et al., 2012), suggesting that environmental cues act as checkpoints for initiation of enhancer activation and T-cell fate. Although H3K4me1 modifications are present at early time points during T-cell differentiation, conversion to “active” status requires acquisition of H3K27ac, which is often not evident until later stages of differentiation (Hawkins et al., 2013). Consistent with a temporal role for enhancers in maintenance of gene expression, RA signaling was not required for initiation of transcription of target genes but rather acted to maintain their expression. These data highlight the importance of enhancers in maintenance of cell identity and plasticity. It is possible that RA-RARα regulation of enhancers represent the major mechanism by which RA regulates cell fate. A recent study identified enrichment of RARα at enhancers in embryonic stem cells (Chen et al., 2012). Given that the RA-RARα axis is a highly conserved signaling pathway, which plays a critical role in regulating cell-fate specification during early embryo and cell differentiation, it will be important to evaluate a broader role for RA-RARα in regulation of enhancer functionality, both in alternative Th-cell subsets and outside of the immune system.

In addition to sustaining expression of Th1-cell-associated genes, we found that RA actively silences genes implicated in Th17-cell differentiation. Among genes known to regulate the Th17-cell program, Runx1 and Il6ra were directly repressed by RA-RARα. In addition, BATF-IRF4 target genes were derepressed in the absence of RA signaling. In Th17 cells, BATF-IRF4 complexes act co-operatively as pioneer factors at key Th17 genes (Ciofani et al., 2012), modulating chromatin accessibility to facilitate binding of STAT3 and RORγt. On the basis of their expression in alternative Th-cell subsets, it has been suggested that BATF-IRF4 complexes play a universal role in establishing binding of lineage-specific TFs (Ciofani et al., 2012). However, BATF deficiency does not impact on Th1-cell differentiation (Schrabl et al., 2009). An alternative model is that upregulation of BATF and IRF4 confers plasticity in early Th1 cells, poising chromatin specifically at Th17-cell-associated genes. IRF8, an alternative binding partner for BATF, negatively regulates Th17-cell differentiation (Ouyang et al., 2011). Our results identified IRF8 as a member of the Th1-cell transcriptional network whose expression was critically dependent on RA signaling. Induction of IRF8 would be expected to limit plasticity of Th1 cells by repressing Th17 differentiation, potentially by competing for binding to BATF. In support of a role for IRF8 in regulation of Th1-Th17 axis, patients with mutations in IRF8 have impaired Th1 responses (Hambleton et al., 2011) and single nucleotide polymorphisms (SNPs) in Irf8 are associated with several autoimmune diseases in which IFN-γ IL17+ Th17 cells play a pathogenic role (Franke et al., 2010; Cunningham Graham et al., 2011). It will be of interest to identify transcriptional targets of BATF, IRF4, and IRF8 in Th1 cells.

RA signaling was critical to maintain appropriate Th1-cell responses and suppress the development of IL-17+ and IFN-γ+IL17+ cells. Hybrid Th1-Th17 cells are implicated in the pathogenesis of several autoimmune diseases. Their development has been attributed to the plasticity of Th17 cells. Our findings suggest that these cells might alternatively reflect Th1 plasticity and suggest a novel developmental pathway for Th17 cells. Th1 derived “Th17” cells expressed high levels of the receptor for IL-23, a critical determinant of Th17 pathogenicity (Basu et al., 2013), and were associated with significant gut inflammation and pathology in a model of oral tolerance. Further experiments are required to test the prediction that pathogenic Th17 and IFN-γ+IL17+ cells which arise in autoimmunity emerge from Th1 cells when RA is deficient or its signaling perturbed.

A range of inflammatory stimuli can induce RA synthesis and signaling during the course of an immune response. Our results suggest that in a Th1-cell instructing microenvironment the dominant action of RA is to repress Th17-cell fate and promote Th1-cell responses. We did not observe enhanced Th17-cell responses during primary Th17-cell differentiation, suggesting that the impact of RA on T-cell stability might vary both temporally and among tissues. Previously we have shown in a model of skin allograft rejection that impaired Th1 responses in dnRara mice were accompanied by increased Th2-cell cytokines (Pino-Lagos et al., 2011). We did not identify direct repression of Th2-cell-associated genes by RARα. However, T-bet suppresses GATA3 (Zhu et al., 2012) and in the presence of a Th2 skewing microenvironment, such as the skin, impaired expression of T-bet in the absence of RA signaling renders cells susceptible to Th2 deviation. Thus, the effects of RA on T-cell fate are likely dependent on external and intrinsic factors that shape T-cell polarity.

In summary, we show that RA signaling plays a critical role in regulating stability and functional plasticity of Th1 cells. Regulation of enhancer activity at lineage determining genes by RA-RARα provides mechanistic evidence for reciprocal regulation of Th1 and Th17-cell programs. In the absence of RA signaling, downstream modulation of T-bet, STAT4, and IFN-γ, and loss of repression of Th17-cell genes, creates a permissive environment for transdifferentiation of Th1 cells to Th17 cells. This study identifies the RA-RARα axis as a potential node for intervention in diseases in which dysregulation of the Th1-Th17-cell axis is observed.

**EXPERIMENTAL PROCEDURES**

**Mice**

C57Bl/6 dnRara mice have been described previously (Pino-Lagos et al., 2011). Ifng<sup>(GREAT)</sup> mice were purchased from the Jackson Laboratory. Mice were bred and maintained at Charles River Laboratory, UK, in pathogen-free conditions. All animal experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. C57Bl/6 OTII(dnRara), OTII, and Rag1<sup>−/−</sup> mice were bred and maintained at the Rockefeller University specific pathogen-free animal facility.
Cell Isolation, Cell Culture, and Flow Cytometry
Sort purified, naive CD4^+CD25^-CD44^-CD62L^- T cells were cultured with T-cell depleted splenocytes (APCs) and anti-CD3 under polarization conditions for Th0, Th1, Th2, and Th17-cell-associated subsets. Details are provided in the Supplemental Experimental Procedures. For analysis of cytokine production, cells were restimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin in the presence of monensin for 4–5 hr at 37°C. Cells were stained with LIVE/DEAD Dead Cell Stain (Invitrogen), followed by staining for cell-surface markers and then fixed and permeabilized (BD Biosciences) for staining of intracellular antigens. Flow cytometry was performed on a LSR Fortessa (BD Biosciences) and analyzed with Flowjo software (Tree Star).

TAT-Cre Transduction
Sort purified naive CD4^+ T cells were differentiated under Th1 conditions. After 5 days, cells were treated with 50 μg/ml TAT-Cre peptide for 45 min at 37°C, then washed and expanded in IL-2-containing medium. After 48 hr cells were retreated with Tat-Cre followed by polarization under Th1 conditions.

Real-Time Quantitative PCR
Total RNA was extracted from cells with RNeasy Mini kit (QIAGEN) and cDNA was synthesized with QuScrit RT kit (Quanta). Quantitative gene-expression analysis was performed using Taqman primer probe sets (Applied Biosystems), listed in Table S4. Expression of target genes was normalized to β-actin.

Microarray Gene-Expression Profiling
For gene-expression analysis Affymetrix (forIfng^-yfp dataset) or Agilent (for the dnRara Th1 dataset) microarray chips were used. Differentially expressed genes were detected using fold-change and t test analysis. See Supplemental Experimental Procedures for further information.

Chromatin Immunoprecipitation and ChIP-Seq
Immunoprecipitation and DNA sequencing was performed by Active Motif. The following antibodies were used: anti-H3K27me3 (Millipore 07–449), anti-H3K4me1 (Active Motif 39287), anti-H3K4me3 (Active Motif 39159), anti-H3K27ac (active Motif 39133), anti-RARα (Diagenode C15310155). Illumina sequencing libraries were prepared from the ChIP and Input DNAs. For ChIP q-PCR, enrichment calculated as binding events per 1,000 cells using Active Motif’s normalization scheme. Detailed methods for ChIP-seq and binding site analyses are provided in the Supplemental Information.

L. monocytogenes Infection
Mice were infected i.v. with 1 × 10^6 cfu L. monocytogenes and spleens were harvested 7 days later. Splenocytes were enriched for CD4^+ T cells with a CD4^+ T-cell negative selection microbead kit (Miltenyi Biotec) and stained with PE labeled, LLO/I-A^d dextramer (Immudex) and cell-surface antibodies. For analysis of intracellular cytokine production, splenocytes were restimulated with LLO peptide (Proteomics) at 10 μg/ml for 6 hr.

Food-Antigen-Induced Diarrhea Model
Naïve CD4^+ T cells from OTI or OTII (dnRara) were intravenously transferred to Rag1^-/- mice. These mice were then maintained on a diet containing OVA for 7 days and challenged with oral OVA on days 9 and 10. Lymphocytes were isolated from the intestinal epithelium, lamina propria, MLN, and spleen at the indicated time points after the start of oral OVA exposure of the recipient mice. Detailed experimental procedures are described in the Supplemental Experimental Procedures.

Statistical Analysis
Statistical significance was calculated by unpaired two-tailed Student’s t test with Graphpad Prism software. p values < 0.05 were considered significant. p values are denoted in figures as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

ACCESSION NUMBERS
Chip-seq and microarray data are available under GEO accession number GSE60336.

SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.02.003.

AUTHOR CONTRIBUTIONS
C.C.B. designed the studies, performed most of the experiments, analyzed the data, and wrote the manuscript. D.E., M.L., and D.M. performed and analyzed gut-inflammation studies. A.S., I.O.-G., and R.a.-B. assisted in processing of samples for in vitro co-culture studies and qPCR. R.E. and C.O. assisted with processing of tissues for phenotyping studies. M.A. provided advice and performed microarrays. V.P. analyzed ChIP sequencing data. E.d.R. supervised ChIP-seq data analysis. G.M.L. provided advice and supervision. D.M. designed gut-inflammation studies and contributed to the writing of the manuscript. R.J.N. supervised the overall study and contributed to the writing of the manuscript.

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