

# The Orphan Nuclear Receptor ROR $\gamma$ t Directs the Differentiation Program of Proinflammatory IL-17<sup>+</sup> T Helper Cells

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## SUMMARY

IL-17-producing T lymphocytes have been recently shown to comprise a distinct lineage of proinflammatory T helper cells, termed Th17 cells, that are major contributors to autoimmune disease. We show here that the orphan nuclear receptor ROR $\gamma$ t is the key transcription factor that orchestrates the differentiation of this effector cell lineage. ROR $\gamma$ t induces transcription of the genes encoding IL-17 and the related cytokine IL-17F in naïve CD4<sup>+</sup> T helper cells and is required for their expression in response to IL-6 and TGF- $\beta$ , the cytokines known to induce IL-17. Th17 cells are constitutively present throughout the intestinal lamina propria, express ROR $\gamma$ t, and are absent in mice deficient for ROR $\gamma$ t or IL-6. Mice with ROR $\gamma$ t-deficient T cells have attenuated autoimmune disease and lack tissue-infiltrating Th17 cells. Together, these studies suggest that ROR $\gamma$ t is a key regulator of immune homeostasis and highlight its potential as a therapeutic target in inflammatory diseases.

## INTRODUCTION

Effective adaptive immune responses to pathogenic and commensal microorganisms require that lymphocytes be endowed with effector properties appropriate for each challenge. In response to microbe-induced cues from the innate immune system, CD4<sup>+</sup> helper T lymphocytes thus differentiate in peripheral tissues to adopt a variety of fates (Seder and Paul, 1994). Much focus has been placed on two T helper cell subsets, the Th1 cells, which produce interferon- $\gamma$  (IFN $\gamma$ ), and the Th2 cells, which produce IL-4, IL-5, and IL-13 (Abbas et al., 1996; Glimcher

and Murphy, 2000). Th1 cells control infections with intracellular pathogens, including viruses and bacteria, but they have also been implicated as the effectors in a variety of autoimmune diseases (Christen and von Herrath, 2004; Gately et al., 1998; Trembleau et al., 1995). Th1 differentiation requires the cytokine IL-12 and the transcription factors, STAT4, STAT1, and T-bet (Szabo et al., 2000). Th2 cells control infections with helminths and other extracellular microbes and are, in part, the effectors that mediate the immunopathology of allergic responses and asthma. Th2 cell differentiation requires the cytokine IL-4 and the transcription factors STAT6 and GATA3 (Zheng and Flavell, 1997).

It has recently been recognized that in many of the mouse autoimmune disease models that have been attributed to Th1 cells, disease severity is actually increased in the absence of these cells. For example, experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) are exacerbated in the absence of IFN $\gamma$ , IFN $\gamma$ -R, STAT1, or IL-12 p35 (Iwakura and Ishigame, 2006; McKenzie et al., 2006). However, mice lacking the IL-12 p40 subunit, which is shared with IL-23, are resistant to these autoimmune diseases (Cua et al., 2003; Murphy et al., 2003). This paradox has now been resolved by the characterization of a third subset of T helper cells that secrete IL-17 (also known as IL-17A) and IL-17F and expand in response to IL-23 independently of T-bet or STAT1 (Aggarwal et al., 2003; Harrington et al., 2005; Iwakura and Ishigame, 2006; McKenzie et al., 2006; Park et al., 2005). Mice lacking IL-17 are resistant to CIA and EAE (Nakae et al., 2003), transfer of cells that produce IL-17 results in severe disease (Langrish et al., 2005), and treatment of mice with a neutralizing anti-IL-17 mAb suppresses CNS autoimmune inflammation (Langrish et al., 2005). The differentiation of the IL-17- and IL-17F-producing cells, now termed Th17 cells, was initially shown to be dependent on the presence, during antigen stimulation, of IL-23 produced by the antigen-presenting cells (Aggarwal et al., 2003; Harrington et al., 2005; Langrish et al., 2005;

Park et al., 2005). Consistent with this observation, mice deficient for the p19 subunit of IL-23 failed to produce Th17 cells and were resistant to EAE, CIA, and inflammatory bowel disease (IBD) (Cua et al., 2003; Langrish et al., 2005; Yen et al., 2006).

Although IL-23 has a key role in Th17-mediated inflammation in vivo, recent studies have demonstrated that in vitro polarization of naïve CD4<sup>+</sup> T cells toward the Th17 lineage requires a combination of T cell antigen receptor (TCR) stimulation and the cytokines TGF- $\beta$  and IL-6 but is independent of IL-23 (Bettelli et al., 2006; Veldhoen et al., 2006). Rather, IL-23 may be required for maintaining the Th17 phenotype or for promoting survival and/or proliferation of these cells. Overexpression of TGF- $\beta$  in vivo also results in a marked increase of encephalitogenic Th17 cells following immunization with MOG antigen and complete Freund's adjuvant (Bettelli et al., 2006). TGF- $\beta$  had been thought of primarily as an anti-inflammatory cytokine, in part because it induces in vitro differentiation of Foxp3-expressing regulatory T cells (Tregs) (Chen et al., 2003; Fantini et al., 2004). Thus TGF- $\beta$  may induce either anti-inflammatory Tregs or proinflammatory Th17 cells, depending on the presence of IL-6.

The intestinal tract harbors a large fraction of the body's lymphatic tissue. Most T lymphocytes within the intestinal epithelium and lamina propria have an effector phenotype and are thought to be involved in maintaining a homeostatic balance between the luminal commensal microflora and the tissues of the intestine (Mowat, 2003). Innate immune signaling pathways involving Myd88 are required for repair of intestinal epithelium, suggesting that increased exposure of cells within the lamina propria to commensal microorganisms results in signals that activate regenerative processes (Rakoff-Nahoum et al., 2004). The cells involved in such processes and the mediators of homeostatic signals have yet to be characterized. Organized lymphoid structures in the lamina propria, including cryptopatches and isolated lymphoid follicles (ILFs), may receive signals from dendritic cells that surround them and can sample luminal content (Hamada et al., 2002; Kanamori et al., 1996; Newberry and Lorenz, 2005; Niess et al., 2005; Rescigno et al., 2001). Differentiation of these lymphoid tissues, as well as lymph nodes and Peyer's patches, is dependent on the orphan nuclear receptor ROR $\gamma$ t. This transcription factor is required for the development of lymphoid tissue inducer (LTi) cells and LTi-like cells that guide development of the lymphoid tissues within the fetus and in the adult intestine, respectively (Eberl and Littman, 2004; Sun et al., 2000).

ROR $\gamma$ t is expressed in double-positive (CD4<sup>+</sup>CD8<sup>+</sup>) thymocytes, extending their survival during clonal selection, and in the LTi and LTi-like cells (Eberl et al., 2004). Here we show that ROR $\gamma$ t is also expressed in populations of intestinal lamina propria T lymphocytes, most of which constitutively produce IL-17, and that these cells are absent in ROR $\gamma$ t-deficient mice. In addition, both cytokine-directed in vitro differentiation of Th17 cells and in vivo Th17-mediated inflammatory disease require induction

of ROR $\gamma$ t. Our results demonstrate that ROR $\gamma$ t is the transcription factor that directs the differentiation of inflammatory Th17 cells.

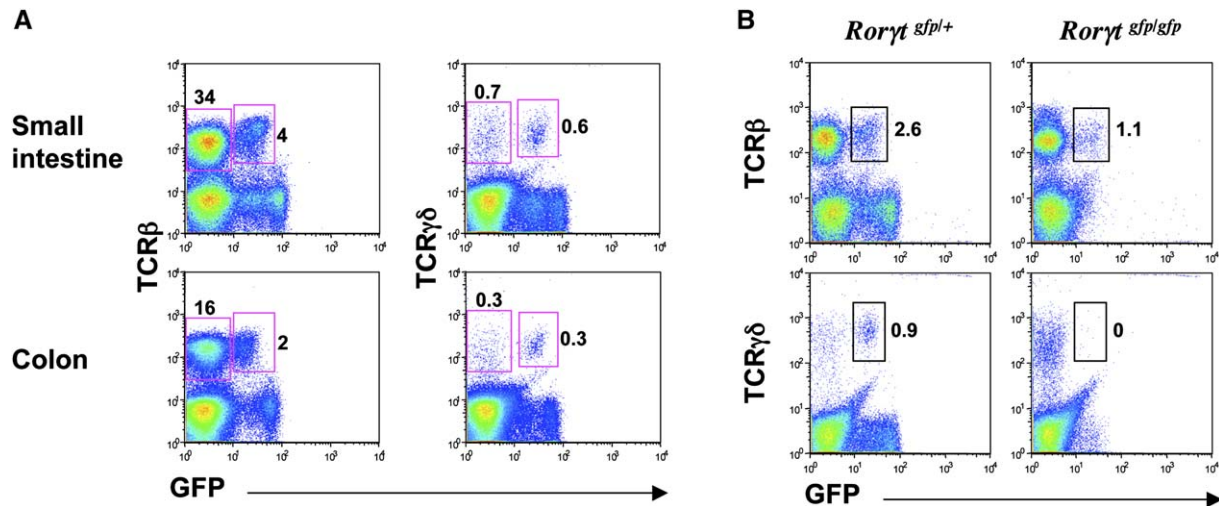
## RESULTS

### ROR $\gamma$ t Is Expressed in a Subset of Lamina Propria T Cells and Is Required for Expression of IL-17

To examine the role of ROR $\gamma$ t in T cell development and lymphoid organogenesis we previously generated mice with a GFP reporter cDNA knocked-in at the site for initiation of ROR $\gamma$ t translation (Eberl et al., 2004). Using these animals we found that ROR $\gamma$ t is expressed at the double-positive stage of T cell development but is absent in more mature thymocytes and in mature T cells in spleen and peripheral LNs (Eberl et al., 2004). In the periphery, we had found that GFP (or ROR $\gamma$ t) expression was limited to a population of ROR $\gamma$ t-dependent lymphoid-tissue inducer cells (LTi) in the fetus and a population with a similar phenotype in adult intestinal cryptopatches and lymphoid follicles (Eberl and Littman, 2004; Sun et al., 2000). Upon closer examination of lamina propria cells from *Ror $\gamma$ t<sup>gfp/+</sup>* mice, we observed subpopulations of TCR $\alpha\beta$ <sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup> cells that expressed a lower level of GFP than the LTi-like TCR-negative cryptopatch cells. Approximately 10% of TCR $\alpha\beta$ <sup>+</sup> T cells, most of which were CD4<sup>+</sup>, and 50% of TCR $\gamma\delta$ <sup>+</sup> T cells expressed GFP (Figure 1A). In *Ror $\gamma$ t<sup>gfp/gfp</sup>* mice, which lack expression of ROR $\gamma$ t, the LTi-like cells, which are Lin<sup>-</sup>GFP<sup>+</sup>, were completely absent. In contrast, the GFP<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> T cells were still present, although reduced to 50%–75%, and the GFP<sup>+</sup>TCR $\gamma\delta$ <sup>+</sup> T cells were no longer observed (Figure 1B). ROR $\gamma$ t (GFP) expression was not detected in the intestinal epithelial cell (IEL) compartment of *Ror $\gamma$ t<sup>gfp/+</sup>* or *Ror $\gamma$ t<sup>gfp/gfp</sup>* mice (Figure S1 available with the Supplemental Data online).

In experiments aimed at investigating the mechanisms that regulate Th17 cell differentiation, we performed Affymetrix gene chip analysis of CD4<sup>+</sup> T cells cultured under conditions favoring Th17 (grown in IL-23) versus Th1 (grown in IL-12) differentiation. In Th17 culture conditions, ROR $\gamma$ t mRNA levels were found to be elevated (B.S.M. and D.J.C., unpublished data). The finding of ROR $\gamma$ t<sup>+</sup> T cells in the intestinal lamina propria therefore raised the possibility that these cells were of the Th17 lineage. When lamina propria lymphocytes (LPL) from *Ror $\gamma$ t<sup>gfp/+</sup>* mice were isolated and stimulated for 5 hr with anti-CD3/CD28, we found that a large proportion of the GFP<sup>+</sup> cells, but not the GFP<sup>-</sup> cells, indeed expressed IL-17 (Figure 2A).

In *Ror $\gamma$ t<sup>gfp/+</sup>* and in wild-type mice, approximately 10% of CD4<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> intestinal T cells expressed IL-17 (Figure 2B and data not shown). However, in ROR $\gamma$ t null (*Ror $\gamma$ t<sup>gfp/gfp</sup>*) mice, IL-17<sup>+</sup> cells in the lamina propria were reduced by at least 10-fold to less than 1% of the CD4<sup>+</sup> T cells, suggesting that ROR $\gamma$ t may be necessary for the generation of Th17 cells in vivo (Figure 2B). Both IL-17 and ROR $\gamma$ t (GFP) were also expressed in CD4<sup>-</sup> T cells in



**Figure 1. ROR $\gamma$ t Is Expressed in a Subpopulation of Lamina Propria T Cells**

(A) Lamina propria lymphocytes (LPL) were isolated from the small intestine and colon of heterozygous ROR $\gamma$ t-reporter mice (*Rorγt*<sup>gfp/+</sup>) and stained for TCR $\beta$  and TCR $\gamma\delta$ . Representative data from multiple experiments are shown.

(B) Expression of ROR $\gamma$ t (GFP) in the small intestinal lamina propria of heterozygous (*Rorγt*<sup>gfp/+</sup>) and homozygous (*Rorγt*<sup>gfp/gfp</sup>) ROR $\gamma$ t-reporter mice.

the lamina propria and were reduced in ROR $\gamma$ t-deficient mice (Figure 2B and Figure S2).

In mice kept under specific pathogen-free conditions, IL-17<sup>+</sup> cells were present in the lamina propria of the small intestine (proximal and distal), cecum, colon, and rectum. However, the small intestinal lamina propria contained the largest proportion of IL-17<sup>+</sup> T cells and the cecum contained the smallest (Figure S3). Very few cells in the IEL compartment (0.1%–0.2% of TCR $\alpha\beta$  and TCR $\gamma\delta$  cells) expressed IL-17 (data not shown). We did not observe ROR $\gamma$ t/GFP<sup>+</sup> or IL-17<sup>+</sup> T cells outside of the intestine in other secondary lymphoid organs. These observations suggest that signals from the intestinal microflora may regulate the differentiation of the intestinal Th17 cells in the lamina propria. In an effort to investigate this possibility, we assessed the presence of IL-17<sup>+</sup> cells in the lamina propria of MyD88-deficient mice. We discovered only a mild decrease in the percentage of these cells in the mutant animals, indicating that if intestinal Th17 cells differentiate in response to signals from the lumen, then other signaling pathways are likely involved (Figure S4).

#### ROR $\gamma$ t Is Required for the In Vitro Induction of IL-17 in T Helper Cells

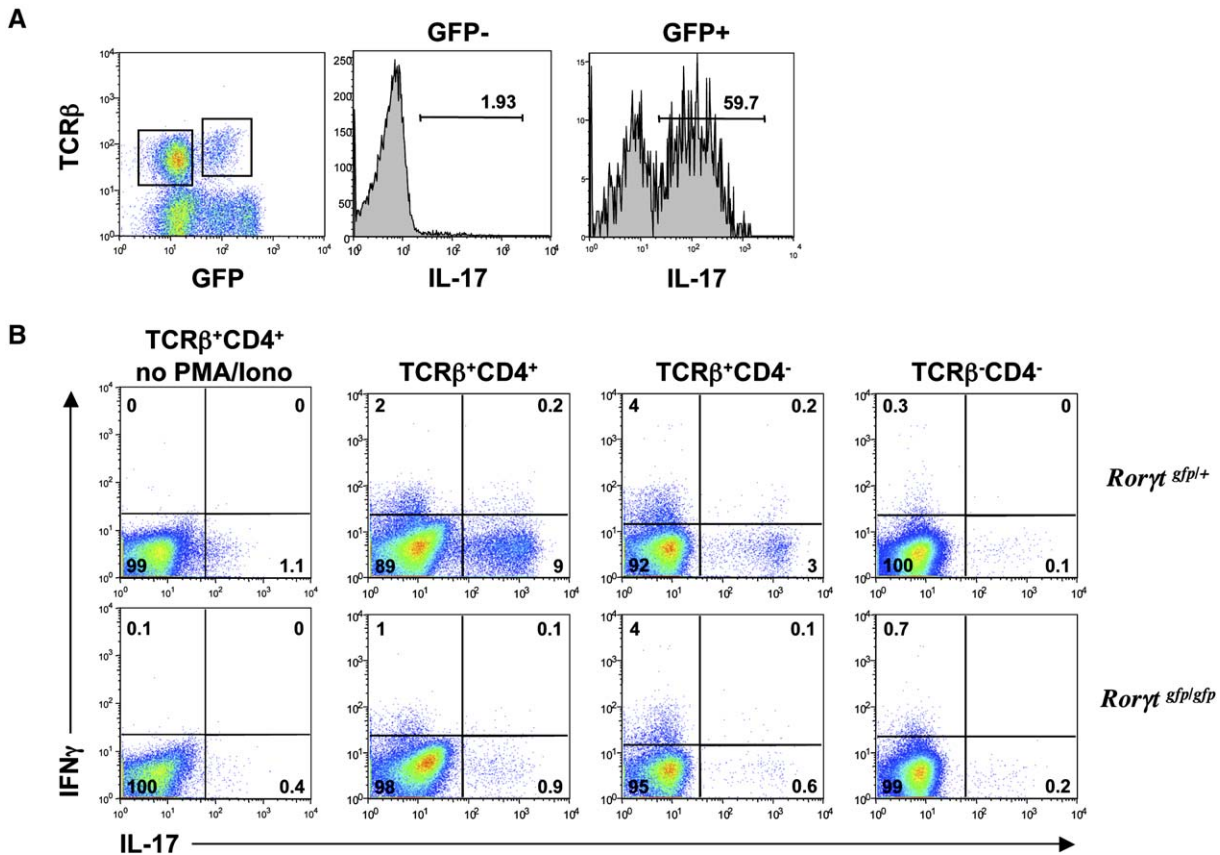
It was recently reported that purified naive CD4<sup>+</sup> T cells are induced to differentiate in vitro into Th17 cells upon antigen receptor ligation in the presence of IL-6 and TGF- $\beta$  (Veldhoen et al., 2006). To test if ROR $\gamma$ t is required for this differentiation program, we purified CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>+</sup>CD44<sup>-</sup> naive splenic T cells from wild-type and ROR $\gamma$ t-deficient mice and stimulated them in vitro with plate bound anti-CD3 and soluble anti-CD28 for 3 days under various polarizing conditions. We confirmed that a combination of IL-6 and TGF- $\beta$  in the presence of neutralizing antibodies

against IFN $\gamma$  and IL-4 resulted in robust induction of IL-17 in wild-type cells, and that either cytokine alone or IL-23 had no effect (Figure 3A and data not shown). In contrast, CD4<sup>+</sup> T cells from *Rorγt*<sup>-/-</sup> mice (which lack both ROR $\gamma$  and ROR $\gamma$ t) displayed a marked reduction in IL-17<sup>+</sup> cells after in vitro polarization. There was typically at least a 50-fold decrease in the number of IL-17<sup>+</sup> cells, and the level of IL-17 staining in these cells was significantly reduced (Figure 3A). Consistent with this observation, incubation of anti-CD3/CD28-stimulated T cells with IL-6 plus TGF- $\beta$  resulted in induction of ROR $\gamma$ t mRNA, as well as transcripts for IL-17 and IL-17F (Figure 3B). The mRNA for ROR $\gamma$ t peaked at 16 hr, whereas those for IL-17 and IL-17F peaked later, at 48 hr, consistent with ROR $\gamma$ t regulation of IL-17 transcription. IL-6 and TGF $\beta$  on their own induced low levels of ROR $\gamma$ t expression but were unable to induce IL-17 mRNA or IL-17-producing T cells (Figure 3C and data not shown).

The defect in ROR $\gamma$ t-deficient mice was confined to IL-17-producing T cells, as differentiation of IFN $\gamma$ -producing Th1 cells upon incubation with IL-12 was normal or even enhanced with cells from the mutant mice as compared to those from wild-type animals (Figure S5).

#### Forced Expression of ROR $\gamma$ t Induces IL-17 in Naive CD4<sup>+</sup> T Cells

As ROR $\gamma$ t appeared to be essential for Th17 cell differentiation in response to cytokines, we wished to determine whether its expression is sufficient to induce the Th17 lineage program in naive T cells. We used retroviral transduction to deliver ROR $\gamma$ t to magnetic bead (MACS)-purified CD4<sup>+</sup> T cells or FACS-sorted CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>+</sup>CD44<sup>-</sup> T cells stimulated with anti-CD3/CD28. In the absence of exogenous polarizing cytokines, overexpression of ROR $\gamma$ t



**Figure 2. ROR $\gamma$ t Is Required for the Generation of Lamina Propria IL-17<sup>+</sup> T Cells**

(A) ROR $\gamma$ t<sup>+</sup> (GFP<sup>+</sup>) T cells from *Ror $\gamma$ t<sup>gfp/+</sup>* mice express IL-17. LPLs were stimulated in vitro with plate bound anti-CD3/anti-CD28 for 5 hr and in the presence of Brefeldin A for the final 2 hr, after which they were fixed and stained for intracellular IL-17 and GFP.

(B) Comparison of lamina propria IL-17<sup>+</sup> and IFN $\gamma$ <sup>+</sup> T cells in *Ror $\gamma$ t<sup>gfp/+</sup>* and *Ror $\gamma$ t<sup>gfp/gfp</sup>* mice. Freshly isolated LPLs from heterozygous (*Ror $\gamma$ t<sup>gfp/+</sup>*) and homozygous null (*Ror $\gamma$ t<sup>gfp/gfp</sup>*) mice were stimulated in vitro with PMA/ionomycin for 5 hr or incubated with Brefeldin A only (no PMA/Iono) and stained for CD4, TCR $\beta$ , and intracellular cytokines. The gating used is shown in Figure S2. Representative data from multiple experiments are shown.

resulted in the induction of IL-17 in a large fraction of the transduced (GFP<sup>+</sup>) T cells from C57BL/6 or Balb/c mice (Figure 4A). In contrast, no IL-17 was observed in cells transduced with T-bet-IRES-GFP and IRES-GFP control vectors. Analysis by quantitative RT-PCR indicated that the forced expression of ROR $\gamma$ t resulted in induced transcription of both IL-17 and IL-17F (Figure 4B).

Transduction of the T-bet-encoding retrovirus resulted, as expected, in the induction of IFN $\gamma$ , but not IL-17. Conversely, there was no IFN $\gamma$  induction upon overexpression of ROR $\gamma$ t. Instead, the number of IFN $\gamma$ -producing cells was reduced in cells expressing ROR $\gamma$ t (Figure 4A). Even when the cells were cultured under Th1 polarizing conditions, ROR $\gamma$ t expression still induced IL-17 expression (data not shown).

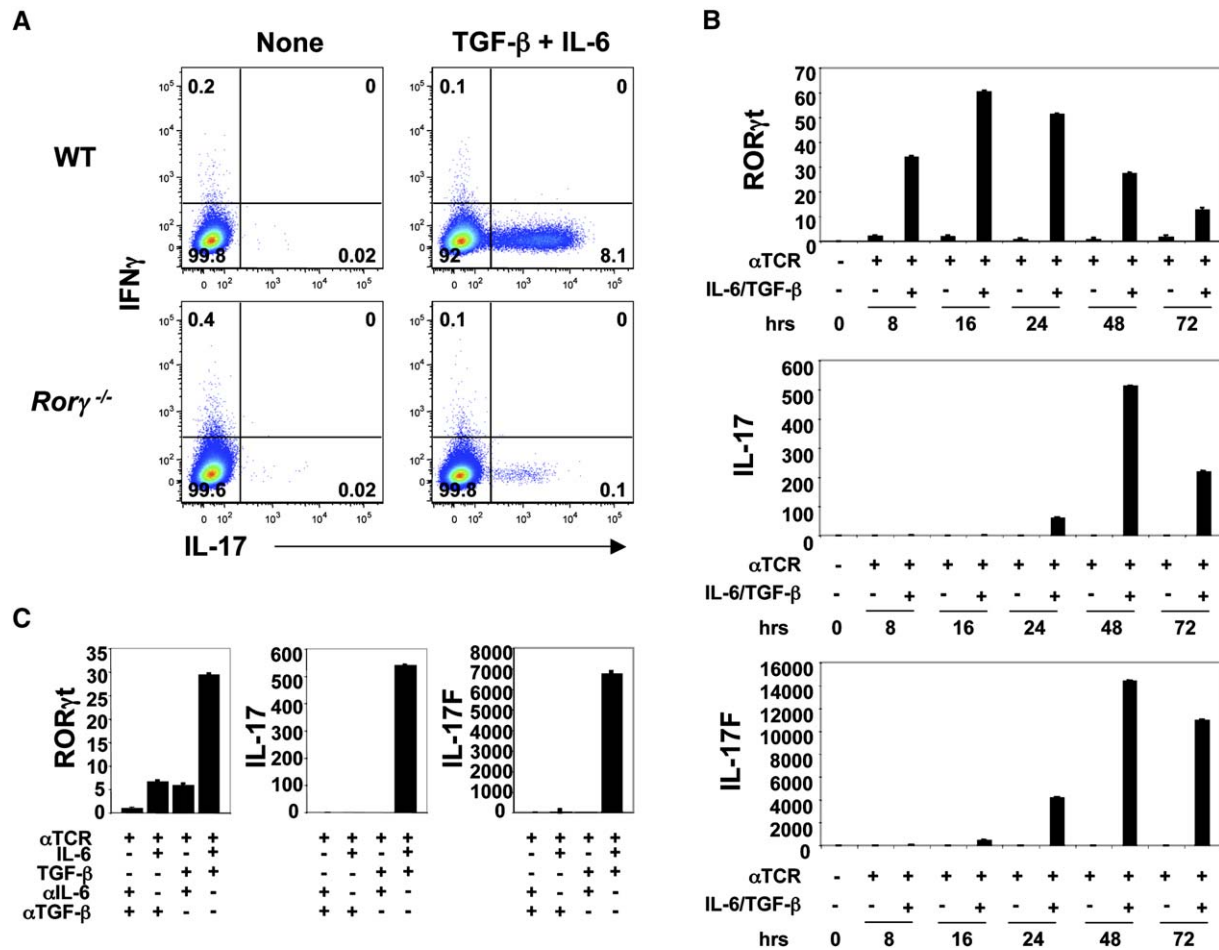
To eliminate any possibility that cytokines produced by contaminating antigen-presenting cells contribute to the induction of IL-17 by ROR $\gamma$ t, we repeated the experiments using highly purified naive CD4<sup>+</sup> T cells. Transduction of ROR $\gamma$ t resulted in IL-17 expression in more than half of the cells in the absence of exogenous cytokines (Fig-

ure 4C). Together, these results indicate that ROR $\gamma$ t is sufficient to induce expression of IL-17 and IL-17F in antigen-stimulated naive CD4<sup>+</sup> T cells and are consistent with a role for ROR $\gamma$ t downstream of its induction by IL-6 and TGF- $\beta$ .

#### IL-6 Is Required for ROR $\gamma$ t Expression in the Lamina Propria

IL-6-deficient mice have been shown to have normal numbers of T cells but are defective in the induction of autoimmune diseases and display increased susceptibility to a variety of pathogens (Eugster et al., 1998; Kopf et al., 1994). To determine if IL-6 is required in vivo for the differentiation of Th17 cells in the lamina propria, we characterized intestinal T cells from IL-6 mutant animals. Although lamina propria T cells were present in normal numbers in IL-6-deficient mice, IL-17<sup>+</sup> cells were reduced by about 10-fold, similar to what was observed with ROR $\gamma$ t-deficient mice (Figure 5A).

Consistent with the flow cytometry data, quantitative RT-PCR performed with RNA from sorted lamina propria



**Figure 3. In Vitro Differentiation of Th17 Cells Requires ROR $\gamma$ t**

(A) Cytokine production by naive CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>+</sup>CD44<sup>-</sup> T cells from wt and *Ror $\gamma$ <sup>-/-</sup>* mice after stimulation with anti-CD3/CD28 for 3 days with or without TGF- $\beta$  and IL-6.

(B) Time course of ROR $\gamma$ t, IL-17, and IL-17F mRNA expression following stimulation as in (A).

(C) ROR $\gamma$ t, IL-17, and IL-17F mRNA expression following stimulation as in (A) at the 48 hr time point. Relative expression levels were measured by quantitative real-time RT-PCR and were normalized to actin expression level using the standard curve method.

In (B) and (C), error bars represent standard deviation obtained using the standard curve method.

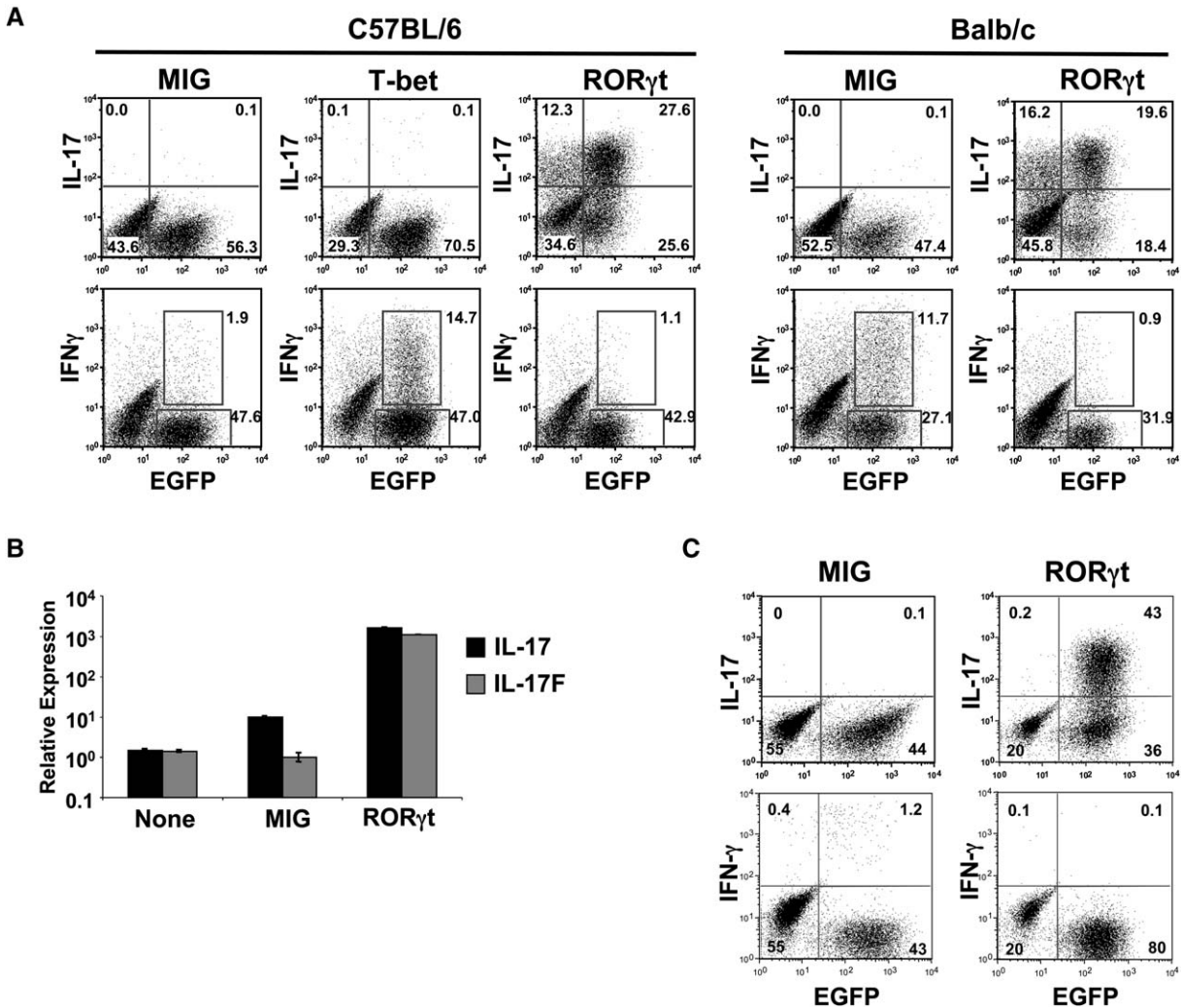
TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> cells demonstrated a reduction in IL-17 expression in the absence of IL-6. Moreover, in contrast to lamina propria CD4<sup>+</sup> T cells from wild-type (wt) mice, those from IL-6-deficient mice did not express ROR $\gamma$ t, IL-17F, and the IL-23-specific chain of the IL-23R (Figure 5B). These data suggest that IL-6 upregulates ROR $\gamma$ t and IL-23R in vivo in the lamina propria, thus promoting the generation of Th17 cells in the intestine.

### ROR $\gamma$ t Is Required for Th17-Mediated Autoimmune Inflammatory Disease

The experiments described above demonstrate that ROR $\gamma$ t is a transcription factor required for differentiation of IL-17-producing Th17 cells in vitro and in the intestinal lamina propria. Since Th17 cells have been shown recently to be the major pathogenic population in several models of autoimmune inflammation, including EAE

(Langrish et al., 2005; Park et al., 2005), we investigated the role of ROR $\gamma$ t in this model. EAE was induced in wild-type and ROR $\gamma$ -deficient mice by injecting MOG 35-55 peptide in complete Freund's adjuvant and pertussis toxin (PTX) at day 0 and PTX at day 2. Wild-type C57BL/6 mice developed disease on days 10–11 and by day 14 reached peak disease manifestation, with 80% reaching a score of 4. ROR $\gamma$ -deficient mice on the C57BL/6 background did not show signs of disease until day 22, at which point some mice developed mild disease (maximum score of 2) that quickly subsided (Figure 6A). Thus, in the absence of endogenous ROR $\gamma$ t, mice are less susceptible to EAE.

Although ROR $\gamma$ -deficient mice contain largely normal splenic architecture, they lack all peripheral lymph nodes. It is therefore possible that the lack of lymph nodes alone may have contributed to the delayed and reduced



**Figure 4. Induction of IL-17 in CD4<sup>+</sup> T Cells by Ectopic Expression of ROR $\gamma$ t**

(A) IL-17 and IFN $\gamma$  production in MACS-sorted CD4<sup>+</sup> T cells isolated from wild-type C57BL/6 and Balb/c mice and transduced with retroviral vectors encoding IRES-GFP (MIG), T-bet-IRES-GFP (T-bet), and ROR $\gamma$ t-IRES-GFP (ROR $\gamma$ t). Cells were analyzed after 5 days in culture.

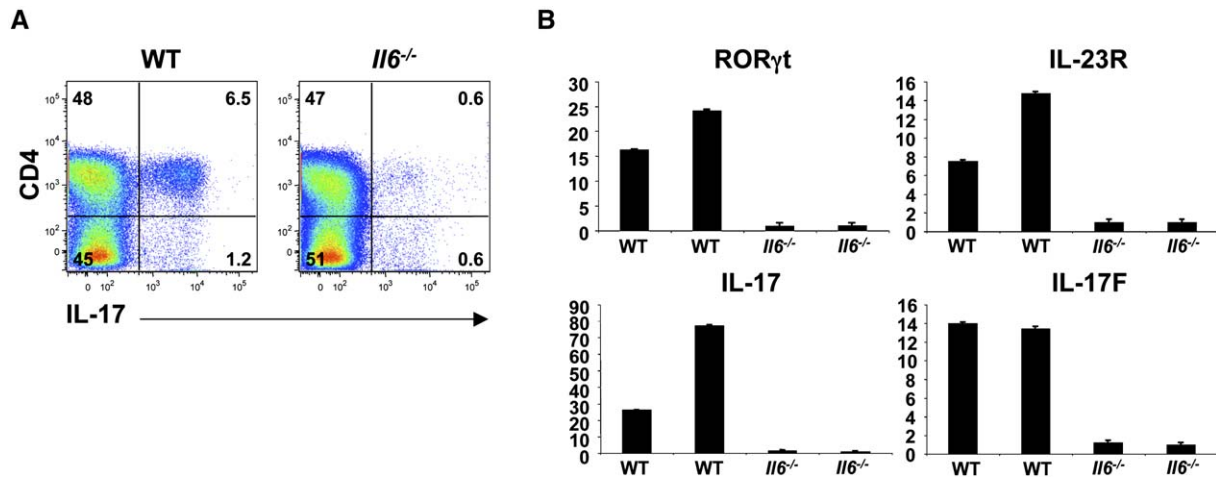
(B) Relative expression levels of IL-17 and IL-17F mRNAs in retrovirally transduced MACS-sorted CD4<sup>+</sup> T cells. Expression levels were monitored by quantitative real-time RT-PCR, and data were normalized to GAPDH expression level using the standard curve method.

(C) IL-17 production in sorted naive CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>+</sup>CD44<sup>-</sup>) transduced with retroviral constructs encoding IRES-GFP (MIG) and ROR $\gamma$ t-IRES-GFP (ROR $\gamma$ t).

In (B), error bars represent standard deviation obtained using the standard curve method.

autoimmunity observed in *Ror $\gamma$ <sup>-/-</sup>* animals. To address this caveat, we employed models in which EAE was induced after reconstitution of RAG2-deficient mice (*Rag2<sup>-/-</sup>*), which contain all secondary lymphoid organs, including lymph nodes, with either CD4<sup>+</sup> splenocytes or bone marrow from wild-type or *Ror $\gamma$ <sup>-/-</sup>* mice. Transfer of  $1 \times 10^7$  CD4<sup>+</sup> splenocytes from wild-type mice, followed by induction of EAE 24 hr later, led to development of disease in all animals by day 15 post-induction. In contrast, transfer of CD4<sup>+</sup> splenocytes from *Ror $\gamma$ <sup>-/-</sup>* mice resulted in only mild disease or, more commonly, in no disease (Figure 6B).

In the bone marrow transfer model, we confirmed that the reconstitution efficiency was similar after 11 weeks in mice that received wild-type or ROR $\gamma$ -deficient cells (Figure S6 and data not shown), and EAE was then induced. RAG-deficient mice reconstituted with wild-type bone marrow cells developed disease as early as day 10 after immunization (Figure 6C). By day 14, 90% of the mice had severe disease (score of 4) and most animals were moribund and had to be sacrificed by day 20. In contrast, less than half of the RAG-deficient animals reconstituted with *Ror $\gamma$ <sup>-/-</sup>* bone marrow cells developed some disease, and this was relatively mild (Figure 6C). Together,



**Figure 5. IL-6 Controls the Differentiation of ROR $\gamma$ <sup>t</sup> Th17 Cells in the Lamina Propria**

(A) Comparison of IL-17<sup>+</sup>CD4<sup>+</sup> T cells in the lamina propria of wt (B6) and IL-6-deficient mice. LPLs were isolated from two 17-week-old mice of each genotype and stimulated for 4 hr with PMA/Ionomycin in the presence of Brefeldin A.

(B) Levels of ROR $\gamma$ <sup>t</sup>, IL-23R, IL-17, and IL-17F mRNA expression in sorted TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> lamina propria T cells from wt and IL-6-deficient mice.

In (B), error bars represent standard deviation obtained using the standard curve method.

these results show that ROR $\gamma$ <sup>t</sup> is required for fulminant EAE.

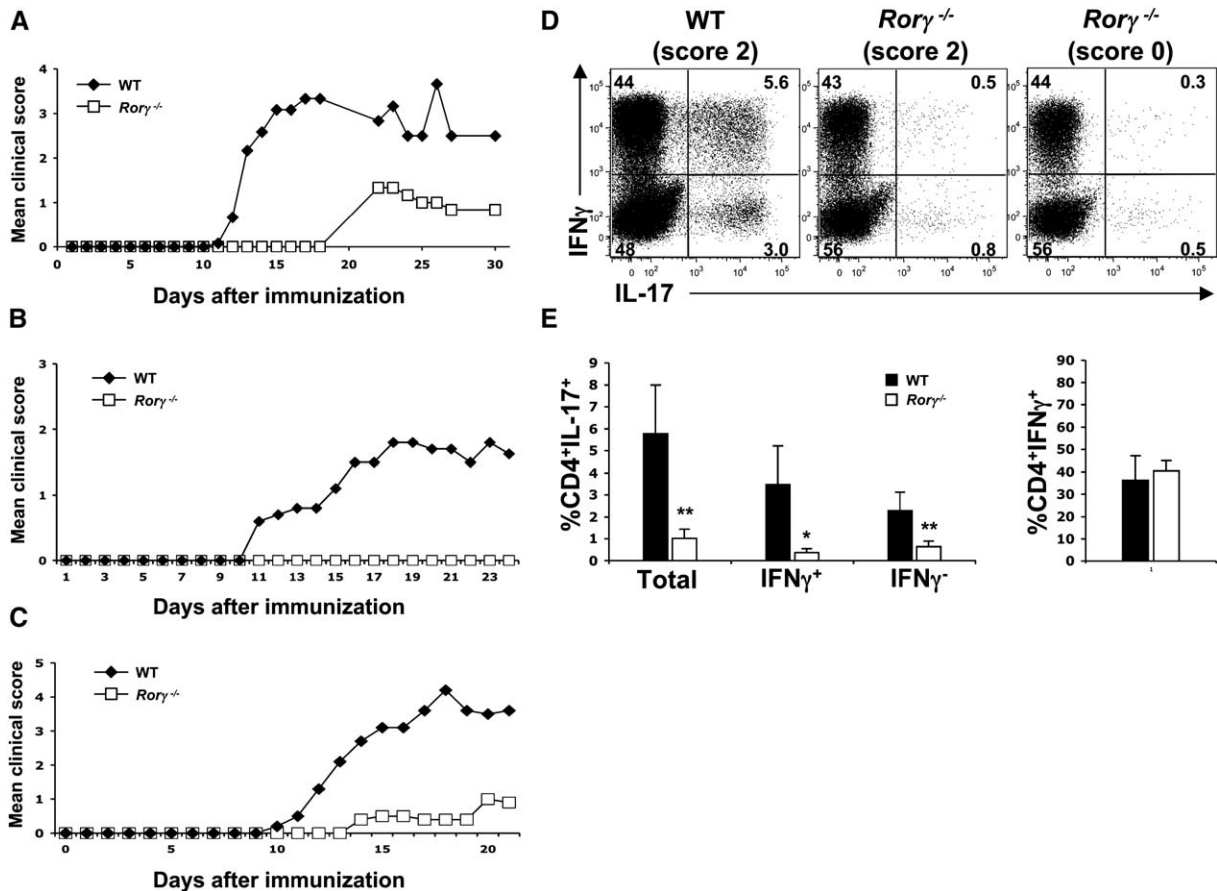
Despite the markedly reduced disease manifestations in mice that received bone marrow cells from *Rorγ*<sup>-/-</sup> animals, infiltrating T cells were present in the spinal cords at levels similar to those in mice reconstituted with wild-type cells (Figure S6). The total number of mononuclear infiltrating cells was slightly lower in the ROR $\gamma$ -deficient cell transfers but did not reach statistical significance ( $3.4 \pm 1.3 \times 10^6$  cells for wt, versus  $2.0 \pm 1.1 \times 10^6$  cells for ROR $\gamma$ -deficient mice on day 21). Mice that received wild-type bone marrow before induction of EAE contained both IFN $\gamma$ - and IL-17-producing T cells in the spinal cord infiltrate (Figures 6D and 6E). Remarkably, more than half of the IL-17-producing cells also expressed IFN $\gamma$ . In contrast, although IFN $\gamma$ -producing T cells were present in the ROR $\gamma$ -deficient T cell infiltrate, few of the infiltrating T cells produced IL-17 (Figures 6D and 6E), irrespective of the presence or absence of disease (Figure 6D). In addition, MOG-specific IL-17<sup>+</sup> cells could not be found in draining lymph nodes from mice reconstituted with bone marrow from mutant mice after 4 days of culture with MOG peptide, although similar numbers of total MOG-specific T cells were found in these cultures and those from control mice, suggesting a similar frequency of MOG-specific T cells in both groups (Figure S7). Similar results were obtained in the analysis of mice in which EAE was induced after transfer of CD4<sup>+</sup> splenocytes. Although infiltrating CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> T cells were present in both groups, only mice that received wild-type CD4<sup>+</sup> T cells contained IL-17<sup>+</sup> cells in the spinal cord and developed disease (Figure S8).

During EAE, lymphoid and myeloid cells in the inflammatory infiltrate elicit increased expression of a number

of proinflammatory cytokines and chemokines locally in the CNS. To further assess the etiology of the residual disease in the ROR $\gamma$ -deficient chimeras, we investigated the local CNS inflammatory responses by real-time PCR. Induction of EAE in wild-type bone marrow chimeras led to increased expression of the proinflammatory cytokine genes previously reported, including IL-17, IL-17F, and IL-6, as well as the chemokines CCL6, CCL9, CCL11, CCL20, and CCL24 and the receptor CCR1 (Figure S9). Consistent with the reduced number of Th17 cells, ROR $\gamma$ -deficient mice displayed significantly decreased levels of the Th17 cytokines and chemokines in the spinal cord during disease. However, IFN $\gamma$  and IFN $\gamma$ -regulated chemokines (including MIG) were unchanged in the ROR $\gamma$ -deficient chimeras (Figure S9), consistent with a primary role of pathogenic Th17, rather than Th1, cells in the disease process.

## DISCUSSION

In this study, we have shown that the orphan nuclear receptor ROR $\gamma$ <sup>t</sup> is the transcription factor that directs the differentiation of IL-17-producing inflammatory T cells. Recent studies have demonstrated that IL-17 is expressed at elevated levels in a variety of allergic and autoimmune diseases in humans (Barczyk et al., 2003; Fujino et al., 2003; Infante-Duarte et al., 2000; Lock et al., 2002; Witowski et al., 2004), and that Th17 cells represent a distinct lineage of T helper cells whose function is required in numerous animal models of autoimmune disease (Dong, 2006; McKenzie et al., 2006). Within tissues, Th17 effector cells stimulate production of a variety of inflammatory chemokines, cytokines, metalloproteases, and other proinflammatory mediators and promote



**Figure 6. Reduced Severity of EAE and Absence of Infiltrating Th17 Cells in Mice with ROR $\gamma$ t-Deficient T Cells**

(A) EAE disease course in wild-type and *Rorγ*<sup>-/-</sup> mice (data are from six C57BL/6 and three syngeneic *Rorγ*<sup>-/-</sup> mice).

(B) EAE disease course in RAG2-deficient mice reconstituted with MACS-purified CD4<sup>+</sup> splenocytes from wild-type (wt) and *Rorγ*<sup>-/-</sup> mice. Data are representative of two independent experiments—n = 5 (wt), n = 3 (*Rorγ*<sup>-/-</sup>).

(C) EAE disease course in RAG2-deficient mice reconstituted with total bone marrow cells from wild-type or *Rorγ*<sup>-/-</sup> animals (five animals in each group). All recipient animals were irradiated with a sublethal dose (400 rads/animal) before reconstitution, and EAE was induced 11 weeks later. To assess similar reconstitution efficiency, blood before disease induction as well as spleen populations on day 21 after induction were compared between the two groups (Figure S6 and data not shown). Data are representative of three independent experiments.

(D and E) Cytokine production by lymphocytes isolated on day 21 after disease induction from the spinal cords of RAG-deficient mice reconstituted with wt and *Rorγ*<sup>-/-</sup> bone marrow (experiment in Figure 6C). The cells were stimulated for 4 hr with PMA/Ionomycin and stained for surface markers and intracellular cytokines. Representative FACS plots (gated on TCRβ<sup>+</sup>CD4<sup>+</sup> cells) from mice from each group are shown in (D). Clinical scores are shown in parentheses. In the *Rorγ*<sup>-/-</sup> group, 3 out of 5 mice did not develop any clinical signs of disease (score 0), but all had considerable spinal cord infiltrate. One is shown in (D). Similar results were achieved in three independent experiments. Tabulated results from all mice are presented in (E) as percentage of TCRβ<sup>+</sup>CD4<sup>+</sup> cells in the spinal cord infiltrate. Total: all IL-17<sup>+</sup> cells; IFNγ<sup>+</sup>: IL-17<sup>+</sup>IFNγ<sup>+</sup> cells; IFNγ<sup>-</sup>: IL-17<sup>+</sup>IFNγ<sup>-</sup> cells; CD4<sup>+</sup>IFNγ<sup>+</sup>: IL-17<sup>-</sup>IFNγ<sup>+</sup> cells. \*\*p = 0.002, \*p = 0.006, unpaired t test. Error bars represent standard deviation.

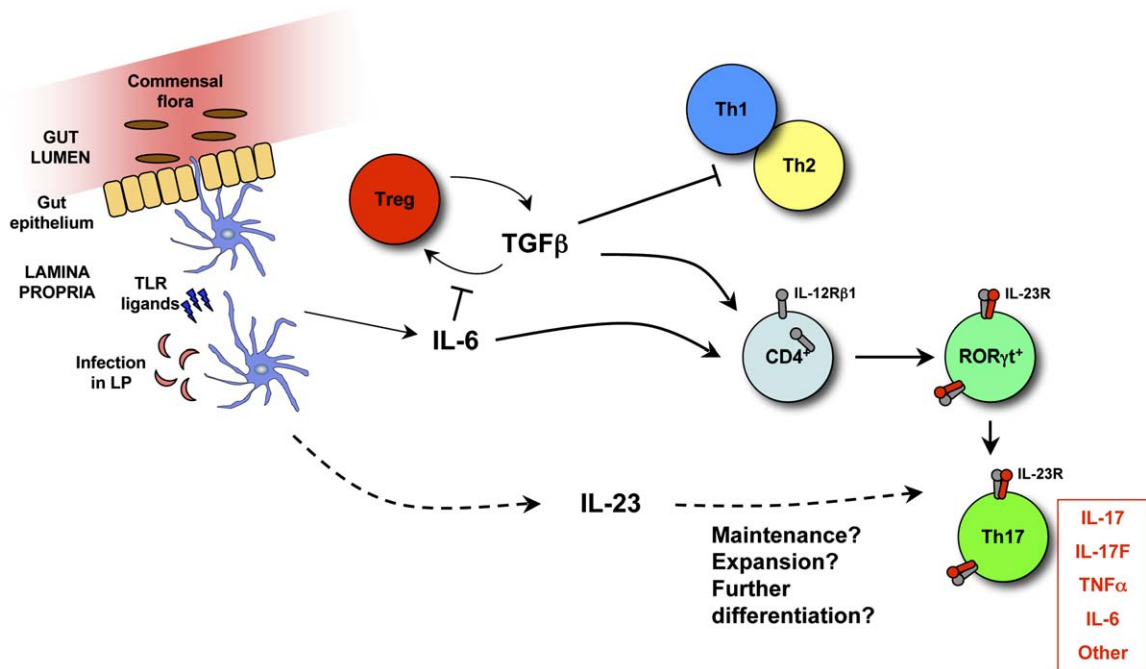
recruitment of granulocytes (Kolls and Linden, 2004; Stamp et al., 2004). We found that ROR $\gamma$ t is required for the constitutive expression of IL-17 in intestinal lamina propria T cells and for the in vitro differentiation of Th17 cells from naïve CD4<sup>+</sup> T cells. Following ligation of the antigen receptor, the cytokines IL-6 and TGF- $\beta$  together induce the transcription of ROR $\gamma$ t, which, in turn, participates in and may be sufficient for the induction of IL-17 expression. We have additionally shown a central role for ROR $\gamma$ t in the in vivo differentiation of pathogenic Th17 cells within the central nervous system in a model for in-

flammatory autoimmune disease, EAE. Together, these studies suggest that manipulation of ROR $\gamma$ t activity may be an attractive therapeutic strategy in a variety of diseases with inflammatory etiology.

#### Regulation of the Th17 Differentiation Pathway by ROR $\gamma$ t

ROR $\gamma$  and ROR $\gamma$ t are both encoded within the *Rorc* locus and differ only in their amino-terminal sequences due to utilization of different promoters (Eberl and Littman, 2003). Both are members of the retinoic acid-related





**Figure 7. Model of Th17 Development in the Intestinal Lamina Propria**

Th17 development in the gut requires ROR $\gamma$ t expression in CD4 $^{+}$  T cells. ROR $\gamma$ t expression results from the action of IL-6 and TGF- $\beta$  (but not IL-23) produced by activated dendritic cells (DCs) and other cells in the lamina propria. DCs can be activated by signals derived from the luminal flora or infectious agents and TLR ligands that gain access to the lamina propria. It is currently unknown if IL-6, TGF- $\beta$ , and IL-23 are produced by different types of DCs or by the same DC. TGF- $\beta$  may also be derived from regulatory T cells (Tregs), which normally suppress Th1 and Th2 cell development. IL-6 may also inhibit TGF- $\beta$ -induced differentiation of Tregs, thus further promoting Th17 development. ROR $\gamma$ t $^{+}$  T cells upregulate IL-23R and thus become responsive to IL-23. IL-23 reinforces the Th17 phenotype by possibly helping in maintenance, expansion, or further differentiation of the cells.

orphan nuclear hormone receptor family that also includes ROR $\alpha$  and ROR $\beta$  (Jetten, 2004). Whereas ROR $\gamma$  is expressed broadly and as yet has no defined function, ROR $\gamma$ t is expressed exclusively in cells of the immune system.

We previously showed that ROR $\gamma$ t, but not ROR $\gamma$ , is expressed in fetal LTI cells, intestinal LTI-like cells, and immature thymocytes (Eberl and Littman, 2004; Sun et al., 2000). In this study, we found that subpopulations of intestinal lamina propria T cells also express low levels of ROR $\gamma$ t, and many of these cells constitutively express IL-17. In vitro, IL-6 and TGF- $\beta$  induced transcription of ROR $\gamma$ t in purified CD4 $^{+}$  T cells prior to the onset of IL-17 and IL-17F expression. The expression of IL-17 in the intestinal T cells and in cytokine-stimulated CD4 $^{+}$  T cells was dependent on the presence of ROR $\gamma$ t. In addition, lamina propria T cells from IL-6-deficient mice did not express ROR $\gamma$ t or IL-17. Thus it is likely that, after T cells migrate into the lamina propria, ROR $\gamma$ t is induced locally by the combination of IL-6 and TGF- $\beta$ , which are abundant at this site, resulting in differentiation of Th17 cells (Figure 7).

It was recently shown that TGF- $\beta$  induces expression of Foxp3 and promotes the differentiation of regulatory T cells (Tregs) (Chen et al., 2003; Fantini et al., 2004). This program was blocked by IL-6, which together with

TGF- $\beta$  induced IL-17 expression instead (Bettelli et al., 2006). The relative balance of IL-6 and TGF- $\beta$  may therefore control the local differentiation of Tregs and Th17 cells at steady state in the intestine and in inflammatory conditions in diverse tissues (Figure 7). Paradoxically, TGF- $\beta$  and IL-6 can individually induce expression of some ROR $\gamma$ t, but neither alone can induce IL-17. This may in part be explained by the ability of Foxp3 to inhibit ROR $\gamma$ t-induced IL-17 expression (L.Z. and D.R.L., unpublished data). It will be important to further dissect the transcriptional networks that govern differential expression of Foxp3 and ROR $\gamma$ t in response to the cytokines. IFN $\gamma$  and IL-4 have also been reported to interfere with Th17 differentiation in vitro, raising the possibility that there is reciprocal inhibition between the different T helper cell differentiation pathways.

IL-23 is also involved in Th17 cell differentiation, but naïve T cells are IL-23 receptor negative and relatively refractory to IL-23 stimulation (Langrish et al., 2005; Oppmann et al., 2000). Only following in vivo priming does in vitro stimulation of T cells with antigen and antigen-presenting cells plus IL-23 result in effective expansion of Th17 cells (Langrish et al., 2005; Murphy et al., 2003; Veldhoen et al., 2006). Our results suggest that IL-23R is upregulated on ROR $\gamma$ t $^{+}$  Th17 cells in an IL-6-dependent manner (Figure 5;

L.Z. and B.S.M., unpublished data). IL-23 may therefore function subsequent to IL-6/TGF- $\beta$ -induced commitment to the Th17 lineage to promote cell survival and expansion and, potentially, continued expression of IL-17 and other cytokines that characterize the Th17 phenotype (Figure 7). It is not yet known if ROR $\gamma$ t is required for sustained IL-17 expression in response to IL-23R-mediated signals in effector/memory T cells.

### Role of ROR $\gamma$ t in Immune System Homeostasis

In healthy adult mice, ROR $\gamma$ t has been detected only in immature thymocytes and in cells residing in the intestinal lamina propria. In the intestine, ROR $\gamma$ t is required for the differentiation of Th17 cells and of LTI-like cells that form cryptopatches and appear to be critical for differentiation of isolated lymphoid follicles (ILFs) (Eberl and Littman, 2004). It is intriguing that two types of ROR $\gamma$ t-dependent cells with very different mechanisms of action are both positioned at a mucosal surface that is in constant interaction with an enormous amount of microbial flora. The cryptopatch cells and the Th17 cells may be regulated coordinately, perhaps by a ligand for ROR $\gamma$ t, to maintain homeostasis of the microflora and integrity of the epithelial barrier. Cryptopatches are located at the base of intestinal crypts and are thus positioned to receive signals from the intestinal lumen and transmit them to other cells in the lamina propria and to crypt epithelial cells. Development of mature ILFs requires signals from the gut flora (Lorenz et al., 2003; Pabst et al., 2005; Taylor and Williams, 2005) that may induce recruitment of B lymphocytes to cryptopatches (Ivanov et al., 2006). Signals from the gut flora may also be responsible for the unique presence of Th17 cells in the intestinal mucosa.

Th17 cells are likely to be required to control infections at mucosal surfaces. IL-23-deficient mice are unable to control orally administered *Citrobacter rodentium*, which stimulates expansion of lamina propria Th17 cells (Mangan et al., 2006). IL-17 and IL-23 have also been shown to be important in protecting mice from lung infection with *Klebsiella pneumoniae* (Happel et al., 2005; Ye et al., 2001). By inducing chemokine and G-CSF expression from surrounding cells, ROR $\gamma$ t-dependent Th17 cells promote expansion and recruitment of neutrophils that can control these microorganisms if they breach the epithelial barrier. In vitro studies using intestinal epithelial cell cultures showed that IL-17 could enhance tight-junction formation that is crucial for the protective function of the gut mucosa (Kinugasa et al., 2000). In an acute model of colitis induced by dextran sulfate sodium, which strips the intestinal mucosal barrier, neutralization of IL-17 exacerbated colonic inflammation (Ogawa et al., 2004). These results suggest that ROR $\gamma$ t-mediated induction of IL-17 expression can also have a protective or regenerative function when epithelial damage occurs.

It is unknown if ROR $\gamma$ t directly induces IL-17 transcription in Th17 cells. ROR response elements (ROREs) consist of the consensus core motif AGGTCA preceded by a 5 bp A/T-rich sequence (Jetten et al., 2001). We per-

formed a search for potential ROREs within the *il17* locus and identified an evolutionarily conserved ROR $\gamma$ t binding site within the 200 bp core promoter region, suggesting that transcription of IL-17 may be directly regulated by ROR $\gamma$ t (data not shown). Whether ROR $\gamma$ t activity is regulated by a ligand is not yet known. Exposure to a ligand in the intestine could potentially regulate the transcriptional activity of ROR $\gamma$ t and may thus govern formation of follicles, differentiation of effector T cells, and even the dynamic equilibrium of the epithelial barrier.

### Central Role of ROR $\gamma$ t in Inflammatory Diseases

During the past year, it has become increasingly clear that T helper cells that secrete IL-17, rather than IFN $\gamma$ -producing Th1 cells, are the major inflammatory cells in a number of disease models, and that function of these cells is dependent on IL-23 (Chen et al., 2006; Cua et al., 2003; Langowski et al., 2006; Mangan et al., 2006; Yen et al., 2006). In EAE, Th17 cells are far more potent than IFN $\gamma$ -producing Th1 cells, with as few as  $1 \times 10^5$  IL-17-producing T cells sufficient to transfer disease to naïve recipients (Langrish et al., 2005). We found that ROR $\gamma$ t is required for pathogenic Th17 responses in EAE. In the CNS of animals with ROR $\gamma$ t-deficient T cells, the cytokines characteristic of Th17 cells were significantly reduced, but Th1 cytokines were normally expressed. The absence of Th17 cells led to a decrease in proinflammatory chemokines, consistent with the role of the IL-23-IL-17 pathway in recruiting inflammatory cells to sites of inflammation (McKenzie et al., 2006; Stark et al., 2005). ROR $\gamma$ t-dependent mechanisms that normally regulate host defense by promoting neutrophil mobilization and tissue repair are therefore also critical in initiating tissue-specific inflammatory responses.

Consistent with a key role for ROR $\gamma$ t-dependent Th17 cells in autoimmunity, we have found that mRNAs for both ROR $\gamma$ t and IL-17 are significantly increased in skin from psoriasis patients (B.S.M. and D.J.C., unpublished data). This and recent findings of elevated IL-17 levels in human autoimmune diseases suggest that intervention with ROR $\gamma$ t activity and, hence, with the differentiation of Th17 cells may be of significant therapeutic benefit. Our results indicate that ROR $\gamma$ t has a role in Th17 differentiation that resembles the roles of T-bet and GATA-3 in the differentiation of Th1 and Th2 cells, respectively. Unlike these other transcription factors, ROR $\gamma$ t is a nuclear receptor with a ligand binding pocket, and it is hence likely to be an excellent target for pharmacologic intervention in inflammatory diseases that result in autoimmunity and cancer progression.

## EXPERIMENTAL PROCEDURES

### Mice

Mice with a GFP reporter cDNA knocked-in at the site for initiation of ROR $\gamma$ t translation (Eberl et al., 2004), as well as *Ror $\gamma$ <sup>-/-</sup>* mice on the C57BL/6 background (Sun et al., 2000), have been described and were kept in SPF conditions at the animal facility of the Skirball Institute. *Myd88<sup>-/-</sup>* mice were provided by Dr. Ruslan Medzhitov

(Yale University). *Ilg<sup>-/-</sup>* mice on the C57BL/6 background were purchased from the Jackson laboratory and were provided by Dr. Joel Ernst (NYU). C57BL/6, Balb/c, and *Rag2<sup>-/-</sup>* mice were purchased from Taconic. All animal experiments were performed in accordance with approved protocols from the NYU Institutional Animal Care and Usage Committee.

#### EAE Induction and Disease Scoring

For induction of EAE, mice were immunized subcutaneously on day 0 with 150  $\mu$ g/mouse MOG 35-55 peptide (Molecular Biology Core Facilities, Dana-Farber Cancer Institute, Harvard University), emulsified in CFA (CFA supplemented with 400  $\mu$ g/ml *Mycobacterium tuberculosis*), and injected intravenously on days 0 and 2 with 200 ng/mouse of pertussis toxin (Calbiochem). The basic scoring system used was 0—no disease, 1—limp tail, 2—weak/partially paralyzed hind legs, 3—completely paralyzed hind legs, 4—complete hind and partial front leg paralysis, 5—complete paralysis/death. Mice with disease levels 4 and 5 were considered moribund and were euthanized.

#### Bone Marrow Reconstitutions

Total bone marrow mononuclear cells were isolated from wild-type and *Rory<sup>-/-</sup>* mice by flushing the long bones. Red blood cells were lysed with ACK Lysing Buffer (BioWhittaker) and the remaining mononuclear cells were resuspended in PBS for injection. 5–10  $\times$  10<sup>6</sup> cells per mouse were injected intravenously into 3- to 5-week-old *Rag2<sup>-/-</sup>* mice that were sublethally irradiated using 400 rads/mouse 4 hr before reconstitution. EAE was induced 11 weeks post bone marrow reconstitution.

#### CD4<sup>+</sup> T Cell Transfers

Single-cell suspensions were prepared from spleens of wild-type and *Rorg<sup>-/-</sup>* mice and CD4<sup>+</sup> cells purified by using anti-CD4 magnetic microbeads (Miltenyi Biotec) and MACS columns (purity was >95%, usually 97%–98%). 10<sup>7</sup> CD4<sup>+</sup> cells per mouse were injected intravenously into unirradiated *Rag2<sup>-/-</sup>* mice. EAE was induced 24 hr after transfer.

#### Isolation of Lamina Propria Lymphocytes and Intraepithelial Lymphocytes

Mice were killed and intestines removed and placed in ice-cold PBS. After removal of residual mesenteric fat tissue, Peyer's patches were carefully excised, and the intestine was opened longitudinally. The intestine was then thoroughly washed in ice-cold PBS and cut into 1.5 cm pieces. The pieces were incubated twice in 5 ml of 5 mM EDTA in HBSS for 15–20 min at 37°C with slow rotation (100 rpm). After each incubation, the epithelial cell layer, containing the intraepithelial lymphocytes (IELs), was removed by intensive vortexing and passing through a 100  $\mu$ m cell strainer and new EDTA solution was added. After the second EDTA incubation the pieces were washed in HBSS, cut in 1 mm<sup>2</sup> pieces using razor blades, and placed in 5 ml digestion solution containing 4% fetal calf serum, 0.5 mg/ml each of Collagenase D (Roche) and DNase I (Sigma), and 50 U/ml Dispase (Fisher). Digestion was performed by incubating the pieces at 37°C for 20 min with slow rotation. After the initial 20 min, the solution was vortexed intensely and passed through a 40  $\mu$ m cell strainer. The pieces were collected and placed into fresh digestion solution and the procedure was repeated a total of three times. Supernatants from all three digestions (or from the EDTA treatment for IEL isolation) from a single small intestine were combined, washed once in cold FACS buffer, resuspended in 10 ml of the 40% fraction of a 40:80 Percoll gradient, and overlaid on 5 ml of the 80% fraction in a 15 ml Falcon tube. Percoll gradient separation was performed by centrifugation for 20 min at 2500 rpm at room temperature. Lamina propria lymphocytes (LPLs) were collected at the interphase of the Percoll gradient, washed once, and resuspended in FACS buffer or T cell medium. The cells were used immediately for experiments.

#### Isolation of Mononuclear Cells from Spinal Cords

Before spinal cord (SC) dissection, mice were perfused with 25 ml 2 mM EDTA in PBS to remove blood from internal organs. The spinal columns were dissected, cut open, and intact SCs separated carefully from the vertebrae. The SCs were cut into several small pieces and placed in 2 ml digestion solution containing 10 mg/ml Collagenase D (Roche) in PBS. Digestion was performed for 45 min at 37°C with short vortexing every 15 min. At the end of the digestion the solution was vortexed intensely and passed through a 40  $\mu$ m cell strainer. The cells were washed once in PBS, placed in 6 ml of 38% Percoll solution, and pelleted for 20 min at 2000 rpm. Pellets were resuspended in FACS buffer or T cell medium and used for subsequent experiments.

#### Isolation of Total and Naïve CD4<sup>+</sup> T Cells

CD4<sup>+</sup> T cells were purified from spleens using anti-CD4 magnetic microbeads (Miltenyi Biotec) and MACS columns (purity was >95%). For naïve CD4<sup>+</sup> T cells, single-cell suspensions were first negatively depleted by staining with anti-B220-PE, anti-CD8 $\alpha$ -PE, anti-CD11b-PE, anti-CD11c-PE, anti-CD49b-PE, all at 1:100 dilution, and anti-Ter119-PE at 1:66 dilution, for 20 min on ice, followed by incubation with anti-PE magnetic microbeads (Miltenyi Biotec) at 1:20 dilution for 20 min on ice. The depleted fraction was stained with anti-CD25-PE, anti-CD4-PECy7, anti-CD62L-FITC, and anti-CD44-APC. Cell sorting was performed on a MoFlo cytometer (DAKO Cytomation) to obtain a pure population of naïve CD4<sup>+</sup>CD25<sup>-</sup>CD44<sup>low</sup>CD62L<sup>+</sup> T cells (>99% purity).

#### Surface and Intracellular Cytokine Staining

For intracellular cytokine staining, cells obtained from *in vitro* culture or from dissection of lamina propria or spinal cords were incubated for 4–5 hr with 50 ng/ml PMA (Sigma), 750 ng/ml Ionomycin (Sigma), and 10  $\mu$ g/ml Brefeldin A (Invitrogen) in a tissue culture incubator at 37°C. Surface staining was performed for 15–20 min with the corresponding cocktail of fluorescently labeled antibodies. After surface staining, the cells were resuspended in Fixation/Permeabilization solution (BD Cytotfix/Cytoperm kit—BD Pharmingen), and intracellular cytokine staining was performed as per the manufacturer's protocol.

#### Flow Cytometry and Antibodies

Flow cytometric analysis was performed on LSR II (BD Biosciences) or FACSCalibur (BD Biosciences) instruments and analyzed using FlowJo software (Tree Star Inc.). All antibodies were purchased from BD Pharmingen or eBiosciences. In cases where intracellular cytokine staining was performed, GFP fluorescence was detected with anti-GFP-Alexa 488 (polyclonal rabbit IgG fraction—Molecular Probes).

#### Plasmids and Retrovirus Production

The ROR $\gamma$ t cDNA was PCR amplified and cloned into pMIG (ROR $\gamma$ t-IRES-GFP). T-bet-IRES-GFP was a kind gift from Dr. Steve Reiner (University of Pennsylvania). Phoenix cells were transfected with 9  $\mu$ g of the indicated plasmids using Lipofectamin 2000 (Invitrogen). Viral supernatant was collected and supplemented with 8  $\mu$ g/ml polybrene (Sigma).

#### Cell Culture and Retroviral Transduction

The T cell culture medium used was RPMI Media 1640 (Invitrogen) supplemented with 10% Fetal Calf Serum (Hyclone), 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 5 mM 2- $\beta$ -mercaptoethanol. 1.5  $\times$  10<sup>6</sup>/well MACS-purified CD4<sup>+</sup> T cells or sorted naïve CD4<sup>+</sup> T cells were cultured in 24-well plates (or 0.7  $\times$  10<sup>6</sup> cells per well in 48-well plates) containing plate bound anti-CD3 (5  $\mu$ g/ml) and soluble anti-CD28 (1  $\mu$ g/ml); cultures were supplemented with 40 U/ml mouse IL-2 (Roche), 10  $\mu$ g/ml anti-IL-4 (BD Pharmingen), 10  $\mu$ g/ml anti-IFN- $\gamma$  (BD Pharmingen) with or without 20 ng/ml IL-6 (eBioscience), and 5 ng/ml TGF- $\beta$  (Preprotech). For flow cytometry cells were analyzed on days 3 and 5.

For viral transduction, sorted naïve CD4<sup>+</sup> T cells were plated as above in the absence of TGF- $\beta$  and IL-6 on day 0. On days 1 and 2, fresh retrovirus supernatant was added and the cells were spun at 2500 rpm for 1.5 hr at 30°C. After spin infection, the cells were cultured in the T cell culture medium and harvested on day 5 or 6 for intracellular cytokine staining and real-time PCR (RT-PCR) analysis.

#### RT-PCR

cDNA was synthesized with RNA prepared by TRIZOL using RNase H-reverse transcriptase (Invitrogen). cDNA was analyzed by real-time quantitative PCR in triplicates by using iQ CYBR Green Supermix (Bio-Rad) or QuantiTect Multiplex PCR mix (Qiagen) in the iCycler Sequence Detection System (Bio-Rad). The starting quantity (SQ) of the initial cDNA sample was calculated from primer-specific standard curves by using the iCycler Data Analysis Software. The expression level of each gene was normalized to actin expression level using standard curve method. The primer sets for real-time PCR can be found in the Supplemental Data.

#### Supplemental Data

Supplemental data include Experimental Procedures, References, and nine figures and can be found with this article online at <http://www.cell.com/cgi/content/full/126/6/1121/DC1/>.

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