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*J Immunol* 2011; 187:4483-4491; Prepublished online 21 September 2011; doi: 10.4049/jimmunol.1101757
http://www.jimmunol.org/content/187/9/4483
The NF-κB Transcription Factor c-Rel Is Required for Th17 Effector Cell Development in Experimental Autoimmune Encephalomyelitis

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Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated autoimmune disease involving effector Th subsets such as Th1 and Th17. In this study, we demonstrate that mice lacking the NF-κB transcription factor family member c-Rel (rel−/−), which are known to be resistant to EAE, show impaired Th17 development. Mixed bone marrow chimeras and EAE adoptive transfer experiments show that the deficiency of effector Th17 cells in rel−/− mice is T cell intrinsic. Consistent with this finding, c-Rel was activated in response to TCR signaling in the early stages of Th17 development and controlled the expression of Rorc, which encodes the Th17 transcription factor retinoic acid-related orphan receptor γt. CD28, but not IL-2, repression of Th17 development was dependent on c-Rel, implicating a dual role for c-Rel in modulating Th17 development. Adoptive transfer experiments also suggested that c-Rel control of regulatory T cell differentiation and homeostasis influences EAE development and severity by influencing the balance between Th17 and regulatory T cells. Collectively, our findings indicate that in addition to promoting Th1 differentiation, c-Rel regulates the development and severity of EAE via multiple mechanisms that impact on the generation of Th17 cells. The Journal of Immunology, 2011, 187: 4483–4491.

CD4+ T cells play an essential role in the adaptive immune responses that protect the host from a broad range of pathogenic microorganisms. When activated by specific pathogens, the cytokine environment generated by the innate immune system and APCs promotes naive CD4+ T cells to differentiate into particular subsets with distinct effector functions geared toward specific pathogen clearance. Based upon their cytokine profiles, two distinct groups of effector CD4+ cells, Th1 and Th2, were initially proposed (1). Recently, this paradigm has been updated following the discovery of new subsets of Th cells, such as Th17 cells, which produce the cytokine IL-17 and exhibit distinct effector functions (2, 3) required for the clearance of particular fungi and extracellular bacteria and are associated with autoimmune disease (4).

TGF-β and IL-6 are now considered to be responsible for the initiation of Th17 cell differentiation, whereas amplification is driven through the autocrine production of IL-21 and stabilization and maintenance achieved by IL-23 (5–7). Th17 development is thought to rely on STAT3 (8) and the lineage-specific transcription factor retinoic acid-related orphan receptor (ROR) γt (9).

Experimental autoimmune encephalomyelitis (EAE) is a CD4+ T cell-mediated demyelinating disease of the CNS that is frequently used as an animal model of human multiple sclerosis (10). Although it was initially thought that EAE was the result of dysregulated, autoantigen-specific Th1 responses, this has now been challenged by several observations. Firstly, IFN-γ knockout mice still develop enhanced EAE (11). Secondly, a deficiency in the IL-12–specific subunit p35 does not alter the progression of EAE, whereas a deficiency in either p19 (the IL-23–specific subunit) or p40 (the shared subunit of both IL-23 and IL-12) results in decreased Th17 levels and protection from EAE (12, 13). Thirdly, EAE induced by the transfer of myelin-reactive Th17 cells expanded in vitro with IL-23 is more severe than that arising from Th1 cells expanded in culture with IL-12 (6). Fourthly, IL-17–deficient mice develop attenuated EAE, a finding consistent with the profound proinflammatory effects and tissue damage caused by IL-17 during EAE (14). Lastly, increased levels of IL-17 have been observed in human multiple sclerosis patients (15).

Together, these data emphasize the important effector function of Th17 cells in EAE development.

Regulatory T cells (Treg) cells, another CD4+ T cell subset, are involved in limiting EAE pathology. Treg depletion results in a reduced threshold for the induction of EAE as well as increased disease severity (16, 17). The involvement of TGF-β in the development of both Treg and Th17 cells raises the likelihood that this cytokine can influence the severity of EAE by altering the balance between effector Th17 and Treg cells (5, 18).
The NF-kB family of transcription factors, important in immune-mediated pathology, comprise five family members: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel, all of which function as homo- or heterodimers, regulating genes important for inducible immune and inflammatory responses (19). Mice lacking c-Rel (rel−/−) mice have been found to be resistant to induction of EAE (20). c-Rel expression is primarily restricted to mature cells of the myeloid and lymphoid lineages and is critical for controlling B cell, T cell, macrophage, and dendritic cell function and regulating the expression of genes encoding various cytokines and transcription factors (21). c-Rel was recently found to play a role in the development of Treg cells (22) and T follicular helper (Thf) cells (23) in addition to its previously described in Th1 differentiation (20). The defects in Treg and Thf cell production in rel−/− mice are at least partially attributable to the direct regulation by c-Rel of Foxp3 (24) and I221 (23), respectively. Although the deficiency of the rel−/− mice to EAE was originally attributed to a defect in Th1 polarization and the decreased expression of IFN-γ (20), with the recognition of the importance Th17 cells in EAE pathogenesis coupled with Th17 cell development being regulated by IL-23, the possibility emerged that c-Rel may also play a role in the development of Th17 cells.

We show in this study that impaired Th17 development, in parallel with protection from EAE, occurs in rel−/− mice. This defect, which appears to be T cell intrinsic, coincides with c-Rel directly controlling the expression of Rorc, the gene encoding the OROry transcription factor. We also established that the Treg cell population remains markedly reduced in rel−/− mice during EAE and that an association may exist between the Th1/Treg balance and EAE clinical scores in rel−/− mice. Finally, we present evidence that c-Rel may play a role in the ability of CD28 to repress Th17 development. Together, these findings point to a novel and critical role for c-Rel in the promotion of Th17 development.

Materials and Methods

Animals, EAE induction, and clinical scoring

All animals were maintained in a specific pathogen-free facility. rel−/− mice were fully backcrossed 10 generations onto C57BL/6. Wild-type C57BL/6 mice (rel+/+) were purchased from the Australian Pharmacology Facility. The Australian National University. Eight- to 12-wk-old female mice were used in all experiments. Myelin oligodendrocyte glycoprotein (MOG) peptide (aa 35–55; MEVGWYRSPFSRCVHLYRNGK, MOG35–55) was synthesized by the Biomolecular Resource Facility, The Australian National University. The MOG35–55 peptide was dissolved in PBS at 2 mg/ml and emulsified in an equal volume of CFA consisting of IFA (Difco) and 100 g/ml and emulsified in an equal volume of CFA consisting of IFA (Difco) and B2, p65 (RelA), RelB, and c-Rel, all of which function

RNA preparation and quantitative PCR

Total RNA was extracted from CD4+ T cells isolated from rel−/− mice and rel−/− mice and reverse transcribed as described previously (26). SYBR Green real-time PCR was performed using an ABI PRISM 7700 sequence detection system (PerkinElmer/PE Biosystems) as described previously (26). To normalize for inefficiencies in cDNA synthesis and RNA input, PCRs for the control gene ubiquitin-conjugating enzyme E2D2 (Ub2d2) were conducted in parallel. The primer pairs used were Il17a forward: 5′-TTT AAC TCT CTT GGC CAA AAA-3′, Il17a reverse: 5′-CTP TCC CTC CTC CGC ATT GAC AC T-3′, Rorc forward: 5′-GAC CCA CAC CTC ACA AAT TGA-3′, Rorc reverse: 5′-AGT AGG CCA CAT TAC ACT GCT-3′, Rel forward: 5′-TTA CCA GAA ATG CCC AGG ATC T-3′, Rel reverse: 5′-AGG CCC TTC TAG GAA TGG AA-3′, Ub2d2 forward: 5′-AAG AGA ATC CAC AAG GAA TGT AAT G-3′, and Ub2d2 reverse: 5′-CAA CAG GAC CTG CTG AAC ACT G-3′.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis of c-Rel binding was performed as previously described (23) with some minor modifications. Briefly, CD4+ splenocytes from rel−/− or rel−/− mice were stimulated with the given stimuli, fixed with 1% formaldehyde for 15 min, and sonicated so that the chromatin was fragmented to 200–1000 bp. c-Rel immunoprecipitation was performed on precleared cell lysates for 1 h at 4°C with 3 μg anti–c-Rel Ab (sc-71; Santa Cruz Biotechnology) or no Ab. Quantitative PCR was performed on purified DNA from the anti–c-Rel or no Ab (control) immunoprecipitations using primers to detect binding site (BS) 1 (forward: 5′-CCC ACC ACA CCA GAA GTA TG-3′, reverse: 5′-CTT GCT GTG TTT CTC CT-3′) and BS2 (forward: 5′-GGG TGG GCC AGT CAC GAG-3′ and BS2 reverse: forward: 5′-CCG CTT CCC TGC TCT TCT G-3′) of the Rorc promoter.

Flow cytometry

Abs against the following markers were used in flow cytometric analysis: CD4, CD45.1, CD45.2, CD44, CD69, CD25, Foxp3, IL-17a, IFN-γ with conjugated fluorochromes (eBioscience), and 7-aminoactinomycin D (A1310; Molecular Probes). For flow cytometric analysis of cytokines, CD4+ cells from rel−/− or rel−/− mice were stimulated with PMA and ionomycin (P/I) plus GolgiStop (Biosciences) for 4–6 h, followed by cell-surface marker staining, then were fixed and permeated before staining with the anti-cytokine Abs. For the absolute cell number counting, cells were stained with 7-aminoactinomycin D to exclude the dead cells and mixed with calibrate beads (BD Biosciences) immediately just before flow cytometric analysis (27). Data were acquired using an FACSCalibur or LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star). The absolute cell number was calculated using a ratio based on the number of beads: the cell number = beads added/beads counted × cell counted (27). The proliferation profiles were analyzed using FlowJo software (Tree Star).

Computational promoter analysis

The DNA sequence 1000 bp upstream and 500 bp downstream of the transcription start site of the Rorc gene was analyzed using Rvista tools
Luciferase reporter assays

The mouse Rorc luciferase reporter, rorc/pXPG, was constructed by inserting the −914 to +19 bp promoter region of the mouse Rorc gene into the pXPG luciferase reporter (29) using a BglII/Sall PCR product amplified from the genomic DNA of a healthy C57BL/6 female mouse. For luciferase assays, EL-4 T cells were transfected in duplicate with 10 μg empty pXPG plasmid or rorc/pXPG reporter plasmids. For overexpression assay, 10 μg human c-Rel expressed plasmid and its mother empty CMV plasmid were cotransfected with the pXPG or rorc/pXPG. Transfected cells were recovered overnight, left unstimulated, or stimulated with PMA and ionomycin for 7.5 h, and cell lysates were harvested and analyzed as described previously (26). A total of 30 μg total protein was analyzed for luciferase activity using a Turner Biosystems microplate luminometer (Turner BioSystems).

Statistical analysis

The CD4+ cells proliferation profiles were analyzed using the CFSE time-series method (27). The cell numbers in each division were measured using FlowJo software (Tree Star) using the level of CFSE fluorescence. The precursor cohort numbers were generated by dividing the cell numbers in the ith division by 2^{i-0.5}, where i is the division number and used for fitting the normal distributions for cells in division. For all experiments, statistical significance was assessed using Student t test, with statistical significance cutoffs of *p < 0.05, **p < 0.01, ***p < 0.001.

Results

Resistance of rel−/− mice to EAE is associated with impaired Th17 development

Initially, we confirmed the previous finding that rel−/− mice are indeed resistant to EAE induced by MOG (Fig. 1A) (20). Although the proportion of splenic CD4+ T cells in the rel−/− EAE mice was only marginally reduced when compared with wild-type EAE mice (Supplemental Fig. 1A, 1C), the proportion of large activated rel−/− CD4+ cells was more significantly reduced (Supplemental Fig. 1B, 1D). Staining for the early and late T cell activation markers, CD69 and CD44, respectively, showed that levels of CD44 but not CD69 were decreased significantly in the rel−/− mice (Supplemental Fig. 1E, 1G), suggesting that the defect occurred later in T cell activation.

When total splenocytes were taken from the MOG-immunized animals and cultured in vitro for 4 d with IL-23 and MOG35–55, the development of IL-17+IFN-γ+ cells (Th17), IL-17+IL-17+ (Th1), and IL-17+IFN-γ+ cells was reduced in the rel−/− cultures (Fig. 1B, 1C). The expression of Il17a and Rorc mRNAs was also decreased in freshly isolated CD4+ splenic T cells from rel−/−-immunized mice when compared with those of wild-type controls (Fig. 1D, 1E). These results demonstrate that EAE-resistant rel−/− mice display impaired Th17 and Th1 development, which coincides with a T cell-activation defect.

Effect of c-Rel–dependent cytokines on the impaired rel−/− Th17 development

When wild-type and rel−/− CD4+ splenic T cells were cultured in vitro with a combination of anti-CD3 Abs, TGF-β, and IL-6, the proportion of rel−/− Th17 cells was significantly lower (Fig. 2A, 2B) and proliferation-independent (Supplemental Fig. 2). Given that the expression of IL-2 and IL-23 are c-Rel dependent (30, 31), we explored the possibility that decreased levels of these cytokines could be at least in part responsible for the Th17 defect by the addition of these cytokines to the Th17-polarizing cultures of rel−/− CD4+ T cells. The results showed that in the presence of anti-CD3, IL-6, and TGF-β, addition of IL-2 and/or IL-23 did not increase the proportion or absolute number of rel−/− Th17 cells when compared with wild-type counterparts (Fig. 2C, 2D). Instead the presence of IL-2 reduced the overall percentage and number of Th17 cells, whereas the addition of anti–IL-2–neutralizing Abs increased the percentage of Th17 cells (Fig. 2E) in both populations. In contrast, CD28 costimulation, which was recently shown to inhibit Th17 development (32) and also regulates IL-2 expression via c-Rel (33), repressed Th17 development in cultures of wild-type but not rel−/− CD4+ T cells (Fig. 2C, 2D), thereby establishing that CD28 but not IL-2 inhibition of Th17 differentiation is c-Rel dependent.
Interestingly, the addition of IL-2 had little impact on Th17 development in the presence of agonistic anti-CD28 Abs (Fig. 2C, 2D), indicating that the c-Rel–dependent inhibition of Th17 development by CD28 may involve the regulation of IL-2 expression. Given that IL-21 is a Th17-amplifying factor (7) that is also c-Rel regulated (23), wild-type and rel−/− CD4+ T cells were costimulated with anti-CD3 Abs and TGF-β, and IL-6 as the standard Th17 conditions with the addition of IL-2 or IL-23 in the presence or absence of an activating anti-CD28 Ab. The results showed that although IL-6–dependent Th17 development was deficient in rel−/− cells, c-Rel had no impact on IL-21 signaling (either in the presence or absence of IL-6). However, the percentage of Th17 cells generated under the IL-21–only condition was considerably lower than that induced by IL-6 (Fig. 2F).

Collectively, these results show that the rel−/− Th17 defect can be recapitulated in culture, but cannot be rescued by the addition of exogenous IL-2 or IL-23, and the defect is not observed in the presence of IL-21. Furthermore, addition of IL-2 or CD28 activation leads to a decrease in wild-type Th17 cell development, with IL-2 but not CD28 activation having a similar effect on both wild-type and rel−/− cells.

**Impaired Th17 development in rel−/− mice is due to a T cell–intrinsic defect**

Because c-Rel influences the development and activation of diverse immune cells as well as nonimmune cell types (19), bone marrow transfer chimeras were generated in which donor bone marrow from wild-type or rel−/− mice was transferred to lethally irradiated wild-type or rel−/− recipients, which were then immunized with MOG 4 wk later. The results showed that wild-type and rel−/− recipients of rel−/− donor bone marrow cells were resistant to EAE as judged by clinical scoring at day 14 postimmunization, whereas wild-type donor cells led to the development of EAE in both wild-type and rel−/− animals (Fig. 3A).

FIGURE 2. The defect in Th17 development in rel−/− mice can be replicated in in vitro cultures. CD4+ splenocytes from rel+/+ and rel−/− mice were stimulated with anti-CD3, TGF-β, IL-6, with and without anti-CD28, and/or various cytokines for 3.5 d. The cells were analyzed for Th17 development by intracellular Ab staining and flow cytometry. A and B, The development of Th17 cells in nonstimulated cells (NS) and cells stimulated under Th17-inducing conditions with anti-CD3, TGF-β, and IL-6 (Th17) was monitored. A representative dot plot of the CD4+ gated cells is shown (A) as are the means and SEM of three independent experiments (B). C and D, An in vitro rescue assay was performed for Th17 development in rel+/+ and rel−/− mice using anti-CD3, TGF-β, and IL-6 as the standard Th17 conditions with the addition of IL-2 or IL-23 in the presence or absence of an activating anti-CD28 Ab. The means of the percentages of Th17 (IL-17a+IFN-γ) in CD4+ cells (C) and the absolute Th17 cell numbers (D) are shown. E, The Th17 in vitro development assay was performed in the presence of anti-CD3, TGF-β and IL-6 with the addition of a neutralizing IL-2 Ab and plus or minus anti-CD28. F, Th17 development in vitro in the presence of anti-CD3 and TGF-β with the addition of IL6 IL-21, or both cytokines. The CD4+ splenocytes from rel+/+ or rel−/− mice stimulated as described were analyzed for Th17 cells using flow cytometry. The mean and SEM percentages of Th17 cells (IL-17a+IFN-γ) are shown (E, F). All experiments were repeated independently at least three times. *p < 0.05, **p < 0.01, ***p < 0.001.
These results indicate that the Th17 defect in rel−/− mouse was associated with the hematopoietic compartment and was T cell intrinsic. CD45.1 congenic C57BL/6 mice (rel−/−) or CD45.2 rel−/− mice were irradiated and i.v. injected with CD45.2 congenic rel−/+ or rel−/−, or a 1:1 mixture of CD45.1 rel−/+ and CD45.2 rel−/− bone marrow cells and immunized with MOG33–55 on week 4 after the reconstruction of the immune system. A. The clinical scores were monitored at day 14 after immunization and are plotted as means with SEM. B and C, Total splenocytes were isolated from rel−/− or rel−/+ mice receiving rel−/+ or rel−/− bone marrow for flow cytometry analysis of Th17 cells at day 14 after immunization. The Th17 cells were gated as IL-17+IFN− or CD45.2+ (from the donor) Th17 cells (Fig. 3B, 3C), and whereas rel−/− recipients of either wild-type or rel−/+ bone marrow showed a higher percentage of Th17 cells than their wild-type recipient counterparts, Th17 numbers were still lower in the rel−/− mice that received rel−/− as opposed to wild-type bone marrow (Fig. 3B, 3C). Collectively, these results indicate that the Th17 defect in rel−/− mice is hematopoietic in nature.

To determine if the rel−/− Th17 defect was cell intrinsic, lethally irradiated wild-type mice were injected with a 1:1 mix of wild-type and rel−/− bone marrow cells and then injected 4 wk later with MOG. Wild-type recipients of wild-type cells (1:1 mix of CD45.1 and CD45.2 cells) showed normal development of EAE, whereas animals that received equal numbers of wild-type (CD45.1) and rel−/− (CD45.2) bone marrow cells developed only mild EAE symptoms (Fig. 3A). In these MOG-immunized mixed chimeras that received a 1:1 ratio of wild-type and rel−/− donor bone marrow cells, the percentage of CD45.2+ (from the rel−/− donor) Th17 cells were reduced compared with CD45.1+ (from the wild-type donor) Th17 cells (Fig. 3D, 3E). These experiments establish that the rel−/− Th17 defect is cell intrinsic and could not be rescued by factors provided by wild-type hematopoietic cells.

**FIGURE 3.** The Th17 defect in rel−/− mouse was associated with the hematopoietic compartment and was T cell intrinsic. CD45.1 congenic C57BL/6 mice (rel−/−) or CD45.2 rel−/− mice were irradiated and i.v. injected with CD45.2 congenic rel−/+ or rel−/−, or a 1:1 mixture of CD45.1 rel−/+ and CD45.2 rel−/− bone marrow cells and immunized with MOG33–55 on week 4 after the reconstruction of the immune system. A. The clinical scores were monitored at day 14 after immunization and are plotted as means with SEM. B and C, Total splenocytes were isolated from rel−/− or rel−/+ mice receiving rel−/+ or rel−/− bone marrow for flow cytometry analysis of Th17 cells at day 14 after immunization. The Th17 cells were gated as IL-17+IFN− or CD45.2+ (from the donor) Th17 cells (Fig. 3B, 3C), and whereas rel−/− recipients of either wild-type or rel−/+ bone marrow showed a higher percentage of Th17 cells than their wild-type recipient counterparts, Th17 numbers were still lower in the rel−/− mice that received rel−/− as opposed to wild-type bone marrow (Fig. 3B, 3C). Collectively, these results indicate that the Th17 defect in rel−/− mice is hematopoietic in nature.

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c-Rel contributes to the development of both the Th17 and Treg cell populations in EAE

To further investigate the T cell-intrinsic nature of the Th17 developmental defect observed in rel−/− mice, adoptive transfer of splenic T cells from MOG-injected mice, cultured with IL-23 and MOG33–55 was performed to test their ability to induce EAE when injected into naive mice. Cells from MOG-immunized rel−/+ mice could induce EAE in both wild-type and rel−/− mice, whereas rel−/− cells were unable to induce EAE in wild-type recipients (Fig. 4A). These findings are consistent with those obtained in the bone marrow chimera experiments and further reinforce the T cell-intrinsic nature of the defect. Interestingly, the EAE that developed in rel−/− animals following the transfer of wild-type cells appeared to be more severe and had a relapsing-remitting disease pattern, with a second wave of disease observed after 30 d (Fig. 4A). To trace donor Th17 T cell infiltration of the CNS, mononuclear cells were isolated from the PBS-perfused brain and spinal cord of the passively induced EAE recipients at day 35 following adoptive transfer and analyzed for donor Th17 (CD45.1+ and CD45.1+IL-17+IFN−) infiltration. Th17 cells infiltrated the CNS...
of wild-type and rel\textsuperscript{−/−} mice injected with rel\textsuperscript{+/+} donor cells to the same extent, whereas there was only a very low level of Th17 T cell infiltration of the CNS in the rel\textsuperscript{−/−} mice injected with rel\textsuperscript{−/−} cells (Fig. 4B, 4C). Importantly, the overall pattern of Th17 T cell infiltration of the CNS reflected the general pattern of disease seen in these animals (Fig. 4A, 4B).

Despite the rel\textsuperscript{−/−} Th17 defect being hemopoietic specific, the observation that the number of Th17 T cells in EAE that were generated from wild-type bone marrow was higher in rel\textsuperscript{−/−} as opposed to wild-type recipients indicated that the rel\textsuperscript{−/−} hemopoietic environment could also exacerbate this disease. Because the balance between Th17 and Treg cells has been shown to play a role in the development of EAE (16, 17) and with the thymic development and peripheral homoeostasis of Treg cells impaired in rel\textsuperscript{−/−} animals (22), we investigated the Treg cell population in these animals following the adoptive transfer of EAE. Recipient splenic Treg (CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+}) numbers measured 35 d after adoptive transfer, and the brain and spinal cord cells were gated on CD4\textsuperscript{+} and the donor marker (CD45.1 or CD45.2), whereas the splenocytes were gated on CD4\textsuperscript{+} and the recipient marker (CD45.1 or CD45.2). The Th17 (IL-17a\textsuperscript{+}IFN-\gamma\textsuperscript{+}) and Treg (CD25\textsuperscript{+}Foxp3\textsuperscript{+}) percentages are shown in a representative dot plot (B) and with the means with SEM (C). D, The ratio of donor Th17 to recipient Treg cells was calculated to elucidate the profiles of passive EAE recipients. One of three independent experiments (n = 3–5) is presented.

\textbf{Discussion}

Although c-Rel has long been shown to have a role in Th1 development (20), more recently, it was also shown to be necessary for the appropriate development of Treg cells (22) and for Th1 cell differentiation (23). In this report, we show that c-Rel is also involved in the differentiation of IL-17\textsuperscript{+}IFN-\gamma\textsuperscript{−} Th1 T cells and also the recently identified IL-17\textsuperscript{+}IFN-\gamma\textsuperscript{−} T cells, which were named as Th17.1 T cells in the human system (34). These results

\textbf{C-Rel directly binds to and controls the rorc gene promoter}

We next examined the possibility that c-Rel regulated the expression of key molecules involved in Th17 signaling. In wild-type cells stimulated under Th17-polarization conditions, Rel expression increased very early (30 min) after the activation, whereas induced Rorc and Il17a expression occurred after 4 h of stimulation (Fig. 5A). The levels of both Rorc and Il17a were lower in rel\textsuperscript{−/−} than wild-type T cells after 24 h of stimulation (Fig. 5A).

Two potential c-Rel BS were identified 579–590 (BS1) and 894–905 (BS2) bp upstream of the transcription start site of rorc, but not Il17a, Il17f, Il22, or stat3 (Fig. 5B, Supplemental Fig. 3), with BS2 located in a highly conserved region. Recently generated c-Rel ChIP-on-chip data (S. Lee, unpublished observations) had also identified this region as a c-Rel binding region (Supplemental Fig. 3). Within 6 h of initiating Th17 differentiation, both sites bound c-Rel in vivo as measured by ChIP assay, with the conserved BS2 showing considerably stronger binding (Fig. 5C). In contrast, no c-Rel binding to these elements was observed during the later stages of Th17 development or in response to P/I co-stimulation (Fig. 5C). Using a luciferase reporter assay, we also demonstrated that whereas the Rorc promoter (950 bp) could enhance the reporter gene expression in both unstimulated and P/I-stimulated cells, c-Rel coexpression significantly increased the promoter function in stimulated cells (Fig. 5D). Together, these data indicate that Rorc is a direct target of c-Rel and that it is through the regulation of Rorc that c-Rel may act to promote Th17 development.
establish an additional role for this transcription factor in the development of distinct T cell subsets. The development of Th17 cells in \( \text{rel}^{-/-} \) mice was markedly reduced in the autoimmune model of MOG-induced EAE as well as in cell-culture models of Th17 differentiation that depend on IL-6 and TGF-\( \beta \), cytokines that are required for Th17 development in vivo. Several lines of evidence suggest that the impaired Th17 development in \( \text{rel}^{-/-} \) mice is due to a hemopoietic defect that is T cell intrinsic. In the bone marrow chimeras, wild-type animals receiving \( \text{rel}^{-/-} \) donor marrow did not develop clinical symptoms of EAE as well as in cell-culture models of Th17 differentiation that depend on IL-6 and TGF-\( \beta \), cytokines that are required for Th17 development in vivo. Several lines of evidence suggest that the impaired Th17 development in \( \text{rel}^{-/-} \) mice is due to a hemopoietic defect that is T cell intrinsic. In the bone marrow chimeras, wild-type animals receiving \( \text{rel}^{-/-} \) donor marrow did not develop clinical symptoms of EAE following MOG immunization, which coincided with the impaired development of the Th17 cell population. This indicates that the wild-type stromal environment could not support the development of Th17 cells. Importantly, in mixed bone marrow chimeras, the Th17 response contributed by \( \text{rel}^{-/-} \) donor cells was still impaired, indicating that the other hemopoietic components that develop from the wild-type bone marrow could not rescue the \( \text{rel}^{-/-} \) Th17 deficiency. Consistent with the findings from the bone marrow chimeras, in the adoptive transfer model of EAE, the T cell-intrinsic nature of the \( \text{rel}^{-/-} \) Th17 defect was confirmed by the failure of EAE to fully develop in wild-type animals injected with \( \text{rel}^{-/-} \) donor CD4+ T cells. These in vivo findings were supported by Th17-differentiation studies in culture. In response to CD3 activation, TGF-\( \beta \) and IL-6, a combination of stimuli that normally promotes efficient Th17 polarization in culture, the generation of \( \text{rel}^{-/-} \) Th17 cells was markedly reduced. This defect could not be rescued by providing IL-23, a maturation and stabilizing factor for Th17 development (30), or IL-2, a c-Rel–regulated T cell proliferation factor (33). IL-21 is a cytokine produced by Th17 cells that is thought to be part of an autocrine positive-feedback loop that amplifies the precursor frequency of Th17 cells. Although IL-21 expression is controlled in part by c-Rel (23), a defect in the expression of this cytokine is unlikely to contribute to the \( \text{rel}^{-/-} \) Th17 phenotype, given \( \text{Il21}^{-/-} \)
and II21r−/− mice display normal Th17 development in culture and remain highly susceptible to EAE (35, 36).

The T cell-intrinsic nature of the Th17 defect in rel−/− animals suggested that c-Rel might be involved in controlling cell-signaling pathways known to be essential for Th17 cell development. Transcription factors reported to regulate Th17 development directly or indirectly include RORγt, RORα, STAT3, IFN regulatory factor 4, B cell-activating transcription factor, and IκB (9, 37–40). RORγt, which is induced by TGF-β and IL-6, and STAT3, induced by IL-21 or IL-23, are considered as master regulators of gene expression and essential for Th17 development. Both transcription factors bind directly to the proximal promoter of the IL17 gene (9, 40). We provide evidence that c-Rel directly regulates the expression of the rorc gene. ChIP-on-chip studies had identified a rel-binding region upstream of the rorc gene that contains two putative c-Rel binding motifs. We show using ChIP that c-Rel binds strongly to at least one of these putative sites under Th17-polarizing conditions but not in response to normal T cell activation. These results were supported by luciferase assays showing that overexpression of c-Rel enhances rorc promoter activity. The rorc gene produces several transcripts with distinct transcription start sites, all of which encode very similar RORγ proteins, one of which is known as RORγt. Given the putative transcription start site for the transcript encoding RORγt has been mapped 5.5 kb downstream of the Rel-binding sites we identified in the promoter (41), it remains to be determined how the expression of the various rorc transcripts are regulated.

Our in vitro Th17 culture experiments demonstrated that addition of IL-2 or activation of the CD28 receptor led to a decrease in the generation of Th17 cells. We also demonstrated that although the neutralization of IL-2 increased the generation of Th17 generation, it did not correct the defect in Th17 development observed in rel−/− cells. In agreement with the findings presented in this study, IL-2 has been demonstrated to antagonize the generation of Th17 cells via activation of STAT5 (42). Our finding that Th17 development was negatively regulated by CD28 costimulation also supports a recent report by others (32). The use of CD28 costimulation in the culture may explain a recent study claiming that c-Rel did not regulate Th17 cell development (43). Interestingly, we found that CD28 activation did not appear to inhibit the generation of rel−/− Th17 cells in culture. A possible explanation for these effects is that the inhibitory impact of CD28 on Th17 development in wild-type cultures is c-Rel dependent, possibly through its contribution to the production of IL-2, which is known to be c-Rel dependent (31). Consistent with such a model is the finding that the addition of IL-2 inhibits Th17 production in both wild-type and rel−/− cultures. Depending on the T cell activation signals encountered by a naive CD4+ T cell, c-Rel may play a dual role in both promoting and antagonizing the development of Th17 cells.

Despite rel−/− mice having reduced numbers of peripheral Treg cells due to defects in natural Treg development and homeostasis (22), rel−/− animals remain resistant to development of EAE (20). Given that residual rel−/− Treg cells appear to function normally, coupled with the fact that c-Rel is dispensable for the TGF-β-dependent generation of induced Treg cells (22), this may indicate that there are sufficient Treg cells in rel−/− mice to help suppress the development of EAE, particularly in light of the reduced numbers of rel−/− Th17 and Th1 cells. This explanation is consistent with our findings in the adoptive transfer model of EAE, in which rel−/− mice receiving wild-type Th17 cells show both increased disease severity and a higher Th17/Treg ratio compared with wild-type recipients of these cells. This finding is in agreement with other reports showing that an alteration in the Th17/Treg balance leads to either an increase in the severity of disease (high Th17 numbers) or resistance to the disease (higher Treg numbers) (16, 17). Interestingly, a similar phenotype was observed in the bone marrow chimera experiment, with a high percentage of Th17 cells observed in the rel−/− recipients compared with rel+/+ recipients (Fig. 3C), indicating that, besides Treg cells, some other repressive function might act in the balance of inflammation and regulation.

The reported resistance of rel−/− mice to EAE has previously been attributed to a reduced Th1 response, coinciding with a decrease in IFN-γ expression (20). Our demonstration that c-Rel regulates Th17 responses in autoimmune EAE suggests that EAE resistance may be associated with a decrease in the Th17 population as well as the Th1 population. An intriguing phenotype of the rel−/− mice is that allogeneic heart or pancreatic islet transplants survive for significantly longer periods in rel−/− mice than in wild-type recipients (44, 45). As with EAE, this was attributed to a defect in the rel−/− Th1 response (44, 45), but recent studies have shown that Th17 cells play more important roles in organ rejection responses than Th1 cells (46). Therefore, both resistance to autoimmune disease and the inability to reject allogeneic transplants could be linked to a Th17 as well as a Th1 defect in the rel−/− mice.

Acknowledgments
We thank Dr. David Lineras (Canberra Hospital) for advice on EAE, Debbie Howard for irradiation work, and Dianna McWilliam and Holly Burke for mouse care.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1.
Deficiency in CD4+ T cell activation in rel-/- mice during EAE development.

rel+/+ or rel-/- mice were immunized with MOG_{35-55} for two weeks and the splenocytes were isolated and analysed using flow cytometry. A-D, The proportion of CD4+ T cells and their size distribution. A is a representative dot plot of staining with anti-CD4 antibody and the forward scatter (FSC), a measurement of cell size for both wild-type and rel-/- splenocytes and the percentage of CD4+ cells is shown for this experiment. B, is a representative histogram of the distribution of CD4+ cell size (as measured by FSC) in rel+/+ (dark line, top value) and rel-/- (light line, bottom value) mice. C, shows the mean percentage +/- SEM of CD4+ cells in the total splenocytes from the immunized rel+/+ or rel-/- mice. D presents the proportion of large (activated) CD4+ T cells, as gated with CD4/FSC<sup>c</sup> from the immunized rel+/+ or rel-/- mice. E-G, Detection of T cell activation markers, CD69 (early activation marker E-F) and CD44 (late activation marker, E and G) in the CD4+ splenocyte population with representative dot plots (E) and summaries (F-G). n = 6-8, Error bars show SEM.
A-D, CD4+ splenocytes from rel+/+ or rel−/− mice were labeled with CFSE, and not stimulated (NS) or stimulated with anti-CD3, TGF-β and IL-6 (Th17 conditions) for three and a half days and the level of CFSE and IL-17 was measured using flow cytometry. The total CFSE distribution (A) for rel+/+ (dark lines) or rel−/− (grey lines) CD4+7AAD- cells was broken up into the component populations based on the number of predicted divisions using the proliferation tool in FlowJo to calculate the cells in each division for cells nonstimulated, polarized under Th17 conditions. The normal distributions are shown as dotted lines (B, rel+/+ closed circles, rel−/− open circles). The percentages of CFSElow (proliferated) cells is shown in representative dot plots (C) and the distributions of IL-17 for the CFSE low population is shown for rel+/+ (dark lines/open) and rel−/− (dashed lines/grey) cells in D. The percentage of cells with IL-17 higher than the value marked is given. One representative experiment of three is shown.

Supplementary Figure 2.
The Th17 deficiency in rel−/− mice was not associated with defective proliferation.
Supplementary Figure 3
c-Rel does not act on the promoters of *Il17a, Il17f, Il22* and *Stat3* directly

Schematic of the promoter of *Il17a* (A), *Il17f* (B), *Il22* (C) and *Stat3* (D) (+/-1.5-2 kb relative to transcription start site) in the UCSC browser (mm8), showing the location of chromatin, the c-Rel and NF-kB binding sites from computational predictions, the c-Rel binding region from ChIP-chip data and the conservation across species.