T Cell-Intrinsic Expression of c-Rel Regulates Th1 Cell Responses Essential for Resistance to *Toxoplasma gondii*[1]

Nicola J. Mason,* Hsiou-Chi Liou,† and Christopher A. Hunter[2]*

The ability of many microbial and inflammatory stimuli to activate members of the Rel/NF-κB family of transcription factors is associated with the regulation of innate and adaptive responses required to control infection. Individual family members play distinct roles during different infectious and inflammatory responses. For example, c-Rel is essential for the production of IL-12 in response to LPS, but dispensable for IL-12 production in response to *Toxoplasma* Ag. To assess the role of c-Rel during immunity to the intracellular pathogen *Toxoplasma gondii*, wild-type (WT) and c-Rel$^{-/-}$ mice were infected with *Toxoplasma* and the immune response was analyzed. c-Rel$^{-/-}$ mice developed severe toxoplasmic encephalitis with increased numbers of parasites compared with WT controls and succumbed to infection within 5–8 wk. Although increased susceptibility of c-Rel$^{-/-}$ mice was associated with decreased T cell activation, proliferation, and production of IFN-γ, these mice were able to generate Th1 effector cells that were present in the brain during chronic infection. In vitro mixing studies using WT and c-Rel$^{-/-}$ dendritic cells and WT and c-Rel$^{-/-}$ TCR transgenic T cells indicated that c-Rel$^{-/-}$ dendritic cells are defective in their ability to stimulate T cell responses. However, when c-Rel$^{-/-}$ T cells were transferred into T cell-deficient hosts, early defects in T cell activation, proliferation, and IFN-γ production persisted, and these mice remained susceptible to infection. Together, these studies indicate that although c-Rel is an important regulator of innate immune responses, it also plays an important role in optimization and maintenance of adaptive T cell responses during infection. *The Journal of Immunology,* **2004, 172: 3704–3711.**

Many infectious and inflammatory stimuli lead to the activation of the Rel/NF-κB family of transcription factors that play an important role in the development of innate and adaptive immune responses (1). For example, direct recognition of microbial products by Toll-like receptors on macrophages and dendritic cells (DCs)[3] results in the activation of NF-κB, which regulates the expression of many cytokines, chemokines, and adhesion molecules necessary for a coordinated immune response (1–4). Furthermore, many stimuli such as CD28, CD40, TNF-α, and TCR engagement lead to NF-κB translocation and initiate T cell activation, proliferation and effector function (5–8). The importance of this pathway in resistance to infection was demonstrated by studies in which NF-κB-deficient mice exhibited enhanced susceptibility to various viral, bacterial, and parasitic infections (9–11). Although specific mutations in NF-κB have not been reported in human patients, a variety of mutations in the Iκβ kinase γ gene leading to decreased NF-κB activation has been reported and is responsible for the rare syndromes of ectodermal dysplasia and incontinentia pigmenti (12). These patients display defects in Ab class switching and cell-mediated immunity associated with an increased susceptibility to bacterial infections and intracellular infections, most likely as a consequence of reduced activation of multiple NF-κB family members.

As part of the NF-κB family, c-Rel is expressed predominantly in immune cells and has a prominent role in the regulation of accessory cell and lymphocyte functions. Early studies demonstrated that in the absence of c-Rel, lymphocyte proliferation in response to polyclonal stimuli was deficient and impaired T cell responses were associated with reduced production of the autocrine growth factor IL-2 (13, 14). Subsequent studies identified a role for c-Rel in the production of IL-12p40 by macrophages (15, 16) and IL-12p35 and p19 by DCs (17), indicating a role for c-Rel in the regulation of innate immune function. However, the role of c-Rel in the regulation of Th1 responses is unclear. Recent studies have suggested that c-Rel is intrinsically required by T cells for the production of IFN-γ, and by accessory cells to direct the development of a Th1-type response (18).

Despite the studies indicating that c-Rel regulates individual aspects of innate and adaptive immune responses, relatively little is known about the role of c-Rel during a coordinated immune response to infection. Previous studies have shown that c-Rel$^{-/-}$ mice have an increased susceptibility to *Leishmania major* and have a delayed clearance of influenza virus, but the basis for these defects remains unknown (9, 10). Furthermore, although c-Rel$^{-/-}$ mice infected with *Trichuris muris* are able to successfully clear the parasite, defects in the generation of Th2 responses and reduced IL-4 and IL-13 production have been identified (11). Despite an important role for c-Rel in the regulation of IL-12 production, studies from this laboratory with the intracellular pathogen *Toxoplasma gondii* revealed the presence of a c-Rel-independent pathway of IL-12 production by macrophages and DCs, which was required for resistance to the acute phase of this infection (15). The studies reported in this work demonstrate that c-Rel$^{-/-}$ mice develop severe toxoplasmic encephalitis associated with decreased T cell activation and proliferation. This defect is

*Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104; and †Cornell University Medical College, Department of Medicine, Division of Immunology, New York, NY 10021

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[2] Address correspondence and reprint requests to Dr. Christopher A. Hunter, Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104. E-mail address: chunter@phl.vet.upenn.edu

[3] Abbreviations used in this paper: DC, dendritic cell; BMDC, bone marrow-derived DC; BMNC, brain mononuclear cell; BrdU, 5-bromo-2′-deoxyuridine; EAE, experimental allergic encephalomyelitis; huIL-2, human IL-2; MLN, mesenteric lymph node; RAG, recombination-activating gene; RF, responder frequency; STAg, soluble *Toxoplasma* Ag; Tg, transgenic; WT, wild type.

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manifest by reduced levels of IFN-γ early after infection and reduced numbers of T cells in the brains of c-Rel$^{-/-}$ mice. However, in vitro and in vivo studies demonstrate that functional Th1 effector cells can be generated in the absence of c-Rel. Nevertheless, T cell-intrinsic expression of c-Rel is required for the optimal activation and expansion of these Th1 effector cells that are required for long-term resistance to *T. gondii*.

Materials and Methods

Animals and infection

Female C57BL/6 mice (age 4–8 wk) and C57BL/6 recombination-activating gene$^{-/-}$ (RAG1$^{-/-}$) mice (age 4–6 wk) were obtained from The Jackson Laboratory (Bar Harbor, ME). Female C57BL/6 nude mice (age 4–6 wk) were obtained from Taconic Farms (Germantown, NY). C57BL/6 c-Rel-deficient (c-Rel$^{-/-}$) mice (13) and OVA peptide 323–339 I-A^b-specific OTII TCR transgenic (Tg) mice on a C57BL/6 background (19) were bred within the University Laboratory Animal Resources facility of the University of Pennsylvania. OTII Tg mice were crossed with c-Rel$^{-/-}$ mice to generate mice with c-Rel$^{-/-}$ TCR Tg CD4$^+$ T cells specific for OVA peptide 323–339 (permission from W. Heath, Walter and Eliza Hall Institute, Melbourne, Australia). Age- and sex-matched mice were inoculated orally with 20 cysts of the ME49 strain of *T. gondii*, which were prepared from the brains of chronically infected CBA/CaJ mice, as previously
described (20, 21).

Histological analysis and assessment of parasite burden

Mice were sacrificed at different time points, and lungs, spleens, and brains were harvested and fixed overnight in Accustain 10% neutral buffered formalin solution (Sigma-Aldrich, St. Louis, MO) and embedded in paraffin. Sections were stained with H&E before microscopic evaluation. To determine cyst number, brains were homogenized in 3 ml of PBS by repeat passage through a 21-gauge needle. For each brain, 30 μl of homogenate was evaluated microscopically, and the number of cysts was counted and used to estimate the total number of cysts per brain.

Cell culture and analysis of T cell responses

Spleens and mesenteric lymph nodes (MLN) from uninfected or infected mice were harvested and dissociated into single cell suspensions in complete RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 10 U/ml penicillin, 100 μg/ml streptomycin (BioWhittaker, Walkersville, MD), 2 mM glutamine, 25 mM HEPES, 0.1 mM nonessential amino acids, and 50 μM 2-ME. Erythrocytes were depleted using 0.86% w/v ammonium chloride (Sigma-Aldrich). In some experiments, CD3$^+$ T cells were purified using T cell enrichment columns (R&D Systems, Minneapolis, MN), following the manufacturer's instructions. Purities obtained using this technique were routinely >85%. Cells were washed, suspended in PBS, and labeled with CFSE (Molecular Probes, Eugene, OR) (1.25 μM) for 5 min at 37°C. Labeling was quenched with FCS, and cells were washed twice and resuspended in complete RPMI 1640. Cells were plated at a concentration of 4×10^5 cells/well in a final volume of 200 μl in 96-well U-bottom plates (Costar, New York, NY) or 96-well flat-bottom plates (Costar) in DC mixing experiments. Cells were cultured in medium alone or stimulated with soluble anti-CD3 (1 μg/ml) (BD PharMingen, San Diego, CA) or soluble *Toxoplasma* Ag (STAg) (25 μg/ml) either with or without exogenous IL-2 (Genzyme, Cambridge, MA) (200–1000 U/ml) for 72–96 h. Before harvesting, supernatants were collected for ELISA analysis. IFN-γ and IL-2 levels were measured using a two-site ELISA, as previously described (22). Cells were pulsed with PMA (Sigma-Aldrich) (50 ng/ml), ionomycin (Sigma-Aldrich) (500 ng/ml), and brefeldin A (Sigma-Aldrich) (10 μg/ml) for 4 h. Cells were harvested, washed in FACS buffer (1× PBS, 0.2% BSA fraction V (Sigma-Aldrich), and 4 mM sodium azide), and incubated with Fc block (anti-FcRII/III mAb (2.4G2) (10 μg/ml) and rat IgG (10 μg/ml)) for 15 min; surface stained with FITC-labeled anti-CD4, PE-labeled anti-CD8, or isotype control mAb (BD PharMingen) for 20 min; and fixed with 1% w/v paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) overnight. In some experiments, cells were washed again, permeabilized using 0.1% saponin (Sigma-Aldrich) in FACS buffer, and intracellularly stained with allophycocyanin-labeled IFN-γ or isotype control mAb (BD PharMingen) for 20 min at 4°C. Cells were washed again with 0.1% saponin and then with FACS buffer before being acquired on a FACSCalibur cytometer (BD Biosciences, San Jose, CA) and analyzed using CellQuest software (BD Biosciences).

Isolation of brain mononuclear cells

Mice were anesthetized using xylazine and ketamine and perfused through the left cardiac ventricle with 40 ml of ice-cold PBS to remove peripheral blood. Brains were removed, minced with scissors, and then digested for 1 h at 37°C with 300 μg/ml collagenase/dispase (Boehringer Mannheim, Indianapolis, IN) and 600 μg/ml DNase I (Boehringer Mannheim) in complete RPMI 1640. Brain tissue was pelleted at 200 × *g* for 10 min, resuspended in a 60% (1.07 g/ml) isotonic Percoll solution (Sigma-Aldrich), and overlayed with a 30% (1.035 g/ml) Percoll solution. Discontinuous gradients were centrifuged for 25 min at 1000 × *g*. After removal of the myelin layer on top of the gradient, brain mononuclear cells (BMNC) were harvested from the gradient interphase and washed twice in complete RPMI 1640 before further analysis.

Analysis of DC function

Bone marrow DCs from wild-type (WT) and c-Rel$^{-/-}$ mice were prepared, as previously described (23), and plated at a concentration of 2×10^4 cells/well in 96-well flat-bottom plates. DCs were pulsed for 24 h with OVA protein (Worthington Biochemical, Lakewood, NJ) at 1 μg/ml. CFSE-labeled WT or c-Rel$^{-/-}$ OTII Tg CD3$^+$ splenic T cells were added to the pulsed DCs at T-DC ratios of 20:1. In wells in which DCs were not pulsed with OVA, 1 μg/ml soluble anti-CD3 was used as a polyclonal stimulus. Cells were cultured for 4 days at 37°C before treatment with PMA, ionomycin, and brefeldin A, and prepared for flow cytometric analysis, as described above.

5-Bromo-2′-deoxyuridine (BrdU) labeling and T cell proliferation in vivo

WT and c-Rel$^{-/-}$ mice were infected orally with 20 cysts of ME49 strain of *T. gondii*. A total of 0.2 ml of 4 mg/ml BrdU (Sigma-Aldrich) was injected i.p. on days 6 and 7 postinfection. MLN and spleens were harvested on day 9 postinfection, and BrdU incorporation was determined using flow cytometric analysis. Briefly, cells were harvested, washed in FACS buffer, and incubated in Fc block for 15 min. Cells were then surface stained with a combination of mAbs against CD4, CD8, CD44, CD25, and CD62L or isotype control mAb (BD PharMingen) for 20 min and fixed with 1% w/v paraformaldehyde overnight. Cells were washed in FACS buffer and permeabilized in 1% paraformaldehyde plus 0.05% Tween for 60 min. Cells were then treated with DNase (Sigma-Aldrich) for 30 min before staining with FITC-conjugated anti-BrdU Ab (BD PharMingen) or an isotype control (IgG1) for 20 min. Cells were washed twice in FACS buffer and analyzed by flow cytometry.

Adoptive transfer experiments

CD3$^+$ T cells were purified from the spleens and peripheral lymph nodes of naive WT and c-Rel$^{-/-}$ mice by negative selection using T cell enrichment columns (R&D Systems). Purified T cells were routinely >85% purity. A total of 5-10 × 10^6 cells was adoptively transferred into either RAG$^{-/-}$ mice or nude mice, and reconstitution was assessed by flow cytometric analysis of CD3$^+$ T cells in PBMCs 21 days later. Reconstituted mice were infected orally with 20 cysts of the ME49 strain of *T. gondii* 3–5 wk after reconstitution.

Statistics

INSTAT software (GraphPad, San Diego, CA) was used for unpaired two-tailed Student's *t* tests. A p value of <0.05 was considered significant.

Results

c-Rel is required for resistance to toxoplasmosis

To determine the role of c-Rel in resistance to *T. gondii*, WT and c-Rel$^{-/-}$ mice were infected orally with 20 cysts of the ME49 strain of *T. gondii* and survival was monitored. Similar to our previous studies (15), infected c-Rel$^{-/-}$ mice survived the acute phase of infection; however, these mice showed an increased susceptibility to *T. gondii* during the chronic phase with most deaths occurring 5–8 wk postinfection (Fig. 1*A*). The increased susceptibility to *T. gondii* was associated with the presence of 10 times more cysts in the brain of c-Rel$^{-/-}$ mice compared with WT controls (*, $p = 0.001$) (Fig. 1*B*). Histopathological analysis of the brains of infected WT and c-Rel$^{-/-}$ mice revealed that c-Rel$^{-/-}$ mice had more inflammation, and severe meningoencephalitis with

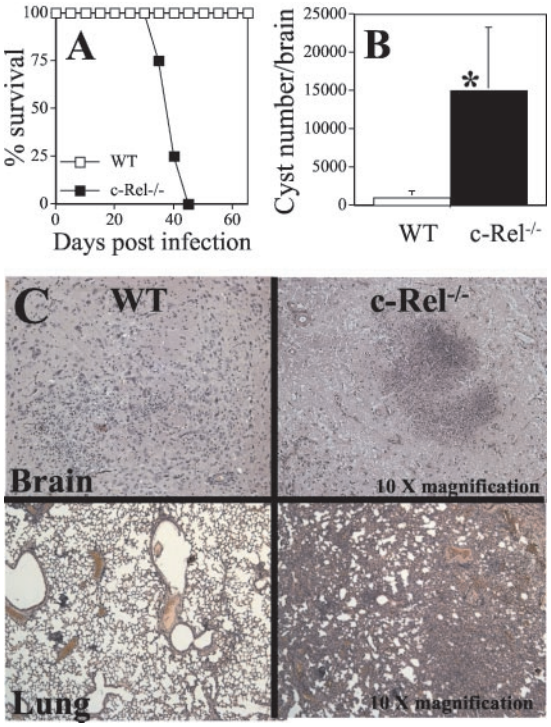

FIGURE 1. c-Rel is required for resistance to toxoplasmosis. *A*, WT ($n = 4$) and c-Rel$^{-/-}$ ($n = 4$) mice were infected orally with 20 ME49 cysts of *T. gondii*, and survival was monitored. Similar results were obtained in two additional experiments with four to six mice per group. *B*, The number of cysts isolated from the brains of chronically infected WT and c-Rel$^{-/-}$ mice was determined. Data show mean values ± SD from four individual mice per group. Similar results were obtained in three additional experiments with three to four mice per group. *C*, Histopathology of brains and lungs harvested from chronically infected WT and c-Rel$^{-/-}$ mice (day 44 postinfection). Similar pathology was found in a repeat experiment with three mice per group.

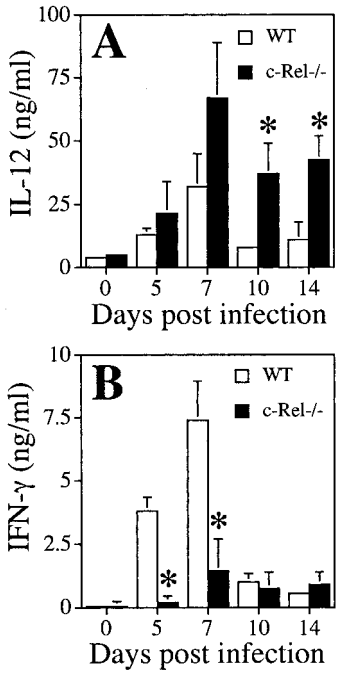

FIGURE 2. c-Rel is required for the early production of IFN-γ during toxoplasmosis. *A*, WT and c-Rel$^{-/-}$ mice were infected orally, and serum levels of IL-12p40 (*A*) and IFN-γ (*B*) were measured 0, 5, 7, 10, and 14 days postinfection by ELISA. Data show mean values ± SD from five individual mice per group and are representative of three different experiments.

areas of coalescing necrosis and abscessation (Fig. 1*C*). Lung parenchyma from chronically infected c-Rel$^{-/-}$ mice showed increased areas of consolidation and inflammatory cell infiltrates compared with WT mice (Fig. 1*C*). No gross differences were observed in splenic histopathology of WT and c-Rel$^{-/-}$ mice (data not shown). Taken together, these data demonstrate that c-Rel is required for resistance to *T. gondii* infection and that increased susceptibility of c-Rel$^{-/-}$ mice is associated with a reduced ability to control parasite replication.

c-Rel is required for optimal production of IFN-γ during infection

The IL-12-driven production of IFN-γ by T cells is essential for long-term resistance to *T. gondii*. Therefore, to determine whether the increased susceptibility of c-Rel$^{-/-}$ mice to infection was associated with a defect in production of IFN-γ, serum levels of IL-12p40 and IFN-γ were measured at days 0, 5, 7, 10, and 14 postinfection (Fig. 2). Consistent with previous findings (15), serum levels of IL-12p40 were comparable between WT and c-Rel$^{-/-}$ mice within the first week of infection and higher in c-Rel$^{-/-}$ mice thereafter (∗, $p = 0.003$). Previous studies with *T. gondii* have shown that serum levels of IL-12 are directly proportional to parasite burden (24). Thus, the increased levels of serum IL-12 detected in infected c-Rel$^{-/-}$ mice are likely to reflect an increase in parasite burden. However, serum levels of IFN-γ were significantly reduced in c-Rel$^{-/-}$ mice during the first week of infection when compared with WT controls (∗, $p < 0.0001$). Nevertheless, as IFN-γ$^{-/-}$ mice infected with *T. gondii* die within 8–10 days of infection (25), these data indicate that c-Rel-independent production of IFN-γ is able to mediate resistance during the acute phase of infection.

To further characterize the basis for the early defect in IFN-γ responses, the ability of T cells from infected WT and c-Rel$^{-/-}$ mice to proliferate and produce IFN-γ in response to parasite Ags was determined. Spleens were harvested from orally infected WT and c-Rel$^{-/-}$ mice at day 7 postinfection and stimulated with STAg either alone or in the presence of human IL-2 (huIL-2) for 4 days. IFN-γ and murine IL-2 production were measured in the culture supernatants by ELISA. In unstimulated cultures and in cultures stimulated with STAg alone, c-Rel$^{-/-}$ lymphocytes produced reduced amounts of IFN-γ when compared with WT controls (∗, $p = 0.0025$) (Fig. 3*A*). This was associated with decreased production of murine IL-2 (WT, 135 ± 49 pg/ml; c-Rel$^{-/-}$, 50 ± 1 pg/ml) (data not shown). MLN were harvested from WT and c-Rel$^{-/-}$ mice at day 7 postinfection, labeled with CFSE, and stimulated with STAg either alone or in the presence of huIL-2 for 4 days. Proliferation of CD4$^+$ and CD8$^+$ T cells and expression of intracellular IFN-γ were determined by flow cytometry. Proliferative responses were assessed in the MLN because there is no NO-mediated suppression of proliferation in this site (26). As shown in Fig. 3*B*, proliferation and intracellular IFN-γ production by c-Rel$^{-/-}$ CD4$^+$ lymphocytes in response to STAg were significantly reduced compared with WT controls. Addition of huIL-2 to these cultures repaired the defect in production of IFN-γ by c-Rel$^{-/-}$ lymphocytes (Fig. 3*A*). This was associated with recovered proliferation of CD4$^+$ c-Rel$^{-/-}$ T cells with positive staining for intracellular IFN-γ (Fig. 3*B*). Similar results were found with c-Rel$^{-/-}$ CD8$^+$ T cells (data not shown). However, even in the presence of IL-2, the percentage of c-Rel$^{-/-}$ IFN-γ$^+$ CD4$^+$ T cells was reduced compared with WT controls. Although IL-2

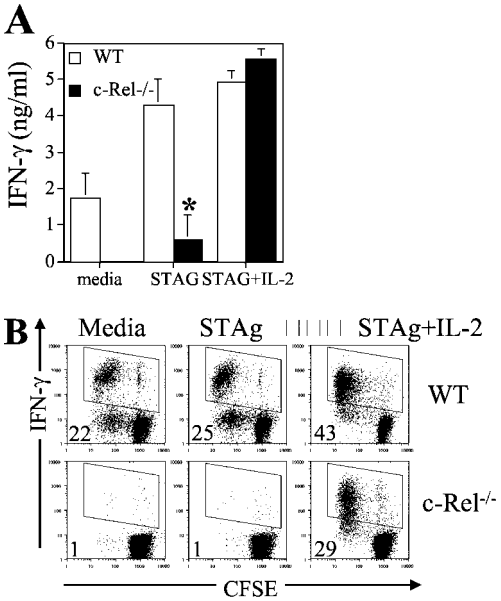

FIGURE 3. Decreased production of IFN-γ by c-Rel$^{-/-}$ T cells is associated with reduced proliferation of CD4$^+$ and CD8$^+$ T cell subsets. *A*, Splenocytes from day 7 infected WT or c-Rel$^{-/-}$ mice were stimulated with STAg alone or in the presence of IL-2 for 72 h. Supernatants were assayed for IFN-γ production by ELISA. Data show mean values ± SD from three individual mice per group. Similar results were found in three experiments. *B*, MLN from day 7 infected WT or c-Rel$^{-/-}$ mice were stimulated with STAg alone or in the presence of IL-2 for 72 h. Proliferation and frequency of CD4$^+$ cells secreting IFN-γ were determined by FACS analysis. Dot plots shown are gated on live CD4$^+$ T cells as indicated. Numbers represent the percentage of CD4$^+$ T cells secreting IFN-γ. Similar results were found in two different experiments.

alone induced IFN-γ production (data not shown), this was associated with the presence of residual parasite Ag from infected mice because comparable cultures from uninfected mice stimulated with IL-2 alone produced significantly less IFN-γ (WT, 0.4 ± 0.1 ng/ml; c-Rel$^{-/-}$, 0.04 ± 0.02 ng/ml). Taken together, these data show that decreased production of IFN-γ by c-Rel$^{-/-}$ T cells during infection is associated with decreased proliferation and decreased IL-2 production. Nevertheless, despite this defect, these results indicate that c-Rel$^{-/-}$ Ag-specific effector cells are present at this time point postinfection in the spleen and MLN, and, although reduced, are most likely sufficient to protect mice beyond the acute phase of infection.

Defects in T cell activation and proliferation in the absence of c-Rel in vivo

To determine whether the reduced capacity of T cells from acutely infected c-Rel$^{-/-}$ mice to produce IFN-γ was a consequence of decreased T cell activation and expansion, the ability of c-Rel$^{-/-}$ T cells to become activated and proliferate in vivo following infection was determined using BrdU incorporation and flow cytometry. WT and c-Rel$^{-/-}$ mice were infected orally, and BrdU was administered i.p., as described in *Materials and Methods*. Mice were sacrificed on days 0 and 9; spleens and MLN were harvested; and CD4$^+$ and CD8$^+$ T cells were analyzed for the expression of the surface activation markers CD25 and CD62L (Fig. 4*A* and data not shown) and BrdU incorporation (Fig. 4*B*). On day 0, the percentage of CD62L^{low} CD25^{high} CD4$^+$ T cells was significantly less in c-Rel$^{-/-}$ mice when compared with WT controls (p = 0.015). Following infection, although there was a significant increased in the percentage of activated CD62L^{low} CD25^{high} CD4$^+$

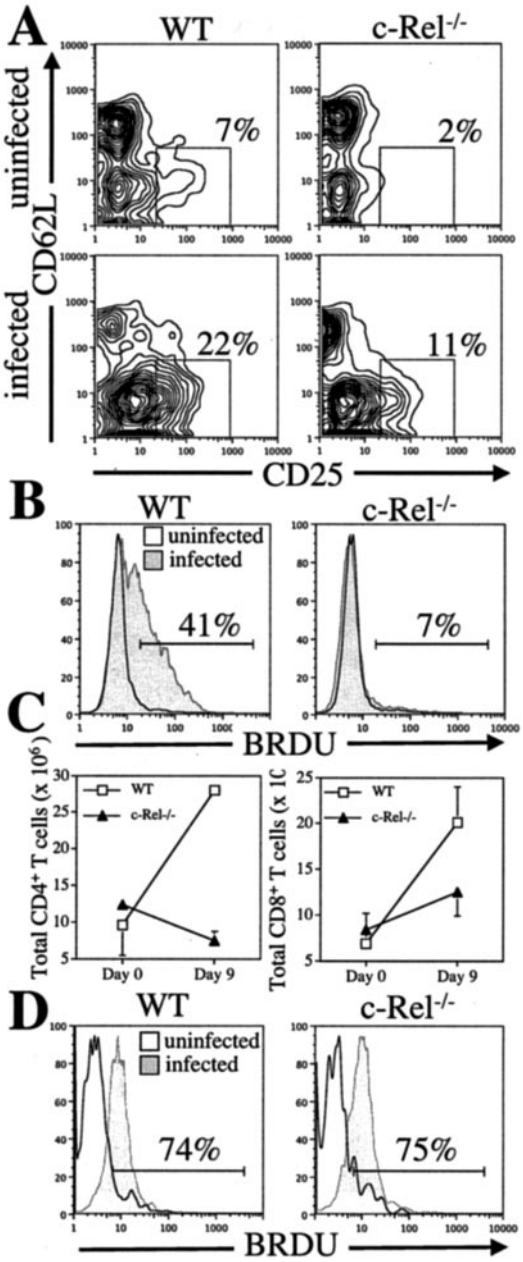

FIGURE 4. c-Rel is required for the early activation and proliferation of T cells during toxoplasmosis. *A*, Splenocytes from uninfected and day 9 infected WT and c-Rel$^{-/-}$ mice were assessed for surface expression of CD25 and CD62L by FACS analysis. Contour plots shown are gated on live CD4$^+$ cells, and numbers represent the percentage of CD4$^+$ cells that are CD62L^{low} and CD25$^+$ as determined by isotype controls. Results are representative of three mice per group. Similar results were obtained in two additional experiments. *B*, MLN from uninfected and day 9 infected WT and c-Rel$^{-/-}$ mice were analyzed for BrdU incorporation. Histograms shown are gated on live CD4$^+$ cells, and numbers indicate the percentage of CD4$^+$ cells that stain positive for BrdU as determined by isotype controls. Results are representative of three mice per group. Similar results were obtained in three separate experiments. *C*, Percentages of CD4$^+$ and CD8$^+$ cells present in the spleens of uninfected and day 9 infected WT and c-Rel$^{-/-}$ mice were determined by flow cytometry and used to determine the total number of CD4$^+$ and CD8$^+$ splenocytes at each time point. Data show mean values ± SD from three individual mice per group and are representative of three different experiments. *D*, Splenic CD62L^{low} CD25$^+$ CD4$^+$ cells from BrdU-treated, uninfected and day 9 infected WT and c-Rel$^{-/-}$ mice were analyzed by flow cytometry for BrdU incorporation. Histograms shown are gated on live CD62L^{low} CD25$^+$ CD4$^+$ cells. The numbers indicate the percentage of CD62L^{low} CD25$^+$ CD4$^+$ cells that stain positive for BrdU as determined by isotype controls. Results from individual mice are shown and are representative of three mice in each group.

T cells in both WT and c-Rel$^{-/-}$ mice (WT, $p = 0.0237$; c-Rel$^{-/-}$, $p = 0.02$), the percentage of activated T cells in c-Rel$^{-/-}$ mice remained significantly less than WT controls ($p = 0.02$) (Fig. 4*A*). Furthermore, following infection, the percentage of BrdU$^+$ CD4$^+$ T cells and BRDU$^+$ CD8$^+$ T cells was significantly greater in WT mice compared with c-Rel$^{-/-}$ mice (Fig. 4*B* and data not shown). The total number of splenic CD4$^+$ and CD8$^+$ T cells was calculated from percentages of CD4$^+$ and CD8$^+$ T cells determined by flow cytometry before and after infection. In WT mice, activation and proliferation of CD4$^+$ and CD8$^+$ T cells resulted in an increase in the total number of splenic CD4$^+$ and CD8$^+$ T cells following infection. However, in c-Rel$^{-/-}$ mice, consistent with the findings of decreased T cell activation and proliferation, total numbers of splenic CD4$^+$ and CD8$^+$ T cells were significantly less than WT controls following infection (CD4$^+$ $p = 0.01$; CD8$^+$ $p = 0.03$) (Fig. 4*C*). Finally, analysis of BrdU incorporation within the activated CD62L^{low} CD25^{high} CD4$^+$ T cell population revealed that comparable percentages of activated CD4$^+$ T cells from WT and c-Rel$^{-/-}$ mice incorporated BrdU when compared with uninfected controls (Fig. 4*D*). Taken together, these data suggest that in the absence of c-Rel, reduced numbers of T cells become activated and proliferate, contributing to decreased total numbers of T cells following infection. Furthermore, these results suggest that in the absence of c-Rel, the reduced capacity to produce IFN-γ following infection is associated with reduced numbers of activated CD4$^+$ and CD8$^+$ T cells. These findings are consistent with previous studies that have shown a role for c-Rel in T cell activation, proliferation, and expression of antiapoptotic factors (13, 14, 27).

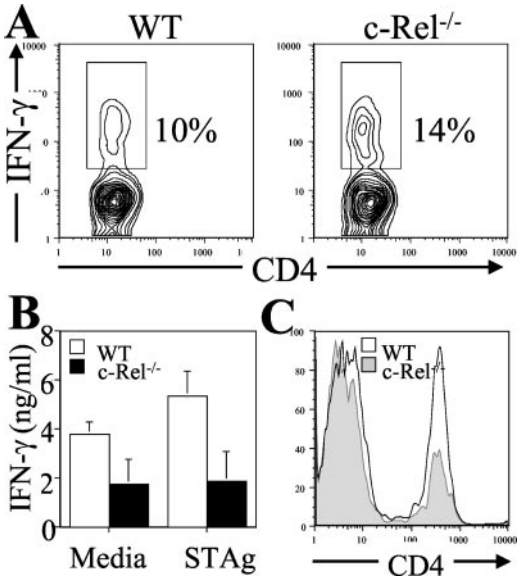

FIGURE 5. c-Rel is not required for the generation of Th1 effector cells during toxoplasmosis. BMNCs from chronically infected WT and c-Rel$^{-/-}$ mice were stimulated with STAg for 72 h. *A*, Frequency of CD4$^+$ BMNC secreting IFN-γ after restimulation was determined by FACS analysis. Contour plots shown are gated on live CD4$^+$ cells. Numbers represent the percentage of CD4$^+$ cells expressing IFN-γ. Similar results were found in two additional experiments. *B*, Supernatants were assayed for IFN-γ production by ELISA. Data show mean values ± SD from three mice per group and are representative of three different experiments. *C*, Ex vivo frequency of CD4$^+$ cells isolated from the brains of chronically infected WT and c-Rel$^{-/-}$ mice (WT, 36%; c-Rel$^{-/-}$, 17%). Similar results were obtained in three additional experiments with three to four mice per group.

c-Rel-independent generation of Th1 effector cells

Recent studies have demonstrated that c-Rel is required for the generation of Th1 effector cells (18, 28). However, that c-Rel$^{-/-}$ mice survive the acute phase of toxoplasmosis suggests that IFN-γ-producing Th1 cells can be generated in the absence of c-Rel and can mediate resistance to *T. gondii*. Therefore, to determine whether functional Th1 effector cells can be produced in the absence of c-Rel in vivo following infection, mononuclear cells were isolated from the brains of chronically infected (day 40–60) WT and c-Rel$^{-/-}$ mice and stimulated with STAg for 3 days. Cultured cells were then analyzed for the presence of intracellular IFN-γ using flow cytometry. Comparable percentages of CD4$^+$ c-Rel$^{-/-}$ lymphocytes stained positive for intracellular IFN-γ when compared with WT controls (Fig. 5*A*). However, in unstimulated cultures and in cultures stimulated with STAg alone, c-Rel$^{-/-}$ lymphocytes produced reduced amounts of IFN-γ in culture supernatants when compared with WT controls (Fig. 5*B*). Finally, although routinely >95% of both WT and c-Rel$^{-/-}$ CD4$^+$ cells isolated from the brains of chronically infected mice expressed an activated phenotype (CD62L^{low} CD44^{high}) (data not shown), there was a 50% reduction in the percentage of CD4$^+$ T cells isolated from the brains of c-Rel$^{-/-}$ mice when compared with WT controls (WT, 29.3% ± 2; c-Rel$^{-/-}$, 15.9 ± 0.9, $p = 0.003$) (Fig. 5*C*). Taken together, these data indicate that functional Th1 effector cells can be produced in the absence of c-Rel in response to infection. However, numbers of CD4$^+$ T cells accumulating at sites of infection are reduced, and this most likely contributes to decreased levels of IFN-γ in the brains of infected c-Rel$^{-/-}$ mice and a reduced capacity to control parasite replication.

c-Rel is intrinsically required by DCs for T cell proliferation

Recent studies have indicated a role for c-Rel in the ability of DCs to induce T cell proliferation and direct a Th1 response (18, 28). However, the studies presented above indicate that Th1 responses can be generated in c-Rel$^{-/-}$ mice following infection with *T. gondii*. Therefore, to further assess the contribution of c-Rel to DC functions required for T cell activation, an Ag-specific system using OVA peptide 323–339 I-A^b-specific OTII TCR Tg mice was adopted. Bone marrow-derived DC (BMDC) from either WT or c-Rel$^{-/-}$ mice were pulsed with OVA plus LPS and used to stimulated CFSE-labeled WT OTII Tg or c-Rel$^{-/-}$ OTII Tg splenic CD3$^+$ T cells either with or without exogenous IL-2. WT DCs were capable of stimulating both WT and c-Rel$^{-/-}$ T cells to proliferate, although the responder frequency (RF) of c-Rel$^{-/-}$ T cells was reduced (WT T cell, 39%; c-Rel$^{-/-}$ T cell, 13%) (Fig. 6*A*). Addition of IL-2 increased, but did not fully recover, the RF of c-Rel$^{-/-}$ T cells (WT T cell, 45%; c-Rel$^{-/-}$ T cell, 24%) (Fig. 6*B*). These results suggest that c-Rel is intrinsically required by CD4$^+$ T cells for optimal Ag-specific proliferation. In contrast to WT DCs, c-Rel$^{-/-}$ DCs were unable to support Ag-specific proliferation of WT T cells (WT T + WT DC RF, 39%; WT T + c-Rel$^{-/-}$ DC RF, 3%) (Fig. 6*C*). Furthermore, this defect was not overcome by the addition of exogenous IL-2 (WT T + WT DC + IL-2 RF, 45%; WT T + c-Rel$^{-/-}$ DC + IL-2 RF, 5%) (Fig. 6*D*) or IL-12 (data not shown). To determine whether these observed defects in c-Rel$^{-/-}$ DC function were associated with defective Ag processing/presentation or costimulation, soluble anti-CD3, instead of OVA, was added to cultures of WT or c-Rel$^{-/-}$ DCs, and WT T cell proliferation was assessed as before (Fig. 6, *E* and *F*). In contrast to Ag stimulation, c-Rel$^{-/-}$ DCs were able to support T cell proliferation in response to anti-CD3 (WT T + WT DC RF, 69%; WT T + c-Rel$^{-/-}$ DC RF, 36%). Together, these data indicate that c-Rel is intrinsically required by DCs for Ag-specific

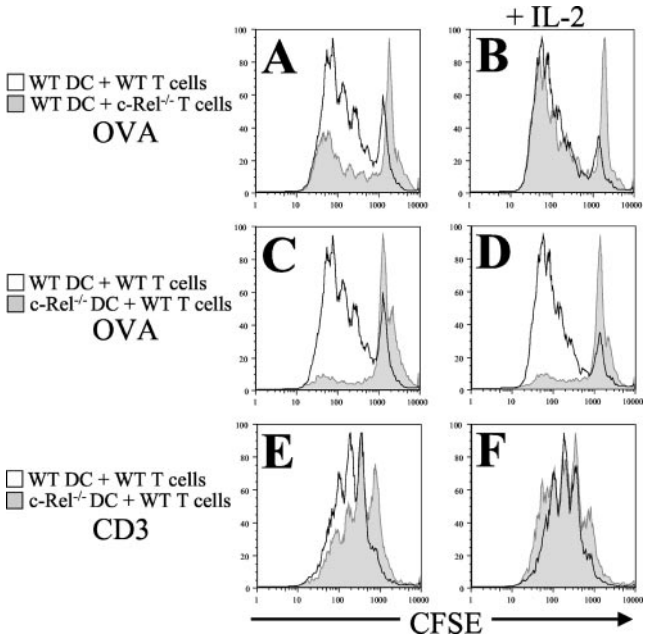

FIGURE 6. c-Rel is intrinsically required by DCs for T cell proliferation. *A* and *B*, Proliferation profiles of WT and c-Rel$^{-/-}$ OTII Tg CD3$^+$ T cells stimulated with WT BMDC pulsed with OVA plus LPS for 96 h without IL-2 (*A*) and with IL-2 (*B*). *C* and *D*, Proliferation profiles of WT OTII Tg CD3$^+$ T cells stimulated with WT or c-Rel$^{-/-}$ BMDC pulsed with OVA plus LPS for 96 h without IL-2 (*C*) and with IL-2 (*D*). *E* and *F*, Proliferation profiles of WT OTII Tg CD3$^+$ T cells stimulated with WT or c-Rel$^{-/-}$ BMDC and soluble anti-CD3 for 96 h without IL-2 (*E*) and with IL-2 (*F*). All experiments were performed at T-DC ratios of 20:1, and all histograms shown are gated on live CD4$^+$ T cells. Similar results were obtained in two additional experiments.

proliferation and suggest that c-Rel is principally required for optimal Ag processing/presentation by DCs.

c-Rel is intrinsically required by T cells for expansion of IFN-γ-producing T cells following infection

The studies in the previous section demonstrate that c-Rel$^{-/-}$ DCs are defective in their ability to support Ag-specific T cell proliferation. Therefore, an intrinsic requirement for c-Rel in APCs may contribute to the decreased expansion of T cells observed in infected c-Rel$^{-/-}$ mice. However, these studies also demonstrate that c-Rel$^{-/-}$ T cells have a reduced capacity to expand even when stimulated with WT DCs. Thus, the observed decreased expansion of T cells in infected c-Rel$^{-/-}$ mice could reflect an intrinsic requirement for c-Rel by DCs and/or T cells for infection-induced T cell proliferation. Therefore, to determine the relative contribution of c-Rel activity in the T cell and accessory cell compartments to the susceptible phenotype of c-Rel$^{-/-}$ mice infected with *T. gondii*, adoptive transfer experiments using RAG$^{-/-}$ or nude mice were performed. To determine whether intact accessory cell function could reverse defects in c-Rel$^{-/-}$ T cell activation, proliferation, and IFN-γ production and improve survival following *T. gondii* infection, T cell-deficient mice were reconstituted with either WT or c-Rel$^{-/-}$ CD3$^+$ T cells and infected orally with 20 cysts of the ME49 strain of *T. gondii*. T cell activation and proliferation in the spleen and MLN were determined using BrdU and flow cytometry. Mice reconstituted with WT or c-Rel$^{-/-}$ CD3$^+$ T cells had comparable percentages of CD4$^+$ and CD8$^+$ T cells in peripheral blood 3 wk after reconstitution and before infection (data not shown). At day 9 postinfection, whereas the total numbers of activated (CD44^{high} CD62L^{low}) CD4$^+$ and CD8$^+$ T cells present within the spleens of mice reconstituted with WT T cells had increased 4–5 times compared with uninfected controls, the increase in the numbers of activated CD4$^+$ and CD8$^+$ T cells in the spleens of infected mice reconstituted with c-Rel$^{-/-}$ T cells was significantly less (infection-induced increase of 1–1.5 times compared with uninfected controls) (data not shown). Furthermore, whereas 28 ± 4% of CD4$^+$ T cells and 35 ± 6% of CD8$^+$ T cells incorporated BrdU within the draining lymph nodes of WT T cell-reconstituted mice following infection, only 8.5 ± 1% of CD4$^+$ T cells and 21 ± 6% of CD8$^+$ T cells incorporated BrdU within the draining lymph nodes of c-Rel$^{-/-}$ T cell-reconstituted mice following infection (Fig. 7*A*). Moreover, whereas the percentages of BrdU$^+$ WT CD4$^+$ and CD8$^+$ T cells following infection were significantly higher than uninfected controls (naive WT reconstituted control: CD4$^+$ BrdU$^+$, 5%; CD8$^+$ BrdU$^+$, 5%), percentages of BrdU$^+$ c-Rel$^{-/-}$ CD4$^+$ and CD8$^+$ T cells following infection were not significantly different from uninfected controls (uninfected c-Rel$^{-/-}$ reconstituted control: CD4$^+$ BrdU$^+$, 8%; CD8$^+$ BrdU$^+$, 20%). Consistent with failure to recover T cell activation and proliferation following infection, T cell-deficient mice reconstituted with c-Rel$^{-/-}$ T cells had significantly reduced levels of serum IFN-γ on day 9 postinfection, when compared with mice reconstituted with WT T cells (Fig. 7*B*). Furthermore, splenocytes harvested from infected RAG$^{-/-}$ mice reconstituted with c-Rel$^{-/-}$ T cells and stimulated with STAg produced less IFN-γ in the culture supernatants than mice reconstituted with WT T cells ($p = 0.05$) (Fig. 7*C*). Finally, to determine whether intact accessory cell function could improve survival following *T. gondii* infection, T cell-deficient mice were reconstituted with either WT or c-Rel$^{-/-}$ T cells and infected orally, and survival was monitored (Fig. 7*D*). Mice reconstituted with c-Rel$^{-/-}$ T cells remained susceptible to *T. gondii* infection and died within 40–50 days, similar to the phenotype of infected c-Rel$^{-/-}$ mice (Fig. 1). Furthermore, histopathological analysis of the brains of infected mice reconstituted with c-Rel$^{-/-}$ T cells showed similar lesions to infected c-Rel$^{-/-}$ mice (Fig. 7*E*). Taken together, these data demonstrate that T cell-intrinsic expression of c-Rel is required for optimal Th1 responses required for long-term resistance to *T. gondii* infection.

Discussion

The studies presented in this work establish an important role for c-Rel in the development of an optimal Th1-type response required for long-term resistance to *T. gondii*. However, c-Rel$^{-/-}$ mice do develop a level of T cell-mediated protective immunity that is required for them to survive into the chronic phase of infection. Nevertheless, the decreased ability of c-Rel$^{-/-}$ T cells to expand contributes to the low systemic levels of IFN-γ observed during the acute phase of this infection as well as the increased levels of parasite replication observed in mice infected via the i.p. route (data not shown). Because even immune competent WT C57BL/6 mice are highly susceptible to toxoplasmic encephalitis, it is likely that the increased parasite numbers observed in c-Rel$^{-/-}$ C57BL/6 mice contribute to an accelerated form of toxoplasmic encephalitis in these mice. In addition, the reduced numbers of CD4$^+$ T cells in the brains of infected c-Rel$^{-/-}$ mice are likely to be a factor in the failure to control parasite replication and the development of lesions similar to those seen in T cell-deficient hosts (29, 30).

Recent studies have identified an important role for c-Rel in the ability of CD4$^+$ T cells to develop into Th1-type cells during experimental allergic encephalomyelitis (EAE) (18, 28), consistent with the presence of c-Rel binding sites within the IFN-γ gene (3, 31). However, the studies presented in this work indicate that although c-Rel is required for optimal T cell activation, proliferation, and production of IFN-γ during the early stages of infection, a

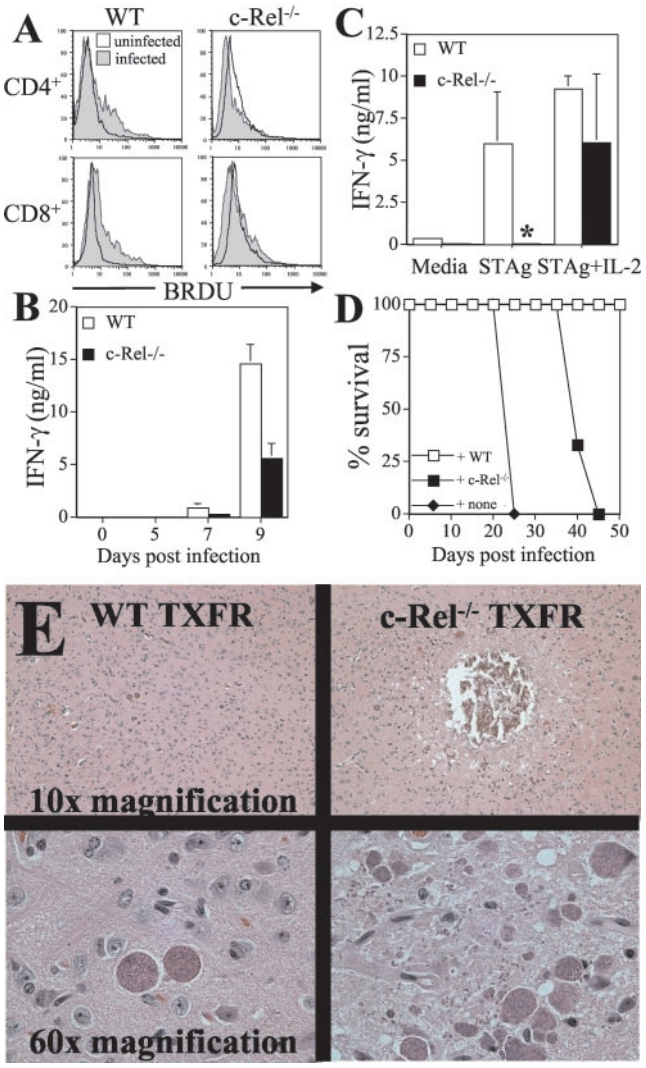

FIGURE 7. c-Rel is intrinsically required by T cells for proliferation and production of IFN-γ following infection. *A*, BrdU incorporation in adoptively transferred WT and c-Rel$^{-/-}$ CD4$^+$ and CD8$^+$ T cells 9 days postinfection. The percentages of BrdU$^+$ T cells were determined using isotype controls. Histograms are gated on live CD4$^+$ or CD8$^+$ cells. Three adoptively transferred mice were in each group, and results are representative of two separate experiments. *B*, Plasma levels of IFN-γ from RAG$^{-/-}$ mice reconstituted with either WT or c-Rel$^{-/-}$ CD3$^+$ T cells were determined by ELISA on days 0, 5, 7, and 9 postinfection. *C*, Splenocytes from day 9 infected RAG$^{-/-}$ mice reconstituted with WT or c-Rel$^{-/-}$ T cells were stimulated with STAg alone or with IL-2 (200 U/ml) for 72 h. Supernatants were assayed for IFN-γ production. Data show mean values ± SD from three individual mice per group and are representative of three different experiments. *D*, Nude mice reconstituted with 1×10^7 purified WT or c-Rel$^{-/-}$ CD3$^+$ T cells were infected, and survival was monitored. Similar results were obtained in an additional experiment with three mice per group. *E*, Histopathology of brains harvested from infected RAG$^{-/-}$ mice reconstituted with either WT or c-Rel$^{-/-}$ T cells and sacrificed on day 30 postinfection.

protective Th1-type response can still develop and mediate partial resistance. These latter findings are consistent with initial studies that suggested that c-Rel is not intrinsically required for the production of IFN-γ (32). Rather, the data presented in this work suggest that because T cell proliferation is required for T cell differentiation and effector function (33), the defects in proliferation observed in infected c-Rel$^{-/-}$ mice most likely contribute to their reduced IFN-γ responses. Nevertheless, the basis for the different requirements for c-Rel in the development of a Th1-type response in different models (EAE vs infection) is uncertain. One possible explanation for these differences is that the relative resistance of c-Rel$^{-/-}$ mice to EAE was in part attributed to decreased IL-12 production (18), whereas during infection, c-Rel$^{-/-}$ mice produced normal levels of IL-12 (15).

The initiation and polarization of an Ag-specific effector T cell response are dependent upon efficient Ag presentation in the context of MHC, appropriate costimulation, and cytokine production by professional APCs such as DCs. Previous studies have demonstrated a role for NF-κB in the development and maturation of DC (34, 35), but c-Rel does not appear to be required for DC development or expression of MHCI and MHCII, B7-2, and ICAM-1 (34). The finding that c-Rel$^{-/-}$ DCs have a reduced capacity to support the proliferation of Ag-specific T cells is in agreement with recent studies (36). Furthermore, the studies presented in this work suggest that this defect is principally associated with impaired Ag processing or presentation as c-Rel$^{-/-}$ DCs can support polyclonal T cell proliferation. Although the in vivo studies reported in this work do not directly address the role of c-Rel in DC function during infection, there were no obvious differences between the phenotype of WT and c-Rel$^{-/-}$ DCs from infected mice in terms of B7-1, B7-2, and MHCII expression (data not shown). Experiments are currently underway to determine whether the absence of c-Rel in DCs affects T cell responses during toxoplasmosis.

The results of in vitro mixing experiments and in vivo adoptive transfer experiments presented in this work identify a T cell-intrinsic requirement for c-Rel in optimal Ag-specific activation, proliferation, and IFN-γ production, and raise the basic question of what the role of c-Rel is in the direct regulation of T cell function in vivo. The activation of c-Rel has been associated with signaling through the costimulatory molecule CD28, an important interaction that regulates T cell production of cytokines, proliferation, and survival (37). There are clear similarities between the T cell responses to *T. gondii* in c-Rel$^{-/-}$ and CD28$^{-/-}$ mice; both deletions result in failure to appropriately expand the CD4$^+$ T cell compartment following infection with *T. gondii*. However, in contrast to c-Rel$^{-/-}$ mice, CD28$^{-/-}$ mice produce near normal levels of IFN-γ, and parasite replication is controlled (38). This is in part attributed to the ability of the CD28 homologue inducible costimulation molecule to provide compensatory signals necessary for the development of protective Th1 responses required for resistance to *T. gondii* (39). It is possible that in addition to CD28, these alternative costimulatory pathways may also signal through c-Rel, and so account for the increased susceptibility of c-Rel$^{-/-}$ mice to *T. gondii* compared with CD28$^{-/-}$ mice.

Although the NF-κB family of transcription factors has been intimately linked to the regulation of innate and adaptive functions required for the recognition and elimination of invading microorganisms, there is still a poor understanding of the function of individual family members during different infections. To better understand the role of NF-κB in immunity to infection, several laboratories have started to define the role of individual NF-κB family members in resistance to *T. gondii*. These studies have revealed that different family members appear to have distinct roles during this infection. For example, RelB is required for the production of IFN-γ and acute resistance to this parasite (40). In contrast, p52$^{-/-}$ mice develop normal IFN-γ responses to infection, but these mice display an increased level of apoptosis and loss of T cells during the chronic phase of infection associated with the development of severe toxoplasmic encephalitis (41). Furthermore, mice deficient in Bcl-3 (an I-κB family member) fail to mount a protective T cell response to *T. gondii* and, despite normal initial production of IFN-γ, die in the chronic phase between 3 and

5 wk postinfection (41). In those reports, it has been difficult to determine whether the defects in T cell function could be attributed to an intrinsic requirement for the different NF-κB members in the T cells, or whether these defects were a consequence of altered accessory cell functions. This question was partially addressed by the use of a lineage-specific transgene to inhibit NF-κB activity, which indicated that NF-κB signaling in T cells was not required for initial activation, but was required for subsequent expansion and the ability to produce IFN-γ (42). The data presented in this work extend our knowledge of the role of the NF-κB family in resistance to infection and indicate that c-Rel-independent pathways of T cell activation and proliferation exist during *Toxoplasma* infection. However, in the absence of c-Rel, T cell expansion is limited and T cell responses are not effectively maintained. Thus, although c-Rel has been implicated as an important regulator of the innate immune response (16), the studies presented in this work establish an important role for this transcription factor in the optimization and maintenance of adaptive T cell responses during infection.

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