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Cutting Edge: Identification of c-Rel-Dependent and -Independent Pathways of IL-12 Production During Infectious and Inflammatory Stimuli[1]

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Cutting Edge: Identification of c-Rel-Dependent and -Independent Pathways of IL-12 Production During Infectious and Inflammatory Stimuli[1]

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The production of IL-12 is required for immunity to many intracellular pathogens. Recent studies have shown that c-Rel, a member of the NF-κB family of transcription factors, is essential for LPS-induced IL-12p40 production by macrophages. In this study, we demonstrate that c-Rel is also required for IL-12p40 production by macrophages in response to *Corynebacterium parvum*, CpG oligodeoxynucleotides, anti-CD40 and low molecular weight hyaluronic acid. However, c-Rel$^{-/-}$ mice infected with *Toxoplasma gondii* produce comparable amounts of IL-12p40 to infected wild-type mice and have an IL-12-dependent mechanism of resistance to this infection. Furthermore, c-Rel was not required for IL-12p40 production by macrophages or dendritic cells in response to soluble *Toxoplasma* Ag, and neutrophils from c-Rel$^{-/-}$ mice contain normal amounts of preformed IL-12p40. Together these studies reveal the presence of c-Rel-dependent pathways critical for IL-12p40 production in response to inflammatory stimuli and demonstrate a novel c-Rel-independent pathway of IL-12p40 production during toxoplasmosis. *The Journal of Immunology*, 2002, 168: 2590–2594.

Interleukin-12 is a critical cytokine required for the production of IFN-γ during the innate and adaptive immune response to many different viral, bacterial, and parasitic infections (1). Many of the microbial products which stimulate the innate production of IL-12 from accessory cells such as macrophages and dendritic cells (DC)[3] signal through Toll-like receptors (TLR) which activate the NF-κB family of transcription factors (2–4). The NF-κB family of transcription factors includes p65 (RelA), RelB, c-Rel, p50, and p52. Several studies have indicated the importance of NF-κB in the regulation of IL-12 production (5–8), consistent with the role of NF-κB as an important regulator of innate responses. More recent studies have suggested that of these family members, only c-Rel is critical for the ability of LPS to stimulate macrophages to produce IL-12, although c-Rel is not required for their ability to produce TNF-α, IL-6, or NO (9). These previous studies are important because they place c-Rel at a critical point in the innate response that leads to the production of IL-12 following stimulation with Gram-negative bacteria. However, as discussed by Sanjabi et al. (9), the biological significance of the c-Rel requirement for IL-12p40 expression is unknown. The studies presented here demonstrate that c-Rel is essential for the ability of macrophages to produce IL-12 in response to numerous proinflammatory stimuli in vitro and in vivo, but identify a c-Rel-independent pathway for the production of IL-12 following infection with the parasite *Toxoplasma gondii*. These results suggest the presence of alternative pathways for the innate recognition of *T. gondii* that are distinct from the TLR pathways that have been implicated in the innate recognition of bacterial, fungal, parasitic, and viral infections (10).

Materials and Methods

Mice and inoculations

Wild-type (WT) C57BL/6 mice (age 6–8 wk) were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 c-Rel-deficient (c-Rel$^{-/-}$) mice were originally obtained from H. C. Liou and bred within the University Laboratory Animal Resources facility of the University of Pennsylvania. Age- and sex-matched mice were inoculated i.p. with either 100 μg of *Salmonella minnesota*-derived LPS (Sigma-Aldrich, St. Louis, MO) in sterile PBS, 25 μg of soluble *Toxoplasma* Ag lysate (STAg) or 200 μg of anti-CD40 (R&D Systems, Minneapolis, MN). STAg was prepared from in vitro-cultured tachyzoites of *T. gondii* strain RH as previously described (11). For live infections, age- and sex-matched WT and c-Rel$^{-/-}$ mice were inoculated orally with 20 cysts of the ME49 strain of *T. gondii* or i.p. with 10,000 tachyzoites of the RH strain of *T. gondii*.

Preparation of macrophages and DC

Bone marrow-derived macrophages (BMMΦ) were prepared as previously described (12). Peritoneal macrophages were elicited using 1 ml of 10% thioglycolate (TG) injected i.p., and cells were harvested 72 h later. Cell purity was assessed by cytospin analysis and >98% of cells were found to be macrophages. CD11c^{+} splenic DC were positively selected from collagenase D-digested spleens using MACS Separation Beads (CD11c MicroBeads; Miltenyi Biotec, Auburn, CA). Isolated cell purity was assessed

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[3] Abbreviations used in this paper: DC, dendritic cell; STAg, soluble *Toxoplasma* Ag; LMW-HA, low molecular weight hyaluronic acid; WT, wild type; TLR, Toll-like receptor; BMMΦ, bone marrow-derived macrophage; TG, thioglycolate; PEC, peritoneal exudate cell.

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by FACS analysis and routinely found to be 85–90%. For stimulation, BMMΦ were incubated at 37°C in 5% CO_2 for 72 h with LPS (250 ng/ml, Sigma-Aldrich), *C. parvum* (10 μg/ml; Trudo Institute, Saranac Lake, NY), CpG containing oligodeoxynucleotides (ODN; 1 μg/ml), soluble anti-CD40 (15 μg/ml; R&D Systems, Minneapolis, MN), low molecular weight hyaluronic acid (LMW-HA, 100 μg/ml; Sigma-Aldrich), or STAg (25 μg/ml). The ODN used in this study was the CpG containing phosphorothioate backbone oligonucleotide 1826 (TCC ATG ACG TTC CTG ACG TT) (synthesized by the University of Pennsylvania Veterinary DNA facility). The stimulants *C. parvum*, CpG-ODN, soluble anti-CD40, LMW-HA, and STAg were pretreated with 10 μg/ml polymixin B (Sigma-Aldrich) to ensure that any LPS contamination would not contribute to IL-12 production by macrophages. Supernatants were harvested and the production of IL-12p40 and IL-6 was measured using ELISAs as previously described (12). Levels of reactive nitrogen intermediates were measured using the Greiss assay as previously described (13).

Flow cytometric analysis

$CD11c^+$ splenic DC from infected mice or mice injected with STAg were cultured overnight with 25 μg/ml STAg or media, respectively, plus 10 μg/ml brefeldin A (Sigma-Aldrich). Cell suspensions were then incubated with Abs against CD11c and CD8α, before staining for intracellular IL-12p40, as previously described (14). Peritoneal cells were harvested from infected mice and stained with anti-Gr-1 or isotype control and IL-12p40 as previously described (15). Cells were immediately recorded on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) and analyzed with CellQuest software (BD Biosciences). One hundred thousand events were recorded for each sample.

Results

c-Rel-dependent production of IL-12 in response to inflammatory stimuli

Previous studies demonstrated an essential role for c-Rel in the ability of macrophages stimulated with LPS to produce IL-12p40 (9). Since there are alternative stimuli which can induce macrophages to produce high levels of IL-12 (16–19), studies were performed to determine whether there was a stimulus-specific requirement for c-Rel in the production of IL-12p40. BMMΦ and TG-elicited peritoneal macrophages from WT and c-$Rel^{-/-}$ mice were stimulated with LPS, LMW-HA, CpG containing ODN, anti-CD40, or *C. parvum* for 72 h and the levels of IL-12p40 produced in the supernatants were measured by ELISA. BMMΦ and TG-elicited peritoneal exudate cells (PECs) from c-$Rel^{-/-}$ mice were deficient in the production of IL-12p40 in response to all of the stimuli used (Fig. 1, *A* and *B*). Furthermore, WT and c-$Rel^{-/-}$ macrophages produced comparable levels of IL-6 and NO in these cultures (data not shown). These data confirm previous studies that c-Rel is essential for LPS-induced production of IL-12p40, which is mediated through TLR4, and extend these studies to show that the production of IL-12p40 mediated through TLR2, TLR9, CD40, and CD44 is also dependent on c-Rel.

To determine the biological significance of c-Rel in the production of IL-12p40 in vivo, WT and c-$Rel^{-/-}$ mice were injected with LPS or anti-CD40 and serum levels of IL-12p40 were measured. Similar to the results observed in vitro, c-$Rel^{-/-}$ mice were deficient in the production of IL-12p40 in response to these stimuli (Fig. 1*C*). These data demonstrate that c-Rel is also important for LPS and anti-CD40 to stimulate innate production of IL-12p40 in vivo.

c-Rel-independent IL-12 production mediates resistance to T. gondii

The production of IL-12 is essential for the development of resistance to many intracellular pathogens including *T. gondii*. Thus, mice infected with *T. gondii* and treated with Abs to deplete IL-12 or that lack the capacity to produce or respond to IL-12 are highly susceptible to toxoplasmosis and succumb to infection within

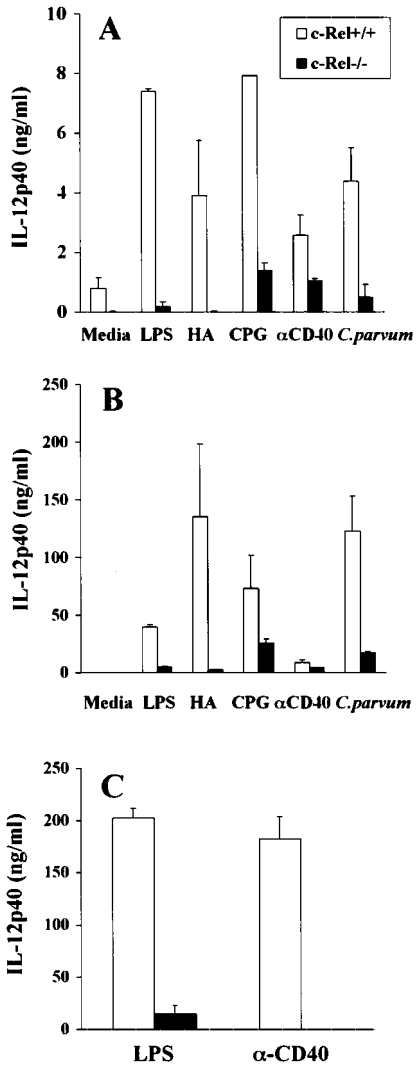

FIGURE 1. c-Rel is required for the production of IL-12p40 in response to different inflammatory stimuli. BMMΦ (2×10^5) (*A*), and TG-elicited PECs (2×10^5) (*B*), from WT and c-$Rel^{-/-}$ mice were stimulated with LPS, *C. parvum*, LMW-HA, CpG containing ODN, or soluble anti-CD40 for 72 h, and the levels of IL-12p40 in the culture supernatants were determined by ELISA. The data presented are the means ± SD from a single experiment with three mice in each experimental group. Similar results were seen in three separate experiments. *C*, WT and c-$Rel^{-/-}$ mice were given LPS or anti-CD40 i.p. and serum levels of IL-12p40 were measured by ELISA at 3 and 24 h after administration, respectively. Similar results were seen in three and two separate experiments, respectively, with three to five mice per experiment.

10–14 days (20–22). To determine whether c-Rel is necessary for the production of IL-12 in response to this infection, c-$Rel^{-/-}$ mice were infected orally with 20 cysts of *T. gondii* and serum levels of IL-12p40 were measured 5 and 7 days after infection. Surprisingly, c-$Rel^{-/-}$ mice produced comparable levels of IL-12p40 to WT mice at both time points (Fig. 2*A*). Infected mice survived for 6–7 wk, suggesting that an IL-12-dependent mechanism of resistance to *T. gondii* exists in c-$Rel^{-/-}$ mice. This was confirmed by studies in which c-$Rel^{-/-}$ mice treated with anti-IL-12 at the time of infection died within 10–14 days (Fig. 2*B*). These studies demonstrate that in c-$Rel^{-/-}$ mice infected with *T. gondii* there is a mechanism that allows normal production of IL-12 which provides protection against this infection.

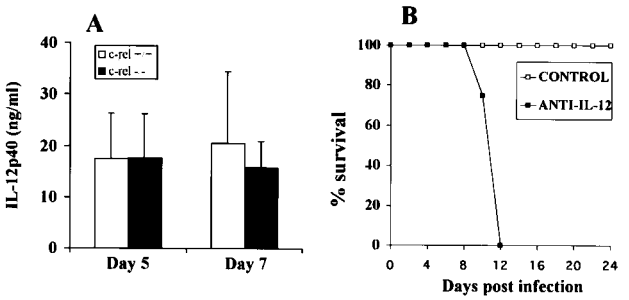

FIGURE 2. c-Rel$^{-/-}$ mice have an IL-12-dependent mechanism of resistance to *T. gondii* infection. *A*, WT and c-Rel$^{-/-}$ mice were infected orally with 20 ME49 cysts of *T. gondii*, and the levels of IL-12p40 in serum were measured by ELISA 5 and 7 days after infection. The data presented are the means ± SD from two experiments, each containing three to four mice per experiment. Similar results were seen in five additional experiments. Serum levels of IL-12p40 in uninfected mice were typically <2 ng/ml. *B*, Eight c-Rel$^{-/-}$ mice were infected orally with 20 cysts of the ME49 strain of *T. gondii*. Infected mice received either 2 mg (200 μl) of C17.8 (anti-IL-12) Ab i.p. every third day, starting at the time of infection, or 2 mg of rat IgG, and survival was monitored.

c-Rel is not required for the production of IL-12p40 by macrophages, DC, or neutrophils in response to T. gondii

Macrophages (23), DC (24), and neutrophils (25) have all been shown to be sources of IL-12p40 in different models of *Toxoplasma*-induced production of IL-12. Therefore, several defined experimental systems were used to examine the capacity of these different cell types to produce IL-12p40 in response to *T. gondii*. As previously described (23), the stimulation of TG-elicited macrophages from WT mice with STAg led to the production of IL-12p40. In contrast to the results obtained with other stimuli, when TG-elicited macrophages from c-Rel$^{-/-}$ mice were stimulated with STAg they produced comparable levels of IL-12p40 to WT (Fig. 3*A*) and similar results were obtained using BMMΦ (data not shown).

Recent studies demonstrated that injection of naive mice with STAg results in the production of IL-12p40 by CD8α^+ DC in the spleen (14, 24). To determine whether DC production of IL-12 in response to *T. gondii* is c-Rel dependent, WT and c-Rel$^{-/-}$ mice were injected i.p. with STAg, and the systemic levels of IL-12p40 were measured 6 h later and intracellular staining for IL-12p40 was performed on splenic DC. In these studies, the systemic levels of IL-12p40 produced after injection of STAg were comparable (WT = 8.855 ng/ml ± 3.2; c-Rel$^{-/-}$ = 13.7885 ng/ml ± 6.3; three mice in each group; similar results were obtained in four separate experiments). FACS analysis revealed that although there were no IL-12p40$^+$ DC from control mice, the injection of STAg resulted in the emergence of a population of CD8α^+ DC that were positive for IL-12p40 and this was similar in WT and c-Rel$^{-/-}$ mice (Fig. 3*B*). In additional experiments, analysis of splenic DC from WT and c-Rel$^{-/-}$ mice infected with *T. gondii* revealed that comparable numbers of CD8α^+ and CD8α^- splenic DC contained IL-12p40 (CD8α^+CD11c$^+$ WT, 18.56%; c-Rel$^{-/-}$, 13.93%; CD8α^-CD11c$^+$ WT, 6.51%; c-Rel$^{-/-}$, 4.44%,) and that the levels of IL-12p40 produced by these cells were comparable (data not shown).

Neutrophils have recently been shown to contain preformed stores of IL-12 (15, 26) and have been identified as an important source of IL-12 during the early immune response to infection with *T. gondii* (25). To determine whether neutrophils require c-Rel for the production of IL-12p40, the approach described by Denkers and colleagues (15) was used. WT and c-Rel$^{-/-}$ mice were infected i.p. with the RH strain of *T. gondii* and PECs were harvested 4 h later and flow cytometry was used to analyze granulocytes (Gr-1$^+$ cells) for the presence of intracellular IL-12p40. As shown in Fig. 3*C*, similar numbers of Gr-1$^+$IL-12p40$^+$ cells were observed in samples from WT and c-Rel$^{-/-}$ mice. Thus, c-Rel is not required for the ability of neutrophils to produce preformed stores of IL-12p40.

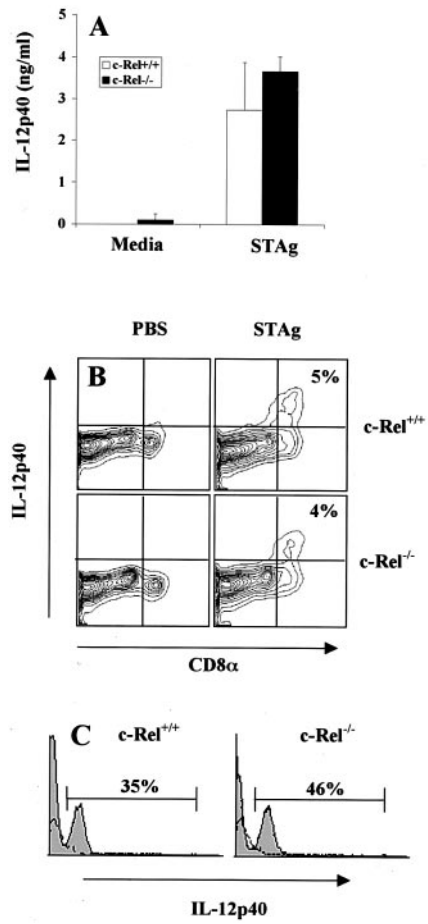

FIGURE 3. c-Rel is not required for the production of IL-12p40 by macrophages, DC and neutrophils in response to *T. gondii*. *A*, TG-elicited PECs (2×10^5) were stimulated in vitro with 25 μg/ml STAg for 72 h. The production of IL-12p40 was measured in culture supernatants by ELISA. Similar results were obtained in three separate experiments with three mice in each group. *B*, Splenic DC were isolated from WT and c-Rel$^{-/-}$ mice 6 h after i.p. administration of 25 μg of STAg. These cells were cultured in vitro for 12 h with brefeldin A (10 μg/ml). Cells were stained with mAbs against CD11c, CD8α, and IL-12p40. CD8α and IL-12p40 expression on CD11c-positive cells was determined using FACS analysis. *C*, WT and c-Rel$^{-/-}$ mice were infected i.p. with 10,000 tachyzoites of the RH strain of *T. gondii*. Four hours later, mice were sacrificed, PECs were harvested, permeabilized, and stained for IL-12 and Gr-1 as previously described (32). Gr-1$^+$ PECs were analyzed for intracellular IL-12p40 by flow cytometry. The Gr-1-expressing subset was gated and IL-12 staining of this population is shown in a histogram format. The gray fill indicates staining with anti-IL-12p40 mAb, and the dashed lines indicate staining with an isotype control. Similar results were obtained in two separate experiments with three mice in each group per experiment.

Discussion

LPS stimulates the release and nuclear translocation of p50/c-Rel and p50/p65 heterodimers in macrophages and both heterodimers

display comparable binding and transactivation potentials for the NF-κB site in the IL-12p40 promoter (9). However, only c-Rel has been shown to be essential for the LPS-induced signaling through TLR4 that leads to the production of IL-12p40 by macrophages (9). The studies reported here extend these findings to include an essential role for c-Rel in IL-12p40 production by macrophages in response to *C. parvum*, CpG containing ODN, anti-CD40, and LMW-HA which signal through TLR2, TLR9, CD40, and CD44, respectively (16–19). TLR are strongly implicated in innate immune recognition and are thought to activate NF-κB via a common pathway (4). Signaling through CD40 and CD44 have also been shown to activate NF-κB (27, 28), and the studies presented here demonstrate a role for c-Rel in their ability to induce IL-12. Thus, together these data place c-Rel at a critical point in innate, TLR-mediated production of IL-12 and indicate that c-Rel is likely to be involved in the regulation of macrophage production of IL-12 during inflammation with consequences for the nature of the subsequently elicited adaptive immune response.

Given the important role of c-Rel in the regulation of innate production of IL-12, the finding that c-Rel$^{-/-}$ mice infected with *T. gondii* produced normal levels of IL-12 and had an IL-12-dependent mechanism of resistance was surprising. The studies presented here demonstrate that the ability of macrophages and neutrophils to respond to *T. gondii* and produce IL-12p40 is independent of c-Rel. In addition, our finding that CD8α^+ DC can produce IL-12p40 in a c-Rel-independent manner is in agreement with the recent findings of Grumont et al. (29). Interestingly, these studies also demonstrated that c-Rel is required for the TLR-mediated production of p35 by CD8α^+ DC (29), suggesting that c-Rel$^{-/-}$ DC produce only inhibitory p40 homodimers and monomers. However, since IL-12p70 is required for resistance to *T. gondii*, the finding that c-Rel$^{-/-}$ mice survive the acute phase of toxoplasmosis indicates that c-Rel-independent production of IL-12p35 occurs in mice challenged with *T. gondii*.

Together, these results suggest the presence of an alternative pathway for the innate recognition of *T. gondii* and production of IL-12 in response to this parasite that is distinct from the Toll-mediated, NF-κB-dependent pathways that have been implicated in the innate recognition of bacterial, fungal, parasitic, and viral infections (1). Support for this conclusion is provided by studies in which mice deficient in NF-κB$_1$, NF-κB$_2$, or RelB can produce normal levels of IL-12 when infected with *T. gondii* (12, 30). Furthermore, recent studies have shown that STAg or infection of cells with *T. gondii* does not induce activation of NF-κB and that this parasite inhibits the ability of infected cells to activate NF-κB in response to LPS (31, 32). However, some pathways that lead to the production of IL-12 in response to LPS and *T. gondii* are shared, since IFN consensus sequence-binding protein, an IFN regulatory factor family member, is required for the production of IL-12 in response to both of these stimuli (33, 34). Since there are a number of intracellular pathogens that evade or actively inhibit activation of NF-κB (35, 36), the identification of a c-Rel-independent pathway for IL-12 production indicates the presence of a unique innate response that allows the development of protective immunity to these pathogens. This c-Rel-independent pathway of IL-12 production may be activated following the engagement of a TLR, but the downstream signaling pathway may be distinct from that observed with TLR2, TLR4, and TLR9. Alternatively, innate recognition of *T. gondii* and its components may occur through a novel receptor that induces production of IL-12 independently of NF-κB.

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