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NF-κB1 Is Required for Optimal CD4+ Th1 Cell Development and Resistance to Leishmania major

David Artis,* Kendra Speirs,† Karen Joyce,* Michael Goldschmidt,* Jorge Caamaño,‡ Christopher A. Hunter,* and Phillip Scott‡*

The NF-κB family of transcription factors regulates the expression of a wide range of immune response genes involved in immunity to pathogens. However, the need for individual family members in regulating innate and adaptive immune responses in vivo has yet to be clearly defined. We investigated the role of NF-κB1 in the induction of protective IL-12-dependent Th1 cell responses following infection with the intracellular protozoan parasite Leishmania major. Whereas wild-type C57BL/6 mice controlled parasite replication, NF-κB1 knockout (KO) mice were susceptible to infection, developing chronic unresolved lesions associated with persistent parasites. There was a profound defect in Ag-specific CD4+ T cell proliferation and IFN-γ production in infected KO mice, although innate responses—including IL-12 production and control of intracellular parasite replication by macrophages—were intact. In vitro polyclonal stimulation of purified naive KO T cells revealed an intrinsic defect in CD4 T cell proliferation associated with reduced IL-2 receptor expression, but operating independently of APC function and IL-2 production. Critically, the frequency of proliferating KO CD4+ T cells secreting IFN-γ matched that of wild-type cells, suggesting that NF-κB1 was not required for efficient transcription of the IFN-γ gene. Taken together, these results identify a novel role for NF-κB1 in CD4+ T cell proliferation and the development of Th1 cell responses required for protective immunity against intracellular pathogens. The Journal of Immunology, 2003, 170: 1995–2003.

The mammalian NF-κB family of transcription factors, composed of NF-κB1 (p50), NF-κB2 (p52), RelA, RelB, and c-Rel, plays a critical role in regulating expression of immune response genes associated with innate and adaptive immunity to pathogens (1, 2). In resting cells, NF-κB is maintained in an inactive form in the cytoplasm bound to a family of proteins termed IκB. After cells are exposed to a broad range of infectious and inflammatory stimuli, these different signals converge at the level of IκB kinase, a complex of three proteins that initiates a cascade of signaling events culminating in the phosphorylation, polyubiquitination, and subsequent degradation of IκB (3–5). Dissociation of NF-κB from IκB unmask the nuclear localization sequence, allowing NF-κB to translocate into the nucleus and initiate gene transcription (6).

The complex role of NF-κB and the specific functions of individual family members in the development and regulation of immune responses following exposure to pathogens have become increasingly apparent as mice with targeted deletions in individual NF-κB family members have become available (reviewed in Refs. 7 and 8). For example, the failure of RelB-deficient mice to control infection with lymphocytic choriomeningitis virus and Toxoplasma gondii (an intracellular protozoan parasite) was associated with defective Th1 cell responses (9, 10). In contrast, IFN-γ responses and the ability to control viral infections were intact in NF-κB2 knockout (KO) (3) and c-Rel KO mice (11–13). Resistance to intestinal helminth infection was also independent of NF-κB2 and c-Rel, but required NF-κB1 (14). Studies have also begun to examine the roles of specific NF-κB family members in the development of protective immunity against the intracellular protozoan parasite Leishmania major. Control of infection requires the production of IL-12 and subsequent expansion of CD4+ Th1 cells secreting IFN-γ (15–17). Mice deficient in NF-κB2 exhibited defective CD40-induced IL-12 responses after L. major infection, resulting in chronic disease, although Th1 cell responses and NO-mediated parasite killing were intact (13). In contrast, susceptibility to L. major infection in c-Rel KO mice was associated with defective NO production and parasite killing (18).

Although the role of NF-κB1 in immunity to L. major has not been examined, previous studies suggest a role for this family member in Th2 cell development. NF-κB1-deficient CD4+ T cells failed to up-regulate GATA-3—a transcription factor critical in Th2 cell development (19, 20)—and exhibited significant defects in secretion of IL-4 and IL-13 following in vitro stimulation (21). KO mice were also defective in the expression of Th2 cytokines in murine models of airway hyperresponsiveness and helminth infection (14, 22). However, the role of NF-κB1 in Th1 cell polarization and IFN-γ production has remained controversial (23). Whereas in vitro studies have reported that expression of T-bet—a critical transcription factor in Th1 cell development (24, 25)—and IFN-γ production were normal in NF-κB1-deficient T cells (21), impaired IFN-γ responses and less severe Th1-mediated pathology have been observed in vivo (26, 27).

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been reported in KO mice in vivo following induction of autoimmunity (26).

Here we investigated the role of NF-κB1 in the development of protective Th1 responses after L. major infection. Whereas wild-type (WT) C57BL/6 mice effectively controlled parasite replication, NF-κB1 KO mice developed chronic lesions associated with persistent parasites. Susceptibility to infection was not associated with defective innate immune responses, as IL-12 production and parasite killing by macrophages were intact. In contrast, CD4+ T cell functions were dramatically impaired in the absence of NF-κB1, with infected KO mice exhibiting low Ag-specific proliferation and IFN-γ responses. In vitro polyclonal stimulation of purified KO T cells demonstrated a proliferation defect in NF-κB1 KO CD4+ T cells associated with defective IL-2R expression. Furthermore, analysis of proliferating KO CD4+ T cells suggested that defects in IFN-γ production were due to defective T cell proliferation, rather than a direct effect on cytokine gene transcription. Taken together, these results suggest that in addition to proliferation, rather than a direct effect on cytokine gene transcription. Signaling event required for optimal entry of CD4+ T cells in the proliferative cycle.

Materials and Methods

Animals

Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). NF-κB1-deficient mice were generated by Dr. W. Sha and Dr. D. Baltimore (Berkeley, CA) (27) and bred at the University of Pennsylvania on a B6 × 129 background or backcrossed at least eight times to C57BL/6. In most experiments, groups of four to six backcrossed female (5–8 wk old) mice were used. No differences were observed between KO mice on a B6 or B6 × 129 background. Animals were maintained in a specific-pathogen-free environment and tested negative for pathogens in routine screening. All experiments were conducted following the guidelines of the University of Pennsylvania institutional animal care and use committee.

Parasite, Ag, and infection

L. major parasites (MHOM/IL/80/Friedlin) were grown in Grace’s insect culture medium (Life Technologies, Gaithersburg, MD) supplemented with 20% heat-inactivated FBS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 100 μM penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO). Amastigote parasites were isolated from lesions of infected recombinant-activating gene KO mice. Soluble leishmanial Ag (SLA) was prepared as previously described (28). Mice were injected in the footpad. To quantify parasites in tissues, single-cell suspensions of lesions were prepared and plated in 10-fold serial dilutions (initial dilution of 1:1000) in Grace’s insect culture medium. Each sample was plated in quadruplicate, and the mean of the negative log parasite titer was determined after 7 days of culture at 26°C.

EMSA

Papolite lymph node (LN) cells were isolated from naive and infected mice, and nuclear extracts were prepared from B cell-depleted LN cultures as previously described (29). In brief, double-stranded oligodeoxynucleotides corresponding to the palindromic B site (5'-GGGATTCCTCC-3') were labeled by filling the overlapping ends with the Klenow fragment of DNA polymerase I and [32P]dCTP. Unincorporated nucleotides were removed, and 10 fmol of labeled oligonucleotide (50,000 cpm) were incubated with 10 μg of protein extracts and 2 μg of poly(dI-dC) in a buffer containing 20 mM HEPEs (pH 7.9), 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, 0.7 mM PMSF, and 17% glycerol in a final volume of 22 μl for 15 min at 20°C. Equivalent free probe was detected in all wells, confirming that probe was added in excess to all samples. Antisera against p50 (SC 114-G) (Santa Cruz Biotechnology, Santa Cruz, CA) were used in supershift assays. Complexes were separated on 5.5% polyacrylamide gels run on 0.25% Tris-borate-EDTA buffer, dried, and exposed to Kodak X-Omat AR film (Rochester, NY) at −70°C.

Histological analysis

Footpad tissues were fixed in 10% neutral buffered formalin, decalcified, routinely processed, and embedded in paraffin. Five micrometer sections were stained with H&E before microscopic evaluation.

Bone marrow cultures, NO production, and in vitro killing assays

Bone marrow-derived macrophages (BMDM) and dendritic cells (DC) were cultured as previously described (30–32). In brief, macrophages were grown in RPMI containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 25 mM HEPEs and supplemented with LPS (400 pg/ml) for 7 days. DC were cultured for 10 days in medium supplemented with rmGM-CSF (PeproTech, Rocky Hill, NJ). Cells were resuspended at 1 × 106 cells/ml in 96-well plates and cultured either in medium alone, in LPS (100 ng/ml) (Sigma-Aldrich), in anti-CD40 mAb (20 μg/ml) (IC10 clone; R&D Systems, Minneapolis, MN), or in cytokine-phosphorothioate-guanine (CpG)- containing oligonucleotide (5'-TTTACGACGTTCGAGCTTGTT-3') (1 μg/ml) (synthesized at the University of Pennsylvania) (33, 34) for 48 h. Nitrite production was determined by the Greiss reaction (35). For in vitro killing assays, adherent macrophages were harvested, resuspended at 1 × 106/ml in polypropylene tubes, and primed with 100 U/ml rmIFN-γ (R&D Systems) for 4 h. Following incubation of suspension cultures with L. major amastigotes (2:1 parasit明年 ratio) for 2 h, parasites were washed to remove extracellular parasites, resuspended in medium containing rmIFN-γ (100 IU/ml), and cultured for 72 h at 34°C as previously described (36). Cytosips were prepared and stained with Hema 3 (Biochemical Sciences, Swedesboro, NJ), and the number of intracellular parasites was determined under light microscopy.

Cell culture and analysis of cytokine secretion

Spleen cells were harvested and single-cell suspensions prepared in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPEs, and 5 × 10−5 M 2-ME. Cells were plated at 4 × 106/ml in 24-well culture plates in medium or SLA (50 μg/ml). In addition, 5 μg/ml anti-IL-1R mAb (M1; a gift from Dr. F. Finkelman, University of Cincinnati, and Dr. C. Maliswjeski, Immunex, Seattle, WA) was added to cultures to enhance detection of IL-4. Supernatants were harvested after 72 h and assayed for cytokine production by sandwich ELISA using paired mAb to detect IL-4 (1B11 and BVD-24G2), IFN-γ (R46A2 and polyclonal rabbit anti-IFN-γ), and IL-12 (C17.8 and C15.6).

CFSE labeling, T cell proliferation, and intracellular cytokine analysis

Cells were harvested from naive and infected mice, and single-cell suspensions prepared. In some experiments, CD3+ T cells were purified using T cell enrichment columns (R&D Systems) following manufacturer’s instructions. Purities obtained using this technique were determined by FACS analysis (see below) and were routinely between 85 and 90%. Proliferation was analyzed by labeling cells with CFSE (Molecular Probes, Eugene, OR), a fluorescent dye that makes cell division visible after stimulation (37). Cells were washed and suspended in PBS at 4 × 106/ml and labeled with CFSE (1.25 μM) for 5 min at 37°C. Labeling was quenched with FBS, and cells were washed twice and resuspended in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPEs, and 5 × 10−5 M 2-ME. Cells were cultured at 2 × 105 cells/well in 96-well U-bottom plates or at 4 × 106/ml in 24-well culture plates (Costar, Cambridge, MA) in medium alone, in SLA (50 μg/ml), or in anti-CD3/anti-CD28 (each at 1 μg/ml) (BD PharMingen, San Diego, CA) for 3–5 days. In some experiments, cells were cultured under Th1 polarizing conditions (rmIL-12, 10 ng/ml; a gift from Dr. S. Wolff and Dr. J. Sypek, Genetics Institute, Cambridge, MA; anti-IL-4 mAb (11B11), 10 μg/ml) or in the presence of exogenous IL-2 (500 IU/ml; Chiron, Emeryville, CA). The reduction in intensity of CFSE labeling, concomitant with cell division, was compared with cells cultured in medium alone. Before harvesting, supernatants were collected for ELISA analysis, and cells were pulsed with PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (10 μg/ml) (Sigma-Aldrich) for 4–5 h. Cells were washed: incubated with Fe block (anti-Fcγ/II/III mAb (2.4G2) (10 μg/ml) and rat IgG (10 μg/ml) for 10 min; surface-stained with anti-CD4, anti-CD25, or isotype control mAb (BD PharMingen); and fixed overnight in 1% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA). Cells were then permeabilized with 0.2% saponin (Sigma-Aldrich) and intracellularly stained with anti-IL-2, anti-IFN-γ, or isotype control mAb (BD PharMingen). Cells were washed, and 1 × 104
live CD4⁺ T cells were acquired on a FACSCalibur cytometer (BD Biosciences, San Jose, CA). Acquisition and analysis were conducted using CellQuest software (BD Biosciences). All dot plots shown have a log axis of 10⁻²⁻¹⁰⁷.

Results

NF-κB1 is activated following L. major infection of resistant B6 mice

Previous studies have shown that NF-κB is activated in vitro or in vivo after exposure to a range of pathogens including viruses, bacteria, and parasites (7, 8). To determine whether infection of resistant B6 mice with L. major resulted in the activation of NF-κB, nuclear extracts were prepared from B cell-depleted lymphocyte suspensions isolated from naive and infected mice, and κB binding activity was analyzed by EMSA. As shown in Fig. 1, NF-κB binding was not detected in nuclear extracts isolated from naive mice (samples from naive mice were collected on day 0 and day 42 p.i.; only those from day zero are included, because equivalent results were obtained with both). In contrast, NF-κB binding was detected in the draining LN of infected mice as early as day 3 p.i. and remained at day 42. A 100-fold molar excess of unlabeled competitor oligonucleotide was routinely used in competition assays and efficiently blocked detection of NF-κB binding (data not shown). Addition of anti-p50 mAb resulted in a shift in NF-κB complexes at both time points, demonstrating that NF-κB-containing complexes were activated after L. major infection (Fig. 1). The presence of two distinct bands implies that at least two different NF-κB complexes were activated following infection.

NF-κB1 KO mice fail to control L. major infection

We investigated whether the deletion of NF-κB1 would affect the outcome of L. major infection using gene-targeted mice. In WT mice, lesion development peaked between wk 3 and 4 and resolved by wk 12 p.i. (Fig. 2A). In contrast, NF-κB1 KO mice developed chronic lesions that failed to resolve by wk 18 p.i. Interestingly, lesion development in infected KO mice was significantly delayed in the first 4 wk p.i. (Fig. 2A). Because infection of KO mice is associated with defective CD4⁺ T cell proliferation (see below), this observation is consistent with previous reports demonstrating that early lesion development after Leishmania infection is a CD4⁺ T cell dependent phenomenon (13, 38–40). At wk 10 p.i., cutaneous lesions in WT mice were resolving (Fig. 2A). Histological evaluation demonstrated the presence of few viable parasites and a small number of inflammatory cells in the dermis, typical of resistant strains at this time point (Fig. 2B). In contrast, foci of inflammatory cells (composed of granulocytes, lymphocytes, and macrophages), tissue necrosis, and fibrin deposition were present in the dermal layer of lesions isolated from infected KO mice (Fig. 2C). The epidermis was also thickened, with evidence of microabscesses, focal necrosis, and spongiosis. Numerous viable intracellular parasites were observed within epidermal keratinocytes (Fig. 2D, arrows), suggesting that a reservoir of infection existed in the epidermal layer of infected KO mice.

Consistent with the development of chronic lesions (Fig. 2A) and the presence of parasites in histological sections from KO mice (Fig. 2, C and D), quantification of parasite numbers in the footpad by limiting dilution demonstrated that KO mice had significantly more parasites than WT mice had beyond wk 4 p.i. (Fig. 2E). By wk 10 p.i., parasite numbers in WT mice had fallen below the limit of detection, while viable promastigotes were detected at 10⁻⁸ dilutions in lesions isolated from KO mice (Fig. 2E). Interestingly, the number of parasites did not become progressively greater in the absence of NF-κB1 (as is the case in susceptible BALB/c or recombination-activating gene KO mice), demonstrating that NF-κB1 KO mice were able to control parasite replication to some extent—although the response was not sufficient to mediate disease resolution.

Intact macrophage function in NF-κB1 KO mice

Previous studies demonstrated that disruption of NF-κB activation can impair the production of NO (41), an effector molecule critical to the control of leishmanial infection. B1 is activated in vitro or in vivo after infection of resistant strains at this time point (Fig. 2A). In contrast, foci of inflammatory cells in the dermis, typical of chronic lesions, were present in cutaneous lesions isolated from infected KO mice (Fig. 2C). The epidermis was also thickened, with evidence of microabscesses, focal necrosis, and spongiosis. Numerous viable intracellular parasites were observed within epidermal keratinocytes (Fig. 2D, arrows), suggesting that a reservoir of infection existed in the epidermal layer of infected KO mice.
to the control of *L. major* infection (42, 43). Therefore, we investigated whether NF-κB1-deficient macrophages were defective in NO production and the control of *L. major* replication in vitro. Following stimulation of IFN-γ-activated BMDM with either LPS or CpG, nitrite production was greater in cultures of KO macrophages than in WT (Fig. 3A). To investigate whether the absence of NF-κB1 altered the control of *L. major* replication in vitro, IFN-γ-activated KO and WT macrophages were infected with *L. major* and cultured for 72 h. Consistent with intact NO responses (Fig. 3A), no significant differences were observed in the number of parasites per 100 KO or WT macrophages examined, suggesting equivalent control of parasite replication by KO and WT cells (Fig. 3B). In addition, equivalent control of parasite replication was observed in infected KO and WT macrophages treated with LPS (data not shown). Together, these results suggested that activated NF-κB1-deficient macrophages were able to produce NO and to control growth of intracellular *L. major*.

**Defective Ag-specific IFN-γ, but not IL-12 responses, in L. major infected NF-κB1 KO mice**

Analysis of cytokine responses at wk 2 and 8 p.i. revealed significantly lower levels of Ag-induced IFN-γ in cultures of KO cells compared with WT cells (Fig. 4, A and B). Flow cytometric analysis of lymphocyte cultures also demonstrated that a significantly lower percentage of KO as opposed to WT CD4+ T cells had shifted in their forward scatter (KO, 10.8 ± 1.5%; WT, 48.6 ± 7.4%) (Fig. 4, C and D), an indication that the proportion of cells undergoing proliferation was lower in KO cultures (see below). In addition, intracellular cytokine staining showed a significantly lower frequency of KO CD4+ T cells staining positive for IFN-γ at wk 8 p.i. (KO, 1.4 ± 0.1%; WT, 23 ± 5%) (Fig. 4, C and D). However, the failure of KO mice to control infection was not due to the development of a nonprotective Th2 response, because the levels of IL-4 were either lower (consistent with previous reports of defective Th2 response in NF-κB1 KO mice) (14, 21) or equivalent following stimulation of KO and WT cells (Fig. 4, E and F).

The development of protective IFN-γ responses following *L. major* infection is dependent on IL-12 production (44–46). Because previous studies have shown that NF-κB family members, notably c-Rel and NF-κB2, can regulate IL-12 p40 production in response to certain stimuli (13, 47, 48), we analyzed infection-induced IL-12 p40 levels in NF-κB1 KO mice. No significant differences in the production of IL-12 were observed between KO and WT mice at wk 4, 8, and 12 p.i. (Fig. 5, A–C), suggesting that NF-κB1 is not essential for IL-12 responses after *L. major* infection. IL-12 responses were also normal following in vitro stimulation of NF-κB1 KO BMDM or DC with LPS or anti-CD40 mAb (Fig. 5, D and E), both potent inducers of IL-12. In addition, in vivo serum IL-12 p40 responses following systemic exposure to LPS or anti-CD40 mAb were equivalent in KO and WT mice (LPS stimulation: KO, 157.0 ± 7.1 ng/ml; WT, 105.3 ± 1.8 ng/ml; anti-CD40 stimulation: KO, 143.0 ± 8.5 ng/ml; WT, 159.0 ± 13.5 ng/ml). Taken together, these results suggested that the defective development of Ag-specific CD4+ T cells secreting IFN-γ in *L. major*-infected NF-κB1 KO mice was not due to a deficiency in IL-12 production.

**NF-κB1 KO CD4+ T cells have an intrinsic defect in proliferation**

Previous studies have shown that the expression of IFN-γ is closely linked to lymphocyte proliferation (49, 50). Therefore, we investigated the proliferative capability of NF-κB1-deficient CD4+ T cells isolated from *L. major*-infected mice. Proliferation following ex vivo exposure to Ag was determined by evaluating CFSE dilution of CD4+ T cells using flow cytometry. A clear shift...
FIGURE 5. IL-12 production is normal in NF-κB1 KO mice. KO and WT mice were infected with 5 × 10⁶ L. major promastigotes in the left hind footpad. At wk 4 (A), 8 (B), and 12 (C) p.i., spleen cells were isolated and cultured in medium (med) or SLA for 72 h. BMDM (D) and DC (E) were isolated from naive KO and WT mice and cultured in medium, LPS, or αCD40 mAb for 48 h. Supernatants were assayed for IL-12 p40 production by sandwich ELISA. Values represent the mean ± SD for four or five mice per group.

in CFSE profile was observed in CD4⁺ T cells from infected WT mice as early as 2 wk p.i., and this shift grew in magnitude as the infection progressed (Fig. 6, A and B). In contrast, a profound defect in proliferation was detected in KO CD4⁺ T cells throughout the course of infection (Fig. 6, A and B).

To further investigate the defect in CD4⁺ T cell proliferation, spleen cells were isolated from naive NF-κB1 KO and WT mice, labeled with CFSE, and stimulated with soluble anti-CD3/anti-CD28 under neutral and Th1 polarizing conditions. As shown in Fig. 7, the dilution of intracellular CFSE staining distinguished individual generations of dividing cells 3 days after stimulation. At least four generations of dividing CD4⁺ T cells were visible in WT cultures, while the majority of NF-κB1 KO CD4⁺ T cells had only progressed as far as the second proliferative generation (Fig. 7A). We observed similar results using anti-CD3 stimulation alone (data not shown). To determine whether the reduced proliferation observed in NF-κB1-deficient CD4⁺ T cells was related to defective growth or survival signals from APC or from accessory cells, CD3⁺ T cells were purified from KO and WT mice and stimulated with plate-bound anti-CD3/anti-CD28 for 3 days. Consistent with our results with whole spleen cell cultures, four generations of proliferating CD4⁺ T cells were visible in cultures of purified T cells from WT mice, while only two generations were discernable in KO cultures (Fig. 7B)—demonstrating that NF-κB1-deficient CD4⁺ T cells had an intrinsic defect in their ability to proliferate following stimulation through the TCR and CD28.

Production of IFN-γ by proliferating NF-κB1 KO CD4⁺ T cells is intact following in vitro polyclonal stimulation

In addition to regulating T cell proliferation, NF-κB can directly regulate expression of IFN-γ through κB binding sites in the IFN-γ promoter (51). We investigated whether the defects in IFN-γ production after stimulation were due to a direct effect on IFN-γ gene transcription or were an indirect effect of defective T cell proliferation. Spleen cells were cultured with CFSE and stimulated with anti-CD3/anti-CD28; the frequency of proliferating CD4⁺ T cells expressing IFN-γ was determined by flow cytometry. Confirming our previous results, the frequency of NF-κB1 KO CD4⁺ T cells undergoing proliferation was lower than that observed in cultures of WT cells (Fig. 8, A and B). However, the frequency of proliferating CD4⁺ T cells that produced IFN-γ was similar in KO and WT cultures under both neutral and Th1 polarizing conditions (Fig. 8, A and B).

To further examine whether IFN-γ responses were equivalent in proliferating KO and WT CD4⁺ T cells, we analyzed the fluorescence intensity of the IFN-γ-positive cells from KO and WT T cell cultures. Despite equivalent frequencies of IFN-γ-positive proliferating cells, the mean fluorescence intensity (MFI) of the IFN-γ signal was lower in proliferating KO than in WT cultures (KO, 41; WT, 51). This difference may have been the result of reduced transcription of the IFN-γ gene in KO T cells or because IFN-γbright T cells (i.e., those with the highest MFI) were found predominantly in the fourth generation in WT cultures, a population that was absent in cultures of KO T cells. Therefore, the reduced MFI of IFN-γ staining in KO T cells may have been the result of impaired proliferation. To test this, we conducted T cell generation-specific analysis of the fluorescence intensity of IFN-γ staining. We found that the geometric mean of IFN-γ staining was...
equivalent in the first, second, and third generations of KO and WT cultures (first generation: KO, 39.7; WT, 34.3; second generation: KO, 40.9; WT, 42.5; third generation: KO, 47.5; WT, 56.5). The intensity of IFN-γ/H9253 staining increased markedly in the fourth generation of proliferating WT T cells (83.9), suggesting that the reduced overall MFI of IFN-γ staining observed in KO T cells was the result of reduced T cell proliferation. Together, the similar frequency of proliferating T cells secreting IFN-γ and the equivalent MFI of the IFN-γ signal between KO and WT CD4+ T cells in early generations provide compelling evidence that defective IFN-γ production in NF-κB1 KO mice was a result of defective CD4+ T cell proliferation and was not related to direct transcriptional regulation of the IFN-γ gene. Furthermore, these results indicated that IL-12R expression and responsiveness were intact in KO CD4+ T cells.

**FIGURE 8.** Production of IFN-γ by proliferating NF-κB1 KO CD4+ T cells is intact after in vitro polyclonal stimulation. Spleen cells were isolated from naive KO and WT mice, labeled with CFSE, and cultured for 3 days with anti-CD3 and anti-CD28 under neutral or Th1 polarizing conditions. Cells were stained for surface CD4 and intracellular IFN-γ. Proliferation and IFN-γ production in WT (A) and KO (B) CD4+ T cells were analyzed by flow cytometry. Generation-specific analysis was conducted to determine the geometric MFI of IFN-γ-positive cells in individual proliferating generations. Numbers represent the frequency of proliferating CD4+ T cells staining positive for IFN-γ. Results are representative of three experiments. ND, Not done.

**FIGURE 9.** IL-2R expression is impaired in NF-κB1 KO CD4+ T cells following in vitro polyclonal stimulation. Spleen cells were isolated from naive KO and WT mice and cultured for 3 days in medium or in anti-CD3 and anti-CD28. Cells were stained for CD4, intracellular IL-2 (A), and surface CD25 (C). Exogenous IL-2 was administered to polyclonally stimulated KO and WT T cells, and proliferation was analyzed by dilution of CFSE. Numbers in A and C denote the frequency of positively stained CD4+ T cells and are representative of two experiments.

**IL-2R expression is impaired in NF-κB1 KO CD4+ T cell following in vitro polyclonal stimulation**

NF-κB family members play a critical role in regulating the expression of IL-2 and IL-2Rα (CD25) genes after T cell activation (52–54). Therefore, we investigated whether a deficiency in the expression of IL-2 or its receptor may have been responsible for the defects in T cell proliferation observed in NF-κB1 KO mice. Following stimulation of whole spleen cultures, the frequency of CD4+ T cells staining positive for intracellular IL-2 was higher in KO cells than in WT cells (66% vs 41%) (Fig. 9A). Elevated levels of secreted IL-2 were also detected by ELISA in culture supernatants from KO cells after polyclonal stimulation for 3 days (KO, 3180 pg/ml; WT, 520 pg/ml). These results suggested that defects in IL-2 production were not the basis for defective CD4+ T cell proliferation in KO T cells. Supporting this hypothesis, administration of exogenous IL-2 enhanced proliferation in WT T cell cultures but failed to reverse the proliferation defect in NF-κB1 KO T cell cultures (Fig. 9B). Because IL-2 production is an early event in T cell activation, occurring in the G0-S phase (55), these results also seemed to show that early T cell activation was normal in KO mice.

In contrast to the intact IL-2 responses in NF-κB1 KO T cells, there was a significant defect in the ability of KO CD4+ T cells to
up-regulate CD25 following stimulation. Whereas 53% of proliferating WT CD4^+ T cells expressed high levels of CD25, only 25% did so in KO cultures, with significantly more KO CD4^+ T cells expressing only intermediate levels of CD25 (63% in KO cultures compared with 38% in WT cultures) (Fig. 9C). Histogram analysis also demonstrated that the MFI of IL-2R staining over background was 39% lower on stimulated KO CD4^+ T cells than on WT CD4^+ T cells (KO, 401.0 ± 17.1; WT, 655.1 ± 118.7). This observation provides a possible explanation as to why KO T cells failed to respond to exogenous IL-2 (Fig. 9B). Collectively, these results identified an intrinsic proliferative defect in NF-κB1-deficient CD4^+ T cells, operating independently of APC function and IL-2 production. Furthermore, while early events in T cell activation remained intact, NF-κB1 KO CD4^+ T cells exhibited a defect in their ability to up-regulate expression of CD25.

**Discussion**

There is increasing evidence that NF-κB family members play unique roles in regulating innate and adaptive immune responses to a range of pathogens. In the case of NF-κB1, previous studies have demonstrated its importance in innate responses to viral and bacterial infection and in the expression of Th2 cytokines following helminth infection or induction of airway hyperresponsiveness (14, 22, 27). In this report we extend these observations, identifying a novel role for NF-κB1 in the development of polarized Th1 cytokine responses and resistance to the intracellular protozoan parasite *Leishmania major*. Mice deficient in NF-κB1 developed chronic infections associated with a failure to control protozoan replication. Although IL-12 responses and parasite killing were independent of NF-κB1, KO mice exhibited a profound defect in the development of Ag-specific CD4^+ T cells secreting IFN-γ. Polyclonal activation of naïve KO T cells demonstrated a specific requirement for NF-κB1 in regulating CD4^+ T cell proliferation in response to stimulation through the TCR and CD28. These results suggest that NF-κB1 may be critical to the upstream signaling events involved in optimal T cell responsiveness to IL-2 and entry into the proliferative cycle, which is necessary for the subsequent development of polarized Th cell responses.

Susceptibility to *L. major* infection has also been reported in mice deficient in NF-κB2 or c-Rel. Chronic infection in NF-κB2 KO mice was associated with defective CD40-induced IL-12 responses, while susceptibility to infection in c-Rel KO mice was attributed to defective NO production and parasite killing (13, 18). Therefore, while innate responses operating against *L. major*, including IL-12 production and parasite killing, were partially dependent on NF-κB2 and c-Rel, respectively, they were independent of NF-κB1. In contrast, the results presented here identify an essential role for NF-κB1 in adaptive immune responses to *L. major*, because susceptibility to infection in NF-κB1 KO mice was associated with a marked defect in the expansion of Ag-specific CD4^+ T cells secreting IFN-γ. Together, these studies highlight the complex regulatory role that different NF-κB family members play in controlling innate and adaptive responses after infection with intracellular pathogens.

Our results demonstrating that NF-κB1 is required for the development of polarized Th1 responses in conflict with a previous in vitro study that concluded that Th1 cell differentiation was intact in NF-κB1 KO CD4^+ T cells (21). However, these differences may reflect the genetic background of the mice, or the magnitude and duration of the stimulation used in the different studies. A role for NF-κB1 in regulating T cell proliferation and Th1 responses is supported by studies in a murine model of collagen-induced arthritis. In the absence of NF-κB1, IFN-γ production and the severity of Th1-mediated pathology were significantly reduced (26). In addition, the original description of NF-κB1 KO mice reported that KO T cells exhibited a proliferation defect, although no data were included (27). To our knowledge, this is the first detailed analysis of proliferation and its effect on IFN-γ production in NF-κB1-deficient CD4^+ T cells.

The importance of other NF-κB family members in regulating T cell proliferation has been documented in a number of studies. Analysis of IκB transgenic T cells that express a degradation-deficient form of IκB, which efficiently blocked c-Rel and RelA activation, demonstrated the importance of NF-κB for optimal T cell proliferation (56–58). Similar defects were observed in T cells isolated from mice individually deficient in RelA or c-Rel (59–61). Here we extend these studies, identifying a critical role for NF-κB1 in optimal CD4^+ T cell proliferation. Although the mechanisms underlying defective T cell proliferation once the NF-κB pathway is disrupted are not fully understood, the presence of κB binding sites in the promoters of the IL-2 and CD25 genes (both crucial for optimal T cell activation and homeostasis) (62, 63) suggest that NF-κB may regulate the expression of these genes following TCR ligation. However, IL-2 and CD25 levels were normal in RelA-deficient T cells, indicating that this family member was not essential to the control of these genes (60). In c-Rel-deficient T cells, IL-2 production was reportedly defective, while conclusions vary on the expression of CD25 and the responsiveness to exogenous IL-2 (59, 61, 64). Unlike T cells deficient in c-Rel or RelA, the frequency of NF-κB1-deficient T cells secreting IL-2 in our experiments was greater than that observed in WT cultures following stimulation through the TCR and CD28. This result is supported by previous studies that suggest that NF-κB1 acts as a transcriptional repressor of cytokine genes under certain circumstances (65–68). Furthermore, while expression of CD25 has been reported to be normal in other NF-κB KO mice, our results show that NF-κB1 is critical for the optimal up-regulation of CD25. A defect in CD25 expression and IL-2 responsiveness is also supported by our results, which demonstrated that proliferation in KO CD4^+ T cell cultures did not improve after administration of exogenous IL-2. Taken together, these results identify distinct mechanisms that underlie the defective proliferation observed in T cells deficient in individual NF-κB proteins, highlighting the elaborate role the NF-κB family plays in the regulation of immune response genes after T cell activation.

NF-κB has also been implicated in the regulation of signaling downstream of the IL-2R (56, 58, 69); in addition to defective CD25 expression, NF-κB1 KO T cells may exhibit defects in the downstream signaling events that follow activation through CD25. Indeed, an attractive hypothesis is that NF-κB1 regulates STAT5a activation, a transcription factor required for IL-2-mediated induction of TD2 (70). In support of this hypothesis, it has been found that CD4^+ T cells deficient in STAT5a were also deficient in CD25 but not in IL-2 expression (70)—an effect similar to the phenotype we observed in NF-κB1 KO T cells. Alternatively, the defects in T cell proliferation in the absence of NF-κB1 may involve pathways independent of CD25 expression and the activation of STAT5a. For instance, NF-κB may directly or indirectly regulate other early events in T cell activation, including control of cyclin-dependent kinases. NF-κB1, in connection with other family members, has been shown to associate with cyclin E/Cdk2, the nuclear abundance of which peaks in G1/S—concomitant with irreversible commitment to DNA synthesis and cell division (71, 72). Other studies have demonstrated that NF-κB activation is linked to cell cycle progression through transcriptional activation of cyclin D, another cyclin required for passage through early cell cycle checkpoints (73, 74). However, the specific role of NF-κB1
in regulating cyclin expression and other cell cycle machinery after T cell activation remains to be defined.

The failure of NF-κB1 KO mice to mount protective Th1 cytokine responses sufficient to resolve L. major infection may also have implications for understanding the molecular mechanisms that underlie chronic leishmaniasis. Murine models of cutaneous and visceral leishmaniasis posited a critical role for IL-2/IL-2R interactions in promoting optimal T cell responses, IFN-γ and parasite killing (75–78). In addition, nonhealing diffuse cutaneous lesions reported in human patients have been associated with reduced IL-2 and/or CD25 expression and defective T cell responses (79, 80). Therefore, aberrant NF-κB1 activation may provide a molecular trigger for the development of anergic T cell responses and chronic disease in humans. Taken together, these results suggest that targeting NF-κB1 levels may prove useful in manipulating a range of Th cell-mediated inflammatory and pathologic conditions associated with anergic responses, autoimmunity, and chronic inflammation.

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References


