

LF15-0195 generates tolerogenic dendritic cells by suppression of NF- κ B signaling through inhibition of IKK activity

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Abstract: LF15-0195 (LF) is a potent, less toxic analog of the immunosuppressant 15-deoxyspergualine, which we previously reported to prevent graft rejection and to induce permanent tolerance in a murine cardiac transplantation model. However, the underlying mechanism of action of LF required elucidation. In this study, dendritic cells (DC) treated with LF before activation with tumor necrosis factor α (TNF- α)/lipopolysaccharide (LPS) failed to express maturation markers (major histocompatibility complex II, CD40, CD86) and interleukin-12. LF prevented, in a concentration-dependent manner, the activation and nuclear translocation of nuclear factor- κ B (NF- κ B) in DC following addition of TNF- α /LPS. Yet-activated and active I κ B kinases (IKKs) were inhibited in cells pretreated with LF, thereby preventing the phosphorylation of I κ B and release of NF- κ B, a key regulator of genes associated with the maturation of DC. LF-induced inhibition of IKK activity was reversed in a dose-dependent manner by the overexpression of IKK. The T helper cell type 2 (Th2) differentiation of naïve T cells promoted by LF-treated DC in vitro correlates with Th2 polarization observed in transplant recipients made tolerant by LF. These data demonstrated that LF-induced blockade of NF- κ B signaling at the level of IKK promoted the generation of tolerogenic DC that inhibited Th1 polarization and increased Th2 polarization in vitro and in vivo. *J. Leukoc. Biol.* 74: 438–447; 2003.

Key Words: transplantation tolerance · immune modulation · cytokines

INTRODUCTION

Dendritic cells (DC) are the most potent stimulators of T cell activation, as the only antigen-presenting cell (APC) that can initiate immune responses from naïve T cells [1]. A unique feature of DC that allows for potent immune stimulation is their

provision of three signals to T cells: an antigen-specific signal (signal 1) provided by major histocompatibility complex (MHC)–T cell receptor (TCR), a costimulatory signal (signal 2) provided by CD40, CD80, and CD86, and a differentiation signal (signal 3) provided by interleukin (IL)-12 [2, 3]. The stage of DC maturation is an important factor for the immune-initiating capacity of these cells. Although mature DC are immune-stimulatory, immature DC possess immune-regulatory properties as a result of low expression of MHC II and costimulatory molecules. Activation of naïve T cells in the absence of signal 2 or signal 3 results in T cell anergy and apoptosis [4]. Furthermore, immature DC express little or no IL-12, which causes the naïve T cell to acquire a T helper cell type 2 (Th2) cytokine profile [5–8]. Thus, immature DC are considered as “tolerogenic DC” and have been used for inducing tolerance to prevent progression of autoimmune diseases and rejection of transplant grafts [9–11].

Recent research revealed that DC maturation is associated with activation of the nuclear factor- κ B (NF- κ B) pathway [12, 13]. Inflammatory stimuli such as lipopolysaccharide (LPS), tumor necrosis factor α (TNF- α), and IL-1 induce DC maturation through activation of this pathway. When NF- κ B activation is inhibited pharmacologically or by overexpression of inhibitory proteins, DC maturation in response to stimuli is prevented [14]. In nonactivated, immature DC, I κ Bs, a family of inhibitory proteins, sequester NF- κ B in the cytoplasm [15]. Upon activation of DC, upstream signaling cascades converge to activate I κ B kinases (IKKs), which phosphorylate I κ Bs. When phosphorylated, I κ B is rapidly ubiquitinated and targeted for proteosomal degradation, thereby releasing NF- κ B, which then translocates to the nucleus. In the nucleus, NF- κ B interacts with κ B sites in regulatory regions of target genes to control expression [15]. Inhibition of NF- κ B activation in DC results in reduced expression of T cell stimulatory molecules such as MHC II, CD40, CD80, and CD86 [13]. Therefore, blockade of NF- κ B signaling in DC using a pharmacologically

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acceptable drug would be a practical approach to generate immature tolerance-promoting DC in vitro and in vivo.

15-Deoxyspergualin (DSG), an immunosuppressive drug that is currently undergoing clinical testing [16], has been recently reported to inhibit NF- κ B activity in a pre-B cell line [17]. Recently, LF15-0195 (LF) was reported to directly induce apoptosis of T cells through facilitating the recruitment of CD95, Fas-associated death domain, and procaspase-8 to the death-inducing signaling complex and through enhancing caspase-8 and -10 activation [18]. However, in contrast to conventional immune suppressants that specifically target T cell function, DSG is a poor inhibitor of direct T cell responses stimulated by mitogens [19] and alloantigens [20]. Therefore, DSG may mediate its immune-regulatory effects by mainly targeting the APC. In support of this notion, DSG-treated APC are poor allostimulators of the mixed lymphocyte reaction (MLR), suggesting inhibition of APC instead of T cell function [21]. Recent studies have suggested that DSG induces tolerance, in part, through targeting DC function by suppressing production of signal 3 by DC [22] or preventing the differentiation of immature DC, which lack signal 2 [23]. However, no mechanistic studies on DC have been performed.

LF, a novel analog of DSG, is less toxic but has greater immune-suppressive activity. We and others have demonstrated that a short course of LF treatment induced donor-specific tolerance in cardiac transplants through generation of immature tolerogenic DC [24–26]. However, the mechanism by which LF prevents DC maturation remains unknown. Therefore, in this study, we investigated the molecular components that LF may target when blocking DC maturation.

In this study, we have demonstrated that LF blocked DC maturation, functionally and phenotypically, through the inhibition of NF- κ B activation. In cell-free reconstitution and IKK overexpression assays, we found LF directly inhibited IKK. Inhibition of IKK activity prevented phosphorylation of I κ B α and thus blocked the nuclear translocation of NF- κ B. Furthermore, LF-treated DC promoted Th2 differentiation in vitro. Th2 polarization was observed in allograft recipients made tolerant by LF. Thus, LF promotes tolerogenic DC in vitro and in vivo through the specific inhibition of NF- κ B activation.

MATERIALS AND METHODS

Reagents

LF was provided by the Laboratoires Fournier (Daix, France). LPS from *Escherichia coli* serotype 0127:B8 was purchased from Sigma (Mississauga, ON).

DC cultures

An immature DC line established from a C57BL/6 mouse, designated JAWS II, was obtained from American Type Culture Collection (Manassas, VA). JAWS II cells were cultured in α -minimal essential medium without ribonucleosides and deoxyribonucleosides (Gibco Invitrogen Life Technologies, Burlington, ON) containing 4 mM L-glutamine, 1 mM sodium pyruvate, 5 ng/ml murine granulocyte macrophage-colony stimulating factor (mGM-CSF; Peprotech, Rocky Hill, NJ), and 20% fetal bovine serum (Gibco Invitrogen Life Technologies). Incubating JAWS II cells with 20 μ g/ml LF for 72 h and performing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Roche Diagnostics, Laval, Quebec) assessed cytotoxicity of LF.

Primary DC were generated from murine bone marrow (BM) as described previously [27]. In brief, BM cells were flushed from the femurs and tibias of tolerant, rejecting, and naive C57BL/6 mice and were washed and cultured at 4×10^6 cells per well in six-well plates (Corning, Corning, NY) in 4 ml RPMI 1640 (Gibco Invitrogen Life Technologies) supplemented with 10% fetal calf serum (Gibco Invitrogen Life Technologies), 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ M 2-mercaptoethanol (Gibco Invitrogen Life Technologies), 10 ng/ml recombinant (r)mGM-CSF (Peprotech), and 10 ng/ml IL-4 (Peprotech). Nonadherent cells were removed after 48 h of culture, and fresh medium was added every 48 h. DC were used for in vitro experiments after 7 days of culture.

Antibodies

Anti-IKK α (H-744), IKK β (H-470), I κ B α (C-21), NF- κ B p65 (C-20), and NF- κ B p50 (nuclear localization sequence) antibodies, as well as secondary antibodies, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse CD11c, anti-mouse I-A^b, anti-mouse CD86, and anti-mouse CD40 were obtained from BD PharMingen (San Diego, CA).

Flow cytometry

Phenotypic analysis of DC was performed on a FACScan (Becton Dickinson, San Jose, CA). DC were pretreated with LF at different concentrations for 4 h followed by addition of TNF- α (10 ng/ml) and LPS (10 ng/ml) for 24 h. The cells were stained with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAb) against marker surface antigens: anti-mouse CD11c, anti-mouse I-A^b, anti-mouse CD86, and anti-mouse CD40. The same isotype immunoglobulins (Cedarlane Laboratories, Mississauga, ON) were used as controls.

MLR

DC were treated with or without LF (10 μ g/ml) for 4 h followed by addition of TNF- α (10 ng/ml), LPS (10 ng/ml), and IL-4 (10 ng/ml) for 24 h. LF-treated DC were irradiated (3000 rad) and plated in a 96-well plate (0.5×10^5 cells/well). BALB/c spleen cells (5×10^5 /well), isolated by gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Baie D'Urfé, Quebec), were added as responders. The MLRs were cultured at 37°C for 72 h and pulsed with 1 μ Ci/well ³H-thymidine (Amersham Pharmacia Biotech) in the last 16 h. Cultures were harvested, and the incorporation of ³H-thymidine was determined with a Wallac Betaplate liquid scintillation counter.

Immunoblot analysis

Cytoplasmic extracts were prepared from LF-treated and nontreated DC mechanically released from tissue-culture plates by scraping in cold phosphate-buffered saline. Cells were collected by centrifugation (800 g) and were then resuspended in buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% Nonidet P-40 (NP-40), 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] with CompleteTM protein inhibitor (Roche Diagnostics). Nuclei were collected by microcentrifugation (10,000 g) at 4°C and were washed twice in buffer A. Nuclei were lysed by shaking vigorously in buffer B [20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF] at 4°C for 20 min, and the resulting nuclear extracts were cleared by microcentrifugation (10,000 g) at 4°C for 10 min. Protein content was determined (Bio-Rad Laboratories, Mississauga, ON), and 40 μ g each cell lysate was resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane (Bio-Rad Laboratories), blocked with 5% fat-free milk (Carnation) in Tris-buffered saline-Tween 20, probed with the appropriate antibodies according to the manufacturer's instructions, and visualized by enhanced chemiluminescence (ECL) assay (Amersham Pharmacia Biotech).

Immunoprecipitation and in vitro kinase assay

rI κ B α substrate was produced from an expression vector for glutathione S-transferase (GST) protein fused to amino acids 1–54 of I κ B α (GST-I κ B α) as described previously [28]. GST-I κ B α full-length (1–317) fusion protein was purchased from Santa Cruz Biotechnology. To analyze IKK activity, 200 μ g cytoplasmic extracts prepared using the procedures described for immunoblot analysis were incubated with 1 μ g IKK α and IKK β polyclonal antibodies

(Santa Cruz Biotechnology) and 60 μg Protein A/G agarose-conjugated beads (Santa Cruz Biotechnology) for 3 h or overnight at 4°C. After washing with buffer C [50 mM HEPES (pH 7.0), 250 mM NaCl, 5 mM EDTA, and 0.1% NP-40] twice and kinase buffer once, the beads were incubated with 20 μl kinase buffer [20 mM HEPES (pH 7.4), 10 mM MgCl_2 , 2 mM MnCl_2 , 25 mM β -glycerophosphate, 4 mM NaF, 0.1 mM sodium orthovanadate, and 1 mM DTT] containing 100 μM adenosine 5'-triphosphate (ATP), 5 μCi γ -[^{32}P] ATP, and 1 μg bacterially expressed GST-I κ B α as a substrate of the IKKs at 30°C for 30 min. The reaction was resolved as for immunoblot analysis and visualized by autoradiography. The same membranes were immunoblotted using the polyclonal antibodies to IKK α and IKK β to normalize the kinase activities.

To overexpress IKK, the JAWS II DC line was grown at 80% confluency in 100-mm culture dishes and transfected with increasing amounts of an IKK β expression vector (pcDNA3.1-IKK β), kindly provided by Dr. Hiroaki Sakurai [29] using FuGENE 6 (Roche Diagnostics) following the manufacturer's protocol. All cells were transfected with equivalent amounts of DNA (10 μg) with the empty parental vector, pcDNA3.1, making up the difference for cells transfected with less than 10 μg pcDNA3.1-IKK β . After 36 h, transfected DC were treated with 10 $\mu\text{g}/\text{ml}$ LF for 4 h followed by TNF- α /LPS (10 ng/ml) for 30 min. Cell extracts were prepared, and IKK activity was determined as described above.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts of LF-treated and nontreated DC were prepared using the NE-PER nuclear extract kit (Pierce, Rockford, IL). Double-stranded NF- κ B consensus oligonucleotide (5'-AGTTGAGGGACTTCCCAGGC-3', Promega, Madison, WI) was labeled with γ -[^{32}P] ATP (Amersham Pharmacia Biotech), and 10^5 cpm-labeled NF- κ B oligonucleotides were used in binding reactions with 5 μg nuclear extract at room temperature for 30 min as per the manufacturer's instructions (Promega). The protein/DNA complexes were resolved in a 4% nonreduced polyacrylamide gel and visualized by autoradiography. Specificity of the DNA/protein complex was examined by adding 100-fold excess unlabeled oligonucleotide or an uncompetitive oligonucleotide AP2 (5'-GATCGAACTGACCGCCCGGCCCGT-3', Promega). Supershift analysis with addition of 1 μg anti-NF- κ B antibody (p65 or p50) to the binding reaction for 30 min before addition of radiolabeled probe was performed to identify the components of the protein/DNA complex.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from DC treated with or without LF (10 $\mu\text{g}/\text{ml}$) and/or TNF- α /LPS (10 ng/ml each) for 4 h using Trizol (Gibco Invitrogen Life Technologies), according to the manufacturer's protocol. To remove DNA contamination, total RNA was further processed using the Message Clean kit (GenHunter Corp., Nashville, TN). Briefly, 20 μg RNA was digested with 10 units DNase I at 37°C for 30 min, extracted with phenol:chloroform (3:1), precipitated with ethanol, washed with 70% ethanol, and finally dissolved in 20 μl RNase-free water. To generate the first-strand cDNA, the SuperScript preamplification system (Gibco Invitrogen Life Technologies) was used. Briefly, 0.5 μg oligo-dT (12–18 bp) and 200 U SuperScript-2 RT were incubated with 2 μg DNA-free total RNA for 50 min at 42°C in the presence of 0.5 mM deoxynucleotide triphosphates, 10 mM DTT, and 1 \times First Strand buffer. For PCR amplification, reactions were performed in a volume of 25 μl PCR Supermix High Fidelity (Gibco Invitrogen Life Technologies). The primers used in this study were: IL-12p40 (451 bp), sense 5'-AAACAGTGAACCTCACCTGTGACAC-3' and antisense 5'-TTCATCTGCAAGTCTTTGGGCG-3'; IL-10 (404 bp), sense 5'-TGCATATGCTGCCTCTTACTGAC-3' and antisense 5'-AATCACTCTTCACCTGCTCCACTG-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 249 bp), sense 5'-TGATGACATCAAGAAGGTGGTGA-3' and antisense 5'-TCCTTGGAGGCCATGTAGCCAT-3'. PCR was conducted as follows: Denature at 95°C for 3 min followed by eight cycles of 95°C for 1 min, anneal primers 1 min at 60°C, extend at 72°C for 1 min and 30 cycles of 95°C for 1 min, anneal primers 1 min at 50°C, and extend at 72°C for 2 min and a final extension at 72°C for 5 min. The PCR products were resolved by electrophoresis in a 2% agarose gel with 1 \times 40 mM Tris - acetate, 2 mM $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ (pH 8.5), buffer and were visualized by ethidium bromide staining.

Enzyme-linked immunosorbent assay (ELISA)

To test *in vitro* Th polarization, allogeneic T cells were isolated from BALB/c spleens and incubated with DC that were pretreated with 10 $\mu\text{g}/\text{ml}$ LF for 4 h. After 48 h incubation, the supernatants were harvested, and ELISA quantified the concentrations of interferon- γ (IFN- γ) and IL-4 according to the manufacturer's instructions (Endogen, Woburn, MA) using a Benchmark microplate reader (Bio-Rad Laboratories). The amounts of IFN- γ and IL-4 produced by the T cells were determined from standard curves generated with rIFN- γ and IL-4 (Endogen).

T cells isolated from cardiac allograft recipients made tolerant by LF treatment were stimulated by anti-CD3 mAb (1 $\mu\text{g}/\text{ml}$) for 48 h. The supernatants were assessed as IFN- γ and IL-4 by ELISA as described above.

Statistical analysis

Statistical evaluation was performed using the Student's *t*-test for unpaired data, and results were considered significant if *P* values were ≤ 0.05 . Data were expressed as mean \pm SD.

RESULTS

LF prevents maturation of DC

We have previously reported that immature DC are found in LF-treated recipients [26, 30]. To determine whether LF prevents DC maturation, we used the C57BL/6-derived DC cell line JAWS II, which expresses similar phenotypic and functional characteristics to immature DC. Similar to primary DC, JAWS II matures upon activation with TNF- α /LPS [31]. JAWS II cells have previously been used to substitute for primary DC in tumor immunity [32] and chemokine production [33] experiments.

As mature DC are associated with stimulation of T cell responses, and immature DC inhibit T cell activation, we first assessed the effect of LF on DC maturation. Changes to the expression of cell-surface markers associated with DC maturation (MHC II, CD40, and CD86) were assessed by flow cytometry. Untreated DC showed high expression of CD11c, a specific DC marker independent of stage of maturation, but low expression of MHC II and costimulatory molecules CD40 and CD86 (Fig. 1A, top panels). Activation of DC with TNF- α (10 ng/ml) and LPS (10 ng/ml) for 24 h increased the expression of MHC II, CD40, and CD86 (Fig. 1A, middle panels). Pretreatment of the cells with LF (10 $\mu\text{g}/\text{ml}$) for 4 h before TNF- α /LPS significantly prevented the expression of MHC II, CD40, and CD86 in terms of percentage of cells expressing these markers and the extent of expression (Fig. 1A, bottom panels), indicating that LF reduced specific components of DC maturation. LF was not toxic to the DC as treatment at higher doses (20 $\mu\text{g}/\text{ml}$) and for longer periods of time (72 h), was not associated with morphological alterations or cellular cytotoxicity as assessed by microscopic analysis and MTT assay, respectively (data not shown).

The ability of DC to activate naive T cells depends on the expression of MHC II and costimulatory molecules on DC. LF-treated and TNF- α /LPS-activated DC (C57BL/6 background, H-2^b) were incubated with allogeneic T cells (BALB/c, H-2^d) to determine LF-mediated alterations of the DC allostimulatory function (Fig. 1B). DC activated by TNF- α /LPS evoked a strong, proliferative response from allogeneic T cells as compared with nonactivated, control DC. Treatment of DC

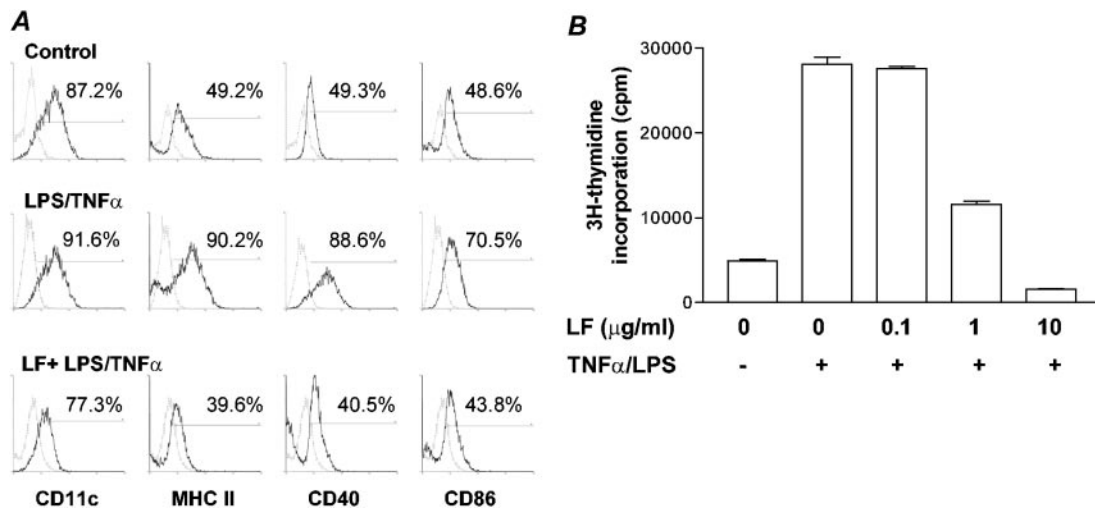


Fig. 1. LF prevents maturation of DC cell line. (A) Phenotypic analysis of LF-treated DC. Nonactivated DC (Control; top panels), TNF- α /LPS (10 ng/ml, 24 h)-treated DC (middle panels), and LF (10 ng/ml, 4 h) followed by TNF- α /LPS-treated DC (bottom panels) were stained with FITC-conjugated mAb as indicated for cell-surface molecules and analyzed by flow cytometry. (B) LF inhibits DC allostimulatory capacity in MLR. DC were pretreated with LF at the indicated concentrations for 4 h and subsequently stimulated with 10 ng/ml TNF- α /LPS for 24 h. LF-treated DC (0.5×10^5 /well) were stimulators, and BALB/c splenocytes (5×10^5 /well) were responders. Stimulators and responders were cocultured, and proliferation was assessed as described in Materials and Methods. Data shown are representative of three independent experiments.

with LF before addition of TNF- α /LPS suppressed the T cell response in the MLR assay in a concentration-dependent manner, and the highest concentration of LF completely prevented the allostimulatory effect of TNF- α /LPS.

Parallel experiments were performed with primary DC derived from BM. LF was added at various concentrations from day 2 to day 4 of culture, an optimal treatment regime for DC with LF established in previous experiments (data not shown). LF treatment of DC precursors resulted in the arrest of maturation induced by LPS/TNF- α . Similar to the DC cell line,

decreased levels of MHC II, CD40, and CD86 were observed in LF-treated, primary DC (Fig. 2A). Using LF-treated DC as stimulators in MLR, the T cell response was inhibited in a dose-dependent manner (Fig. 2B).

LF inhibits NF- κ B activation in DC

The maturation of DC occurs in response to diverse stimuli such as cytokines, tissue damage, and recognition of pathogen-associated molecular motifs, all of which converge to activate the NF- κ B pathway [34]. Activated NF- κ B binds to κ B con-

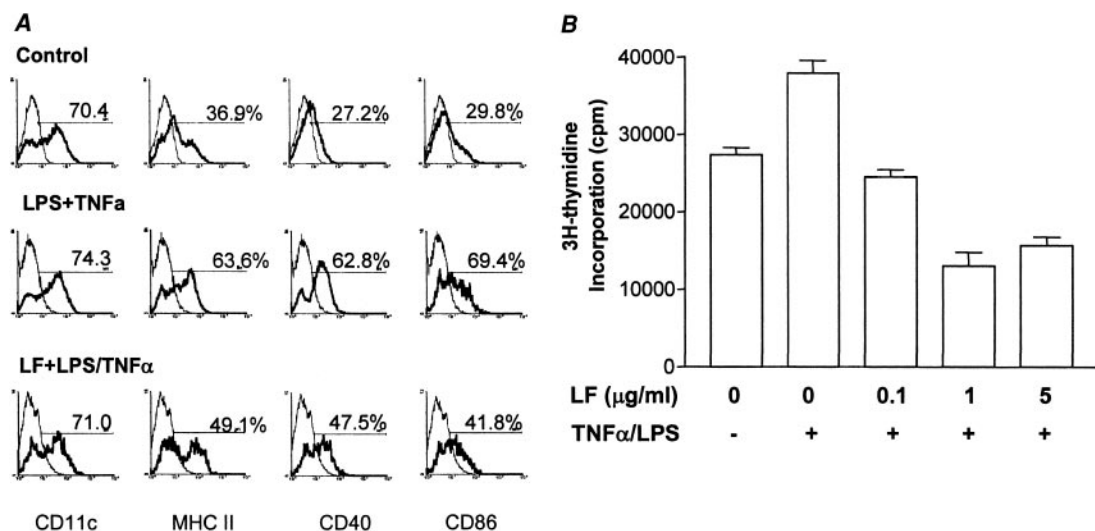


Fig. 2. LF prevents maturation of primary DC. (A) Phenotypic analysis of LF-treated DC. DC were generated from BM progenitors in the presence of GM-CSF and IL-4 for 7 days. LF (10 μ g/ml) was added from day 2 to day 4 of culture. Nonactivated DC (Control; top panels), TNF- α /LPS (10 ng/ml, 24 h)-treated DC (middle panels), and LF-treated and TNF- α /LPS-activated DC (bottom panels) were stained with FITC-conjugated mAb as indicated for cell-surface molecules and analyzed by flow cytometry. (B) LF inhibits DC allostimulatory capacity in MLR. DC were cultured and pretreated with LF at the indicated concentrations as described above and subsequently, were stimulated with 10 ng/ml TNF- α /LPS for 24 h. LF-treated DC (0.5×10^5 /well) were stimulators, and BALB/c splenocytes (5×10^5 /well) were responders. Stimulators and responders were cocultured, and proliferation was assessed as described in Materials and Methods. Data shown are the mean \pm SD of three independent experiments.

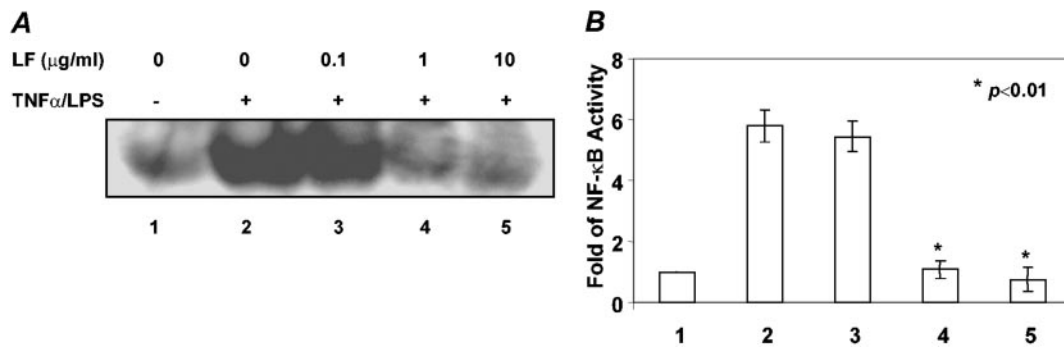


Fig. 3. LF inhibits NF- κ B activity. DC were pretreated for 4 h with LF at the indicated concentrations before addition of TNF- α /LPS (10 ng/ml) for 30 min. (A) Nuclear extracts were prepared, and activation of NF- κ B was assayed by EMSA. Data shown are representative of three independent experiments. (B) Relative intensity of bands was obtained by densitometry and normalized to unactivated DC. Data shown are the mean \pm SD of three independent experiments.

sensus motifs in the regulatory regions of target genes. To determine whether the prevention of DC maturation by LF is a result of the inhibition of the NF- κ B signaling pathway, EMSAs were conducted with nuclear extracts of DC pretreated with LF and subsequently activated with TNF- α /LPS (Fig. 3, A and B). A fivefold increase in the formation of protein/DNA complex was observed when DC were treated with TNF- α /LPS alone (Fig. 3, A and B, lane 2). Pretreatment with LF resulted in a concentration-dependent reduction in NF- κ B activation with concentrations of 1.0 and 10 μ g/ml LF, preventing any increase in NF- κ B activation (Fig. 3, A and B, lanes 4 and 5).

LF blocks nuclear translocation of NF- κ B

The p65/p50 heterodimers play a crucial role in the transcriptional regulation of target genes associated with the induction of DC maturation by inflammatory cytokines or LPS, which is thereby dependent on the nuclear translocation of NF- κ B [12, 35]. Although we have demonstrated that LF inhibited NF- κ B activity by EMSA, LF may act on NF- κ B in the nucleus or cytoplasm. To clarify this matter, nuclear translocation of NF- κ B following TNF- α /LPS activation was assessed by immunoblotting cytoplasmic and nuclear extracts of DC with or without LF pretreatment (Fig. 4). Levels of p65 and p50 in the cytoplasmic fractions were comparable for all conditions. Activation of DC with TNF- α /LPS led to an increase in levels of p65 and p50 in the nucleus. However, levels of nuclear p65 and p50 were comparable with those of nonactivated DC when DC were treated with LF before addition of TNF- α /LPS. Taken together, these results indicate that the failure of NF- κ B to translocate to the nucleus is responsible for the reduction in nuclear NF- κ B activation in LF-treated, TNF- α /LPS-stimulated DC observed by EMSA (Fig. 3).

LF inhibits IKK activity

Nuclear translocation of NF- κ B is dependent on the dissociation of NF- κ B from I κ B. To determine whether LF altered phosphorylation of I κ B, the activity of IKKs was investigated (Fig. 5). IKKs were immunoprecipitated from cell extracts of DC activated with TNF- α /LPS in the presence or absence of LF pretreatment. Subsequent *in vitro* kinase assays revealed low levels of endogenous IKK activity in untreated cells (Fig. 5, A and B, lane 1) but a significant increase in IKK activity

following activation of DC (Fig. 5, A and B, lane 2). Pretreatment of DC with LF at different concentrations before activation resulted in a concentration-dependent inhibition of IKK activity (Fig. 5A, lanes 3–5). The IKK activity of DC pretreated with 10 μ g/ml LF before activation with TNF- α /LPS was comparable with that of nonactivated DC. These results indicate that in LF-treated DC, NF- κ B remains associated with I κ B as a result of failure of the IKK to phosphorylate I κ B.

To determine whether IKK is a specific target for the actions of LF, IKK was overexpressed in DC by transfecting pcDNA3.1-IKK β vectors. Increasing amounts of the IKK β vector reversed the inhibition of the I κ B phosphorylation, thereby restoring the activation of NF- κ B in DC treated with TNF- α /LPS (Fig. 6A). This result suggests that IKK is acting as a necessary molecular switch and a rate-limiting component in DC maturation.

To assess whether the inhibition of IKK by LF was a direct or secondary effect, a cell-free kinase reconstitution assay was performed (Fig. 6B). IKKs were immunoprecipitated from ac-

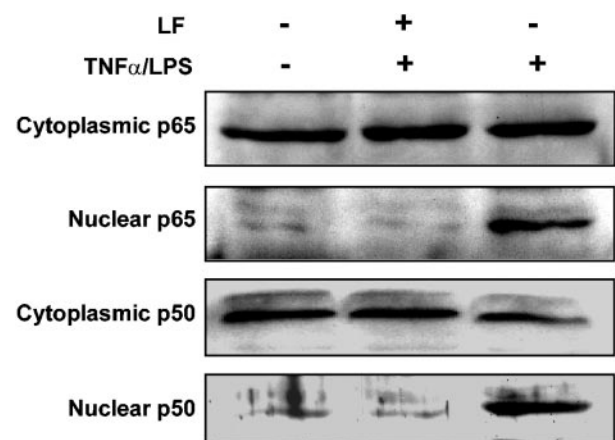


Fig. 4. LF inhibits nuclear translocation of NF- κ B. DC were pretreated with 10 μ g/ml LF for 4 h before addition of TNF- α /LPS (10 ng/ml) for 30 min. Cytoplasmic and nuclear extracts were prepared as described in Materials and Methods, and 40 μ g protein of each was resolved on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were probed with anti-NF- κ B/p65 and anti-NF- κ B/p50 polyclonal antibody and visualized by ECL assay. Data shown are representative of three independent experiments.

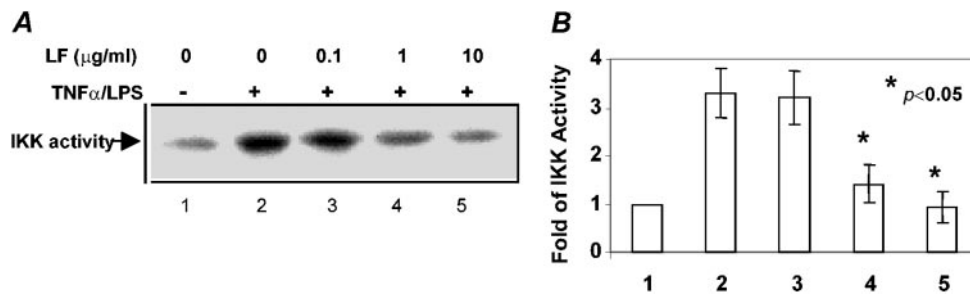


Fig. 5. LF inhibits IKK activity. (A) IKK activity assay. DC were treated with LF at the concentrations indicated for 4 h before addition of TNF- α /LPS (10 ng/ml, 30 min). Cytoplasmic IKKs were immunoprecipitated with anti-IKK α and anti-IKK β polyclonal antibodies. IKK activity was assessed by phosphorylation of a GST-I κ B α (amino acids 1–54) substrate as described in Materials and Methods. (B) Quantification of IKK activity by densitometry. Data shown are the mean \pm SD of three independent experiments.

tivated DC and were incubated with a GST-I κ B substrate in the presence or absence of LF, which inhibited IKK activity *in vitro* in a concentration-dependent manner. This suggested that LF inhibited the activation of the NF- κ B pathway through direct interaction with IKK or sites on I κ B involved in IKK interaction to reduce the phosphorylation of I κ B rather than interfering with an upstream event.

LF down-regulates IL-12 mRNA and up-regulates IL-10 mRNA in DC

IL-12 and IL-10 are key mediators in the immune reaction associated with allograft rejection or survival, respectively. In DC, IL-12 production is stimulated by NF- κ B [36]. To determine whether LF alters the expression profile of cytokines in DC, we examined the expression of IL-12 and IL-10 mRNA following activation with TNF- α /LPS with or without LF pretreatment (**Fig. 7A**). In the absence of activation, DC express a low baseline level of IL-10 and an undetectable level of IL-12p40 mRNA. Treatment of DC with TNF- α /LPS increased the level of IL-12p40 mRNA. Pretreatment of DC with LF before activation with TNF- α /LPS prevented increases in the level of IL-12p40 mRNA but increased the level of IL-10 mRNA.

To further examine the functional relevance of LF treatment on DC, we performed an *in vitro* Th polarization test. Naïve, allogeneic CD4⁺ T cells were incubated with LF-treated DC for 48 h. Supernatants of T cells incubated with untreated DC expressed high levels of IFN- γ but low levels of IL-4 (**Fig. 7B**). Supernatants from cultures with the LF-treated DC had in-

creased levels of IL-4 and decreased levels of IFN- γ (**Fig. 7B**), suggesting that LF-treated DC exert a Th2 polarization effect on naïve T cells.

LF-induced tolerance is associated with Th2 differentiation

We previously reported that a short course of monotherapy with LF achieved tolerance and permanent acceptance of allografts in rodent models [26]. To investigate whether LF-induced tolerance is associated with Th2 differentiation *in vivo*, we tested cytokines levels from T cells of rejecting and tolerant recipients. The recipients (BALB/c, H-2^d) were treated with LF (2 mg/kg/day, s.c., from days 0 to 20) following heterotopic cardiac transplantation using fully MHC-mismatched, allogeneic (C57BL/6, H-2^b) hearts. CD4⁺ T cells isolated from spleens of control, LF-induced long-term survivors (>100 days) and untreated, allograft-rejecting recipients (8 days) were activated by anti-CD3 mAb for 48 h. ELISA assessed the change in levels of IFN- γ and IL-4 (**Fig. 8**). Compared with T cells from control mice, T cells from rejecting mice produced a lower level of IL-4 and a higher level of IFN- γ , suggesting a prevalence of Th1 differentiation in the allograft-rejecting mice. In contrast, T cells derived from LF-treated mice (graft survival >100 days) produced a lower level of IFN- γ and a higher level of IL-4, suggesting that allograft survival was associated with Th2 differentiation. In support of our *in vitro* data, DC from tolerant recipients expressed an immature phenotype and decreased IL-12 (data not shown). These data

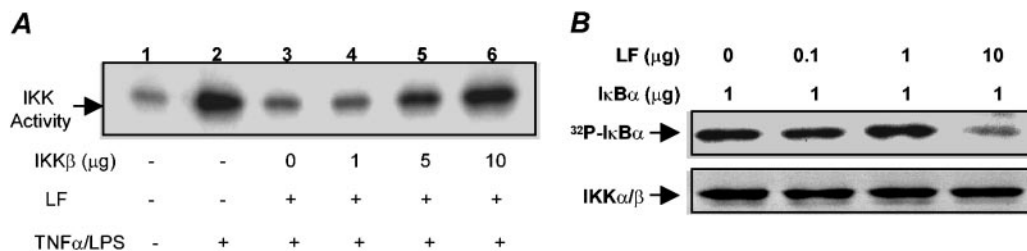


Fig. 6. LF directly blocks IKK activity. (A) IKK overexpression reduces sensitivity to LF. JAWS II cells were transfected with increasing amounts of an IKK β expression vector and were subsequently treated with LF (10 μ g/ml) and TNF- α /LPS (10 ng/ml) as in Figure 4. The IKK activity was assessed as described in Materials and Methods. (B) LF directly blocks IKK activity in cell-free extract. IKKs were immunoprecipitated from DC treated with TNF- α /LPS (10 ng/ml, 30 min) and were added to a GST-I κ B α (amino acids 1–317) substrate *in vitro*. The indicated concentrations of LF and buffer control were added to the reaction mixture. Phosphorylation of the GST-I κ B α substrate was assessed by autoradiography. Data shown are representative of three independent experiments.

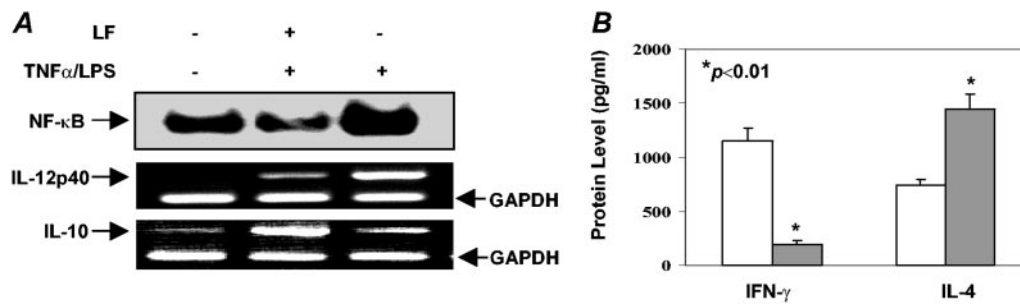


Fig. 7. LF treatment alters cytokine production by DC and promotes Th2 polarization. (A) LF regulates expression of cytokine mRNA in DC, which were treated with LF (10 $\mu\text{g/ml}$, 4 h) before addition of TNF- α /LPS (10 ng/ml) for 24 h. Total RNA was prepared, and the expression of IL-12p40 and IL-10 mRNA was analyzed by RT-PCR as described in Materials and Methods. In parallel, the activation of NF- κ B was monitored by EMSA. (B) LF-treated DC promote Th2 polarization in vitro. Allogeneic T cells were isolated from BALB/c spleens and incubated with untreated DC (open bars) or LF-treated DC (10 $\mu\text{g/ml}$, 4 h, solid bars). After 48 h, the supernatants were assayed for IFN- γ and IL-4 by ELISA as described in Materials and Methods. Each lane is representative of three experiments per experimental group.

suggest that LF induces tolerance by promoting the generation of tolerogenic DC, which are capable of guiding the generation of Th2 cells, which contribute to tolerance.

DISCUSSION

In this study, we defined how LF averts graft rejection by preventing DC maturation and subsequent activation of T cells. Inhibition of IKK activity in DC by LF prevented the expression of key molecules (MHC II, CD40, CD86, and IL-12) required for T cell activation by DC. Furthermore, DC acquired a tolerogenic phenotype (i.e., expression of IL-10) that would promote acceptance of the graft. DC are the pivotal cell population in graft rejection, as mature DC are the most potent presenters of alloantigens leading to strong activation of naïve T cells during transplant rejection [37]. In contrast, immature DC inhibit T cell responses as a result of their ability to induce T cell apoptosis or anergy [1]. The capacity of DC to present antigen is related to high surface expression of MHC and costimulatory molecules [1]. Previous approaches using immature DC to induce tolerance have reduced graft rejection [10, 38]. Thus, by preventing maturation of DC, LF optimizes tolerogenicity within grafted tissue by minimizing the stimulus for T cell activation and number of cells capable of responding.

To demonstrate the effects of LF on DC maturation, we used the established, immature DC cell line JAWS II, which expresses similar phenotypic and functional characteristics to immature DC [31] and has previously been used to substitute for primary DC in tumor immunity [32] and chemokine production [33] experiments. DC cell lines have also been used to model primary DC in studies requiring large numbers of a homogenous cell population [39]. Various DC lines have been developed to replace primary DC for in vitro and in vivo studies. For example, the DC line XS106 was transfected with Fas ligand to generate killer DC, which block T cell activation in an antigen-specific manner [40]. Additionally, the DC lines DC1.2, DC2.4, and DC4.1 have been used to study molecular aspects of antigen cross-presentation [41]. In the present study, JAWS II cells activated with TNF- α /LPS acquired a mature DC phenotype and function that was similar to that of acti-

vated, primary DC. Furthermore, we demonstrated comparable effects of LF on DC maturation in the JAWS II cell line and primary DC.

Recently, activation of NF- κ B has been identified as a key signaling event driving the maturation of DC [12, 13]. Up-regulation of MHC II and the expression of costimulatory

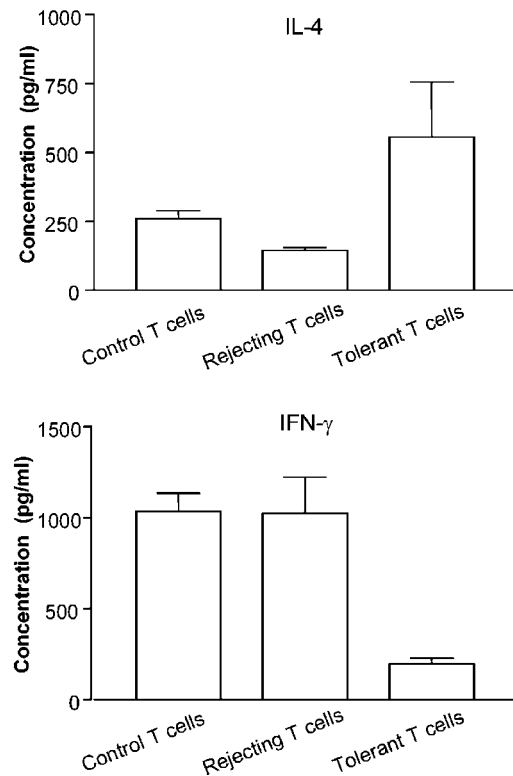


Fig. 8. LF-induced tolerance is associated with Th2 polarization in vivo. Recipients (BALB/C, H-2^d) were treated with LF (2 mg/kg/day, s.c., between day 0 and day 20) following allogeneic cardiac graft (C57BL/6, H-2^b) transplantation. ELISA determined production of cytokines by T cells. CD4⁺ T cells isolated from normal mice, long-term survival mice (>100 days), and untreated, allograft-rejected mice were stimulated with anti-CD3 antibody for 48 h. Supernatants were harvested and assessed for IL-4 (upper panel) and IFN- γ (lower panel). Data shown are representative of three mice per experimental group.

molecules observed in mature DC follow increased activation of NF- κ B in response to maturation-inducing stimuli [42]. Overexpression of I κ B α [43], inhibition of proteasome activity to prevent I κ B degradation [14], or interfering with nuclear NF- κ B DNA-binding activity [38] induces DC tolerogenicity by selectively suppressing the cell-surface expression of costimulatory molecules, as well as production of IL-12. Mice with a functionally inactivated Rel B (p68), a subunit of NF- κ B, fail to produce differentiated DC, indicating the critical importance of functional NF- κ B in DC physiology [44]. Furthermore, when immature DC generated by inhibition of NF- κ B using double-stranded oligodeoxyribonucleotides containing κ B-binding sites were administered to transplant recipients, prolonged allograft survival was seen in a murine cardiac transplantation model [38]. Multiple receptors initiate NF- κ B signaling by activating a common downstream IKK complex (IKK α , - β , and - γ). IKKs have the gatekeeper function of phosphorylating I κ Bs, which act to sequester NF- κ B in the cytoplasm, away from target genes. Thus, IKK is a key factor regulating the shuttling of NF- κ B from the cytoplasm to the nucleus and consequently, NF- κ B-dependent gene transcription.

DSG, the more toxic, parent compound of LF, has been shown previously to inhibit expression of human leukocyte antigen-DR, CD80, and CD83 [23], as well as to suppress NF- κ B translocation in B cells [17] and lymph node cells [45]. Immunohistochemical staining of DC in interstitial lymph nodes of allografted Rhesus macaques treated with DSG revealed a lack of nuclear translocation of Rel B, a subunit of NF- κ B [23]. DSG binds to heat-shock cognate proteins 70 [46] and 73 [47], which are involved in degradation of I κ B [48] and thereby could impair NF- κ B translocation to the nucleus. However, the mechanism of action of DSG has not been elucidated. Other studies examining tolerance induction or immunosuppression via inhibition of NF- κ B have identified impaired translocation [49, 50] but have not characterized changes to the upstream signaling cascade. The present study identified IKK as a direct target of LF, which inhibited yet-activated and already active IKK, thereby preventing translocation of NF- κ B into the nucleus and restricting the maturation of DC.

Classically, it is believed that T cell activation requires two signals: signal 1 MHC-TCR interaction, which provides antigenic stimulation, and signal 2, costimulatory molecule that enables the T cell to proliferate, clonally expand, and produce various cytokines. T cells are subject to anergy or apoptosis when receiving signal 1 in the absence of signal 2 [51]. Immature DC have been classified as tolerogenic as a result of lack of signal 2 [11]. Reduction in the expression of costimulatory molecules promotes tolerance (reviewed in ref. [52]). More recently, immature DC interacting with naïve T cells were found to give rise to T regulatory cells that inhibit activation of other T cells [53]. Reduced expression of MHC causes incomplete TCR activation that is known to result in anergy or apoptosis. Recently, T cell activation was blocked by another NF- κ B inhibitor (N-benzyloxycarbonyl-Ile-Glu(O-tert-butyl)-Ala-leucinal) through suppression of MHC and costimulatory molecules on APC [14].

In the present study, suppression of NF- κ B signaling by LF not only prevented the APC function of DC by restricting the expression of MHC II and costimulatory molecules (signal 2) but also promoted tolerance by altering the complement of cytokines expressed by DC (signal 3). Th1 differentiation of naïve T cells is dependent on the expression of IL-12 by mature DC [2, 3]. Th1 cytokines (e.g., IFN- γ and IL-2) are associated with T cell-mediated rejection, and Th2 cytokines (e.g., IL-4, IL-10) promote allograft acceptance [37, 54]. Thus, the ratio of Th1-to-Th2 polarization is an important component of transplant survival. Production of IL-12 by DC is transcriptionally regulated by NF- κ B through κ B binding sites in the IL-12 promoter [55]. Previous studies demonstrated that inhibition of NF- κ B by pharmacological inhibitors or overexpression of I κ B α in DC diminished the ability of DC to produce and secrete IL-12 in response to activation signals such as TNF- α and LPS [56]. In the present study, our data suggest that LF induces Th2 polarization. Not only did LF suppress IL-12 gene expression in DC and IFN- γ production by T cells but also LF-stimulated IL-10 gene expression in DC and IL-4 production by T cells. Furthermore, T cells isolated from LF-treated, tolerant recipients expressed cytokines consistent with a Th2 phenotype. Recent studies in our laboratory found that DC isolated from allograft recipients made tolerant by LF treatment had an IL-12-deficient phenotype [24].

In conclusion, this is the first study, to our knowledge, demonstrating that LF inhibits NF- κ B in DC through blocking IKK activity. The suppression of IKK was mediated by a direct interaction with LF, as demonstrated by cell-free reconstitution and IKK-overexpression experiments. The blockade of this pathway was associated with the generation of tolerogenic DC that primed Th2 polarization, which may account for tolerance induced by LF.

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