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Immunomodulatory activity of curcumin: suppression of lymphocyte proliferation, development of cell-mediated cytotoxicity, and cytokine production in vitro

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Abstract

Curcumin (diferuloylmethane), a major curcumanoid found in the spice turmeric, exhibits anti-inflammatory, anti-oxidant, and chemopreventive activities. However, the effect of curcumin on the development of T cell-mediated immunological responses largely remains unknown. In this study we have investigated the effect of curcumin on mitogen/antigen induced proliferation of splenic lymphocytes, induction of cytotoxic T lymphocytes (CTLs), lymphokine activated killer (LAK) cells, and the production of cytokines by T lymphocytes and macrophages. We found that mitogen, interleukin-2 (IL-2) or alloantigen induced proliferation of splenic lymphocytes, and development of cytotoxic T lymphocytes is significantly suppressed at 12.5–30 μmol/L curcumin. The generation of LAK cells at similar concentrations was less sensitive to the suppressive effect of curcumin compared to the generation of antigen specific CTLs. Curcumin irreversibly impaired the production of these immune functions, since lymphoid cells failed to respond to the activation signals following 8 h pretreatment with curcumin. Curcumin also inhibited the expression/production of IL-2 and interferon-gamma (IFN-γ) by splenic T lymphocytes and IL-12 and tumor necrosis factor-alpha (TNF-α) by peritoneal macrophages irreversibly. Curcumin inhibited the activation of the transcription factor nuclear factor kappaB (NF-κB) without affecting the levels of constitutively expressed NF-κB. The latter result suggests that curcumin most likely inhibits cell proliferation, cell-mediated cytotoxicity (CMC), and cytokine production by inhibiting NF-κB target genes involved in induction of these immune responses.

Keywords: Curcumin; Lymphocytes; Proliferation; Cytotoxicity; Cytokines

1. Introduction

Epidemiological and laboratory studies suggest that diet plays an important role in preventing the development of human cancers and other diseases [1,2]. Several essential and nonessential dietary constituents found in common plant-derived foods have been recognized for their anticarcinogenic properties, and high dietary intake of dark green leafy vegetables, fruits, beans, and soy products has been linked to a low incidence of cancer and coronary heart disease [3–6]. The disease-preventing effects of these foods are attributed in part to the presence of bioflavonoids, a group of naturally occurring polyphenolic substances [4]. Laboratory data have demonstrated that chemopreventive effects of some of these compounds are attributed to their strong antioxidant and anti-inflammatory properties, their ability to promote metal chelation, scavenging of free radicals, and detoxification of carcinogens [7–9]. At the cellular levels, these polyphenolic substances regulate a range of biochemical pathways that affect cell proliferation and signaling [6].

Abbreviations: CTL, cytotoxic T lymphocytes; LAK cells, lymphokine activated killer cells; IL-2, interleukin-2; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; ELISA, enzyme-linked immunoabsorbant assay; ConA, concanavalin A

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Curcumin (1,7-bis(4-hydroxy-3-methoxy phenyl)-1-6-heptadine-3,5-dione) is a naturally occurring yellow pigment found in the spice turmeric derived from the rhizome of the plant Curcuma longa. Besides its culinary appeal for color and flavor, turmeric or curcumin has been widely used for centuries in the Indian subcontinent for the treatment of a variety of illnesses such as common colds, coughs, jaundice, inflammatory bowl conditions, and arthritis [10]. Because of its ability to scavenge free radicals and inhibit inflammation [11,12], curcumin has been investigated for cancer chemoprevention and tumor growth suppression. Curcumin was shown to prevent the development of cancers of the forestomach, duodenum, tongue, colon, and mammary glands in models of chemical carcinogenesis in mice and rats [13–19]. Exposure of tumor cell lines to curcumin in vitro has resulted in inhibition of cell proliferation or induction of apoptotic cell death [20–24].

We have previously shown that curcumin inhibits proliferation and induces apoptosis in several murine and human leukemia cell lines [25]. In addition, curcumin inhibited the IL-1α or tumor necrosis factor-alpha (TNF-α) induction of activation protein-1 (AP-1) and nuclear factor kappaB (NF-κB) in bone marrow stromal cells [26]. Recently, we also reported that curcumin sensitizes human prostate cancer cells for induction of apoptosis by TNF-α related apoptosis-inducing ligand (TRAIL) [27]. On the other hand, little is known about the effect of curcumin on the development of cell-mediated immune responses and production of cytokines involved in inflammation and antitumor immune responses. In the present study, we examined the effect of curcumin on mitogen/antigen-induced proliferation of murine lymphocytes, the development of cell-mediated cytotoxicity (CMC), and production of cytokines by lymphocytes and macrophages.

2. Materials and methods

2.1. Agents

Curcumin, mouse interleukin-2 (mIL-2) (2.5×10^8 U/mg), and Concanavalin A (Con A) were purchased from Sigma Chemical Co. (St. Louis, MO). A 100 mmol/L solution of curcumin was prepared in DMSO and all test concentrations were prepared by diluting the appropriate amount of stock solution in tissue culture medium.

2.2. Mice

Eight to ten-week-old male C3H ($H\text{-}2^k$) and C57BL/6J ($H\text{-}2^b$) were purchased from Taconic Laboratories (Germantown, NY). Mice consumed Breeder Diet (W) 8626 (protein, 20.0%; fat, 10.0%; fiber, 3.0%) and water ad libitum and were housed in the Bioresource Facility of the Henry Ford Health System. They were housed for at least one week before experimental use and age-matched animals were used within any given experiment. The treatment of mice and all experimental protocols were according to the Institutional Animal Care and Use Committee guidelines.

2.3. Tissue culture medium

All in vitro cell cultures were carried out in RPMI-1640 medium (Grand Island Biological Company, Grand Island, NY), supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 1% penicillin/streptomycin, 25 mmol/L HEPES buffer, and 5×10^{-5} M 2-mercaptoethanol. Hereafter, this medium will be referred to as complete RPMI-1640 medium.

2.4. Preparation of spleen cells

Mice were euthanized by CO_2 inhalation and spleens were removed aseptically. Spleens were placed in cold Hank's balanced salt solution (HBSS) and teased apart with a pair of forceps and a needle. Single-cell suspension from the teased tissue was obtained by passing it through a 20 G needle. Cells were washed two times in cold HBSS and finally resuspended in complete RPMI-1640 medium.

2.5. Measurement of cell viability

Viability of spleen cells was determined by trypan blue dye exclusion using a hemocytometer or by colorimetric MTS assay. For MTS assay, 1×10^5 untreated spleen cells (control) or those treated with curcumin for 96 h were added to each well of a 96-well plate in 100 μl tissue culture medium in triplicate. Cell viability was determined by the colorimetric MTS using CellTiter 96 AQueous Assay System from Promega (Madison, WI). In this assay, the quantity of formazan product formed is directly proportional to the number of viable cells in the cultures.

2.6. ^{3}H-thymidine incorporation assay

To determine the effect of curcumin on the proliferation of lymphocytes, 5×10^6 spleen cells were cultured in 5 ml of RPMI-16 in a 25 cm^2 tissue culture flask in the absence or presence of Con A (1 μg/ml) or mIL-2 (100 ng/ml) or allogeneic spleen cells (1:1 ratio) as stimulators. Curcumin was added to the cultures in concentrations as described in the individual experiments. After incubation for 4 days at 37 °C, 95% humidity, and 5% CO_2, cells were harvested, washed once with cold PBS, and resuspended in RPMI-1640. Cell viability was determined and cell concentration adjusted to 2×10^6 cells/ml. 0.1 ml of cell suspension was added to each well of a 96-well microtiter tissue culture plate in triplicate. 0.25 μCi of ^{3}H-thymidine in 20 μl of HBSS was added to each well and the plate was incubated for additional 8 h. Cultures were harvested with

an automatic cell harvester using distilled water. The amount of radioactivity incorporated into DNA was determined in a liquid scintillation spectrometer.

2.7. Generation of cytotoxic T lymphocytes (CTLs)

For the generation of alloantigen specific CTLs, 10^7 spleen cells of C3H/HeN (responders) mice and an equal number of irradiated (20 Gy) allogeneic spleen cells of C57BL/6 (stimulators) mice were cultured in 10 ml of RPMI-1640 tissue culture medium supplemented with 10% FBS. After incubation for 5 days, cells were harvested and viability determined as described. Cells were tested for cytotoxicity against ^{51}Cr labeled EL-4 lymphoma cells of C57BL/6 origin as target cells in 4 h ^{51}Cr release assay.

2.8. Generation of lymphokine activated killer (LAK) cells

For the generation of nonspecific cytotoxic LAK cells, C3H splenic cells (5×10^6 cells/5 ml) were cultured in medium alone or in the medium containing IL-2 (150 ng/ml). After incubation for 72 h, cells were harvested, viability determined, and tested for cytotoxicity against YAC-1 target cells in 4 h ^{51}Cr release assay.

2.9. Cytotoxicity assay

Target cells were resuspended at 1×10^7 cells/ml RPMI-1640 and 100 μCi of Sod. ^{51}Chromate was added to cells. Cells were incubated for 90 min at 37 °C. Following incubation, cells were washed three times in PBS to remove unbound radioactivity. The effector and labeled target cells were adjusted to desired cell concentrations and added to wells of a U bottomed 96-well microtiter plate in triplicate to obtain effector:target (*E*:*T*) ratios of 100:1 to 12.5:1. For maximum release of radioactivity, target cells were lysed in 1% SDS solution. For minimal release of radioactivity (spontaneous release), target cells were incubated in medium alone. Plates were centrifuged at 800 rpm for 2 min and incubated at 37 °C for 4 h. The 100 μl of the supernatant from each well was removed to measure the amount of radioactivity released. Percent cytotoxicity was determined by the formula

$$\text{percent cytotoxicity} = \frac{\text{experimental release (cpm)} - \text{spontaneous release (cpm)}}{\text{maximum release (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

2.10. Spleen cell supernatant

Spleen cells (2×10^6 cells/ml RPMI-1640) were treated with Con A (1 μg/ml) in the absence or presence of 30 μmol/L curcumin for 20 h. In separate cultures, spleen cells were first treated with 30 μmol/L curcumin for 8 h and then washed with PBS three times. Cells were then treated with Con A (1 μg/ml) for 20 h. Culture supernatants were collected by centrifugation and IL-2 was determined using commercially available cytokine-specific enzyme-linked immunoabsorbant assay (ELISA) kits (Bioresource International, Camarillo, CA).

2.11. Macrophage supernatant

Thioglycolate induced peritoneal exudate cells (1×10^6) were plated in 60 mm petri dishes and allowed to adhere to the plastic surface for 1 h. Nonadherent cells were removed and the adherent cells were treated with LPS (500 ng/ml) for 20 h in the absence or presence of curcumin (30 μmol/L). Separately, cells were treated with curcumin (30 μmol/L) for 8 h followed by its removal by washing cells with PBS. Cells were then stimulated with LPS and supernatant collected after 20 h as described. The amount of mTNF-α in supernatants was determined by ELISA.

2.12. RT-PCR

Total cellular RNA was extracted from cells with TRI-zol reagent (GIBCO) according to the manufacturer's recommendation. 2.5 μg of RNA was then reverse transcribed by using random primers (Boehringer Mannheim) and reverse transcriptase to generate cDNAs. The primer sequences for PCR amplification were as follows. IL-2, upper, 5′-GTCACATTGACACTTGTCGTCC-3′, and lower, 5′-AGTCAAATCCAGAACATGCCG-3′; interferon-gamma (IFN-γ), upper, 5′-AACGCTACACACTGCATCTTGG-3′, and lower, GACTTCAAAGAGTCTGAGG-3′; and IL-12 (p40), upper, 5′-GATGTGTCCTCAGAAGCTA-3′, and lower, 5′-TTGCATCCTAGGATCGGACC-3′. The 1 μg of cDNA was amplified by PCR for 35 cycles of denaturation (94 °C for 1 min), annealing (61 °C for 1 min), and polymerization (72 °C for 2 min). The PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. These primers amplified DNA fragments of 294 bp (IL-2), 267 bp (IFN-γ) and 1025 bp (IL-12, p40).

2.13. Measurement of NF-κB

Nuclear extracts were prepared by the modified procedure of Dignam et al. [28]. Following treatment, cells were washed three times with PBS, resuspended and incubated on ice for 15 min in hypotonic buffer A (10 mM HEPES, pH 7.9,10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 0.6% NP−40). Cells were vortexed gently for lysis and nuclei were separated from the cytosol by centrifugation at $12{,}000 \times g$ for 1 min. Nuclei were resuspended in buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM

EGTA, 1 mM DTT, 0.5 mM PMSF) and shaken for 30 min at 4 °C. Nuclear extracts were obtained by centrifugation at 12,000 × *g* and protein concentration measured by Bradford assay (Bio−Rad, Richmond, CA). Nuclear extract was fractionated on 12% SDS-PAGE gel, transferred to nitrocellulose membrane, and probed with anti-NF-κB (p65) antibody using enhanced chemiluminescence detection system from Amersham Corp. (Arlington Heights, IL).

In addition, NF-κB (p65) binding activity in whole cell lysates was measured using TransAM NF-κB kit from Active Motif (Carlsbad, CA). In this assay, NF-κB present in extracts binds to the immobilized oligonucleotide containing the NF-κB consensus sequence (5′-GGGACTTTCC-3′), which is then detected by sandwich ELISA. The detection limit for TransAM NF-κB kit is <0.4 ng purified p65 ml^{-1}.

2.14. Statistical analysis

Data were analyzed by one-way and two-way analysis of variance. When statistically significant interaction between curcumin dose and treatment was present, separate treatment group comparisons were made within each dose. Fisher's protected LSD method was used to address multiple comparisons.

3. Results

3.1. Antiproliferative effect of curcumin

The effect of curcumin on proliferation of splenic lymphocytes was examined in ^{3}H-thymidine uptake assay. The desired concentration of curcumin was incorporated into culture medium at the initiation of cultures. The results presented in panels A–C of Fig. 1 demonstrate the effect of curcumin on Con A (A), IL-2 (B), and alloantigen (C) induced proliferation of splenic lymphocytes. There was significant increase in Con A induced proliferation of splenic cells at 6.25 μmol/L curcumin ($P < 0.001$) followed by a significant decrease in proliferation at 12.5 μmol/L curcumin (Fig. 1A, $P < 0.0001$). The proliferative response was completely blocked at 25 μmol/L curcumin. Curcumin also inhibited the IL-2 induced proliferation of splenic cells. The inhibition of IL-2 induced proliferation of cells was dose-dependent, since increasing suppressive effect was observed at increasing concentration of curcumin from 6.25 to 25 μmol/L (Fig. 1B). IL-2 induced proliferation of spleen cells was completely inhibited by curcumin at 25 μmol/L. The effect of curcumin on alloantigen induced proliferation of spleen cells (C3H anti-C57BL/6) was modest at 20 μmol/L. However, at 30 μmol/L, proliferation of cells was significantly suppressed ($P < 0.001$). These data demonstrate that at 12.5 μmol/L and above curcumin significantly inhibits the mitogen and IL-2 induced proliferation of splenic cells, but alloantigen induced proliferation of splenic lymphocytes is suppressed only at higher concentration of curcumin (30 μmol/L).

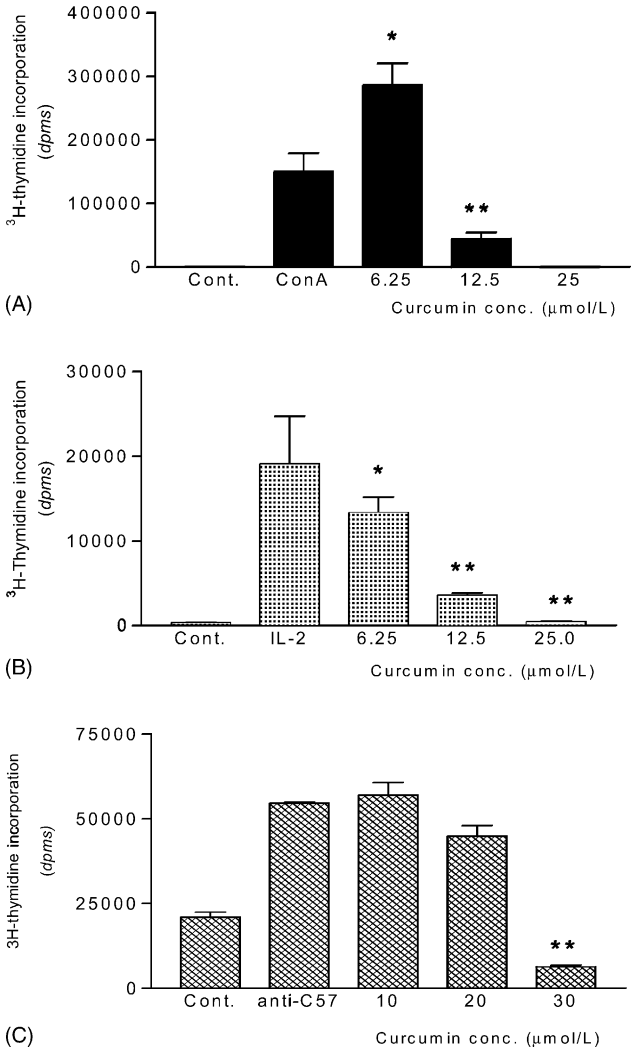

Fig. 1. Effect of curcumin on proliferation of spleen cells. C3H spleen cells (1×10^6 cells/ml) were stimulated with Con A (1 μg/ml) (A) or IL-2 (100 ng/ml) (B), or irradiated C57BL/6 spleen cells (1:1) (C) for 4 days in the absence or the presence of curcumin at concentrations as indicated. 2×10^5 viable cells from each culture were transferred to the wells of a 96-well microtiter tissue culture plate in triplicate. Cultures were pulsed with ^{3}H-thymidine (0.25 μCi/well) for 8 h. ^{3}H-thymidine incorporation was determined by liquid scintillation spectrometry. Data are presented as mean (dpms) ± S.E.M. of three to four experiments. (*) Significantly increased compared to Con A stimulated cells ($P < 0.001$); (**) significantly reduced compared to Con A stimulated cells ($P < 0.0001$).

3.2. Curcumin inhibits the development of cell-mediated cytotoxic responses

The suppression of mitogen/alloantigen induced proliferation of splenic lymphocytes suggested that this compound may also affect the generation of cell-mediated cytotoxic responses. In order to test this, we examined the effect of curcumin on the production antigen specific cytotoxic T lymphocytes (CTLs) and IL-2 induced non-specific cytotoxic cells. For the alloantigen induced CTLs,

spleen cells from the C3H/Hen strain of mice (H-2^k) were incubated with the spleen cells (irradiated) of C57BL/6 (H-2^d) mice for five days in the absence or the presence of curcumin. The cytolytic activity of effector cells against EL-4 lymphoma cells of C57BL/6 origin as target cells was determined in 4 h ^{51}Cr release assay. At 10 μmol/L curcumin, the generation of CTLs was only insignificantly reduced (Fig. 2A), but it was significantly reduced at 20 μmol/L curcumin ($P < 0.007$). The response was, however, completely abrogated at 30 μmol/L curcumin. To investigate further the effect of curcumin on the development cell-mediated cytotoxicity, we examined the generation of IL-2 induced non-specific cytotoxic LAK cells from spleen cells in the presence of curcumin. The cytotoxic activity of LAK cells was determined against YAC-1 target cells in 4 h ^{51}Cr release assay. Curcumin exhibited minimal effect on the generation of LAK cell-mediated cytotoxicity at 10–20 μmol/L (Fig. 2B). At a concentration of 30 μmol/L LAK cell production was significantly inhibited ($P < 0.001$). These data demonstrate that both CTL and LAK cell generation is significantly inhibited at 30 μmol/L, however, at lower concentrations of curcumin only the generation of CTLs is affected.

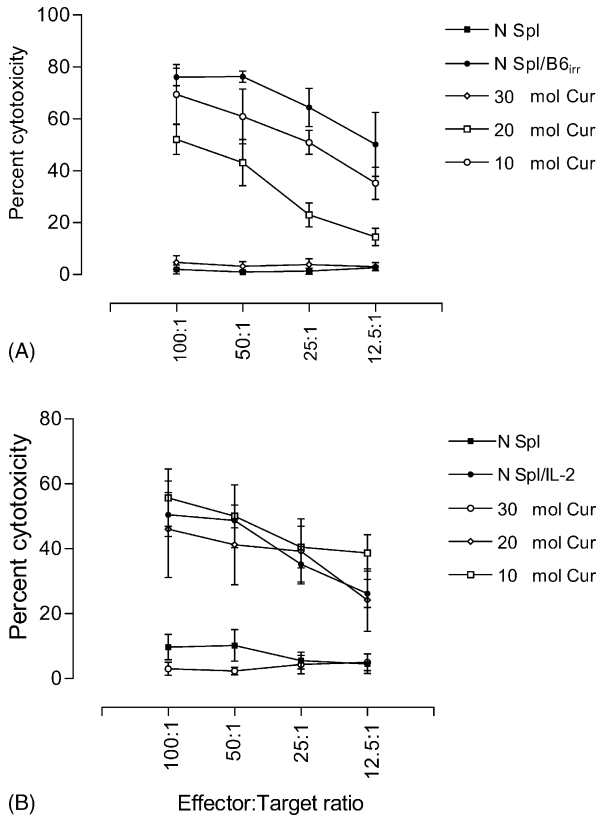

Fig. 2. Effect of curcumin on the development of cell-mediated cytotoxicity. For effect of curcumin on the generation of CTLs, 10^7 C3H spleen cells were co-cultured with an equal number of irradiated C57BL/6 spleen cells in 10 ml RPMI-1640 medium for 5 days in the absence or the presence of curcumin (10, 20, and 30 μmol/L). Cytotoxicity of the viable effector cells against EL-4 target cells of C57BL/6 origin was determined in 4 h ^{51}Cr release assay (A). Effect of curcumin on LAK cell generation was examined by incubating C3H spleen cells (10^6 cells/ml) with IL-2 (150 ng/ml) for 3 days in the absence or the presence of curcumin (10, 20, and 30 μmol/L). Cytotoxicity of effector cells against YAC-1 target cells was measured in 4 h ^{51}Cr release assay (B). In each panel, the results are presented as mean percent cytotoxicity ± S.D. of three to four experiments. CTL generation was significantly inhibited at 20 μmol/L curcumin, $P < 0.001$. LAK cell generation was significantly suppressed at 30 μmol/L curcumin, $P < 0.001$.

The inhibitory effect of curcumin on proliferation of lymphocytes or generation of cytotoxic cells was not due to DMSO used for dissolving curcumin, since equivalent concentrations of DMSO alone had no effect on the development of proliferative or cytotoxic responses (not shown).

In all of our bulk spleen cell cultures whether treated or not with curcumin, we observed significant cell losses (30–40%) as determined by trypan blue dye exclusion. To make certain that the lack of development of immune responses in spleen cells treated with curcumin was not due to lack of viable cells, we measured the viability of spleen cells treated with curcumin by MTS assay. Spleen cells (1×10^6 ml^{-1}) were cultured in the presence of curcumin at concentrations ranging from 10 to 30 μmol/L for 96 h. Cells were washed once and viability determined by MTS assay as described in Section 2. As shown in Fig. 3, despite an overall decrease cells in all of the cultures, including untreated control cultures, as determined by trypan blue dye exclusion, the viability of cells recovered from these cultures was comparable whether cells were treated or not with curcumin. This result demonstrates that although cells treated with curcumin are viable but fail to respond to mitogenic or antigenic stimulation.

3.3. Curcumin irreversibly inhibits proliferation of lymphocytes

In order to investigate whether the inhibitory effect of curcumin on proliferation of lymphocytes is transient or

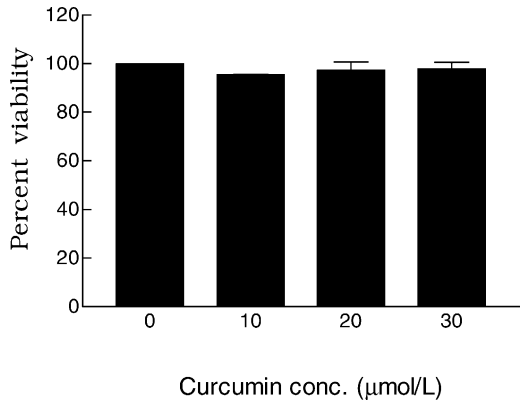

Fig. 3. Effect of curcumin on viability of spleen cells. Spleen cells (2×10^6 ml^{-1}) were cultured in the absence or presence of curcumin at concentration ranging from 10 to 30 μmol/L for 96 h. Cells were washed once and 100 μl of cell suspension was added to each well of a 96-well microtiter plate in triplicate. Cell viability was measured by the MTS assay using CellTiter AQueous Assay System from Promega. Data presented are mean percent viability from two experiments.

permanent, spleen cells were pre-treated with curcumin prior to induction of proliferation. For this purpose, spleen cells were treated with 30 μmol/L curcumin for 8 h and then washed three times to remove curcumin. Untreated and pretreated spleen cells were stimulated with Con A, IL-2 or allogeneic stimulator cells and proliferation was examined as described before. There was no effect of pretreatment with curcumin on the basal proliferative rate of splenic lymphocytes (Fig. 4A–C). In contrast, cells pretreated with curcumin remained completely unresponsive to each of the three stimuli following the removal of curcumin from the cultures. In additional experiments, when pretreated cells were incubated in medium for 24 h prior to stimulation with mitogen/antigen also failed to recover from the suppressive effect of curcumin (data not shown). These results suggested that curcumin irreversibly inhibits the induction of lymphocyte proliferation by mitogens and alloantigens.

Consistent with the observation that curcumin irreversibly impairs the proliferative responses, Fig. 5 demonstrates that inhibition of the development of alloantigen specific CTLs or nonspecific IL-2 induced LAK cells by curcumin is also irreversible. The cytolytic activity of effector cells (i.e., CTLs and LAK cells) generated from spleen cells pre-treated with curcumin for 8 h was significantly lower compared to the untreated control cells ($P < 0.001$). This was also true when pretreated cells were incubated for 24 h before stimulation to generate CTLs or LAK cells (not shown). Thus, suppression of both proliferative and cell-mediated cytotoxic immune responses by curcumin is irreversible.

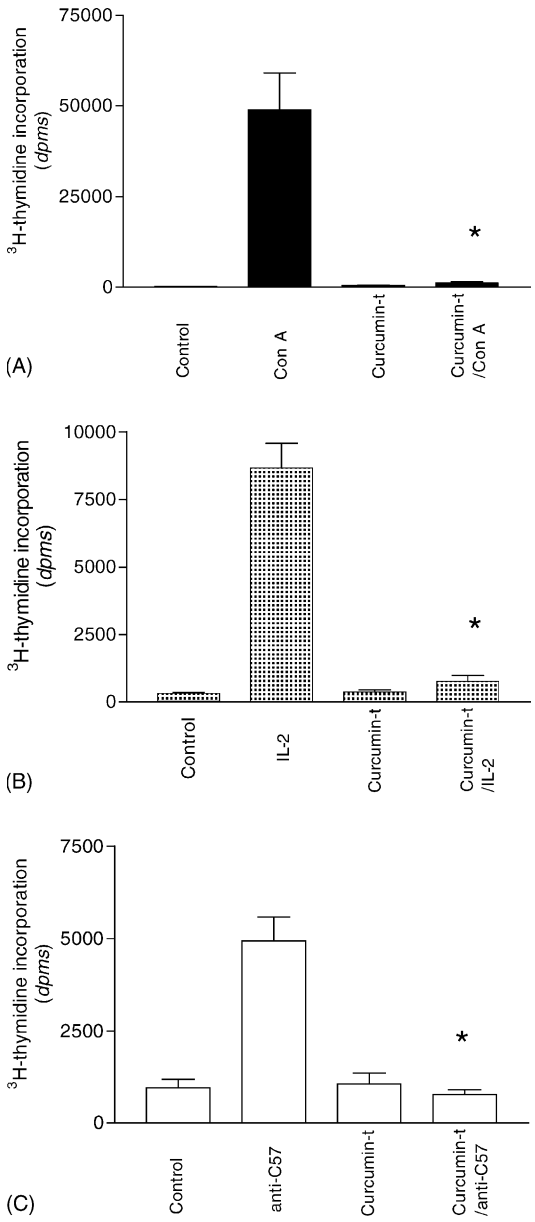

Fig. 4. Irreversibility of the antiproliferative effect of curcumin. Spleen cells (1×10^6 cells/ml) were treated or not with curcumin at 30 μmol/L for 8 h. Cells were washed three times in PBS (Curcumin-T) and then stimulated with Con A (A) or IL-2 (B) or irradiated C57BL/6 spleen cells (1:1) (C) for 4 days as described in Fig. 1. Proliferation of cells was determined by ^{3}H-thymidine incorporation as described. Data represent mean of three experiments ± S.D.(*) Significantly different from Con A or IL-2 or anti-C57 positive responses, $P < 0.001$.

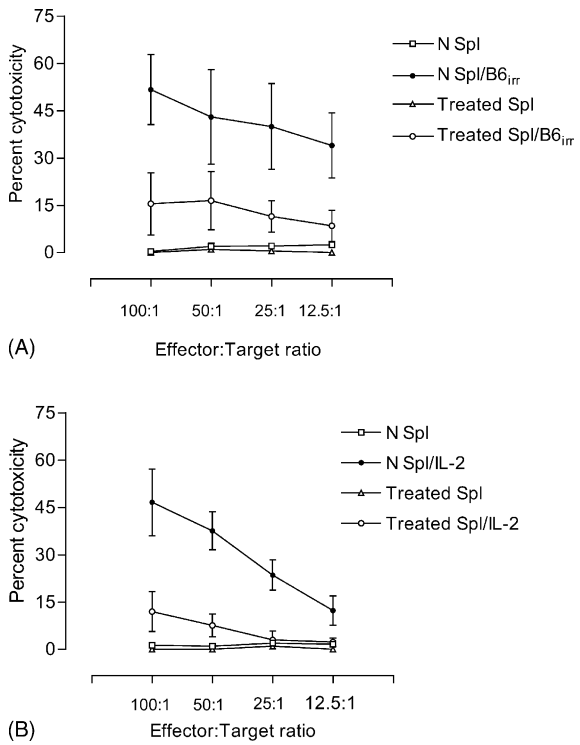

Fig. 5. Irreversibility of curcumin effect on CTL and LAK cell development. C3H spleen cells (1×10^6 cells/ml) were treated or not with curcumin at 30 μmol/L for 8 h. Cells were washed with PBS three times prior to stimulation with irradiated C57BL/6 spleen cells (1:1 responder/stimulator ratio) for 5 days or with IL-2 (150 ng/ml) for 3 days as described in Fig. 2. Cytotoxicity of effector cells against ^{51}Cr-labeled EL-4 (CTLs) or YAC-1 (LAK cells) target cells was determined in a 4 h ^{51}Cr release assay. Data represent mean ± S.D. of three experiments. CTL and LAK cell generation from spleen cells pretreated with curcumin was significantly reduced compared to untreated spleen cells ($P < 0.001$).

3.4. Curcumin inhibits cytokine gene expression

To evaluate the effect of curcumin on cytokine expression in lymphocytes and macrophages, we measured induction of IL-2 and IFN-γ gene expression in spleen cells and of IL-12 in macrophages by RT-PCR. For this purpose, spleen cells were treated with curcumin for 1 h before stimulating them with Con A for 1 h. Similarly, peritoneal macrophage monolayers were treated with curcumin for 1 h prior to stimulation with LPS for 1 h. Total cellular RNA was isolated and reverse transcribed, amplified, and fractionated on 1% agarose DNA gel. Low level of IL-2 and IFN-γ gene expression was detected in normal spleen cells (Fig. 6, lane 1), which increased following stimulation of cells with Con A (lane 2). The expression of both genes was markedly suppressed in cells treated with 20 and 10 μmol/L curcumin (lanes 3 and 4). At 5 μmol/L curcumin, there was no effect of curcumin on IL-2 gene expression, but the expression of IFN-γ was suppressed (lane 5).

The effect of curcumin on expression of IL-12 in macrophages was tested at 20 and 5 μmol/L curcumin. As shown in Fig. 6B, the expression of IL-12 is markedly increased upon stimulation with LPS (lane 1 versus lane 2). However, the expression IL-12 was dramatically reduced at 20 μmol/L (lane 3), but was only slightly reduced at 5 μmol/L curcumin (lane 4).

3.5. Curcumin inhibits the production of cytokines

Since lymphocyte proliferation and the development of CTLs is cytokine dependent, the suppression of these immune responses by curcumin suggests that it may also impair the production of cytokines. To determine the effect of curcumin on cytokine production, we examined the production of IL-2 by splenic T lymphocytes or TNF-α by peritoneal macrophages in the continuous presence of curcumin or by cells that were pretreated with curcumin for

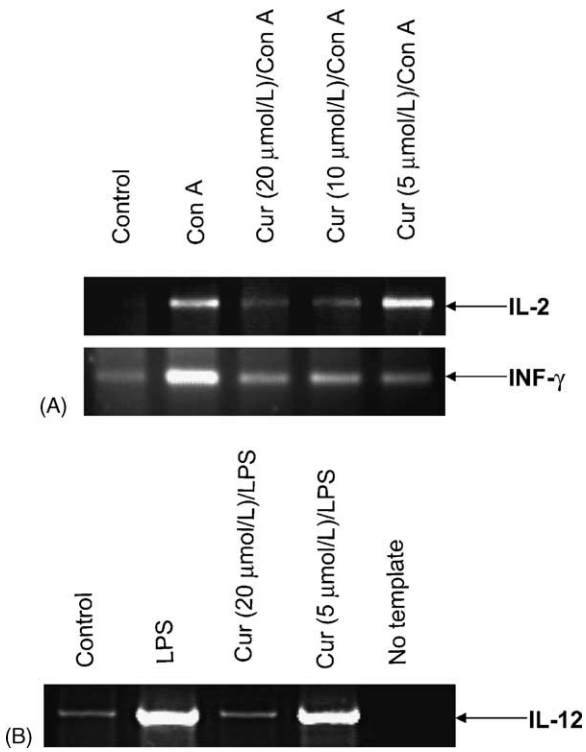

Fig. 6. Effect of curcumin on cytokine gene expression. Treatment of spleen cells with curcumin (5,10, or 20 μmol/L) was started 1 h prior to stimulating cells with Con A (1 μg/ml) for 1 h. Similarly, macrophage monolayers were treated with curcumin at 5 or 20 μmol/L starting 1 h prior to stimulation with LPS (500 ng/ml) for 1 h. Total cellular RNA was isolated and reverse transcribed using random primers to generate cDNAs. cDNA (1 μg) was amplified by PCR using gene specific primers (mIL-2, mIFN-γ, or mIL-12, p40). PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Expected amplified gene products of sizes 294 bp (IL-2), 267 bp (IFN-γ), and 1025 bp (IL-12, p40) were obtained. Similar results were obtained in two separate experiments.

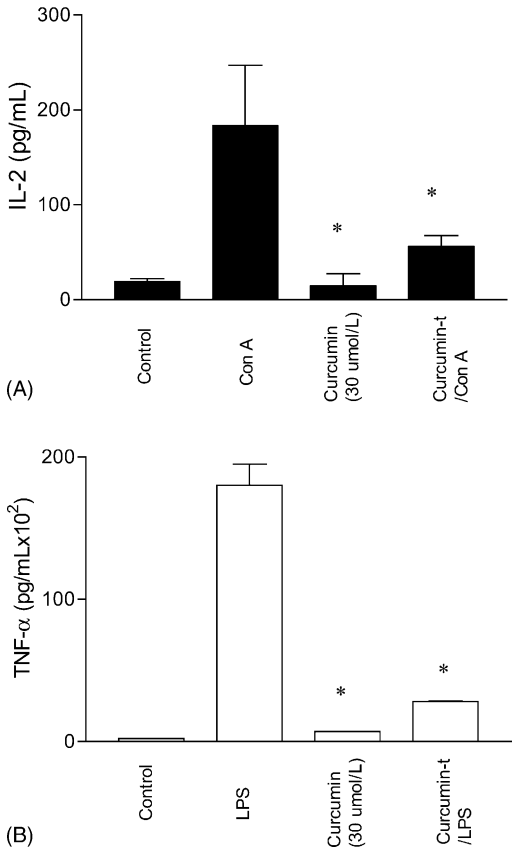

Fig. 7. Effect of curcumin on cytokine secretion. Spleen cells (2×10^6 ml^{-1}) or peritoneal macrophages (1×10^6 ml^{-1}) were treated or not with curcumin (30 μmol/L) for 8 h. Cells were washed with PBS three times and then stimulated with Con A (1 μg/ml) (A) or LPS (500 ng/ml) (B) for 20 h. Culture supernatants were collected by centrifugation and concentration of mIL-2 and mIFN-γ (spleen cells) or TNF-α (macrophages) was measured using commercially available cytokine-specific ELISA kits. Data are means ± S.D. of two experiments. (*) Production of IL-2 and TNF-α is significantly suppressed by curcumin at 30 μmol/L ($P < 0.001$); production of both cytokines by cells pretreated with curcumin is also significantly reduced compared to untreated cells ($P < 0.001$).

8 h and then washed free of it before inducing the production of cytokines. As shown in Fig. 7A, stimulation of spleen cells with Con A for 20 h caused several-fold increase in IL-2 production (183.5 ± 63 ng/ml by stimulated cells versus 19.2 ± 2.9 ng/ml by unstimulated cells), which was completely inhibited at 30 μmol/L curcumin. The inhibitory effect of curcumin on IL-2 production was irreversible since spleen cells pretreated with curcumin (30 μmol/L) for 8 h produced markedly reduced amount of IL-2 compared to untreated control cells upon stimulation with Con A ($P < 0.001$)). Curcumin also significantly ($P < 0.001$)) reduced the production of TNF-α by macrophages at 30 μmol/L (Fig. 7B). The suppressive effect of curcumin on TNF-α production by macrophages was also irreversible, since suppression of TNF-α production was not reversed following the removal of curcumin from cultures (Fig. 7B).

3.6. Curcumin impairs NF-κB activation

Activation and nuclear translocation of transcription factor NF-κB is a critical step in the transcription of cytokine genes involved in the development of immune and inflammatory responses [29]. To investigate whether inhibition of the cytokine gene expression by curcumin results from inhibition of NF-κB activation, we measured the NF-κB DNA binding activity in spleen cells treated with curcumin. Spleen cells were pretreated with curcumin at 20, 10, and 5 μmol/L for 1 h followed by stimulation with Con A for 45 min. Nuclear protein was isolated and level of NF-κB was measured by western blotting. Fig. 8A demonstrates marked increase in NF-κB level in the nucleus following stimulation of cells with Con A. Treatment with curcumin at 20 μmol/L reduced the level of NF-κB more than 60%. There was no effect of curcumin on nuclear level of NF-κB at 10 or 5 μmol/L. Measurement of NF-κB in whole cell lysate by ELISA also demonstrated suppression of NF-κB by curcumin (Fig. 8B). In these measurements, curcumin inhibited the inducible but not the constitutively expressed NF-κB at 20 and 10 μmol/L ($P < 0.001$). At 5 μmol/L curcumin, NF-κB was insignificantly increased compared to cells treated with Con A alone. Together, these results indicate that suppression of various immune responses by curcumin may, at least in part, result from suppression of the activation of NF-κB activity by curcumin.

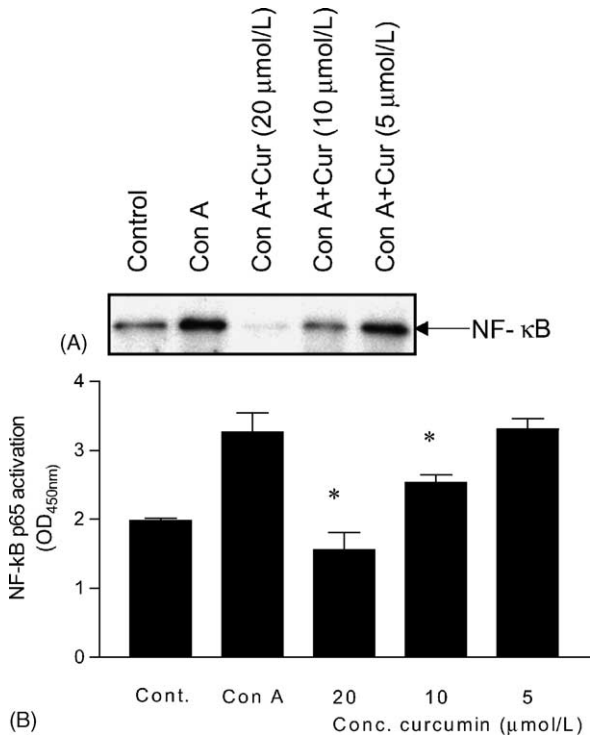

Fig. 8. Effect of curcumin on activation of NF-κB. Spleen cells were pretreated with curcumin (5–30 μmol/L) for 1 h before stimulating with Con A (1 μg/ml) for 45 min. Nuclear extracts were prepared from control and treated cells and NF-κB was analyzed by immunoblotting using anti NF-κB (p65) antibody (A). Separately, whole cellular lysates were prepared from control and treated cells and NF-κB was measured using ELISA based TransAm NF-κB kit from Active Motif (B). Similar results were obtained in two independent experiments. (*) Significantly reduced compared to Con A ($P < 0.001$).

4. Discussion

Curcumin has been used in traditional medicine for centuries in the Indian subcontinent to treat inflammatory disorders such as rheumatoid arthritis, intestinal inflammatory conditions, atherosclerosis, skin wounds, and hepatic and biliary disorders [10,30]. However, the mechanism of anti-inflammatory action of curcumin is not well understood. In the present study we considered the possibility that the beneficial effects of curcumin against inflammatory disorders are attributed to the suppression of T cell-mediated immune functions that play a pivotal role in the pathogenesis of the chronic inflammatory disorders. We investigated the effect of curcumin on the development of several lymphocytic responses in vitro, including mitogen/antigen induced T cell proliferation, cell-mediated cytolysis, and production of cytokines. Curcumin, for the most part, inhibited the proliferation of normal spleen cells induced with Con A, IL-2, or alloantigens. With the exception of significant enhancement of Con A induced proliferation of spleen cells at 6.5 μmol/L curcumin, the proliferative response induced with each of the three inducers was significantly suppressed at 25 μmol/L and abolished at 50 μmol/L curcumin. The inhibition of proliferation of spleen cells corroborated previously reported antiproliferative effect of curcumin on myelin basic protein-specific T lymphocytes [31] and proliferation of several mouse and human leukemia cell lines previously reported by us and others [25,32,33]. Although the exact mechanism of the antiproliferative action of curcumin is not known, the possibility exists that curcumin suppresses proliferation of cells by inhibiting ribonucleotide reductase

and DNA polymerase activation, two key enzymes involved in DNA synthesis as well as processes that are essential to allow cells to progress through the S phase of the cell cycle [34,35]. In addition, the antiproliferative effect of curcumin on lymphocytes could also be attributed due to the anti-oxidant properties of curcumin, since reactive oxygen species have been shown to have a role in cell response to cytokines and growth factors [36,37].

Cytotoxic T lymphocytes play an important role in the pathogenesis of autoimmune diseases. The development of cytotoxic T cells is a complex process involving antigen induced activation of Th1 helper cells which results in production of essential cytokines needed for the differentiation and maturation of precursors of cytotoxic T cells [38]. We investigated the effect of curcumin on both the generation of cell-mediated cytotoxicity and production of cytokines. A concentration related effect of curcumin was observed on the production of cytotoxic responses. The generation of alloantigen specific CTLs was only slightly reduced at 10 μmol/L curcumin, whereas production of these cells was significantly to completely abolished at 20–30 μmol/L curcumin. The production of broadly non-specific cytotoxic LAK cells by IL-2 was somewhat resistant to suppression by curcumin compared to the suppression of CTL production. LAK cell production was significantly reduced only at the highest concentration of curcumin (30 μmol/L). Curcumin did not affect the cytotoxic activity of CTLs or LAK cells, since preincubation of effector cells with curcumin (30 μmol/L) for 90 min had no effect on lysis of the target cells (not shown). This result indicates that curcumin inhibits the development of cytotoxic cells most likely by interfering with the differentiation and/or maturation of the precursors of cytotoxic cells.

To study whether the effect of curcumin was transient or permanent, spleen cells were treated with curcumin (30 μmol/L) for 8 h and then the compound was removed by extensively washing the cells. The results of these experiments demonstrated that the suppressive effect of curcumin on the proliferation of lymphocytes is not reversed upon removal of curcumin from the cultures. Even when pretreated cells were incubated for 24 h prior to induction of proliferation the suppression by curcumin was not reversed. This suggests that curcumin irreversibly interferes with the molecular processes involved in proliferation of lymphocytes. Although the exact mechanism by which curcumin irreversibly blocks the proliferation of lymphocytes is not known, it may involve inhibition of D type cyclins that are required for the progression of cells from the G1 phase of the cell cycle to S phase (DNA synthesis). Indeed, curcumin has been shown to inhibit cyclin D1 and arrest multiple myeloma cells in G_1/S phase of the cell cycle [32,33]. Consistent with the effect on T cell proliferation, the inhibitory effect of curcumin on the development of cytotoxic effector cells was also irreversible. Whether clonal deletion of the precursors of cytotoxic cells by curcumin plays a role in irreversibility of the inhibitory effect of curcumin on the generation of cytotoxic responses remains unknown at present.

IL-2 and IL-12 play a crucial role in the progression of antigen activated T cells from G_1 to S/G_2/M phase of the cell cycle [39,40], and TNF-α and IFN-γ are required for the production and cytolytic effects of cytotoxic cells. The inhibition of T cell activation/proliferation and generation of cytotoxic effector by curcumin may involve suppression of the production of these cytokines. Our data demonstrated that curcumin inhibits the expression of IL-2 and IFN-γ mRNAs in splenic T cells, and IL-12 mRNAs in macrophages. In addition, the secretion of IL-2 by T cells and TNF-α by macrophages was irreversibly inhibited by curcumin. These findings corroborate the results of earlier studies in which curcumin was shown to inhibit IFN-γ by $CD4^+$ T cells [41] and IL-12 by macrophages [31,42]. Thus, inhibition of T cell proliferation and the development of cytotoxic effector cells by curcumin are attributed, at least in part, to the suppression of cytokine production by curcumin.

The molecular targets involved in the anti-inflammatory and anticarcinogenic effects of curcumin are less well understood. Others have shown that curcumin inhibits NF-κB and JAK-STAT signaling pathways [31,43,44]. Since NF-κB plays a critical role in transcription of several genes involved in immune and inflammatory responses [29,45], cell proliferation/differentiation [46] and cell transformation [47], we considered the possibility that suppression of lymphocyte proliferation, CTL development, and cytokine production may result from the suppression of NF-κB by curcumin. In resting cells, NF-κB remains sequestered in the cytoplasm in a functionally inactive form noncovalently bound to an inhibitory protein, IκBα [48]. Upon stimulation of cells with mitogens, antigens, or cytokines, IκBα is phosphorylated and degraded allowing NF-κB to translocate to the nucleus where it binds to the κB motifs in the promoter region of the responsive genes. Consistent with the previously reported suppression of NF-κB activation by curcumin [43,44], our studies also demonstrated the inhibition of Con A induced activation of NF-κB in spleen cells by curcumin. Whether curcumin inhibited NF-κB signaling in T cells by interfering with the degradation of IκBα remains to be determined. Thus, suppression of proliferation of T cells, generation of cytotoxic effector cells, and production of cytokines by curcumin may result from inhibition of the transcription of NF-κB responsive genes involved in the production of these immune responses.

In conclusion, we have demonstrated that curcumin inhibits the mitogen/antigen induced lymphocyte proliferation, development of cell-mediated cytotoxicity, and the production of cytokines. The inhibition of the development of these responses results at least in part from suppression of the activation of transcription factor NF-κB by curcumin.

Acknowledgments

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