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Curcumin inhibits Th1 cytokine profile in CD4$^+$ T cells by suppressing interleukin-12 production in macrophages

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1 Interleukin-12 (IL-12) plays a central role in the immune system by driving the immune response towards T helper 1 (Th1) type responses which are characterized by high IFN-γ and low IL-4 production. In this study we investigated the effects of curcumin, a natural product of plants obtained from *Curcuma longa* (turmeric), on IL-12 production by mouse splenic macrophages and the subsequent ability of these cells to regulate cytokine production by CD4$^+$ T cells.

2 Pretreatment with curcumin significantly inhibited IL-12 production by macrophages stimulated with either lipopolysaccharide (LPS) or head-killed *Listeria monocytogenes* (HKL).

3 Curcumin-pretreated macrophages reduced their ability to induce IFN-γ and increased the ability to induce IL-4 in Ag-primed CD4$^+$ T cells. Addition of recombinant IL-12 to cultures of curcumin-pretreated macrophages and CD4$^+$ T cells restored IFN-γ production in CD4$^+$ T cells.

4 The *in vivo* administration of curcumin resulted in the inhibition of IL-12 production by macrophages stimulated *in vitro* with either LPS or HKL, leading to the inhibition of Th1 cytokine profile (decreased IFN-γ and increased IL-4 production) in CD4$^+$ T cells.

5 These findings suggest that curcumin may inhibit Th1 cytokine profile in CD4$^+$ T cells by suppressing IL-12 production in macrophages, and points to a possible therapeutic use of curcumin in the Th1-mediated immune diseases.

Keywords: Curcumin; interleukin-12; macrophage; T helper cell; cytokine profile

Abbreviations: Ag, antigen; APC, antigen-presenting cell; ELISA, enzyme-linked immunosorbent assay; HKL, heat-killed *Listeria monocytogenes*; IFN-γ, interferon-gamma; IL, interleukin, KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; MAb, monoclonal antibody; NF, nuclear factor; rIL-12, recombinant interleukin-12; Th, T helper

Introduction

T helper lymphocytes can be divided into two distinct subsets of effector cells, Th1 and Th2 cells, based on their functional capabilities and the profile of cytokines they produce (Mosmann, 1991). The Th1 subset of CD4$^+$ T cells secretes cytokines usually associated with inflammation such as interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β), and induces cell-mediated immune responses. The Th2 subset produces cytokines such as IL-4 and IL-5 that help B cells to proliferate and differentiate and is associated with humoral immune responses (Seder & Paul, 1994; Constant & Bottomly, 1997). Recent studies indicate that the ratio of these two Th cell types, Th1 and Th2, is closely correlated with the outcome of many diseases (Romagnani, 1996a; D'Elios & Del Prete, 1998). Polarized Th1-type and Th2-type responses play different roles in protection, Th1 being effective in the defence against intracellular pathogens and Th2 against intestinal nematodes (Else & Finkelman, 1998). Moreover, Th1 responses predominate in organ-specific autoimmune disorders, acute allograft rejection, unexplained recurrent abortions and in some chronic inflammatory disorders of unknown etiology (Hayashi *et al*., 1995; Lafaille, 1998). In contrast, Th2 responses predominate in Omenn's syndrome, transplantation tolerance, chronic graft *versus* host disease, systemic sclerosis, and allergic diseases (Grewe *et al*., 1998).

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The nature of Th1 or Th2 polarizing signals is not yet fully understood. However, the factors that seem to play a role in driving naive CD4$^+$ T cells toward Th1- or Th2-dominated populations are the type and the amount of antigen as well as the density and affinity of the peptide ligand, the nature of antigen-presenting cells and of their co-stimulants, and hormones released into the micro-environment, and genetic background of the T cell (Romagnani, 1996b). Most importantly, the cytokines that are present in the environment of the CD4$^+$ T cell at the time it encounters the antigen regulate the differentiation of Th cells into either Th1 or Th2 subsets (O'Garra, 1998). IL-12 promotes Th1 differentiation, while IL-4 plays a key role in the differentiation of the precursor CD4$^+$ T cells toward a Th2 phenotype.

Interleukin-12 (IL-12), a heterodimeric cytokine composed of two disulphide-linked subunits of 35 (p35) and 40 (p40) kDa encoded by two separate genes, is produced by phagocytic cells and other antigen-presenting cells in response to stimulation by a variety of microorganisms as well as their products (Benjamin *et al*., 1996; Kang *et al*., 1996; Lawrence and Nauciel, 1998). IL-12 exerts multiple biological activities mainly through T and natural killer (NK) cells by inducing their production of interferon-γ (IFN-γ), which augments their cytotoxicity, and by enhancing their proliferation potential. IL-12 production is critical for the development of Th1 cells and the initiation of cell-mediated immune responses (Trinchieri, 1998a). Recent evidence points to a critical role

for IL-12 in the pathogenesis of rodent models of Th1-mediated autoimmune diseases such as type-1 diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and acute graft–*versus*–host disease. IL-12 has also been implicated in the pathogenesis of sarcoidosis, a multi-system disorder characterized by non-caseating granulomatous inflammation and dominant type-1 cytokine expression (Gately *et al*., 1998; Trinchieri, 1998b). Thus, pharmacological control of IL-12 production may be a key strategy in modulating specific immune-mediated diseases dominated by type-1 cytokine responses.

Curcumin, widely used as a spice and responsible for the yellow colour of curry, is a natural product of plants obtained from *Curcuma longa* Linn (turmeric). Curcumin is known to exhibit a variety of pharmacological effects including anti-tumour, anti-inflammatory, anti-HIV and anti-infectious activities (Mazumder *et al*., 1996; Allen *et al*., 1998; Chan *et al*., 1998; Vlietinck *et al*., 1998), and is under preclinical trial evaluation for drug development of cancer prevention and anti-inflammation (Gescher *et al*., 1998).

In this study, we investigated the effect of curcumin on IL-12 production in mouse macrophages. Here we demonstrate that pretreatment with curcumin inhibits IL-12 production in macrophages stimulated with LPS or HKL. Importantly, inhibition of IL-12 production in curcumin-pretreated macrophages resulted in the inhibition of Th1 cytokine profile (decreased IFN-γ and increased IL-4 production) in Ag-primed $CD4^+$ T cells.

Methods

Preparation of splenic macrophages stimulated with either LPS or HKL

Splenic macrophages were isolated from DBA/2 mice at 6–10 weeks of age (Japan SLC, Tokyo, Japan) and stimulated, as previously described (Na *et al*., 1999). In brief, spleen cells were cultured at 10^6 cells ml^{-1} for approximately 3 h in DMEM containing 10% foetal bovine serum (FBS) (GIBCO BRL, Grand Island, NY, U.S.A.) at 37°C in a 5% CO_2 humidified air atmosphere. The non-adherent cells were removed by washing with warm DMEM until visual inspection revealed a lack of lymphocytes (>98% of the cell population). The adherent cells were removed from plates by incubating for 15 min with ice-cold phosphate-buffered saline solution and rinsing repeatedly. The isolated adherent cell population was stimulated with 5 μg ml^{-1} LPS (from *E. coli* 0111 : B4; Sigma, St. Louis, MO, U.S.A.) in the absence or presence of curcumin (Sigma) at 0.5, 1.0, 2.5, 5.0 μg ml^{-1} at 1×10^5 cells per well in 96-well culture plates for 48 h. For some experiments, the cells were stimulated with HKL at 2×10^6 bacteria per well.

Purification and induction of cytokine synthesis in $CD4^+$ T cells

Draining axillary, popliteal and inguinal lymph nodes were removed from mice 9 days after priming with 100 μg KLH (Calbiochem., San Diego, CA, U.S.A.) in CFA in the footpads. Lymph node cells were depleted of B cells by adherence to goat anti-mouse Ig-coated dishes for 1 h at 4°C. Nonadherent cells were depleted of $CD8^+$ T cells and other antigen-presenting cells by treating the cells with a mixture of anti-CD8 and anti-class II MAbs on ice for 30 min, followed by addition of low toxicity rabbit complement (Pel Freeze, Rogers, AR, U.S.A.) and incubation at 37°C for 45 min. More than 95% of the cells were $CD4^+$ T cells, as demonstrated by cytofluorometric analysis using anti-CD4 MAb (PharMingen, San Diego, CA, U.S.A.). Purified $CD4^+$ T cells were incubated in 96-well plates at 4×10^5 cells per well with macrophages (1×10^5 per well) and KLH (10 μg ml^{-1}). Culture supernatants were harvested after 2 days (for IL-12 p70) or after 4 days (for IFN-γ and IL-4), and assayed by ELISA.

Cytokine assays

The quantities of IFN-γ, IL-4, IL-10, and IL-12 p70 in culture supernatants were determined by a sandwich ELISA using MAbs specific for each cytokine as previously described (Kim *et al*., 1997). The MAbs for coating the plates and the biotinylated second MAbs (PharMingen) were as follows: for IFN-γ, rat anti-mouse IFN-γ (HB170) and biotinylated rat anti-mouse IFN-γ (XMG1.2); for IL-4, rat anti-mouse IL-4 (BVD4-1D11) and biotinylated anti-mouse IL-4 (BVD6); for IL-10, rat anti-mouse IL-10 (JES-2A5) and biotinylated rat anti-mouse IL-10 (SXC-1); for IL-12 p70, anti-mouse IL-12 (p35/p70) (Red-T/G297-289) and rat anti-mouse IL-12 p40 (C17.8). Standard curves were generated using recombinant cytokines. Murine rIL-12 was provided from Dr S. Wolf (Genetics Institute, Cambridge, MA, U.S.A.), and rIFN-γ, rIL-4 and rIL-10 were purchased from PharMingen. The lower limit of detection was 125 pg ml^{-1} for IFN-γ, 3 pg ml^{-1} for IL-4, 0.2 ng ml^{-1} for IL-10, and 50 pg ml^{-1} for IL-12 p70.

Statistical analysis

Student *t*-test was used to determine the statistical differences between various experimental and control groups. A P value of <0.01 was considered as significant.

Results

Pretreatment with curcumin inhibited IL-12 production from mouse macrophages stimulated with either LPS or HKL

To determine whether curcumin could affect the production of T cell cytokine indirectly *via* an effect on IL-12 production by antigen-presenting cells (APCs) such as macrophages, we first investigated the effect of curcumin on IL-12 production in mouse macrophages. Macrophages were treated with varying amounts of curcumin for 4 h. After washing out the curcumin in the cultures, the cells were stimulated with either LPS or HKL for 48 h. As shown in Figure 1, LPS or HKL readily induced IL-12 production, as expected. Pretreatment of macrophages with curcumin significantly inhibited this induced IL-12 production in a dose-dependent manner ($P<0.01$ at 2.5 and 5.0 μg ml^{-1} curcumin, relative to an untreated group). In contrast, treatment with curcumin did not significantly influence IL-10 production from macrophages, suggesting that the inhibition of IL-12 production by curcumin was not the result of a general dampening of cellular activation.

Pretreatment of macrophages with curcumin inhibited IFN-γ production and enhanced IL-4 production by antigen-primed $CD4^+$ T cells

Since IL-12 has been known to potently enhance IFN-γ and inhibit IL-4 in $CD4^+$ T cells, we asked if the cytokine profiles of $CD4^+$ T cells responding to Ag presented by curcumin-

treated macrophages would be altered. Direct effects of curcumin on $CD4^+$ T cells were eliminated by pretreatment of macrophages *in vitro* with curcumin for 4 h and washing them to remove curcumin, before culture with syngeneic $CD4^+$ T cells purified from lymph nodes of KLH-primed mice and the Ag KLH. Figure 2A shows that IL-12 production in cultures of curcumin-treated macrophages was significantly decreased in comparison with untreated macrophages. In the absence of curcumin treatment, stimulation with KLH resulted in the development of T cells producing high levels of IFN-γ. However, pretreatment of macrophages with curcumin for 4 h greatly inhibited their capacity to induce IFN-γ production by $CD4^+$ T cells (Figure 2B) and significantly increased IL-4 production (Figure 2C). No cytokine production by $CD4^+$ T cells was detected in the absence of macrophages, demonstrating that the curcumin-treated macrophages regulated the cytokine production by $CD4^+$ T cells. Thus, pretreatment of macrophages with curcumin enhances their capacity to preferentially inhibit Th1 and enhance Th2 cytokine synthesis.

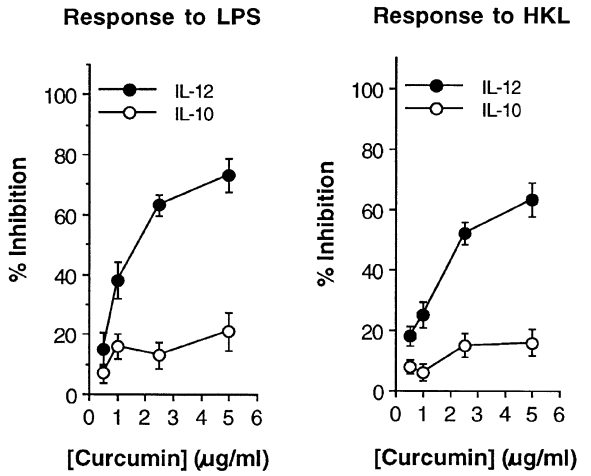

Figure 1 Inhibition of IL-12 production in macrophages by curcumin. Primary macrophages were treated with varying concentrations of curcumin or left untreated. After 4 h, the cells were washed and stimulated with either LPS or HKL for 48 h. Culture supernatants were harvested and cytokine levels were evaluated by ELISA. The results are presented as mean $\pm$ s.d. of the % inhibition of cytokine production of curcumin-treated macrophages compared with untreated control macrophages stimulated with either LPS or HKL. Mean cytokine levels in the absence of curcumin were 1730 pg ml^{-1} (IL-12), 1250 ng ml^{-1} (IL-10) for LPS-stimulated macrophages, and 3650 pg ml^{-1} (IL-12), 1580 ng ml^{-1} (IL-10) for HKL-stimulated macrophages.

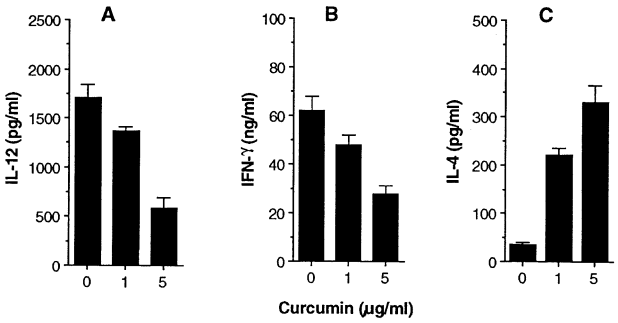

Figure 2 Macrophages pretreated with curcumin inhibit IFN-γ and enhance IL-4 production by Ag-primed $CD4^+$ T cells. Macrophages (1×10^5/well) were pretreated with media or 5 μg ml^{-1} curcumin. After 4 h, the cells were washed and incubated with KLH-primed $CD4^+$ T cells (5×10^5/well) and KLH (10 μg ml^{-1}). Supernatants were harvested after 2 days for IL-12 (A) or after 4 days for IFN-γ (B) and IL-4 (C), and assayed by ELISA. The results shown are representative of three separate experiments.

Addition of recombinant IL-12 (rIL-12) to cultures of curcumin-pretreated macrophages and $CD4^+$ T cells restored the levels of IFN-γ production in $CD4^+$ T cells

To determine whether the reduced ability of curcumin-pretreated macrophages to induce IFN-γ synthesis in $CD4^+$ T cells was a result of their diminished production of IL-12, we reconstituted cultures of curcumin-pretreated macrophages and $CD4^+$ T cells with rIL-12. Figure 3 shows that addition of rIL-12 to cultures of curcumin-pretreated macrophage and $CD4^+$ T cells significantly increased IFN-γ and reduced IL-4 production to levels seen in untreated control cultures. These results suggest that reduction of IL-12 synthesis by curcumin-treated macrophages was a major effect that affected the ability of macrophages to regulate cytokine synthesis in $CD4^+$ T cells.

Macrophages from mice treated in vivo *with curcumin also inhibited Th1 cytokine profile by Ag-primed $CD4^+$ T cells*

To demonstrate that curcumin had a consequential effect on macrophages in an *in vivo* setting, mice were injected intraperitoneally (i.p.) with 500 μg curcumin per mouse. After 24 h, splenic macrophages were purified from the curcumin-treated mice, or from saline-injected control mice. Figure 4 shows that macrophages from curcumin-treated mice produced much lower amounts of IL-12 in response to either LPS or HKL than macrophages from control mice.

To determine whether cytokine production by Ag-primed $CD4^+$ T cells would differ in the presence of Ag presented by macrophages from mice treated *in vivo* with curcumin, macrophages were purified from DBA/2 mice 24 h following i.p. injection with curcumin (500 μg), and cultured with $CD4^+$ T cells from KLH-primed mice, and KLH. Figure 5 demonstrates that production of IL-12 in cultures containing Ag-primed $CD4^+$ T cells and macrophages from curcumin-treated mice was greatly reduced compared with those from saline-injected control mice. Moreover, macrophages from mice injected with curcumin significantly induced lower

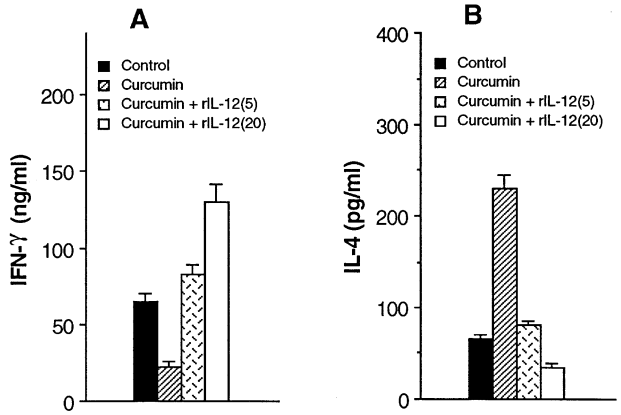

Figure 3 Effect of rIL-12 on cytokine production of T cells in cultures of curcumin-pretreated macrophages and Ag-primed $CD4^+$ T cells. $CD4^+$ T cells were cultured with curcumin-pretreated macrophages in the presence of rIL-12 (5 or 20 pg ml^{-1}). Supernatants were harvested 4 days later and assayed for their IFN-γ and IL-4 content by ELISA. The results shown are representative of three separate experiments.

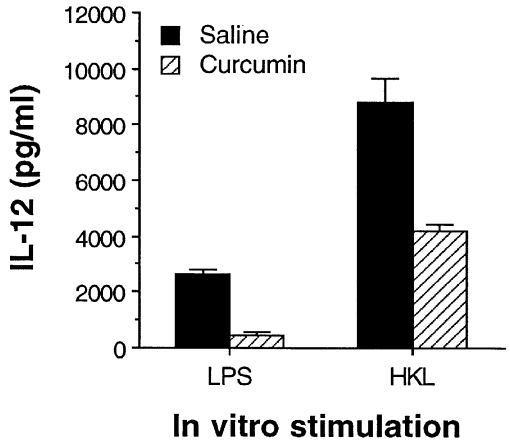

Figure 4 Decreased levels of IL-12 production in macrophages exposed to curcumin *in vivo*. Mice were treated *in vivo* with curcumin (500 μg, i.p.). After 24 h, macrophages were purified and stimulated with either LPS or HKL. Culture supernatants were harvested 48 h later and IL-12 content was assayed by ELISA. The experiment is representative of three experiments.

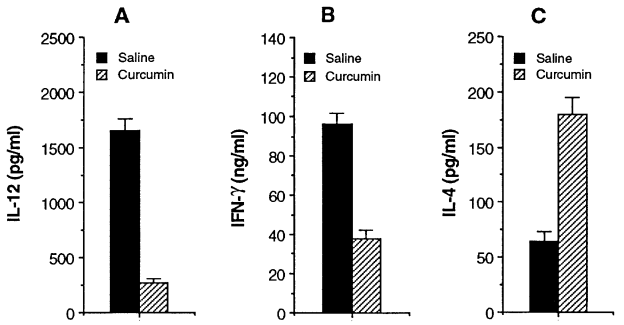

Figure 5 Regulation of cytokine production in Ag-primed CD4$^+$ T cells by macrophages purified from mice treated *in vivo* with curcumin. DBA/2 mice were treated *in vivo* with either curcumin (500 μg, i.p.) or saline. After 24 h, macrophages were purified and incubated with KLH-primed CD4$^+$ T cells and KLH (10 μg ml^{-1}). After 4 days, culture supernatants were harvested and assayed for IL-12 (A), IFN-γ (B) and IL-4 (C) content by ELISA. This experiment is representative of two experiments.

amounts of IFN-γ and greater amounts of IL-4 than macrophages from control mice. These results show that *in vivo* treatment with curcumin regulates the ability of macrophages to induce IFN-γ and IL-4 production.

Discussion

Inhibiting the action of IL-12 has been shown to prevent development and block progression of disease in experimental models of autoimmunity (Caspi, 1998). These findings have raised great interest in identifying inhibitors of IL-12 production for the treatment of Th1-mediated diseases such as type-1 diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease and acute graft–*versus*–host disease. In this study we demonstrated that pretreatment of macrophages with curcumin, a natural product of plants obtained from *Curcuma longa* Linn (turmeric), inhibited IL-12 production from macrophages, resulting in a reduced ability to induce IFN-γ and an increased ability to induce IL-4 in CD4$^+$ T cells. These results suggest that curcumin-mediated inhibition of IL-12 production led to the inhibition of Th1 and enhancement of Th2 cytokine synthesis in CD4$^+$ T cells. Since the cytokine profile of Th cells plays an important role to determine the outcome of many diseases, curcumin may have therapeutic potential to treat Th1-mediated diseases. Conversely, curcumin may trigger or enhance Th2-mediated diseases because of its ability to increase IL-4 production.

Although curcumin may affect cytokine production in CD4$^+$ T cells in several ways, we believe that inhibition of IL-12 production in macrophages is a major mechanism by which curcumin affects cytokine production in CD4$^+$ T cells, particularly since IL-12 is extremely potent in enhancing IFN-γ and inhibiting IL-4 in CD4$^+$ T cells (Marshall *et al*., 1995; Gerosa *et al*., 1996; Umetsu *et al*., 1996). In our cultures, the effect of curcumin on cytokine production in CD4$^+$ T cells was indirect since the CD4$^+$ T cell in these cultures were never directly exposed to the curcumin. Similar studies showed that β2-adrenergic compounds, including salbutamol, inhibited IL-12 production from human monocytes or dendritic cells by increasing intracellular cyclic AMP levels, leading to the inhibition of Th1 development while promoting Th2 cell differentiation (Panina-Bordignon *et al*., 1997). Corticosteroids have been known to enhance the capacity of macrophages to induce IL-4 synthesis in CD4$^+$ T cells by inhibiting IL-12 production (DeKruyff *et al*., 1998).

The mechanism(s) by which curcumin inhibits IL-12 production in LPS- or HKL-activated macrophages is not known. One possible mechanism is that curcumin may inhibit IL-12 production in macrophages by down-regulating NF κ B activity in IL-12 p40 gene, which was known as the highly inducible and tightly regulated component of IL-12 (Kang *et al*., 1996). Our studies showed that the inhibitory effect of curcumin on a series of 5′ deletions of the IL-12 p40 promoter was retained within -185 bp upstream of the transcription initiation site, suggesting that curcumin may interfere with NF κ B-mediated activation at position $-121/-131$ bp (κB site) in the IL-12 p40 promoter (data not shown). Earlier reports also indicated that in TNF-activated Jurkat T lymphoma cells curcumin inhibited NF κ B signalling by interfering with IkBα degradation (Brennan & O'Neill, 1998).

In conclusion, we have shown that curcumin inhibited IL-12 production in macrophages in a dose-dependent manner, leading to the inhibition of Th1 cytokine profile in CD4$^+$ T cells. These results suggest that curcumin-induced inhibition of IL-12 production in macrophages may explain a variety of known biological effects of curcumin including anti-inflammatory activity, and curcumin may be useful in treatment of Th1-mediated immunological disorders.

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