Curcumin modulation of IFN-β and IL-12 signalling and cytokine induction in human T cells

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Abstract

Curcumin is a polyphenol derived from the dietary spice turmeric. It possesses diverse anti-inflammatory and anti-cancer properties. Curcumin has been shown to exhibit an inhibitory effect on the production of inflammatory cytokines by human monocytes and has inhibited the animal model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE) in association with a decrease in interleukin 12 (IL-12) production and signal transducer and activator of transcription 4 (STAT4) activation. The type I interferon (IFN) IFN-β has the ability to suppress IL-12. Both IL-12 and IFN-α/β signal through the activation by phosphorylation of STAT4. Our aim was to investigate the effects of curcumin on the ability of T cells to respond to IL-12 or IFN-α/β. We report that curcumin decreases IL-12-induced STAT4 phosphorylation, IFN-γ production, and IL-12 Rβ1 and β2 expression. IFN-β-induced STAT4 phosphorylation, IL-10 production and IFN receptor (IFNAR) subunits 1 and 2 expression were enhanced by curcumin. Curcumin increased IFN-α-induced IL-10 and IFNAR1 expression. Prior exposure to curcumin decreased IFN-α-induced IFNAR2 expression and did not modify the level of IFN-α-induced pSTAT4 generation. Thus, the effect of curcumin on STAT4 activation in T cells is dependent upon the stimulus to which the T cells have been exposed.

Keywords: human • T cells • curcumin • interleukin-12 • interferon • signal transduction

Introduction

Curcumin (diferuloylmethane) is a naturally occurring yellow pigment isolated from the rhizomes of the plant Curcuma Longa Lin, found in south Asia, and is an important ingredient of the dietary spice, turmeric. Its medicinal value has been well recognized with its antioxidant, anti-tumour, and anti-inflammatory activities and is under pre-clinical trial for the treatment of cancer and inflammation. Curcumin is a potent inhibitor of tumour initiation in vivo [1] and possesses anti-proliferative and apoptotic activities against tumour cells in vitro [2]. Curcumin has been shown to facilitate apoptosis of cancer cells via inhibition of signal transducer and activator of transcription 3 (STAT3) activation and can also suppress interferon (IFN)-α-induced STAT1 phosphorylation, without affecting STAT5 phosphorylation [3]. Curcumin has been shown to have an inhibitory effect on the production of inflammatory cytokines by human monocytes and alveolar macrophages [4]. Natarajan and Bright (2002) examined the effect and mechanism of action of curcumin on the pathogenesis of central nervous system (CNS) demyelination in
experimental autoimmune encephalomyelitis (EAE), a T cell-mediated demyelinating disease of rodents, widely used as an animal model of multiple sclerosis (MS). They found that curcumin inhibited EAE in association with a decrease in interleukin (IL)-12 production. In vitro treatment of activated T cells with curcumin inhibited IL-12-induced tyrosine phosphorylation of STAT4 [5].

The type I interferon, IFN-β, is the best characterized and most used disease-modifying treatment for MS. It has been demonstrated that IFN-β significantly increases the expression of the anti-inflammatory cytokine IL-10 [6], a major suppressor of Th1 cytokines [7, 8]. Like IL-12, IFN-β and IFN-α also acts via the STAT4 pathway [9]. In this study, we investigated whether the inhibitory effects of curcumin were specific to IL-12 or whether similar effects would be observed with the type I interferons IFN-α/β. Here, we report that curcumin has a differential effect on IL-12 and IFN-α/β not only through differential effects on STAT4 phosphorylation, but also upstream at the receptor level. This in turn results in a differential modulation on the level of cytokine induction by IFN-α/β and IL-12.

Materials and methods

Cell preparation

Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by standard gradient centrifugation with Histopaque 1077 (Sigma-Aldrich, Dorset UK). The mononuclear cells were prepared at 1 x 10^6 cells/ml in media consisting of Roswell Park Memorial Institute (RPMI) 1640, 2 mM glutamine, 20 mM HEPES, 0.1 mg/ml penicillin and streptomycin and 10% foetal calf serum (Sigma-Aldrich). The cells were co-cultured with 10 µg/ml phytohaemagglutinin (PHA) (Sigma-Aldrich) at 37°C and 5% CO2 for 72 hrs. Following PHA-induced proliferation, the cells were washed with media and stimulated with 100 U/ml IL-2 (R&D systems, Minneapolis, MN, USA) at 37°C and 5%CO2 for a further 24 hrs. The cells were then allowed to rest for 24 hrs in serum-free media under the same conditions. In other experiments, PBMCs were co-cultured with 0.5 µg/ml of anti-CD3 for 72 hrs at 37°C and 5% CO2. Following incubation, the cells were also allowed to rest for 24 hrs in serum-free media under the same conditions. PBMC were also obtained from four patients with relapsing remitting MS who had had no relapses and no steroid treatment for at least 3 months prior to blood donation, and had never had any immunomodulatory or immunosuppressive treatment (including IFN-α/β) at the time of blood collection. The study was approved by the Nottingham Research Ethics Committee.

Cell stimulation

PHA/IL-2-induced T cell blasts (1 x 10^6 cells/ml) or anti-CD3 stimulated T cells (1 x 10^6 cells/ml) were either left untreated or pre-treated with 20 µg/ml Curcumin (Sigma-Aldrich) for 30 min at 37°C. Both sets of cells were then either left unstimulated or incubated with 10 ng/ml IFN-β, 10 ng/ml IFN-β or 0.1 µg/ml IL-12 for 30 min at 37°C. The method used was refined so that the optimum concentrations of cytokines and curcumin were used. Varying concentrations of IFN-β (a gift from Serono International, London, UK), IFN-α (Sigma-Aldrich) and IL-12 and prior incubation with curcumin were analysed for their effect on STAT4 phosphorylation; the concentrations used in this experiment were those that produced the peak pSTAT4 value and consistent suppression for curcumin. The effect of the duration of stimulation on STAT4 phosphorylation was also observed. The results generated suggested that the best stimulation was achieved after 30 min. For the ELISA, cells were stimulated for 18 hrs.

Intracellular staining

Following incubation, the cells were fixed in 1 ml of ice cold 70% ethanol and incubated on ice for 20 min. The cells were then washed by centrifugation once in PBA (PBS, 0.5% bovine serum albumin and 1% sodium azide [Sigma-Aldrich]), once in saponin buffer (PBA + 0.1% saponin [Sigma-Aldrich]) and once in 10% foetal calf serum (FCS) in saponin buffer at 300 g for 5 min. The supernatant was pored off and the cells re-suspended in the residue, to which 0.5 µg of the primary antibody, rabbit polyclonal anti-phosphorylated STAT4 or rabbit polyclonal STAT4 (Zymed, San Francisco, CA, USA) (method adapted for intracellular staining specific for STAT4 and phosphorylated STAT4 described by Uzel et al. [2001] [10]) or 10 µl monoclonal anti-human IFN-γ fluoroscein isothiocyanate isothiocyanate (FITC) antibody or monoclonal anti-human IL-10 phycoerythrin (PE), was added and incubated at room temperature for 30 min. Following incubation the cells were washed by centrifugation with saponin buffer. The cells incubated with non-conjugated primary antibody were incubated with the secondary antibody. 1 µg of PE conjugated goat anti-rabbit IgG for 30 min at room temperature. All cells were washed with 1 ml saponin buffer before resuspension in 500 µl of 0.5% formaldehyde for flow cytometry.
Surface staining

Following incubation, the cells were washed by centrifugation in 2% FCS RPMI, the supernatant was poured off and the cells re-suspended in the residue, to which 10 μl of IFN receptor (IFNAR) 1, or IL-12 Rβ1 PE labelled (R&D systems, Oxford, UK) antibodies were added and incubated on ice for 30 min. The cells were again washed by centrifugation in 2% FCS RPMI. Those cells incubated with the non-conjugated IFNAR antibody were incubated with a secondary antibody, goat antimouse FITC (Caltag, Laboratories, CA, USA) for a further 30 min on ice. All cells were washed twice in 1 ml 2% FCS RPMI before re-suspension in 500 μl of 0.5% formaldehyde for flow cytometry.

Quantitative real-time polymerase chain reaction (PCR)

Quantitative reverse transcriptase real-time PCR was used to assess IL-10, IL-12Rβ1, IL-12Rβ2, IFNAR1 and IFNAR2 mRNA abundance in human PHA/IL-2 T cell blasts that had been stimulated with curcumin and/or IFN-β, IFN-α or IL-12. RNA was extracted using RNeasy miniprep kit (Qiagen, Valencia, CA, USA) following the manufacturers' instructions. The RNA concentration was determined at 260 nm, and purity was assessed by measuring the 260/280 nm ratio. Only samples within a 1.70–1.95 range were used. First-strand cDNA synthesis was initiated from 0.5 µg total RNA, using random hexamers (Promega, Madison, WI, USA), and avian myeloblastosis virus reverse transcriptase (Promega) using conditions as described by the manufacturer in a final volume of 25 µl. Specific oligonucleotide primers were designed for the published sequence: IL-10, (Genbank accession no. NM 000619), IL-12Rβ1, (Genbank accession no. BC029121), IL-12Rβ2 (Genbank accession no. HSU64198), IFNAR1 (Genbank accession no. NM 207585) and β2 microglobulin (Genbank accession no. AF072097). The primers used were as follows: IL-10 forward 5’ CAACCTGCCTAACATGCTTC 3’; reverse 5’ CGCAAAGCAATACATGAACTC 3’; IFN-γ forward 5’ CGCAAGCAATACATGAACCTC 3’; reverse 5’ GTAATGG TTGTCCTGCCTG 3’; IL-12Rβ1 forward 5’ACGAGTGCTCTGCCAGTAT3’; reverse 5’ TACACTCTGGTGGAAATCCT; IL-12Rβ2 forward 5’ GAGGATGCTCATGTATT 3’; reverse 5’ TACATGCTTTTAACAAAGTTG 3’; IL-12Rβ2 forward 5’ CAAGATGGTGTCTTGGCAGTAT3’; reverse 5’ TCA CACTCTGGTGGAAATCCT; IFNAR1 forward 5’ TGGCAGGAAGCTTCTTCTTGA 3’; reverse 5’ TGCTGCTGCTTCTGGAGACAAAC 3’; IFNAR2 forward 5’ CACCATAGTGACACTGAAATG 3’; reverse 5’ TCTGGATGAGGATGTATGCTG 3’; β2 microglobulin forward 5’ CTGGTGGCCCT AGCTGTG 3’; reverse 5’ ATGTGCTGGTGTGCATCATC 3’. All real-time PCR was carried out using the SYBR green fluorescence method with SYBR green qPCR master mix (Stratagene, La Jolla, CA, USA) as specified by the manufacturer. The real-time PCR reactions were carried out in triplicate on a MX4000® Multiplex Quantitative QPCR System (Stratagene) using standard default thermal cycling conditions. Non-template controls were loaded in triplicate and were prepared by replacing the cDNA fraction of the PCR reaction with an equivalent volume of nuclease-free water. Quantification of transcripts was carried out using the relative standard curve method as described by Applied Biosystems (Foster City, CA, USA; 1997) [11]. An equal aliquot of undiluted cDNA from each sample was pooled together. This cDNA pool was serially diluted (neat, 1:2, 1:5, 1:10 1:20) to produce a set of standards, from which the Ct value (cycle number at which the reporter dye emission intensity rises above background noise) of a particular variant could be converted to nanograms of total RNA equivalent used for first-strand synthesis. β2 microglobulin expression was monitored as an internal standard on the cDNA template, therefore mRNA expression for each gene is normalized to the internal standard expression.

Cytokine assay using ELISA

PHA/IL-2-induced T cell blasts (1 x 10⁶ cells/ml) or anti-CD3 stimulated T cells (1 x 10⁶ cells/ml) were either left untreated or pre-treated with 20 μg/ml curcumin (Sigma-Aldrich) for 18 hrs at 37°C. Both sets of cells were then either left unstimulated or incubated with either 10 ng/ml IFN-β or 0.1 μg/ml IL-12 for a further 18 hrs at 37°C. The cell supernatants were removed for cytokine assay. IFN-γ and IL-10 levels were measured using a solid-phase sandwich ELISA purchased from Pharmingen (BD OptEIA™ BD Biosciences, Franklin Lakes, NJ, USA) and were performed following the manufacturers’ instructions.

Measurements

The PHA/IL-2 blasts were evaluated following antibody staining on an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA), and the results were analysed using the computer software WINMDI 2.8. IFN-γ and IL-10 levels were also measured using a solid-phase sandwich ELISA. Each experiment was repeated, using four independent donors. Statistical analysis was performed using a paired T test.

Results

Curcumin differentially affects the activation of STAT4 by IFN-β, IFN-α and IL-12

We examined whether curcumin affected IFN-β, IFN-α or IL-12-induced STAT4 phosphorylation. As shown in Figure 1, exposure to IFN-β, IFN-α or IL-12 resulted...
in increased pSTAT4 generation when compared to unstimulated cells. However, pre-treatment of the cells with curcumin produced contrasting results depending on whether the cells were subsequently exposed to IFN-β, IFN-α or IL-12. Pre-incubation with curcumin and then IFN-β resulted in an increase in pSTAT4 generation (Fig. 1), whilst prior treatment with curcumin followed by IFN-γ resulted in similar pSTAT4 generation compared with IFN-γ alone. However, prior treatment with curcumin resulted in a decrease in pSTAT4 generation (Fig. 1).

**Effects of curcumin on the level of receptor expression**

We next investigated whether curcumin also exerted its effects upstream at the receptor level. Quantitative real-time PCR was used to assess IL-12R and IFNAR mRNA expression in PHA/IL-2 T cell blasts. Both IFN-β and IFN-α modestly increased IFNAR1 expression; this was further increased by prior exposure to curcumin (P <0.05). Prior exposure to curcumin also induced a moderate increase of IFNAR2 by IFN-β (Fig. 2). However, although IFN-α alone increased IFNAR2 expression, prior exposure to curcumin had no effect on IFN-α-induced IFNAR2 expression. In this study, prior exposure to IL-12 alone up-regulated both IL-12 Rβ1 and β2 expression (Fig. 3). However, pre-exposure to curcumin appeared to reduce this induction (Fig. 3, IL-12Rβ1 P = 0.074, IL-12Rβ2 P <0.05). Similar results were also obtained when measuring IL-12Rβ1 and IFNAR protein expression using flow cytometry. The median fluorescence intensity (MFI) for IFNAR was increased from 5.4 (range, 5.4–11.2) to 11.12 (10.35–15.5) when comparing unstimulated cells with cells incubated with IFN-β, respectively. Pre-treatment with curcumin followed by IFN-β enhanced the MFI of IFNAR to 18.47 (11.2–28.0). Exposure to IL-12 alone increased β1 receptor whilst pre-exposure to curcumin decreased the IL-12 receptor β1 expression. The MFI was increased from 16.5 (9–28) to 29.0 (25.5–40) when comparing unstimulated cells with cells incubated with IL-12, respectively. Pre-treatment with curcumin reduced the MFI of IL-12Rβ1 to 21.5 (19–31). These medians (range) are from three independent experiments. All these differences above were statistically significant (P < 0.05).

**Curcumin inhibits IL-12-induced IFN-γ and enhances IFN-β-induced IL-10**

In this study, both IFN-β and IFN-α are shown to induce IL-10 production in T cells (Fig. 4), which was enhanced by prior treatment with curcumin (P < 0.05). These results were confirmed by ELISA (Fig. 6A).
dependent production of IFN-γ is important in the generation of a cell-mediated immune response. Here, we show increased IFN-γ mRNA production by IL-12 in human T cells. However, pre-treatment of these cells with curcumin reduced this induction (Fig. 5A, P < 0.05). We also investigated whether IL-12-induced IFN-γ protein production was also decreased by prior exposure to curcumin; the results were consistent with what we observed at the mRNA level. IL-12 stimulation increased IFN-γ protein expression compared to unstimulated cells, an induction that was reduced by prior treatment of the cells with curcumin (Fig. 5B). These results were confirmed using ELISA (Fig. 6B).

To determine whether the immunomodulatory effects of curcumin are similar in T cells from normal donors and patients with MS, we performed the above studies on T cells from PBMC of MS patients. We showed the same effects on IFN-β and IL-12 induction of cytokines, STAT4 phosphorylation (Fig. 7A and B) and IL-10 and IFN-γ modulation (see Fig. 7C and D).

**Discussion**

As signal transducers, STAT molecules play an important role in many cellular events, including a
role in differentiation, cell growth, apoptosis and inflammation. We have shown previously that the modulation of both STAT1 and STAT4 by glucocorticoids is dependent on the cytokine by which their activation is induced, and that this modulation is different for IFN-α, IFN-β and IL-12 [12]. This observation is consistent with the results obtained in the current study for modulation by curcumin.

Here, we have shown curcumin to be a potent regulator of IL-12 signalling. The reduction of IL-12-induced STAT4 activation, and IL-12Rβ1 and β2 expression, was also associated with a reduction in IL-12-induced IFN-γ production. However, prior exposure to curcumin appears to increase the ability of T cells to respond to IFN-β by enhancing IFN-β-induced STAT4 activation, IFNAR 1 and 2

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**Fig. 5** Modulation of IL-12 induced IFN-γ by curcumin. Quantitative real-time PCR and flow cytometry were used to assess IFN-γ mRNA and protein expression, respectively, in PHA/IL-2 T cell blasts. IL-12 (100 ng/ml) increased IFN-γ production, however, pre-treatment of these cells with curcumin (20 µg/ml) reduced this induction (A). IL-12 stimulation increased IFN-γ protein expression (shaded curve) compared to unstimulated cells (US), an induction that was reduced by prior treatment of the cells with curcumin (20 µg/ml) (unshaded curve) (B).

**Fig. 6** Modulation of IFN-β induced IL-10 and IL-12 induced IFN-γ protein expression by curcumin. IFN-γ and IL-10 levels were measured using a solid-phase sandwich ELISA. IFN-β (10 ng/ml) induces IL-10 production in human T cells (A). IL-10 production is enhanced by prior exposure to curcumin (20 µg/ml). IL-12 (0.1 µg/ml) induces IFN-γ production in human T cells (B). Pre-treatment of these cells with curcumin (20 µg/ml) reduces this induction. The duration of exposure of the cells to IFN-β, IL-12 and curcumin was 18 hrs.
expression, and IL-10 production. Although IFN-α and IFN-β use the same receptor and signalling pathway, differences in their biological effects have been documented [12, 13] and, therefore it is perhaps not surprising that curcumin modulation of IFN-α signalling, as observed in this study, is slightly different to IFN-β. Although curcumin increases IFN-α-induced IL-10 mRNA and IFNAR1 expression, prior exposure to curcumin decreased IFN-α-induced IFNAR2 expression and resulted in similar pSTAT4 generation comparable to IFN-α alone.

Signal transduction through the IL-12 receptor induces tyrosine phosphorylation of the Janus family kinases JAK2 and TYK2, which in turn phosphorylate STAT4 [14]. Phosphorylated STAT4 (pSTAT4) dimerizes, translocates to the nucleus and instigates DNA transcription [15]. Curcumin can completely inhibit the expression of JAK2 at the level of transcription [16]. Signal transduction through the IFNAR induces tyrosine phosphorylation of JAK1 and TYK2. The effect of curcumin on JAK1 expression could be an interesting target for future investigation.

Previous studies have looked at the direct effect of curcumin on IL-12 itself [17]. Here we are more interested in the relationship between curcumin and IL-12 with regard to STAT4 activation, how the

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**Fig. 7** Curcumin modulation in multiple sclerosis (MS) patients. PBMC’s were isolated from MS patients (n = 4) and as with the in vitro samples were stimulated with either IFN-β (10 ng/ml) or IL-12 (100 ng/ml). Both induced pSTAT4 expression (unshaded curve on each graph). Prior incubation with curcumin (20 µg/ml) and then IFN-β resulted in an increase in pSTAT4 generation, whilst prior stimulation with curcumin (20 µg/ml) followed by incubation with IL-12 resulted in a decrease in pSTAT4 generation. (Shaded curve on each graph represents unstimulated cells). IFN-β and IL-10 levels were measured using a solid-phase sandwich ELISA. IFN-β (10 ng/ml) induces IL-10 production in human T cells (A). IL-10 production is enhanced by prior exposure to curcumin (20 µg/ml). IL-12 (0.1µg/ml) induces IFN-γ production in human T cells (B). Pre-treatment of these cells with curcumin (20 µg/ml) reduces this induction. The duration of exposure of the cells to IFN-β, IL-12 and curcumin was 18 hrs.
ability of T cells to respond to IL-12 by producing IFN-γ is affected by prior exposure to curcumin, and the differences between the effects of curcumin on IL-12, IFN-β and IFN-α signalling, since they all activate STAT4. Interestingly, this modulation was observed to be stimulus dependent as prior exposure to curcumin enhanced the ability of T cells to respond to IFN-β, not only at the level of cytokine induction but also upstream at the signalling pathway and receptor level. The type of modulation by glucocorticoids is dependent upon the T cell phenotype [18, 19], and perhaps this is the same for curcumin. We found that prior treatment with curcumin enhanced IFN-β-induced IL-10 production (an anti-inflammatory cytokine), whilst the induction of IFN-γ (a pro-inflammatory cytokine) by IL-12 in human T cells was reduced by pre-treatment with curcumin.

Deregulated signal transduction pathways, such as activation protein-1 (AP-1) and nuclear factor kappaB (NFκB) contribute to carcinogenesis, [20] and play a critical role in the transcription of several genes involved in immune and inflammatory responses [21]. Curcumin has been shown to inhibit induction of AP-1 and NFκB in bone marrow stromal cells [22]. Curcumin is also thought to down-regulate matrix metalloproteinase-9 (MMP-9) expression, which is implicated in the growth and invasiveness of brain tumours by inhibition of NFκB and AP-1 binding to the DNA promoter region [23].

STAT4 has been demonstrated to recruit AP-1 to the IFN-γ promoter to synergistically enhance IFN-γ mRNA expression induced by IL-12 and IL-18 [24]. The reduction of IL-12-induced IFN-γ mRNA expression by curcumin as shown in this study may not only be due to a decrease in STAT4 activation but also a reduction of the recruitment of AP-1 to the IFN-γ promoter, perhaps resulting from the decrease in STAT4 phosphorylation.

Although it is clear that the differentiation of Th1 cells is crucial for an effective immunity to a wide variety of intracellular pathogens, Th1 cells and IL-12 may also contribute to the pathogenesis of a variety of immune-mediated inflammatory disorders, including MS and rheumatoid arthritis [25]. It has already been shown that curcumin inhibits IL-12 production and signalling in EAE [5]. This, combined with the inhibition of IL-12 signalling in human T cells and curcumin synergistic effect with IFN-β observed in this study on immune cells from both normal volunteers and MS patients, could place curcumin as a potential therapeutic agent in the treatment of MS.

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