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Curcumin prevents human dendritic cell response to immune stimulants

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Abstract

Curcumin, a compound found in the Indian spice turmeric, has anti-inflammatory and immunomodulatory properties, though the mechanism remains unclear. Dendritic cells (DCs) are important to generating an immune response and the effect of curcumin on human DCs has not been explored. The role curcumin in the DC response to bacterial and viral infection was investigated *in vitro* using LPS and Poly I:C as models of infection. CD14⁺ monocytes, isolated from human peripheral blood, were cultured in GM-CSF- and IL-4-supplemented medium to generate immature DCs. Cultures were incubated with curcumin, stimulated with LPS or Poly I:C and functional assays were performed. Curcumin prevents DCs from responding to immunostimulants and inducing naïve CD4⁺ T cell proliferation by blocking maturation marker, cytokine and chemokine expression and reducing both migration and endocytosis. These data suggest a therapeutic role for curcumin as an immune suppressant.

Keywords

Curcumin; dendritic cells; immunosuppression

Introduction

Curcumin is a biologically active compound found in the Indian spice turmeric. It belongs to a family of compounds called curcuminoids and usually comprises about 3% of turmeric powder. The spice is commonly used as a preservative for foods and a yellow dye or coloring for textiles and as an ingredient in pharmaceuticals and cosmetics. For many centuries curcumin has been used as an antiseptic, analgesic, appetite suppressant, anti-inflammatory agent, antioxidant, anti-malarial and insect repellent [1; 2]. The pharmacological potential of curcumin is under investigation. Researchers found that it has antiinflammatory, antioxidant, anti-parasitic, anti-viral and anticancer properties [3; 4; 5; 6].

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It targets transcription factors, cytokines, cell adhesion molecules, surface receptors, growth factors and kinases, among other molecules [7; 8; 9; 10], and directly binds to a variety of surface and intracellular proteins causing direct cellular pathway inhibition or activation of secondary cellular responses [2; 11].

Dendritic cells are the sentinels of the immune system and regulate the immune response. Immature or resting dendritic cells reside in peripheral organs where they monitor the surrounding tissue for invading microorganisms. They alert the immune system to the presence of pathogens by engulfing them, processing the foreign proteins and presenting the peptide fragments on their surface. After DCs are activated, they mature and migrate to the lymphoid tissue where they prime naïve T lymphocytes and stimulate a specific or adaptive response [12; 13]. Maturation of dendritic cells involves changes in gene expression and activation of signaling pathways, and it is reasonable to hypothesize that curcumin can modulate some of these pathways and thereby prevent DC maturation and alter function. While a study by Kim *et al.* reveals that curcumin impairs the immunostimulatory function of murine dendritic cells [14], its effects on human dendritic cells remain unknown. Curcumin's effects on human DC stimulation are examined in this study. Modulating the DC response could provide an effective approach to treat and control unwanted inflammation.

Materials and Methods

Reagents

Curcumin (from *Curcuma longa*) was obtained from Sigma Aldrich (St. Louis, MO) and dissolved in DMSO (11mg/ml). Buffy coats were obtained from Florida Blood Services (St. Petersburg Florida). Six donors, four males and two females, in good health and ranging in age from 18 to 50 were used for the study. Cell isolation reagents CD14 microbeads and CD4+ T cell isolation kit were obtained from Miltenyi Biotec (Auburn, CA). Histopaque®-1077 was obtained from Sigma Aldrich and recombinant human cytokines GM-CSF and IL-4 were obtained from PeproTech (Rocky Hill, NJ). All other cell culture reagents were obtained from GIBCO Invitrogen (Carlsbad, CA). LPS, poly I:C and PHA were obtained from Sigma Aldrich (St. Louis, MO). CFSE and Alexa-647 conjugated dextran (molecular weight 10,000) were obtained from Molecular Probes Invitrogen (Carlsbad, CA). LINCOplex Multiplex cytokine assay kits were purchased from Millipore (Temecula, CA). All CD11c, HLA-DR, CD40, CD86, CD83 and CD54 antibodies were obtained from BD Biosciences (San Jose, CA). CCL19 and CCL21 were obtained from PeproTech (Rocky Hill, NJ).

Cell isolation and culture

CD14+ monocytes were isolated and cultured as described by Picki *et al.* [15]. Briefly, leukocytes were extracted from buffy coats using Histopaque-1077. Monocytes expressing CD14 were positively selected with magnetic microbeads. Purity (>90%) was verified by staining with anti-CD14 antibodies and analyzing by flow cytometry. Cells were cultured at 1×10^6 cells/ml in complete RPMI (10% FBS, 1% pen/strep, 10mM Hepes, non-essential amino acids and 5mM sodium pyruvate) with 20 ng/ml each rh IL-4 and GM-CSF for five to six days, (supplementing at day three with fresh medium). Non-adherent and loosely adherent cells were removed on day five for analysis or stimulation. On day 5, more than 90% of the harvested cells expressed CD11c and HLA-DR. Naïve CD4+ T cells were isolated from the CD14- fraction remaining after monocyte depletion and cultured in complete RPMI. Purity was confirmed by flow cytometry after CD4 and CD45RA staining.

Cell treatment and stimulation

Curcumin was added to cell culture (1×10^6 cells/ml and 3 ml/well in 6-well plates) at concentrations of 20 μ M or 30 μ M. DMSO was used as a control. After a 1hr incubation, LPS (1 μ g/ml) or Poly I:C (25 μ g/ml) was added to the appropriate wells. Control wells received neither. Cultures were incubated overnight at 37°C and 5% CO₂/95% air. Cell viability was 95% $\pm$ 0.06 after 24hours of culture under all conditions listed above as determined by a viability assay using 7AAD incorporation.

Flow cytometry

Cells were collected, washed and stained with fluorochrome-conjugated antibodies specific for DC surface markers. Cells were analyzed using the Becton Dickinson (BD) Canto II with HTS sampler and BD FACSDiva™ software.

Cytokine assay

Culture supernatant was collected and cytokine levels measured using the LINCOplex multiplex assay. Assays were performed in duplicate according to the manufacturer's instructions.

Chemotaxis assay

Treated and stimulated cells were collected, counted and re-suspended at a concentration of 1×10^6 cells/ml. 50 μ l of cell suspension was placed in the upper chambers of 5 μ m pore size polycarbonate filter inserts in a 96 well microchemotaxis plate (Chemicon). The lower chambers contained 40 μ l of either CCL19 or CCL21 in 150 μ l of medium. Control wells had medium only. Input wells (in triplicate) contained 1×10^4 cells in the lower chambers without chemokines. Cells were incubated at 37°C and 5% CO₂/95% air overnight. Migration was stopped by the removal of the inserts. 1×10^4 polystyrene beads were added to each well (lower chamber) and analyzed by flow cytometry. The number of cells in each sample and input was calculated using the following equation:

Number of cells/well = (number of cell events $\div$ number of bead events) $\times 10^4$.

Input cells = average [number of input cells/well $\times 5$ (dilution factor)]. The percentage migration for each sample (% input) is determined by the following equation:
Percent migration = (migrating cells $\div$ input cells) $\times 100$.

Mixed Leukocyte Reaction

CFSE labeling of CD4⁺ T cells was carried out by resuspending cells in 1ml PBS containing 5% (v/v) FBS. 1.1 μ l of the CFSE stock (5 μ M) was diluted in 110 μ l of PBS and quickly mixed with the cell suspension. After a 5 minute incubation at room temperature, the reaction was stopped by adding ten volumes of room temperature PBS containing 5% (v/v) FBS and centrifuging at 300 $\times$ g for 5 minutes at 20°C. Cells were washed twice and resuspended in complete medium (1×10^6 cells/ml). The dendritic cell-T cell co-culture was set up at a ratio of 1:16. Curcumin treated and stimulated DCs were removed from culture and placed in 96 well plates in triplicate (6.25×10^3 cells in 100 μ l per well). 100 μ l of T cells were added to each well and cultures incubated at 37°C and 5% CO₂ /95% air for 5 days. CFSE fluorescence intensity was measured by flow cytometry using the BD Canto II with HTS attachment and BD FACS Diva software.

Endocytosis assay

Cells were collected, washed and incubated with 1mg/ml (per 1×10^6 cells) Alexa 647 conjugated dextran at either 4°C or 37°C for 1 hour. Cells were washed with cold PBS and either analyzed by flow cytometry or plated on gelatin coated cover slips and imaged by

confocal microscopy. The change in mean fluorescence intensity (MFI) is calculated as the difference between the MFI of 37°C and 4°C cultures.

Microscopy

All bright field images were captured using the 4x objective of an Olympus IX71 inverted fluorescent microscope with an attached DP70 camera. Fluorescent images were captured using the 63x objective of a Leica scanning confocal microscope.

Statistical analysis

Data was log transformed to ensure normal distribution. Significance was determined using paired *t* tests (repeated measures) for planned comparisons with modified Bonferroni correction. Data are presented as the average of six donors. Error bars represent SEM with *p* values less than 0.05 considered statistically significant.

Results and Discussion

This is the first study to examine the effects of curcumin on human dendritic cells *in vitro*. Donors for the study were selected at random and supplied by Florida Blood Services, St. Petersburg, Florida. The concentrations of curcumin used were based on those previously found efficacious in the literature and confirmed not to be toxic to the cells by viability assays (data not shown). All cultures remained more than 90% viable up to 24hrs after curcumin addition. The pharmacokinetics and pharmacodynamics of curcumin have been more extensively studied in rodents than in humans [16]. From the limited human data available, the low bioavailability of curcumin limits its clinical usefulness when administered orally. High doses can be administered without adverse effects but the systemic distribution may not be sufficient to exert pharmacological activity. Combining curcumin with other compounds, or using drug delivery systems such as liposomes and nanoparticles provide an alternative approach to overcome these issues [17; 18; 19]. The immunostimulants lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (poly I:C) were used in this study to independently stimulate DC activation. LPS via the toll-like receptor 4 (TLR4) pathway and poly I:C mimics viral infections through TLR3. These compounds were chosen to ensure the immunostimulatory effects were not TLR dependent. Observed stimulant effects were found to be significant with *p* values < 0.05 by paired *t* test compared to non-stimulated controls.

Curcumin prevents increased maturation marker expression and cytokine secretion

Mature dendritic cells express elevated levels of co-stimulatory and antigen presenting molecules such as CD86, CD83 and HLA-DR on their surface. If the antigen presenting machinery of DCs are impaired, they can not effectively engage the T cells to initiate a response. Stimulated curcumin-treated DCs do not significantly increase their surface expression of CD86 and CD83 above the control (Fig 1a). HLA-DR surface expression is not significantly inhibited by 20μM curcumin. We can surmise that curcumin affects the antigen presenting machinery by reducing co-stimulatory molecule expression but not the antigen presenting molecules. Mature monocyte-derived DCs secrete IL-12, IL-10 and other inflammatory cytokines. Stimulated curcumin-treated DCs produce significantly lower levels of IL-12, IL-10 and TNFα when compared to the controls (Fig 1b) creating a Th2 permissive environment. IL-6 was also significantly reduced by curcumin (data not shown). Though the reduction of TNFα was significant, they were not reduced to the levels of the controls. These findings correlate with those from the study by Kim *et al.* [14] which shows curcumin prevents immunostimulatory function of murine bone marrow-derived cells. They along with others show curcumin is a potent inhibitor of NF-κB and AP-1 activation as well as MAPK signaling [20; 21]. This provides a reasonable explanation for the observed

reduction of IL-12 and IL-10 levels in this study. TNF α expression is controlled by other transcription factors such as lipopolysaccharide-induced TNF factor (LITAF) [22] or interferon regulatory-factor 3 (IRF3) [23] that may not be affected by curcumin, allowing the transcription of some TNF α independent of the NF κ B pathway.

Curcumin prevents DC-induced CD4⁺ T cell proliferation in the mixed lymphocyte reaction

The mixed lymphocyte reaction (MLR) is used as the basic test of DC function since it measures their ability to stimulate proliferation of an allogeneic T cell population. Studies show curcumin can inhibit MLR [24; 25; 26; 27]. Immature DCs will weakly stimulate proliferation, while the mature DCs will induce a significantly more robust response (Fig 2a). Increased expression of co-stimulatory markers is essential for T cell interaction and proliferation. Curcumin-treated DCs, both stimulated and non-stimulated, show muted T cell proliferation (Fig 2a). These observations and those in Fig 1a imply there are factors at play other than the expression levels of co-stimulatory and antigen presenting molecules. The regulation of the DC cytoskeleton is important in DC – T cell interactions [28]. Little is known about the effect of curcumin on cytoskeletal rearrangement. One study reveals curcumin significantly alters the actin cytoskeleton in prostate cancer cells [29]. Based on this premise, curcumin-induced alterations in DC cytoskeleton could account for the observations in Fig 2a. Analysis of the CD4 T cell cytokines produced in the MLR revealed a Th2-biased response evidenced by the increase in the IL-4:IFN γ ratio between untreated and curcumin treated cells (Fig 2b).

Curcumin reduces endocytosis

Capture and presentation of antigen is an important feature of DC biology. This provides the link between innate and adaptive immunity. Immature DCs are highly endocytic, a feature which is lost when cells become mature. We find curcumin reduces endocytosis in non-stimulated DCs (Fig 2c,d). There is a significant decrease in dextran uptake by non-stimulated cells treated with curcumin similar to stimulated cells, but not in stimulated cells. There are conflicting reports on the effects of curcumin on antigen capture; a few studies show increased endocytosis, while others show suppression [30]. Our findings indicate curcumin interferes with antigen handling in human DCs.

Curcumin prevents homotypic clustering and surface adhesion marker expression

DCs aggregate in clusters in response to stimuli as a visual sign of maturation [31]. Cluster formation correlates with increased CD86, CD54 and CD80 expression. Here we show curcumin impairs homotypic DC cluster formation in response to both LPS and poly I:C in a concentration-dependent manner (Fig 3a). Adhesion molecules such as ICAM-1 (CD54) are important in cellular interactions and in generating T cell response. Murine antigen presenting cells (APCs) deficient in ICAM-1 have impaired ability to induce T cell responses [32; 33]. CD11c, a member of the integrin family of proteins, is also important for cell attachment and found in high levels on DCs. Curcumin significantly reduces expression of both markers on the DC surface (Fig 3b-c). The reduced CD11c could be the result of curcumin-induced AP-1 inhibition [34].

Curcumin prevents DC migration and chemokine secretion

Mature DCs travel to the lymph nodes where they present processed antigen to T cells. Migration towards chemoattractants is a feature of mature DCs [35]. They also secrete chemokines to attract responder cells to the site of injury or inflammation. Monocyte-derived DCs migrate in response to CCL19 or macrophage-inflammatory protein-3 β (MIP-3 β) and CCL21 or exodus-2, which are expressed in the lymph nodes. Both chemokines bind to the CCR7 receptor on the DC surface. CCR7 expression is not affected

by curcumin (data not shown). Curcumin prevents migration towards CCL19 and CCL21 in a chemotaxis assay (Fig 4a) and also reduces the levels of chemokines fractalkine (CX3CL1) and interferon producing factor (IP-10) (Fig 4b, c). Both fractalkine and IP-10 attract inflammatory cells to sites of inflammation. Poly I:C stimulated cells did not migrate in response to the chemokines, even in the absence of curcumin (data not shown). By preventing DC migration, curcumin reduces the probability of the DC encountering T cells to initiate a specific immune response. Reduced chemokine secretion will stem the flow of inflammatory cell traffic to sites of inflammation.

Conclusions and perspectives

Curcumin acts in several ways as an immune suppressor of human peripheral CD14⁺ monocyte-derived DCs. It renders them non-responsive to the immuno-stimulants LPS and poly I:C by reducing expression of co-stimulatory and antigen presentation molecules expression and dampening the Th1-type response while promoting a Th2 permissive environment. It also reduces migration and adhesion molecule expression and reduces DC-induced proliferation of allogeneic CD4⁺ T cells. The inhibition of transcription factors NFκB and AP-1 and other cell signaling pathways by curcumin provide a plausible explanation for most of observations; however curcumin may be targeting other essential cellular pathways as well. Based on our observations and reports from other studies, we speculate curcumin may be disrupting the antigen handling and presenting machinery of DCs in addition to transcription factor and signaling pathway inhibition [30]. Elucidation of the mechanism of action of curcumin immunosuppression could lead to clinical applications of this novel anti-inflammatory agent.

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Figure 1.

Curcumin reduces maturation marker expression (A) and cytokine production (B) in human monocyte-derived dendritic cells. Surface marker expression is measured as mean fluorescence intensity (MFI) by flow cytometry. Cytokine levels were measured from culture supernatant using a multiplex assay kit and expressed as log concentration.

* indicates significance by one-tailed *t*-test of planned comparisons $p < 0.05$.

Figure 2.

Curcumin reduces DC-induced proliferation of allogeneic CD4⁺ T cells (A) and promotes a Th2-permissive environment with an increased IL4:IFN γ ratio (B) in mixed lymphocyte reaction. Curcumin also reduces the endocytic capability of DCs. Endocytosis of fluorescently labeled dextran was measured by change in mean fluorescence intensity (MFI) between cells incubated at 37°C and 4°C (C) and imaged by confocal microscopy of a representative donor (D).

* indicates significance by one-tailed *t*-test of planned comparisons $p < 0.05$.

Figure 3.

Curcumin prevents homotypic clustering of DCs in response to stimuli (A) and reduces surface adhesion molecules CD54 (B) and CD11c (C). Surface marker expression is measured as mean fluorescence intensity (MFI) by flow cytometry.

* indicates significance by one-tailed *t*-test of planned comparisons $p < 0.05$.

Figure 4.

Curcumin prevents DC migration towards CCL19 and CCL21 (A) and chemokine secretion in culture (B-C). Chemotaxis assay performed in 96 well microchemotaxis plate with 5 μ m pore size polycarbonate filters. Chemokine levels were measured from culture supernatant using a multiplex assay kit.

* indicates significance by one-tailed *t*-test of planned comparisons $p < 0.05$.