The Role Of Curcumin In Human Dendritic Cell Maturation And Function

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The Role Of Curcumin In Human Dendritic Cell Maturation And Function

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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DEDICATION

This dissertation is dedicated to my family, my parents Byron and Lowalean, my sister Leisle and my husband Karl, for their sacrifice and unconditional support. Their confidence in me and their encouragement motivated me to persevere.
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LIST OF ABBREVIATIONS

5-LOX:  5-lipoxygenase
7-AAD:  7-amino-actinomycin
µM:   micro molar
AP-1:   activator protein 1
APC:   allophycocyanin
Arp:   actin related protein
BCA:   bicinchoninic acid
BSA:   bovine serum albumin
CCL19:  chemokine (c-c motif) ligand 19 (MIP-3β)
CCL21:  chemokine (c-c motif) ligand 21 (Exodus-2)
CCR7:  chemokine (c-c motif) receptor 7
CFSE:  carboxyfluorescein succinimidyl ester
CD:   cluster of differentiation
Cdc42:  cell division cycle 42
cDNA:  complementary deoxyribonucleic acid
COX-2:  cyclooxygenase 2
CX3CL1: chemokine (c-x3-c motif) ligand 1 (fractalkine)
CytoB:  cytochalasin B
DAPI:   4′,6-diamidino-2-phenylindole
DC: dendritic cell
DMSO: dimethyl sulfoxide
Exodus-2: chemokine (c-c motif) ligand 21 (CCL21)
FITC: fluorescein isothiocyanate
Foxp3: forkhead box p3
GAPDH: glyceradehyde-3-phosphate dehydorgenase
GDP: guanosine diphosphate
GM-CSF: granulocyte macrophage colony stimulating factor
GTPase: guanosine triphosphate hydrolase enzyme
HLA-DR: human leukocyte derived antigen
HRP: horseradish peroxidase
HTS: high throughput system
ICAM1: intracellular adhesion molecule 1 (CD54)
iDC: immature dendritic cell
IFNγ: interferon gamma
IP-10: interferon-inducing protein 10 (CXCL10)
IPA: ingenuity pathways analysis
IL: interleukin
LITAF: lipopolysaccharide-induced tumor necrosis factor
Log: logarithm base 10
LPS: lipopolysaccharide
µg: microgram
MFI: mean fluorescence intensity
MIP-3β: chemokine (c-c motif) ligand 19 (CCL19)
MHC: major histocompatibility complex
ml: milliliter
MLR: mixed lymphocyte reaction
mdDC: monocyte-derived dendritic cells
NFκB: nuclear factor kappa B
ng: nano gram
NSAID: non-steroidal anti-inflammatory drug
PAK: p21 activated kinase
PBMC: peripheral blood mononuclear cells
PBS: phosphate buffered saline
PE: phycoerythrin
PFA: paraformaldehyde
pg: pico gram
PHA: phytohemmaglutinin
Poly I:C: polyinosinic-polycytidylic acid
Rho: Ras homolog family member
RNA: ribonucleic acid
ROS: reactive oxygen species
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM: standard error of the mean
STAT: signal transducer and activator
TNF-α: tumor necrosis factor alpha
WASP: Wiskott-Aldrich syndrome protein
THE ROLE OF CURCUMIN IN HUMAN DENDRITIC CELL
MATURATION AND FUNCTION
Shawna A. Shirley, M.S.

ABSTRACT

Curcumin is the yellow pigment found in the Indian spice curry. It has anti-inflammatory, ant-oxidant, anti-cancer, anti-viral, anti-bacterial and wound healing properties. It is widely used in industry for its flavor as a spice and as a coloring agent because of its brilliant yellow color. It is also used as a dye for textiles and as an additive to cosmetics. Dendritic cells (DCs) are the sentinels of the immune system and functions as the bridge between the innate and adaptive immune response. The effect of curcumin on DCs is poorly understood. A study shows curcumin prevents the immuno-stimulatory function of bone marrow-derived murine DCs, but no study examines the effects on human DCs. This study investigates the effects of curcumin on immature human DC maturation and function in response to immune stimulants lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (poly I:C).

Human CD14+ monocytes isolated from the peripheral blood of donors are cultured with GM-CSF and IL-4 supplemented media to generate immature DCs. The cultures are treated with curcumin, stimulated with the above
mentioned stimulants then functional assays performed. These assays include homotypic cluster formation, surface marker expression, cytokine production, chemotaxis, endocytosis, DC-induced allogeneic CD4+ T cell proliferation after mixed lymphocyte reaction, gene expression analysis and immuno-fluorescence labeling and imaging. Curcumin-induced changes in gene expression indicate the actin cytoskeleton signaling pathway is a target. Immuno-fluorescence labeling and imaging of f-actin was carried out.

Curcumin reduces DC maturation in response to the stimulants used in the study. Expression of surface markers, cytokines and chemokines is reduced as well as DC-induced stimulation of allogeneic CD4+ cells after MLR. Curcumin prevents chemotaxis without affecting chemokine receptor expression and significantly reduces endocytosis in non-stimulated cells. Curcumin-treated DCs do not induce a Th1 or Th2 population in allogeneic MLR but induces a CD25+Foxp3+ regulatory cell population. Immuno-fluorescence imaging shows curcumin causes the cell to become more rounded. These data imply that curcumin inhibits f-actin polymerization and thereby prevents DC maturation and function in response to stimulation. This outlines a novel role for curcumin as an immune suppressant and shows its therapeutic potential as an anti-inflammatory agent.
PREFACE

I would first like to acknowledge The Creator; through him all things are possible. I would like to thank my major professor, Dr. Shyam S. Mohapatra for his continued guidance, support and encouragement and for giving me the wonderful opportunity to explore the basic sciences. Thanks to my co-major professor, Dr. Richard Heller, for is advice, encouragement and motivating spirit. When I needed someone to give me another perspective, his door was always open. To my committee members, Dr. Thomas Klein and Dr. Mark Glaum, thanks for your invaluable advice and support throughout the process. I would like to extend a special thank you to Dr. Richard F. Lockey. His faith in my abilities has meant a lot to me. I acknowledge the Joy McCann Culverhouse endowment to the University of South Florida as well as the Mabel and Ellsworth Simmons professorship to Dr. S.S. Mohapatra for funding the research. Thanks to wonderful researchers and staff at the Joy McCann Culverhouse Center for Airway Disease and Nanomedicine. You were always happy to help me and answer my questions. Special thanks to Bobby, Weidong, Sonya, P.K. and Sandyha. I would like to extend special thanks to Homero San-Juan Vergara for his friendship, support and advice.
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INTRODUCTION

Turmeric is a bright yellow spice derived from the root of the perennial *Curcumin longa*, a member of the ginger family. Curcumin, also called diferuloylmethane, is the most biologically active compound turmeric and belongs to a family of compounds called curcuminoids which usually comprises about 3% of turmeric powder. The spice is widely used for its culinary flavor as it gives curry its characteristic taste. Its use also extends to medicine. For centuries it has been used in Asian cultures in Ayurvedic systems of medicine most likely because of its properties as an antiseptic, analgesic, appetite suppressant, anti-inflammatory agent, antioxidant, anti-malarial and insect repellant (3, 49). In industry it is commonly used as a food preservative, a yellow dye or coloring for food and textiles and as an ingredient in pharmaceuticals and cosmetics.

Research shows curcumin has anti-inflammatory, antioxidant, anti-parasitic, anti-viral and anti-cancer properties (12, 74, 88, 91). It targets transcription factors, cytokines, cell adhesion molecules, surface receptors, growth factors and kinases, among other molecules (9, 72, 78, 152), and directly binds to a variety of surface and intracellular proteins causing direct cellular pathway inhibition or activation of secondary cellular responses (3, 48).
A Natural History of Curcumin

Curcumin was first isolated from turmeric in 1815 and its chemical structure was determined as C_{21}H_{20}O_{6} by Vogel and Pellatier (145). The history of curcumin however, dates back over 5000 years to the time of Ayurveda. Ayurveda, or the science of good life, is an ancient system of healthcare practiced in India. In this system of medicine curcumin was used for wound healing, blood cleansing and to cure stomach illnesses. Also known as Haldi, curcumin has been used for centuries without known side effects as a food preservative or additive for coloring and flavor. It can be ingested to treat a host of internal ailments or mixed into a paste and applied topically to treat wounds, bruises, boils pains, sprains, swellings and other disorders of the skin (61).

Curcumin is a valuable export crop and is widely cultivated by Asian countries. In addition to its culinary and medicinal uses, it is also commonly used in industry as a food preservative, a yellow dye or coloring for food and textiles and as an ingredient in pharmaceuticals and cosmetics (128). In Hindu religious ceremonies it is mixed with sandalwood powder and applied to the forehead (61).

The earliest entry about curcumin in PubMed is from 1949, a study published in Nature explored the antibacterial action of curcumin (115). To date there are 2438 articles in PubMed related to curcumin.
Curcumin Classification and Structure

Taxonomically, turmeric belongs to Class Liliopsida; Subclass Commelinids; Order Zingiberales; Family Zingiberaceae; Genus Curcuma; Species Curcuma longa (ref). It is a hydrophobic, polyphenolic compound that is soluble in ethanol, dimethyl sulfoxide, acetone, chloroform and oils. Curcumin exists in both keto and enol forms, but the keto form is more energetically stable (Figure 1). Its absorption maxima is around 420 nm and therefore it fluoresces at the wavelength of FITC. Most commercially available preparations of curcumin contain other compounds such as its analogs demethoxycurcumin and bisdemethoxycurcumin (3). Though curcumin is thought to be the most potent of the three, it is unclear whether the other analogs have similar activity (4, 127). It is suggested that the combination of all three is more potent than each individually (58).

Molecular Targets and Receptors of Curcumin

The lipophilic property of curcumin allows it to rapidly permeate cell membranes and enter the cytoplasm (62). There are proteins to which curcumin binds and initiates secondary cellular responses. These include, among others, 5-LOX, serum albumin, iron, IKK, PKC, PKA, GST and autophosphorylation-activated protein kinase (3). Due to its size and chemical composition, it is possible that curcumin enters cells by passive diffusion though the lipid bi-layer. Its anti-inflammatory properties are ascribed to its ability to inhibit transcription factors such as NFκB, AP-1 and STATs, its ability to reduce COX-2 and 5-LOX
expression and its ability to reduce the expression of surface adhesion molecules. Its role as a potent antioxidant may also contribute to its anti-inflammatory actions (3, 15, 118, 143). Antioxidants are compounds that delay or arrest disease progression. Curcumin functions as an antioxidant by inducing the expression of reactive oxygen species (ROS) and binding iron (39, 155). The structure of curcumin is rich in hydroxyl and methoxyl groups. There is some debate over whether it is the methylenic group at center or the phenol groups that contribute the hydrogen atom that confer its antioxidant property (16, 65, 107).

Figure 1. Structure of curcumin (diferuloylmethane) in both keto and enol forms (reprinted from Wikipedia)
Anti-inflammatory Activity of Curcumin

The anti-inflammatory property of curcumin is thought to be responsible for many of the activities associated with curcumin. It has been suggested that this property is directly related to the structure of the molecule, though there is debate over which portion is responsible; the diene bonds in the center or the phenol groups at either end (12, 27, 28, 70). Another notable property of curcumin related to its role as an anti-inflammatory agent is its antitumor activity. Curcumin can alter multiple signaling pathways by interacting with a number of molecular targets (3, 10) and function as a chemopreventative agent. It suppresses multiple forms of cancer including cancers of the breast, colon, liver, oral cavity and prostate.

Curcumin inhibits proliferation of an array of tumor cell types \textit{in vitro} (2) and prevents metastasis in an \textit{in vitro} model of mouse melanoma (93). It interferes with angiogenesis by inhibiting fibroblast growth factor (FGF)-induced neovascularization and inhibits vascular endothelial growth factor (VEGF) expression (13, 52, 94). Adhesion molecule expression is important for tumor metastasis. Curcumin may mediate its anti-tumor effects partially by reducing adhesion molecule expression (19, 138). Curcumin also modulates matrix metalloproteinases (MMPs) that regulate endothelial cell migration and attachment (82). Curcumin affects multiple signaling pathways including key pathways regulated by transcription factors NF-κB, AP-1, Akt and Nrf2 (2, 54). These pathways control the production of cytokines and other inflammatory
mediators, cell proliferation and apoptosis. Modulation of these pathways by curcumin has a direct impact on tumor progression and survival.

**Pharmacology and Pharmacokinetics of Curcumin**

Turmeric has been classified by The Food and Drug Administration among substances Generally Recognized as Safe (GRAS). It has been shown to be safe at high doses in humans, rats, mice, monkeys and guinea pigs (26, 116, 117). It has also been tested for mutagenicity using the Ames test and has been shown to be nonmutagenic (97). Curcumin has been safely used as a dietary spice for centuries without adverse effects. An estimated 200mg of curcumin is ingested daily by Indian adults (29, 49). The only adverse effects noted in the literature are rare cases of allergic contact dermatitis (46, 53). No adverse drug interactions have been reported (49).

Turmeric contains turmerin, essential oils (turmerones, altanones and zingiberene), curcumin, flavanoids, resins, proteins and sugars. Curcumin is the most biologically active compound found in turmeric and comprises about 2 to 8 percent of turmeric preparations (137). It is estimated that about 40-85% of curcumin remains unaltered after ingestion in the gastrointestinal tract where it is absorbed by the intestinal mucosa (109, 146). The oral toxicity of curcumin is low. Human clinical trials indicate no toxicity at doses as high as 12g/day (11). The oral bioavailability of curcumin is also very low due to its rapid metabolism in the intestinal mucosa and liver. Humans given about 3g/day had undetectable or very low serum levels (119, 122). One study reports a patient with a serum
concentration of 58ng/ml two hours and 51ng/ml four hours after receiving 12g of curcumin. Another patient had serum levels of 51ng/ml four hours after receiving a 10g dosage (79).

The degradation kinetics and stability of curcumin in physiological conditions vary. Degradation is pH dependent. At neutral or basic pH, the degradation is rapid, while more acidic conditions promote slower degradation. In 0.1M phosphate buffer and serum free media curcumin is degraded by about 90% over 30 minutes. In culture medium containing 10% fetal calf serum and in human blood only 20% of curcumin is degraded after 1 hour. After 8 hours 50% still remained (148). Byproducts of curcumin metabolism differ based on the route of delivery; when given orally, curcumin sulfonate and curcumin glucuronide are produced but when given intraperitoneally or systemically tetrahydrocurcumin is produced. It is unclear whether these metabolites are biologically active, though tetrahydrocurcumin is active in some systems (103, 104, 130) but not others (60, 98).

The rapid metabolism and poor bioavailability of curcumin impedes its use as an orally delivered drug. A study by Shoba et al. showed that combining piperine, a known inhibitor of hepatic and intestinal glucuronidation, with curcumin significantly increases its oral bioavailability in humans and rats (122). A more recent approach is to use polymeric nanoparticle-encapsulated curcumin or “nanocurcumin” as a novel formulation to deliver curcumin (20).
Curcumin in the Clinic

The ability of curcumin to modulate multiple molecular targets, coupled with its pharmacological safety and low cost make it attractive for clinical research. There are currently about twenty-five clinical trials examining the therapeutic potential and efficacy of curcumin (www.clinicaltrials.gov). These are outlined in a few review articles (10, 45, 54). Initial results are positive in some subsets of patients when curcumin is used to treat cancer and inflammatory conditions including idiopathic inflammatory orbital pseudo tumors, post-operative inflammation, external cancerous lesions and pancreatic cancer (33, 75-77, 112). Other disease targets being considered include psoriasis and Alzheimer’s disease.

Dendritic Cell Biology

Dendritic cells are the sentinels of the immune system and regulate the immune response. They are widely distributed throughout the body and exist in two functionally distinct states; immature and mature. 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 (50, 92, 129). Immature DCs express relatively low levels of co-stimulatory and antigen
presenting molecules but have a high endocytic capacity while mature DCs express higher levels of these markers and a reduced endocytic capacity. Maturation of dendritic cells involves changes in gene expression, activation of signaling pathways and substantial cytoplasmic reorganization. mDCs extend long dendritic processes that increase the cell surface area that enhances the opportunity for T cell interaction (92). The changes that occur in the DC during the maturation process are regulated by actin assembly and disassembly mediated by the Rho family of GTPases which include Rho, Rac and Cdc42 (24, 43, 100, 124, 133, 151).

Maturation of DCs is a key step in the initiation of immunity. Decreased endocytosis, increased migration and the increased ability to stimulate proliferation and differentiation of T cells are characteristics of mature DCs. Another characteristic feature of mature DCs is their ability to form cellular aggregates or homotypic clusters (37). Cluster formation has been observed in vivo with cutaneous Langerhan’s cells as well as in vitro (31, 84, 150). It has been shown that cluster formation is not an accidental encounter between migrating cells, but rather has a physiological function to enhance maturation. Clustering results in increased CD86, CD80 and CD54 marker expression, and a modest increase in the ability to stimulate T cells in a syngeneic MLR (31). The authors also suggest clustering facilitates antigen transfer between maturing DCs.

Immature DCs are quite different from mature cells. They can take up particles, antigen and microorganisms by phagocytosis and they express
receptors that mediate endocytosis (64, 132). Once iDCs have captured antigens or particles, their ability to capture more quickly decreases. The antigens enter the endocytic pathway where they are processed and presented on the surface of the cell in the context of MHCII molecules (25, 106).

Primed DCs will migrate to secondary lymphoid organs and present the antigen-peptide-MHC complexes to naïve CD4+ T cells and cytotoxic CD8+ T cells which induce differentiate into memory and effector cells. The mixed lymphocyte reaction (MLR) can be used as an in vitro model of DC-TC interaction. DCs will stimulate proliferation of the T cells in co-culture and drive a specific phenotype based on the cytokine environment and the type and activation state of the DCs (32, 135). The ratio of DCs to TCs will influence the phenotype of T cells produced. A low ratio induces a Th2 population, while a high ratio induced mixed Th1/Th2 cell development (135).

DC migration is regulated by chemokine and chemokine receptor interactions with the aid of accessory proteins (38). The chemokine receptor CCR7 plays an important role in DC migration. Mature DCs upregulate CCR7 expression to improve their responsiveness to its ligands CCL21 and CCL19. CCL21 is important in guiding the maturing DCs to the lymphatic vessels and CCL19 guides cells to the T-cell zones of the lymphoid tissues (30, 35, 36, 51, 85).
The Actin Cytoskeleton

The actin cytoskeleton provides the scaffolding of the cell that helps to maintain its shape. Most dendritic cell functions are controlled by cytoskeletal rearrangement. Individual units of actin, globular actin (g-actin), assemble in long polymer filaments to form filamentous actin (f-actin). Two parallel strands of f-actin twist around each other to form the microfilaments of the cytoskeleton. Antigen capture, antigen presentation, cell adherence and cell migration is regulated by the Rho family of GTPases which regulate actin cytoskeleton organization (14, 43, 95, 100). The engagement of T cells by DCs is also dependent on cytoskeletal rearrangement for formation of the immunological synapse (5, 6). The Rho family of GTPases belongs to the larger Ras superfamily of GTP-binding proteins. The Rho subfamily consist of more than twenty distinct proteins including RhoA, RhoB, RhoC, RhoD, RhoE, Rac1, Rac2 and Cdc42 (124). The Rho GTPases function by cycling between the active GTP-bound state and the inactive GDP-bound state (22). Regulation of endocytosis is in part due to the levels of activated Cdc42 (43). Cdc42, Rac and Rho are involved in antigen presentation in DCs as well as motility, adhesion and chemotaxis (7, 8, 124). Regulation of the DC cytoskeleton is largely developmentally regulated (24).

The Wiskott-Aldrich syndrome protein (WASP) is the specific effector of Cdc42 (141). It is expressed mainly in hematopoietic cells and its functions as a signal transducer to the actin cytoskeleton (140). WASP is also important in filapodia formation, adhesion marker expression and DC chemotaxis (7, 8, 140).
WASP binds the actin related protein (Arp) 2/3 complex. Together they regulate the actin cytoskeleton by nucleating the actin filament assembly to create a branching network at podosomes that govern the directional movement of DCs (21, 96, 154).

Nexilin is an f-actin binding protein localized at the cell-matrix adherens junction that was first described in 1998 in rat brain and fibroblasts by Ohtsuka et al (102). Nelin (nexilin-like protein) is the human homolog of nexilin found primarily in the heart, skeletal muscle, artery and vein. Based on structural analysis, it can regulate the formation of stress fibers and focal adhesions (156). In HeLa cells it stimulates migration and adhesion and so mediates cell motility (147). The role nelin plays in dendritic cell function is unknown.

Curcumin and the Actin Cytoskeleton

Little is know about how curcumin affects cytoskeleton of the cell. A few studies have examined the effects of curcumin on the actin cytoskeleton in neurons, hepatic cells and cancer cells but none have outlined these effects in dendritic cells. In an in vitro study using prostate cancer cells, curcumin shows profound effects on actin-based motility and microfilament organization (56). The actin inhibitor chytochalasin B was used in this study as a control. Curcumin shows similar inhibitory effects. Cyclin-dependent kinase 1A (p21) functions as a regulator of cell cycle progression at the S phase. p21-activated kinases (PAKs) also participate in the regulation actin filaments along with the Rho GTPases. Curcumin suppresses PAK translocation in aged Tg2576 transgenic mice with
Alzheimer amyloid pathology (86). Although this was an Alzheimer’s study and curcumin was not the focus, it demonstrates the role curcumin plays in the regulation of actin organization. In another study, curcumin affected the formation of actin stress fibers which helps to suppress the intra-hepatic metastasis in an orthotopic implantation model (101).

Curcumin and Dendritic Cells

Outside of the work published from this study, there is only one article to date that explores the role of curcumin dendritic cells. The authors show curcumin inhibits the immuno-stimulatory function of murine bone marrow-derived dendritic cells (69). The authors show that at non-toxic concentrations, curcumin is a potent inhibitor of DC maturation. Curcumin suppresses the expression of surface maturation markers CD80, CD86 and MHC class II in a concentration dependent manor and reduces the production of IL-12 and other pro-inflammatory cytokines IL-6, IL-1β and TNF-α in LPS-matured DCs. Studies have chronicled the effects of curcumin on antigen presenting cells other than dendritic cells. An in vivo model of murine latex allergy shows CD80 and CD86 levels are reduced on lung B cells treated with curcumin (73). Another study reports reduced CD80 and CD86 expression on macrophages treated with curcumin (121). The immunomodulatory properties of curcumin also extend to its effects on cytokine production in dendritic cells and other antigen presenting cells (1, 44, 66, 69, 152). Curcumin reduced levels of IL-12, IL-6, IL-1β, TNF-α in murine DCs, monocytes and macrophages. Pre-treatment with curcumin also
inhibits transcription of IL-1α, IL-1β, IL-2, IL-6, IL-10 and TNF-α mRNA in rat liver (40). In this study we investigate the effect of curcumin on human dendritic cell maturation by pre-treating the cells with curcumin and then inducing maturation with immune stimulants.

**Significance of the Study**

A study by Kim et al. reveals that curcumin impairs the immunostimulatory function of murine dendritic cells (69), but the effects of curcumin on human dendritic cells remain unknown. In this study we investigate the effects of curcumin on human monocyte-derived DC maturation and function. Dendritic cells direct the adaptive immune response to pathogens and allergens so we hypothesize they play a critical role in mediating curcumin’s systemic effects. We examine effects of curcumin on human dendritic cell maturation and function. Modulating the DC response could provide an effective approach to treat and control unwanted inflammation and could provide an effective approach to treating inflammatory diseases. Elucidation of the underlying mechanism of curcumin modulation will have a direct impact on allergic disease control as recent studies point to its great potential for protection against lung diseases and allergic asthma (71, 73, 110, 131, 144). Curcumin shows promise as an immunomodulatory compound for the treatment of allergic diseases. Kobayashi et al. reports that curcumin inhibits IL-5, GM-CSF and IL-4 production and inhibits the proliferation and IL-2 production in lymphocytes obtained from atopic asthmatics in response to *Dermatophagoides farinea* (71). Curcumin diminishes
the Th2 response, reduces lung inflammation and reduces eosinophilia in a murine model of latex allergy (73) and also reduces histamine release from rat basophilic leukemia cells (131). In guinea pigs it is shown to attenuate airway hyper-responsiveness (110). Curcumin presents itself as an interesting molecule for further investigation. The findings of this study provide novel treatment and control strategies for allergic diseases. Due to its low toxicity, curcumin would offer itself as a safe alternative to non-steroidal anti-inflammatory drugs (NSAIDs) and other inflammatory drugs currently available.
GOALS AND OBJECTIVES

Purpose

The purpose of this study is to examine the effect of curcumin on immature human dendritic cell development and function in response to external stimulants that mimic infection and stimulate cell maturation or activation. This study utilizes in vitro cultures of primary dendritic cells obtained from a number of donors assumed to be in good health. Lipopolysaccharide (LPS), polyinosinic:polycytidylic acid (poly I:C) or tumor necrosis factor alpha (TNF-α) were used to stimulate or induce DC maturation through independent cellular pathways.

Hypothesis

Curcumin has potent anti-inflammatory properties and prevents the immunostimulatory function of murine dendritic cells. We hypothesize that the same is true for human dendritic cells. We expect curcumin will prevent DC maturation in response to a variety of stimulants and impede cell function.
Specific aims

**Aim 1:** To determine the effect of curcumin on human mdDC maturation in response to immune stimulants.

- Does curcumin affect visual signs of DC maturation such as cell morphology and homotypic clustering?
- Can curcumin alter the expression of the surface markers on iDCs?
- Does curcumin affect the level of expression of the surface markers on DCs in response to immune stimulants? If so, is it dependent on the pathway propagated by the stimulant?
- Can curcumin alter cytokine production in stimulated cells?

**Figure 2.** Aim 1 experimental outline. Designed to investigate the effects of curcumin on human mdDCs with and without stimulation. Dashed boxes represent independent variables and solid boxes represent dependent variables.
**Aim 2: To determine the effect of curcumin on human mdDC function in the presence of immune stimulants.**

- Does curcumin affect iDC endocytosis?
- Does curcumin affect endocytosis of stimulated DCs?
- Does curcumin affect DC chemokine secretion and chemokine receptor expression?
- Does curcumin affect DC chemotaxis?
- Does curcumin affect the ability of DCs to induce proliferation of allogeneic donor CD4+ T cells in co-culture?
- How does curcumin affect the phenotype of proliferated T cells in co-culture?

![Diagram](attachment:image.png)

**Figure 3.** Aim 2 experimental outline. Designed to examine the effects of curcumin on stimulated DC function.
**Aim 3:** To determine the effect of curcumin on actin rearrangement in human mdDCs.

- Does curcumin affect the expression of actin and actin pathway-associated proteins in human DCs?
- Does curcumin affect actin polymerization and cytoskeleton rearrangement in human DCs?
- Does the inhibition of actin result in similar functional observations to that of curcumin?

![Figure 4. Aim 3 experimental outline. Designed to examine the effect of curcumin on the actin cytoskeleton in dendritic cells.](image)
MATERIALS AND METHODS

Materials

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). Donors in good health and ranging in age from 18 to 50 were used for the study (Table 1). The cell isolation reagents CD14 microbeads and naïve CD4+ T cell isolation kit were obtained from Miltenyi Biotec (Auburn, CA). For cell isolation and culture, Histopaque®-1077 and 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 antibodies used for flow cytometry CD11c, HLA-DR, CD40, CD86, CD83 and CD54 were obtained from BD Biosciences (San Jose, CA). The antibodies used for western blotting: CD83, CD86 and HLA-DR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Chemokines CCL19 and CCL21 were obtained from PeproTech (Rocky Hill, NJ).
Table 1. Age and gender of donors used in the study

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Monocyte Isolation and culture

In the human body DCs are generated via multiple pathways. They can be derived from CD34+ stem cell precursors or derived from blood monocytes (123). Monocyte-derived DCs generated from CD14 + cells obtained from peripheral blood are phenotypically and functionally similar to circulating DCs in the human body (47, 134). For the purposes of this study mdDCs provide a suitable in vitro model. CD14+ monocytes were isolated and cultured as described by Picki et al. (105). 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 $1 \times 10^6$ cells/ml in complete RPMI (2% L-Glutamin, 10% fetal bovine serum, 1% penicillin/streptomycin, 10mM Hepes, non-essential amino acids and 5mM sodium pyruvate) with 20 ng/ml each rh IL-4 and GM-CSF for five days at 37°C in an atmosphere of 5% CO$_2$ and 95% air, (supplementing at day three with fresh medium). On day 5, most cells express an immature DC phenotype expressing high CD11c, low CD40, CD86, CD83 and HLA-DR (113) (Figure 6). Mature DCs were induced by adding either LPS, Poly I:C or the cytokine TNF-α to the culture for 24 hours (23). The mature phenotype was confirmed by surface marker expression (high CD11c, CD40, CD86, CD83 and HLA-DR) measured by flow cytometry (Figure 6). DCs were identified by forward and side scatter morphology; those cells were gated and subjected to further analysis to quantify marker expression (Figure 5).

![Figure 5. Dendritic cell morphology gating based on forward and side scatter from flow cytometry after culture with GM-CSF and IL-4. 93.6 percent of the total events recorded were assumed to be DCs.](image-url)
Figure 6. Surface marker expression on cultured immature and mature DCs. Cells display both immature and mature dendritic cell phenotype under the specified culture conditions. Surface marker expression determined by antibody staining of cell surface markers and flow cytometry analysis. The mature DCs express higher intensity surface marker expression than immature DCs.
**T cell isolation and culture**

CD4+ T cells were isolated from the non-CD14 expressing fraction remaining after monocyte depletion and cultured in complete RPMI. The untouched cells were negatively selected using magnetic beads (Miltenyi Biotec).

**Curcumin treatment and cell stimulation**

Curcumin, an ingredient of Indian curry powder, supplied as a powder (Sigma) is a polyphenolic compound insoluble in water. Dimethylsulfoxide (DSMO) is used as a solvent in this study and therefore used as a control. Curcumin was added to iDC culture (1 \times 10^6 cells/ml and 3 ml/well in 6-well plates) at the indicated concentrations (20µM or 30µM in most experiments). Cultures were incubated for 1 hour at 37°C in an atmosphere of 5% CO₂ and 95% air after which a stimulant (LPS, Poly I:C or TNF-α) was added to the appropriate wells. Control wells received no stimulants. Cultures were incubated overnight at 37°C and 5% CO₂ and 95% air. Curcumin toxicity was assessed by 7AAD incorporation and measured by flow cytometry. Cells were found to be 95% (± 0.06) viable after 24 hours of culture under all conditions listed above.

**Cell viability by flow cytometry**

In order to assess the toxicity of curcumin in DC culture after a prolonged period, cell viability was assessed. Cells were collected and stained with 7-amino-actinomycin D (7AAD), a nuclear dye, and analyzed by flow cytometry. 7AAD is used to discriminate living cells from dead cells. Live cells with intact membranes
will exclude the dye, while damaged cells will allow the dye to enter the cell (114). The gating strategy was similar to that mentioned above (Figure 5). Measurements were taken at three time points using six concentrations of curcumin. The 30µM concentration is the highest at which viability does not fall below 90% after 12 hr culture (Figure 7). The 10µM concentration did not show significant changes in preliminary studies and so was not included in this work. Only the non-toxic 20µM or 30µM concentrations of curcumin were used for the experiments in this study.

![Graphs showing dendritic cell viability](image)

**Figure 7.** Dendritic cell viability measured by 7AAD staining and flow cytometry after 3 hrs, 12 hrs and 24 hrs of culture with curcumin at various concentrations.

**Surface marker analysis by flow cytometry**

Cells were collected, washed, re-suspended (1 x 10^6 cells/ml) and stained with fluorochrome-conjugated antibodies specific for DC surface markers CD11c, HLA-DR, CD40, CD86, CD83 and CD54. After staining, cells were washed and fixed with 4% paraformaldehyde (PFA) then re-suspended in staining buffer, protected from light and stored at 4°C until flow cytometry analysis. Cells were
analyzed using the Becton Dickenson (BD) Canto II with HTS sampler and BD FACSDiva™ software. Figures were generated using FlowJo software (Tree Star Inc.)

**Multiplex bead assay**

Cytokines produced by DCs in culture were measured by multiplex bead assay (Millipore). Culture supernatant was collected, centrifuged to remove any particulates and stored at -20°C. Cytokine levels measured from the supernatant using the LINCOplex human multiplex assay. Six cytokines (IL-12p70, IL-10, IL-6, IL-8, TNFα and IFNγ) and two chemokines (IP-10 and fractalkine) were measured from DC supernatant using this method. Six cytokines (IL-2, IL-4, IL-6, IL-10, IL-13 and IFNγ) were measured from DC – TC co-culture supernatant after the mixed lymphocyte reaction (MLR). Assays were performed in duplicate according to the manufacturer’s instructions. In summary, samples were diluted with an equal volume of medium and 25µl aliquots were used per assay well. Culture medium was used as the blank for the assay. Samples were incubated with antibody coated capture beads for 1hr, wells were washed and the cocktail of biotin labeled anti-human cytokine antibodies were added to all wells. After a 2hr incubation at room temperature streptavidin-phycoerythrin was added for 30 minutes. Samples were analyzed using the Luminex 100 IS system and IS 2.3 software (Luminex, Austin, TX). Data was generated as mean fluorescence intensity (MFI) for each cytokine. Standard curves were generated using 5 parameter logistic regression based on known concentrations of the recombinant
cytokines provided by the manufacturer. This was used to calculate the concentration (pg/ml) for the samples that were assayed.

**Homotypic clustering**

Dendritic cell clustering is a hallmark of activated or mature cells. It allows cells to communicate with each other as well as responder cells such as T cells and B cells. Cell to cell contact is critical for antigen presentation and the propagation of the adaptive immune response (31). The size and density of the cluster may be indicative of the activation state of the cells. Strongly activated cells form larger and more dense clusters than weakly activated or immature cells. Clusters were observed by light microscopy at low power magnification (4x). Images were recorded using an Olympus digital camera.

**Western Blotting**

Cells were collected, washed and lysed using NP-40 lysis buffer containing protease and phosphatase inhibitors. Proteins were quantified using a BCA protein assay kit and 100µg loaded onto an SDS-PAGE gel. After electrophoresis, proteins were transferred onto a PVDF membrane by electrophoresis. The membranes were blocked using 5% non-fat milk for 1 hr at room temperature and subsequently probed with the appropriate antibodies in 5% bovine serum albumin (BSA) buffer overnight at 4°C. The membranes were washed and incubated with horseradish-peroxidase (HRP)-conjugated secondary antibodies. The bands were visualized by incubating the membranes in West
Pico chemiluminescent reagent (Thermo Scientific) for 5 minutes protected from light and exposing the membranes to x-ray film (Kodak).

**Endocytosis assay**

Immature DCs have the intrinsic ability to capture foreign materials by endocytosis. Stimulated or mature DCs do not possess this ability (113). This inherent property is used as a measure of DC maturity. Treated and stimulated cells were collected, washed and incubated with 1mg/ml (per $1 \times 10^6$ cells) Alexa 647 conjugated dextran at either $4^\circ C$ or $37^\circ C$ 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 $37^\circ C$ and $4^\circ C$ cultures.

**Chemotaxis assay**

Another measure of DC function is its ability to migrate towards chemo-attractants (81). Mature DCs are more motile than iDCs. Treated and stimulated cells were collected, counted and re-suspended at a concentration of $1 \times 10^6$ cells/ml. 50µl of cell suspension was placed in the upper chambers of 5µm pore size polycarbonate filter inserts in a 96 well microchemotaxis plate (Chemicon). The lower chambers contained 40µl of either CCL19 or CCL21 in 150µl of medium. Control wells had medium only. Input wells (in triplicate) contained $1 \times 10^4$ 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 x 10⁴ 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 ÷ number of bead events) x 10⁴. Input cells = average [number of input cells/well x 5 (dilution factor)]. The percentage migration for each sample (% input) is determined by the following equation: Percent migration = (migrating cells ÷ input cells) x 100.

Mixed lymphocyte reaction

Mature DCs are able to stimulate proliferation of allogeneic T cells and induce a helper response. In order to determine the effect of curcumin on DC function after stimulation, T cell proliferation and polarization was assayed using a mixed lymphocyte reaction. In order to measure proliferation, T cells were loaded with an intracellular dye carboxyfluoroscein succinimidyl ester (CFSE). CFSE passively diffuses into cells and reacts with amines in the cytoplasm forming highly fluorescent conjugates. As the cells divide the conjugates are distributed to the daughter cells. Fluorescence intensity was measured by flow cytometry, with each generation of cells emitting approximately half the fluorescence intensity of the parent. CFSE labeling of CD4+ T cells was carried out according to published procedures (108). Cells were suspended in 1ml PBS containing 5% (v/v) FBS. 1.1µl of the CFSE stock (5µM) was diluted in 110µl of PBS and then 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 20ºC. Cells were washed twice and re-suspended in complete medium ($1 \times 10^6$ cells/ml). The DC-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 ($6.25 \times 10^3$ cells in 100µl per well). 100µl of T cells were added to each well and cultures incubated at 37ºC and 5% CO$_2$/95% air for 5 days. Unstimulated T cells were used as the negative control, mitogen (phytohemaglutinin: PHA at a concentration of 5µg/ml) stimulated T cells were used as the positive control. CFSE fluorescence intensity was measured by flow cytometry using the BD Canto II with HTS attachment and BD FACS Diva software.

*Immunofluorescence labeling*

Cells were collected, washed and fixed with 4% PFA. For intracellular staining, cells were permeabilized with CytoFix/CytoPerm solution (BD Pharmingen). Non-specific antigens were blocked by using staining buffer containing FBS. The staining buffer also contained saponin (BD PermWash buffer) which maintains cell permeabilization. Cells were incubated with the appropriate antibodies, washed thoroughly and mounted in a glycerol-based mounting medium that contains DAPI. Slides were stored at 4ºC, protected from light until imaging.
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 either the 63x or the 40x objective of a Leica scanning confocal microscope.

RNA extraction from human DCs

Monocyte-derived DCs obtained from human peripheral blood were placed in experimental groups, treated with curcumin and stimulated with LPS (Figure 8). Total RNA was extracted from cells using the RNeasy isolation kit (Qiagen) as per the manufacturer’s instructions. RNA was quantified by optical density measurements and its purity and integrity determined by agarose gel electrophoresis of samples stored at room temperature and samples heated to 42ºC for 1 hr and 70ºC for 10 minutes.

Microarray

All microarray experiments and analysis were carried out at the H. Lee Moffitt Cancer Center Microarray Core Lab. Affymetrix HG U133 Plus 2 array GeneChips were used for the experiment. A separate chip was used for each of the four samples (Figure 8). Isolated RNA was biotinylated as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix). 5µg total RNA was converted to double stranded DNA using 100pmol of an oligo-dT primer that contains a T7 promotor. The resulting cDNA was used in a transcription reaction
with biotinylated nucleotides. The product of this reaction was fragmented and hybridized to the GeneChips. After 16 hours of hybridization, the chip was washed and stained with streptavidin-phycoerythrin and then read using an Affymetrix GeneChip scanner. Data was processed using the GeneChip Operating Software (GCOS) Microarray Suite 5.0 (Affymetrix). Based on criteria set by Affymetrix GCOS software, only genes that are considered to be “present” will be used for further analysis. “Present” calls are made by comparing the 11 perfect matches and mismatches for each probe set. The data generated was normalized to control genes and filtered by present and absent calls or by gene increase or decrease calls. Genes were annotated using software provided by the Microarray Core Facility. To identify cellular pathways affected by curcumin treatment and LPS stimulation, the data was examined using Ingenuity Systems Pathways Analysis 6.3 (Ingenuity Systems®, www.ingenuity.com) software which categorizes identified genes based on biological function and signaling pathways. Canonical Pathways Analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to the dataset. The significance of the association between the dataset and the canonical pathway was measured in 2 ways: 1) A ratio of the number of genes from the dataset that map to the pathway divided by the total number of molecules that exist in the canonical pathway is displayed. 2) Fischer’s exact test was used to calculate a $p$-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.
Statistical analysis

In this study a 4 x 3 factorial design was used. All values are reported as mean and standard error of the mean (SEM). Most data was transformed using a base 10 logarithm to ensure normal distribution, with the exception of viability and proliferation values in which case the raw data was reported. All donors displayed similar trends in response to treatment and stimulation, however due to individual variation there were differences in the magnitude of the values. Paired $t$-tests (repeated measures) for planned comparisons were conducted. Significance was determined using a modified Bonferroni correction (55). Each test of significance was ranked and the range of observed $p$ values were compared to critical alpha values $a$ ($a = \alpha 0.05$ divided by the number of
planned comparisons). Values are presented as the average of six donors. Error bars represent SEM. All statistical calculations were performed using the Statistical Package for Social Sciences (SPSS version 15). Figures were generated using GraphPad Prisim (version 3.03). A concentration dependent reduction in values was observed in almost all cases. Though significance was calculated for only the 20μM concentrations, the change in values of the 30μM concentration compared with the control were therefore thought to be significant as well.
RESULTS

Aim 1: Curcumin Prevents Dendritic Cell Maturation

Curcumin Affects DC Morphology

Dendritic cells have a characteristic morphology. They are slightly irregular in shape and have finger-like projections or dendrites that increase their surface area to maximize cell-cell contact. Curcumin appears to cause the cells to become more spherical and to lose their dendrites (Figure 9). This change is evident in both the absence and presence of LPS. Similar effects were noted for poly I:C and TNF-α stimulated cells (data not shown).
Figure 9. Curcumin affects cell morphology. Cells were cultured for 24hs with or without curcumin (20µM) and LPS (1µg/ml). Images were captured using an inverted light microscope with a 40x objective lens. Red arrows indicate cell dendrites. Images are from a single representative of eight donors.

Curcumin Prevents Homotypic Cluster Formation

Homotypic cluster formation correlates directly with phenotypic DC maturation. Stimulated or mature DCs will form clusters due to their increased marker expression and enhanced motility. In order to determine the effects of curcumin on DC clustering, cells were cultured in the presence of curcumin for 24 hrs and then examined under an inverted light microscope using a low power objective (4x). Curcumin prevents DCs from forming large, dense clusters characteristic of mature DCs in response to stimulants such as LPS, poly I:C and TNF-α (Figure 10). This effect is concentration dependent as the 30µM
concentration abrogates cluster formation completely. There are some clusters formed in the experimental groups that received 20µM curcumin and were stimulated with poly I:C and TNF-α, but these were much smaller than those formed in the similarly stimulated control groups. The clusters formed in response to LPS were larger and more dense that those formed in response to poly I:C and TNF-α.

Figure 10. Curcumin prevents homotypic clustering of stimulated DCs. Cells were cultured for 24 hrs with or without curcumin (20µM or 30µM) and LPS, poly I:C or TNF-α added to the appropriate wells. Images were captured using an inverted light microscope with a 4x objective lens. Images are of a single representative of eight donors.
**Curcumin Reduces Surface Marker Expression**

Immunofluorescence labeling and flow cytometry was used to determine the effects of curcumin on surface marker expression. Cells were evaluated using two concentrations of curcumin (20µM and 30µM) and three immune stimulants (LPS, poly I:C and TNF-α). Data is reported either as percentage positive cells or mean fluorescence intensity (MFI) of all donors \( (n = 8) \). Table 2 shows \( p \) values of significance for each experimental group assessed. A similar number of cells were stained for the surface markers CD11c, HLA-DR, CD83, CD86 and CD40 and analyzed in each experimental group. In order to determine the effects of surface marker expression on iDCs, cells were treated with curcumin for 24 hrs and no stimulant was introduced into culture. There was no significant change in surface marker expression for all donors (Figure 12). In figure 11 the unstimulated cells treated with 30µM curcumin show reduced CD86 expression, this was only seen in this donor and not the trend across all donors. In the presence of all three stimulants, the cells that were treated with curcumin had reduced expression of CD83, CD86, CD54 and CD40 when compared to stimulated controls (Figures 11, 12). HLA-DR surface expression was not significantly affected by the 20µM concentration of curcumin; however the 30µM concentration of curcumin showed significant results (Figure 11).

Immunofluorescece staining shows that curcumin-treated DCs retain some HLA-DR in the cytoplasm (Figure 13a). Western blot shows curcumin-treated DCs have a lower level of expression of the antigen presenting molecules CD86, CD83 and HLA-DR (Figure 13b). All cells expressed high levels of CD11c, but by
measure of fluorescence intensity, the LPS and TNF-α stimulated cells treated with 30µM concentrations and the LPS stimulated cells 20µM showed significantly reduced expression of CD11c compared to stimulated controls.
Figure 11. Curcumin reduces dendritic cell surface marker expression in stimulated cells. Flow cytometry histograms are a single representative of 6 donors. The yellow-green line represents the unstained cells and used to designate the negative population.
Figure 12. Curcumin reduces dendritic cell surface marker fluorescence intensity in stimulated cells. The y-axis represents the average log₁₀ mean fluorescence intensity (MFI) ± SEM for 8 donors. * indicates significance by one-tailed t-test of planned comparisons p < critical alpha value.
Table 2. Significance of curcumin effects on surface marker expression (mean fluorescence intensity): Comparison of DMSO vs. Curcumin (Cur 20µM)

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<tr>
<td>HLA-DR</td>
<td>3.544 ± 0.195</td>
<td>3.542 ± 0.211</td>
<td>0.0170</td>
<td>0.1326</td>
<td>No</td>
</tr>
<tr>
<td>CD86 *</td>
<td>5.013 ± 0.102</td>
<td>4.317 ± 0.145</td>
<td>0.0085</td>
<td>0.0000</td>
<td>Yes</td>
</tr>
<tr>
<td>CD83</td>
<td>3.868 ± 0.152</td>
<td>3.260 ± 0.208</td>
<td>0.0085</td>
<td>0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td>CD40</td>
<td>3.793 ± 0.222</td>
<td>3.399 ± 0.336</td>
<td>0.0085</td>
<td>0.0005</td>
<td>Yes</td>
</tr>
<tr>
<td>CD54 *</td>
<td>5.052 ± 0.087</td>
<td>4.792 ± 0.107</td>
<td>0.0085</td>
<td>0.0000</td>
<td>Yes</td>
</tr>
<tr>
<td>Poly I:C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11c</td>
<td>4.920 ± 0.076</td>
<td>4.621 ± 0.105</td>
<td>0.0170</td>
<td>0.0019</td>
<td>Yes</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>3.412 ± 0.167</td>
<td>3.319 ± 0.197</td>
<td>0.0085</td>
<td>0.0013</td>
<td>Yes</td>
</tr>
<tr>
<td>CD86 *</td>
<td>4.644 ± 0.514</td>
<td>4.120 ± 0.446</td>
<td>0.0170</td>
<td>0.0000</td>
<td>Yes</td>
</tr>
<tr>
<td>CD83</td>
<td>3.542 ± 0.088</td>
<td>2.873 ± 0.111</td>
<td>0.0170</td>
<td>0.0004</td>
<td>Yes</td>
</tr>
<tr>
<td>CD40</td>
<td>3.854 ± 0.595</td>
<td>3.454 ± 0.589</td>
<td>0.0170</td>
<td>0.0011</td>
<td>Yes</td>
</tr>
<tr>
<td>CD54</td>
<td>4.659 ± 0.516</td>
<td>4.307 ± 0.615</td>
<td>0.0170</td>
<td>0.0011</td>
<td>Yes</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11c</td>
<td>4.926 ± 0.093</td>
<td>4.744 ± 0.076</td>
<td>0.0250</td>
<td>0.0022</td>
<td>Yes</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>3.415 ± 0.198</td>
<td>3.409 ± 0.190</td>
<td>0.0250</td>
<td>0.4483</td>
<td>No</td>
</tr>
<tr>
<td>CD86</td>
<td>4.173 ± 0.523</td>
<td>4.006 ± 0.490</td>
<td>0.0250</td>
<td>0.0024</td>
<td>Yes</td>
</tr>
<tr>
<td>CD83</td>
<td>3.445 ± 0.106</td>
<td>2.995 ± 0.106</td>
<td>0.0250</td>
<td>0.0006</td>
<td>Yes</td>
</tr>
<tr>
<td>CD40</td>
<td>4.463 ± 0.633</td>
<td>4.282 ± 0.569</td>
<td>0.0250</td>
<td>0.0188</td>
<td>Yes</td>
</tr>
<tr>
<td>CD54</td>
<td>3.884 ± 0.799</td>
<td>3.679 ± 0.819</td>
<td>0.0250</td>
<td>0.0033</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* observed p values were less than 0.0001
Figure 13. Curcumin reduces the expression of the antigen presentation molecules.

Immunofluorescence labeling of fixed and permeabilized DCs (A) and western blot of whole cell lysate (B) showing reduced expression of marker expression in the presence of curcumin and LPS.
Curcumin Reduces Cytokine Production

Cytokine production by dendritic cells is indicative of maturation. Cytokines produced will begin the cascade of immune reaction and induce cell–cell interactions which initiate the adaptive response. In order to assess the effects of curcumin on cytokine production, iDCs were treated with two concentrations of curcumin and stimulated with either LPS or poly I:C. Culture supernatants were collected and analyzed by multiplex bead assay. IFNγ, IL-12p70, the immunomodulatory cytokine IL-10 as well as pro-inflammatory cytokines IL-6, IL-8 and TNF-α were measured. The assay was carried out in duplicate and the average of 6 donors is represented in figure 14. Due to the innate variations in donor response to the stimulants, the data are averaged and represented as log_{10} concentration. Table 3 shows the p value significance for the curcumin effects noted in this assay. Table 4 shows the p value significance of the stimulant effects. Characteristically low levels of all cytokines were produced by non-stimulated cells. These levels were not affected by curcumin at either concentration. Stimulation with LPS and poly I:C resulted in substantial production of all cytokines. IL-10, IL-6 and IL-12p70 were significantly reduced in the curcumin treated cells stimulated with LPS and poly I:C in a concentration dependent manor. These levels were more or less reduced to the levels of the iDC cells that received no stimulation (Figure 14). In cells stimulated with LPS, higher levels of IL-8 were produced than the poly I:C stimulated groups. Some values were outside of the dynamic range of the assay. The 20µM curcumin concentration significantly increased IL-8 production in poly I:C stimulated cells.
above the stimulated controls. Due to the fact that the LPS-stimulated 20µM curcumin treated cells were out of the range of the assay, the curcumin effects were not significant. The 30µM concentration did not reduce levels below the untreated control in response to either stimulant. There was a significant increase in IL-8 levels however in the unstimulated control group treated with curcumin (20µM). The level of IFNγ produced in this assay was minimal. In LPS-stimulated cells, curcumin caused a significant reduction in the amount of this cytokine that was produced. No significant reduction was observed in the poly I:C stimulated group. Stimulated DCs showed significantly reduced TNF-α levels in when curcumin was present. Though this reduction was concentration dependent, unlike with the other cytokines, the TNF-α levels were not reduced to that of the unstimulated controls (Figure 14).
Figure 14. Curcumin reduces cytokine production by human DCs in response to stimulants. Cells were cultured in the presence of curcumin (20µM and 30µM) and stimulated with either LPS or poly I:C. Data is represented as log (base 10) concentration and the average of 6 donors. Error bars are SEM and * indicates significance (p < critical alpha)
Table 3. Significance of curcumin effects on cytokine production: Comparison of DMSO vs. Cur 20µM

<table>
<thead>
<tr>
<th></th>
<th>Mean (DMSO)</th>
<th>Mean (Cur 20µM)</th>
<th>Critical p</th>
<th>Observed p</th>
<th>Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No Stimulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>1.196 ± 0.454</td>
<td>0.977 ± 0.459</td>
<td>0.025</td>
<td>0.003</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.477 ± 0.000</td>
<td>0.477 ± 0.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.194 ± 0.676</td>
<td>1.099 ± 0.831</td>
<td>0.05</td>
<td>0.199</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-8</td>
<td>2.022 ± 0.428</td>
<td>2.553 ± 0.899</td>
<td>0.025</td>
<td>0.0078</td>
<td>Yes</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.234 ± 0.113</td>
<td>0.869 ± 0.548</td>
<td>0.025</td>
<td>0.0665</td>
<td>No</td>
</tr>
<tr>
<td>IFN&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>0.627 ± 0.423</td>
<td>0.477 ± 0.000</td>
<td>0.05</td>
<td>0.1753</td>
<td>No</td>
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<tr>
<td><strong>LPS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>3.453 ± 0.825</td>
<td>2.078 ± 1.011</td>
<td>0.017</td>
<td>0.0005</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>2.626 ± 0.983</td>
<td>0.964 ± 0.596</td>
<td>0.025</td>
<td>0.002</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-6</td>
<td>4.132 ± 0.257</td>
<td>3.372 ± 0.926</td>
<td>0.025</td>
<td>0.009</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-8</td>
<td>4.162 ± 0.306</td>
<td>4.314 ± 0.022</td>
<td>0.05</td>
<td>0.097</td>
<td>No</td>
</tr>
<tr>
<td>TNF-α</td>
<td>4.157 ± 0.388</td>
<td>3.623 ± 0.398</td>
<td>0.017</td>
<td>0.0065</td>
<td>Yes</td>
</tr>
<tr>
<td>IFN&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>1.557 ± 0.764</td>
<td>0.832 ± 0.612</td>
<td>0.017</td>
<td>0.0074</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Poly I:C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>1.705 ± 0.394</td>
<td>1.151 ± 0.585</td>
<td>0.05</td>
<td>0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>2.467 ± 1.296</td>
<td>0.865 ± 0.809</td>
<td>0.05</td>
<td>0.003</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-6</td>
<td>3.309 ± 0.497</td>
<td>2.285 ± 0.808</td>
<td>0.017</td>
<td>0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-8</td>
<td>3.309 ± 0.497</td>
<td>3.630 ± 0.282</td>
<td>0.017</td>
<td>0.0007</td>
<td>Yes</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.839 ± 0.918</td>
<td>2.362 ± 0.702</td>
<td>0.05</td>
<td>0.0145</td>
<td>Yes</td>
</tr>
<tr>
<td>IFN&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>0.780 ± 0.424</td>
<td>0.557 ± 0.225</td>
<td>0.025</td>
<td>0.1376</td>
<td>No</td>
</tr>
</tbody>
</table>
Table 4. Significance of stimulant effects: Comparison DMSO (No stim) vs. Stimulant (Stim)

<table>
<thead>
<tr>
<th></th>
<th>Mean (No Stim)</th>
<th>Mean (Stim)</th>
<th>Critical p value</th>
<th>Observed p value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LPS</strong>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>1.196 ± 0.454</td>
<td>3.453 ± 0.825</td>
<td>0.025</td>
<td>0.0000</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.477 ± 0.00</td>
<td>2.626 ± 0.983</td>
<td>0.025</td>
<td>0.0000</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.194 ± 0.676</td>
<td>4.132 ± 0.257</td>
<td>0.025</td>
<td>0.0000</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-8</td>
<td>2.022 ± 0.428</td>
<td>4.162 ± 0.306</td>
<td>0.025</td>
<td>0.0000</td>
<td>Yes</td>
</tr>
<tr>
<td>TNFα</td>
<td>1.234 ± 0.113</td>
<td>4.157 ± 0.388</td>
<td>0.025</td>
<td>0.0000</td>
<td>Yes</td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.627 ± 0.423</td>
<td>1.557 ± 0.764</td>
<td>0.025</td>
<td>0.0210</td>
<td>Yes</td>
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<tr>
<td><strong>Poly I:C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>1.196 ± 0.454</td>
<td>1.705 ± 0.394</td>
<td>0.05</td>
<td>0.0010</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.477 ± 0.00</td>
<td>2.467 ± 1.296</td>
<td>0.05</td>
<td>0.0015</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.194 ± 0.676</td>
<td>3.309 ± 0.497</td>
<td>0.05</td>
<td>0.0005</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-8</td>
<td>2.022 ± 0.428</td>
<td>3.309 ± 0.497</td>
<td>0.05</td>
<td>0.0035</td>
<td>Yes</td>
</tr>
<tr>
<td>TNFα</td>
<td>1.234 ± 0.113</td>
<td>2.839 ± 0.918</td>
<td>0.05</td>
<td>0.0010</td>
<td>Yes</td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.627 ± 0.423</td>
<td>0.780 ± 0.424</td>
<td>0.05</td>
<td>0.2719</td>
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*Observed p values were less than 0.0001
Aim 2: Curcumin Prevents Dendritic Cell Function

**Curcumin Reduces Endocytosis**

Endocytosis is a feature of immature dendritic cells which are usually found in the periphery. Maturing migrating DCs lose their ability to endocytose. Therefore, the test of endocytic capacity is thought of as the gold standard in assessing DC maturity as a measure of function. In order to assess the effects of curcumin on iDC endocytosis, cells were cultured in the presence of curcumin for 24hrs and then cultured with fluorescently labeled dextran (MW 10,000). Dextran uptake was measured by flow cytometry and confocal microscopy. Data is represented as the average change in mean fluorescence intensity (MFI) of six donors (Figure 15b). Confocal microscope imaging of a representative donor is shown in figure 15a. The significance of the effects of curcumin are shown in table 4 and the significance of stimulant effects are shown in table 5.

Curcumin prevents iDCs from taking up dextran in a concentration dependent manner (Figure 15b). In the curcumin treated cells, dextran accumulates along the cell membrane and not throughout the cytoplasm as in the untreated control (Figure 15a). LPS stimulated cells show characteristically reduced dextran uptake. The level of endocytosis for both LPS and poly I:C stimulated DCs were significantly reduced when compared to the unstimulated control (open bars in figure 15b). Pre-treated stimulated DCs show dextran uptake similar to that of the unstimulated pre-treated controls (black and hatched bars in Figure 15b). Similar to curcumin treated DCs, LPS stimulated DCs show
dextran accumulated along the cell membrane and not throughout the cytoplasm (Figure 15a). There was no significant difference between curcumin-treated and untreated DCs in the LPS and poly I:C treated experimental groups (Table 5).

Figure 15. Curcumin reduces endocytosis in human dendritic cells. Confocal microscopy image (a) and change in mean fluorescence intensity (MFI) as measured by flow cytometry (B). Confocal images were captured using a 64x objective lens. Change in MFI was calculated by subtracting the MFI at 4°C from the MFI at 37°C. * indicates significant curcumin effects $p < 0.05$; † indicates significant stimulant effects $p < 0.05$. 

![Confocal images and flow cytometry graph](image-url)
Table 5. Significance of curcumin effects on endocytosis: Comparison of DMSO vs. Cur 20µM

<table>
<thead>
<tr>
<th></th>
<th>Mean (DMSO)</th>
<th>Mean (Cur 20µM)</th>
<th>Critical p value</th>
<th>Observed p value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Stim</td>
<td>23,729 ± 6196</td>
<td>13,596 ± 6353</td>
<td>0.017</td>
<td>0.0126</td>
<td>Yes</td>
</tr>
<tr>
<td>LPS</td>
<td>12,726 ± 12,237</td>
<td>9,850 ± 8,543</td>
<td>0.025</td>
<td>0.1165</td>
<td>No</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>14,242 ± 9,198</td>
<td>12,964 ± 6,060</td>
<td>0.050</td>
<td>0.2960</td>
<td>No</td>
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</table>

Table 6. Significance of the effects of stimulants on endocytosis: Comparison of DMSO (No Stim) vs. Stimulant (Stim)

<table>
<thead>
<tr>
<th></th>
<th>Mean (No Stim)</th>
<th>Mean (Stim)</th>
<th>Critical p value</th>
<th>Observed p value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>23,729 ± 6196</td>
<td>12,726 ± 12,237</td>
<td>0.050</td>
<td>0.0418</td>
<td>Yes</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>23,729 ± 6196</td>
<td>14,242 ± 9,198</td>
<td>0.050</td>
<td>0.0382</td>
<td>Yes</td>
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</table>

**Curcumin Reduces Chemokine Secretion**

Chemokines are cytokines that induce cellular migration. Two key cytokines produced by DCs to attract inflammatory cells that are fractalkine (CX3CL1) and IP-10 (CXCL10). The supernatant of DC experimental cultures were analyzed for these chemokines by multiplex bead assay. Data are reported as the average log_{10} transformed concentration of four donors. The significance of the curcumin effects are shown in table 7. A lower concentration of curcumin (20µM) was used in this experiment, since for previous experiments the changes at this concentration were found to be significant. LPS was the only stimulant used as curcumin effects do not seem to depend on the stimulant used.
Curcumin reduces the levels of IP-10 and fractalkine produced in LPS-stimulated DCs (Figure 16). The reduction in IP-10 production was found to be significant, however, due to the low levels of fractalkine produced, the reduction was not found to be significant (Table 7).

![Figure 16. Curcumin reduces chemokine production by dendritic cells. Error bars represent SEM and * indicates p values < critical value.](image)

Table 7. Significance of curcumin effects on chemokine production: Comparison DMSO vs. Cur 20µM

<table>
<thead>
<tr>
<th></th>
<th>Mean (DMSO)</th>
<th>Mean (Cur 20µM)</th>
<th>Critical p</th>
<th>Observed p</th>
<th>Significant</th>
</tr>
</thead>
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<tr>
<td><strong>No Stimulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td>1.588 ± 0.769</td>
<td>1.144 ± 0.726</td>
<td>0.05</td>
<td>0.0828</td>
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<tr>
<td>Fractalkine</td>
<td>0.486 ± 0.015</td>
<td>0.382 ± 0.166</td>
<td>0.05</td>
<td>0.2113</td>
<td>No</td>
</tr>
<tr>
<td><strong>LPS</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td>4.278 ± 0.036</td>
<td>3.008 ± 0.705</td>
<td>0.025</td>
<td>0.0009</td>
<td>Yes</td>
</tr>
<tr>
<td>Fractalkine</td>
<td>0.980 ± 0.456</td>
<td>0.486 ± 0.015</td>
<td>0.025</td>
<td>0.0970</td>
<td>No</td>
</tr>
</tbody>
</table>
Curcumin Prevents DC Chemotaxis

DC chemotaxis is a characteristic function of mature DCs. After they acquire antigen they migrate towards lymphoid organs to stimulate T lymphocytes. In order to assess the effects of curcumin on DC chemotaxis, treated and stimulated cells were placed in a chemotaxis chamber and allowed to migrate towards chemo-attractants CCL19 and CCL21. Data is reported as the average percent cell migration of two donors. The significance of the effects of curcumin are shown in table 8. The samples were normalized to the percentage of cells migrating towards medium only. Curcumin treated cells are unable to migrate towards the chemo-attractants CCL19 and CCL21 in response to LPS (Figure 17). The chemokine receptor CCR7, expressed on the surface of DC binds both the CCL21 and CCL19 ligands. The expression of CCR7 is not affected by curcumin (Figure 18). CCR7 expression on DCs was evaluated by surface marker immunofluorescence staining followed by flow cytometry (Figure 18a,b) and by western blot of whole cell lysate (Figure 18c).
Figure 17. Curcumin prevents chemotaxis of dendritic cells in response to LPS. Cell migration was normalized using percent cell migration towards culture medium. Error bars represent SEM and * indicates p values < critical value. CCL21 – chemokine ligand 21 or exodus-2; CCL19 – chemokine ligand 19 or macrophage inflammatory protein-3-beta (MIP-3β)

Table 8. Significance of curcumin on dendritic cell chemotaxis: Comparison of DMSO vs. Cur 20µM

<table>
<thead>
<tr>
<th></th>
<th>Mean (DMSO)</th>
<th>Mean (Cur 20µM)</th>
<th>Critical p value</th>
<th>Observed p value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Stim</td>
<td>0.599 ± 0.765</td>
<td>0.160 ± 0.089</td>
<td>0.050</td>
<td>0.2999</td>
<td>No</td>
</tr>
<tr>
<td>LPS</td>
<td>25.219 ± 1.457</td>
<td>0.176 ± 0.083</td>
<td>0.025</td>
<td>0.0123</td>
<td>Yes</td>
</tr>
<tr>
<td>CCL19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Stim</td>
<td>0.780 ± 0.052</td>
<td>0.117 ± 0.087</td>
<td>0.025</td>
<td>0.0120</td>
<td>Yes</td>
</tr>
<tr>
<td>LPS</td>
<td>22.431 ± 3.225</td>
<td>0.319 ± 0.439</td>
<td>0.050</td>
<td>0.0371</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Figure 18. Expression of the chemokine receptor CCR7 is not affected by curcumin. CCR7 expression is determined both on the cell surface by immunofluorescence labeling and flow cytometry (a and b) and intracellularly by western blot of whole cell lysate of a representative donor (c). Histograms shown are from a single donor (a) and the bar chart shows the average mean fluorescence intensity (MFI) of four donors with error bars representing SEM (b).

CCR7 – chemokine receptor 7; GAPDH – glyceraldehyde 3-phosphate dehydrogenase.
Curcumin Reduces DC-induced T Cell Proliferation in an Allogeneic Mixed Lymphocyte Reaction

The mixed lymphocyte reaction can be used as a measure of DC maturation. Although iDCs can weakly stimulate T cell proliferation, mDCs initiate a more robust proliferation of the T cell population in a co-culture. In order to study the effects of curcumin in this process, iDCs were treated with curcumin, stimulated with either LPS or poly I:C and then co-cultured with allogeneic CD4+ T helper cells from mismatched donors. The T cells were pre-loaded with CFSE, an intracellular dye and proliferation was assessed after 5 days of co-culture by flow cytometry. Flow cytometry data is represented as either the average mean fluorescence intensity of six donors or of a representative donor. The significance of curcumin effects and stimulant effects on the experimental populations are shown in tables 8 and 9 respectively. For the flow cytometry analysis, gates were set based on side scatter vs. forward scatter morphology (figure 19a) and those cells were evaluated for CFSE expression. Proliferated cells have lower fluorescence intensity of CFSE than unproliferated cells. Mature DCs induce significantly higher proliferation by CD4+ T cells than iDCs (Figure 20). Though iDCs induce some proliferation in the T helper cell population, curcumin significantly impairs this ability as the number of proliferated cells is reduced from 25.28 (± 10.84) % to 4.83 (± 4.20) % on average. Even in the presence of the stimulants LPS or poly I:C curcumin-treated DCS are unable to induce proliferation of T helper cells at levels greater than 6.03 (± 3.50) % (Figure 19).
Figure 19. Curcumin reduces DCs ability to induce proliferation in allogeneic CD4+ helper T cells in a MLR. Data is representative of six donors. The gating strategy is shown in panel (a) and the histograms of gated cell proliferation based on CFSE fluorescence is show in panel (b). CFSE – carboxyfluorescein succinimidyl ester; MLR – mixed lymphocyte reaction.
Figure 20. Curcumin-treated DCs show reduced CD4+ T cell proliferation in allogeneic MLR. Data represents the average of six donors.

Table 9. Significance of curcumin effects on inducing proliferation of CD4+ T cells: Comparison of DMSO vs. Cur 20µM

<table>
<thead>
<tr>
<th></th>
<th>Mean (DMSO)</th>
<th>Mean (Cur 20µM)</th>
<th>Critical p value</th>
<th>Observed p value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Stim</td>
<td>25.275 ± 10.838</td>
<td>4.825 ± 4.195</td>
<td>0.050</td>
<td>0.0215</td>
<td>Yes</td>
</tr>
<tr>
<td>LPS</td>
<td>38.750 ± 10.376</td>
<td>4.700 ± 1.337</td>
<td>0.017</td>
<td>0.0040</td>
<td>Yes</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>36.900 ± 13.422</td>
<td>6.025 ± 3.491</td>
<td>0.025</td>
<td>0.0055</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 10. Significance of stimulated DCS inducing T cell proliferation: Comparison DMSO (No stim) vs. Stimulated cells (Stim)

<table>
<thead>
<tr>
<th></th>
<th>Mean (No Stim)</th>
<th>Mean (Stim)</th>
<th>Critical p value</th>
<th>Observed p value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>25.275 ± 10.838</td>
<td>38.750 ± 10.376</td>
<td>0.05</td>
<td>0.0045</td>
<td>Yes</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>25.275 ± 10.838</td>
<td>36.900 ± 13.422</td>
<td>0.05</td>
<td>0.0570</td>
<td>No</td>
</tr>
</tbody>
</table>
**Curcumin Induces a CD4+ CD25+ T Regulatory Cell Population**

Mature DCs will induce T helper cell differentiation. In order to assess the effects of curcumin on the phenotype of proliferated T cells in co-culture, cells were immunofluorescently labeled for FoxP3 and CD25. Proliferated T cells were gated and only that population was analyzed for surface marker expression (Figure 22a). Curcumin-treated DCs, both stimulated and unstimulated induce a CD4+ CD25+ FoxP3+ population from the proliferated T cell population (Figures 21 and 22b). Culture supernatant was also assessed for T helper cytokines IL-2, IL-4, IL-5, IL-10, IL-13 and IFN\(\gamma\). Data is reported as average log\(_{10}\) concentration of four donors. Curcumin-treated DCs induced significantly lower cytokine production from the T cells in co-culture.

![Graph showing the percentage of FoxP3+ CD25+ cells](image)

**Figure 21.** Curcumin-treated DCs induce regulatory T cells in MLR. Data represents the average percentage of FoxP3+ CD25+ T cells of four donors. Only the proliferated cell population was analyzed. Error bars represent the SEM.
Figure 22. Curcumin induces CD4+ CD25+ FoxP3+ regulatory T cells. After 5 days of co-culture with curcumin-treated and stimulated DCs in an allogeneic MLR, T cells were immuno-fluorescently labeled and analyzed by flow cytometry. The gating strategy is outlined in panel (a); cells are gated based on morphology and then proliferated T cells are gated and analyzed for FoxP3 and CD25 expression. The numbers are the relative percentages of proliferated cells (b)
Figure 23. Curcumin reduces T helper cytokine production after five days of co-culture in allogeneic MLR with DCs. Cytokines were measured from the supernatant after T cell co-culture with curcumin-treated, stimulated DCs. Data is reported as average log_{10} concentration ± SEM.
Table 11. Significance of the effects of curcumin on DC-induced T cell cytokine production

<table>
<thead>
<tr>
<th></th>
<th>Mean (DMSO)</th>
<th>Mean (Cur 20µM)</th>
<th>Critical p</th>
<th>Observed p</th>
<th>Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No Stimulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>1.270 ± 0.201</td>
<td>0.477 ± 0.000</td>
<td>0.017</td>
<td>0.0021</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.941 ± 0.226</td>
<td>0.925 ± 0.383</td>
<td>0.017</td>
<td>0.0013</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.886 ± 0.217</td>
<td>1.425 ± 0.146</td>
<td>0.050</td>
<td>0.0320</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-13</td>
<td>2.463 ± 0.433</td>
<td>0.843 ± 0.486</td>
<td>0.025</td>
<td>0.0075</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.485 ± 0.286</td>
<td>0.477 ± 0.000</td>
<td>0.025</td>
<td>0.0029</td>
<td>Yes</td>
</tr>
<tr>
<td>IFNγ</td>
<td>2.213 ± 0.142</td>
<td>0.944 ± 0.464</td>
<td>0.025</td>
<td>0.0027</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>LPS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>1.777 ± 0.350</td>
<td>0.477 ± 0.000</td>
<td>0.025</td>
<td>0.0025</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-10</td>
<td>3.100 ± 0.279</td>
<td>1.213 ± 0.517</td>
<td>0.025</td>
<td>0.0034</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-4</td>
<td>2.054 ± 0.251</td>
<td>1.452 ± 0.142</td>
<td>0.017</td>
<td>0.0061</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-13</td>
<td>2.792 ± 0.407</td>
<td>0.826 ± 0.394</td>
<td>0.017</td>
<td>0.0013</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.979 ± 0.319</td>
<td>0.477 ± 0.000</td>
<td>0.017</td>
<td>0.0013</td>
<td>Yes</td>
</tr>
<tr>
<td>IFNγ</td>
<td>2.722 ± 0.230</td>
<td>0.933 ± 0.121</td>
<td>0.017</td>
<td>0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Poly I:C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>1.516 ± 0.715</td>
<td>0.477 ± 0.000</td>
<td>0.050</td>
<td>0.0311</td>
<td>Yes</td>
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<tr>
<td>IL-10</td>
<td>2.571 ± 1.003</td>
<td>1.403 ± 0.440</td>
<td>0.050</td>
<td>0.0154</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.904 ± 0.343</td>
<td>1.458 ± 0.136</td>
<td>0.025</td>
<td>0.0219</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-13</td>
<td>2.230 ± 1.185</td>
<td>0.815 ± 0.394</td>
<td>0.050</td>
<td>0.0390</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.506 ± 0.705</td>
<td>0.477 ± 0.000</td>
<td>0.050</td>
<td>0.0308</td>
<td>Yes</td>
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<tr>
<td>IFNγ</td>
<td>2.086 ± 0.926</td>
<td>0.921 ± 0.268</td>
<td>0.050</td>
<td>0.0408</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Aim 3: Curcumin Modulates the Actin Cytoskeleton

*Microarray Analysis of Total RNA Reveals the Effects of Curcumin on Gene Expression in Dendritic Cells*

Curcumin has anti-inflammatory, anti-oxidant and anti-viral properties. It is reasonable to speculate that changes in gene expression as well as protein expression are in part responsible for these properties. In order to assess the effect of curcumin on human dendritic cells, DCs were cultured with curcumin and then stimulated with LPS. Total RNA was analyzed using an Affymetrix HG U133 plus 2 genome array. Ingenuity pathways analysis (IPA) software was used to identify pathways that may be affected. Table 12 shows some pathways that were most significantly affected by curcumin treatment as identified by IPA software. Many of the pathways affected are associated with cell structure, motility and function. These pathways also have many of the same molecules in common. Upon closer inspection of the actin cytoskeleton pathway, though many genes are unaffected, there are some genes that show an increase or decrease in expression in response to curcumin-treated and stimulated cells when compared to LPS-stimulated cells (Table 13). Of interest are those genes that are down-regulated in curcumin-treated and stimulated cells such as nexilin which decreased 129 fold. A few GTPases and Rho family members were down-regulated along with WASP interacting protein, plexin and a tubulin-specific chaperone.
Table 12. Some pathways affected by curcumin as determined by Ingenuity pathways analysis software.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>-Log(P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axonal Guidance Signaling</td>
<td>0.01540</td>
</tr>
<tr>
<td>Purine Metabolism</td>
<td>0.01220</td>
</tr>
<tr>
<td>Glucocorticoid Receptor Signaling</td>
<td>0.01060</td>
</tr>
<tr>
<td>Xenobiotic Metabolism Signaling</td>
<td>0.00966</td>
</tr>
<tr>
<td>Huntington's Disease Signaling</td>
<td>0.00904</td>
</tr>
<tr>
<td>Actin Cytoskeleton Signaling</td>
<td>0.00868</td>
</tr>
<tr>
<td>G-Protein Coupled Receptor Signaling</td>
<td>0.00828</td>
</tr>
<tr>
<td>Integrin Signaling</td>
<td>0.00810</td>
</tr>
<tr>
<td>Protein Ubiquitination Pathway</td>
<td>0.00801</td>
</tr>
<tr>
<td>Leukocyte Extravasation Signaling</td>
<td>0.00779</td>
</tr>
<tr>
<td>LPS/IL-1 Mediated Inhibition of RXR Function</td>
<td>0.00766</td>
</tr>
<tr>
<td>ERK/MAPK Signaling</td>
<td>0.00766</td>
</tr>
<tr>
<td>NRF2-mediated Oxidative Stress Response</td>
<td>0.00735</td>
</tr>
<tr>
<td>Calcium Signaling</td>
<td>0.00735</td>
</tr>
<tr>
<td>Ephrin Receptor Signaling</td>
<td>0.00726</td>
</tr>
<tr>
<td>Acute Phase Response Signaling</td>
<td>0.00717</td>
</tr>
<tr>
<td>RAR Activation</td>
<td>0.00703</td>
</tr>
<tr>
<td>Wnt/β2-catenin Signaling</td>
<td>0.00681</td>
</tr>
<tr>
<td>cAMP-mediated Signaling</td>
<td>0.00663</td>
</tr>
<tr>
<td>PPARα/RXRα Activation</td>
<td>0.00659</td>
</tr>
</tbody>
</table>
Table 13. Actin cytoskeleton pathway genes affected by curcumin.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene description</th>
<th>Fold change Cur 20µM + LPS vs LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHOBTB1</td>
<td>Rho-related BTB domain containing 1</td>
<td>11.761</td>
</tr>
<tr>
<td>PLEC1</td>
<td>plectin 1, intermediate filament binding protein</td>
<td>5.332</td>
</tr>
<tr>
<td>SVIL</td>
<td>supervillin</td>
<td>4.815</td>
</tr>
<tr>
<td>FGD6</td>
<td>FYVE, RhoGEF and PH domain containing 6</td>
<td>4.408</td>
</tr>
<tr>
<td>MAP7</td>
<td>microtubule-associated protein 7</td>
<td>4.292</td>
</tr>
<tr>
<td>CDC42EP3</td>
<td>CDC42 effector protein 3</td>
<td>4.081</td>
</tr>
<tr>
<td>ARHGDIB</td>
<td>Rho GDP dissociation inhibitor (GDI) beta</td>
<td>3.836</td>
</tr>
<tr>
<td>RHOBTB2</td>
<td>Rho-related BTB domain containing 2</td>
<td>3.727</td>
</tr>
<tr>
<td>SPTAN1</td>
<td>spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)</td>
<td>3.4278</td>
</tr>
<tr>
<td>SPTBN1</td>
<td>spectrin, beta, non-erythrocytic 1</td>
<td>2.3971</td>
</tr>
<tr>
<td>RASA4</td>
<td>RAS p21 protein activator 4</td>
<td>1.994</td>
</tr>
<tr>
<td>PAK1</td>
<td>p21/Cdc42/Rac1-activated kinase 1</td>
<td>1.172</td>
</tr>
<tr>
<td>VCL</td>
<td>vinculin</td>
<td>1.155</td>
</tr>
<tr>
<td>FGD4</td>
<td>FYVE, RhoGEF and PH domain containing 4</td>
<td>-1.074</td>
</tr>
<tr>
<td>CDC42SE1</td>
<td>Cdc42 small effector 1</td>
<td>-1.408</td>
</tr>
<tr>
<td>RND3</td>
<td>Rho family GTPase 3</td>
<td>-1.442</td>
</tr>
<tr>
<td>PHACTR2</td>
<td>phosphatase and actin regulator 2</td>
<td>-1.454</td>
</tr>
<tr>
<td>PHACTR4</td>
<td>phosphatase and actin regulator 4</td>
<td>-1.582</td>
</tr>
<tr>
<td>RAB12</td>
<td>RAB12, member RAS oncogene family</td>
<td>-1.659</td>
</tr>
<tr>
<td>MYOZ3</td>
<td>myozenin 3</td>
<td>-1.687</td>
</tr>
<tr>
<td>ARHGAP25</td>
<td>Rho GTPase activating protein 25</td>
<td>-1.835</td>
</tr>
<tr>
<td>RAB30</td>
<td>RAB30, member RAS oncogene family</td>
<td>-1.888</td>
</tr>
<tr>
<td>TBCD</td>
<td>tubulin-specific chaperone d</td>
<td>-2.161</td>
</tr>
<tr>
<td>WASPIPI</td>
<td>Wiskott-Aldrich syndrome protein interacting protein</td>
<td>-2.214</td>
</tr>
<tr>
<td>MASTL</td>
<td>microtubule associated serine/threonine kinase-like</td>
<td>-2.885</td>
</tr>
<tr>
<td>PLXNA1</td>
<td>plexin A1</td>
<td>-3.426</td>
</tr>
<tr>
<td>ARHGAP25</td>
<td>Rho GTPase activating protein 25</td>
<td>-4.224</td>
</tr>
<tr>
<td>RHOH</td>
<td>ras homolog gene family, member H</td>
<td>-4.681</td>
</tr>
<tr>
<td>SCIN</td>
<td>scinderin</td>
<td>-4.695</td>
</tr>
<tr>
<td>RABGAP1L</td>
<td>RAB GTPase activating protein 1-like</td>
<td>-4.976</td>
</tr>
<tr>
<td>NEXN</td>
<td>nexilin (F-actin binding protein)</td>
<td>-129.149</td>
</tr>
</tbody>
</table>
Curcumin Alters the Actin Cytoskeleton in Human DCs

The actin cytoskeleton of DCs is responsible for its shape and function. We hypothesize that curcumin can alter the conformation of the cytoskeleton and so provide a mechanism for its observed anti-inflammatory effects. Fluorescently labeled phalloidin was used to visualize actin in this experiment. In order to assess the effect of curcumin on the cell architecture, DCs were treated with curcumin, washed, fixed with 4% paraformaldehyde and stained with Alexa 555-phalloidin in suspension. The cell nucleus was visualized using mounting medium containing the nuclear dye 4’, 6-diamidino-2-phenylindole (DAPI). Images were captured using a confocal microscope and representative z slices are shown in figure 24. Cytochalasin B (CytoB) prevents actin polymer elongation and causes cells to become more rounded. It was used as a control in this study. Increasing concentrations of curcumin causes DCs to become more rounded and less filamentous. The rounding and loss of protrusions is similar to what was observed in the presence of the actin inhibitor CytoB (Figure 25).

The actin cytoskeleton plays an important role in cell adhesion and motility. In order to assess curcumin effects, iDCs were allowed to adhere to gelatin coated cover slips for 2 hrs at 37°C (5%CO₂ and 95% air). iDCs were either treated with curcumin or stimulated. Actin accumulates at podosomes towards the leading edge of the iDCs (Figure 24, left). Curcumin-treated cells show podosome formation, but the cells are not elongated (Figure 24, center). Maturing LPS-stimulated DCs do not show podosomes but rather stress fiber formation (Figure 24, right). In order to assess the effect of curcumin on cell
attachment, DCs are treated with curcumin and stimulated before they are allowed to attach to poly L-lysine coated slides. Curcumin-treated DCs have a rounded morphology and do not adhere tightly to the substrate (Figure 26, center). iDCs and LPS-stimulated DCs adhere and are polarized with leading-edge lamellapodia (Figure 26, left). DCs treated with an actin inhibitor, cytochalasin B, were rounded and loosely adherent (Figure 26, right). The Rho GTPases play an essential role in cellular organization. Curcumin reduces the activity of Rac1 and Cdc42 GTPases evidenced by western blotting of whole cell lysate using phospho-antibodies. The expression of Cdc42 was not affected (Figure 27).

![Figure 24](image)

**Figure 24.** Curcumin interferes with DC motility and attachment. DCs were allowed to adhere to gelatin for 2 hrs at 37°C, 5% CO₂ and 95% air. Curcumin and LPS were added to appropriate wells. After 1 hour, cells were fixed with 4% paraformaldehyde and stained with phalloidin to visualize f-actin. Images were captured with a confocal microscope using a 63x objective lens. 

*Red – Alexa 555-phalloidin; blue – 4′, 6-diamidino-2-phenylindole (DAPI); green – curcumin fluorescing in FITC channel.*
Figure 25. Curcumin changes DC morphology in a concentration dependent manner. DCS were fixed, stained and imaged in suspension to maintain their three-dimensional shape. Curcumin inhibits actin similar to cytochalasin B. The nucleus of the cell is represented by the blue color 4', 6-diamidino-2-phenylindole (DAPI), the actin is stained red (Alexa 555), curcumin fluoresces at about 435nm and is detected in the FITC channel. This accounts for the increasing green color in the cytoplasm of the cells as the concentration of curcumin increases. iDC – immature DC (untreated); DMSO – dimethyl sulfoxide; Cytochalasin B – an actin inhibitor; Cur - curcumin
Figure 26. Curcumin affects DC adhesion. DCs were cultured with curcumin and stimulated with LPS overnight, washed and allowed to adhere to poly L-lysine coated slides for 2 hrs. Cells were fixed with 4% paraformaldehyde and stained for actin (Alexa 555-phalloidin). Images were captured with a confocal microscope using a 40x objective lens.

Figure 27. Curcumin reduces the activity of the Rac1/Cdc42 kinases. Western blots from whole cell lysate of curcumin-treated stimulated DCs.
Curcumin-Induced Reduction in Endocytosis May be Due to Actin Inhibition.

The actin cytoskeleton plays a key role in iDC endocytosis. In order to assess whether the curcumin-induced reduction in phagocytosis is mediated by actin inhibition, a known actin inhibitor, cytoB, was used as a control. CytoB-treated cells showed a similar reduction in phagocytosis to curcumin treated cells (Figure 28).

Figure 28. Disruption of the actin cytoskeleton causes reduced endocytosis in immature DCs.

DCs cultured with curcumin for 24hrs and then endocytosis assay was performed.
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. 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 (120). 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 (20, 122, 142). 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. TNF-α signals through its receptor, TNF-R, which is a TLR-
independent pathway. These compounds were chosen to ensure the immunostimulatory effects were not pathway specific or TLR dependent.

The inhibition of transcription factors NF\(\kappa\)B and AP-1 and other cell signaling pathways by curcumin explains some of the observations made in this study, but curcumin may be targeting other essential cellular pathways as well. The observations of this study suggest curcumin functions as an inhibitor of actin signaling. Since f-actin reorganization is responsible for dendritic cell maturation and function, its inhibition may be the mechanism by which curcumin prevents DC response to stimulants. Elucidation of the underlying mechanism of curcumin immunosuppression could lead to clinical applications of this novel anti-inflammatory agent.

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 LPS poly I:C and TNF-\(\alpha\) in a concentration-dependent manner. 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 an impaired ability to induce T cell responses (41, 126). 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. The reduced CD11c could be the result of curcumin-induced AP-1 inhibition (99).
Mature or activated dendritic cells express elevated levels of co-stimulatory and antigen presenting molecules on their surface such as CD86, CD83 and HLA-DR. If the antigen presenting machinery of DCs are impaired, they can not effectively engage the T cells to initiate a response. HLA-DR surface expression is only significantly inhibited at the 30µM concentration of curcumin in LPS and TNF-α stimulated cells but in both concentrations stimulated with poly I:C show a significant reduction in HLA-DR surface expression. These data suggest curcumin may be interfering with the antigen presenting machinery of DCs by affecting the expression of key presentation molecules.

Mature monocyte-derived DCs secrete IL-12, IL-10 and other pro-inflammatory cytokines. Stimulated curcumin-treated DCs produce significantly lower levels of IL-12, IL-10, IL-6 and TNF-α when compared to the controls creating a Th2 permissive environment. Though the reduction of TNF-α was significant, they were not reduced to the levels of the unstimulated controls. These findings correlate with those from the study by Kim et al. (69) which shows curcumin prevents immunostimulatory function of murine bone marrow-derived cells. They along with others show curcumin is a potent inhibitor of NFκB and AP-1 activation as well as MAPK signaling (59, 149). This provides a reasonable explanation for the observed reduction of IL-12 and IL-10 levels in this study. This is the first study to report that curcumin decreases IL-10 in human DCs. TNF-α expression is controlled by other transcription factors such as lipopolysaccharide-induced TNF factor (LITAF) (136) or interferon regulatory-
factor 3 (IRF3) (111) that may not be affected by curcumin, allowing the transcription of some TNF-α independent of the NFκB pathway.

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. 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 (44). Our findings indicate curcumin interferes with antigen handling in human DCs.

Mature DCs travel to the lymph nodes where they present processed antigen to T cells. Migration towards chemo-attractants is a feature of mature DCs (81). 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-3beta (MIP-3β) and CCL21 or exodus-2, which are expressed in the lymph nodes. Both chemokines bind to the CCR7 receptor on the DC surface. Though CCR7 expression is not affected, curcumin prevents migration towards CCL19 and CCL21 in a chemotaxis assay and also reduces the levels of chemokines fractalkine (CX3CL1) and interferon producing factor (IP-10). Both fractalkine and IP-10 attract inflammatory cells to sites of inflammation. Fractalkine attracts T cells, monocytes and microglia and mediates firm cell adhesion (17, 87). It also induces actin polymerization in human
dendritic cells (34). IP-10 is produced in response to IFNγ and LPS. This chemokine plays an important role in effector T cell trafficking (68). Poly I:C stimulated cells did not migrate in response to the chemokines, even in the absence of curcumin. 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.

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 (42, 57, 125, 153). Immature DCs will weakly stimulate proliferation, while the mature DCs will induce a significantly more robust response. This was observed in this study as there was a significant increase in the amount of T cell proliferation in the stimulated groups compared to the non-stimulated group. Increased expression of co-stimulatory markers on the surface of DCs is essential for T cell interaction and proliferation. Curcumin-treated DCs, both stimulated and non-stimulated, show muted T cell proliferation (Figures 19 and 20). Curcumin inhibits the Th1 profile in antigen-primed CD4+ T cells while promoting the Th2 profile by suppressing IL-12 production in macrophages (66, 67). We observe that curcumin suppresses IL-12 production in DCs, but the cytokines produced after the MLR were mixed Th1/Th2. There was no clear delineation in either direction and the levels were very low. The low level of expression is most likely due to the reduced number of proliferated T cells generated by the co-culture. The mixed
phenotype could also be due to the ratio of DC to T cells used in the reaction (135). Curcumin-treated DCs induce a low level of proliferation and those proliferated cells express CD25 and Foxp3. This implies that curcumin is conferring a tolerogenic property to DCs. A measure of this propensity is the capacity to expand Foxp3 expressing lymphocytes (83, 139). It is suggested that an environment containing IL-10 can induce tolerogenic DCs (63, 89, 90) but in this study, the levels of IL-10 are only slightly (not significantly) elevated above control levels in both DC culture and co-culture, suggesting there may be an alternative mechanism at play.

We show that curcumin is able to exert profound effects on the expression of genes involved with multiple signaling pathways associated with cytoskeleton organization and function. These pathways include axonal guidance signaling, glucocorticoid receptor signaling, actin cytoskeleton signaling. Immunofluorescence staining reveals curcumin interferes with cell actin based cell motility, attachment and microfilament organization on human dendritic cells \textit{in vitro}. The actin inhibitor cytochalasin B was used as a control in most experiments to confirm the effects noted are most likely due to curcumin’s effects on f-actin organization.

The regulation of the DC cytoskeleton is important in DC – T cell interactions (18). Little is known about the effect of curcumin on cytoskeletal rearrangement. One study reveals curcumin significantly alters the actin cytoskeleton in prostate cancer cells (56). Based on this premise, curcumin-induced alterations in DC cytoskeleton could account for our observations. DC
migration involves regulation of the actin assembly. The cells must form protrusions such as filapodia and lamellapodia, form adhesions and retract its tail (80). Curcumin reduces expression of adhesion molecules and though lamellapodia and podosome formation appears to be unaffected, the cells are more rounded and we conclude less motile as there is the absence of the trailing edge seen in the non curcumin-treated cells and these cells do not seem to polarize in any particular direction. Cells were allowed to adhere to coated slides before curcumin treatment and LPS stimulation.

For a more three-dimensional viewpoint, the cells that were imaged in suspension show a dramatic change in morphology. As the concentration of curcumin increases, the surface of the cell becomes more rounded and smooth and less elongated projections are visible. This results in an overall decrease in the cell surface area and so will affect the ability of the cell to interact with other cells such as T cells. The changes observed are similar to those induced by CytoB therefore we can infer that curcumin is inhibiting f-actin polymerization. Curcumin-treated DC show reduced attachment. They did not polarize or adhere strongly to the surface of the poly L-lysine coated slides as the non-treated cells did. The morphology was similar to that of the CytoB treated cells which clearly indicates the reduced attachment is due to inhibition of f-actin.

The Rho family of GTPases is central to the reorganization of the actin cytoskeleton. Two key members are Rac1 and Cdc42. They participate in the control of cell migration, endocytosis and antigen presentation (43, 124). As determined by western blot analysis, curcumin reduces the activity of these
GTPases and so modulates the function of DCs in part through inhibition of actin signaling. The reduction in endocytosis observed in curcumin-treated cells may be due in part to the inhibition of actin pathway signaling. This is evidenced in figure 28 in which curcumin-treated cells show a similar level of endocytosis to that of CytoB-treated cells.
CONCLUSION

Curcumin reduces the DC response to immune stimulants by reducing cell maturation and preventing normal cell function. It causes reduced expression of the surface markers CD86, CD84, HLA-DR, CD40 as well as the adhesion molecules CD11c and CD54 in response to a variety of external stimulants when compared to the untreated controls. Stimulant-induced cytokine and chemokine production was also reduced as a result of curcumin treatment. Functional aspects of DC maturation were also affected by curcumin. The high endocytic capacity of iDCs was significantly reduced in curcumin culture. Chemotaxis and DC-induced T cell proliferation was also abrogated. The few T cells that proliferated in co-culture expressed CD25 and Foxp3 indicating a regulatory population and curcumin may induce a tolerogenic DC. Curcumin affects cell architecture by inhibiting f-actin.
LIMITATIONS OF THE STUDY

The major limitation of this study was the use of donors from Florida blood services. Without access to medical records or individual medical history, we are left to assume the participants were in good health. There is also the aspect of human subject variability. We elected to use a repeated measures analysis approach for data analysis in an effort to control for this variability. There is no established standard for the response of a donor to curcumin. The data was log transformed to ensure a normal distribution for statistical analysis. The age, gender and ethnicity of the donors may have played a role in the noted variation in responses. A larger sample size would be needed to address these aspects of the study.

The concentrations of curcumin used in the study, though non-toxic, are not physiological. Due to the pharmacokinetic properties of curcumin and low bioavailability, the experimental concentrations can not be achieved through daily ingestion. In order to achieve these levels in the circulation, a special formulation may need to be engineered to prevent biodegradation. The method of delivery needs to be taken into account as well. When administered systemically curcumin will affect multiple cell types. To reproduce the DC-specific effects observed here, a formulation needs to be developed that will target curcumin specifically to DCs.
FUTURE DIRECTIONS

Further investigation into the generation of tolerogenic DCs by curcumin would be important in elucidating the immunosuppressive role of curcumin. This would include further characterization of the regulatory T cell population generated in co-culture. The DC to T cell ratio can be adjusted to examine the effects on the phenotype of these cells. This phenomenon needs to be examined in vivo using a mouse model of allergic asthma. If curcumin-DCs induce a regulatory T cell population, then the Th2 type response should be reduced. Using curcumin to target DCs and induce immune tolerance would make it an effective treatment for inflammation.

Further investigation into the effects of curcumin on the actin cytoskeleton needs to be carried out as well. It would be important to determine if curcumin binds to any members of the actin cytoskeleton pathway. Disruption of f-actin organization affects cell attachment and function. Elucidation of the underlying mechanism is important for the development on novel curcumin based therapeutics.
LIST OF REFERENCES


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Appendix A: List of Definitions

Adaptive immunity: host defenses that are mediated by T and B cells after exposure to antigen that generate a specific or memory response or self/non-self recognition.

Allergen: non-infectious antigen that induces a hypersensitivity or allergic reaction.

Allogeneic: individuals of the same species that differ genetically.

Antigen: any molecule that can bind to an antibody. Some have the ability to stimulate antibody production.

Chemokine: small proteins that induce that mediate chemotaxis by regulating the expression of leukocyte integrins.

Chemo-attractant: a substance that attracts leucocytes

Chemotaxis: directional movement of cells.

CD4: cluster of differentiation 4 is present on thymocytes, monocytes, macrophages and is a co-receptor of MHC Class II

CD11c: cluster of differentiation 11c is present on myeloid cells and binds fibrinogen. It can be used to identify dendritic cells.

CD80: cluster of differentiation 80 is present on dendritic cells and is a ligand for CD28 on T cells. It is important in the activation of naïve T cells through co-stimulation (signal 2).
Appendix A (Continued)

**CD83:** cluster of differentiation 83 is present on dendritic, B and Langerhans' cells. It is a marker for activated dendritic cells and may assist in antigen presentation or cellular interactions that follow lymphocyte activation.

**CD86:** cluster of differentiation 86 is present on dendritic, monocytes and activated B cells and it is a ligand for CD28 on T cells. It is important in the activation of naïve T through co-stimulation (signal 2).

**CD54:** cluster of differentiation 54 is present on hematopoietic and non-hematopoietic cells. Also known as intercellular adhesion molecule (ICAM-1) and is involved in adhesion of neutrophils and is a receptor for rhinovirus.

**Cluster of differentiation (CD):** a monoclonal antibody that identifies surface molecules, used to identify various immune cells.

**Cytokine:** low molecular weight proteins that regulate the intensity and duration of the immune response by affecting the functions on other immune cells as well as the cell that produces it.

**Dendritic cell (DC):** professional antigen presenting cell and the most potent activator of T and B cells. They are link the innate and adaptive immune systems and reside in tissues exposed to the external environment such as the skin, lungs and intestines.

**Dimethyl sulfoxide (DMSO):** a chemical compound that is a polar aprotic solvent. It is an excellent solvent and cryoprotectant.
Flow cytometry: a technique used to count, examine and sort microscopic particles or cells suspended in a fluid stream. It uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data.

Homotypic cluster formation: clustering of dendritic cells with each other that is an indicator of increased expression of adhesion molecules, chemokines and chemokine receptors.

Human leukocyte antigen DR (HLA-DR): human leukocyte antigen -DR, the term for MHC in humans is required for antigen presentation to T cells.

Innate immunity: non-specific host defense to invading pathogens such as bacteria, virus and allergens that includes various recognition systems such as toll-like receptors, endocytic, phagocytic and inflammatory mechanisms. The innate immune response does not increase with repeated exposure.

Interferon gamma (IFNγ): cytokine that can induce cells to resist viral replication. It is produced by CD4+ Th1 cells and is a type II interferon and has antiviral, immunoregulatory, and anti-tumor properties.

Interferon-inducing protein 10 (IP-10): chemokine that selectively attracts Th1 lymphocytes and monocytes, and inhibits cytokine-stimulated hematopoietic progenitor cell proliferation. Also called CXCL10.
Interleukin 2 (IL-2): a T cell derived cytokine that stimulates growth and differentiation of T cells, B cells, NK cells, monocytes, macrophages. A central cytokine in the development of an adaptive immune response.

Interleukin 4 (IL-4): a cytokine that is secreted by Th2 cells. It enhances both secretion and cell surface expression of IgE and IgG1. An important cytokine in allergic disease and has overlapping functions with IL-13.

Interleukin 5 (IL-5): secreted by Th2 and mast cells that is a key mediator in eosinophil activation. An important cytokine in allergic disease.

Interleukin 6 (IL-6): both a pro-inflammatory and anti-inflammatory cytokine. Important mediator of fever and of the acute phase response and secreted in response to activation of the innate immune system. High IL-6 is associated with both Th1 and Th2 responses and low IL6 is associated with Treg responses.

Interleukin 8 (IL-8): inflammatory chemokine produced by many cell types. Mainly functions as a neutrophil chemoattractant.

Interleukin 10 (IL-10): secreted by Th0 cells blocks cytokine synthesis by Th1 cells.

Interleukin 12 p70 (IL-12p70): Th1 polarizing type cytokine secreted mainly by dendritic cells. Induces IFNγ production and proliferation/differentiation of Th1 cells.

Interleukin 13 (IL-13): secreted by Th2 cells, involved in the up-regulation of IgE secretion by B cells. An important cytokine in allergic disease.
Ligand: general term for a molecule recognized by a receptor.

MHC Class II: major histocompatibility complex class II present on antigen presenting cells with the primary function to present peptide antigens, both self and non-self, to lymphocytes (T cells) for the purpose of eliciting an immune response.

Mixed lymphocyte reaction (MLR): lymphocytes from two individuals are cultured together for several days in order to induce T cell proliferation.

Monocyte: mononuclear phagocytic leukocyte

Monocyte-derived dendritic cells (mdDCs): dendritic cells obtained by culturing CD14+ monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL4).

Phagocytosis: the engulfment of particles, bacteria, cell debris, etc. by cells. A characteristic function of macrophages and dendritic cells.

Polyinosinic-polycytidylic acid (Poly I:C): a commercially available synthetic double stranded RNA. A toll-like receptor 3 ligand that mimics viral infection and induces a Th1 response.

T cells (TC): thymic lymphocytes that developed in the thymus.

T cell proliferation: The reproduction of a T cell to produce two daughter cells.

Naïve T helper lymphocyte (Th0): T lymphocytes that have never engaged a specific antigen.
Appendix A (Continued)

*T helper type 1 lymphocyte (Th1):* T lymphocyte characterized by the cytokines they produce (especially IFNγ). An increase in Th1 cytokines are associated with autoimmune diseases.

*T helper type 2 lymphocyte (Th2):* T lymphocyte characterized by the cytokines they produce (especially IL4, IL5, IL13). An increase in Th2 cytokines are associated with allergic diseases.

**Toll-like receptor (TLR):** recognize pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents. They mediate the production of cytokines necessary for the development of effective immunity.

**Toll-like receptor 3 (TLR3):** one of the toll-like receptors that recognizes double stranded RNA of viruses.

**Toll-like receptor 4 (TLR4):** one of the toll-like receptors that recognize bacterial lipopolysachharide on Gram-negative bacteria.

*T regulatory lymphocyte (Treg):* T lymphocyte characterized by the cytokines they produce (IL10, TGF-β). They have the ability to inhibit T cell responses and induce tolerance.

**Tumor necrosis factor alpha (TNF-α):** a pleiotropic inflammatory cytokine involved in apoptotic cell death/proliferation, differentiation, inflammation, tumor growth, and viral replication.

**Viability:** ability of the cell to survive.
Appendix B: List of Publications by the Author

Primary Articles


Review Articles

Appendix B (Continued)

Abstracts/Posters – Conference Proceedings

**S. Shirley**, A.J. Montpetit, R.F. Lockey, S.S. Mohapatra, Curcumin Modulates LPS-Induced Inflammation in Human Dendritic Cells, *The Journal of Allergy and Clinical Immunology*,
February 2008 (Vol. 121, Issue 2, Page S10)


X. Kong, S. Song, X. Wang, W. Xu, **S. Shirley**, R.F. Lockey, S.S. Mohapatra, Bone Marrow Derived Stem Cells Reduce Lung Inflammation in a Mouse Asthma Model, *The Journal of Allergy and Clinical Immunology*, February 2008 (Vol. 121, Issue 2, Page S132)

Shyam S. Mohapatra; Xiaoyuan Kong; Xiaoqin Wang; Weidong Xu; Jia-Wang Wang; Gary Hellermann; Raji Singham; **Shawna Shirley**; Prasanna Jena; Weidong Zhang; Subhra Mohapatra; Richard F. Lockey; William Gower, A critical role for atrial natriuretic peptide receptor signaling in allergic disease. *Abstracts of the XX World Allergy Congress (TM) 2007 December 2-6, 2007, Bangkok, Thailand: ORAL ABSTRACT SESSIONS: MECHANISMS OF ASTHMA I: 26*


Appendix B (Continued)


**Shawna Shirley**, Weidong Zhang, Xiaoyuan Kong, Richard F. Lockey and Shyam S. Mohapatra, Development of a Murine Model of Chronic Airway Disease, *University of South Florida Health Sciences Center Research Day*, 2005
Appendix C: First Author Publication

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 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.
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, anti-oxidant, anti-malarial, and insect repellant [1,2]. The pharmacological potential of curcumin is under investigation. Researchers found that it has anti-inflammatory, anti-oxidant, anti-parasitic, anti-viral, and anti-cancer properties [3–6]. It targets transcription factors, cytokines, cell adhesion molecules, surface receptors, growth factors, and kinases, among other molecules [7–10], 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 T lymphocytes and stimulate a specific or adaptive response
Appendix C (Continued)

[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.
Appendix C (Continued)

MATERIALS AND METHODS

Reagents. Curcumin (from Curcuma longa) was obtained from Sigma Aldrich (St. Louis, MO) and dissolved in DMSO. 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 and 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). LINCOpex Multiplex cytokine assay kits were purchased from Millipore (Temecula, CA). All CD11c, HLA-DR, 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 ×
Appendix C (Continued)

$10^6$ cells/ml in complete RPMI (10% FBS, 1% pen/strep, 10 mM Hepes, non-essential amino acids and 5 mM sodium pyruvate) with 20 ng/ml each rh IL-4 and GM-CSF for 5–6 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. 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 staining.

*Cell Treatment and Stimulation.* Curcumin was added to cell culture ($1 \times 10^6$ cells/ml and 3 ml/well in 6-well plates) at concentrations of 20 or 30 µM. DMSO was used as a control. After a 1 h incubation, LPS (1 µg/ml) or Poly I:C (25 µg/ml) was added to the appropriate wells. Control wells received neither. Cultures were incubated overnight at 37 ºC and 5% CO$_2$/95% air. Cell viability was 95% ± 0.06 after 24 h 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 Dickenson (BD) Canto II with HTS sampler and BD FACSDivaTM software.
Appendix C (Continued)

**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 $1 \times 10^6$ cells/ml. Fifty microliters of cell suspension was placed in the upper chambers of 5 µm pore size polycarbonate filter inserts in a 96-well microchemotaxis plate (Chemicon). The lower chambers contained 40 µl of either CCL19 or CCL21 in 150 µl of medium. Control wells had medium only. Input wells (in triplicate) contained $1 \times 10^4$ cells in the lower chambers without chemokines. Cells were incubated at 37 ºC and 5% CO₂/95% air overnight. Migration was stopped by the removal of the inserts. Polystyrene beads ($1 \times 10^4$) 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 × number of bead events) × $10^4$. Input cells = average [number of input cells/well × 5 (dilution factor)]. The percentage migration for each sample (% input) is determined by the following equation: Percent migration = (migrating cells × input cells) × 100.

**Mixed Lymphocyte Reaction.** CFSE labeling of CD4+ T cells was carried out by resuspending cells in 1ml PBS containing 5% (v/v) FBS. 1.1 µl of the CFSE stock (5 µM) was diluted in 110 µl of PBS and quickly mixed with the cell suspension. After a 5 min incubation at room temperature, the reaction was
stopped by adding ten volumes of room temperature PBS containing 5% (v/v) FBS and centrifuging at 300g for 5 min 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 µl per well). T cells (100 µl) 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 1 mg/ml (per 1 × 10^6 cells) Alexa-647 conjugated dextran at either 4 °C or 37 °C for 1 h. 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
Appendix C (Continued)

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 24 h 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 [17–19]. 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.
Appendix C (Continued)

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 20 µM 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 immuno-stimulatory 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.
TNFα 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 TNFα independent of the NF-κB 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 [24–27]. 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.
Appendix C (Continued)

Cell cytokines produced in the MLR revealed a Th2–biased response evidenced by the increase in the IL-4:IFN\(\gamma\) 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 and 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 and 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-3beta (MIP-3b) 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 and C). Both fractalkine and IP-10 attract inflammatory cells to sites of inflammation. Poly
Appendix C (Continued)

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 NF-kB 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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REFERENCES


Appendix C (Continued)


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FIGURE LEGENDS

Fig. 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. *Significance by one-tailed t-test of planned comparisons p < 0.05.

Fig. 2. Curcumin reduces DC-induced proliferation of allogeneic CD4+ T cells (A) and promotes a Th2-permissive environment with an increased IL4:IFN\textsubscript{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). *Significance by one-tailed t-test of planned comparisons p < 0.05.

Fig. 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. *Significance by one-tailed t-test of planned comparisons p < 0.05.
Appendix C (Continued)

Fig. 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 μm pore size polycarbonate filters. Chemokine levels were measured from culture supernatant using a multiplex assay kit.

*Significance by one-tailed t-test of planned comparisons p < 0.05.
Figure 1.
Appendix (Continued)

Figure 2.
Figure 3.
Figure 4.
ABOUT THE AUTHOR

Shawna Ann Shirley was born in St. Andrew, Jamaica in 1980. She earned a Bachelor’s degree in Biochemistry with a minor in Chemistry from the University of the West Indies, Mona campus (Honors) in 2001. She then became a teacher of Mathematics and Science at Ardenne High School in St. Andrew Jamaica. After two years in the profession she decided to pursue higher education in research. She moved to Tampa, Florida in 2003 to join the Multidisciplinary Biomedical Sciences Ph.D. program at the University of South Florida, College of Medicine. She earned a Master of Science in Medical Sciences in 2007 and went on to complete the Ph.D. degree in Medical Sciences with a specialty cognate in Molecular Medicine. Shawna is married to Karl Gilman, a teacher who is currently pursuing a career as a mental health counselor.