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Major Tea Catechin Inhibits Dendritic Cell Maturation in Response to Microbial Stimulation

by

James L. Rogers

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Molecular Medicine
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Keywords: Dendritic cells, EGCG, IL-12, TNFα, CD86, CD40, MHC, Toll-like receptors

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DEDICATION

This dissertation is dedicated to my mother whose loving support has made my studies possible.

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LIST OF ABBREVIATIONS

ACK:	Ammonium chloride potassium bicarbonate
APCs:	Antigen presenting cells
APC:	allophycoerythrin
Ag:	antigen
BMDCs:	bone marrow derived dendritic cells
DCs:	dendritic cells
EGCG:	(-)-Epigallocatechin-3-Gallate
ERK:	extracellular signal-regulated kinase
FBS:	fetal bovin serum
FITC:	fluorescein isothiocyante
FSC:	forward scatter
GM-CSF:	granulocyte-macrophage colony stimulating factor
HBSS:	Hank's balanced salt solution
iDC:	immature DC
Jnk	c-Jun N-terminal kinase
mDC:	mature DC
MHC II:	class II MHC
2-Me:	2-mercaptoethanol
IFNγ:	interferon gamma
IL-12:	interleukin-12
Lp	*Legionella pneumophila*
LPS:	lipopolysaccharide
MAPK	mitogen-activated protein kinases
MIP-1alpha/CCL3:	macrophage inflammatory protein-1alpha
MCP-1/CCL2:	monocyte chemoattractant protein-1
MDP:	muramyldipeptide
Ml:	milliliter
MIP:	macrophage inflammatory protein
NF-κB:	nuclear factor kappa B
NK:	natural killer cell
PBS:	phosphate buffered salin
PE:	phycoerythrin
PGN:	peptidoglycan
PI:	propidium iodide
RANTES:	regulated on activation normal T cell expressed and secreted
ROS	reactive oxygen species
RPMI1640:	medium supplemented with 10% serum
SSC:	side scatter

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TLR	Toll-like receptor
TNFα:	tumor necrosis factor alpha
μg:	microgram

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MAJOR TEA CATECHIN INHIBITS DENDRITIC CELL MATURATION IN RESPONSE TO MICROBIAL STIMULATION

JAMES L. ROGERS

ABSTRACT

Dendritic cells (DCs) are a migratory group of bone-marrow-derived leukocytes specialized for uptake, transport, processing and presentation of antigens to T cells. Exposure of DCs to bacterial pathogens can induce DC maturation characterized by cytokine production, up-regulation of co-stimulatory molecules and an increased ability to activate T cells. DCs have the ability to restrict growth of *L. pneumophila* (Lp), an intracellular Gram-negative bacillus that causes a severe form of pneumonia known as Legionnaires' disease, in murine ER-derived organelles (121) but replicate in human DCs (145). Even in human cells, however, lysis of the DCs does not occur for at least 24 hours which may allow DCs time to participate in the transition from innate to adaptive immunity (145). The primary polyphenol in green tea extract is the catechin (-)-epigallocatechin-3-gallate (EGCG) which accounts for most of the numerous reported biological effects of green tea catechins, including anti-bacterial, anti-tumor, and neuroprotective effects. Primary murine bone marrow derived DCs from BALB/c mice were treated *in vitro* with Lp, or stimulated for comparison with *Escherichia coli* lipopolysaccharide (LPS). CD11c, considered an important marker of mouse DCs, and surface expression of co-stimulatory molecules CD40, CD80, CD86, as well as class I/ II MHC molecules was determined by flow cytometry. Treatment of the cells with EGCG

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inhibited the microbial antigen induced up-regulation of CD11c, CD40, CD80, CD86 and MHC I/ II molecules. EGCG also inhibited, in a dose dependent manner, induced production of the Th1 helper cell activating cytokine, IL-12, and the chemokines RANTES, MIP1α, and MCP-1. However, EGCG upregulated TNFα production. In addition, EGCG inhibited both Lp and LPS induced expression of both TLR2 and TLR4 as well as LPS-induced NF-κB activation; all of which are important mediators of DC maturation. The modulation of phenotype and function of DCs by EGCG has implications for host interaction with microbial pathogens like Lp, which involve TLR interaction.

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INTRODUCTION

EGCG

Sources and Structure

Polyphenols are natural substances found in abundance in fruits, vegetables and plant-derived beverages such as tea and consist of an aromatic ring that is condensed to a heterocylic ring and attached to a second aromatic ring (90). Flavonoids are the largest group of polyphenols, which include the subcasses of flavones, isoflavones, flavanols, flavans and flavonols. Catechins are a further subcategory of flavanols (166)(Figure 1).

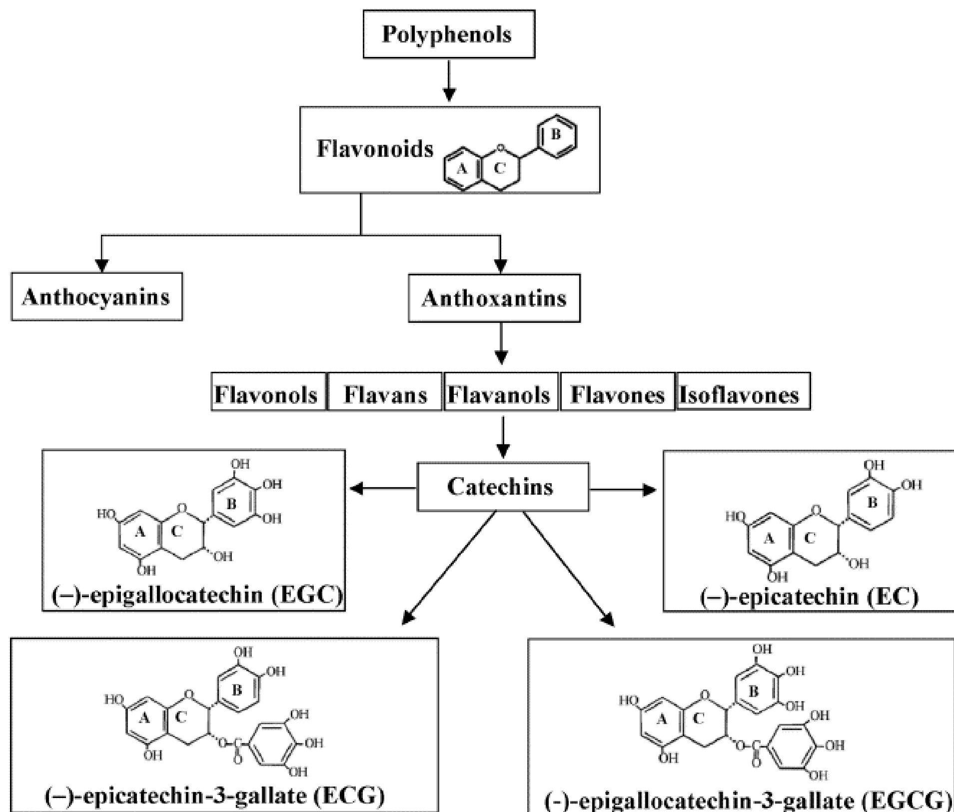

Figure 1. Diagram of the natural polyphenol classification and the chemical structure of green tea catechins. Reproduced with permission of Elsevier Limited.

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(-)-epigallocatechin-3-gallate (EGCG) is one of several catechins found in many natural products, particularly both green and white tea. The other major catechins are (-)-epicatecin (EC), (-)-epigallocatechin (EGC), and (-) epicatechin-3-gallate (ECG) (166). EGCG is the major catechin in green tea, and it also accounts for most of the reported biological effects of green tea, especially its reported anti-tumor effects (115). These biological effects of EGCG may relate to the presence of the trihydroxyl group on the B ring and the gallate moiety at the 3' position in the C ring (120).

Antibacterial Activity of EGCG

EGCG reportedly also has potent antimicrobial activity. For example, a report published in 2001, from our own laboratory, showed that the growth of Lp in permissive macrophages could be selectively inhibited by small amounts of EGCG. These antimicrobial effects were not due to direct effects on the bacteria, since EGCG could not alter *Lp* growth in medium regardless of the concentration used (106). Instead, antimicrobial effects were mediated by indirect effects of EGCG on the macrophages themselves which were activated to induce the observed antimicrobial activity. This activation was also mediated, at least in part, by induction of TNFα and IFNγ production from the macrophages, since treatment of the macrophage cultures with anti-TNFα and anti-IFNγ monoclonal antibodies markedly abolished the antibacterial effects of EGCG (106).

Effects on Cytokine Production

Cytokines are soluble proteins secreted by cells of the immune system. They have pleiotropic effects in that they act on many cell types to modulate the host's immune

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response (150). Various studies have shown that EGCG has immunomodulatory effects upon pro-inflammatory cytokines. For example, EGCG inhibits LPS-induced TNFα production by peritoneal macrophages from BALB/c mice (179). In the murine macrophage cell line, RAW264.7, EGCG decreases LPS induced TNFα production in a dose-dependent fashion as well as LPS-induced TNFα mRNA expression. The mechanism of action was reported to be due, in part, to the down regulation of NF-kB, an oxidative stress –sensitive nuclear transcription factor, since EGCG also inhibited LPS induced nuclear NF-kB-binding activity (179). EGCG combined with EC also reportedly inhibits TNFα production by BALB/3T3 cells treated with the tumor promoter, okadaic acid (152).

However, in cultured human peripheral blood mononuclear cells, EGCG stimulates production of TNFα (143). Moreover, Matsunaga showed that EGCG selectively upregulated production of TNFα by macrophages induced by bacterial infection (106). Other studies from Matsunaga show that EGCG attenuates nicotine-induced inhibition of TNFα production in Lp infected macrophages (105) as well as attenuates suppression by cigarette smoke condensate of TNFα in response to infection with Lp (104).

The effects of EGCG on IL-12, another pro-inflammatory cytokine, has also been investigated. For example, Ahn and company reported that EGCG inhibits IL-12 production by BMDCs stimulated with LPS (3). However, in the MH-S murine alveolar macrophage cell line, EGCG selectively upregulates production of IL-12 (106). EGCG also attenuates nicotine inhibition of IL-12 production in Lp infected macrophages (105). Topical application of EGCG before UVB exposure also reportedly upregulates UVB-

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induced production of IL-12 in skin as well as in draining lymph nodes from C3H/HeN mice (75).

EGCG has been reported to have immunomodulating effects on various other cytokines. In the MH-S murine alveolar macrophage cell line, EGCG selectively down regulates IL-10 production by macrophages induced by bacterial infection and upregulates macrophage gamma interferon (IFN-γ) mRNA by EGCG but does not alter IL-6 production (106). Topical application of EGCG before UVB exposure reportedly decreases UVB-induced production of IL-10 in skin as well as in draining lymph nodes in C3H/HeN mice (75). However, EGCG attenuates nicotine inhibition of IL-6 production in Lp infected macrophages (105) as well as attenuates suppression by cigarette smoke condensate of IL-6 in response to infection with Lp (104). Using normal human keratinocytes stimulated with TNFα, EGCG has also been reported to inhibit production of VEGF and IL-8 (160). In cultured human peripheral blood mononuclear cells, EGCG stimulates production of IL-1α/β (143).

The results of all of these studies establish that EGCG has inhibitory effects on pro-inflammatory cytokines such as TNFα and IL-12. However, the effects of EGCG upon such pro-inflammatory cytokines, as well as other cytokines, varies depending upon both the host cell studied as well as the stimulus used in the study.

Dendritic Cells

Functions in Immunity

DCs are potent APCs because of their unique characteristic features such as very high MHC class II expression, costimulatory molecules B7-1/2, and the ability to capture antigen at an immature stage and efficiently present to T cells at a mature stage (13, 22).

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Although T and B cells of the adaptive immune system express antigen receptors of enormous diversity, activation of these cells depends on their induction by co-stimulatory molecules and secretion of cytokines and chemokines by APCs such as DCs (126). As DCs mature, they migrate to the T cell areas of lymphoid organs, where they translate tissue-derived information into language that T helper (Th) cells can understand. DCs do this by providing Th cells with an antigen-specific "signal 1," a costimulatory signal 2, and a signal 3 which determines the polarization of naïve Th cells into Th1 or Th2 cells. Thus, DCs provide a critical link between innate and adaptive immunity (129).

DCs are also often said to "direct" the type of immune response delivered in response to the detected pathogen. LPS, dsRNA and oligodeoxynucleotides containing immunostimulatory CpG motifs (CpG ODN) promote maturation of DCs that direct naïve T cells to a Th1 subtype. By contrast, phosphorycholine-containing glycoproteins derived from nematode parasites, cholera toxin or yeast hyphae activate DCs that selectively induce Th2 cells (109)(Figure 2)

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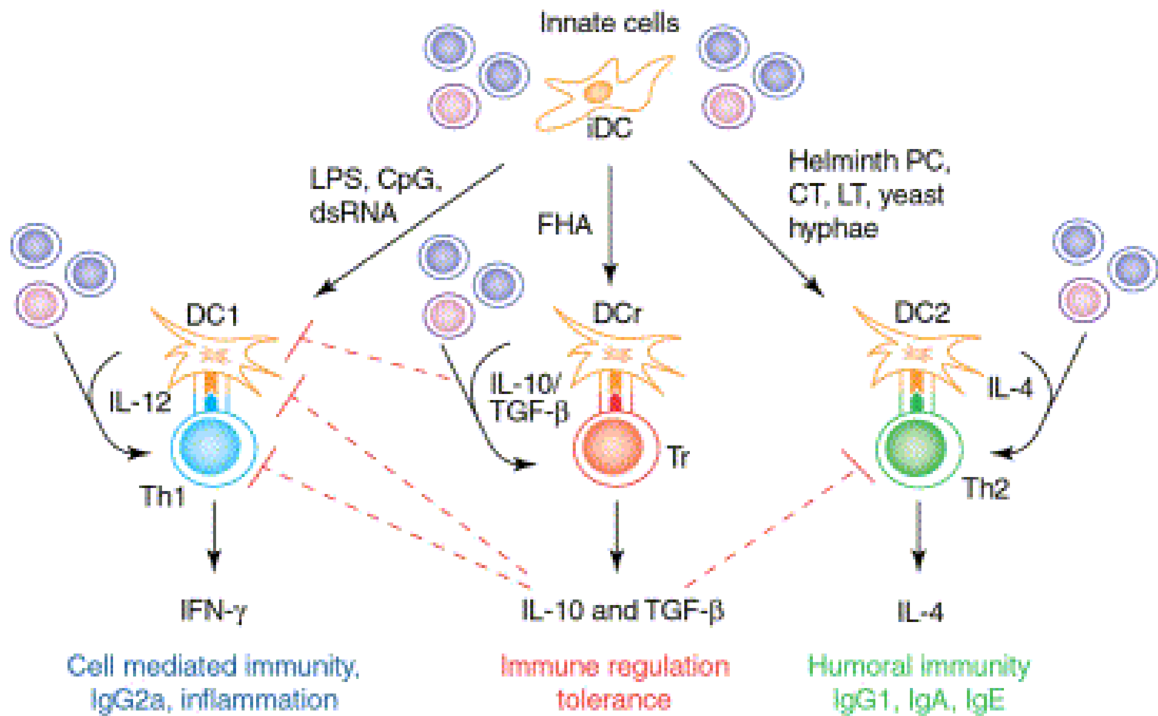

Figure 2. DCs Direct an Immune Response. Reproduced with permission of Elsevier Limited.

DC Maturation and the Immune Response

The ability of DCs to "direct" an immune response is linked to their maturation state. In the mature state, DCs represent a potent APC for helper (CD4+) T cell activation. Interaction with activated CD4+ T cells may also result in the delivery of additional stimuli that render the DC "hyper-mature." These DCs can subsequently induce activation of cytotoxic (CD8+) T cells (88). In addition, it is becoming increasingly clear that DCs, in an immature state, play a central role in peripherally expressed self and non-threatening foreign antigens. For example, immature DCs within peripheral tissues capture cells dying by apoptosis and migrate to the draining lymph node where they present self-peptide-MHC complexes, in the absence of costimulation

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signals, to the circulating naïve autoreactive T cells. This results in their inactivation either by anergy or deletion (151).

There is also evidence that DCs can control peripheral tolerance through induction and maintenance of regulatory T cells. For example, fusion proteins targeted to DCs lead to antigen-specific tolerance induction when DCs are left immature (17), and CD4+ T cells repetitively stimulated with allogeneic immature DC differentiate into IL-10 producing regulatory cells, which inhibit the proliferation of alloreactive T cells (69). Injection of immature DCs pulsed with influenza matrix peptide into healthy human volunteers also leads to the appearance of MP-specific IL-10 producing CD8+ T cells and silencing of MP-specific CD8+ T cell effector function in freshly isolated T cells (33). It is important to keep in mind that the induction of T cell responses versus tolerance is a complex process which depends on much more then whether DCs are "mature" or "immature." The outcome of an immune response depends on the phenotypic and functional change which occurs as DCs mature.

Phenotypic Changes Associated with DC Maturation

Introduction

During the process of DC maturation, DCs lose the ability to phagocytosize, but they also produce large amounts of cytokines and chemokines. Simultaneously, MHC class II molecules are translocated to the membrane, and costimulatory molecules such as CD86 and CD40 are up-regulated. Mature DCs demonstrate a characteristic morphology with enlarged size and numerous cytoplasmic processes ((148)(Figure 3).

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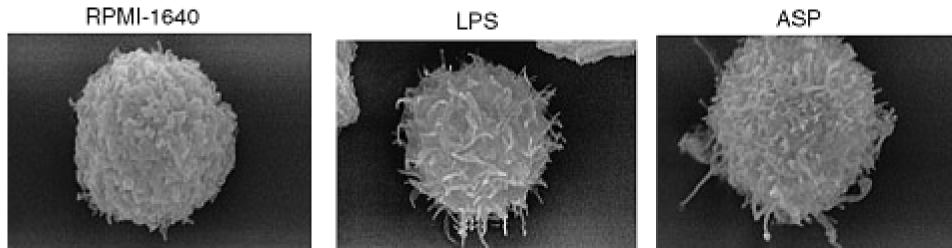

Figure 3. Compared with the RPMI-1640 (untreated control), Astragalus mongholicus polysaccharides (ASP) or LPS treated DC show characteristic morphology of mature DC (needle-like protrusions). Reproduced with permission of Elsevier Limited.

MHC Molecules

Whereas, in immature DCs, class II molecules are rapidly internalized and have a short half-life, maturation stimuli lead to a burst of MHC class II synthesis and translocation of the MHCII peptide complexes to the cell surface where they remain stable for days and are available for recognition by CD4+ T cells (12). To generate CD8+ cytotoxic killer cells, DCs present antigenic peptides on MHC class I molecules (12). Although most cells use their MHC class I molecules to present peptides derived from endogenously synthesized proteins, DCs have the capacity to deliver exogenous antigens through the MHC class I pathway, a phenomenon known as cross-presentation (55). Increased MHC class II expression has been shown to occur in several autoimmune diseases, including multiple sclerosis and rheumatoid arthritis (49).

Co-Stimulatory Molecules

During DC maturation, several co-stimulatory molecules are also expressed, with especially high levels of CD86. The MHC-peptide complexes are found in clusters at the DC surface together with CD86 (161). It is believed that these high levels of antigen-presenting and co-stimulatory molecules, in a clustered distribution, initiate the formation

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of the immunologic synapse, bringing together essential elements, such as the T cell receptor (TCR) and CD28, that are required for T cell activation (89). Low levels of the costimulatory molecules CD80 and CD86 expression on APCs leads to T cell anergy. This reportedly occurs because CTLA-4, which inhibits T cell responses, has a higher affinity for CD80 and CD86 than CD28, which promotes T cell responses (119).

DCs from CD40-/- mice do not make IL-12 or elicit CD4+ and CD8+ T cell responses, even though they are able to present peptide Ag (44). DCs lacking cell surface expression of CD40, due to inhibited RelB function, reportedly also suppress ongoing immune responses by inducing IL-10-secreting Tregs (102). Moreover, CD40/CD40L interactions release immature DCs from suppression by CD4+CD25+ T cells, further suggesting that CD40 ligation is necessary and sufficient to abrogate tolerance and inhibit the action of Tregs (147). There is also evidence that suggests that costimulatory molecules on APCs may selectively influence T helper cell differentiation: antibodies against CD80 or CD86 selectively inhibit the development of Th1 and Th2 responses, respectively (157).

Functional Changes Associated with DC Maturation

Cytokine Induction and Associated Biological Functions

In the DC maturation process, cytokine genes are expressed with distinct kinetics in mice. Following appropriate stimulation, TNFα is released rapidly (peaking at 3 h), whereas IL-6, IL-10, IL-12 and IL-23 are produced between 6 and 18 h after stimulation (87). The nature of the immune response is also dependent upon the types of cytokines secreted by maturing DCs. A prime example of this is the Th1/Th2 dichotomy. Naïve Th cells differentiate into Th1 or Th2 cells depending on the cytokine microenvironments

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after activation through their antigen-specific receptors. In particular, IL-12 is a pro-inflammatory cytokine with immunoregulatory function that bridges innate resistance and antigen-specific adaptive immunity (159) and, when produced by DCs, induces Th1 differentiation and, hence, cellular immunity. This cytokine acts in concert with natural killer (NK) cell-derived IFNγ to further promote Th1 responses (159). Secretion of cytokines by DCs is also important for induction or reversal of tolerance. For example, attenuation by DCs of T regulatory cells depends, at least in part, on DC secretion of IL-6 (127).

In BMDCs, there is one report associating a possible anti-inflammatory role with TNFα. In particular, BMDCs produced less IL-12p40 when preincubated with TNFα and then stimulated with LPS (1 ng/ml) (184). In general, however, TNFα is recognized as a proinflammatory cytokine as well as associated with antigen-specific, cell-mediated immune responses (57). TNFα also promotes DC migration from tissues into lymph nodes, can induce chemokines that are important in the recruitment of APCs, and upregulates antigen presentation(84).

Chemokines

Chemokines are potent chemoattractants that can be divided into four highly conserved but distinct families: CXC, CC, C, and CX3C, based on the position of the first two cysteines in the amino terminus as well as the remaining cysteines in the carboxy portion of the molecule. Maturing DCs are also an abundant and strategic source of chemokines, which are produced in a precise time-ordered fashion. Following stimulation with LPS, DCs show an initial burst of MIP-1α (CCL3), MIP-1β (CCL4) and IL-8 (CXCL8) production, which cease within a few hours. RANTES (CCL5) and MCP-1 are

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also induced, but in a more steady manner. At later time points DCs produce mainly lymphoid chemokines, such as CCL17 (TARC), CCL18 (DC-CD1), CCL19 (MIP-3β) and CCL22 (MDC), that attract T and B lymphocytes (108, 144).

Chemokines are produced by DCs in response to microbial antigens through TLRs. For example, TLR4 is activated by LPS from Gram-negative bacteria. Activation of different TLRs induces expression of different sets of chemokines that recruit distinct subsets of leukocytes (Figure 4). Many different chemokines are produced through TLR activation in DCs including IL-8 (also known as CXCL8), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5) and IP-10 (CXCL10). MIP-1α, MIP-1β and RANTES are reported to be induced by agonists of both TLR2 and TLR4 whereas IP-10 is preferentially induced by TLR4 agonists and IL-8 preferentially induced by TLR2 specific agonists. These studies suggest that pathogens can determine the nature of the immune response through differential activation of TLRs and the subsequent patterns of chemokines expression (97).

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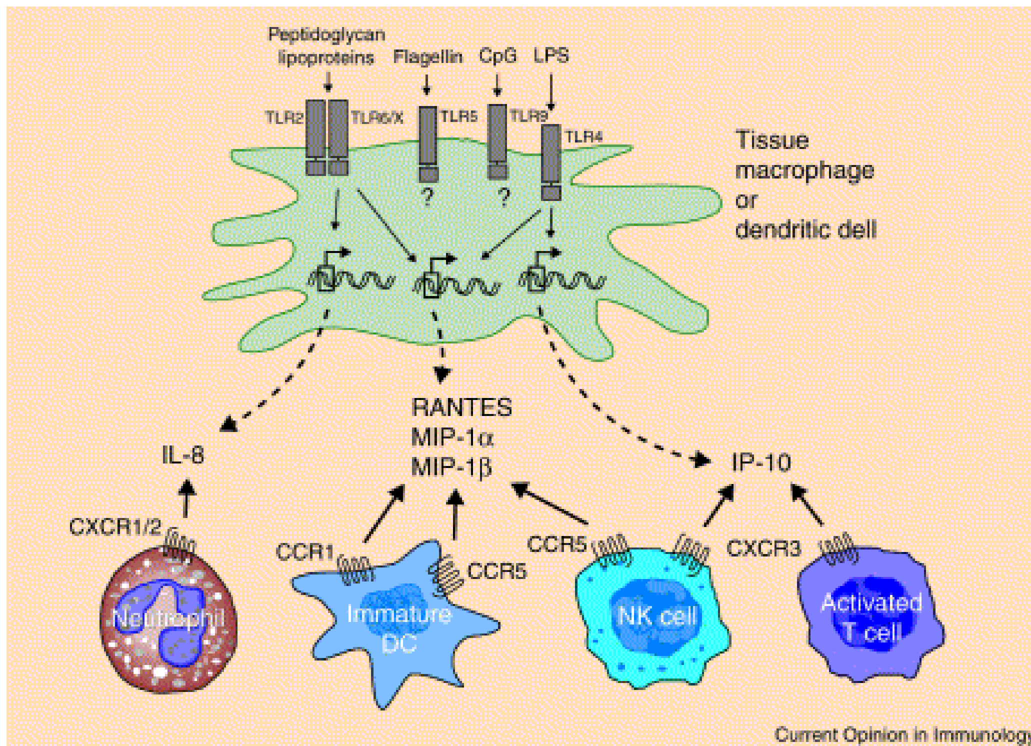

Figure 4. Pathogens Induce Different Patterns of Chemokine Expression . Reproduced with permission of Elsevier Limited.

Thus, in similarity to production of cytokines, the early production of chemokines is essential in shaping the immune response that follows in the tissue. For example, the production of IL-8 will induce the recruitment of neutrophils, and MIP-1α and MIP-1β will induce the influx of NK cells, macrophages and immature dendritic cells (97). The stimulation of select TLRs by the pathogen and the subsequent production of a specific subset of chemokines may be the first point at which the immune system is tailored to a specific pathogen (97).

As with cytokines, the types of chemokines produced by DCs have been associated with Th1/Th2 immune response. In particular, fractalkine and IP-10 have been associated with a Th1 phenotype, whereas MDC and TARC with a Th2 phenotype (30, 32, 43, 62, 66, 92, 108, 187). MIP-1α also reportedly upregulates Th1-type cytokine responses (74) and downregulates Th2 (96), while IP-10 selectively up-regulates antigen-

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driven IFN-γ synthesis suggesting an important role in maintaining bias toward a Th1 response (45). Some of these effects of chemokines on T helper biasing may be direct or indirect through the action of cytokines. For example, MIP-1α-driven Th1 differentiation was not abrogated by anti-IFN-γ suggesting that the effects of MIP-1α are either direct or operating through undertermined cofactors. In contrast, anti-IL-4 abrogated the ability of MCP-1 to drive Th2 differentiation suggesting that MCP-1 enhanced T cell-mediated IL-4 production which in turn supported the Th2 phenotype (73).

Chemokines can also directly influence the polarizing potential of DCs. For example, CCL19 reportedly programmed DCs for the induction of Th1 rather than Th2 responses. Migrating DCs isolated form mice genetically deficient in CCL19 and CCL21 also presented an only partially mature phenotype, highlighting the importance of these chemokines for full DC maturation *in vivo* (100).

Chemokine Receptors

The type of chemokine receptor expressed is associated with the maturation state of the DC. Immature DCs respond to MIP-3α, RANTES, and MIP-1α via chemokine receptors CCR1, 5 and 6, whereas mature DCs respond to MIP-3β/ELC and SLC via CCR7. Down-regulation of receptors for the inflammatory chemokines and up-regulation of receptors on mature DCs for chemokine that are expressed in secondary lymphoid organs allow DCs to leave the sites of inflammation and migrate to regional lymph nodes (10, 21, 35) ((Figure 5).

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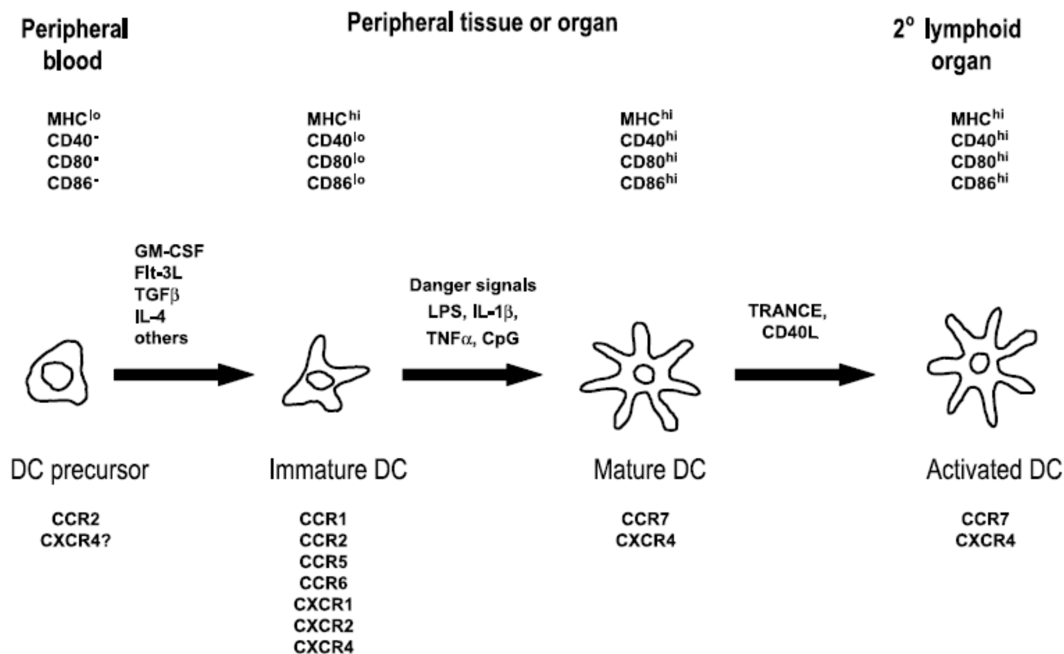

Figure 5. Chemokine Receptor Expression on Dendritic Cells. Reproduced with permission of Nature Publishing Group.

Each immature DC population also displays a unique spectrum of chemokine responsiveness. For example, Langerhans cells migrate selectively to MIP-3α (via CCR6), blood, CD11C+ DC, to MCP chemokines (via CCR2), monocyte derived-DCs respond to MIP-1 alpha/beta (via CCR1 and CCR5), while blood CD11c+DC precursors do not respond to any of these chemokines (21, 108).

A number of chemokine receptors are also found on Th1 and Th2 cells. CCR5 and CXCR3 have been associated with the Th1 phenotype, while CCR3, CCR4, and CCR8 have been associated with the Th2 phenotype (124). Mice which are defective for CCR2, the receptor for MCP-1, reportedly have significant defects in production of Th1-type cytokines as well as delayed type hypersensitivity responses (18). Interestingly, the expression of chemokine receptors may change depending on the activation status of the T cell. For example, CCR8 is only strongly expressed in activated Th2 cells (185).

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Microbial Factors and Dendritic Cell Maturation

Lipopolysacharide (LPS)

Lipopolysaccharide (LPS), a major component of the Gram-negative bacterial envelope, elicits immediate proinflammatory responses in the host (47). LPS is captured by LPS-binding protein (LBP) and subsequently transferred to CD14 (53). However, because CD14 lacks intracellular signaling domains, the complex interacts with TLR4 providing the necessary intracellular signaling capacity (111). LPS can induce DC maturation *in vitro and in vivo*, resulting in increased expression of costimulatory molecules and production of proinflammatory cytokines that influence the subsequent immune response (110, 136, 139, 164).

Peptidoglycan/Murymyldipeptide (MDP)

Myramyldipeptide (N-acetyl-muramyl-L-alanyl-D-isogluatamine; MDP) is the smallest structural unit responsible for the immunoadjuvant activity of the peptidoglycan (PGN) in bacterial cell walls (170). (Audibert). Although Gram-negative bacterial cell walls also contain PGN, its concentration is far greater in the walls of Gram-positive bacteria (Traub).

MDP has been shown to exert diverse biological effects on immunocompetent cells in vitro (24). It enhances phagocytic and microcidal activities of monocytes and macrophages (29, 138). It can also augument the expression of immunostimulatory molecules such as MHC class II and CD40 on monocytes and B cells (28, 56).

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L. pneumophila (Lp)

Lp is a Gram-negative intracellular pathogen that often causes serious and life-threatening pneumonia in humans known as Legionnaires' disease with an estimated 17,000 to 50,000 patients hospitalized annually in the United States (101) (183). Unlike macrophages, DCs have the ability to restrict Lp growth which has been suggested as a factor allowing DCs ample time to present antigens for a cell-mediated immune response (121). In contrast to murine DCs, human DCs support Lp replication; however, lysis of the DC does not occur for at least 24 hours allowing DC-mediated transition from innate to adaptive immunity (145). Alterations in maturation parameters such as co-stimulatory and MHC molecules induced by Lp are essential for effective antigen presentation by DCs and enhanced cellular immunity against Lp.

An alteration in chemokine production caused by Lp infection is another maturation parameter important in host immunity. Lp infection of cultured mouse peritoneal macrophages reportedly increases the levels of cellular mRNAs for the neutrophil-attracting CXC chemokines, such as keratinocyte-derived chemokine and macrophage inflammatory protein 2 (116, 176). Lp infection also reportedly induces the gene expression of monocyte chemotactic protein 3 (CCL7) by mouse alveolar macrophage MH-S cells (112). Neutrophil accumulation in Lp infected mouse lungs is reportedly mediated by CXC chemokines such as keratinocyte-drived chemokine, macrophage inflammatory protein 2 and lipopolysaccharide-induced CXC cehmokine (CXCL6) (116, 156). Moreover, DC-mediated immune response to Lp reportedly is attributed at least in part to the DC-derived expression of the membrane-bound Th1 attractant fractalkine, which may promote both the chemotaxis of T cells toward Lp-

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capturing DCs and the adhesion between them, leading to clonal expansion and a Th1-polarized differentiation of T cells recognizing Lp antigens (80).

Toll-Like Receptors

DCs have been shown to express TLRs 2, 3, 4, 5, 6 and 9. The activation of TLRs on DCs induces DC maturation which is characterized by the production of proinflammatory cytokines, upregulation of co-stimulatory molecules and altered expression of chemokine receptors (58, 97). TLR activation ultimately leads to the activation of NF-κB which is essential for the induction of chemokines and cytokines (97). TLR activation on DCs downregulates the expression of CCR1, CCR5 and CCR6, and upregulates the expression of CCR7. Because TLR stimulation occurs when a DC is likely to have internalized microbial pathogens, this switch in chemokine receptor expression ensures that DCs loaded with antigens leave the tissue and are attracted into the lymphoid organs. This modulation of chemokine-receptor expression and subsequent pattern of DC migration are crucial for the induction of an adaptive immune response (97).

Structurally, TLRs are members of the type I transmembrane receptor family, first described in *Drosophila*, and share homology to components of the IL-1 signaling pathway (14). TLR signaling is initated by dimerization of TLRs, which can form homodimers (such as TLR4) or heterodimers (such as TLR2 and TLR1) (6). TLRs and other members of the IL-1 receptor family share a homologus intracellular domain, designated as the toll/IL-1R-like region (TIR), and have been reported to share common intermediate signaling molecules such as myeloid differentiation factor 88 (MyD88), IL-1 receptor-associated kinase (IRAK), and tumor necrosis factor (TNF) receptor-

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associated factor 6 (TRAF6), for activation of NFκB, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase pathways (20, 118, 155).

In addition to the enormous diversity of the adaptive system, there also exists considerable diversity of recognition within innate immunity through the TLR superfamily which recognizes conserved structures called pathogen-associated molecular patterns (PAMPs) such as LPS. TLR4, for example, recognizes bacterial LPS whereas TLR2 recognizes acylated outer membrane lipoproteins of Gram-positive bacteria. The various TLRs also have a diversity of function through the selective use of intracellular adaptor molecules (125, 173, 174). For example, the adaptor MAL is vital for TLR1 through 9 with the exception of TLR3 for the activation of NF-κB (41, 172). TLR3 uses instead the adaptor molecule TRIF to induce NF-κB and IFN-α synthesis through IFN-regulatory factor (IRF) 3 and 7, a signaling pathway that is crucial for anti-viral immunity (77, 158). This pathway is sometimes referred to as the MyD88-independent pathway. TLR4 can also activate the IRF3 signalling pathway in a process that requires the adaptors TRIF and TRAM. There are also other pathways that contribute to TLR function, such as those involving Jun N-terminal kinase (JNK) and the mitogen-activated protein kinases (MAPKs) (36, 59).

TLR2

TLR2 is capable of recognizing a much broader range of pathogen components compared to TLR4. For example, TLR2 can recognize components derived from both Gram-positive and Gram-negative bacteria and mycobacteria such as peptidoglycan (PGN), lipoteichoic acid (LTA), bacterial lipoproteins, lipopeptides, and lipoarabinomannan (40, 146, 181). TLR2 signalling can induce activation of NF-κB and

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MAPK cascades in a MyD88-dependent manner (155). Murine DCs deficient in TLR2 do not undergo maturation upon stimulation with PGN (113).

TLR2 has been shown to be an important molecule responsible for resistance to intracellular growth of Lp in bone marrow-derived macrophages. In particular, intracellular growth was enhanced within TLR2(-/-) compared to wild type and TLR4(-/-) macrophages. There was, however, no difference in the bacterial growth with dendritic cells from WT or TLR-deficient mice (5).

TLR4

TLR4 is a critical receptor and signal transducer for LPS, a prominent PAMP of Gram-negative bacteria, in coordination with CD14 and MD2 molecules (133, 135, 154, 162). LPS ligation induces NF-κB activation (118) and TLR4-deficient mice are hyporesponsive to LPS (12) and derived DCs do not undergo maturation upon stimulation with TLR4 ligands such as LPS and lipid A (113).

LPS-induced TLR4 activates two downstream pathways; the MyD88-dependent pathway that leads to the production of proinflammatory cytokines with quick activation of NF-kB and MAPK, and the MyD88-independent pathway, associated with activation of IRFs, subsequent induction of IFN, and maturation of DCs, with delayed activation of NF-kB and MAPK (70). Although cytokine production is severly restricted in MyD88-deficient mice, some responses to LPS, including the induction of interferon-inducible genes and the maturation of DCs are still observed (70, 76, 77).

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TLR5

Both humans and mice detect Lp flagellin to mount an immune response. In humans, its recognition by TLR5 correlates with resistance to Legionnaires' disease (54). When injected into mice, Lp flagellin triggers a robust inflammatory response (137).

TLR9

Recent results from our own laboratory suggest that TLR9 is also important in sensing Lp in DCs from both BALB/c and A/J mice. As evidence for the importance of TLR9, chloroquine treatment suppressed IL-12p40 production in response to Lp infection, and the TLR9 inhibitor ODN2088 suppressed Lp-induced IL-12 production in DCs from both strains (122).

Molecular Mechanisms of Action of EGCG

TLR Signaling Effects

As mentioned above, microbial antigens trigger the activation of two downstream signaling components of TLRs including MyD88 and TRIF leading to activation of NF-κB. EGCG has been shown to inhibit both of these signaling pathways. For example, EGCG reportedly inhibits IKKβ and TBK1 in the MyD88 and TRIF-dependent signaling pathways, respectively (182) .

MAPKs

The MAPKs are central to receptor signal transduction in the activation of many immune cell genes. They are activated upon phosphorylation, which then allows them to phosphorylate and activate other intracellular factors. The major subgroups of MAPKs comprise ERK, JNK, and p38. Whereas ERKs are predominantly activated by mitogenic signals, JNK and p38 are primarily activated by environmental stresses such as UV

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radiation, inflammatory cytokines, heat shcok and DNA-damaging agents (23, 72, 85). Activation of the p38 pathway is involved in IL-12 p40 promotor activity and cytokine release in DCs (4, 95, 165). However, there are some data indicating that activation of the ERK pathway acts to suppress IL-12 secretion as well as DC maturation (169, 177).

EGCG has previously been shown to inhibit the ultraviolet-B-induced activation of p38-MAPK in a human keratinocyte cell line (27), while others have shown that EGCG activates ERK1/2, JNK and p38 in HeLa cells (25). In vascular smooth muscle cells, EGCG inhibited the platelet-derived growth factor-β-induced activation ERK1/2 in a dose-dependent manner (2). In addition, EGCG selectively inhibited IL-1β-induced activation of JNK, but not ERK1/2 or p38 MAPK, in human osteoarthritis chondrocytes (149). EGCG inhibited LPS-induced IL-12p40 production in murine macrophages by inhibiting p38 MAPK while enhancing p44/p42 ERK, leading to the inhibition of Iκβα degradation and NF-κB activation (61). In DCs, EGCG inhibited LPS-induced MAPKs, ERK1/2, p38 and JNK (3) Thus, it appears that MAPK activating or inhibitory effects of EGCG may be stimulus and/or cell type-dependent.

NF-κB

NF-κB is the common downstream signaling component for all TLRs and plays a critical role in immune and inflammatory responses. Most genes of inflammatory mediators such as TNFα and IL-12 are regulated by NF-κB because they have a κB site in their 5' flanking region (46). NF-κB is sequestered in the cytoplasm of most cell types by virtue of its association with the IκB family of inhibitor proteins, which includes IκBα and IκBβ. The IκBs bind to the Rel homology domain, which contains the dimerization, nuclear transfer, and DNA binding functions of the NF-κB/Rel protein (11). At least two

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of the IκBs (IκBα and IκBβ) undergo rapid phosphorylation at two conserved N-terminal residues in response to cell stimulation by proinflammatory cytokines or bacterial LPS. This phosphorylation targets them for rapid polyubiquitination followed by degradation through the 26S proteasome pathway, thereby liberating NF-κB, which is then free to translocate to the nucleus and bind to DNA (34)

EGCG is known to inhibit NF-κB activation induced by many pro-inflammatory stimuli. In DCs, EGCG has previously been shown to inhibit LPS-induced NF-κB p65 translocation (3). Interestingly, EGCG-mediated inhibition of NF-κB constitutive expression was reportedly found to occur at much higher doses of EGCG in normal human keratinocytes compared to human epidermal carcinoma cells suggesting that cancer cells were more sensitive to the effects of this compound (1).

Antioxidant Properties of EGCG

EGCG is a potent antioxidant, and this catechin has been associated with most of the biological effects of tea catechins, including reduced risk of cancer, diabetes and cardiovascular disease (86). The ability of green tea polyphenols such as EGCG to act as oxygen radical scavengers and chelate transitional metals such as iron and copper may also be of major significance for treatment of neurodegenerative diseases such as multiple sclerosis, Parkinsons disease and Alzheimer's disease (99). EGCG also reportedly elevates the activity of two major oxygen-radical species metabolizing enzymes, superoxide dismutase and catalase in mice striatum which may also be significant for its reported neuroprotective effects (91).

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ROS and Redox Environment

NF-κB can be activated through the generation of exogenous and endogenous reactive oxygen species (67, 71, 134) which includes mechanisms of involving TLR4 activation and function (9). In addition, LPS-induced NFκB activation and consequent TLR4-induced TLR2 expression in endothelial cells is reportedly mediated by NADPH oxidase (39). The involvement of ROS is postulated to regulate the activity of the upstream kinases that converge onto the NF-κB signaling activation pathway (51). DC maturation has also been reported to be regulated by the redox environment. For example, DCs grown under tightly regulated O_2 in the absence of exogenous reducing agents, e.g., 2-Me, induces DC maturation (48).

Tea preparations have been shown to trap reactive oxygen species, such as superoxide radical, singlet oxygen, hydroxyl radical, peroxyl radical, nitric oxide, nitrogen dioxide, and peroxynitrite. Among tea catechins, EGCG is most effective in reacting with most reactive oxygen species (178). H_2O_2-induced erythrocyte membrane damage has been reported to be inhibited by EGCG treatment (141), and EGCG inhibits deoxycholate induced oxidative stress as well as activation of NF-κB in HCT-116 cells derived from a colon carcinoma (9). EGCG in hydrophilic ointment before UVB exposures also reportedly resulted in significant prevention of induced depletion of antioxidant enzymes such as glutathione peroxidase and catalase in mouse skin (163). In tumor cells, a differential oxidative stress environment and induction of apoptosis by tea polyphenols compared to the normal cells have been reported (175, 180).

Under certain conditions, catechins may undergo autooxidation and behave like prooxidants (178). It has been reported that higher concentrations of tea polyphyenols in

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cell culture systems produce H_2O_2, which may be an important factor responsible for cellular toxicity (68, 94, 142, 175, 180).

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PROJECT SIGNIFICANCE

A vast amount of literature exists linking EGCG to many different beneficial biological effects. Within this literature, many studies also support an anti-inflammatory role of EGCG, although results depend upon the type of immune cell studied and stimulus used. Dendritic cells are critical to linking innate to adaptive immunity by initially detecting PAMPs on invading pathogens and activating naïve T cells. DCs are often said to “direct” an immune response, and they are important in directing a inflammatory response. The type of immune response which DCs direct depends upon their maturation state, and more specifically, upon a range of parameters such as cytokine production and costimulatory surface molecule expression which change as DCs mature in response to microbial stimulation. Enhanced inflammation is known to be a critical step in the cascade of events leading to the development of many chronic diseases such as Alzheimer’s disease and multiple sclerosis, and it is widely believed that newer therapies are needed in the management of these diseases. Recent evidence also suggests the involvement of TLRs in these chronic inflammatory diseases. The studies are significant because DC maturation parameters such as cytokine/chemokine production and TLR expression are important in inflammation, and the type of immune response directed by DCs.

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OBJECTIVES

These studies examine effects of EGCG upon important parameters of DC maturation in response to microbial products such as LPS and Lp. In this respect, an objective of the following studies is to investigate effects of EGCG on phenotypic maturation parameters of DCs such as costimulatory and MHC molecule surface expression. A second goal is to examine effects of EGCG on functional characteristics of DC maturation such as cytokine and chemokine production. A third goal of the following studies is to examine mechanistic effects of EGCG on DC maturation and in particular, its effects on TLR signaling pathways. EGCG is one of the most widely consumed natural products in the form of tea, particularly green tea. In addition, there is a vast reservoir of literature attributing many beneficial biological effects to this natural compound, particularly its anti-cancer effects. However, EGCG has also been reported to have anti-inflammatory properties and DCs play a central role in inflammatory and immune responses. The **hypothesis** to be tested is that EGCG exerts its anti-inflammatory effect in part by suppressing the activation and maturation of DCs.

Aim 1: Determine the effects of EGCG treatment on costimulatory and MHC molecule expression in response to microbial stimulation.

Various phenotypic changes occur upon maturation of DCs. Among changes which occur are upregulation of costimulatory molecule expression, particularly CD80 (B7-1) and CD86 (B7-2). DCs also upregulate MHC class I/II molecule expression upon maturation. Whereas immature DCs express chemokine receptors 1-6, mature DCs express CCR7-8 and CXCR4. These phenotypic changes or the lack thereof have been implicated in the type of immune response which DCs direct. For example, antibodies

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against CD80 reportedly inhibit Th1 responses whereas antibodies against CD86 reportedly inhibit Th2 responses (157). Low levels of CD80 and CD86 on DCs are also known to lead to T cell anergy because CTLA-4 reportedly has a higher affinity for low expression of CD40 and CD86 compared to CD28 (119). In this aim, we will measure co-stimulatory and MHC surface molecule expression on mouse bone marrow-derived DCs by flow cytometry following microbial stimulation (i.e., LPS treatment and Lp infection) with or without EGCG treatment.

Aim 2: Determine the effects of EGCG on DC cytokine and chemokine production in reponse to microbial stimulation.

Various functional changes also occur upon maturation of DCs. Among these are shifts in endocytic and or phagocytic ability from one of high capacity to one of low capacity. Other changes associated with DC maturation are cytokine and chemokine production important in determining what type of immune response DCs will direct. For example, DC production of IL-12 drives differentiation of CD4 T cells to Th1 effector cells, while IL-4 production drives naïve T cells to become Th2 effectors. Among chemokines reported as important for a Th1 response are CX3CL1 (fractalkine), CXCL10 (IP10) and MIP-1α. Chemokines implicated as being important for a Th2 response are CCL17 (TARC) and CCL22 (MDC). In particular, MIP-1α reportedly induces Th0 cells to differentiate into Th1 effectors whereas MCP-1 induces Th0 cells into Th2 effects (73). In this aim, we will examine effects of microbial stimulation (e.g., LPS, Lp) either with or without EGCG treatment on cytokine (IL-12, TNFα) and inflammatory chemokine (MIP-1α, MCP-1, RANTES) production by DCs using ELISA technology.

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Aim 3: Determine the molecular signaling mechanisms involved in effects of EGCG on DC maturation.

TLRs are an evolutionary conserved family of cell surface proteins that recognize PAMPs. These PAMPs can include such microbial products as LPS from Gram-negative bacteria as well as teichoic acid from Gram-positive bacteria. Once engaged, TLRs interact with a host of signaling proteins which culminates in activation of different sets of genes including cytokine and co-stimulatory marker genes. In this respect, the TLR molecular signaling pathway is crucial to the ability of DCs to direct an immune response. A major transcription factor induced by TLRs is NFkB; the activation of this factor has also been shown to be modulatated by EGCG. Therefore, in this aim we will examine the modulation of TLRs and NKkB in microbial stimulated and EGCG-treated cells. In particular, we will stimulate DCs with Lp or LPS and study TLR expression by flow cytommetry. We will use ELISA to determine NFκB protein levels following stimulation and treatment with EGCG.

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MATERIAL AND METHODS

Catechins and Stimulants

EGCG was obtained from Sigma Chemical Co. (St. Louis, MO) and stored as 5 mg/ml stock solutions. LPS from *E. coli* was also obtained from Sigma. The vehicle for all solutions was sterile pyrogen-free water.

Animals

BALB/c mice from NCI (Frederic, MD) were utilized. They were 8-10 weeks of age at the start of an experiment and kept in groups of 4 in plastic mouse cages with barrier filters and fed Purina mouse chow and water *ad libitum*. They were housed and cared for in the University of South Florida animal facility, which is fully accredited by the American Association of Laboratory Animal Care.

Preparation of DCs

DCs were prepared as described previously (63) with several modifications. Briefly, bone marrow cells were flushed from the femurs and tibias of the mice and the red cells lysed with ACK lysing buffer to deplete red blood cells. Pooled BM cells were plated in six-well culture plates (10^6 cells/ml; 3 ml/well) and cultured overnight in RPMI 1640 medium (Sigma, Saint Louis, Mo) supplemented with 10 % heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1% 2-mercaptoethanol, 1% antibiotic/ antimycotic solution (Sigma), and 10 ng/ml recombinant GM-CSF (BD Pharmingen, San Diego, CA). Non-adherent cells were removed and the adherent cells were incubated with fresh GM-CSF-containing medium for an additional 7-9 days, during which time the BMDCs became non-adherent and were harvested. The cells were typically about 97 % positive for CD11b and 60-70 % positive for CD11c, as measured by flow-cytometry analysis.

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Bacteria

A virulent strain of Lp (M124), serogroup 1, was obtained from a case of Legionellosis from Tampa General Hospital (Tampa, FL) and was grown on buffered charcoal-yeast extract agar (BCYE, Difco, Detroit, MI) for 48 hr. The bacterial suspensions were prepared in pyrogen-free saline, and the concentration of bacteria determined by spectrophotometry.

Infection

DCs were infected with Lp at a ratio of 10 bacteria per cell for 30 min., washed to remove non-phagocytized bacteria and incubated in RPMI 1640 medium containing 10 % FCS with no antibiotics. In certain experiments, DCs were infected with Lp at a ratio of 20 bacteria per cell for 40 min., washed to remove non-phagocytized bacteria and incubated in RPMI 1640 medium containing 10 % bovine calf serum with no antibiotics. The cultures were then incubated for 48 hr at 37°C under 5 % CO_2 humidified atmosphere.

Treatment

BMDCs, either infected or non-infected, were added at a concentration of 2 x 10^5 cells/ml to 24-well plastic plates for bioplex cytokine analysis or 1 x 10^6 cells/ml to polypropylene tubes for flow cytommetry analysis and various concentrations of EGCG (0, 10, 50 µg/ml) were then added to each well. For ELISA, DCs, either infected or non-infected, were added at a concentration of 2 x 10^5 cells/ml to 24-well plastic plates (CoStar, Cambridge, MA) and various concentrations of EGCG (0, 10, 50 and 100 µg) were then added to each well. For DNA binding assays, DCs were added, at a concentration of 2x10^5 cells/ml (total volume of 5 ml for LPS stimulation), or at a

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concentration of $1x10^6$ cells/ml (total volume of 1 ml for Lp infection), to polypropylene tubes with 50 μg/ml of EGCG. For stimulation of non-infected cells, *E. coli* LPS (10 ng/ml or 100 ng/ml or1 μg/ml) was added to each well/tube with the various concentrations of EGCG. In some experiments, DC cultures treated with LPS or infected with bacteria and treated or not either EGCG were incubated with purified rat anti-mouse/rate TNFα monoclonal antibody (Cat No. 554640, Pharmingen, San Diego, Calif.).

Cell Viability

The XTT assay was used to assess the effects of EGCG on cell viability (In Vitro Toxicology Assay Kit XTT Based, TOX-2, Sigma, Saint Louis, MO). This assay is based on the ability of mitochondrial dehydrogenases of viable cells to cleave the tetrazolium ring of XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt) yielding orange formazan crystals which are soluble in aqueous solutions. DCs were harvested as outlined above and dispensed in triplicates at a density of $1x10^6$ cells/ml into a 96-well flat bottom tissue culture plate. Plates were incubated with EGCG at various concentrations (0, 10, 50, and 100 μg/ml) in 5% CO_2 at 37^0C for 24h. Because EGCG produced an orange color at higher doses, the culture medium was replaced on day 2 with fresh culture medium (200 μl) before adding 20 μl XTT (20% of the medium volume) and incubated at 37^0C for another 4h. The plates were read on an Emax microphage reader (Molecular Devices, Menlo Park, CA), using a wavelength of 450 nm and a reference wavelength of 650 nm. Control wells contained cells alone. Cell survival was calculated as a percentage of MTT inhibition by the following formula: survival (%) = (mean experimental absorbance/mean control absorbance) X 100%.

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Flow Cytometry

DCs were harvested as outlined above and analyzed for expression of various cell surface molecules by tri-color immunofluorescent staining with fluorescein isothiocyanate (FITC)-conjugated rat anti-major histocompatibility complex (MHC) class II (I-A^b) and class I (H-2^K), phycoerythrin (PE)-conjugated rat anti-CD86, CD40 and CD80 and allophycocyanin (APC)-conjugated rat anti-CD11c (all from PharMingen, San Diego, CA), as well as FITC anti-mouse-TLR2 and PE anti-mouse-TLR-4 (all from eBioscience, San Diego, CA). Cells in PBS containing 2% heat-inactivated bovine growth serum were blocked with anti-FCR antibody (CD16/ CD32) for 15 min. Staining was performed for 30 min on ice with the various conjugated antibodies. Cells were fixed with 1% paraformaldehyde and the fluorescent-labeled cells were analyzed by flow cytommetry (Becton Dickinson, Mountain View, CA). The instrument is equipped with lasers tuned to 488 nm and to 635 nm. In all analyses, dead cells were gated out and cells of the phagocytic lineage were identified by forward and orthogonal light-scattering signals.

ELISA

The amount of IL-12 p40/p70 and TNFα in the culture supernatants of DC cultures, 24 hours after treatment, was determined by sandwich ELISA using matched antibody pairs and protein standard for ELISA (BD Pharmagen) for IL-12 and Duoset® ELISA development system (R&D Systems, Minneapolis, MN) for TNFα. For this purpose, medium-bind, 96-well Costar enzyme immunoassay (EIA) plates were coated with specific monoclonal anti-cytokine antibody for IL-12 p40/p70 or TNFα overnight at 4°C. Plates were blocked for 1 h at 37°C with PBS plus 3% BSA (IL-12 p40/p70) or 1%

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lipid free BSA (TNFα) and 0.05% Tween 20. Culture supernatants or serial dilutions of murine cytokine standard were added for 1 h, followed by biotinylated anti-murine IL-12 p40/p70 or TNFα, and then followed by streptavidin-alkaline phosphatase (1:1,000; BD Pharmagen) for 30 min. After the substrate was added, plates were allowed to develop. The plates were washed between additions with three to five changes of nanopure water. The plates were read at 450 nm on an Emax microphage reader (Molecular Devices, Menlo Park, CA). Units were calculated form the cytokine standard curve, which was performed for each plate.

The amount of MCP-1, CCL5/RANTES and CCL3/MIP-1α in the culture supernatants of DC cultures, 24 hours after treatment, was determined by sandwich enzyme-linked immunosorbent assay ELISA using matched antibody pairs and protein standard for ELISA (BD Pharmagen) for MCP-1 and Duoset® ELISA development system (R&D Systems, Minneapolis, MN) for RANTES and MIP-1α. For this purpose, medium-bind, 96-well Costar enzyme immunoassay (EIA) plates were coated with specific monoclonal anti-cytokine antibody for MCP-1, RANTES or MIP-1α overnight at 4°C for MCP-1 and at room temperature for RANTES and MIP-1α. Plates were blocked for 1 h at 37°C with PBS plus 0.5% BSA (MCP-1) or 1% BSA (RANTES & MIP-1α) and 0.05% Tween 20 in the case of MCP-1. Culture supernatants or serial dilutions of murine cytokine standard were added for 1 h, followed by biotinylated anti-murine MCP-1, RANTES or MIP-1α, and then followed by streptavidin-alkaline phosphatase (1:200; R&D Systems) for 30 min. After the substrate was added, plates were allowed to develop. The plates were washed between additions with three to five changes of nanopure water. The plates were read at 450 nm on an Emax microphage reader

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(Molecular Devices, Menlo Park, CA). Units were calculated form the cytokine standard curve, which was performed for each plate.

Bioplex Cytokine Assay

Briefly, 50 µl of the culture supernatant or cytokine standard was plated in a 96 well filter plate coated with a multiplex of beads coupled to antibodies against the above mentioned cytokines and incubated for 30 min on a platform shaker at 300 rpm at RT. After a series of washes to remove the unbound proteins, a mixture of biotinylated detection antibodies, each specific for a different epitopes, was added to the reaction resulting in the formation of antibodies assimilated around the target proteins. Streptavidin-phycoerythrin (streptavidin-PE) was then added to bind to the biotinylated detection antibodies on the bead surface. The data from the reaction were then collected and analyzed by using the Bio-Plex suspension array system (or Luminex 100 system) from Bio-Rad Laboratories (Hercules, CA).

P65/RelA Dna-binding activity

DNA-binding activity of the p65/RelA subunit of NFκB was determined using Trans Am™ NFκB colorimetric kit (Active Motif®). An equal amount of cellular extracts was added to incubation wells precoated with the DNA-binding consensus sequence. The presence of translocated p65/RelA subunit was then assessed by using the Trans Am™ kit according to manufacturer instructions. Plates were read at 450 nm, and results were expressed as OD.

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Statistics

The results were expressed as means ± SD of indicated number of experiments. Statistical significance was determined using Student's t test for unpaired observations. A value of $p < 0.05$ was considered significant.

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RESULTS

Aim 1: Determine the effects of EGCG treatment on co-stimulatory and MHC molecule expression in response to microbial stimulation.

Lp Infection Induces CD11c, Co-stimulatory Molecule and MHC Surface Molecule Expression

To characterize effects of EGCG on phenotypic maturation of BMDCs after infection with Lp, we investigated the expression of maturation markers MHC class I and II, CD40, CD86 and CD80 on gated populations of DCs from BALB/c mice. For this purpose, donor cells were differentiated into DCs with GM-CSF. DCs were greater than 97% positive for the myeloid cell-surface antigen, CD11b, and typically between 60-70% positive for CD11c as determined by flow cytometry (Figure 6). On days 7-8 of culture, DCs were infected with Lp at an MOI of 10 for 30 minutes and various concentrations of EGCG (10, 30 and 50 μg/ml) were added to either the Lp infected or non-infected groups.

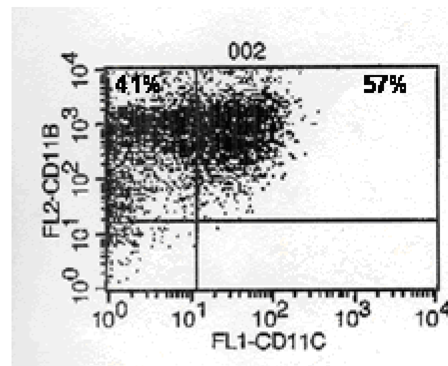

Figure 6. Flow cytometric dot plot of CD11b and CD11c surface molecule expression by DCs.

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DCs infected with Lp in the absence of EGCG were activated, as indicated by a increase in percentage of cells expressing both CD11c and the co-stimulatory molecules CD40 (71% versus 13%), and CD86 (68% versus 20%), indicating maturation of DCs (Figure 7).

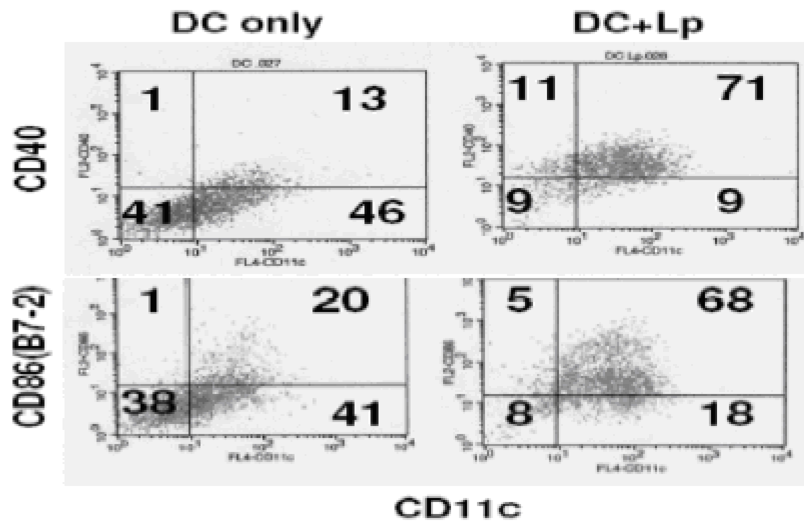

Figure 7. Lp infection up-regulates CD40 and CD86 expression by DCs. Flow cytometric dot plots of CD11c and co-stimulatory molecule expression. Numbers in quadrants reflect percentages rounded to next greater whole integer. Results are 1 of 5 independent experiments with similar results.

Lp was also a potent inducer of both MHC class I and class II surface molecule expression. Cells which were double positive for MHC and CD11c increased from 14% to 32% for MHCII and from 48% to 80% for MHCI (Figure 8).

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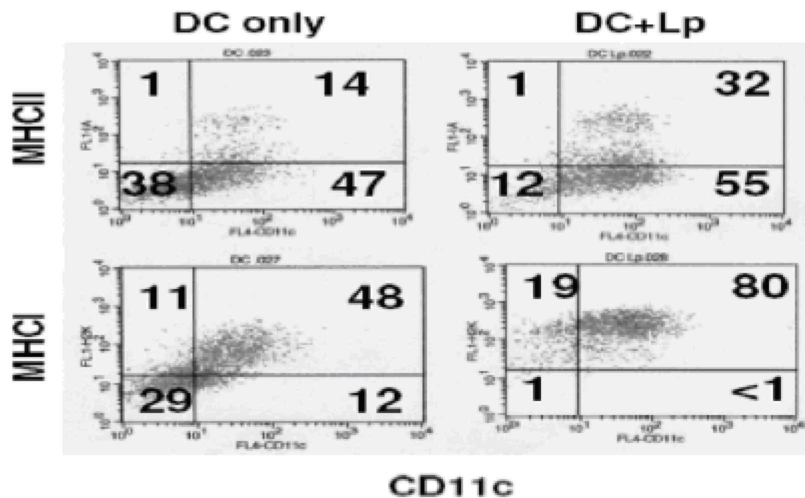

Figure 8. Lp infection up-regulates MHC class I/II epxression by DCs. Flow cytometric dot plots of CD11c and MHC I/II surface molecule expression. Numbers in quadrants reflect percentages rounded to next greater whole integer. Results are 1 of 5 independent experiments with similar results.

EGCG Inhibits CD11c, Co-stimulatory Molecule and MHC Surface Molecule Expression Induced by Lp Infection

Incubation of DCs with various concentrations of EGCG (10, 30 and 50 μg/ml) reduced in a dose dependent manner the upregulating effect of Lp on the percentage of cells expressing MHC I and II molecules (Figure 9).

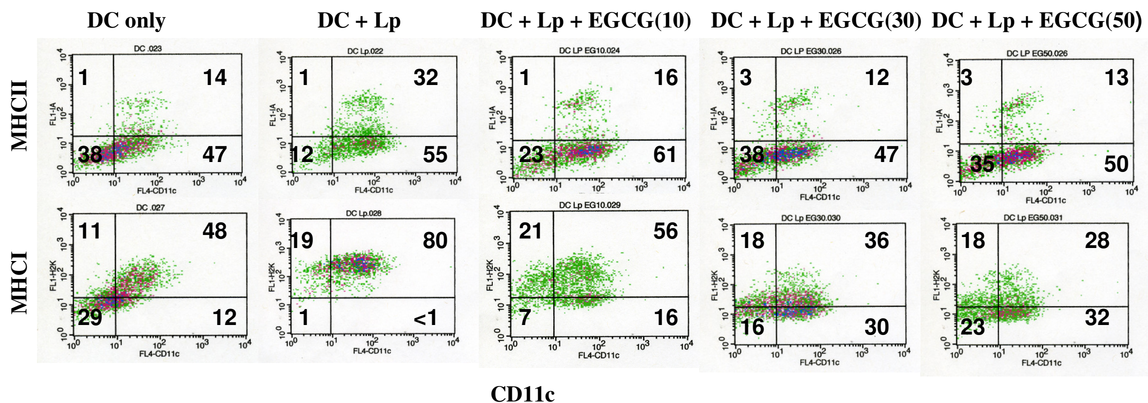

Figure 9. EGCG inhibits Lp upregulation of MHC surface molecule expression by DCs infected with Lp and treated with various concentration of EGCG and analyzed by flow cytommetry. Flow cytometric dot plots of CD11c and MHC surface molecule expression. Number in quadrants reflect percentages rounded to next greater whole integer. Results shown are 1 of 5 independent experiments with similar results.

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In a similar manner, incubation of DCs with various concentrations of EGCG (10, 30 and 50 µg/ml) reduced the upregulating effect on co-stimulatory molecules CD40 and CD86 (Figure 10).

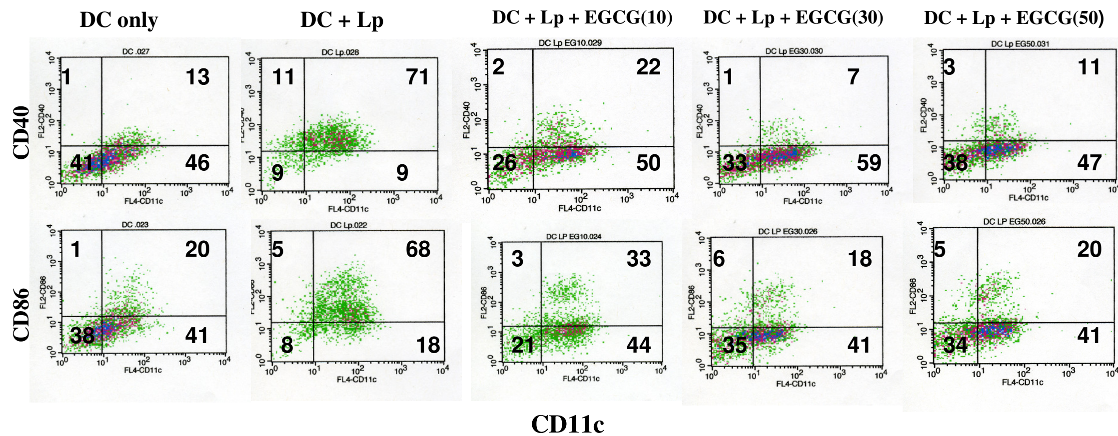

Figure 10. EGCG inhibits Lp upregulation of co-stimulatory molecule CD40 and CD86 expression by DCs infected with Lp and treated with various concentrations of EGCG and analyzed by flow cytometry. Flow cytometric dot plots of CD11c and co-stimulatory surface molecule expression. Numbers in quadrants reflect percentages rounded to next greater whole interger. Results shown are 1 of 5 independent experiments with similar results.

EGCG µg/ml	Percentage of $CD11^+$cells			
	MHCII	MHCI	CD40	CD86
DC only	10 ±3	47 ±6	9 ±5	17 ±7
DC + Lp	28 ±12	68 ±15	20 ±3	52 ±19
EGCG10	13* ±4	52* ±10	18 ±8	30* ±11
EGCG30	8* ±3	44* ±12	8* ±3	17* ±6
ECGC50	12* ±1	42* ±14	11* ±4	23* ±12

Table 1: MHC I/II and Costimulatory molecule CD40, C86 surface molecule expression by DCs infected with Lp (10:1) and treated with various concentrations of EGCG and analyzed by flow cytometry. Results expressed as mean ±SEM from 5 independent experiments. The asterisks indicate statistically significant differences of P<.05 from values of Lp infected cells.

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As shown in Table 1, the standard error mean (SEM) from 5 independent experiments was significantly lower for EGCG groups then values of Lp infection alone for each of the key maturation markers MHC I/II, CD40 and CD86**.**

LPS Induces CD11c, Co-stimulatory Molecules and MHC Surface Molecules that are inhibited by EGCG Treatment

Microbial products such as LPS can also activate immature DCs and induce DC maturation, characterized by up-regulation of co-stimulatory molecules and increased ability to activate T cells (12). EGCG treatment suppressed LPS-induced MHC and co-stimulatory molecule DC surface expression similar to the effect followint Lp treatment. In particular, LPS increased the percentage of DCs double positive for CD11c and CD40/CD86 molecule surface expression whereas in the presence of 50 µg /ml EGCG surface expression was not increased following LPS treatment (Figure 11-12).

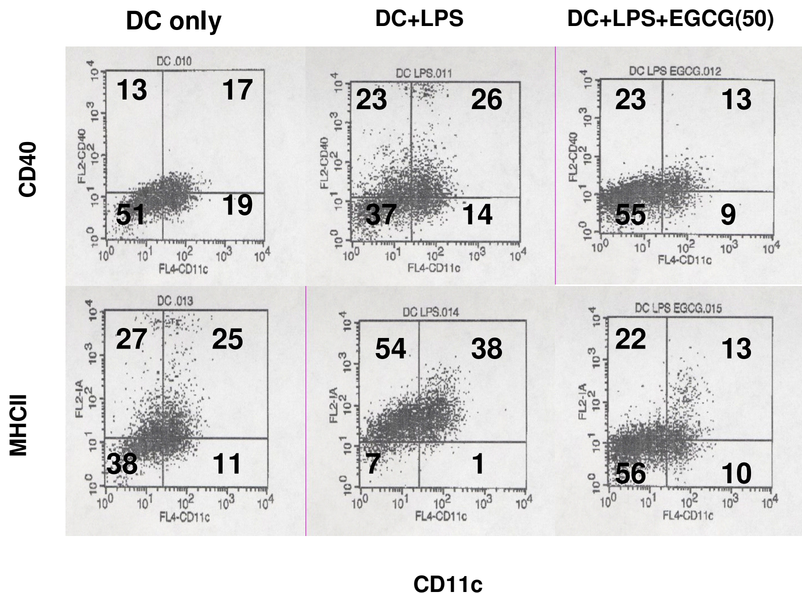

Figure 11. EGCG inhibits CD40 and MHCII surface molecule expression by DCs stimulated with LPS and treated with 50 µg of EGCG and analyzed by flow cytometry. Numbers in quadrants reflect percentages rounded up to next greater whole integer. Results shown are from 4 independent experiments with similar results.

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Similarly, EGCG inhibited the percentage of cells double positive for CD11c and MHCI and II surface molecule expression by DCs induced by LPS (Figure 12**).**

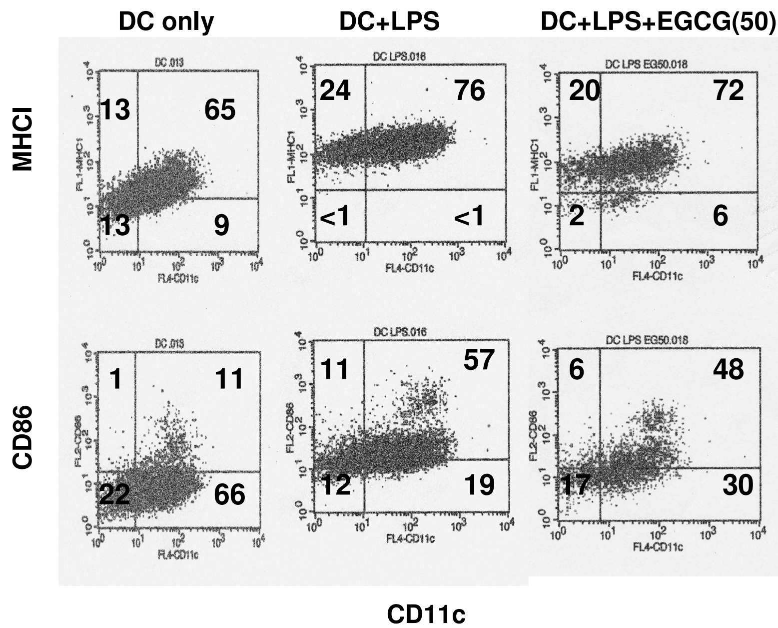

Figure 12. EGCG inhibits MHCI and CD86 surface molecule expression by DCs stimulated with LPS and treated with 50 µg of EGCG and analyzed by flow cytometry. Numbers in quadrants reflect percentages rounded up to next greater whole integer. Results shown are from 4 independent experiments with similar results.

EGCG treatment of DCs alone does not affect CD11c, costimulatory molecule or MHC surface expression.

To determine if the inhibitory effect of EGCG observed above on MHC and co-stimulatory molecule expression was one of drug toxicity rather than an inhibition of the microbial stimulation response, we tested the effect of EGCG, without microbial

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stimulation, on the DC response. As is shown in (Figure 13) and in (Figure 14), EGCG had virtually no effect on surface marker expression of either MHC or co-stimulatory molecules. Thus, the effects of EGCG appeared to involve the EGCG prevention of microbial-induced upregulation of these maturation markers as opposed to a toxic effect of EGCG on the cell.

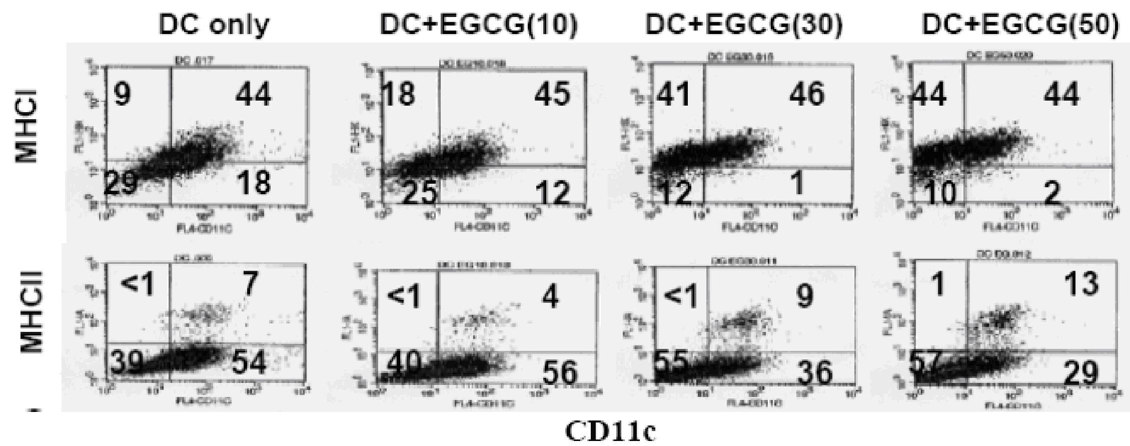

Figure 13. Effects of EGCG on MHC class I/II molcule expression by DCs as analyzed by flow cytometry. Numbers reflect percentages rounded to next greater whole integer. Results shown are 1 of 3 independent experiments with similar results.

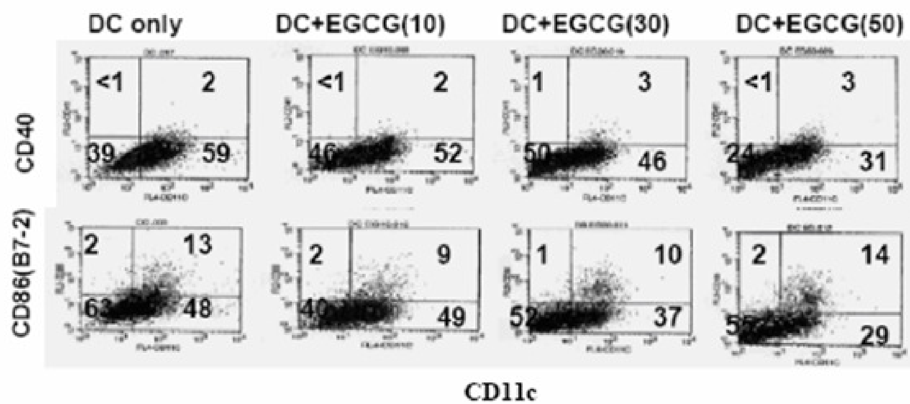

Figure 14. Effects of EGCG on co-stimulatory molecule expression by BMDCs as analyzed by flow cytometry. Numbers in quadrants reflect percentages rounded to next greater whole integer. Results shown are 1 of 3 independent experiments with similar results.

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Inhibitory Effects not Due to Cytotoxity of EGCG

As a direct test of drug toxicity, cells were treated with varying concentrations of EGCG and viability measured by an XTT assay (Figure 15). The results show that EGCG did not reduce vaiblity at 50 µg/ml and only slightly reduced it at 100 µg/ml. Moreover, no measurable effect on DC viability occured over a period of 48 hr following infection with Lp (data not shown).

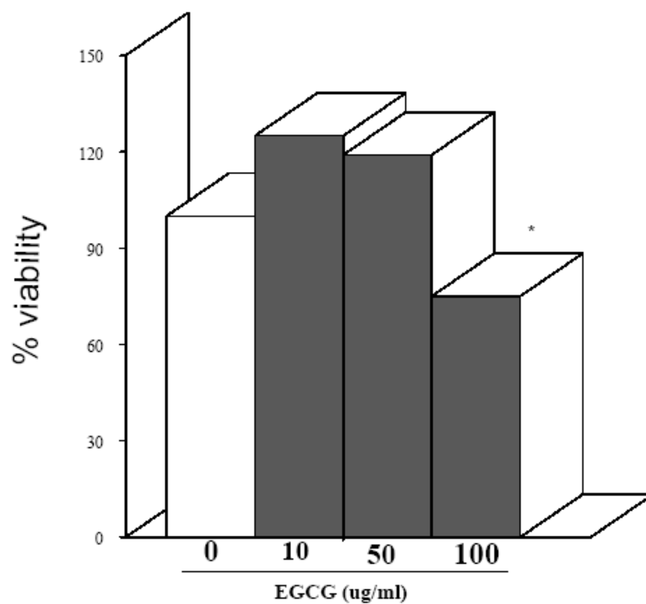

Figure 15. BM derived DCs were exposed to various concentrations (0, 50, 100 µg/ml) of EGCG for 24 h. Cell viability was analyzed with XTT assay. Percent (%) viability was determined by measuring the OD at 450 nm and a reference wavelength of 650 nm in a microplate reader. The results are expressed as an average of 3 independent experiments performed in triplicate. The asterisks indicate statistically significant differences of P<0.05 from values obtained with non-EGCG treated DCs.

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EGCG treated DCs Exhibit the Morphology of Immature DCs

In all cultures, cells infected with Lp or stimulated with LPS and which had the greatest co-stimulatory/ MHC/ CD11c molecule surface expression tended to be larger and more granular, indicative of a more mature DC phenotype. Conversely, EGCG treated infected/stimulated cells, which showed suppression of co-stimulatory/MHC/ CD11c molecule surface expression, tended to be smaller and less granular, indicative of a less mature DC phenotype comparable to the non-infected/ EGCG treated control group as shown by flow cytommetry (Data not shown).

Aim 2: Determine effects of EGCG on DC cytokine and chemokine production in reponse to microbial stimulation.

EGCG Up-regulates TNFα Production by DCs Stimulated with LPS, MDP or Infected with Lp.

Murine derived DCs stimulated with LPS (10 ng/ml) produced detectable levels of TNFα in the culture supernatants 24 hr after stimulation. The DC cultures treated with increasing amounts of EGCG showed marked enhancement, after 24 hours, of TNFα when treated with a concentration of 50 μg/ml (13). In contrast, a higher concentration (100 μg/ml) markedly inhibited TNFα production in the LPS stimulated cultures after 24 hours (Figure 16).

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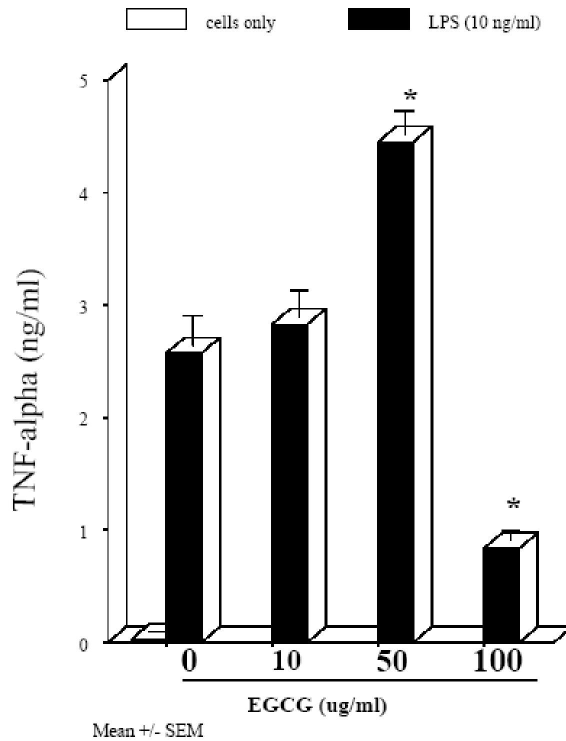

Figure 16. Effects of increasing concentrations of EGCG on TNFα production in cultures of BM derived dendritic cells stimulated with LPS. Results expressed as mean value in ng/ml ± SEM from 5 independent experiments. The asterisks indicate statistically significant differences of $P<0.05$ from values of the non-EGCG treated LPS stimulated cells.

The effects of EGCG were examined further to determine effects on responses to other microbial stimulators. For this purpose, DC cultures were treated with MDP (10 µg/ml) and the results showed DCs stimulated with MDP and treated with the 50 µg/ml concentration of EGCG had approximately a 3 fold increase in TNFα production. Furthermore, a 100 µg/ml concentration also resulted in a significant increase, but less than that induced by the lower concentration (Figure 17).

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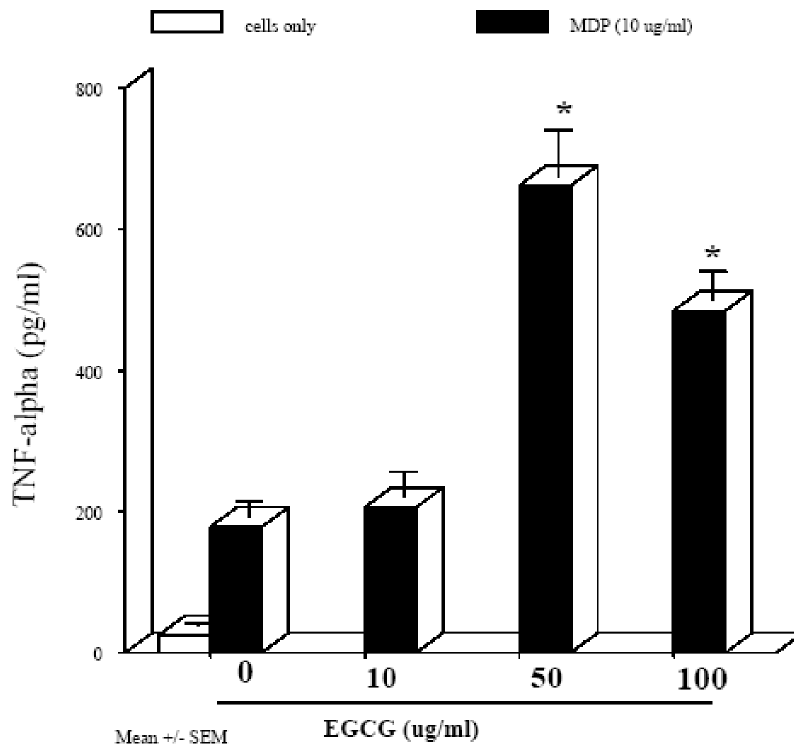

Figure 17. Effects of increasing concentrations of EGCG on TNFα production in cultures of BM derived dendritic cells stimulated with MDP. Results expressed as mean value in pg/ml ± SEM from 5 independent experiments. The asterisks indicate statistically significant differences of P<0.05 from values from non-ECGG treated MDP-stimulated cells.

Next, we examined the effect of EGCG on cytokine production by DCs after infection with Lp. The effects of EGCG on the pattern of production of TNFα in DCs infected with Lp was similar to that observed following stimulation with LPS or MDP. In particular, the 50 µg/ml EGCG concentration enhanced production of TNFα to approximately 2.5 ng/ml, a level several fold higher than observed in Lp infected DCs treated with 100 µg/ml of EGCG (Figure 18).

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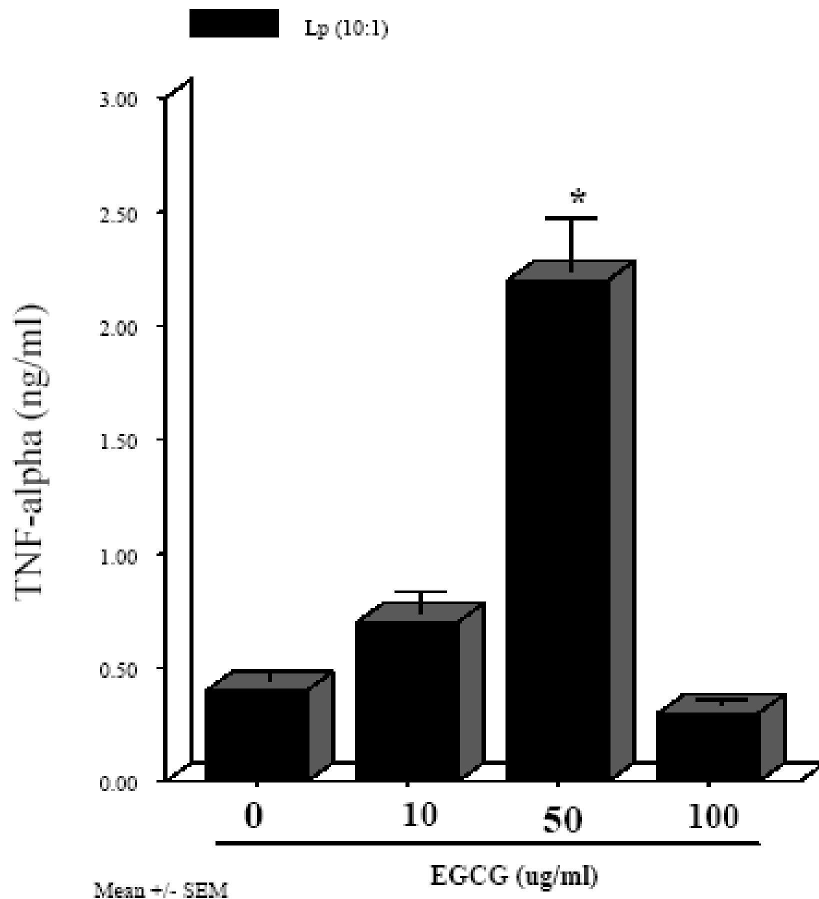

Figure 18. Effects of EGCG on TNFα production by dendritic cells infected 24 hr with Lp. TNFα levels in culture supernatants determined by ELISA and results expressed as mean value in ng/ml ± SEM from 3 independent experiments. The asterisk indicates statistically significant differences ($p<0.05$) from values obtained with non-EGCG treated Lp infected DCs.

EGCG inhibits IL-12 production by DCs stimulated with MDP or LPS or infected with Lp.

EGCG also had marked effects on production of IL-12 p40/p70 in the stimulated DC cultures. LPS treated cells without EGCG evinced marked production of this cytokine after 24 hours. However, addition of EGCG to the cultures inhibited IL-12 p40/p70. The 10 µg/ml concentration of EGCG had a slight inhibitory effect. Moreover,

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the 50 μg/ml and 100 μg/ml concentrations markedly depressed IL-12 p40/p70 production (Figure 19).

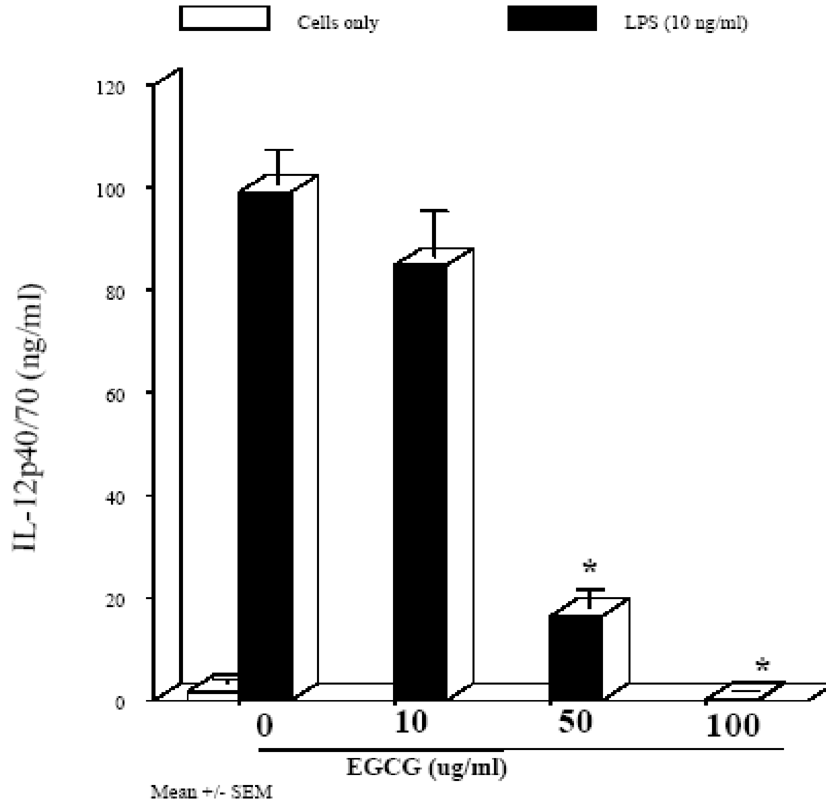

Figure 19. Effects of ECGG on IL-12 p40/p70 production by BM derived dendritic cells stimulated by LPS. Results expressed as mean value in ng/ml ± SEM from 5 independent experiments 24 hrs after stimulation of cells. The asterisk indicates statistically significant differences ($p<0.05$) from values obtained with non- treated EGCG LPS-stimulated cells.

Similar suppressive effects were observed by EGCG treatment of MDP stimulated DCs. The 10 μg/ml concentration reduced by 50% IL-12 production, while the 50 and 100 μg/ml concentrations essentially abolished the response (Figure 20).

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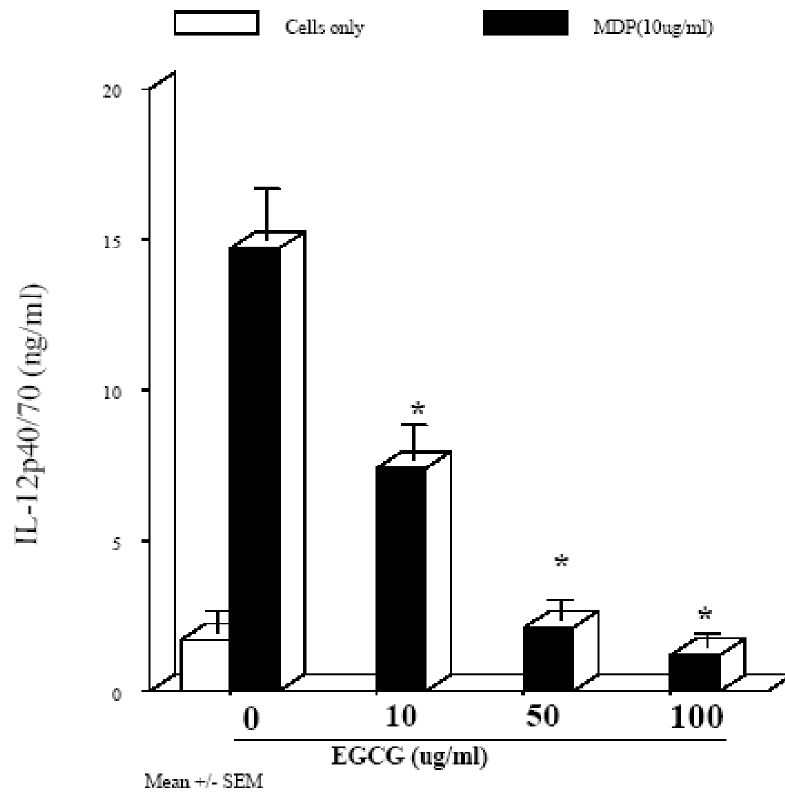

Figure 20. Effects of increasing concentrations of EGCG on IL-12 p40/p70 production in cultures of BM-derived dendritic cells stimulated with MDP. Results expressed as mean value in ng/ml ± SEM from 5 independent experiments. The asterisks indicate statistically significant differences ($p<0.05$) from the values of the non-EGCG treated MDP-stimulated cells.

Similarly, DCs infected with Lp and treated with EGCG showed a marked reduction (50 µg/ml) or essentially abolished (100 µg/ml) the response (Figure 21).

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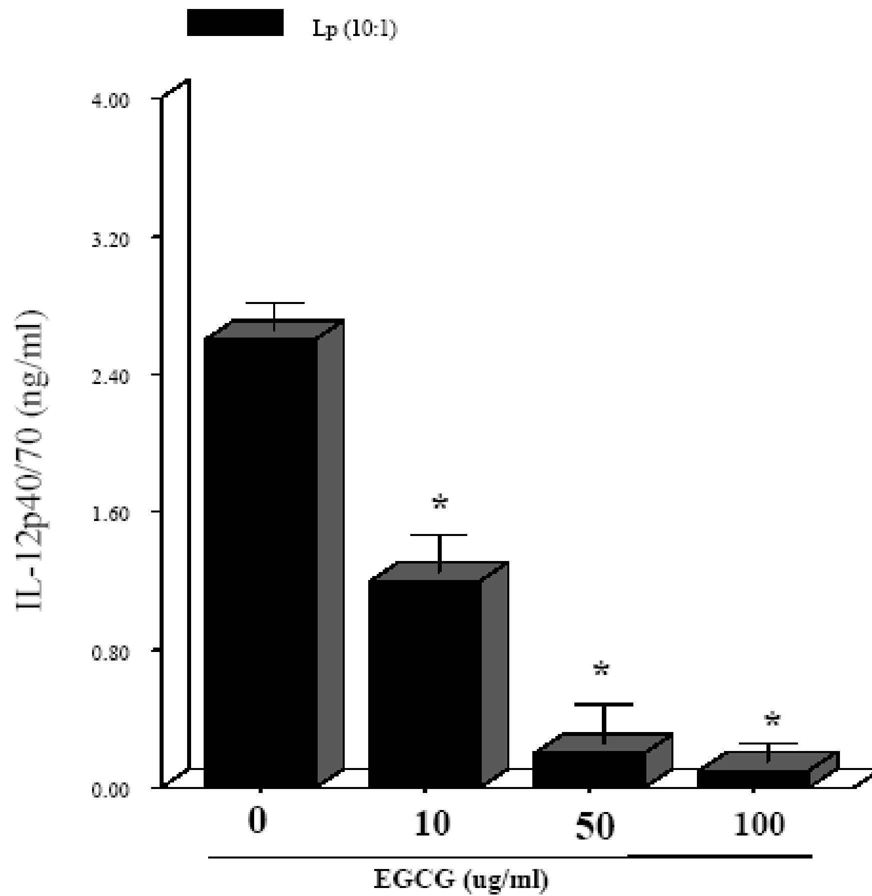

Figure 21. Effects of EGCG on IL-12 p40/p70 production by dendritic cells infected 24 hr with Lp. Results expressed as mean value in ng/ml ± SEM from 3 independent experiments. The asterisks indicate statistically significant differences of P<0.05 from values obtained with non-EGCG treated Lp infected DCs.

As shown previously in cell viability studies (see Figure 15), treatment of DCs with EGCG at 10 and 50 μg/ml did not decrease cell viability, which indicates that increased TNFα and decreased IL-12 production levels were not due to EGCG toxicity at these concentration levels. However, a significant (p<.05) decrease in cell viability (75% of control) was observed when DCs were treated with the higher concentration of 100 μg/ml which may explain why TNFα production levels did not continue to increase at 100

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μg/ml. This suggests that some of the decrease of IL-12 production at 100 μg/ml may be due to cytotoxic effects of EGCG on the DCs.

Inhibition of IL-12 by EGCG does not depend on TNFα

To determine whether inhibition of EGCG inhibited IL-12 production depended on induced TNFα production, DCs were stimulated with LPS either alone or in the presence of neutralizing antibody to TNFα and production of IL-12 was determined. As shown in Figure 22**,** TNFα production by LPS stimulated DCs was decreased about 3 fold with neutralization antibody.

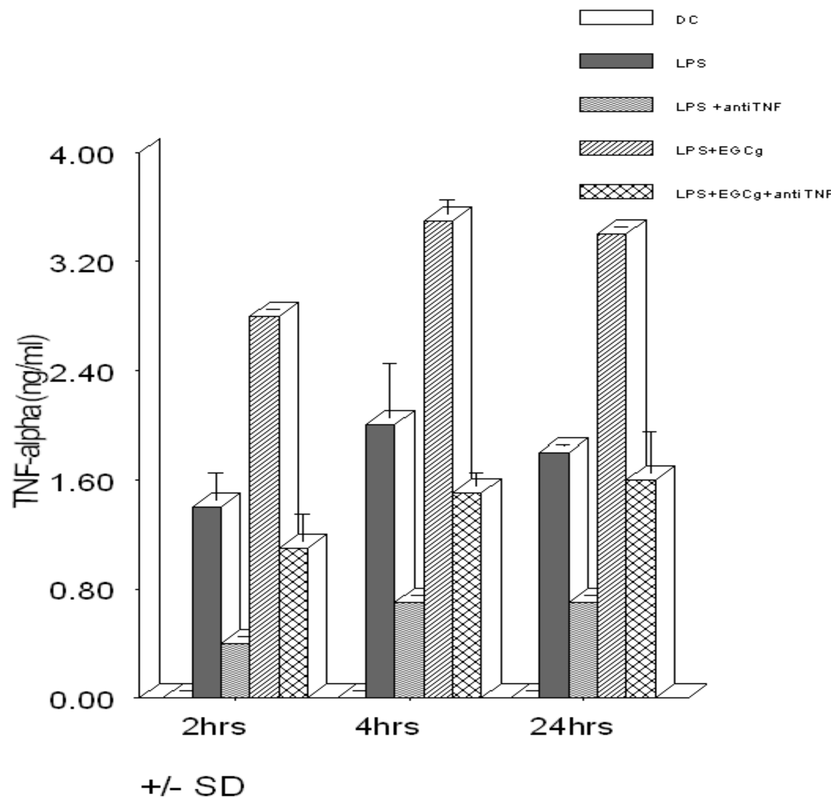

Figure 22. Effects of EGCG (50 μg/ml) on TNFα production in cultures of DCs stimulated with LPS (10 ng/ml) with or without anti- TNFα neutralization antibody (20 μg/ml).

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However, as shown in Figure 23, anti-TNFα had no effect on IL-12 production by DCs at 2 and 4 hours and minimallt decreased the effect at 24 hours in contrast to EGCG treatment which markedly diminished LPS induced IL-12 production.

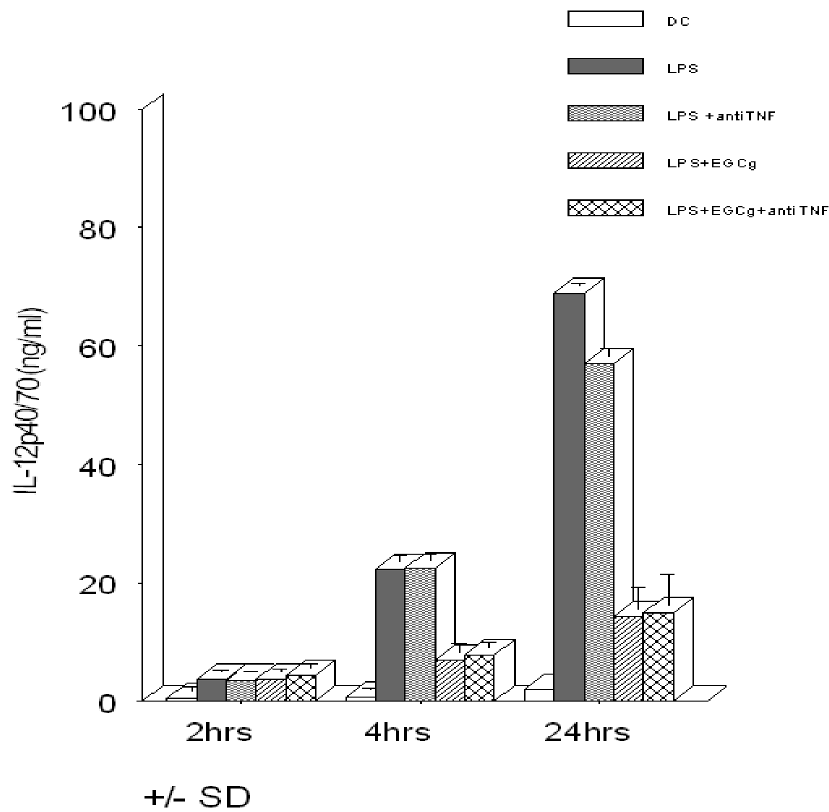

Figure 23. Effects of EGCG (50 µg/ml) on IL12 production in cultures of DCs stimulated with LPS (10 ng/ml) with or without anti- TNFα neutralization antibody (20 µg/ml).

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EGCG inhibits RANTES, MCP-1 and MIP1-α production by DC stimulated with LPS.

DC maturation is often accompanied by production of chemokines that assist DCs in attracting T cells for efficient antigen presentation (108). EGCG inhibited LPS-induced RANTES (Figure 24), MCP-1 (Figure 25), and MIP1-α (Figure 26). For the most part, significant differences were observed only at the 50 µg/ml concentration.

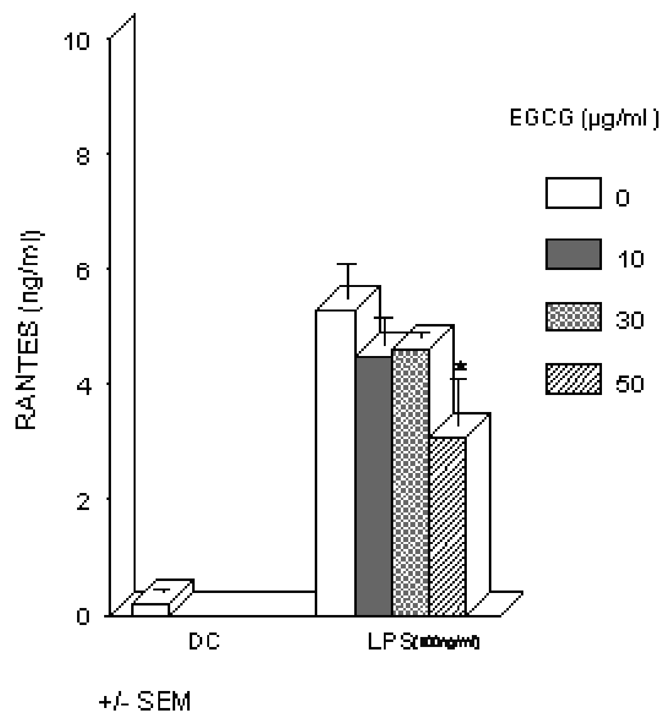

Figure 24. Effects of EGCG on RANTES production by DCs stimulated by LPS (100 ng/ml). Results are expressed as mean value in ng/ml ± SEM from 3 independent experiments 24 hours after stimulation of cells. The asterisk indicates statistically significant differences of P<0.05 from values obtained non-treated EGCG LPS-stimulated cells.

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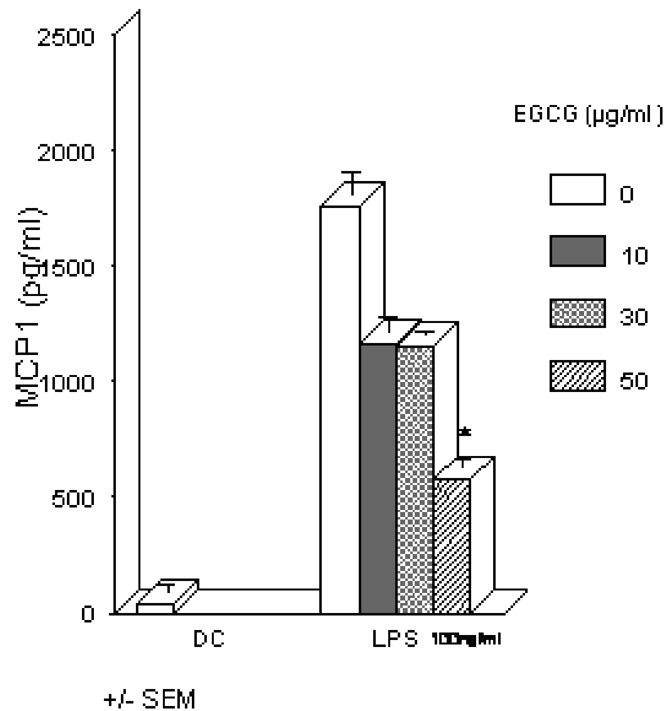

Figure 25. Effects of EGCG on MCP-1 production by DCs stimulated by LPS (100 ng/ml). Results are expressed as mean value in pg/ml ± SEM from 3 independent experiments 24 hours after stimulation of cells. The asterisk indicates statistically significant differences ($p<0.05$) from values obtained in non-treated EGCG, LPS-stimulated cells.

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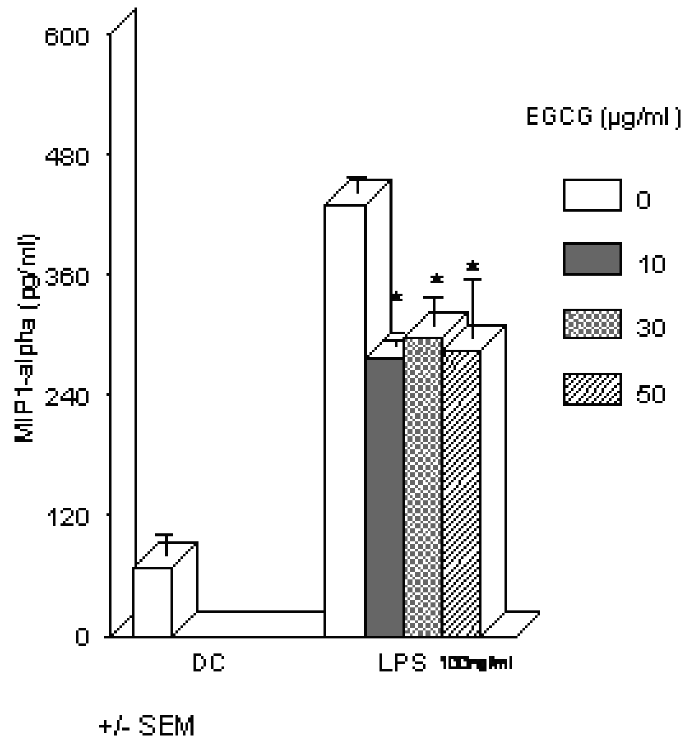

Figure 26. Effects of EGCG on MIP1-α production by DCs stimulated by LPS (100 ng/ml). Results are expressed as mean value in pg/ml ± SEM from 3 independent experiments 24 hours after stimulation of cells. The asterisk indicates statistically significant differences ($p<0.05$) from values obtained in non-treated EGCG, LPS-stimulated cells.

EGCG inhibits RANTES, MCP1 and MIP1α production by DCs infected with Lp.

EGCG also attenuated Lp-induced RANTES (Figure 27), MCP1 (Figure 28) and MIP1α (Figure 29) chemokine production, which was significant at higher doses of EGCG.

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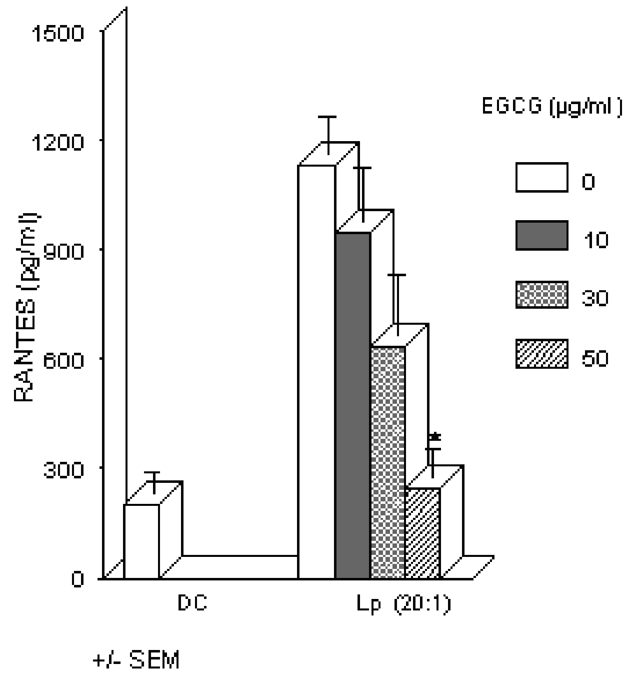

Figure 27. Effects of EGCG on RANTES production by DCs after infection by Lp (20:1). Results are expressed as mean value in pg/ml ± SEM from 3 independent experiments 24 hours after stimulation of cells. The asterisk indicates statistically significant differences ($p<0.05$) from values obtained in non-treated EGCG, Lp infected cells.

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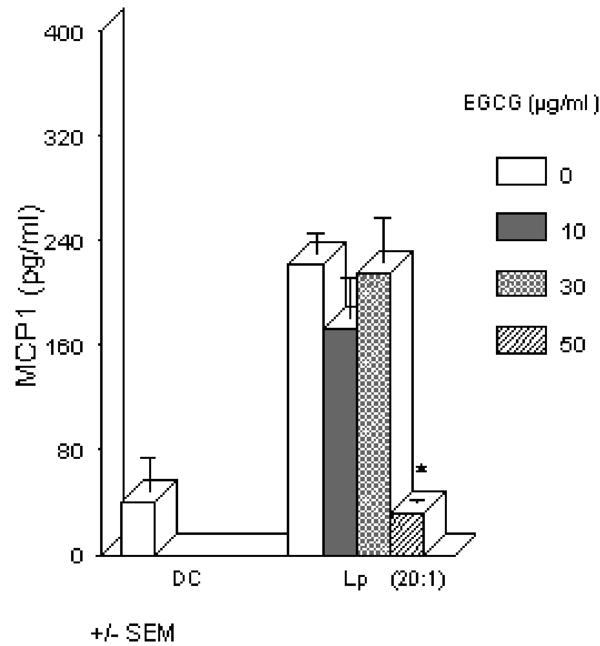

Figure 28. Effects of EGCG on MCP1 production by DCs infected with Lp (20:1). Results are expressed as mean value in pg/ml ± SEM from 3 independent experiments 24 hours after stimulation of cells. The asterisk indicates statistically significant differences ($p<0.05$) from values obtained in non-treated EGCG, Lp infected cells.

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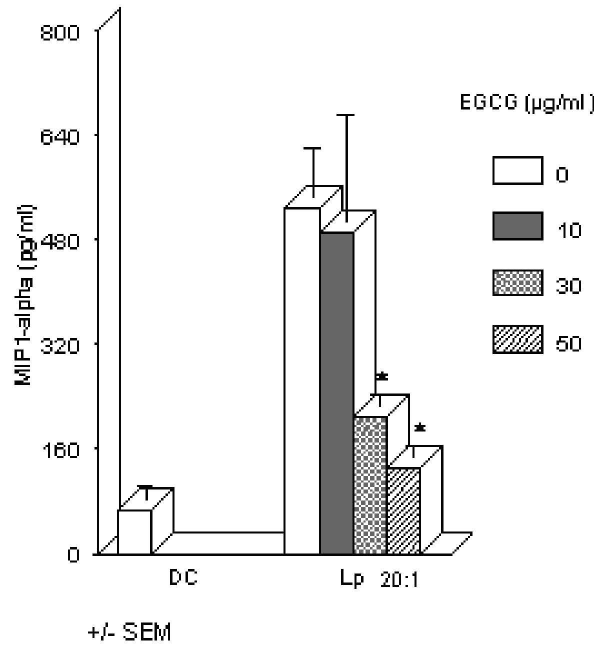

Figure 29. Effects of EGCG on MIP1α production by DCs infected with Lp (20:1). Results are expressed as mean value in pg/ml ± SEM from 3 independent experiments 24 hours after stimulation of cells. The asterisk indicates statistically significant differences ($p<0.05$) from values obtained in non-treated EGCG, Lp infected cells.

Aim 3: Determine molecular signaling mechanisms involved in effects of EGCG on DC maturation.

Lp and LPS are potent inducers of TLR2 and/or TLR4 surface molecule expression.

Lp was a potent stimulator of TLR2 surface molecule expression in DCs. In particular, Lp increased the percentage of cells double positive for CD11c and TLR2 to 64% from 19% (Figure 30). Lp also upregulated surface molecule expression of the TLR4 from16% to 34% (Figure 30).

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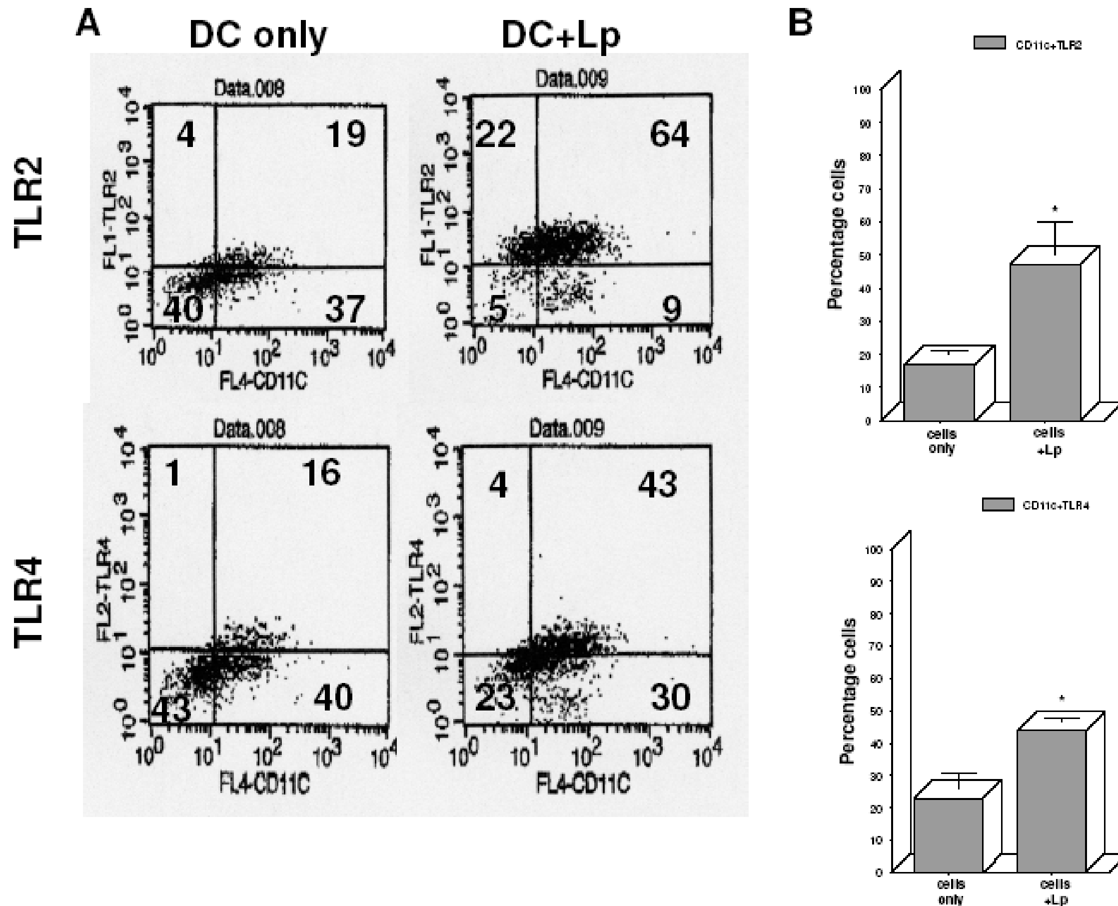

Figure 30. Lp infection up-regulates TLR2/TLR4 surface expression on DCs infected with Lp. DCs were infected at 10 bacteria per cell and cultured at $1x10^6$ cells/ml. (A) Flow cytometric dot plots of CD11c and TLR 2/4 surface molecule expression. Numbers in quadrants reflect percentages rounded to next greater whole integer. Results shown are 1 of 3 independent experiments with similar results. (B) Bar graphs of percentage of CD11c+ and TLR2/4 surface molecule expression. Data represent mean ± SD from three independent experiments. Asterisks indicate statistically significant differences ($p<0.05$) from non-Lp infected cells.

LPS was also a very potent inducer of TLR2 surface molecule expression by DCs. In particular, LPS increased the percentage of cells double positive for CD11c and TLR2 from 28% to 76% (Figure 31). In contrast, LPS actually downregulated TLR4 surface expression (data not shown) which is in accord with previous reports that LPS stimulation of DCs leads to TLR4 internalization and degradation (60).

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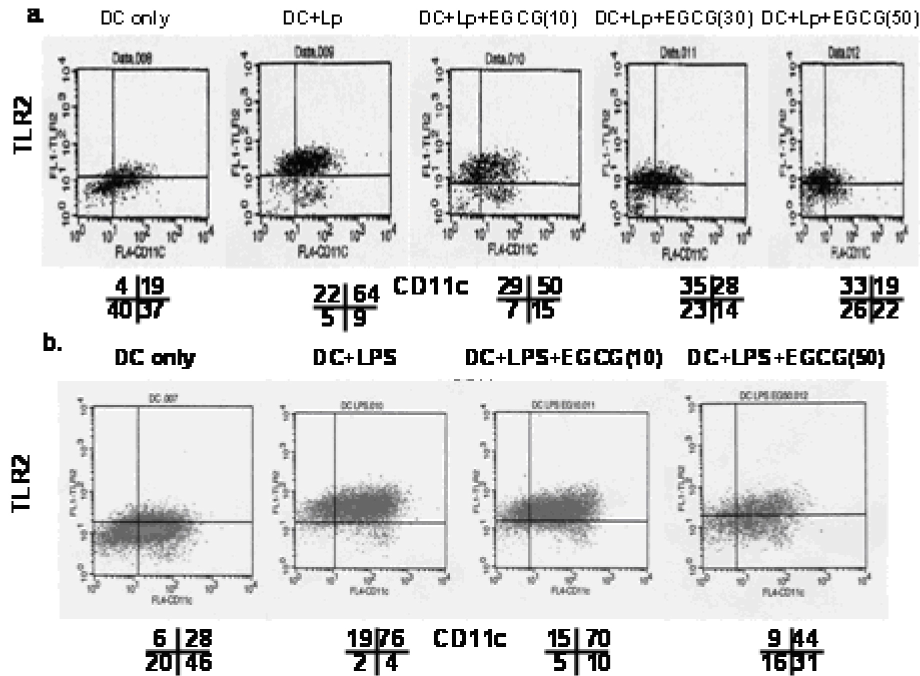

Figure 31. EGCG inhibits induced TLR2 on DCs infected with Lp or stimulated with LPS and treated with various concentrations of EGCG analyzed by flow cytometry. Numbers in quadrants reflect percentages rounded to next greater whole integer. Results shown are 1 of 3 independent experiments with similar results.

EGCG Inhibits Upregulation of TLR2/TLR4 Surface Expression Induced by Lp and LPS.

This upregulation of TLR2 by both Lp and LPS was dramatically inhibited by increasing doses of EGCG (Figure 31). EGCG treatment in a dose dependent manner also inhibited TLR4 up-regulation caused by Lp infection (Figure 32).

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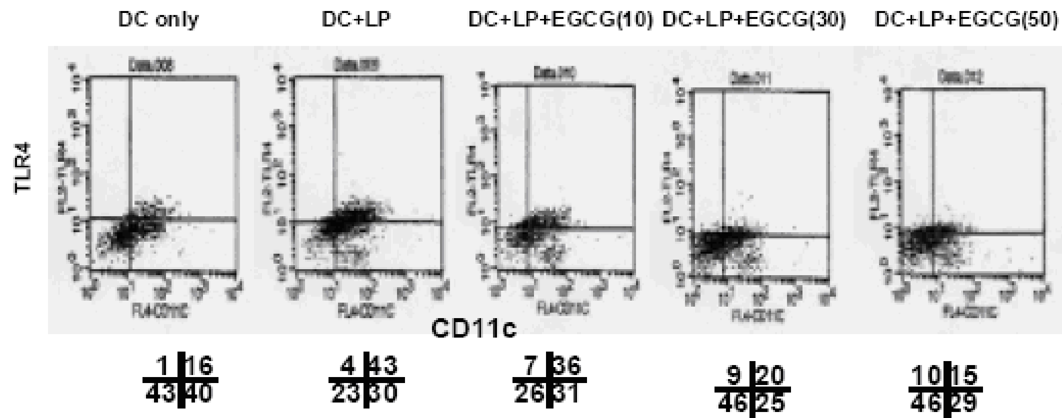

Figure 32. EGCG inhibits induced TLR4 on DCs infected with Lp and treated with various concentrations of EGCG analyzed by flow cytometry. Numbers in quadrants reflect percentages rounded to next greater whole integer. Results shown are 1 of 3 independent experiments with similar results.

EGCG Inhibits NFκB Activation by LPS

Most genes of inflammatory mediators such as TNFα and IL-12 are regulated by NFκB because they have a κB site in their 5' flanking region (46). Inhibition of NFκB has also been reported to suppress induction of TLR4 and TLR2 mRNA expression in mouse DCs stimulated with LPS (8). To determine whether EGCG inhibition of inflammatory mediators and TLR up-regulation involved inhibition of NFκB translocation, DCs exposed to LPS were simultaneously treated with EGCG. As shown in (Figure 33), LPS stimulation resulted in enhanced activation of NFκB whereas this stimulation was significantly inhibited by EGCG (50 μg/ml).

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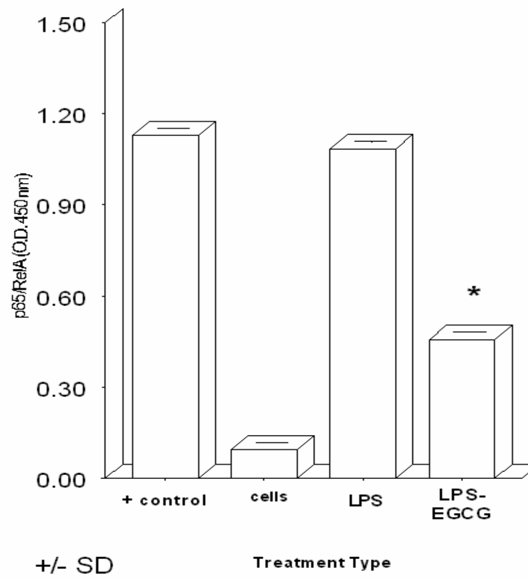

Figure 33. EGCG inhibits DNA binding activity of p65/Rel A subunit from DCs stimulated with LPS. Cellular extracts (16 µg) obtained from DCs treated with 10 ng/ml of LPS without EGCG treatment showed increased binding of p65/Rel A subunit to NFκB binding sequence when compared to EGCG (50 µg/ml; 45 minute incubation) treated DCs.

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DISCUSSION

The mechanisms underlying maturation and immunogenicity of DCs are starting to be elucidated. Immature DCs capture antigens and, during maturation, MHC peptide complexes begin to form within the MHC class II compartments, followed by transport in non-lysosomal vesicles to the cell surface (132). MHC class I is also upregulated upon maturation (161). Several co-stimulatory molecules, such as CD40 and CD86, are also expressed. The MHC-peptide complexes are found in clusters at the DC surface together with CD86 (161). It is believed that these high levels of antigen-presenting and co-stimulatory molecules, in a clustered distribution, initiate the formation of the immunologic synapse, bringing together essential elements like the TCR and CD28 required for T cell activation (89). Maturing DCs change in many other ways, including changes in chemokine receptor expression which contributes to their migration to the T cell areas of lymphoid tissue (30).

In this study, we examined various parameters of DC maturation in response to several microbial products and the effects of EGCG on these parameters. For example, we observed that EGCG inhibits Lp induced surface expression of co-stimulatory molecules by BALB/c mouse DCs. Up-regulation of these proteins is a central feature of DC maturation and is associated with their enhanced ability to activate resting T cells. We additionally showed that EGCG inhibited Lp induced up-regulation of both class MHC I and II molecules. DCs process exogenous antigens intracellularly and present them to CD4 T cells via MHC class II molecules (168). Although most cells use their MHC class I molecules to present peptides derived from endogenously synthesized

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proteins, DCs have the capacity to deliver exogenous antigens to the MHC class I pathway, a phenomenon known as cross-presentation (55).

Up-regulation of CD11c surface expression on BMDCs by bacterial products has been reported. For example, both mycoplasma lipoprotein FL-1 and LPS have previously been reported to up-regulate CD11c on the surfaces of C57BL/6-derived mouse BMDCs (82). Our results also show an increase in CD11c in response to microbial stimulation by Lp or LPS and in addition we observed an increase in double positive DCs which expressed both CD11c and the various MHC/costimulatory molecules. Treatment with EGCG, however, suppressed the expression of all of these developmental markers following stimulation by microbial products.

The inhibitory effects of EGCG on maturation of DCs by infection is further substantiated by our results showing that EGCG inhibits IL-12 p40 production in DCs after Lp infection (140). IL-12p40 is a subunit of IL-12p70 whose expression is inducible and correlated with production of bioactive p70 by DCs (8). IL-12 production is widely regarded as an essential indicator of a fully activated DC phenotype (98). EGCG, as well as other catechins have also reportedly suppressed IL-12 p40 production by murine peritoneal macrophages and the macrophage cell line, J774.1(61). In other studies with EGCG, the compound upregulated important innate immune stimulating cytokines such as IFNγ and TNFα. (106). In our studies, we also show that EGCG upregulates TNFα production by DCs after stimulation by LPS, MDP and Lp (140).

Other studies have reported dependence of IL-12 on TNFα, as well as possibly other cytokines. For example, IL-12 production by murine macrophages in response to *Mycobacterium bovis* Bacillus Calmette-Guérin reportedly depends on IFNγ and TNFα

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production (42). Moreover, administration of anti- TNFα monoclonal antibody diminished the lung levels of IL-12 and IFNγ induced by *Cryptococcus neoformans* infection in CBA/J mice (57). In order to determine dependence of IL-12 production by DCs on TNFα in our system, we treated LPS stimulated DCs with TNFα neutralization antibody. We show that neutralization of TNFα did not significantly affect IL-12 production levels. The differences between our results and those of other thus likely depends upon differences in DC biology compared to other cell types studied such as macrophages.

Zakharova recently reported that addition of TNFα reduced IL-12p40 production in DCs, suggesting a possible anti-inflammatory role for TNFα (184). Our studies do not indicate a role of TNFα in reduction of IL-12p40 because neutralization of TNFα either with or without EGCG treatment did not affect IL-12p40 production levels by DCs. The differences between our results and those of Zakharova may thus relate to differences in cell culture conditions such as levels of LPS stimulation (1 ng/ml used by Zakharova versus 10 ng/ml in our studies), cell number and/or culture medium used. Moreover, Zakharova preincubated DCs with TNFα followed by LPS stimulation whereas we did not add exogenous TNFα. In addition, the majority of Zakharova studies were done with macrophages.

Maturing DCs are also an abundant and strategic source of chemokines which are produced in a precise time-ordered fashion. Following stimulation with LPS, DCs have an initial burst of MIP1α (CCL3), MIP1β (CCL4) and IL-8 (CXCL8) production, which cease within a few hours. RANTES (CCL5) and MCP1 are also induced, but in a more steady manner. At later time points, DCs produce mainly lymphoid chemokines, such as

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CCL17 (TARC), CCL18 (DC-CD1), CCL19 (MIP-3β) and CCL22 (MDC), that attract T and B lymphocytes (107, 144). As shown in this study, LPS induced up-regulation of the early inflammatory chemokines RANTES, MCP1 and MIP1α. This up-regulation was significantly inhibited by EGCG, particularly at higher concentrations of EGCG.

Several other important pharmaceutical agents have been shown to suppress DC maturation and activation such as 1 Alpha, 25-dihydroxyvitamin D3 (15, 128), resveratrol (3), aspirin (50), and glucocorticoids (130). On a molecular level, these agents typically block DC maturation by inhibiting relB, a subunit of the NFκB pathway (98).

As shown in this study, EGCG inhibited both LPS and Lp up-regulation of TLR2 and TLR4 by DCs. EGCG also inhibited activation of the p65/RelA NFκB subunit in DCs treated with LPS. TLRs are critical for induction of downstream effecter functions in monocytes (7), and control expression of co-stimulatory molecules, as well as induction of cytokine and chemokine production by DCs (65, 153). TLR4 is a signal transducer for LPS, whereas TLR2 is a common transducer for a diverse array of bacterial products (93) such as PGN from Gram-positive bacteria (93). Lp is a Gram-negative pathogen and due to its LPS would be expected to activate TLR4 which is a receptor for Gram negative LPS, whereas TLR2 is a receptor for other bacterial products (93). However, related studies suggest that TLR2, rather than TLR4 plays a prominent role in Lp infection since purified Lp LPS as well as Lp, either viable or formalin-killed, are able to activate DCs from TLR4-deficient C3H/HeJ mice but fail to activate DCs from TLR2-knockout mice(19).

In our study, we found that infection with viable Lp resulted in marked up-regulation of TLR2 on DCs, and this may be related to TLR4, since microbial stimulation

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leads to NFκB activation, and the promoter of TLR2 contains NFκB sites known to up-regulate TLR2 gene transcription(117). Inhibition of ERK or NFκB has also been reported to suppress induction of TLR4 and TLR2 mRNA expression in mouse DCs stimulated with LPS (8).

Contrary to our results, the expression of maturation surface markers CD40, CD86 and MHC class II, was strikingly lower than was previously reported in DCs from A/J mice infected with live Lp compared to non-infected cells (81). The differences between these results and ours may be related to the different strains of mice used. A/J mice are relatively more susceptible to Lp infection whereas BALB/c mice used in this study are relatively resistant. The differing results also suggest that co-stimulatory and MHC class II up-regulation on BALB/c DCs may account for increased resistance to infection with *Lp* in this mouse strain. Although not examined in the A/J model, TLR up-regulation in BALB/c mice may serve as an additional important factor in differences between the two strains in susceptibility to *Lp* infection.

In addition to the importance of both TLR2 and TLR4 in sepsis (103, 167), emerging data support contribution of these TLRs in diseases like atherosclerosis (123). For example, mice deficient in MyD88, a TLR-signalling adaptor protein, are less prone to atherosclerosis (16, 114) and patients with a D299G polymorphisms of TLR4 have reduced risk of atheroscelorsis (79). The association between TLR4 function and atherosclerosis is consistent with findings showing that TLR4 mRNA and protein are more abundant in plaques in atherosclerotic lesions than in unaffected vessels (171). TLR2 also reportedly potentiates microglial interaction with Aβ42, a key pathogenic factor in Alzheimer Disease (AD), via the induction of the G-protein-coupled receptor

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mFPR2 (26). TLR signaling may also contribute to dilated cardiomyopathy, a common heart failure in young patients, by elevating dendritic cell function (38). TLRs might also be responsible for the development of diabetes (83, 186) and experimental autoimmune encephalomyelitis (78). TLRs also play a crucial part in the induction and progress of chronic inflammatory disorders such as asthma, a T helper 2 mediated chronic airway disorder (31, 37), and rheumatoid arthritis, a TH1-related inflammatory joint disease (64, 131).

Thus, the inhibitory effects of EGCG on TLR up-regulation as shown in this study may have therapeutic applications. However, both TLR2 and TLR4 are likely regulated differently in human cells by EGCG. This may be particularly the case with TLR2 since the proximal promoter regions of mouse and human TLR2 genes does not reveal a significant level of homology (52). Assessment of the physiological relevance of the findings presented here must also take into account maximum achievable EGCG concentrations attainable in vivo.

In summary, our results show that microbial products from LPS, MDP and Lp infection of DCs can significantly impact key DC maturation markers. These maturation markers include important co-stimulatory and MHC molecules as well as pro-inflammatory cytokines such as IL-12 and TNFα. In addition, EGCG has significant inhibitory effects on DC production of the pro-inflammatory chemokines, RANTES, MIP1α and MCP1. These studies show that DCs are susceptible to immune modulation following Lp infection which is likely important in transition from innate to adaptive immunity. In addition, these studies show that the polyphenol EGCG is a potent anti-inflammatory small molecular weight molecule which may have potential therapeutic

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uses against diseases implicated in inflammation and up-regulation of TLRs. The molecular mechanisms for the action of EGCG likely involve inhibition of ROS and TLR signaling transduction pathways which lead to downstream activation of NFκB (Figure 34).

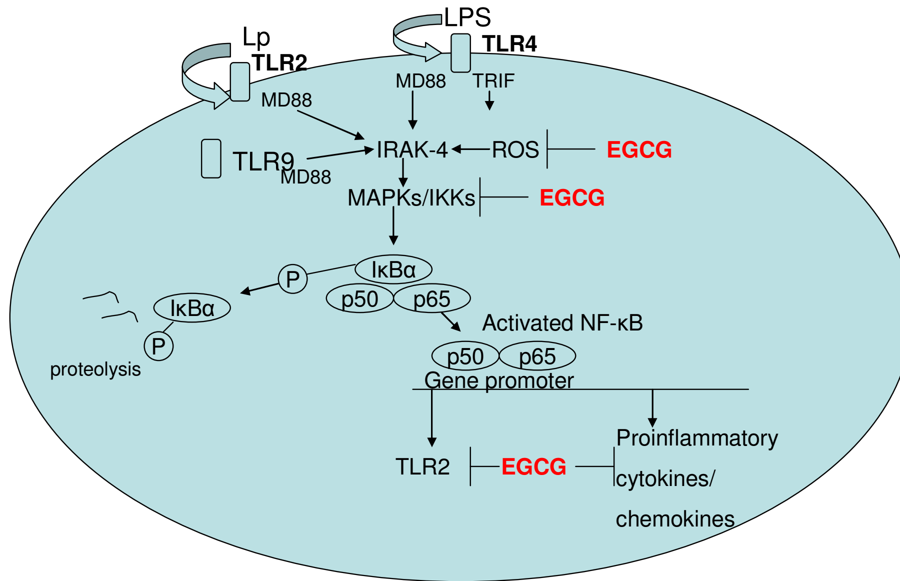

Figure 34. Schematic diagram of proposed effects of EGCG on DCs. Bacterial products such as LPS and Lp interact with TLRs thereby activating TLR signalling transduction and/or ROS which activates MAPKs/IKKs leading to activation of NFκB. NFκB activates many pro-inflammatory genes for pro-inflammatory cytokines/chemokines. TLRs are upregulated themselves in response to NFκB which serves to further heighten the immune response. There is also cross-talk between TLRs as in the case of where LPS activates NFκB which then activates the promoter for TLR2 thereby up-regulating TLR2 in response to LPS stimulation. EGCG inhibits ROS and/or MAPKS and NFκB which downregulates many pro-inflammatory cytokines/chemokines as well as TLRs such as TLR2.

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APPENDICES

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James L. Rogers received his bachelor's degree at Union College in Schenectady, N.Y. in 1987, his J.D. degree from Suffolk University in Boston, MA and his M.S. in biology from New York University in 1999. After several years of law practice in the biotechnology field as a patent attorney, he entered the Ph.D. program in the Department of Medical Microbiology and Immunology (now the Department of Molecular Medicine) in May, 2003.

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