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Exosomal curcumin: its bioavailability and potential anti-inflammation activity

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EXOSOMAL CURCUMIN: ITS BIOAVAILABILITY AND POTENTIAL ANTI-INFLAMMATION ACTIVITY

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ABSTRACT

Curcumin, a pleiotropic polyphenol derived from turmeric plants *Curcuma Longa* with rhizome, exhibits strong evidence as an anti-inflammation, anti-oxidant and anti-cancer reagent both in vitro and in vivo. Due to its low stability and systemic bioavailability, a large amount of curcumin has to be administrated to achieve therapeutic effects both in animal and in human. The major effort to overcome this barrier is making by encapsulation of curcumin into nanoparticles such as polymers and liposomes; some promising data have demonstrated the effectiveness of such method.

Exosomes are vesicle-like nanoparticles secreted by living cells. Exosomes contain both cell surface molecules and cytoplasmic molecules due to endo- and exocytosis formation pathways. These properties may make exosomes a well-armed nanoparticles drug carrier when formulated with curcumin. In this study, we showed evidence that curcumin can be encapsulated into exosomes and this encapsulation didn't disrupt the structure of exosomes. Exosomal curcumin may combine exosomes, curcumin and nanoparticles functions together to be a powerful triple threat. To test this hypothesis, we evaluate the anti-inflammatory activity of exosomal curcumin in comparison with free curcumin alone. Our *in vitro* studies showed that encapsulation curcumin into exosomes can increase stability and solubility of curcumin, and when RAW cells were treated with

LPS, exosomal curcumin dramatically inhibited cytokines IL6 and TNF-α secretion. Meanwhile, we adapted the LPS septic shock model to evaluate the anti-inflammatory activity *in vivo*. Exosomes encapsulation significantly increased bioavailability of curcumin in mice plasma when 100mg/kg exosomal curcumin and free curcumin were injected (i.p) into C57BL/6j mice. Interestingly, 4mg/kg of exosomal curcumin fully protected mice from LPS induced septic shock. Meanwhile, 4mg/kg of free curcumin didn't show any protection to the mice since this dosage was far away from its effective dosage. Significant decreased MDSCs numbers were found in the lungs of exosomal curcumin treated mice but not other organs. Our previous and others data indicate that exosomes can be preferentially uptaken by circulating MDSCs. When exosomal curcumin was co-incubated with MDSCs, the cellular concentration of curcumin was much higher than free curcumin, and exosomal curcumin induced more MDSCs apoptosis. Taken together, exosomal curcumin can target and kill circulating MDSCs during acute inflammation, thereby protects mice from LPS induced life threaten.

KEYWORDS: curcumin, exosomes, IL6, TNF-α, MDSCs, LPS

DEDICATION

This dissertation is dedicated to my parents, Yingcheng Sun and Xiaolin Hua, whose love, understanding and support have encouraged me in every step of my Ph.D study. This dissertation is also dedicated to my two sweet and beautiful daughters, Yuxi (Lucy) Xie and Allison (Ally) Xie.

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LIST OF ABBRIEVIATIONS

ATF2	Activating transcription factor 2
ARE	Antioxidant Response Element
ASK1	Apoptosis signal-regulating kinase 1
ARG1	Arginase 1
BBB	Blood brain barrier
CNS	Central nervous system
CD80	Cluster of differentiation 80
CSF1	Colony stimulating factor 1 receptor
COX-2	Cyclooxygenase-2, Prostaglandin-endoperoxide synthase 2
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
EPR	Enhanced Permeability and Retention Effect
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
Gclc	Glutamate-cysteine ligase catalytic
GCLM	Glutamate-cysteine ligase, modifier
GOT/GPT	Glutamic oxaloacetic transaminase/glutamic pyruvic transaminase

GST	Glutathione S-transferase
gp130	Glycoprotein 130
GMP	Good manufacturing practice
GMCSF	Granulocyte-macrophage colony-stimulating factor
Hsp70	70kD heat shock proteins
HO-1	Heme oxygenase-1
HPLC	High performance liquid chromatography
HNSCC	Human head and neck squamous cell carcinoma
HLA-DR	Human leukocyte antigen, MHC class II cell surface receptor
IKK	IkB kinase
IkB	Inhibitor of kB
ICAM	Inter-cellular adhesion molecule 1
IFN-γ	Interferon-gamma
IL6	Interleukin-6
IL-6R	Interleukin-6 receptor
JAK	Janus kinase
Keap1	Kelch-like ECH-associated protein 1
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MMP	Matrix metalloproteinase
mDC	Mature dendritic cells
MEKK1	Mitogen-activated protein kinase kinase kinase 1
MD-DCs	Monocyte-derived DC

MVBs	Multivesicular bodies
MD2	Myeloid differentiation protein 2
MDSCs	Myeloid derived suppressor cells
NQO	NAD(P)H quinone oxidoreductase
NK cells	Nature killer cells
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NOS2	Nitric oxide synthase
PLGA	Poly(lactic-co-glycolic acid)
RES	Reticulo-endothelial system
STAT3	Signal transducer and activator of transcription 3
Treg	Regulatory T cells
TRAF2	TNF receptor-associated factor 2
TRADD	TNF receptor type 1-associated DEATH domain protein
TRAIL	TNF-related apoptosis inducing ligand
TLR	Toll-like receptor 4
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor-alpha
TSG101	Tumor susceptibility gene 101
UGT	UDP-glucuronosyltransferase
VCAM	Vascular cell adhesion molecule-1
WBC	White blood cells

INTRODUCTION

Exosomes

Exosomes are 50-100nm, cup-like multivesicular bodies (MVBs) secreted by cells into extracellular milieu, although the secretion mechanism is not clear yet. During the endocytosis pathway, MVBs are formed in the late endosome by "inward budding and scission of vesicles from the limiting membranes into the endosomal lumen"(1-4). As a result, transmembrane and peripheral membrane proteins are incorporated into the vesicle membrane, and cytosolic components are included in the vesicles. In the cytoplasma, some of the MVBs will undergo degradation during the transition from late endosome to lysosome, and some of them will be secreted out of the cells as exosomes, conveying cellular communication during their circulation in addition to eliminating unwanted molecules. Exosomes secretion represents another mechanism of exocytosis; their endocytic origin distinguishes them from constitutive exocytosis, a process that happens in all cells and releases extracellular matrix proteins or newly synthesized membrane proteins into the plasma membrane through transporters (5).

The protein composition of exosomes varies by cell type and cell origin, and the diversity brings different functions of exosomes *in vivo* and *in vitro* (6). In general, they contain cytosolic and plasma membrane proteins, such as tubulin, actin, annexins and Rab proteins. Dendritic cell derived exosomes (Dexs) include almost all the antigen-presenting molecules of original cells, such as MHCI, II and CD86. These exosomes can induce immune response upon other cells (7, 8). Tumor derived exosomes, containing

tumor specific antigen, MHCI molecules and Hsp70/90, can transfer tumor antigen to DCs to induce a CD8+ T cell dependent anti-tumor immune response (4). A recently paper showed that even Hsp60, a resident intracellular mitochondrial chaperone, and Hsp70 are found in extracellular exosomes secreted by tumor cell lines(9). Exosomes also contain some signal transduction associated molecules (protein kinases, 14-3-3 and heterotrimeric G protein) although it's not clear if they are functional. Tetraspanins family proteins (Tspan8, CD9, CD63, CD81 and CD82) are highly enriched in exosomes, suggesting the involvement of exosomes in the signal transduction, endothelial cell activation and tumor angiogenesis(10-12).

Many types of cells can release exosomes, including major hematopoietic origin cells, such as B and T lymphocytes, DCs, mast cells and platelets, and non-hematopoietic cells, such as intestinal epithelial cells, Schwan cells, neuronal cells and tumor cells(4). Among these, the secretion and function of DCs and tumor cell derived exosomes are well studied. Exosomes can also be purified from physiological fluids, such as blood, urine and ascites from tumor patients(13). Through a series of differential centrifugation to remove dead cells and cell debris, exosomes will be collected by a final ultracentrifugation. Sucrose gradients will be utilized to purify exosomes and get rid of other contaminating materials, such as protein aggregates and nucleosomal fragments(14). For quality control, FACS, ELISA and western blot are used to monitor some exosomes associated proteins expression (such as MHCII, CD80, CD86 and TSG101)(15), and the morphology is examined by electron microscope (EM) of different preparations. Recently, a Good Manufacturing Practice (GMP) protocol has been

developed to guarantee large amount and high quality exosomes available for clinical studies and research in the future(16).

Exosomes were first investigated during the maturation of reticulocytes to erythrocytes while elimination of the transferrin receptors was required in 1987(17). Afterwards, numerous studies identified that exosomes are not only as an obsolete protein remover, but rather a signaling communicator. When exosomal RNAs isolated from a human (HMC-1) and mouse (MC/a) mast cell lines were transferred to neighboring cells, they conferred new functions in the new cells(18). After secretion, exosomes will be engulfed by neighboring cells. Exosomes containing bacterial coating components from the infection cells or bacteria can be detected and induce a proinflammatory response in the new macrophages(19, 20). Further, Protein urinary Fetuin-A associated with acute kidney injury (AKI) can be detected in a high level from exosomes isolated from cisplatin injected animals(21); Some disease-related proteins such as aquaporin-2, non-muscle myosin II and epithelial sodium channel (ENaC) are found in urine exosomes, making exosomes good prediction markers of some diseases(22).

Dendritic cell derived exosomes (Dexs), containing all the antigen-presenting molecules (MHCI and II) and some costimulatory molecules, can be used as cell-free vaccines for cancer therapy(8, 23, 24). Early pioneer studies showed that Dexs loaded with tumor specific peptides showed specific cytotoxic T lymphocytes (CTL) response and inhibited tumor growth(8). Dexs pulsed with HLA-A2/Mart1 peptides complex can shuttle functional MHCI and HLA-A2/Mart1 complex to DCs to active CTL, and this activation needs mature DC (mDC, natural adjuvants) as a boost(8). Dexs pulsed with a synthetic adjuvant that replaces mDC *in vivo*, such as CpG oligonucleotides (ODN) can

also induce naïve Tc1 lymphocytes activation and inhibit tumor growth(25). Exosomes contain heat shock proteins, such as Hsp70 and Hsp90, which may help MHC molecules loading to mDC. Preclinical and clinical studies from Dr. Zitvogel's group provide promising results, showing that cyclophosphamide synergies with Dexs to promote T cell priming and recovery T/NK cell functions in stage IV patients(26). A phase II clinical trial is now undergoing to test the response of Dexs on non-small cell lung cancer end stage patients(23).

Exosomes display anticancer activities mainly through immune cells. In the same time, exosomes may directly target tumor cells. Tumor cell derived exosomes (Texs) can induce tumor cell death through increasing of Bax and decreasing of BCl-2 expression. Furthermore, exosomes can activate PTEN and Gsk-3β. Together with actin and β-catenin, PTEN and Gsk-3β can inactivate constitutively activated PI3K/AKT survival pathway in pancreatic tumors leading to tumor cell apoptosis(27). Texs can also induce tumor cell apoptosis through the interaction between exosomes and Hes-1 (Hairy and enhancer-of-split homolog-1), an intracellular target of Notch-1 survival pathways in the target cells(28).

Although there were some discrepancies among evidence for the immunogenic activity of exosomes, a phase I clinical trials of patient ascites-derived exosomes showed that the combination of exosomes with GMCSF had more potential to induce tumor antigen (CEA) specific CTL response and anti-tumor activity(29). Dai et al found that malignant effusions derived exosomes contained antigen presenting molecules tetraspanins and tumor antigens (Her2/Neu, Mart1, TRP and gp100), and tumor antigens

Mart1 could be transferred to DCs derived monocytes to induce Mart1 specific CTL activation.

Although so many functions of exosomes are observed, it seems that the power of exosomes is still obscure. Exosomes are lipid bilayer vesicles with a density of 1.13-1.19 g/ml(30). The particular size property may give exosomes nanoparticle-like functions. "Nanoparticles are solid, colloidal particles consisting of macromolecular substances that vary in size from 10nm to 100nm"(31). The size dependent nanoparticle delivery system can well permeate cells and bring drugs to targeted tissues. Nanoparticles are extensively used as vectors to deliver drugs in clinical studies(32, 33). In this study, we will explore the possibility of exosomes carrying an anti-inflammation and anti-cancer drug, in terms of curcumin, to multiple cellular targets and evaluate the bioactivity of formulated curcumin.

Nanoparticles and nanotechnology

In 2000, the National Nanotechnology Institute (NNI) defined nanotechnology as "The understanding and control of matter at dimensions of roughly 1 to 100nm, where unique phenomena enable novel applications" (35). The application of nanoscaled or nanostructured material in medicine according to their unique structure, in terms of nanomedicine, has extended the objects size up to 1,000nm, and a lot of materials (34), such as polymers, liposomes, metals and carbon nanotubes, have been developed as drug deliver vectors(35-40) or *in vitro* BioNanoimaging(34, 41). With the aid of these drug delivery vectors, small molecule drugs, peptides, proteins(42), DNA(43) and even siRNAs(44, 45) are assembled into nanoparticles to enhance drug bioavailability and

bioactivity to fight against multiple fungal infections, inflammatory diseases, bone defects and cancers.

Currently, there are a lot of nanodevices understudied, including polymer(46, 47), dendrimer(20, 48), liposome(48-50), carbon nanotubles(51) and metal nanoparticles (34, 41). Polymers and liposomal nanoparticles have been well studied and have a potential for clinical studies. As drug vectors, the general requirement for nanoparticles is the high biocompatibility and biodegradability. Polymeric nanoparticles contain hydrophobic cores that have high capacity to hold hydrophobic drugs and a hydrophilic sphere that interacts with aqueous environment. Through physical entrapment and chemical conjugation, polymeric nanoparticles can hold targeting drugs, such as myoglobin(52, 53), hemoglobin(54) and doxorubicin(55, 56), with encapsulation efficiency as high as 90%. Liposomal nanoparticles are lipid bilayers consisting of phosphatidylcholine with the size varying from 50 to 1000nm. Liposomal nanoparticles have been studied for several decades. Liposome formulated doxorubicin (Doxil, Myocet) and daunorubicin (DaunoXome)(57, 58) have been approved by FDA and used to treat cancers and Kaposi sarcoma in clinic.

In general, nanoparticle delivery systems can increase intracellular drug concentration, and enhance drug activity. Meanwhile, the encapsulation of drugs into nanoparticles can achieve therapeutic effects at low doses leading to minimized side effects and toxicity. Nanoparticles can also bypass g-glycoprotein efflux pump to overcome drug resistance. More importantly, the high penetration property will allow nanoparticles pass the barrier of transdermal(59) and BBB(60, 61) to deliver drugs.

Exosomes as nanoparticles are MVBs released by living cells in organs or in culture. Exosomes, containing lipid bilayers that are similar to liposomal nanoparticles, may be loaded with curcumin through physical entrapment. Through the hydrophobic interaction between the hydrophobic tails of exosomes and the hydrophobic drug, curcumin can be self-assembled into the lipid bilayers.

EPR effect and passive targeting

Passive targeting of nanoparticles is achieved through the particular size of nanoparticles and leakage property of targeting vasculature. Nanoparticles as drug delivers can increase drug bioavailability through accumulating in the reticulo-endothelial system (RES) and achieving enhanced permeability and retention effect (EPR effect). To achieve this goal, considerable circulation time in the blood stream for nanoparticles to get more chance to reach tumor or inflammation site is very important. Large sized particles (0.25-10μm) are preferentially uptaken by specialized macrophages and neutrophils that are lining in the livers and spleens. To escape the capture by phagocytosis in the RES, nanoparticles should be very small(33). However, to prevent the elimination into blood capillaries through endothelial gap, nanoparticles also need to be above a certain size. This is why particular sized nanoparticles favor drug delivery. Abnormal angiogenesis during tumor initiation and growth leads to defective hypervasculature and inefficient lymphatic drainage system, giving rise to an enlarged endothelial gap that allows nanoparticles to target tumors through EPR effect(62). When the particular sized nanoparticles meet with the unique leaky targeting vasculatures, the carrying drugs can easily get through the blood vessels into the cancer or inflammation sites.

Exosomes are natural nanoparticles with particular size and secreted endogenously by cells. It's reasonable to speculate that they have all size dependent properties of other nanoparticles. When formulated with curcumin, exosomes can increase its water solubility and stability. This may also happen *in vivo* and prolong circulation half-life of curcumin. When exosomal curcumin (100mg/kg) was i.p injected into C57BL/6j mice, the concentration of curcumin in the periphery blood reached 1250ng/ml, which was 5-10 folds higher than free curcumin in one hour, and exosomal curcumin is much more stable during circulation.

Active targeting

To achieve active targeting, nanoparticles are incorporated with some targeting moieties to navigate their aims. This combination system can localize and reach cancer cells in tissues more efficiently and specifically. Lectin-carbohydrates interaction is well studied and applied to achieve active targeting. Lectin is differentially expressed in normal and malignant cells, and this sugar-binding protein recognizes specific carbohydrates in the solution or the surface of tumor cells or pathogens. Lectin or carbohydrates can be incorporated into nanoparticles to enhance targeting specificity(63). There are several efforts to try to target the transferrin receptors, which are overexpressed in many types of cancers. Apotransferrin nanoparticles loaded doxorubicin can be internalized into cells through transferrin receptor mediated endocytosis, and localized in the target organ very quickly and efficiently(64). Paclitaxel-loaded PLGA polymers conjugated with transferrin ligand showed sustained anti-proliferative activity in MCF7 cells due to the increased cellular uptake and decreased exocytosis(65). When Bcl-2 antisense

oligonucleotide G3139 was assembled into the transferrin conjugated lipopolyplexes, the complex showed higher efficiency on the inhibition of tumor growth and survival than free G3139 with longer half-life and higher bioavailability(66).

As a potent carrier of nanoparticles, several characteristics of exosomes indicate that exosomes may be a perfect candidate for delivery of drugs to achieve active targeting. First, in contrast to polymeric and liposomal nanoparticles, exosomes are biological structures with a unique set of proteins. These proteins are mainly cell membrane and cytosolic proteins due to the formation pathways of exosomes. Exosomes from immune cell origin contain all the antigen-presenting molecules (MHCI and II), therefore, exosomes can be used as cell free vaccines to suppress established tumor growth (7, 8). Exosomes also express antigen presenting helper molecules, such as tetraspanin and heat shock proteins Hsp70 and Hsp90. Death receptors (FasL and TRIAL), cytokines, cognate receptors (TNF-α and TGF-beta), drug transporters (ATP7A, ATP7B and MRP2)(6) and signaling transduction molecules (14-3-3, heterotrimeric G proteins) are also found in exosomes. Whether these molecules are functional is not clear, however, this set of proteins does provide exosomes with a high potential to recognize antigens or receptors on the tumor cell surface. More significantly, Texs frequently contain tumor antigens (e.g. MelanA/Mart-1, gp100, CEA and HER2) and these antigens can be presented to specific CTL by transferring to MD-DCs. The protein composition and specific size guarantee that exosomes are a well-armed nanodevice to recognize cancer cells and destroy them.

Second, exosomes can be more powerful by pulsing with functional molecules. Dexs can be pulsed with tumor antigen HLA-A2/Mart1 complex to induce production of IFN-γ by melanoma-specific helper T cells. CpG adjuvants pulsed exosomes can also

induce CTL priming instead of native mDC pulsed exosomes. It has been identified that TS/A exosomes carrying tumor rejection antigens can be cross-presented to MC38 adenocarcinoma host to induce immunogenicity(67). In other words, tumor rejection antigens may be shared between tumors and this is raising the possibility that pulsed or natural exosomes can be used to treat multiple tumors.

Lastly, in contrast to polymeric and liposomal nanoparticles, exosomes are secreted by host cells, and will not induce a host response when they are used to deliver drugs to the original host and can be eliminated through exocytosis pathways.

More importantly, a GMP protocol has been developed, and large amounts of high quality exosomes should be available for clinical studies and therapeutic treatment.

Curcumin

Curcumin is a yellow polyphenol compound purified from the root of turmeric plant *Curcuma Longa*. Its molecular formula is $C_{21}H_{20}O_6$ with a molecular weight 368.37g/mol. Curcumin is soluble in organic solvents such as ethanol, methanol, dimethylsulfoxide (DMSO), acetone and acetonitrile but not in water. Curcumin is light sensitive and unstable in neutral and basic environments. It's relatively stable at acidic pH solutions and in cell culture medium containing serum or in human blood. There are two forms of curcumin, an enolate and a bis-keto form, and these two forms can reach equilibrium in physiological conditions. The significant structure property of curcumin is the two methyl groups and two phenolic hydroxyl groups in the phenol-rings. The hydroxyl groups provide its strong antioxidant activity. The pleiotropic functions of curcumin have been well studied(68). Among these functions, the anti-inflammatory activity of curcumin is

most significant. Through targeting several conserved molecules, curcumin shows impressive power in the treatment of inflammation and inflammation related diseases, such as cancers.

NF-kB and COX-2 signaling

NF-kappaB (NF-kB) is a transcription factor that is found in almost all animal cell types and regulates cellular responses to multiple stimuli such as stress, cytokines, UV radiation and bacterial reagents(69). NF-kB activation is believed to be a master player during multiple diseases, particularly cancer and inflammatory diseases due to the induction of expression of numerous inflammatory mediators and of multiple immune responses as a transcription factor. In most cells, NF-kB is present in the cytoplasm in a latent, inactive and IkB-bound form. NF-kB activation can be considered as the regulation of IKK-modulated IkB-NF-kB interaction. IKK contains three distinct subunits: IKKα, IKKβ (catalytic kinase subunits) and IKKγ (regulatory subunit). Upon stimuli, the IKK complex is activated. Activated IKKβ can phosphorylate the specific serine residues within the N-terminal regulatory domain of IkBα, leading to IkBα ubiquitination by beta-TrCP and degradation by the 26S proteasome. Upon release by inhibitory IkB proteins, NF-kB will translocate to nucleus and regulate target gene expression. In the same time, new synthesized IkBα can enter the nucleus, bind to NF-kB and shuttle NF-kB back to the cytoplasma. The activation of NF-kB will up-regulate several downstream molecules that may play crucial roles in diseases pathogenesis. Numerous reports have demonstrated that NF-kB is constitutively activated in some

cancers due to the chronic stimulation of the IKK pathway or mutated or defective genes encoding of IkB.

Cyclooxygenases are NF-kB downstream regulated enzymes that catalyze the formation of important chemical messengers, prostaglandins, which is responsible for inflammation and pain(70). There are three isoforms of cyclooxygenases, COX-1, 2 and 3. COX-1 is constitutively expressed in most mammalian cells, however, COX-2 is undetectable and inducible to stimuli, and becomes abundant in activated macrophages and inflammation sites. When COX-2 activity is inhibited, inflammation will be reduced. Thus, any COX-2 inhibitor, such as aspirin and ibuprofen, are successfully used to treat patients with inflammation and pain. COX-2 overexpression has been found in many tumors, such as cancers of the colon, rectum, breast, lung, pancreas and prostate. It has been shown that several transcription factors can regulate COX-2 expression, and NF-kB is the crucial up-stream regulator of COX-2.

Curcumin can suppress NF-kB activation through inhibition of IKK activity, IkB phosphorylation and subsequent NF-kB translocation to the nucleus, and downstream protein expression (Fig 1). Due to such suppression, curcumin shows a high potential for the treatment of inflammation related diseases and cancers. Curcumin can inhibit proliferation and induce apoptosis of HNSCC cells through inhibition of NF-kB activations; similarly it can suppress the expression of cell survival and proliferation genes, such as Bcl-2, cyclin D1, IL6, COX-2 and MMP-9(71). Curcumin can also down-regulate COX-2 and PEG2 expression, and prevent colon cancer progression, through inhibition of cell growth and promotion of cell apoptosis(72).

COX-2 and iNOS are important regulators during inflammatory disorders and cancer progression. Curcumin and other chemo-preventive phytochemicals can suppress NF-kB activation and down-regulate COX-2 and iNOS expression(73). Curcumin can down-regulate COX-2 expression in protein and mRNA levels of several inflammatory diseases and cancers. Curcumin treatment (360mg/dose, 3-4 times/day for three months) can inhibit COX-2, LOX, TNF-α, INF-γ and NF-kB expression in inflammatory bowel diseases (IBD) patients(74). Mono-carbonyl analogues of curcumin (beta-diketone moiety deleted) inhibited LPS induced IL6 and TNF-α secretion in a dose-dependent manner, and they showed strong inhibitory effects on the expression of LPS-induced TNF-α, IL1-beta, IL6, MCP-1, COX-2, PGES, iNOS and p65 NF-kB mRNA(75). Meanwhile, curcumin may inhibit VEGF mediated endothelial cell angiogenesis through inhibition of COX-2 expression and PGE2 production through inhibition of MAPKs(76).

There are few studies about how curcumin inhibits NF-kB activation. The expression of Hsp70 in lung can protect body from the possible damage due to the acute respiratory distress. Restoring expression of Hsp70 can minimize the pulmonary inflammatory responses of the acute respiratory distress syndrome in the CLP model that is similar to human sepsis(77). Wong et al showed that curcumin can induce Hsp70 by activation heat shock factor-1(78), a chaperone protein that binds to NF-kB-IKB complex to prevent IkB phosphorylation by IKK(79). Whether Hsp70 expressed on exosomes contributes to enhanced activity of exosomal curcumin is a new mechanistic study in the future.

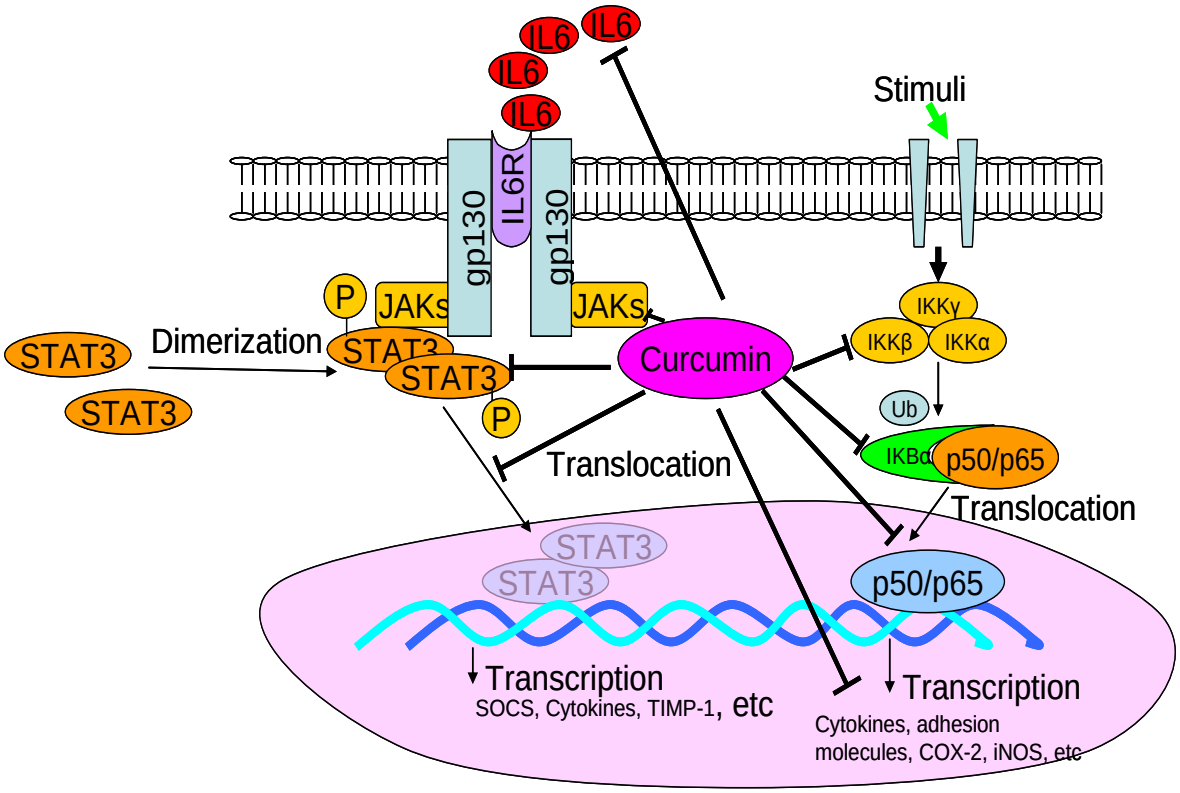

Figure 1. The regulation of curcumin on STAT3 and NF-kB (p50/p65) signaling pathways.

IL6-gp130-STAT3 signaling

IL6-gp130-STAT3 signaling is an important cellular target of curcumin. Interlukin-6 (IL6) is a pro-inflammatory and anti-inflammatory cytokine secreted by T and B cells and macrophages that is involved in the regulation of immune responses and hematopoiesis. In addition, other types of cells, such as fibroblasts, keratinocytes, mesangial cells and some cancer cells can also secrete IL6 in response to multiple stimuli or during diseases conditions, such as Rheumatoid arthritis, Castleman disease and Crohn's disease. IL6 has believed to be a master factor in disease initiation and progression. Inhibition IL6 production or blocking IL6 function could be an effective therapeutic approach to treat these diseases(80).

IL6 receptor complex contain an 80kD ligand-binding chain IL6R and signaling transducing chain, gp130. Upon IL6 binds to IL6R, signal transducer gp130 and Janus kinase (JAKs) are phosphorylated and this activation causes recruitment, dimerization of the nuclear factor signal transducer and activator of transcription 3 (STAT3) and subsequent translocation of STAT3 into nucleus. Activated STAT3 can induce expression of several cytokines, chemokines and other mediators.

Curcumin can inhibit IL6 and other cytokines secretion upon various cellular stimuli (**Figure 1**). Curcumin can also interfere with STAT3 activation through inhibition of JAKs activity, STAT3 dimerization and STAT3 translocation to the nucleus. LPS (500ng/ml) and live bacteria can induce STAT3 phosphorylation dependent IL1-β and IL6 secretion of RAW cells (81). Curcumin can inhibit LPS induced IL6 and TNF-α secretion of RAW cells and acid-induced IL6 and IL-8 secretion of human esophageal epithelial cells during gastroesophageal reflux diseases(82). In addition to inhibition of

IL6 release during inflammation, curcumin can also inhibit STAT3 activation independently or coordinately in the progression of several diseases. Curcumin can inhibit STAT3 phosphorylation and nucleus translocation in STAT3 constitutively activated U266 cells and HNSCC cells. Curcumin can also inhibit IL6-induced STAT3 phosphorylation. The inhibitory effect is even stronger than AG490, a well-characterized STAT3 phosphorylation inhibitor(83-86). Curcumin treatment decreased nuclear expression of STAT3, 5a and 5b, but not STAT1 and phosphorylation of STAT1, 3 and 5 in K563 cells of human chronic myelogenous leukaemia(87).

Similar to NF-kB, STAT3 is constitutively activated in cancers and acts as a signal shuttle between the cytoplasma and nucleus. As a nuclear transcription factor, STAT3 can stimulate inflammatory mediator such as IL6 gene expression and regulate expression of several genes associated with tumor initiation, progression and invasion. In addition, STAT3 interacts with NF-kB at several levels since IL6 is a signal inducer of STAT3, and NF-kB can regulate IL6 gene expression. Curcumin can inhibit tumor growth through inhibition of STAT3 and NF-kB in several cancers. Constitutively activation of NF-kB and STAT3 leads to Hodgkin and Reed-Sternberg (H-RS) cell hyper-proliferation and less programmed cell death. Curcumin down-regulated several proliferation and apoptosis related protein expression, such as Bcl-2, Bcl-xl, XIAP, c-IAP1, survivin, c-myc and cyclin D1 through inactivation of NF-kB and STAT3(88). NF-kB and STAT3 are constitutively activated in CD138+ cells isolated from multiple myeloma patients. Curcumin can effectively inhibit the activation of NF-kB and STAT3 leading to increased cell apoptosis(71). Activated STAT3 prolonged constitutively activated NF-kB through delaying NF-kB nuclear export in cancer cells and tumor –

associated hematopoietic cells. Curcumin can block cell proliferation and induce cell apoptosis of Primary effusion lymphoma (PEL) (89) and T cell leukemia (90) and the regulation is through inhibition of constitutively JAK1-STAT3 signaling pathway.

Nrf2-HO-1 signaling

Transcription factor nuclear factor (erythroid-derived-2)-like -2 (NFE2L2 or Nrf2) is another cellular target of curcumin. Nrf2 is ubiquitously expressed in the kidney, muscle, lung, heart, liver and brain, and shows strong evidences to fight against various oxidative stresses resulted from cancer, cardiovascular diseases, neurological diseases and renal diseases. Nrf2 and downstream gene products are becoming novel drug targets in the treatment of diseases.

As a nuclear transcription factor, Nrf2 is bound in the cytoplasma by Keap1 (Kelch like ECH associated protein 1) in normal conditions (**Figure 2**). Keap1 is also associated with cullin-3 ligase, where the cullin-3 based ubiquitination will degrade Nrf2, and gives Nrf2 a short half-life of about 20 minutes. Upon oxidative stress, the Keap1-Cullin3 association is disrupted and Nrf2 is released from the complex. Unbound Nrf2 will translocate into the nucleus, and dimerize with a small Maf protein. The heterodimer will then bind to the Antioxidant Response Element (ARE) in the upstream promoter region of many genes of antioxidative or detoxifying enzymes and initiate their transcription. The downstream genes include detoxifying enzymes NQO(1), Gclc, HO-1, GST and UGT. These enzymes can catalyze the breakdown of highly reactive substrates and inhibit oxidative stresses (Table 1) (91).

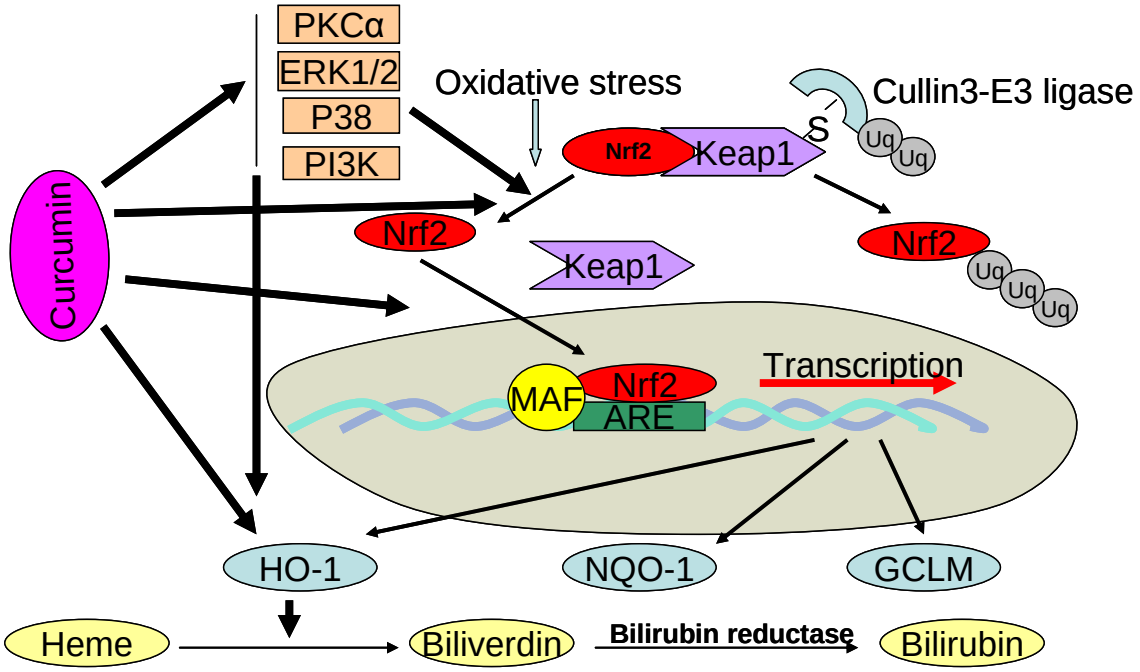

Figure 2. The regulation of curcumin on Nrf2 signaling pathways.

Table 1. The downstream enzymes of transcription factor Nrf2

Enzyme	Substrate
Nqo1 NAD(P)H quinone oxidoreductase 1	Quinones
Gclc Glutamate-cysteine ligase, catalytic	L-cysteine, L-glutamic acid and glycine
GCLM glutamate-cysteine ligase, modifier	
Heme oxygenase-1	Heme
GST glutathione S-transferase	Electrophilic or lipophilic compound conjugated with reduced Glutathione
UGT UDP-glucuronosyltransferase	UGT UDP-glucuronosyltransferase
Mrps Multidrug resistance-associated proteins	

Product	Function	Ref
H_2O_2	Chemo-protection, cancer susceptibility and anti-tumor drug action	(92)
Tripeptide Glutathione	Antioxidant, protect cells from reactive oxygen species	(93)
Biliverdin, CO and Fe2+	Antioxidant, anti-inflammation	(94)
Solubilize compound to get out of body	detoxify endogenous compounds, transport proteins	(95)
Solubilize compound to get out of the body	Glucuronidation, detoxification	(96)
	Membrane transportors, alter the pharamacokinetics and toxicity of compounds	(97)

Abbreviations: NADH, Nicotinamide adenine dinucleotide hydrolyse; UDP, Uridine diphosphate

Numerous evidences have shown that Nrf2 is a critical regulator of cellular antioxidant pathways and detoxifying enzymes that protect the body from various stresses. At the early stage of inflammation related tissue damage, activated Nrf2 may work through the inhibition of synthesis or expression of some proinflammatory cytokines, chemokines, cell adhesion molecules, MMPs, COX-2 and inducible nitric oxide synthase (iNOS) to achieve its cytoprotective function. Disruption of Nrf2 causes increased susceptibility to severe airway inflammation and asthma in response to multiple oxidative stresses in mice (98), and leads to increased mortality of mice due to septic shock. LPS and TNF-α stimuli induced severe pulmonary inflammation in Nrf2 deficient mice, and pretreatment of mice with exogenous antioxidants (N-acetyl cysteine or GSH-monoethyl ester) dramatically reduced inflammation and rescued mice from death. CDDO-im, a potent small molecule activator of Nrf2, can stimulate Nrf2-dependent antioxidative genes expression (HO-1, GCLC, GCLM and NQO1), reduce LPS-induced proinflammatory cytokine expression (TNF-α and IL6) *in vitro* and *in vivo,* and protect mice from septic shock in Nrf2 normal mice(99). CDDO-im and CDDO-Me also showed protection from LPS induced inflammatory response and ROS accumulation in human periphery blood mononuclear cells and neutrophils in preclinical *ex vivo* studies(100).

Hemo oxygenase-1 (HO-1) is an important Nrf2 downstream gene. HO-1 can catalyze the decomposition of heme into the anti-inflammation agent carbon monoxide (CO), ferrous iron (Fe2+) and biliverdin, which will be converted into another potent antioxidant bilirubin by biliverdin reductase. HO-1 is inducible, and increased expression of HO-1 will reduce the inflammation response and protect host through its three by-products(101). LPS can induce HO-1 expression in multiple organs, and show self-

protection effects in LPS-induced septic shock model. A subseptic dose (2.0mg/kg) of LPS periphery injection can induce the expression of pro-inflammatory cytokine IL-1β, IL6, nitric oxide synthase iNOS and HO-1 in the hypothalamus and hippocampus of rats(94). LPS injection dramatically induces the expression of HO mRNA and protein levels and the enzyme activity in liver, lung and kidney(102). In HO-1 deficient mice, LPS induced more WBC and circulating neutrophils. Severe hepatic and renal dysfunction in correlation with high mice mortality were found in the HO-1 deficient mice (103). These evidences indicate that HO-1 activity is very important for host self-protection and survive. Thus, induction of HO-1 activity may represent a therapeutic idea to follow in the clinical studies.

Curcumin can induce HO-1 and Nrf2 expression and activity *in vitro* and *in vivo*, and shows strong evidence to be an antioxidant. Curcumin inhibits LPS induced IL-1 and IL6 release dependent on HO-1 activation(104). High doses of curcumin (e.g. 100 mg/kg) increased Nrf2 and HO-1 expression in MCAO treated brain, and protected mice from MCAO induced focal ischemia(105). Curcumin and its synthetic analogue dimethoxycurcumin induced HO-1 expression and activation through Nrf2 activation(106, 107). In a gene screening experiments, more than 822 and 222 Nrf2 dependent genes respectively in the liver and small intestine are modified by curcumin treatment (1g/kg) in comparison to wild type and Nrf2 knockout C57BL/6J mice. These groups of genes contain genes that encode cellular ubiquitination and proteolysis, electron transport, detoxification, transport, apoptosis, cell cycle control, cell adhesion, kinases and phosphatase, and transcription factors (108). For example, through the activation of

PKCα, PKCδ/ERK1/2, p38α and PI3K kinase, curcumin induced HO-1 expression and activity in a dose dependent manner(104).

As an anti-inflammation and antioxidative polyphenol, curcumin shows protective effects on multiple organs during oxidative stresses through the regulation of Nrf2 and HO1. In a rat model of light induced retinal degeneration, curcumin showed a strong retinal neuroprotective effect when rats were fed with diets supplemented with 0.2% curcumin for 2 weeks, and this protection is through inhibition of NF-kB and induction of HO-1 and thioredoxin(109). Oral administration of curcumin (200mg/kg dose for 4 consecutive days) leads to increased nuclear translocation and ARE-binding of Nrf2 and HO-1 expression, showing protective effect on dimethylnitrosamine (DMN)-induced hepatic injury(110). To protect rat vascular structures, curcumin promoted Nrf2 translocation into nucleus, activated ARE and increased downstream HO-1 expression. Increased HO-1 then inhibited the growth of vascular smooth muscle cells (VSMCs) through up-regulation of cyclin-dependent kinase inhibitor p21 (WAF1/CIP1), which inhibits proliferation of VSMCs. In the same manner, curcumin can also inhibit the growth of human aortic smooth muscle cells induced by TNF-α(111). Curcumin induced HO-1 expression and achieved anti-inflammation activity through inhibition of expression of intercellular adhesion molecule-1 (ICAM-1) *in vitro* and *in vivo*. Decreased endothelial ICAM-1 then hampered transmigration of inflammatory neutrophils into lungs in LPS treated mice and reduced pulmonary inflammation(112). Curcumin treatment induces HO-1 mRNA and protein expression in cultured hippocampal neurons. Similarly, low dose of curcumin treatment leads to significant increased expression of the phase II detoxification enzymes, quinone reductase and glutathione S transferase, in

astrocytes. Taken together, curcumin exhibits strong protective effect on neurons in CNS under oxidant challenge(113).

Systemic bioavailability

Curcumin's low systemic bioavailability after consumption compromises its biological functions, leading to limited application in clinical trials. Low systemic bioavailability means very low detectable level of curcumin in plasma after curcumin administration. A high dose of curcumin administration to rats led to a maximum serum concentration of 1.35ug/ml at 0.83hr, whereas only a trace amount of curcumin was detected in the serum of human volunteers given 2g of curcumin(114). After oral dosing, curcumin will stay and be digested in the stomach. Along with other food emulsion, curcumin will then pass through the intestinal wall into the enterohepatic circulation, arrive to liver for detoxification (the first pass effect) and finally get into blood stream. It's obvious that curcumin's low bioavailability is due to the rapid first pass effect and the fast intestinal metabolism (glucuronidation of curcumin).

To overcome this barrier, chemical analogues and novel delivery methods are under development. Several approaches have been reported to increase curcumin's bioavailability. There are a lot of structural analogues of curcumin available from nature or biosynthesis. FLLL11 and FLLL12, new structure analogues of curcumin, can increase curcumin's bioavailability and induce more cell death than curcumin in human pancreatic cancer cells(115). Piperine is an inhibitor of hepatic and intestinal glucuronidation. When it was co-administrated with curcumin, it increased bioavailability about 154% in rats and 2000% in human. Recently, researchers have focused on encapsulation of curcumin with

Table 2. Approaches to improve bioavailability of curcumin

Nanoparticle encapsulated curcumin	**Particle size**	**Species or cell lines**	**Dose of curcumin**
Piperine		Rat (20mg/kgPP)	2g/kg
		Human 20mg PP	2g
Liposome Lecithins	263nm	Rat	100mg/kg oral
Liposomal curcumin 9:1 [b]	NA	Mice	1mg i.v
Synthetic analogues FLLL1 and FLLL2		Human pancreatic cancer cells	IC(50) 0.28-3.2/8.67-20.35 mM/l
Milk casein	<200nm	Hela cells	NA
Nanosphere PLGA	35-100nm	Prostate cancer cells	NA
Phosphatidylcholine	NA	Rat, oral	360mg/kg
Silk fibroin	50-100nm	Breast cancer	
Phospholipid		Rat	100mg/kg
Phospholipid		Rat	1g/kg
Polymer (PLGA-PEG5000) [c]	80.9nm	Mice	2.5mg/kg i.v
Nanoparticle encapsulation	264nm	NA	NA

Table 2. Approaches to improve bioavailability of curcumin (Continue)

Cmax of NP curcumin over control [a]	**Bioactivity**	**Ref**
Increase154%		(114)
Increase2000%		
319.2/64.6 μg/l	Increase plasma antioxidant activity	(50)
8.79pmol/ND	Suppress growth of HNSCC *in vitro* and *in vivo*	(48)
	Inhibit STAT3/AKT activation, increase PARP and Cas-3 cleavage	(115)
NA	Similar cytotoxicity	(117)
NA	Robust intracellular uptake, induce more cell death	(118)
12.5/2.5 ng/ml		(119)
	Increase cellular uptake and induce more cell death	(120)
600/267 ng/ml		(121)
1.0/0.5 μg/ml	Protect liver from oxidative stress	(116)
450ng/325 ng/ml	Rapid and more efficient cellular uptake, induce apoptosis, inhibit proliferation	(68)
9 fold / cur+PP		(122)

Abbreviations: NA, not applicable; ND, not detectable; PP, piperine.

[a] The maximal increase of bioavailability of NP curcumin treatment in serum in comparison with free curcumin.

[b] Lipids 1,2-dimyristoyl-sn-glycero-phosphocholine: 1,2-dimyristoyl-sn-glycero-3-phospho-Rac-(1-glycerol) = 9:1.

[c] PLGA, poly (lactide-co-glycolide) (PLGA); (PEG)-5000, a stabilizer polyethylene glycol.

nanoparticles to improve its bioavailability and achieve enhanced bioactivity. A curcumin phospholipid complex can increase its bioavailability and bioactivity(116). Casein is a nanoparticle structure isolated from milk, and it can form a complex with curcumin and stabilize curcumin although the complex has similar cytotoxicity to cells compared with free curcumin *in vitro*(117). Liposomal curcumin (curcumin was encapsulated into liposomes) can dramatically increase bioavailability and stability of curcumin. It can also inhibit cell proliferation and induce cell apoptosis, and show even stronger inhibitory effects on ConA-stimulated human lymphocytes, splenocytes and LCL proliferation than free curcumin(49, 50, 118). More nanoparticles encapsulated curcumin and their bioactivities have been summarized in Table 2.

LPS induced septic shock

Sepsis is a severe infection with high mortality, characterized by virulent organism initiation, in-appropriate innate immune responses and serious tissue and organ damage or dysfunction. The combination of bacteremia and systemic inflammatory response syndrome (SIRS) leads to myocardial injury (hypotension), acute respiratory failure, tissue damage, organ dysfunction or death(123). Sepsis is ranking 13th of leading causes of death in the United States.

The LPS septic shock model is a frequently used animal model to investigate sepsis and possible treatment. LPS (Lipopolysaccharides) are endotoxins found in the outer layer of the membrane of Gram-negative bacteria, and can induce acute and strong immune responses from macrophages and neutrophils through TLR4-NF-kB (Toll-like receptor 4) signaling pathway. In plasma, LPS binds to LPS-binding protein (LBP), and

forms a ternary complex with CD14/MD2. The complex then binds TLR4 in the cell surface and activates downstream NF-kB and JNK signaling pathway. Upon activation, these transcription factors will activate expression of downstream proteins, chemokines and cytokines expression. A subseptic dose (2.0mg/kg) of LPS injected into peripheral tissues can induce the expression of pro-inflammatory cytokines IL-1β and IL6, as well as nitric oxide synthase (iNOS) and HO-1 in the hypothalamus and hippocampus of rats(94). Intraperitoneal administration of LPS (10mg/kg) leads to dramatic increases in body temperature, WBC counts and IL6 levels in serum of rats. HO-1 mRNA, protein expression and enzyme activity in liver, lung and kidney increased dramatically in 12hrs after injection. Significant changes in lung congestion, edema, alveolar septal thickening and influx of inflammatory cells indicated that lung is the primary response organ of septic shock(102).

As an anti-inflammation and antioxidant reagent, curcumin showed strong evidence in reduction of the secretion of cytokines and protection of mice from septic shock. Previous *in vitro* studies show that curcumin (5μM) inhibited LPS induced TNF-α, IL1 production and NF-kB activation(124). Curcumin can inhibit nitric oxide synthase (NOS) induction of RAW 264.7 cells treated with LPS and IFN-γ, showing strong evidence of effect as an antioxidant(125).

Curcumin can protect animals from septic shock when mice or rats are administered with sufficient doses of curcumin. Orally treatment of curcumin (40-60 mg/kg body weight) dramatically improved mice mortality from 90% to 30% in mice stimulated with 40mg/kg LPS. Curcumin treated mice showed less diarrhea and watery eyes. LPS treatment induced high expression of the neutrophils adhesion and migration

molecules, ICAM-1 and VCAM-1, in livers and lungs. Curcumin treatment significantly inhibited the expression of ICAM-1 and VCAM-1, leading to less neutrophils migration and infiltration into these organs from blood vessels(126). Pretreatment of rats with curcumin (60mg/kg body weight) prior to LPS (10mg/kg body weight) infusion protected them from septic shock by decreasing serum TNF-α and depletion of peripheral platelets and plasma fibrinogen(127).

To identify how curcumin plays role on LPS induced inflammation, Dr. Roman Jerala's group demonstrated that endotoxin cell surface receptor, myeloid differentiation protein 2 (MD2), is one of the cellular targets. Curcumin may mask the binding site of LPS to TLR4/MD2, leading to the inhibition of MyD88 dependent or independent signaling pathways of LPS signaling(128). Another group also showed that curcumin inhibited ligand (LPS) dependent or independent dimerization of TLR4 to regulate TLR mediated signaling pathways and downstream immune responses. These studies indicate that TLR4 receptor complex could be another cellular target of curcumin in addition to NF-kB/IKKβ(129), STAT3 and NrF2.

Myeloid derived suppressor cells (MDSCs)

Myeloid-derived suppressor cells (MDSCs) are a population of immature myeloid cells with T cell suppression activity, which exhibits inhibitory effects both on innate and adaptive immune systems. The suppression activities are accomplished through a diversity of mechanisms, and targeting cells include CD4+/CD8+ T cells, Treg, DCs, NK cells and macrophages(130). MDSCs were first identified from patients with cancers 20

years ago(131), and the biological significance in the immune system has been established recently by multiple studies.

MDSCs consist of myeloid progenitor cells, immature macrophages, granulocytes and DCs. Myeloid cell lineage differentiation antigen CD11b and Gr1 can well define MDSCs in mice. Macrophage and neutrophil markers Ly6G and Ly6C can further seperate them into granulocytic MDSCs and monocytic MDSCs, and these two subsets may have different functions in immune-suppression. In naïve mice, MDSCs are a main population (20-30%) in bone marrow and are myeloid precursors of mature granulocytes, macrophages and DCs without immunosuppressive properties. Under pathological conditions, such as cancers and some infectious diseases, MDSCs are activated and the number of MDSCs can be dramatically increased to 5-10 fold in the peripheral blood in human, and upregulated to 20-40% in spleen of mice. Other factors, such as bacterial and parasitic infections, drug treatment and even stress, can also induce assembly of MDSCs *in vivo*. Large numbers of MDSCs also accumulate in the tumor sites and the lymph nodes of tumor-bearing mice, contributing to immune dysfunction of the host which favors tumor progression and metastasis(130).

Molecular markers are more complicated and variable due to the lack of a Gr1 gene homolog in humans. Due to the increased circulating MDSCs that are found in cancer patients' blood and expanded from stage I to IV, $CD11b^{+}CD33^{+}CD34^{+}CD14^{-}$ $HLA\text{-}DR^{-}$ as a MDSCs marker in humans is acceptable with some variations. For example, a new population of MDSCs, CD14+/CD11b+/HLA-DRlow/neg, was found in metastatic melanoma patients but not healthy donors(132), and was detected in peripheral

blood and tumor infiltrating lymphocytes of hepatocellular carcinoma patients to induce CD4(+)CD25(+)Fox3(+) Treg cells(133).

MDSCs are an important regulator, linking inflammation and cancer. Acute or chronic inflammation induces accumulation and activation of MDSCs. Activated MDSCs will suppress the host immune system, interrupt immunosurveillance and produce a favorable environment for tumor growth. Several factors induce accumulation and activation of MDSCs, many of which are proinflammatory molecules. Inflammation related molecules or proinflammatory cytokines, such as IFN-γ, TNF-α(134), IL1-β(135) and IL6(136), can induce MDSCs accumulation. Thereby, reduced inflammation achieved by IL1 receptor-deficient mice leads to delayed accumulation of MDSCs and less primary and metastasis tumor initiation, and *vise versa*(136). The potent inflammatory mediator, prostaglandin E2 (PGE2), binding to receptors on MDSCs, induces the differentiation of MDSCs from bone marrow stem cells(137). Proinflammatory heterodimeric protein S100A8/A9 complex can induce MDSCs accumulation and enhance MDSCs migration, which is regulated by NF-kB pathways(138).

Animal model studies show that MDSCs activation may represent a conserved response to inflammation, and the regulation of this population may lead to new therapeutic approaches to eliminate MDSCs and prevent tumors in clinical studies. LPS together with IFN-γ treatment activated MDSCs and suppressed DCs development *in vivo* (139). LPS desensitization led to $CD11b^{+}Gr1^{+}$ activation in bone marrow, spleen and lymph nodes. $CD11b^{+}Gr1^{+}$ cells suppressed T-cell proliferation and Th1 and Th2 cytokine production through IL-10 production and HO-1 expression(140). LPS

instillation in human lung activated $CD11b^+$ neutrophils and increased secretion of chemokines. LPS activated alveolar macrophages, leading to increased proinflammatory mediator expression and cytokine release *ex vivo*. There are several studies which demonstrate that exosomes can regulate MDSCs accumulation and activation. Texs can induce MDSCs accumulation, expression of COX-2, IL6, VEGF and arginase-1 and promote tumor progression(141). Chalmin *et al* and Liu *et al* found that tumor derived exosomes activate STAT-3 upon IL6 induction through TLR2/MyD88 pathway in MDSCs (142), and Hsp72 expressed at the surface of tumor derived exosomes bound to TLR2 and promoted MDSCs suppressive functions(143). Exosomes induced $CD14^+$ monocytes to differentiate into HLA-DR cells and these cells were responsible for significant increased release of IL6, TNF-α and TGF-β and the suppressive effects on activated T cell proliferation and cytolytic functions. Both *in vitro* and *in vivo* data indicate that MDSCs activity is mediated by TGF-β, a circulating proinflammatory cytokine; thereby, tumor cells can achieve their progression and metastasis through circulating exosomes, growth factors and inflammatory cells without cell-cell contact.

Due to the suppressive activity of MDSCs, MDSCs are potential therapeutic targets to treat inflammatory and inflammation related diseases in clinical studies. The efforts are focused on using drugs to reduce activated MDSCs and on interfering with the inhibitory function of MDSCs.

One way to decrease MDSCs is to force the differentiation of MDSCs into mature cells. High levels of ROS are essential to keep an immature phenotype of MDSCs. All trans-retinoic acid (ATRA), a derivate of vitamin A, induced MDSCs differentiation into DCs, macrophages and granulocytes through decreasing glutathione levels in myeloid

cells by increasing the expression of glutathione synthase gene(144). Vitamin D3 treatment has also been shown to protect the lung of HNSCC patients through decreasing MDSCs and improving T cell function (145). Interestingly, NKT cells have the ability to convert immunosuppressive MDSCs into immunogenic APCs, making MDSCs a potential anti-tumor cell-based vaccine(146).

Another way to decrease activated MDSCs is to disrupt MDSCs maturation. STAT3 activity is a critical regulator of MDSCs function. Inhibition of activated STAT3 in cancer patients by small peptides or STAT3 inhibitors can eliminate MDSCs (147, 148). In addition, the tyrosine kinase inhibitor, sunitinib, can inhibit STAT3 activation in renal cell carcinoma cells (149), leading to elimination of MDSCs. Sunitinib also proved to reduce MDSCs through decreasing IL-10, TGF-β, and Foxp3, increasing Th1 cytokine IFN-γ expression and inducing CTL responses in isolated tumor infiltrating leukocytes(150, 151). Several other molecules that may induce differentiation of MDSCs have been studied experimentally. Interferon regulatory factor-8 (IRF-8), a member of the IFN-γ regulatory factor family, regulates normal cell myelopoiesis, and is also an important regulator of tumor induced accumulation of MDSCs (152-154). Anti-VEGF receptor therapy can inhibit MDSCs and Treg infiltration, and thereby increase DC maturation through downregulation IL-1β, IL6 and CXCL1(155). Arginine metabolism modulators, N(G) nitro-L-arginine methyl ester (L-NAME) and sildenafil, can decrease the population of MDSCs, and thereby inhibit tumor growth(156). Accumulated reports show that MDSCs play critical roles in tumor growth and progression. Targeting maturation of MDSCs is feasible and may represent a strategy for chemotherapy in the future.

The suppressive activity of MDSCs depends on the intracellular metabolism of L-arginine, a substrate for two enzymes, inducible-nitric oxide synthase (iNOS/NOS2), which generate nitric oxide (NO), and arginase 1 (ARG1), which catalyze the metabolism of L-arginine into urea and L-ornithine. ARG1 exerts its immuno-suppressive function through decreasing the expression of CD3ξ chain T cells and arresting T cells in G0-G1 phase. Meanwhile, NOS2 regulated NO production will disrupt T cell activation. More severely, super-activated ARG1 leads to L-arginine starvation, and this stimulates NOS2 to produce reactive oxygen species (ROS) and additional toxic reactive nitrogen species, such as peroxynitrites, to disrupt intracellular signaling pathways and induce T lymphocytes apoptosis. ARG1 and NOS2, co-expressed in MDSCs, are potent molecular markers of MDSCs and potential molecular targets that can interfere with the functions of MDSCs (157). COX-2, overexpressed in most human cancers, can stimulate ARG1 and NOS2 secretion through prostaglandin E2. To disrupt the suppressive activity of MDSCs, inhibition of COX-2 activity should be an effective approach. COX-2 inhibitors can decrease IL10 and MDSCs through increasing lymphocytic infiltration into tumors or increase IL12 and IFN-γ secretion (158). Curcumin can inhibit COX-2 activity, and may have the potential to eliminate MDSCs. In addition, the phenotypic expression of Hsp72 on tumor-derived exosomes may enhance the suppressive activity of MDSCs through STAT3 activation. Amiloride, a drug that inhibit the formation of exosomes, can restore immunosurveillance by interfering with the suppressive function of MDSCs (143). CSF1R signaling is important for MDSCs recruitment and function. A selective CSF1R signaling inhibitor, GW2580, can inhibit tumor recruitment of CD11b(+)Gr-1(lo)Ly6C(hi)

mononuclear MDSCs(159). Interestingly, a chemotherapy agent Gemcitabine can directly inhibit MDSCs and tumor growth although the mechanism is not clear(158, 160).

Significance

This study identified a novel natural nanoparticle delivery system. Encapsulation of curcumin into exosomes can increase solubility and stability *in vitro* and bioavailability *in vivo* of curcumin. More importantly, exosomal curcumin shows higher anti-inflammatory activity than free curcumin. For the first time, we identify that curcumin can induce selected death of $Gr1^+$ cells and down-regulate this population in the lungs of mice. Exosomal curcumin can increase this down-regulation.

This is just a fundamental study of exosomes-curcumin drug delivery system. The use of exosomal curcumin may take advantage of functional properties of curcumin, exosomes and nanoparticles in an additive way to fight various diseases. Exosomal curcumin will be a powerful and highly efficient treatment of inflammatory diseases and even cancers with few side effects; however, uncovering all the power of exosomal curcumin and other drugs contained in exosomes will require further studies.

A NOVEL NANOPARTICLE DRUG DELIVERY SYSTEM-THE ANTI-INFLAMMATORY ACTIVITY OF CURCUMIN IS ENHANCED WHEN ENCAPSULATED IN EXOSOMES

by

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ABSTRACT

Monocyte-derived myeloid cells play vital roles in inflammation-related autoimmune/inflammatory diseases and cancers. Here, we report that exosomes can deliver anti-inflammatory agents, such as curcumin, to activated myeloid cells *in vivo*. This technology provides a means for anti-inflammatory drugs, such as curcumin, to target the inflammatory cells as well as to overcome unwanted off-target effects that limit their utility. Using exosomes as a delivery vehicle, we provide evidence that curcumin delivered by exosomes is more stable and more highly concentrated in the blood. We show that the target specificity is determined by exosomes, and the improvement of curcumin activity is achieved by directing curcumin to inflammatory cells associated with therapeutic, but not toxic, effects. Furthermore, we validate the therapeutic relevance of this technique in a lipopolysaccharide (LPS)-induced septic shock mouse model. We further show that exosomes, but not lipid alone, are required for the enhanced anti-inflammatory activity of curcumin. The specificity of using exosomes as a drug arrier creates opportunities for treatments of many inflammation-related diseases without significant side effects due to innocent bystander or off-target effects.

INTRODUCTION

The treatment of inflammatory disorders must rely on targets that are present in both healthy and diseased tissues. Achieving therapeutic effects on the inflammatory cells often requires high drug doses that can induce unwanted effects on other tissues. Thus, although the current target-based drug design paradigm efficiently finds candidate drugs

that are selective in a molecular sense, cell-type specificity is lacking that prevents their use *in vivo*at effective doses.

Monocyte-derived myeloid cells represent a large pool of scavenger and potential effector cells during inflammatory processes (1–4). Uncontrolled activation of myeloid cells leads to chronic inflammation. The process of myeloid cell–mediated inflammation has been shown to play a major role in most chronic illnesses, including neurodegenerative (5–7), cardiovascular (8–12), pulmonary (13–15), metabolic (15–17), autoimmune (18–22) and neoplastic diseases (23–28).

Exosomes released from many different types of cells can transfer information to recipient cells. Activated monocytederived myeloid cells act as scavengers and have a high capacity for taking up vesicles, like exosomes circulating in the peripheral blood, through their phagocytic function (29–33).

Curcumin, a natural polyphenol found in the rhizomes of *Curcuma longa* (turmeric), exhibits anti-inflammatory, antineoplastic, antioxidant, and chemopreventive activity (34–38). Several clinical trials dealing with cancers have addressed the pharmacokinetics, safety, and efficacy of curcumin in humans (36). Despite extensive research and development, poor solubility of curcumin, due to its hydrophobic property and preferential interaction with lipid membranes, remains a major barrier in its bioavailability and clinical efficacy (39). To increase its solubility and bioavailability, attempts have been made through encapsulation in liposomes, polymeric nanoparticles, biodegradable microspheres, cyclodextrin, and hydrogels (40–49).

In this study, we show that exosomes released from a number of different cell types form a complex with curcumin. Mice treated with curcumin complexed with

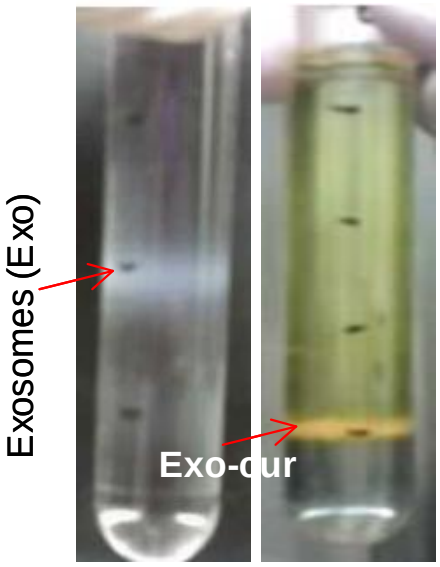
exosomes are protected against lipopolysaccharide (LPS)-induced septic shock. These data show that curcumin complexed with exosomes are taken up by activated monocyte-derived myeloid cells circulating in the peripheral blood and that curcumin subsequently induces apoptosis in these monocytes. In addition, the formation of exosome–curcumin complexes also leads to an increase in the stability of curcumin *in vitro* and bioavailability *in vivo*. We consider these findings to have significant impact on target-based drug development for successful *in vivo* drug delivery to treat inflammatory related diseases.

RESULTS

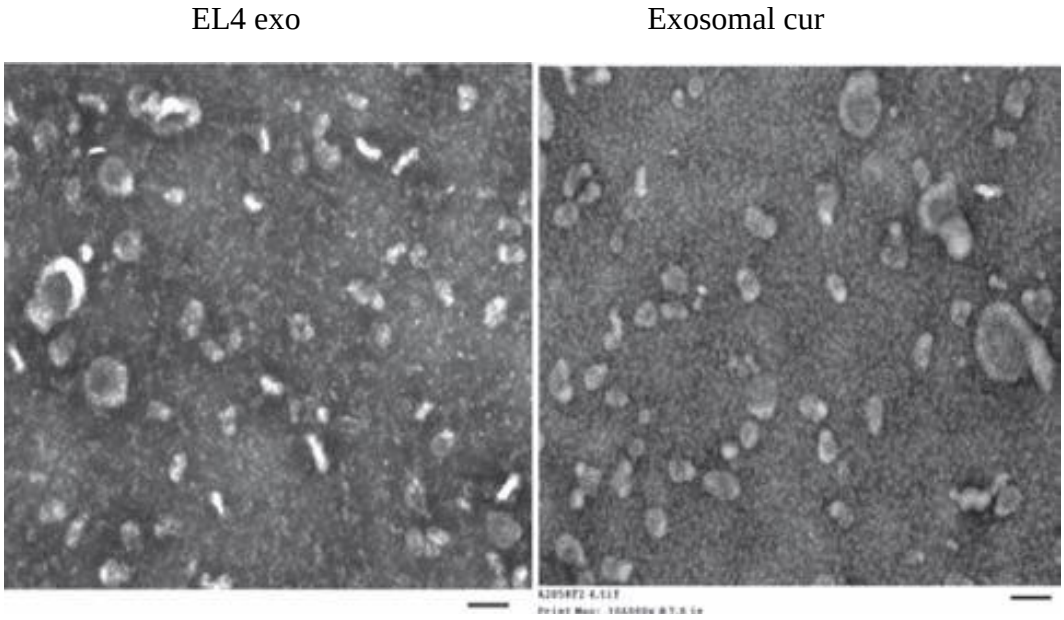
Incorporation of curcumin into exosomes

Exosomes are 30–100 nm nanoparticles secreted by cells into the extracellular environment. To exploit the possibility of nanoparticle exosomes as a carrier to entrap curcumin, curcumin was mixed with EL-4-derived exosomes at 22 °C, and then subjected to sucrose gradient centrifugation. A yellowish band (Figure 1a, right) appeared between the 45 and 60% sucrose gradients, with a weak band appeared between the 30 and 45% gradients (original exosomes, Figure 1a, left). The yellowish band was collected, washed and dissolved in phosphate-buffered saline (PBS). This fraction was designated exosomal curcumin. The morphology and the size of exosomal curcumin were similar to the original exosomes (Figure 1b). Exosomal protein markers, such as TSG101 and CD81 (Figure 1c), were identified in the exosomal curcumin. The binding capacity was estimated to be 2.9 g curcumin to 1 g exosomes. Exosomes isolated from other types of cell lines, including MDAMB231 (human adenocarcinoma), 4T-1 (murine breast tumor

a


Exosomes (Exo)
Exo-cur

b


EL4 exo
Exosomal cur

c

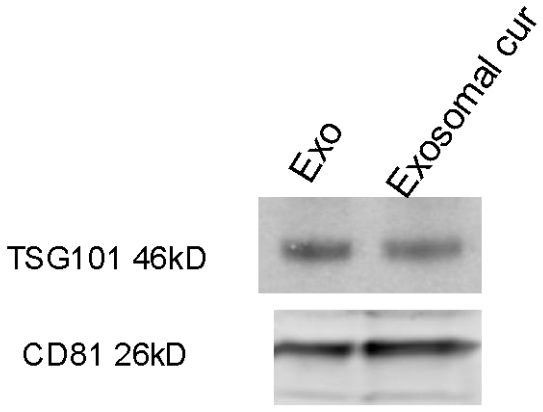

Exo
Exosomal cur
TSG101 46kD
CD81 26kD

Figure 1 Encapsulation of curcumin into exosomes. (**a**) Exosomal curcumin purification. Five minutes after co-incubation of curcumin with EL-4 exosomes, the mixture was loaded on a sucrose gradient (8, 30, 45, and 60%, respectively) for centrifugation. A representative photo of exosomal curcumin is shown between the sucrose gradient layers of 45 and 60% (right, exosomal cur), instead of the 30 and 45% layers (left, original exosomes, Exo). (**b**) Electron microscopic images of EL-4 exosomes and exosomal curcumin. Purified exosomes and exosomal curcumin were imaged by a Hitachi H7000 electron microscope. Bar = 100 nm. (**c**) Western blot analysis of exosomes and exosomal curcumin protein expression. Equal amounts of exosomal protein and exosomal curcumin were loaded onto a 10% SDS-PAGE gel. TSG101 and CD81 expression were determined by blotting with monoclonal antibodies against TSG101 and CD81 (Santa Cruz, CA).

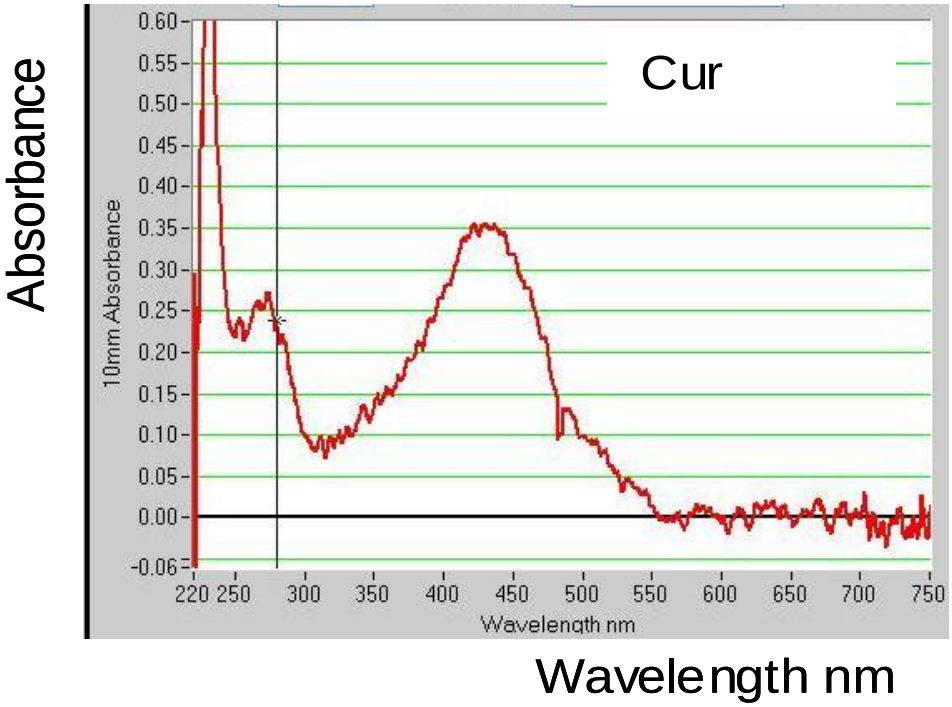
cell line), and primary mouse embryonic fibroblasts, also have similar efficiency in terms of binding curcumin (data not shown).

Incorporation of curcumin into exosomes can increase the solubility, stability and bioavailability of curcumin

Curcumin is a hydrophobic polyphenol compound that is insoluble in aqueous solution. To determine whether the binding of curcumin to exosomes can increase curcumin's solubility, an identical amount of curcumin was mixed in an equal volume of PBS or exosomes in PBS, and the mixtures were placed on ice for 30 minutes. To estimate curcumin solubility, the concentration of curcumin in the supernatant was determined using a Nanodrop 1000 spectrophotometer. The curcumin concentration in the mixture of curcumin and exosomes was appropriately fivefold higher than curcumin alone (Figure 2a). Thus, the solubility of exosomal curcumin is higher than free curcumin and this is due to the binding of curcumin to exosomes.

Curcumin is relatively unstable, and this is one of the major barriers for clinical use of curcumin to treat cancer and other inflammation-related diseases.15 To determine whether exosomal curcumin is more stable, free curcumin and exosomal curcumin were incubated at 37 °C over a period of 150 minutes and sampled periodically to determine the concentration of curcumin. After incubation for 150 minutes at 37 °C, we found that free curcumin in PBS degraded quickly and only 25% remained after the 150 minute incubation when compared to the 0-minute sample (set as 1.0). Curcumin in exosomal curcumin was protected from degradation with >80% remaining after the 150-minute incubation in PBS (pH 7.4, Figure 2b).

a.


Absorbance
Cur
10mm Absorbance
0.60
0.55
0.50
0.45
0.40
0.35
0.30
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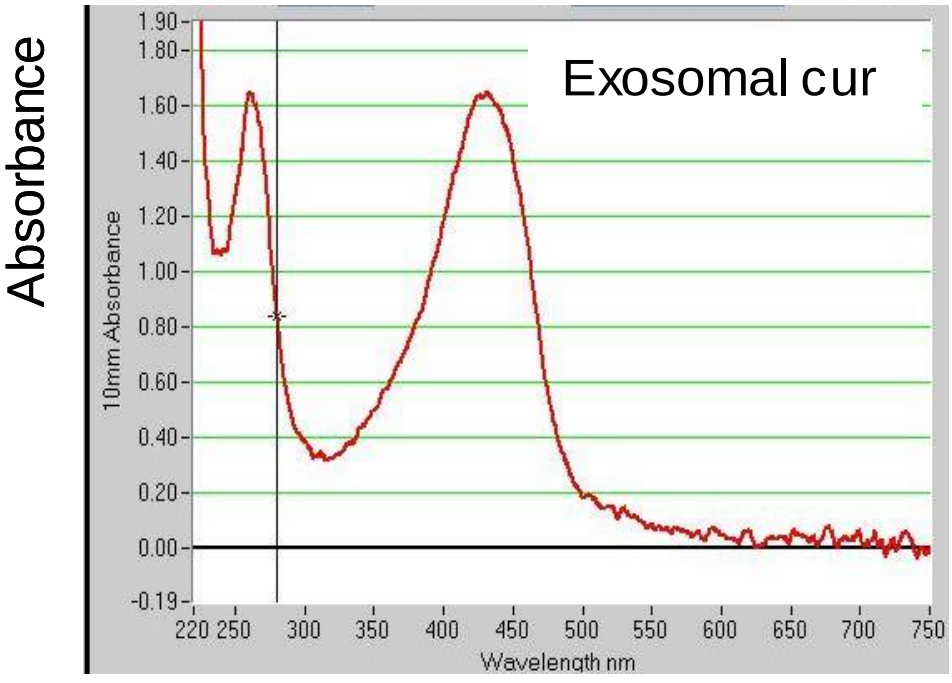
220 250 300 350 400 450 500 550 600 650 700 750
Wavelength nm
Wavelength nm


Absorbance
Exosomal cur
10mm Absorbance
1.90
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1.60
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1.20
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220 250 300 350 400 450 500 550 600 650 700 750
Wavelength nm
Wavelength nm

b.

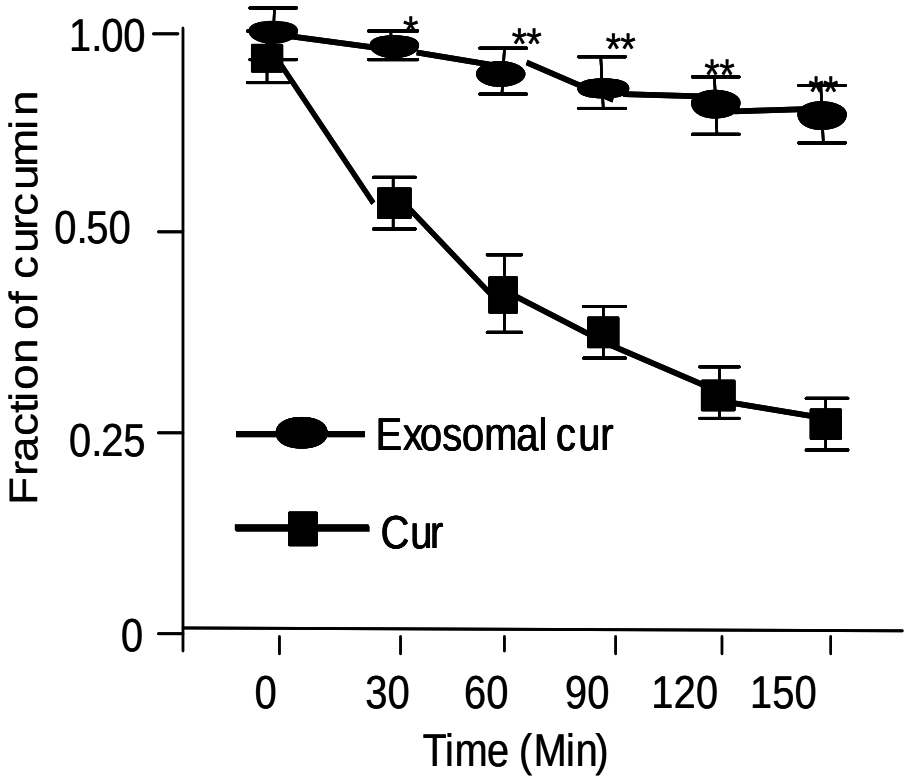

1.00
0.50
0.25
0
Fraction of curcumin
*
**
**
**
**
Exosomal cur
Cur
0
30
60
90
120
150
Time (Min)

Figure 2 Encapsulation of curcumin into exosomes can increase curcumin's solubility and stability *in vitro*. (**a**) Exosomal curcumin is more soluble in phosphate-buffered saline (PBS) than free curcumin. An identical amount of curcumin was mixed with an equal volume of PBS or exosomes. Representative spectrographs of curcumin (Cur) and exosomal curcumin (exosomal cur) in the supernatant are shown, and the spectrographs were obtained using a Nanodrop 1000 spectrophotometer at OD420. (**b**) Exosomal curcumin is more stable than free curcumin. Curcumin and exosomal curcumin were added to 2 ml PBS to achieve a final concentration of 30 μmol/l and incubated in the dark in a 37 °C water bath. At 30, 60, 90, 120, and 150 minutes, 100 μl of each sample was taken to determine the concentration of curcumin. The concentrations of curcumin or exosomal curcumin at the beginning were set as 1.00. The fold reduction of the concentration at each time point compared to the beginning is shown. The experiments were repeated three times for each time point (*$P < 0.05$, **$P < 0.01$).

Another major barrier for clinical use of curcumin is its low systemic bioavailability (15). After administering orally, curcumin is digested in the stomach. Along with other food, curcumin then passes through the intestinal wall into the enterohepatic circulation, arriving in the liver for detoxification (the first-pass effect) and eventually gets into the blood stream. Curcumin's low bioavailability may be due to the rapid first-pass effect and the fast intestinal glucuronidation metabolism. To assess whether exosomal curcumin can increase bioavailability of curcumin, free curcumin, and exosomal curcumin (before purification, see Materials and Methods) was administered intraperitoneally (i.p.) or orally at a dose of 100 mg/kg of body weight. Due to the low bioavailability of curcumin, we used an extremely high dose of curcumin to achieve detectable curcumin in curcumin-treated mice. Curcumin in plasma was quantified at 0.5, 1, 2, 4, 8, and 12 hours after administration using an established high performance liquid chromatography (HPLC) method. Figure 3 and Supplementary Figure S1 show that at 30 minutes, i.p. administration of exosomal curcumin led to a five- to tenfold higher curcumin accumulated in peripheral blood than that of curcumin alone. At 12 hours after i.p. injection, curcumin in the plasma still remained at a much higher level in the group of mice injected with exosomal curcumin. In contrast, there was no detectable curcumin circulating in the blood of mice treated with curcumin alone. Similar results were obtained when mice were administrated with curcumin or exosomal curcumin orally (data not shown). Collectively, our data suggest that exosomal curcumin is stable in the circulation.

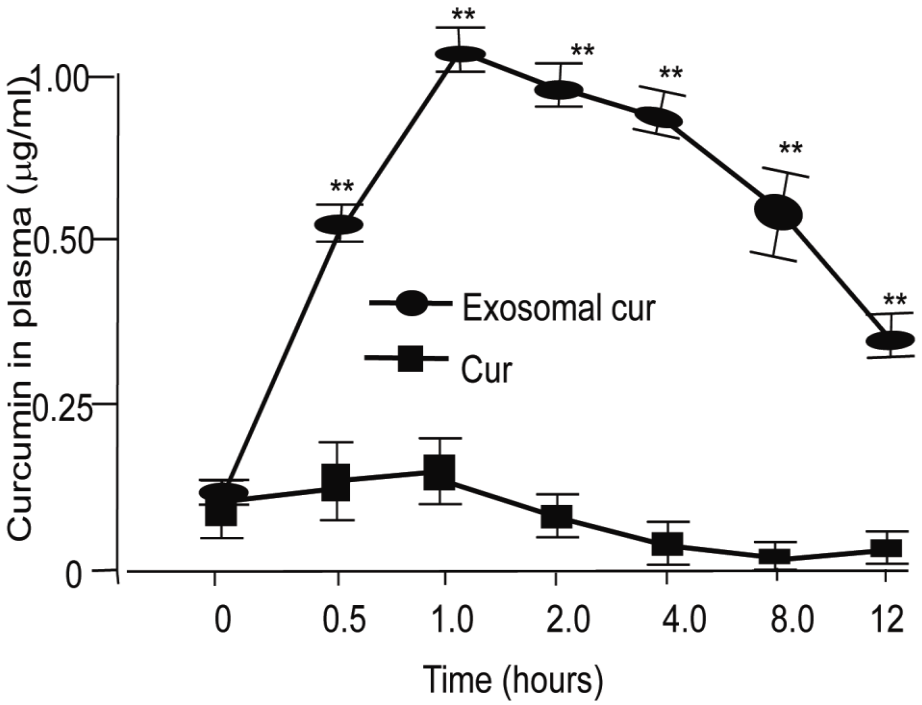


Figure 3 Encapsulation of curcumin into exosomes can increase bioavailability of curcumin *in vivo*. Two groups (five per group) of C57BL/6j mice were injected i.p. with 100 mg curcumin or exosomal curcumin/kg of body weight. Thirty minutes, 1, 2, 4, 8, and 12 hours after injection, blood samples were taken, and the concentration of curcumin in the plasma was determined by high performance liquid chromatography as described in the Materials and Methods. Naive mice without any treatment were used as controls. A Tukey's pairwise comparison with analysis of variance was performed to determine the difference between the groups. Standard deviation (±SD) is presented as error bars. $^{**}P < 0.01$.

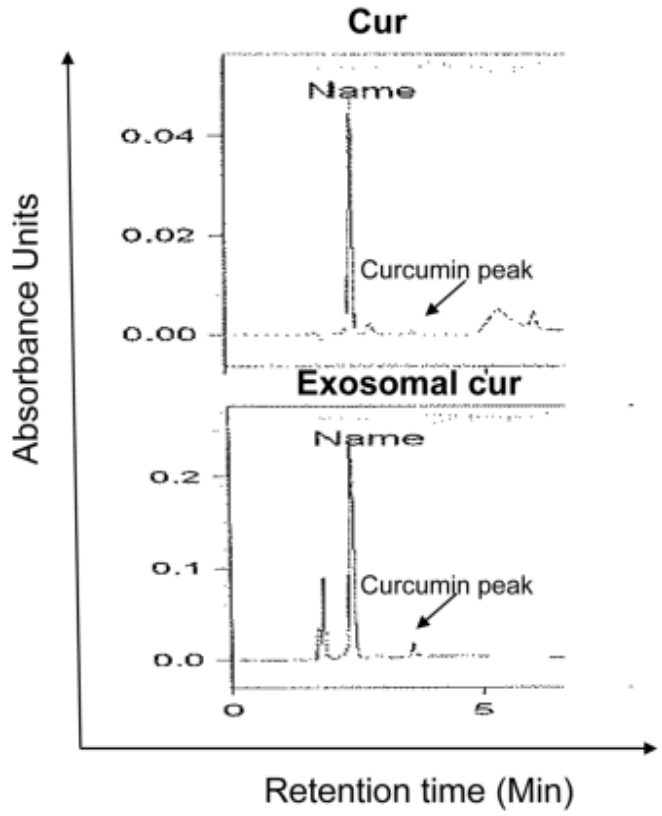


Figure S1 Exosomal curcumin is more stable than free curcumin in vivo.

Anti-inflammation activity of exosomal curcumin

Our data show that, as a nanoparticle drug carrier, exosomes can increase curcumin's solubility and stability *in vitro*, and bioavailability *in vivo*. We speculate that exosomal curcumin can enhance the anti-inflammatory activity of curcumin by accumulating curcumin to high levels in cellular targets. To evaluate the anti-inflammatory activity of exosomal curcumin *in vitro*, RAW 264.7 cells were treated with curcumin or exosomal curcumin at a concentration of 20 μmol/l for 1 hour. Subsequently, treated cells were stimulated with LPS (50 ng/ml) for an additional 6 hours. Cytokine production in the supernatant was measured 6 hours after treatment. As shown in Figure 4, exosomal curcumin– treated macrophages produced significantly less IL-6 and TNF-α in comparison with curcumin treatment alone.

To assess the anti-inflammatory activity of exosomal curcumin in *vivo*, we adapted a LPS-induced septic shock model. To monitor mice mortality, a LD50 (median lethal dose) of LPS was determined first. LPS (5 mg/ml, Sigma-Aldrich) was prepared with sterile H2O, and varied amounts of LPS were i.p. injected into the same batch of commercially supplied C57BL/6j mice. Each group contained 10 mice. The LD50 was 18.75 mg of LPS/kg of body weight. C57BL/6j mice were i.p. injected with LPS (18.75 mg/kg) together with curcumin, or exosomal curcumin (4 mg/kg body weight) treatments. There was a significant survival advantage for mice treated with exosomal curcumin as compared to mice treated with an equivalent concentration of free curcumin over a 4-day period (Figure 5a). Exosomes and PBS injections serving as controls had no effects on LPS-induced mouse mortality. Sixteen hours after i.p. injection of LPS, the sera levels of IL-6 and TNF-α were similar in mice treated with free curcumin, exosomes,

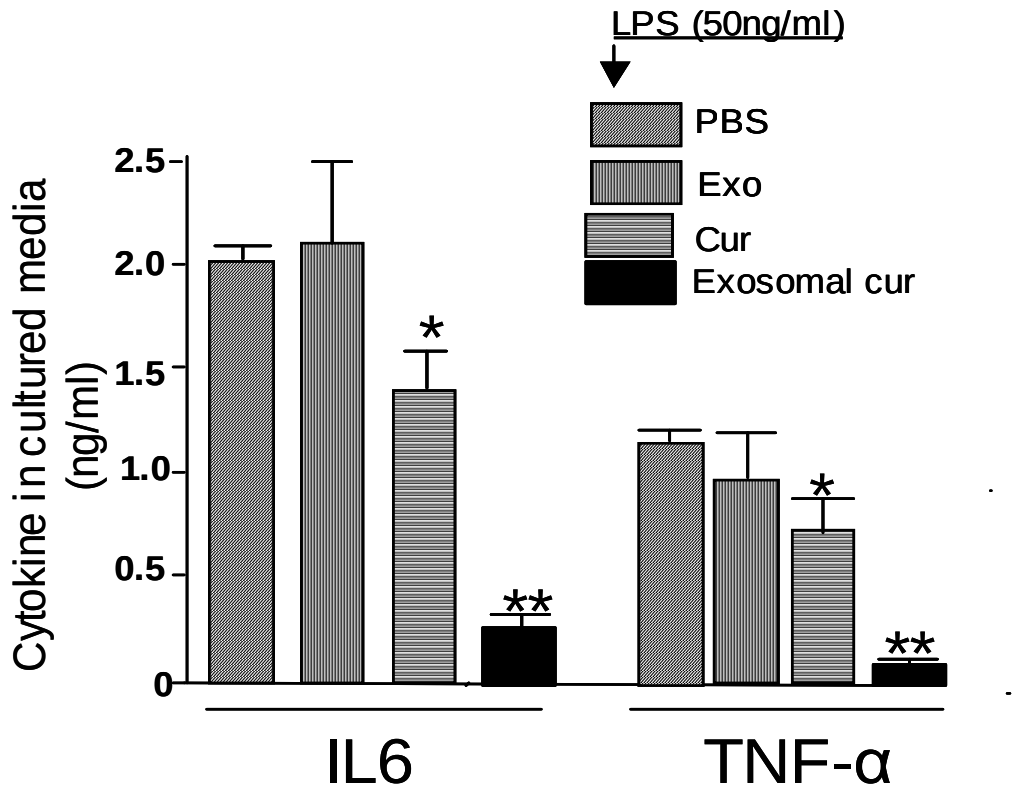


Figure 4 Encapsulated curcumin had a higher inhibitory effect on IL-6 and TNF-α secretion than free curcumin *in vitro*. RAW 264.7 cells were plated into 24-well plates and incubated overnight. The cells were then treated with curcumin or EL-4 exosomal curcumin at a concentration of 20 μmol/l for 1 hour and then stimulated with LPS (50 ng/ml) for an additional 6 hours. RAW 264.7 cells treated with PBS or exosomes served as controls. TNF-α and IL-6 in the culture supernatant were determined using a standard enzyme-linked immunosorbent assay. Data are represented as mean ± SEM (n = 6). *P < 0.05, **P < 0.01. LPS, lipopolysaccharide; PBS, phosphate-buffered saline.

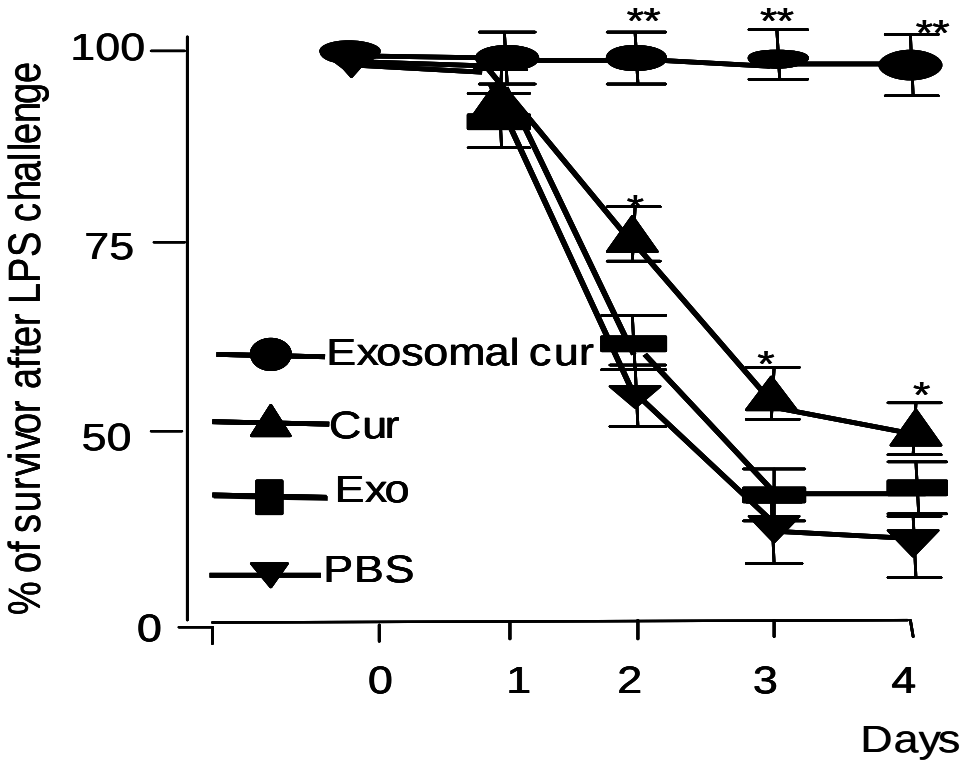
and PBS. However, both cytokines were significantly lower in the exosomal curcumin–treated group of mice (Figure 5b) and this finding correlated with mortality data.

Exosomal curcumin decreased $CD11b^{+}Gr1^{+}$ cells in the lungs of mice

To further identify specific targeting of organs by exosomes, *in vivo*biodistribution of fluorescent dye IRDye800-labeled EL-4 exosomes was conducted in mice using Odyssey imaging. For these studies, we evaluated distribution i.p. injection of IRDye800-labeled EL-4 exosomes. Within an hour after injection, fluorescent signals were predominantly detected in liver, lung, kidney, and splenic tissues (Figure 6a, right panel). As we demonstrated before (32), flow cytometric analysis revealed that PKH67 fluorescent dye–labeled exosomes were taken up by CD11b+Gr-1+ cells (data not shown).

One of the features of LPS-induced septic shock is a robust increase of the number of CD11b+Gr-1+ cells that are sequestered in the lungs leading to acute lung inflammation (26, 27). In LPS-treated mice, there were significantly fewer CD11b+Gr-1+ cells in the lungs of the mice treated with exosomal curcumin when compared to mice treated with curcumin, exosomes, or PBS (Figure 6b). We and others have reported previously that tumor exosomes are taken up by CD11b+Gr-1+ cells circulating in the peripheral blood (25, 28). To find whether exosomal curcumin can take advantage of this property, Gr-1+ cells were isolated from bone marrow cells and cocultured with curcumin, or exosomal curcumin at the same concentration of curcumin. At 1, 2, and 3 hours, cellular curcumin concentration was measured (**Figure 6c**); an increase in curcumin did not occur in cells treated with an equivalent amount of exosomes and curcumin together (data not shown). Interestingly, curcumin induces Gr-1+ cell apoptosis when Gr-1+ cells are treated with

a


100
75
50
0
% of survivor after LPS challenge
Exosomal cur
Cur
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PBS
0
1
2
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Days
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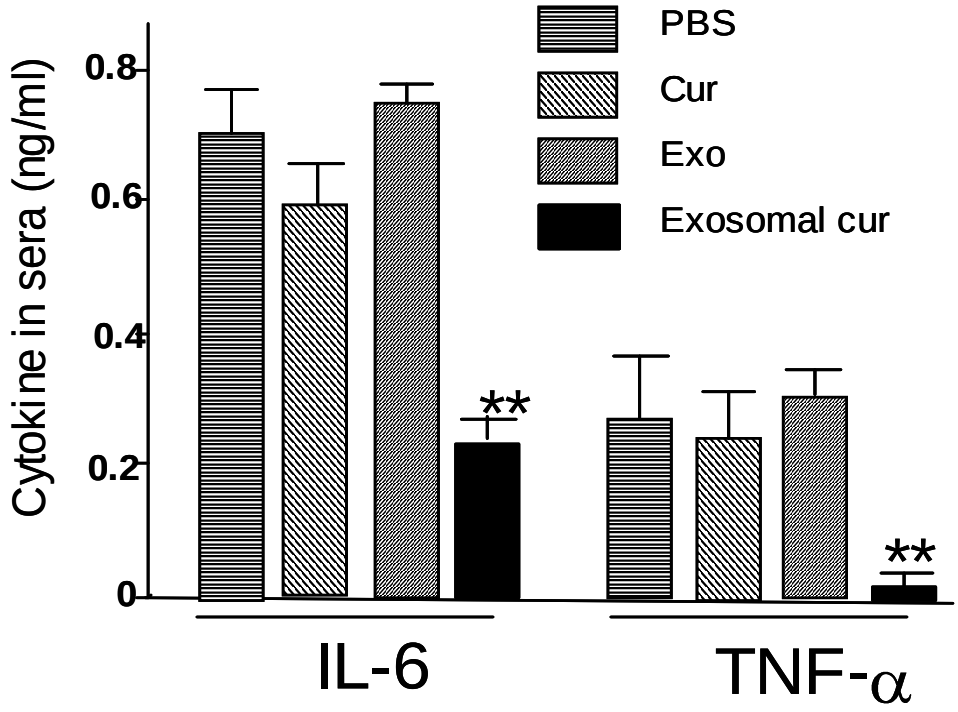
b


Cytokine in sera (ng/ml)
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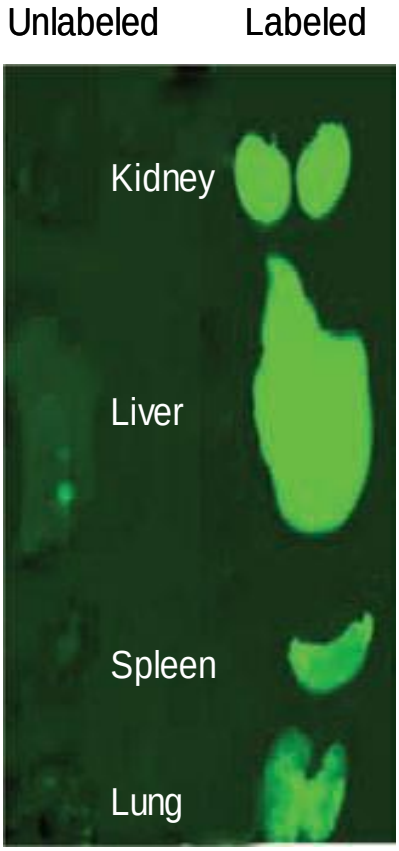
0.6
0.4
0.2
0
PBS
Cur
Exo
Exosomal cur
**
**
IL-6
TNF-α

Figure 5 Exosomal curcumin can protect mice from LPS-induced septic shock. (**a**) Curcumin or exosomal curcumin (4 mg/kg) was injected i.p. into C57BL/6j mice together with LPS (18.5 mg/kg, Sigma-Aldrich). An equal amount of EL-4 exosomes used in the exosomal curcumin was used in the control. Mouse mortality was monitored over a period of 4 days. Data represented 10 mice/group and pooled from two experiments (**$P < 0.01$, *$P < 0.05$). (**b**) Exosomal curcumin can decrease IL-6 and TNF-α secretion in LPS-stimulated mice. The sera were collected 16 hours after LPS injection, The sera were collected 16 hr after LPS injection, and IL-6 and TNF-α concentrations in the sera were determined using an enzyme-linked immunosorbent assay (**$P < 0.01$).

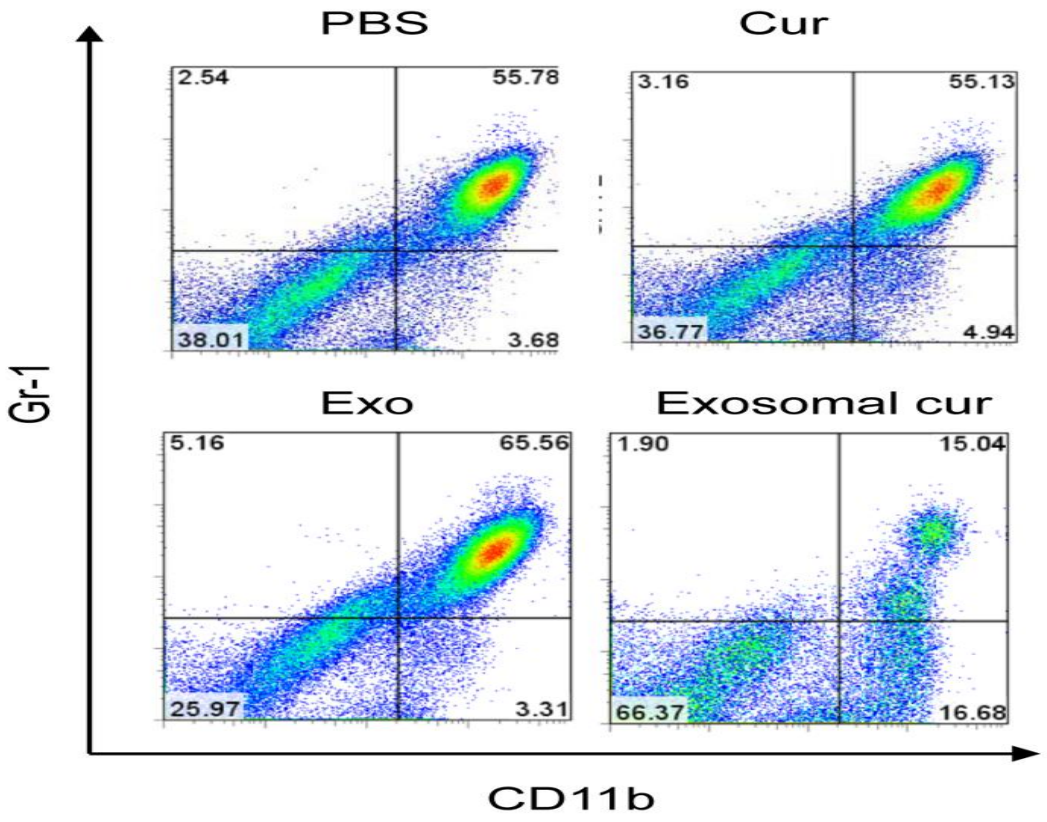
5 and 10 μmol/l curcumin, and exosomal curcumin can significantly enhance this induction as determined by fluorescence-activated cell sorting (FACS) analysis (**Figure 6d**). Gr-1+ cells treated with exosomes alone had a similar apoptosis percentage as the controls (data not shown).

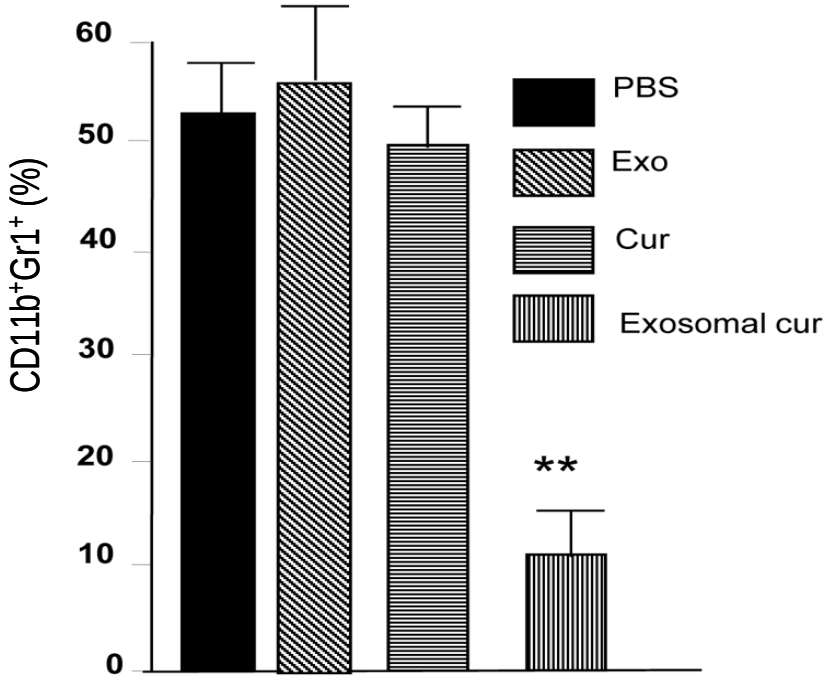
To determine whether the exosomes are functioning as a delivery vehicle or are contributing to the therapeutic effects, an *in vivo* liposome curcumin treatment of LPS-challenged mice was conducted. The results indicate that mice treated with exosomal curcumin have much lower mortality than mice treated with an equivalent concentration of liposome curcumin (**Figure 7**). The inefficiency of liposome curcumin to prevent LPS-induced septic shock is most likely not due to an insufficient inhibition of anti-inflammatory pathways because liposome curcumin treatment of bone marrow–derived macrophages has a similar effect as exosomal curcumin on the prevention of IκBα degradation as well as prevention of Cox2 induction after LPS stimulation (**Supplementary Figure S2**). These data suggest that exosomes are most likely not simply functioning as curcumin carrier-like liposomes. The increase in curcumin uptake by Gr-1+ cells with an increase in cell apoptosis caused by exosomal curcumin treatment may be one of the mechanisms underlying protection of mice from LPS-induced septic death.

a



b


PBS
Cur
Exo
Exosomal cur
2.54
55.78
38.01
3.68
3.16
55.13
36.77
4.94
5.16
65.56
25.97
3.31
1.90
15.04
66.37
16.68
Gr-1
CD11b


60
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CD11b+Gr1+ (%)
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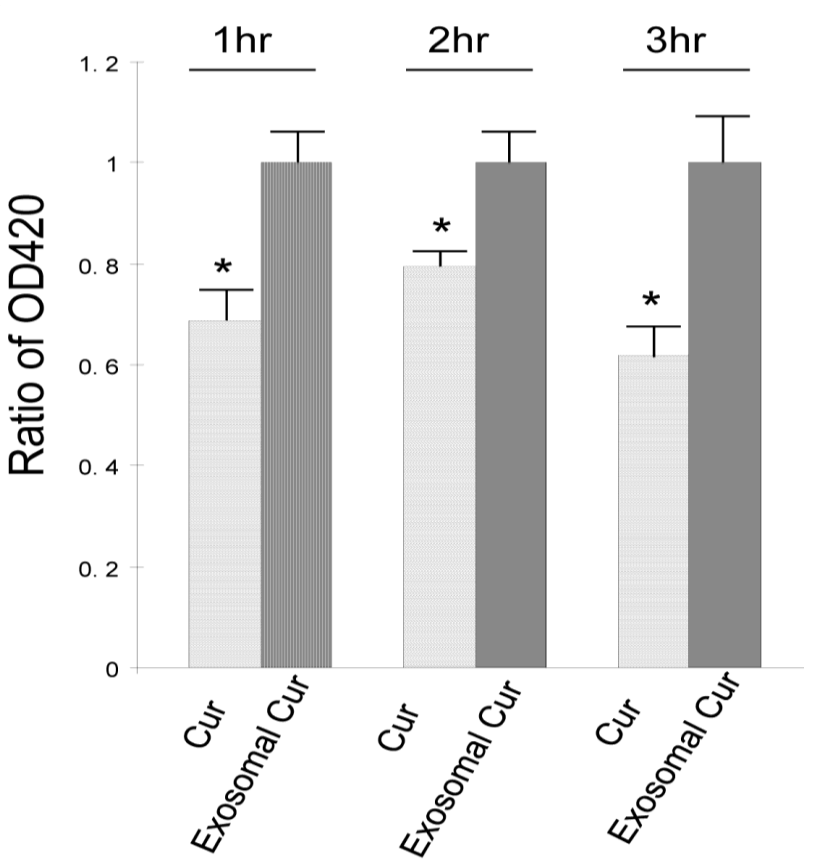
1hr
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Ratio of OD420
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1
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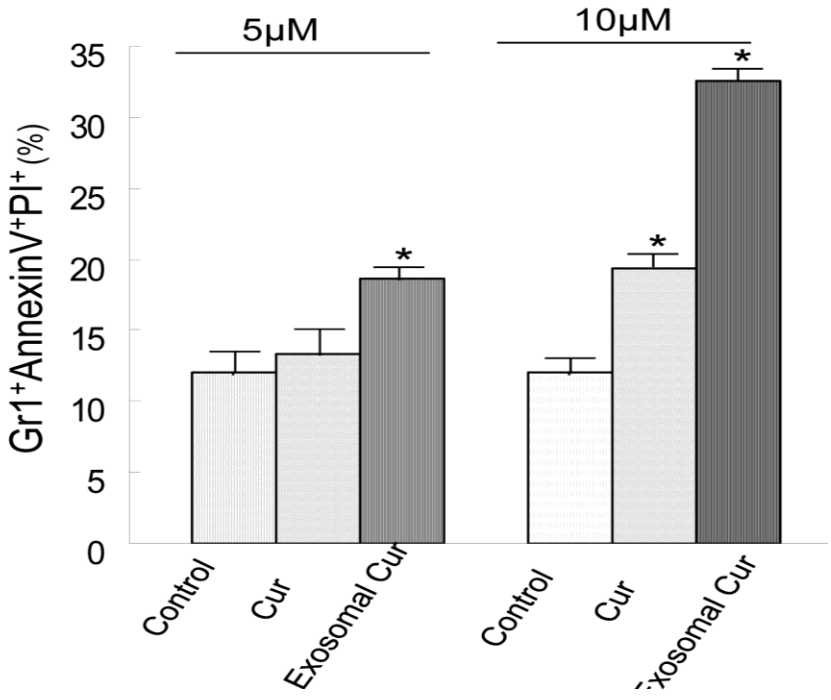
5μM
10μM
Gr1+AnnexinV+PI+ (%)
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Figure 6 Exosomal curcumin can downregulate the CD11b+Gr-1+ cell population during lipopolysaccharide (LPS)-induced inflammation. (**a**) Biodistribution of i.p.-injected EL-4 exosomes. A total of 100 μg of IRDye 800-labeled EL-4 exosomes or unlabeled EL-4 exosomes were injected i.p. into C57BL/6j mice. Each organ imaging was done at 1 hour after injection. (**b**) Exosomal curcumin treatment dramatically decreased the CD11b+Gr-1+ cell population in the lungs. At day 1 after LPS challenge, mice were killed, and the leukocytes in the lungs were isolated. The percentage of cells in the lung was determined by fluorescence-activated cell sorting (FACS) analysis, and representative analyses are shown (left). Statistical significance was analyzed as before (**$P < 0.01$, right). (**c**) Gr-1+ cells incorporated more exosomal curcumin than free curcumin. Gr-1+ cells were selected from bone marrow cells and then treated with the same concentration of free curcumin and exosomal curcumin. At 1, 2, and 3 hours, cells were collected and washed with PBS. Curcumin in the cells was extracted by methanol, and the concentration was determined using a Nanodrop 1000 spectrophotometer. At each time point, curcumin concentration in exosomal curcumin–treated cells was set as 1.0. The ratio of curcumin concentration in free curcumin–treated cells compared to exosomal curcumin–treated cells was reported. Triplicates of each sample were performed. *$P < 0.05$. (**d**) Exosomal curcumin induced more Gr-1+ cell death in culture. Gr-1+ cells were selected from bone marrow cells and then treated with free curcumin and exosomal curcumin. After 8 hours, cells were collected and immunostained with AnnexinV-FITC and PI. The percentage of AnnexinV+PI+ Gr-1+ cells was determined by FACS analysis. Triplicates of each sample were performed, and the significance compared to controls and analyzed as before (*$P < 0.05$).

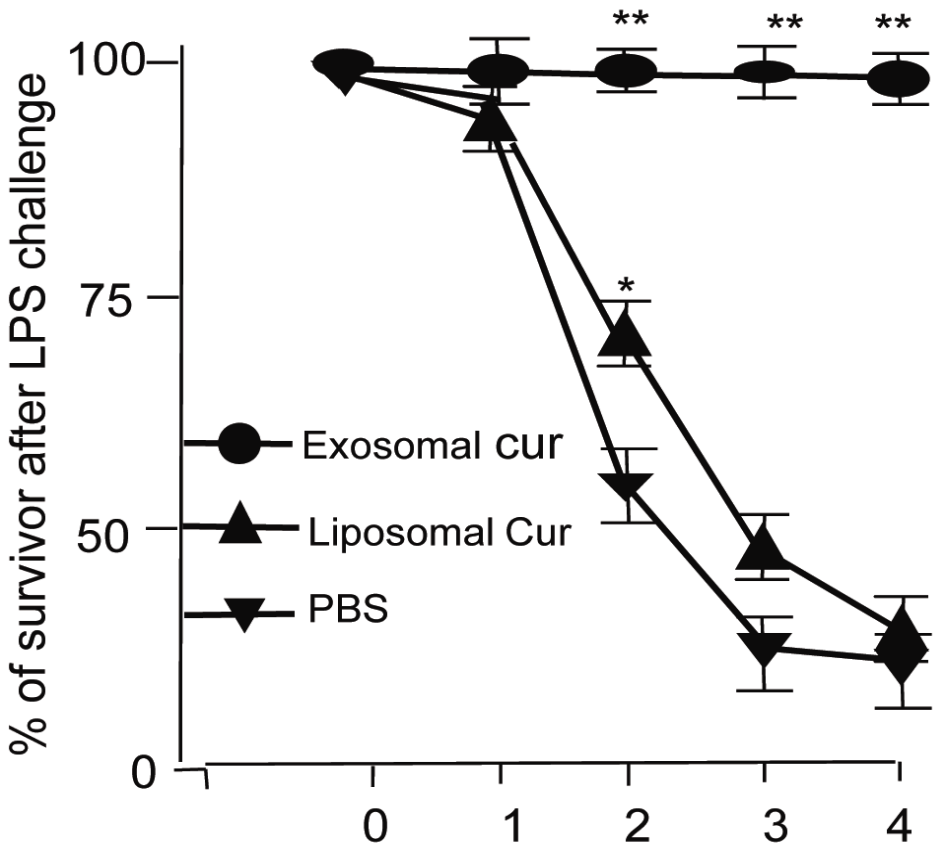

Figure 7 Exosomes function as more than a curcumin carrier. Liposome curcumin or exosomal curcumin (4 mg/kg) was injected i.p. into C57BL/6j mice together with lipopolysaccharide (18.5 mg/kg, Sigma-Aldrich). An equal amount of PBS was used as diluents in the control. Mouse mortality was monitored over a period of 4 days. Data represented six mice/group and were pooled from two experiments (**$P < 0.01$, *$P < 0.05$).

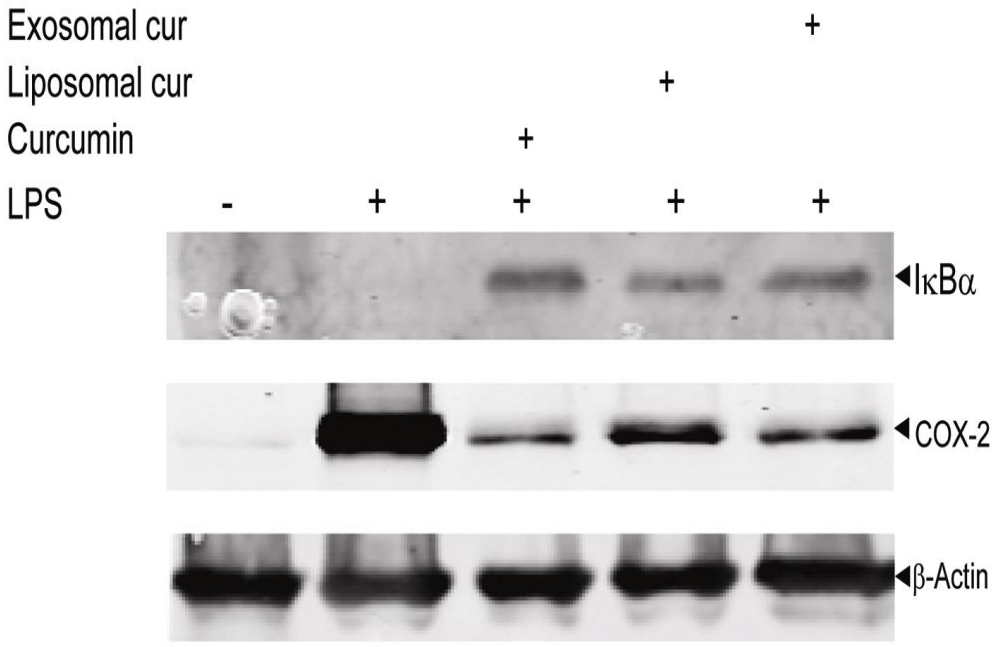

Figure S2 Liposomal curcumin has equal effect as curcumin or exosomal curcumin on the in vitro inhibition of IkB-α degradation.

DISCUSSION

In this study, we provide evidence that naturally existing nanoparticle exosomes can carry and deliver curcumin that in turn enhances its anti-inflammatory activity through (i) increasing the solubility, stability, and bioavailability of curcumin and (ii) enhancing/increasing delivery of curcumin to activated monocytes. Our approach leads to the protection of mice from LPS-induced septic shock. Furthermore, we have extended previous knowledge by demonstrating that exosomes target not only CD11b+Gr-1+ cells in the peripheral circulation but also enhance/increase delivery of exosomal curcumin to CD11b+Gr-1+ cells thus inducing more cell death. Finally, unlike other non-host delivery vehicles, host-derived exosomes have an advantage as a potential delivery vehicle because they would not induce an immune response with subsequent side effects.

CD11b+Gr-1+ cells are one of the major cellular populations associated with disease pathogenesis. Accumulation CD11b+Gr-1+ cells can suppress host immune responses and interrupt immunosurveillance, which could provide an explanation as to why long-term inflammation promotes tumor progression. Exosome-directed curcumin targeting to CD11b+Gr-1+ cells may provide a means to treat inflammation-related diseases, and perhaps even cancers.

Our data indicate that encapsulation of curcumin into exosomes can increase solubility, stability, and bioavailability of curcumin. There are two ways to load drugs into nanoparticles: (i) physical entrapment and (ii) chemical conjugation. Exosomes, containing a lipid bilayer, which is similar to liposomal nanoparticles, may load curcumin through physical entrapment. Through the hydrophobic interaction between the hydrophobic tails and hydrophobic drug, curcumin can be self-assembled into the lipid

bilayer of exosomes and this may protect curcumin from degradation. Nanoparticles are distributed to organs in a size-dependent selective manner.46 Sizes <5 nm nanoparticles are preferentially distributed to kidney and liver. Larger size exosomes likely stay in the vasculature for an extended time. Distribution of this nature may favor exosome-drug combinations, such as curcumin, being delivered as "brick dust–candidate" drugs. "Brick-dust candidates" are small molecule drugs with poor water solubility. When combined with exosomes, an increase in curcumin water solubility and stability is achieved, resulting in better delivery to the blood stream and increased bioavailability. This observation is also supported by data published by other groups, demonstrating that increased solubility and stability can be achieved by packing curcumin into liposomes or phospholipids (40. 40).

The present study is fundamental research on an exosomal curcumin drug delivery system that could be a milestone for a new powerful drug delivery system. The use of exosomal curcumin may take advantage of functional properties of curcumin, exosomes, and nanoparticles in an additive way to fight various inflammation related diseases. Curcumin has been well documented as a potent anti-inflammatory, antioxidant, and anticancer reagent through regulation of multiple pathways.35,37,50 Although our data show that exosomal curcumin enhances apoptosis of CD11b+Gr-1+ cells, additional mechanisms underlying the protection of mice against LPS-induced septic shock could also play a role because curcumin modulates a number of pathways. For an example, curcumin regulates the activity of a number of crucial transcription factors, including NF-κB, STAT3, and Nrf2, and therefore their biological effects on cell proliferation, apoptosis, cytokine induction, and antioxidation may also contribute to the prevention of

LPS-induced septic shock. Second, unlike liposome as a carrier, exosomes, such as dendritic and tumor cell–derived exosomes, exhibit strong tendencies to regulate immune responses and tumor progression. Therefore, selecting different types of exosomes in combination with therapeutic drugs achieves targetable and additive/syngeneic therapeutic effects. Third, because most diseases including cancer involve dysregulation of multiple pathways, exosomes can carry multiple therapeutic drugs to modulate the activity of multiple pathways in the same targeted cells. It is conceivable that using exosomes as a delivery vehicle has advantages over other carrier systems in terms of modulation of multiple pathways simultaneously in the targeted cells.

MATERIALS AND METHODS

Mice

In this study, 7- to 10-week female C57BL/6j mice (Jackson Laboratory, Bar Harbor, ME) were used. All animal studies were conducted within the guidelines established by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Chemicals and reagents

Curcumin and LPS were purchased from Sigma-Aldrich (St Louis, MO). Liposome curcumin was purchased from Encapsula NanoSciences (Nashville, TN).

Cell culture

The EL-4 (mouse lymphoma cell line) and RAW 264.7 (murine macrophage cell line) were maintained *in vitro* at 37°C in a humidified 5% CO2 atmosphere in air with complete RPMI1640 and DMEM medium (supplemented with 10% fetal bovine serum). Fetal bovine serum used in cell cultures to isolate exosomes was exosomes-depleted prior to use by differential centrifugation using a method described previously (29).

Preparation of exosomes

The cell culture supernatants were collected and used for exosome purification by differential centrifugation using a previously described method.22 Purity and integrity of sucrose gradient–isolated exosomes was analyzed using a Hitachi H7000 electron microscope (Electronic Instruments, Akishima, Japan) as previously described (29). The concentration of exosomes was determined by analyzing protein concentration using the Bio-Rad protein quantitation assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The protein expression of exosomes was determined by western blotting analysis as described previously (29).

Preparation of exosomal curcumin

Exosomal curcumin was prepared by mixing curcumin with EL-4 exosomes in PBS. After incubation at 22 °C for 5 minutes, the mixture was subjected to sucrose gradient (8, 30, 45, and 60%, respectively) centrifugation for 1.5 hours at 36,000 rpm. The exosomal curcumin, distinguished as a yellowish band in the sucrose gradient between 45 and 60%, was subsequently collected, washed, and dissolved with PBS. The concentration of

exosomes and curcumin in the complex was determined as described. Based on the morphology and protein expression, the vesicles were determined to be exosomes (29).

Analysis of curcumin concentration *in vitro* and *in vivo*

The *in vitro* concentration of curcumin in samples was determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE) at 420 nm. Briefly, to evaluate the concentration of curcumin, a standard curve of curcumin was plotted first. A stock solution of curcumin was diluted to a range of 5–50 μmol/l. A standard calibration curve was obtained by plotting the concentration of standard curcumin versus fluorescent absorbance at 420 nm (OD420). The curcumin quantity in cell culture supernatant or PBS was calculated based on the OD420 with respect to the concentration of curcumin in the diluted standards.

To determine the concentration of curcumin in plasma, a standard curve of curcumin was plotted first. Briefly, a stock solution of curcumin (0.5 mg/ml) in acetonitrile was diluted to a range of 0.1–5 μg/ml with acetonitrile, and then 10 μl of diluted curcumin were added to 90 μl of plasma isolated from naive C57BL/6j mice (a range of 1–500 ng/ml). The mixture was added to an equal volume of emodin (0.15 μg/ml, Sigma‐Aldrich) and vortexed for 5 minutes at 22 °C. After centrifugation at 2500 *g* for 15 minutes to remove precipitated plasma proteins, 50 μl of each of the working solutions containing 1–500 ng/ml of curcumin was analyzed by HPLC. The chromatographic separation was performed on a C18 column (5 μm, 250 × 4.6 mm; AAPPTec, Louisville, KY) with the mobile phase composed of acetonitrile-5% acetic acid (75:25, vol/vol) at a flow rate of 1.0 ml/minute. The wavelength of detection was at

420 nm. A standard calibration curve was obtained by plotting the concentration of standard curcumin versus absorbance units.

To determine the concentration of curcumin in the samples, plasma samples collected from mice treated with exosomal curcumin or free curcumin were precipitated with emodin to remove proteins and analyzed using an identical method as described above. The concentration was calculated using the absorbance units with respect to the concentration of curcumin in the standard curve.

FACS analysis

For cell surface marker staining, isolated cells were blocked at 4 °C for 5 minutes with 10 μg/ml mouse Fc block (BD Biosciences, San Jose, CA) and then reacted with various fluochrome-labeled antibodies including appropriate isotype controls for 30 minutes at 4 °C. After washing twice, cells were fixed and analyzed using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR). The following antibodies were used for immunostaining: FITC-AnnexinV (Invitrogen, Carlsbad, CA), APC anti-mouse CD11b and PE anti-mouse Gr-1 (eBiosciences, San Diego, CA), and propidium iodide (Sigma-Aldrich) was used for staining apoptotic cells.

***In vitro* stability assays**

To determine the stability of free curcumin and exosomal curcumin in PBS (pH 7.4), curcumin and exosomal curcumin were added to 2 ml PBS to achieve a final concentration of 30 μmol/l and incubated in the dark in a 37 °C water bath. At different

time points, 100 μl of each sample were taken to determine the concentration of curcumin. The concentrations of curcumin or exosomal curcumin at the beginning were considered as 1.00. The fold reduction of the concentration at each time was determined by comparison to the beginning value. The experiments were repeated three times for each time point ($n = 3$).

In *vivo* bioavailability assays

To determine the bioavailability of free curcumin and exosomal curcumin *in vivo*, two groups (five per group) of C57BL/6j mice were i.p. injected or administrated orally with 100 mg curcumin or exosomal curcumin/kg body weight. (To achieve a higher dose of curcumin treatment, curcumin prebinding with an appropriate amount of EL-4 exosomes was considered as exosomal curcumin). At 0.5, 1, 2, 4, 8, and 12 hours, blood samples were taken through eye sinus bleeding, and the concentration of curcumin in the plasma was determined by HPLC as described above. Naive mice without treatment were used as blank controls.

In *vitro* pro-inflammatory cytokine induction assays

RAW 264.7 cells were plated in 24-well plates and incubated overnight. The cells were treated with curcumin or exosomal curcumin at a concentration of 20 μmol/l for 1 hour and then stimulated with LPS (50 ng/ml) for an additional 6 hours. RAW 264.7 cells treated with PBS or exosomes served as controls. TNF-α and IL-6 levels in the cell culture supernatant were measured using a standard enzyme-linked immunosorbent assay (eBiosciences).

LPS mouse septic shock model

Curcumin or exosomal curcumin (4 mg/kg of body weight) was injected i.p. into C57BL/6j mice together with LPS (18.5 mg/kg, Sigma-Aldrich). EL-4 exosomes equal to the amount in exosomal curcumin and PBS were used as controls. Mouse mortality was monitored over a period of 4 days. The sera were collected 16 hours after LPS injection and used to determine IL-6 and TNF-α levels using an enzymelinked immunosorbent assay as before. At day 1 after LPS challenge, three mice from each treated group were killed, and the leukocytes in the lungs were isolated using a method described previously.32 The percentage of CD11b+Gr-1+ cells in the lung was determined by FACS analysis.

Isolation of $Gr1^+$ cells from mouse bone marrow cells

Mouse bone marrow cells were isolated as described previously.39 The isolated bone marrow cells were resuspended to a concentration of 1 × 108 cells/ml using RPMI 1640 medium supplemented with 10% fetal bovine serum. Following the addition of a mouse FcR blocking specific antibody (5 μl/ml), a Gr-1-PE conjugated antibody (3 μg/ml) was added, mixed thoroughly and incubated at 4 °C for 15 minutes. After centrifugation at 1,500 rpm for 5 minutes, cells were resuspended in fresh medium to a concentration of 1 × 108 cells/ml. EasySep PE selection cocktail (25 μl/ml; StemCell Technologies, Seattle, WA), was added to the cells and incubated at 4 °C for 15 minutes. Afterward, EasySep magnetic nanoparticles (25 μl/ml) were added and incubated at 4 °C for another 15 minutes. Culture medium was added to a final volume of 2.5 ml and the cells mixed by gentle pipetting of the mixture 2–3 times. The uncapped polystyrene tube was placed into

the EasySep magnet and set aside for 5 minutes. The supernatant containing unbound cells was removed leaving the magnetically bound Gr-1+ cells. A second round of magnetic separation was done on the supernatant. Positively selected (magnetically bound) cells were collected from the tubes, counted and cultured in RPMI 1640 supplemented with MCSF (20 ng/ml) for curcumin uptake and apoptosis assays.

Labeling of exosomes

Exosomes were labeled using an odyssey fluorescent dye IRDye800 kit (LI-COR Biosciences, Lincoln, NE). To localize EL-4 exosomes injected i.p. into mice, the IRDye 800CW-labeled EL-4 exosomes (100 μg) were injected i.p. into C57BL/6j mice, and mice were imaged over a 48-hour period using a prototype LI-COR imager (LI-COR Biosciences). Mice (five mice/group) receiving nonlabeled EL-4 exosomes served as controls.

Western blot analysis

C57BL/6 mouse bone marrow–derived macrophages were stimulated with LPS (50 ng/ml) or PBS for 15 minutes. Equal amounts of protein extract (50 μg of total protein) were electrophoresed on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The western blot was carried out with the anti-IκBα polyclonal antibody or anti-Cox2 or β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistics analysis

Statistical differences between groups were determined by analysis of variance with multiple comparisons using Fisher's *post hoc* analysis. The Student's *t*-test was used for comparisons when only two parameters were evaluated. $P < 0.05$ was considered significant.

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SUMMARY

Curcumin, an active polyphenol compound, has been used as food condiment or traditional plant medicine to treat numerous diseases in Asian and Western countries. Curcumin is safe as a food supplement and no toxicity has been reported even when oral administrated 8g/day for three months. Among these numerous diseases, various infection-related inflammations, such as cardiovasculature diseases, pulmonary diseases, diabetes, inflammation bowel diseases and cancers are highlighted and the effects of curcumin on these diseases have been proven by numerous medical researches. However, the low stability and bioavailability of curcumin limit its application, since large doses of curcumin (50-100mg/kg body weight) have to be administrated to animals for a therapeutic effect. Nanoparticles delivery systems via encapsulation of small molecular drugs, peptides or proteins into nanoparticles, showed promising effects on improving the bioavailability and efficacy of drugs. Curcumin has been incorporated into liposomes, polymers and dendrimers to overcome these limitations. When we realized that exosomes are natural nanoparticle vesicles, we hypothesized that exosomes may incorporate curcumin into the vesicles in physical condition. The encapsulation may increase systemic bioavailability and enhance bioactivity, in terms of anti-inflammation and further anticancer activity of curcumin.

My study showed that encapsulation curcumin into exosomes can increase solubility, stability, and bioavailability of curcumin. Exosomal curcumin displayed higher anti-inflammatory activity than free curcumin. Exosomal curcumin can protect mice from

LPS induced septic shock, and this may be due to the down-regulation of the $CD11b^{+}Gr1^{+}$ cell population in the lungs.

Exosomal curcumin can protect mice from septic shock in a low dose of 4mg/kg. Previous report showed that effective dose of curcumin to protect mice from septic shock was as high as 40-60 mg/kg body weight (126). Pretreatment of mice with 100mg/kg curcumin (i.p) before administration of LPS and GalN (D-galactosamine, a hepatotoxic agent) decreased acute serum elevation of TNF-α and GOT/GPT, and reduced hepatic necrosis and mice death (161). Sompamit et al showed that LPS induced host hypotension, tachycardia and vascular hyporeactivity when mice were treated with non-lethal dose of LPS. Curcumin (50 or 100mg/kg) treatment showed protective effect on hearts of mice in a dose dependent manner(162). Taken together, there is a significant dose advantage of exosomal curcumin to rescue LPS induced sepsis. In other words, encapsulation curcumin into exosomes will dramatically increase its efficacy.

Exosomal curcumin showed enhanced anti-inflammation activity both *in vitro* and *in vivo*, in terms of inhibition of IL6 and TNF-α secretion. As a proinflammatory and anti-inflammatory cytokine, IL6 is involved in the progression of several diseases, such as diabetes, atherosclerosis, systemic lupus erythematosus, rheumatoid arthritis and prostate cancer. A high level of IL6 is detected in the blood of advanced or metastasis cancer patients. Targeting IL6 is a developing therapeutic interest in clinical studies. TNF-α is another proinflammatory cytokine that plays critical roles during inflammation and tumorigenesis. The binding of TNF-α to its two receptors leads to release of inhibitory protein SODD from the intracellular death domain, recruitment of adaptor protein TRADD, and initiation of downstream signaling pathways. TRADD will recruit

TRAF2 and RIP. The recruitment of IKK by TRAF2 will allow serine-threonine kinase RIP to activate IKK, leading to activation of NF-kB signaling pathway. TRAF2 can also activate MEKK1 and ASK1. Through the phosphorylation of MKK7, TRAF2 will activate JNK, which will translocate to the nucleus and activate downstream transcription factors, such as c-JUN and ATF2. TNF-α overexpression is implicated in some autoimmune disorders; thus, TNF inhibitors are developed to treat several autoimmune diseases in clinic. Exosomal curcumin showed significant inhibition of IL6 and TNF-α both *in vitro* and *in vivo*, and may be adapted in the future to treat inflammation related diseases, such as Rheumatoid arthritis, Ankylosing spondylitis, Crohn's disease, Psoriasis and Refractory asthma.

My study showed that exosomal curcumin targeted inflammatory cells and thereby protected mice in a model of LPS-induced septic shock. To the best of our knowledge, this is the first study to show that curcumin can target MDSCs. Curcumin alone had minor effect on down-regulation of MDSCs in the lungs. However, exosomal curcumin dramatically decreased MDSCs in LPS stimulated mice. Increased accumulation of MDSCs in the lungs of LPS induced mice, leading to acute lung inflammation, is a major phenomenon of the infection. To determine how exosomal curcumin down-regulated MDSCs, my *in vitro* data showed that $CD11b^{+}GR1^{+}$ cells uptake more exosomal curcumin than free curcumin in the same conditions. Thereby, more curcumin induces the death of more cells in exosomal curcumin treated cells. Our previous study and others showed that tumor derived exosomes are preferentially uptaken by MDSCs. If exosomal curcumin takes advantage of this property leading to high accumulation in MDSCs *in vivo* will be addressed in future studies.

Liposomes have similar lipid bilayer structure with exosomes. Liposomal curcumin, encapsulation curcumin into liposomes, can increase stability (49) and bioavailability (48) of curcumin, however, liposomal curcumin didn't show much higher bioactivity than free curcumin[(48, 49, 163) and our data]. When we treated LPS stimulated mice with liposomal curcumin, it didn't show enhanced protection to the mice as exosomal curcumin. This observation raises a possibility that the enhanced protection of exosomal curcumin requires functional exosomes involvement. Curcumin treatment (40-60 mg/kg body weight) partially rescued mice from LPS induced septic shock, with mice mortality decreasing from 90% to 30%(126). My data showed that exosomal curcumin with a dose as low as 4mg/kg can fully protect mice from septic shock. In future studies, we will compare the dose response of curcumin, liposomal curcumin and exosomal curcumin to the LPS treatment. We will treat mice with LPS and dose increased curcumin (5, 10, 20, 30, 40, 50 and 60mg/kg of body weight), liposomal curcumin (same as curcumin), exosomal curcumin (1, 2, 3 and 4mg/kg of body weigh) and exosomes (100, 200, 300, 400 and 500μg/mouse), and watch the mice mortality. We want to identify the effective dosage of curcumin and liposomal curcumin in our system and rule out the effects of liposomes and exosomes alone. Particularly, there are controversial evidences of exosomes and our preliminary data didn't show improved protection to the mice of exosomes alone. Meanwhile, we want to exclude the dramatically increased response of liposomal curcumin based on the published data and our preliminary data. We expect similar dose response of curcumin and liposomal curcumin, and this will confirm that the function of exosomal curcumin requires

biological vesicles of exosomes but not solely stabilizing curcumin due to the encapsulation into lipid bilayer vesicles.

Nanoparticle drug delivery system achieves its enhanced drug efficiency through passive targeting and active targeting. Passive targeting will increase bioavailability of delivered drug, likewise, active targeting will increase specificity of the drug and this is accompanied through receptor-mediated endocytosis or specific antigen-antibody binding at the cell surface. First of all, we will compare *in vitro* stability and *in vivo* bioavailability and half-lives of liposomal curcumin and exosomal curcumin. We will incubate exosomal curcumin and liposomal curcumin in PBS, serum or medium to compare their stability. Meanwhile, we will inject mice with identical amount of liposomal and exosomal curcumin (100mg/kg of body weight), and take the blood to determine the curcumin concentration in the plasma over a period of 12hrs as described before. In comparison with curcumin treatment alone, exosomal curcumin can dramatically increase bioavailability of curcumin and maintain a relative high level in plasma for 8hrs. We expect increased bioavailability and longer half-life due to the stabilization; however, we have no idea if there are large differences between the two forms of encapsulated curcumin. Whether liposomal curcumin has similar improvement as exosomal curcumin will be identified in future studies. In different with nanoparticle liposomes, exosomes are biological active vesicles, containing all membrane and some cytosolic proteins. These proteins may help exosomes to achieve active targeting when exosomes are incorporated with curcumin. To identify if phagocytosis or receptor-mediated endocytosis play critical roles in active targeting, we will treat cells with several phagocytosis or endocytosis inhibitors such as Concanamycin A, Bafilomycin A and

Cytochalasin D before liposomal curcumin and exosomal curcumin administration. If the treatment shows decrease uptake by cells, this will imply that endocytosis pathway is critical for their function. To identify if specific antigen - antibody binding plays critical roles in active targeting, we will screen several cell surface antigens, such as CEA, Her2/Neu, Mart1, TRP and gp100(29). By blocking with specific antibody in the cell cultures, we will determine if the binding is important for cellular uptake.

My data showed that exosomal curcumin targeted $CD11b^{+}Gr1^{+}$ and decreased this population in the lungs of LPS treated mice. For further study, we will identify if the lung is the critical target by screening for organ damage in LPS treated mice, and if other populations of cells are affected. Sixteen hours after treatment, we will sacrifice mice and take the organs such as lung, liver, heart, kidney and spleen. First, we will purify lymphocytes from lung, liver and spleen, and label these cells with Annexin V and cellular markers of DCs (MHCII and CD11c), T cells (CD4, CD8 and CD44), B cells (CD45RB and CD19), and macrophages (CD11b and F4/80), in addition to MDSCs (CD11b and Gr1), to determine if the induction of apoptosis of $CD11b^{+}Gr1^{+}$ is the key mechanism of protection of exosomal curcumin and if there are other cells involved in. After LPS stimulation, mice lung may show significant congestion, edema, alveolar septal thickening and influx of inflammatory cells(102). We will stain lungs and other organs with hematoxylin and eosin for microscopic examination to find the differences of the structures before and after the treatment to determine if lung is the primary response organ to LPS and how exosomal curcumin protect it. The extensive studies of curcumin showed that curcumin exert its anti-inflammation, antioxidant and anti-cancer functions through the regulation of several transcription factors, such as NF-kB, STAT3, Nrf2 and

their downstream proteins COX-2, HO-1. My *in vitro* study showed that in vitro curcumin, liposomal curcumin and exosomal curcumin treatment can equally regulate the expression of STAT3, NF-kB, COX-2 and HO-1 even though there are big differences of IL6 secretion and mice mortality between these treatments. In other words, curcumin, liposomal curcumin and exosomal curcumin can sufficiently regulate anti-inflammatory pathways *in vitro* cell cultures. However, only exosomal curcumin alone can effectively regulate IL6 and TNF-α secretion and further protect mice from the infection. This indicates that exosomes are not only functioning as a delivery vehicle, but also contributing to the therapeutic effects. We will do immunohistochemical staining of these organs with several protein markers (NF-kB, STAT3 and NRf2) or determine the proteins levels by western blot to find if exosomal curcumin treatment can down-regulate their expressions more significantly than free curcumin treatment along. Through these studies, we hope to determine the difference between treatments and identify the specific mechanism of exosomal curcumin in the cell signaling regulation due to the encapsulation by exosomes.

Nanoparticles distribute themselves in an organ and size specific manner. Our preliminary data showed that EL4 and 4T1 derived exosomes distribute to different organs. EL4 exosomes distributed extensively in lung, liver and spleen, meanwhile, 4T1 exosomes accumulated in spleen (data not shown). We are wondering if the distribution of exosomes directs their targeting, or correlates with their direction of cancer metastasis. In my further study, we may extend this study to more exosomes from different cell lines. A particular interest could be Dexs since Dexs show even more promising functions, and they have been used in clinical studies as cell free vaccines.

In comparison with other nanoparticles, exosomes are endogenously secreted by various living cells, such as DCs, T/B cells, fibroblasts and tumors. The abundant resource can give us more choices to select safe and no toxic exosomes as the vector. Due to their cellular origin, exosomes may not induce any side effects and will be degraded as their own origin. More importantly, exosomes, such as Texs and Dexs have promising immuno-regulatory and tumor-prevention functions. The combination of curcumin, exosomes and nanoparticles properties will make exosomal curcumin a well-armed triple threat to fight against multiple diseases and cancers in the future.

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APPENDIX

Notice of Approval for Protocol Modification

DATE: March 24, 2009

TO: Huang-Ge Zhang, M.D., D.V.M., Ph.D.
LHRB-473 0007
FAX: 975-6648

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Curcumin Reverses Tumor Exosomes-Mediated Inhibition of Myeloid Differentiation
Sponsor: NIH
Animal Project Number: 080608155

On March 24, 2009, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the modification as described: Additional Personnel to protocol: Spandan Shah, Xiaoyu Xiang, and Dongmei Sun The sponsor for this project may require notification of modification(s) approved by the IACUC but not included in the original grant proposal/experimental plan; please inform the sponsor if necessary. The following species and numbers of animals reflect this modification.

Species	Use Category	Number in Category
Mice	A	Zero - Procedural modification only

Animal use is scheduled for review one year from June 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files.

Refer to Animal Protocol Number (APN) 080608155 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

NOTICE OF APPROVAL

DATE: June 26, 2008

TO: Huang-Ge Zhang, M.D., D.V.M., Ph.D.
LHRB-473 0007
FAX: 975-6648

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Curcumin Reverses Tumor Exosomes-Mediated Inhibition of Myeloid Differentiation
Sponsor: NIH
Animal Project Number: 080608155

On June 25, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	A	480

Animal use is scheduled for review one year from June 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 080608155 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.