Morphine Inhibition of Macrophage Phagocytosis and Bactericidal Functions

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ABSTRACT

For centuries, opioids have been implicated in increasing susceptibility to infection, reducing bacterial clearance, and increasing bacterial dissemination. Macrophages as key cells of innate immunity play an essential role in pathogen clearance and antigen presentation. Macrophage phagocytosis is a key mechanism responsible for host defense against bacterial pathogens. Although it is known that opioid addicts are prone to both bacterial and viral infections, the molecular and cellular mechanisms underlying these processes remain to be elucidated. Therefore the goal of this research was to investigate mechanisms of decreased bacterial clearance as a contributing factor in the increased susceptibility to infection in opiate drug abusers. To this end, first set of studies examined the role of morphine on inhibition of key mechanisms involved in Fc-gamma receptor mediated phagocytosis. It was demonstrated that morphine inhibits phagocytosis by inhibiting actin polymerization through a cAMP, PKA and MAPK dependant pathways. By superactivation of adenylyl cyclase morphine increases intracellular cAMP leading to inhibition of actin polymerization. Furthermore, morphine by inhibiting p38 MAPK and ERK 1/2 MAPK causes inhibition of actin polymerization and phagocytosis. By modulating TLR4 receptor function morphine was also able to increase macrophage phagocytosis, indicating that morphine might have a differential effect on internalization of Gram-positive, versus Gram-negative pathogens. These effects were mediated through a MyD88 and p38 MAPK dependant pathways leading to changes in actin polymerization and phagocytosis. In addition to macrophage's ability to internalize pathogens, elimination of internalized pathogen is essential for effective bacterial clearance. We therefore set out to investigate morphine's modulation of macrophage bactericidal mechanisms. We note

that morphine inhibits bacterial killing by inhibiting essential mechanisms involved in this process such as formation of reactive oxygen intermediates, reactive nitrogen intermediates, as well as phago-lysosomal fusion. Morphine by inhibiting these essential mechanisms impedes eradication of bacterial infections and leads to detrimental consequences for the host. These series of studies have extended our knowledge in an underrepresented yet clinically significant field of study, however many questions still remain to be addressed and it is crucial to investigate the answers given the prevalence of morphine use today.

<u>TABLE OF CONTENTS</u>

Page

CHAPTER 1. BACKGROUND-LITERATURE REVIEW

CHAPTER 2. CHRONIC MORPHINE TREATMENT MODULATES MACROPHAGE PHAGOCYTOSIS

CHAPTER 3. DIFFERENTIAL MODULATION OF FC-GAMMA RECEPTOR MEDIATED PHAGOCYTOSIS BY MORPHINE FOLLOWING TLR2 AND TLR4 ACTIVATION

CHAPTER 4. MORPHINE INHIBITS MACROPHAGE BACTERICIDAL MECHANISMS

LIST OF FIGURES

CHAPTER 3. DIFFERENTIAL MODULATION OF FC-GAMMA RECEPTOR MEDIATED PHAGOCYTOSIS BY MORPHINE FOLLOWING TLR2 AND TLR4 ACTIVATION

CHAPTER 4. MORPHINE INHIBITS MACROPHAGE BACTERICIDAL MECHANISMS

CHAPTER 1.
BACKGROUND-LITERATURE REVIEW

1. IMMUNE SYSTEM

Immune system is a network of organs, tissues, and cell types, which provide an individual with protection against invading organisms. Immune system is divided in two distinct arms that generate two distinct modes of defense, innate and adaptive immunity.

Innate immune system is the first line of defense, and it provides the initial response to microbial invasions. The cells and mechanisms of innate immune system defend the host from infection by pathogens, in a non-specific manner. Generation of appropriate innate immune response is essential for host's defense for two reasons. First, cells of the innate immunity consist of granulocytes and phagocytes, such as einosinophils, basophils, neutrophils and macrophages whose role is to internalize and kill pathogens, induce inflammation as well as to recruit additional cell types to the site of infection. Inflammations is triggered when bacteria cross the endothelial barrier and activate cell surface receptors on cells of the innate immune system, namely neutrophils and resident macrophages. Following microbial internalization and pathogen recognition, macrophages produce cytokines (such as IL-1 and TNF) that activate the endothelial cells of the nearby venules to produce selectin, ligands for integrins and chemokines. Selectins facilitate tethering and rolling of leukocytes on the endothelium, while integrins mediate firm adhesion and chemokines stimulate extravasations through the endothelium and result in migration to the site of infection (**Figure 1.1**). This mechanism is used by blood neutrophils, monocytes and activated T-lymphocytes to migrate to the

FIGURE 1.1
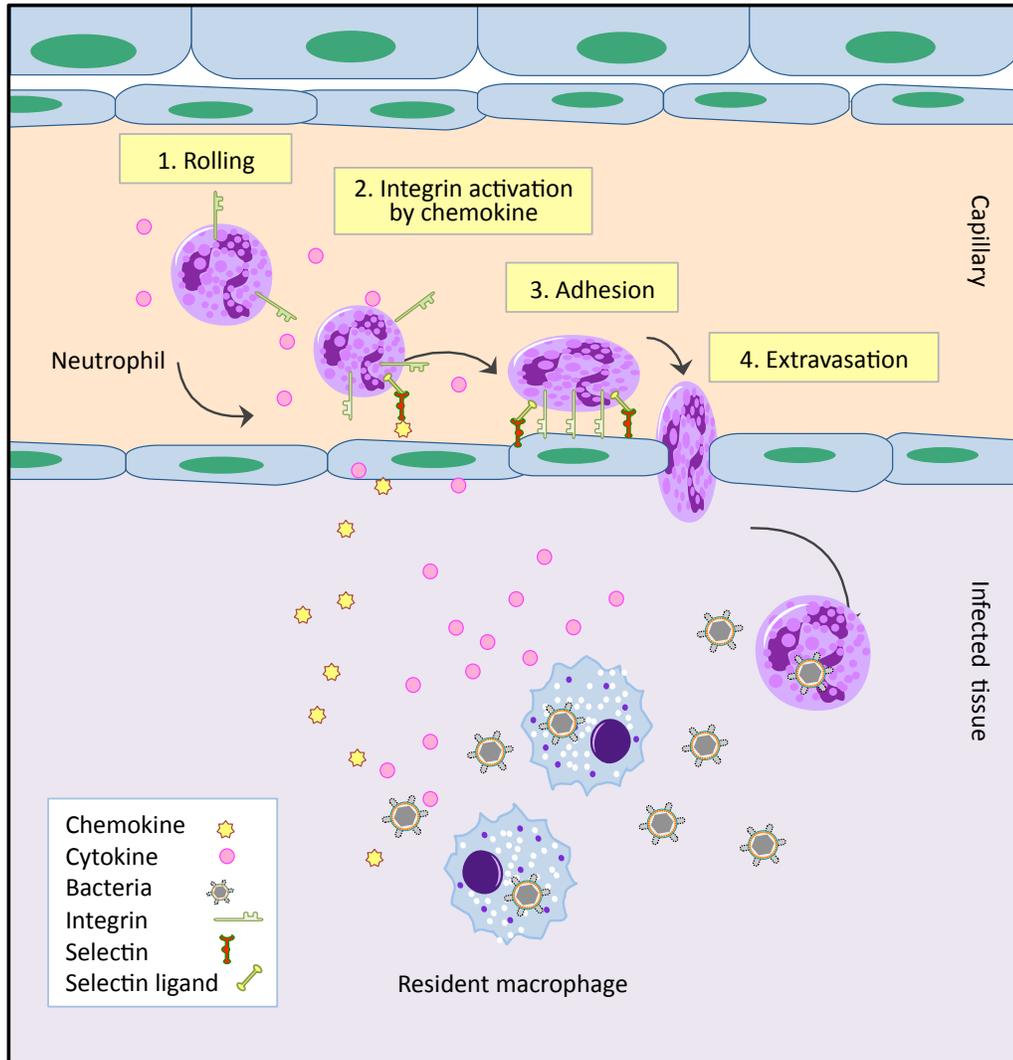
Figure 1.1. Diagram of leukocyte recruitment to the site of infection. Following pathogen infiltration across the endothelial membrane, resident macrophages begin producing cytokines and chemokines leading to neutrophil recruitment. As the neutrophil approaches the site of infection, chemokines act on endothelial cells to activate integrins and produce selectin ligands. This facilitates rollling, adhesion and extravasation to the infected tissue for enhanced bacterial clearance.

sites of infection. Chemokines and cytokines play an important role in cell signaling and inflammation, with the end result of promoting pathogen clearance and leading to effective elimination of infection. Phagocytosis and bacterial killing are essential in immune system's ability to clear infections. Professional phagocytes include neutrophils, macrophages, monocytes, dendritic cells and mast cells. These cells express specific phagocytic receptors that enable them to internalize pathogens and kill them using various mechanisms which will be discussed later in more detail. As the innate immune system is indispensable in initial steps of infection, generation of adequate innate immune response is critical for appropriate overall functioning of the immune system.

Second arm of the immune system defense is the adaptive immunity. Adaptive immune system is composed of highly specialized, systemic cells such as T and B cells, as well as processes that eliminate or prevent pathogenic challenges. Adaptive immunity is activated by innate immune system, and is capable of recognizing and remembering specific pathogens. While innate immunity is non-selective in its pathogen defenses, adaptive immunity consists of highly specialized systemic cells. Adaptive immune system is activated by the innate immune system and it is able to remember specific pathogens in order to generate immunity, and to mount a stronger response each time the pathogen is encountered. Adaptive immunity is highly adaptable because of somatic hypermutation (a process of accelerated somatic mutations), and V(D)J recombination (an irreversible genetic recombination of antigen receptor gene segments). This mechanism allows a small number of genes to generate a diverse repertoire of antigen receptors, which are then uniquely expressed on each individual lymphocyte.

Interactions of innate and adaptive immunity are essential for adequate immune response. Cells of the innate immune system engulf and eliminate pathogens, which is essential in early phases of infection as well as in later stages when adaptive response

3

has been generated and has targeted organisms for destruction. Innate immune system is responsible for initiation of the adaptive immune response, and it is vital in fighting off the infection in the 4-7 day period it takes to generate the adaptive immune response. This brief discussion of the immune system does not fully illustrate complexities of immune functioning, but provides a general overview of the processes involved in clearance of infections.

2. MACROPHAGE PHAGOCYTOSIS

A. ROLE OF Fcγ RECEPTOR IN PHAGOCYTOSIS

Macrophages are professional phagocytes, critical for uptake and degradation of infectious agents and senescent cells. Phagocytosis by macrophages plays an important role in bacterial clearance, tissue remodeling, immune response and inflammation. Macrophage phagocytosis of pathogens initiates the innate immune response, which in turn orchestrates the adaptive immunity. In order to internalize a wide variety of pathogens, macrophages have evolved to express a variety of phagocytic receptors, including the mannose receptor, scavenger receptor, complement receptor and Fcγ receptors.

Extracellular bacteria cannot be internalized unless they are coated with serum opsonins such as IgG antibodies (Underhill and Ozinsky, 2002). These IgG-opsonized pathogens are recognized and phagocytosed by surface receptors called Fcγ receptors (FcgRs) that bind to Fc regions of IgG antibodies (Aderem and Underhill, 1999). After pathogens bind to the phagocytic receptors, a sequence of events follows that leads to induction of rearrangements in actin cytoskeleton, phagosome formation and

internalization of the opsonized particle (**Figure 1.2**) (Aderem and Underhill, 1999; Niedergang and Chavrier, 2005). Most of our understanding of the signaling pathways leading to phagocytosis in macrophages comes from studies of Fc gamma receptors (FcgR) (Ravetch and Bolland, 2001). FcgRs fall into two classes: (*a*) high affinity (FcgRI)-(CD64) and low affinity (FcgRIIIA)-(CD16), containing immunoreceptor tyrosine-based activation motifs (ITAM) in their intracellular domains which recruit kinases and activate phosphorylation cascades, and (*b*) inhibitory receptors (FcgRIIB; CD32), containing immunoreceptor tyrosine-based inhibition motifs (ITIM) which recruit phosphatases that inhibit signaling (Daeron, 1997; Ravetch and Bolland, 2001; Nimmerjahn and Ravetch, 2006). FcgRI and FcgRIII are dependent on expression of accessory γ chain that carries an ITAM; thus, deletion of this receptor subunit leads to the functional loss of all activating Fcγ receptors (Ravetch, 1997). On macrophages, FcgRIIB is coexpressed with activating FcgRs of varying affinities and isotype specificities. They negatively regulate activating signals delivered by these receptors. Animals deficient in FcgRIIB manifested enhanced immune complex-mediated inflammation, phagocytosis, and IgG-mediated clearance of pathogens (Nimmerjahn and Ravetch, 2006).

As shown in **Figure 1.3,** immediately after IgG coated particles bind the FcgRs and induce their crosslinking and phosphorylation of ITAM motifs, Src family of tyrosine kinases is activated and phosphorylates Cdc42 and Syk kinase. Activated Cdc42 then recruits WASP and results in filopodial extensions in cell periphery. Src also activates Syk which activates Vav, which activates Rac and leads to formation of lamellipodia (Niedergang and Chavrier, 2005). The Vav family of Rho GEFs (guanosine exchange

FIGURE 1.2
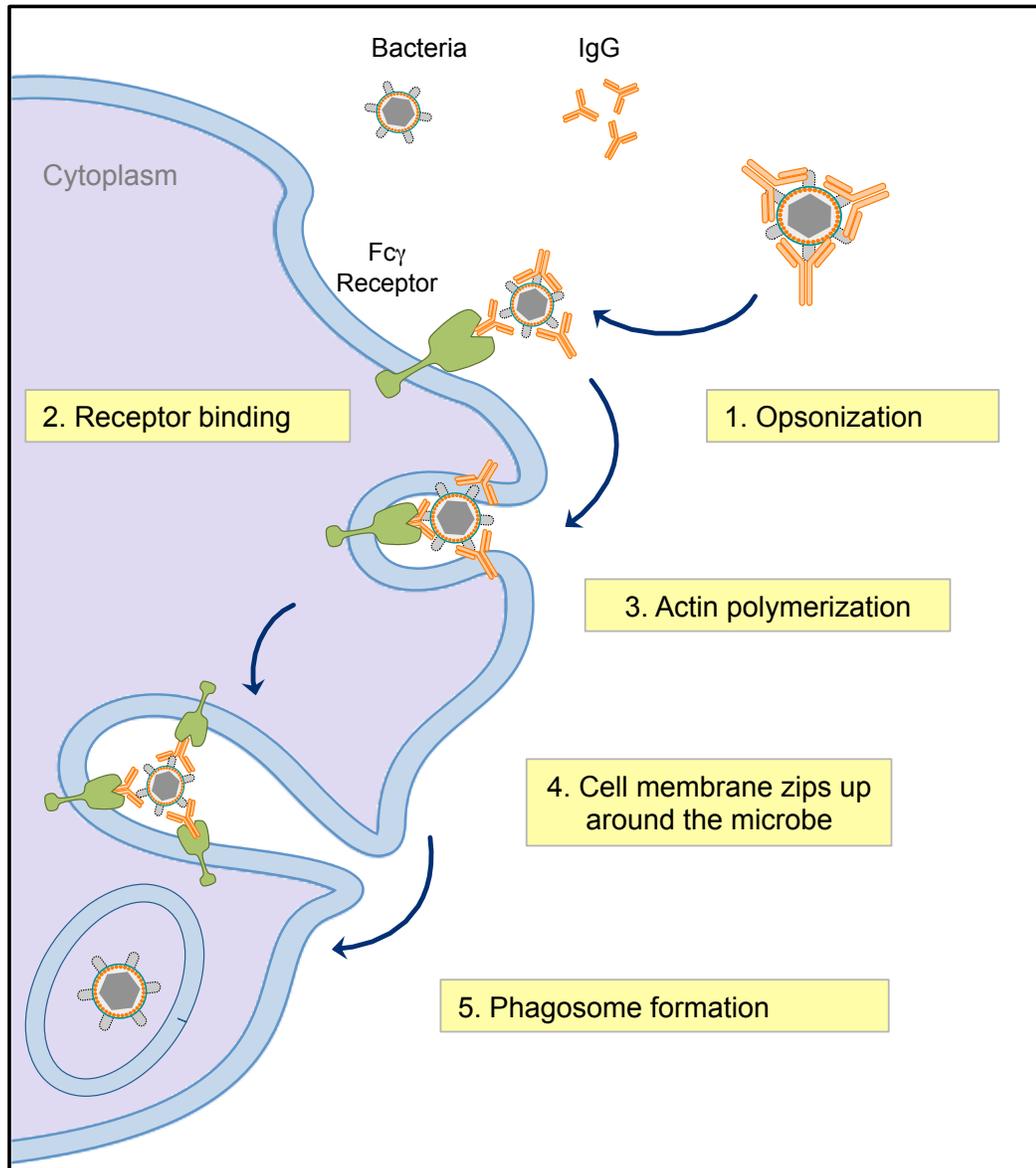
Figure 1.2. Diagram of FcgR mediated macrophage phagocytosis. Following pathogen opsonization with IgG, the opsonized bacteria binds to the FcgR. FcgR receptor activation triggers a signaling cascade leading to actin polymerization, membrane enclosing around the pathogen leading to phagocytosis and phagosome formation.

FIGURE 1.3
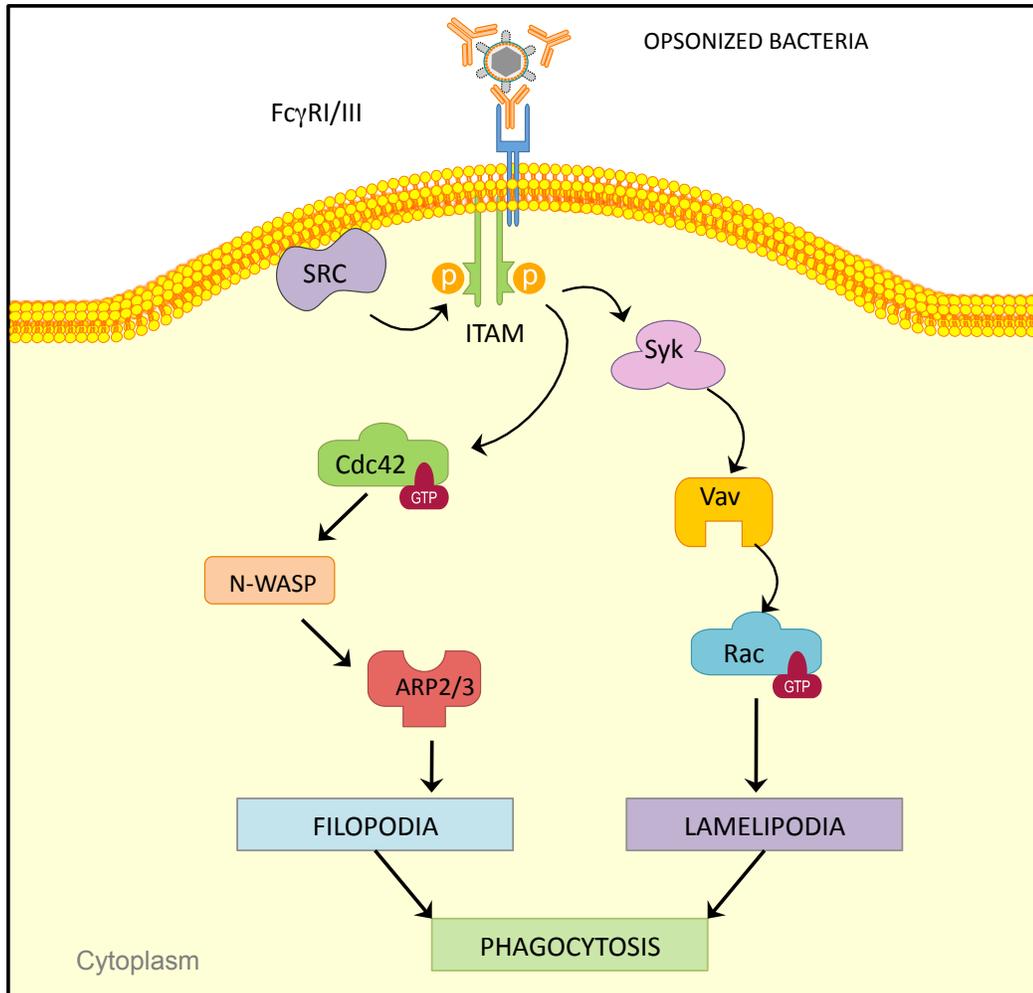
Figure 1.3. Diagram of FcgR signaling following receptor activation. Following binding of the opsonized pathogen to the receptor, FcgR crosslinks, leading to SRC mediated phosphorylation of ITAM motifs. Recruitment of Syk kinase follows via it's SH2 domain, leading to activation of Vav a GTP/GDP exchange factor. Vav activates Rac leading to lamellipodia formation, while Cdc42 activation, acivates WASP and ARP2/3 leading to filopodia formation. Both lamellipodia and filopodia play an essential role in phagocytosis.

factors) is essential for phagocytosis. Their inhibition prevents Rac1 activation and FcgR mediated phagocytosis (Patel et al., 2002). Nucleotide exchange ability of Vav proteins is modulated by tyrosine phosphorylation, and Syk-mediated phosphorylation of Vav on FcgR ligation is instrumental for Rac activation during phagocytosis (Niedergang and Chavrier, 2005).

B. ACTIN POLYMERIZATION AND PHAGOSOME FORMATION

Actin polymerization is obligatory in FcgR phagocytosis. Rho family of guanosine triphosphatases (GTPases) is a family of small signaling G protein, and is a subfamily of the Ras superfamily. Rho GTPases act as molecular switches that control the organization of actin cytoskeleton, and have been found to play a critical role in phagocytosis (Bokoch, 2000). Three members of the Rho family of small GTPases, Rho A, Rac1 and Cdc42, have been studied a great deal and each control a signal transduction pathway linking membrane receptors to the assembly and disassembly of the actin cytoskeleton as shown in **Figure 1.4**. Rho regulates stress fiber and focal adhesion assembly, Rac regulates the formation of lamellipodia protrusions and membrane ruffles, and Cdc42 triggers filopodia extension at the cell periphery (Caron and Hall, 1998). Both Cdc42 and Rac, but not Rho, activities are required for particle internalization.

Rac mediated formation of lamellipodia and filopodia is essential for cell motility, the organization of membrane domains, phagocytosis and the development of substrate adhesions. When Abrecrombie et al. first identified actin as the primary "organelle" of motility he referred to the protrusions parallel to the substrate as the leading edge or lamellipodium, and when the protrusions curled upwards he referred to them

FIGURE 1.4
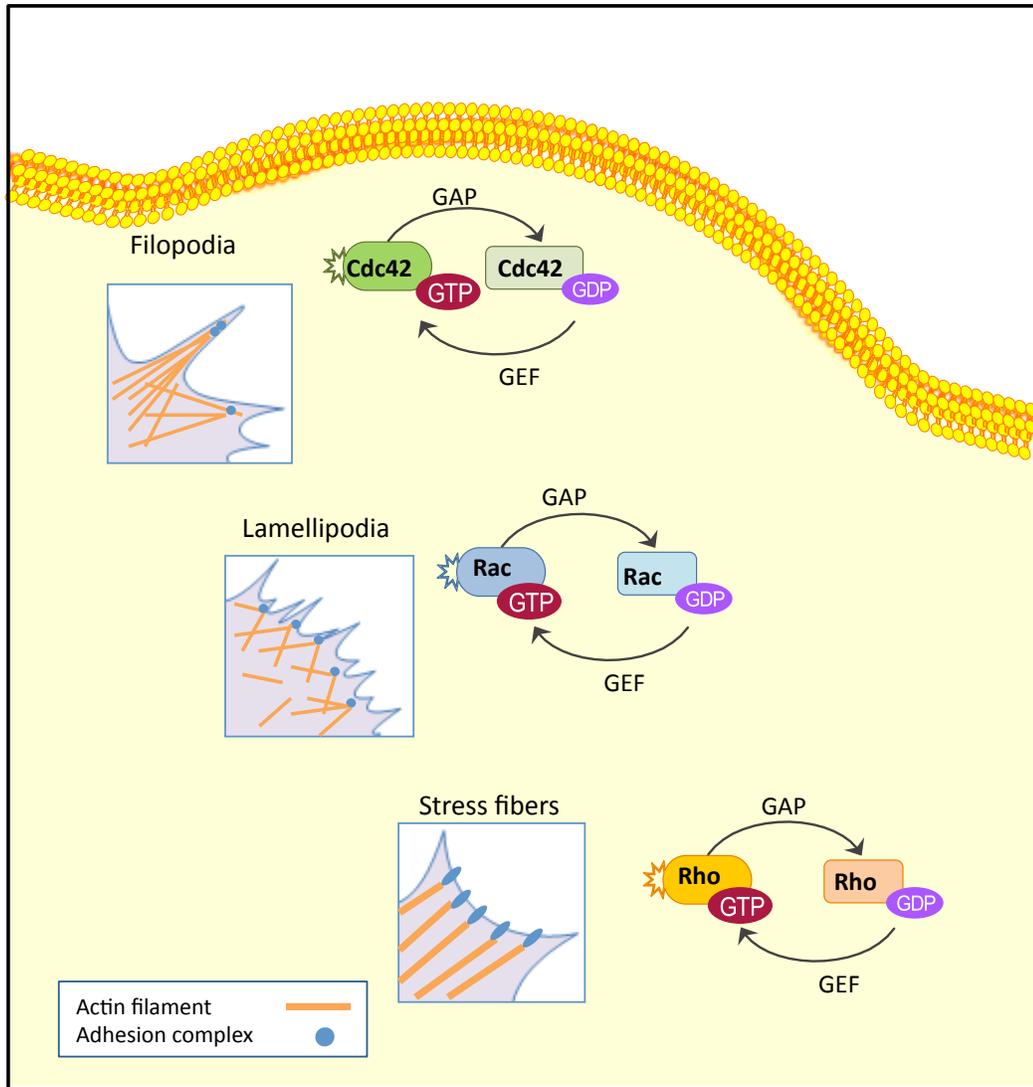
Figure 1.4. Diagram of Rho GTPase function.

Diagram illustrates differential role of individual members of RhoGTPase family in actin polymerization. Following GDP exchange with GTP, activation of Cdc42-GTP leads to actin rearrangement resulting in filopodial extensions, Rac-GTP results in lamellipodia and Rho-GTP leads to stress fiber formation. Adapted and modified from (Underhill and Ozinsky, 2002).

as membrane "ruffles" (Small et al., 2002). The role of lamellipodia is the primary site of actin incorporation and is the major filament of the cell. They are involved in the development of adhesions to the substrate and, as ruffles, serve in phagocytosis (Mejillano et al., 2004).

While lamellipodia seem designed for persistent protrusion over a cell surface, filopodia perform sensory and exploratory functions to steer cells depending on cues from the environment (Mejillano et al., 2004). Filopodia are thin, dynamic cell extensions comprising tight bundles of long actin filaments covered with cell membrane, and are often associated with lamellipodia. They can change their length rapidly, span many cell diameters, and interact with other cells. Filopodia seem to explore the extracellular matrix and surfaces of other cells, identify appropriate targets for adhesion, and then generate guidance cues and traction forces to move the cell body (Jacinto and Wolpert, 2001).

By directing formation of lamellipodia and filopodia Rac and Cdc42 play an essential role in phagocytosis. Furthermore, other than the role it plays in phagocytosis Rac GTPase is also important in mechanisms of phagosome formation and bacterial killing. Rac GTPase is key in recruitment of NADPH oxidase, which is essential in formation of superoxide, a potent bactericidal mechanism, and will be discussed later under mechanisms of bacterial killing. The central role of Rho GTPases in phagocytosis and bacterial killing provides at least a partial explanation of why they are such common substrates for bacterial evasion of killing, and potential sites of drug action on macrophage phagocytosis (Aktories et al., 2000; Galan, 2001).

3. MACROPHAGE BACTERICIDAL ABILITY

A. PHAGOSOMAL MATURATION

Following internalization, phagosomes undergo maturation process, during which, they interact with early and late endosomes and ultimately fuse with lysosomes (Araki, 2006). When phago-lysosomes fuse, low pH lysosomal contents are emptied out into the phagosome and this initiates bacterial killing **(Figure 1.5)**. After the fusion takes place, production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) follows. These are primary mechanisms of bacterial killing and are considered to be a relatively effective host-defense mechanism against microbial pathogens (Chan et al., 2001).

Lysosomes are highly acidic organelles found in all eukaryotic cells and are in charge of digesting macromolecules. In macrophages, their major role is to kill phagocytosed pathogens in mature phagocytes. Lysosomes contain hydrolytic enzymes which are released upon lysosomal fusion with other vacuoles such as phagosomes. Important enzymes that can be found within the lysosomal compartment are: lipase (digests lipids), amylase (digests amylase and starch), protease (digest proteins), and nucleases (digest nucleic acids). These enzymes are synthesized in the cytosol and the endoplasmic reticulum, where they receive a mannose-6-phosphate tag that targets them to lysosome (Kornfeld, 1987).

Hydrolytic enzymes digest phagosomal contents and kill bacteria. High acidity of the lysosome is maintained by membrane bound proton pumps, which pump the protons from the cytosol into the lysosomal compartment, and by chloride ion channels **(Figure 1.6**). Lysosomal enzymes are able to maintain their function at pH as low as 4.5,

FIGURE 1.5
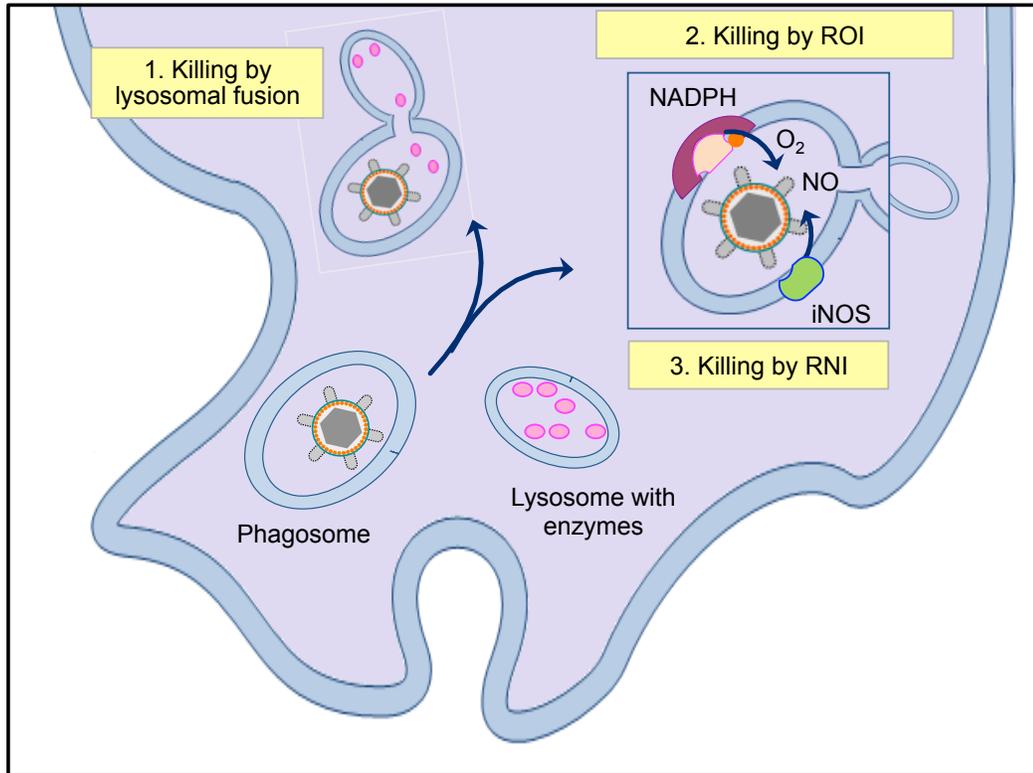
Figure 1.5. Diagram of macrophage bactericidal mechanisms. Following internalization, phagosomes undergo maturation process, during which, they interact with early and late endosomes and finally fuse with lysosomes. Following phago-lysosomal formation, low pH lysosomal contents are emptied out into the phagosome initiating bacterial killing. After the fusion takes place, production of reactive oxygen intermediates (ROI) such as superoxide and reactive nitrogen intermediates (RNI) such as NO.

FIGURE 1.6
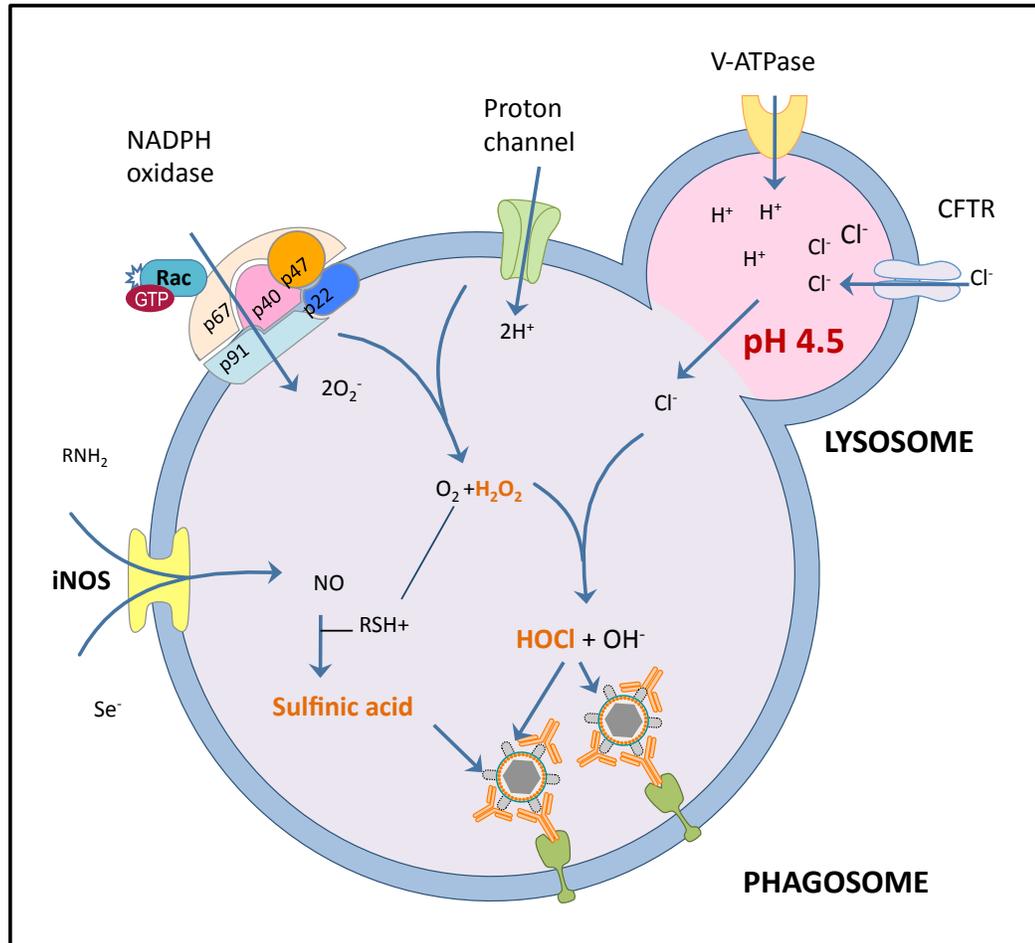
Figure 1.6. Diagram of morphine mediated modulation of macrophage bactericidal mechanisms. Following phago-lysosomal fusion, lysosomal enzymes and low pH lysosomal contents are emptied out into the phagosome initiating bacterial killing. After the fusion takes place, reactive oxygen intermediates (ROI) such as superoxide are synthesized via NADPH oxidase complex, and reactive nitrogen intermediates (RNI) such as NO are synthesized via iNOS. These three mechanisms in combination are essential in bacterial killing by macrophages.

however as the pH increases in alkaline environment of the cytosol their function is impaired. This serves as a protective mechanism of the cell in order to limit tissue damage due to enzyme presence in cytosol or other tissues, and this mechanisms is often used by pathogens to evade killing by lysosomal enzymes (Tapper and Sundler, 1990).

B. SUPEROXIDE FORMATION

Nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase complex is an electron transport chain found in lymphocytes and in the wall of the endocytic vacuole of "professional" phagocytic cells. NADPH is used as an electron donor to reduce oxygen to superoxide and hydrogen peroxide (**Figure 1.6**) (Segal and Abo, 1993). Superoxide is produced within the phagosomes containing ingested bacteria and is capable of killing them by its ability to react with other compounds to generate a diverse family of reactive oxygen intermediates (ROI) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2-), and hydroxyl radical (OH) (Gamaley et al., 1994; Gamaley et al., 1994; Caron and Hall, 1998; Bokoch, 2000; Segal et al., 2000).

Upon stimulation of phagocytic cells, Rac enhances the activity of the NADPH oxidase, resulting in the production of superoxide radicals. Activation of the NADPH oxidase requires the assembly of a multimolecular complex at the plasma membrane consisting of two integral membrane proteins, gp91phox and p21phox, and two cytosolic proteins, p67phox and p47phox (**Figure 1.6**). Rac1 interacts directly with p67phox in a GTP-dependent manner (Diekmann et al., 1994). In addition Rac 2 GTPase plays an important role in NADPH recruitment (Knaus et al., 1991). Activation of NADPH complex is essential for microbial killing, therefore any disruptions in recruitment or the

complex assembly will affect macrophage's ability to kill the microbe which may have deleterious consequences for the host.

C. NITRIC OXIDE

Nitric oxide (NO) conveys a variety of messages between cells, including signals for vasorelaxation, neurotransmission, and cytotoxicity. In macrophages, reactive nitrogen intermediates (RNI) such as NO play an essential role in killing of microbes. NO is a short-lived, gaseous radical that is the smallest biosynthetically derived secretory product of mammalian cells. RNIs can be invoked as either immediate products of the nitric oxide synthase (NOS) reaction (NO radical, NO^-, NO^+) or their adducts or conversion products. The latter category includes NO_2, NO_2^-, NO_3^-, N_2O_3, N_2O_4, S^- nitrosothiols (S-NO), peroxynitrite ($ONOO^-$) and nitrosyl−metal complexes (Bogdan, 2001).

In some endothelial cells and neurons, a constitutive NO synthase is activated transiently by agonists that elevate intracellular calcium concentrations and promote the binding of calmodulin. In contrast, in macrophages, NO synthase activity appears slowly after exposure of the cells to cytokines and bacterial products, is sustained, and functions independently of calcium and calmodulin (Xie et al., 1992).

NO can damage DNA and several chemical moieties on which its propagation and protection depend, including Fe-S clusters, tyrosyl radicals, hemes, sulfhydryls, thioethers, and alkenes. Macrophages have the opportunity to produce superoxide (O_2^-) and nitric oxide (NO) in nearly equimolar amounts and thus can be prolific generators of their joint and particularly destructive product, peroxynitrite ($OONO^-$). Nitroxyl anion a one-electron reduction product of NO, is unlikely to arise from NO under physiologic conditions, but is considered by some investigators to be a primary and more toxic

15

product of NOS (Ma et al., 1999). Reaction of RNI with cysteine sulfhydryls can lead either to S-nitrosylation or to oxidation to the sulfenic acid, as well as to disulfide bond formation, all of which are potentially reversible. OONOH spontaneously decomposes via species resembling the reactive radicals, hydroxyl (OH) and/or nitrogen dioxide (NO_2). When L-arginine is limiting, NOS can produce superoxide (O_2^-) along with NO, favoring the formation of peroxynitrite (Nathan and Shiloh, 2000). There has been considerable controversy as to whether human macrophages express NO. Expression of functional inducible NO synthase (iNOS) in mouse macrophages has been clearly demonstrated and iNOS mRNA is readily induced by IFN-gamma and LPS (Bogdan, 2001). However, these same inflammatory mediators have failed to show consistent effects on human macrophages. Recent work suggests that other mediators, such as IL-4 and anti-CD23, and various chemokines, are actually far more efficient in inducing iNOS in human macrophages (Weinberg, 1998; Mestas and Hughes, 2004). Therefore although iNOS is differentially modulated in mice and humans, its presence in both organisms is undisputed.

4. ROLE OF TOLL LIKE RECEPTORS IN PHAGOCYTOSIS AND BACTERIAL KILLING BY MACROPHAGES

The Toll-like receptor (TLR) family plays an important role in innate immune responses against pathogens, as well as the subsequent induction of adaptive immune responses. TLRs recognize specific molecular patterns found in a broad range of microbial pathogens such as bacteria and viruses, triggering inflammatory and antiviral responses, which result in the eradication of invading microbes. Individual TLRs interact with different combinations of adapter proteins and activate various transcription factors

such as nuclear factor (NF)-κB, activating protein-1 (AP-1) and interferon regulatory factors (IRF), driving a specific immune response (Akira and Takeda, 2004).

A. TLR SIGNALING

MyD88 dependent and independent signaling

Engagement of IL-1R triggers a cell signaling cascade involving myeloid differentiation primary response gene 88 (MyD88), IL-1 receptor activated kinase (IRAK), and tumor necrosis factor receptor associated factor 6 (TRAF6) activation leading to NF-κB translocation to the nucleus and transcription of proinflammatory cytokines (**Figure1.7**). The cytoplasmic portion of IL-1R is highly homologous to that of TLR. Therefore these two receptors utilize similar signaling molecules (Akira, 2006). Role of MyD88 in TLR signaling was further confirmed by several studies where MyD88-defficient mice or macrophages were completely defective in response to LPS and other microbial components in terms of cytokine production and B cell proliferation (Adachi et al., 1998; Kawai et al., 1999). Early studies considered all TLR signaling to be identical and dependant on MyD88. However with discovery of TLR ligands, stimulation of cells with specific TLR ligands revealed signaling pathways through individual TLRs differ in that they can be MyD88 dependent or independent (Akira and Takeda, 2004).

TLR4 signaling

Toll-like receptor 4 (TLR4), as a member of TLR family, plays a fundamental role in pathogen recognition and activation of innate immunity. TLR4 is activated

FIGURE 1.7. Diagram of Toll-like receptor signaling
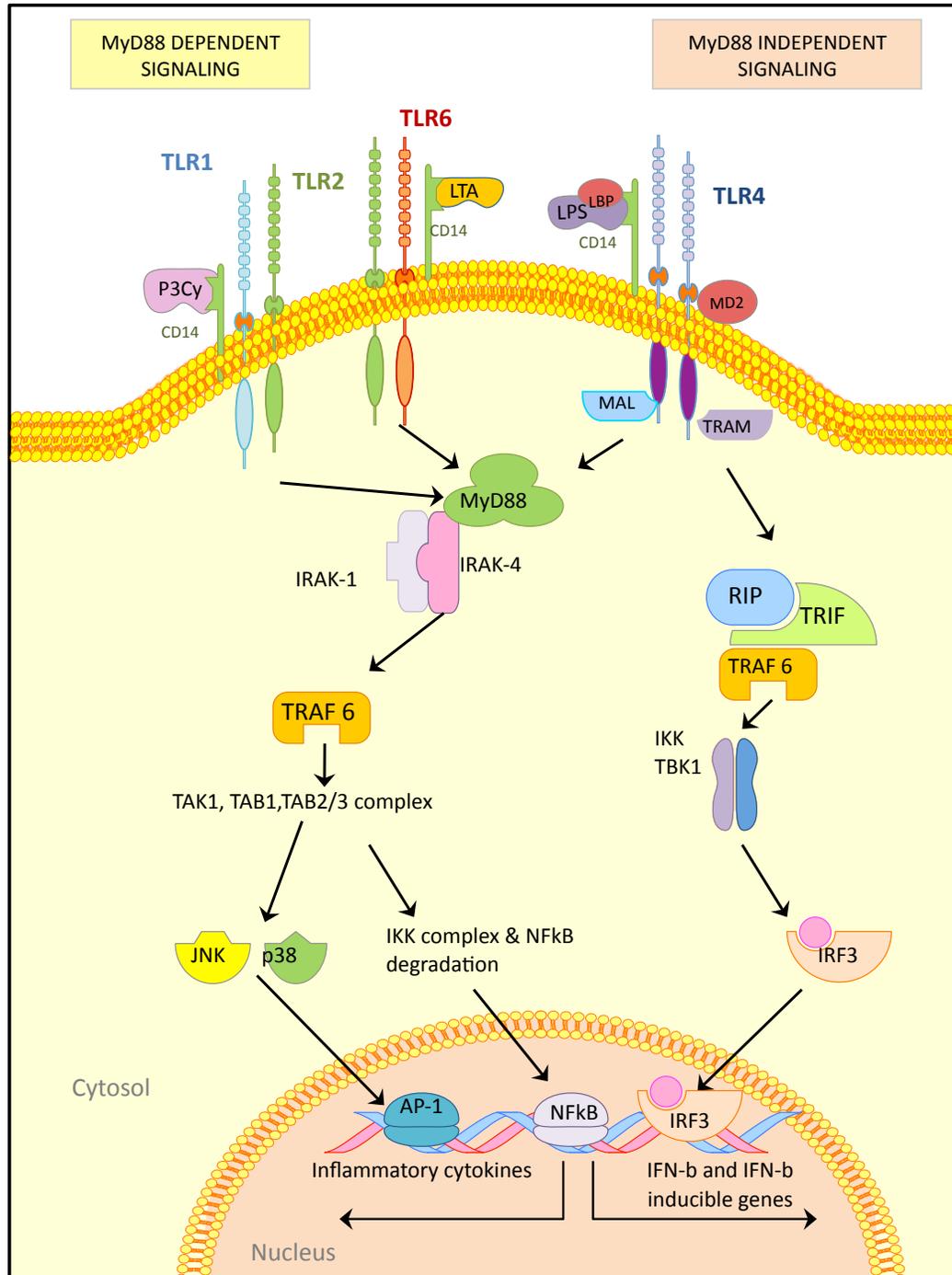
bacterial lipopolysaccharide (LPS) a component of Gram-negative bacteria cell wall. Recognition of LPS by the innate immune system elicits strong pro-inflammatory responses that can eventually cause a fatal sepsis syndrome, therefore any disruption of the TLR4 signaling pathway may lead to deleterious consequences for the host (Triantafilou and Triantafilou, 2002).

LPS is a complex glycolipid composed of a hydrophilic polysaccharide and a hydrophobic domain, known as lipid A, which is responsible for the biological activity of LPS. A complex of LPS and the serum protein LPS-binding protein (LBP) initiates signals by binding to and activating membrane-bound CD14, an opsonic receptor for complexes of LPS (**Figure 1.7)** (Aderem and Ulevitch, 2000; Akira et al., 2001). After LPS and LBP bind to CD14 the complex is then transferred to an immobile receptor such as TLR4, triggering MyD88 dependant pathway as described above, or the MyD88 independent pathway (Triantafilou et al., 2001). First evidence of MyD88 independent signaling came from studies by Kawai et al., (1999) where activation of signaling molecules such as MAPKs and NF-κB was abolished in MyD88 deficient macrophages in response to TLR2 (lipoprotein), TLR7 (imidazoqinolones) or TLR9 (Cpg DNA). However although MyD88 was absent, activation of NF-κB and MAPKs was observed following TLR4 activation by LPS. This was the first evidence of MyD88 independent pathway by TLR receptor. To date, TLR4 and TLR3 are the only known TLRs that utilizes MyD88 independent pathway. After the TRL4 activation the pathway can either act in a MyD88 dependant or independent manner (Alexopoulou et al., 2001).

TLR2 signaling

Of all the mammalian TLRs, and perhaps of all pathogen recognition receptors, TLR2 recognizes the largest number of ligands (Janeway and Medzhitov, 2002). TLR2

primarily recognizes lipoproteins and glycolipids. Lipoproteins are proteins containing lipid that is covalently linked to the NH2-terminal cysteines; they are present in a variety of bacteria, including Gram-negative and Gram-positive bacteria and mycoplasmas. Lipoproteins possess immunostimulating activities that are attributed to the presence of their lipoylated NH_2 termini. Unlike TLR4, TLR2 signal transducing ability is determined by heterodimeric interactions with other TLRs, such as TLR6 and TLR1 (Ozinsky et al., 2000). Expression of dominant-negative TLR2 or dominant-negative TLR6 in the macrophage cell line showed that TLR6 and TLR2 function together to detect Gram-positive bacteria, PGN and zymosan, whereas TLR2 functions either alone or with TLRs other than TLR6 to detect bacterial lipopeptides (Takeuchi et al., 1999). Some of the ligands known to lead to TLR2 heterodmerization are lipoteichoic acid (LTA), a major immunostimulatory component of Gram-positive bacteria, which requires a TLR2 and TLR6 dimerization, while Pam3CSK4 (P3C), a synthetic triacylated lipoprotein requires dimerization of TLR2 and TLR1 **(Figure 1.7**) (Triantafilou et al., 2006).

Toll-like receptors 2 and 4 have been suggested to be the signaling receptors for lipopolysaccharide (LPS) (Kawai et al., 1999). Several observations indicate that TLR2 is also involved in LPS signaling (Kirschning et al., 1998; Yang et al., 1998). However, neither human nor murine TLR2 play a role in LPS signaling, so it was suggested that overexpression of TLR2 may cause cell lines to become extremely sensitive to minor non-LPS contaminations in LPS preparations (Takeuchi et al., 1999);(Underhill et al., 1999). Highly purified LPS does not activate cells through TLR2 (Hirschfeld et al., 2000). Other data indicate that TLR2 may be involved in the response to LPS from Leptospira and Prophyromonas, which are structurally different from Gram-negative LPS (Akira et al., 2001). Although early studies indicate that LPS can activate

both TLR2 and TLR4, more recent studies confirm that this is not the case and that LPS from Gram-negative bacteria remains to be a standard TLR4 ligand.

B. TLRs AND PHAGOCYTOSIS

The phagocytosis of bacteria and other pathogens by macrophages is one of the initiating events of the innate immune response. Phagocytosis is an important step for host defense against microbial pathogens, since it triggers both degradation of pathogens and subsequent presentation of pathogen-derived peptide antigen. Microbial internalization is often accompanied by inflammatory responses that are driven by TLRs, suggesting that phagocytosis and TLR activation may be functionally linked. TLR recognition of pathogens leads to expression of genes such as inflammatory cytokines and co-stimulatory molecules. Phagocytosis-mediated antigen presentation together with TLR-dependent gene expression of inflammatory cytokines and co-stimulatory molecules, leads to development of antigen-specific acquired immunity. Therefore, it is of interest to characterize the relationship between phagocytosis and TLRs. There are three principle steps where TLR signaling can affect phagocytosis (**Figure 1.8**). First, TLR signaling may modulate the efficiency of phagosome formation. Second, TLR signaling may affect maturation of newly formed phagosomes. Third, TLR-mediated transcriptional responses may affect genes and gene product involved in all steps of phagocytosis (Underhill and Gantner, 2004).

Phagosome formation

TLR activation modulates phagosome formation, and hence the rate of internalization. Seminal study by Blander and Medzhitov (2004) revealed the role of TLR in regulation of phagocytosis. In this paper, bone marrow derived macrophages deficient

FIGURE 1.8
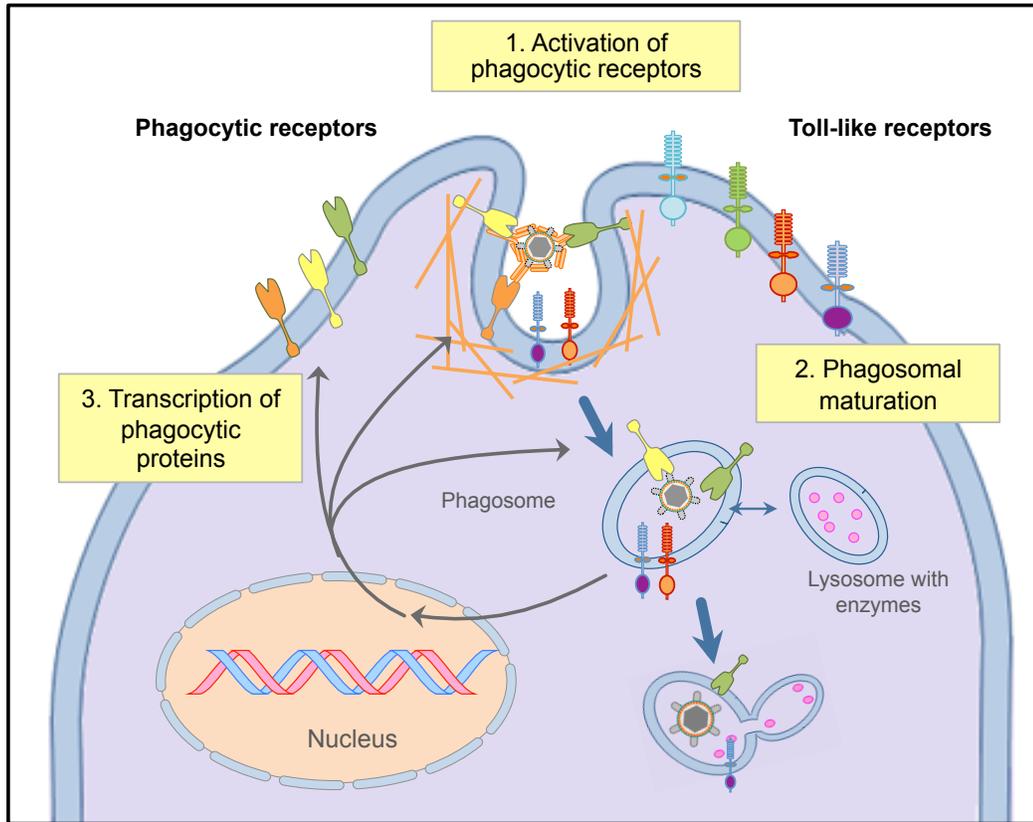
Figure 1.8. Diagram of TLR modulation of phagocytosis.

TLRs may modulate phagocytosis through three mechanisms:

1. TLR signaling may modulate activation of other phagocytic receptors.
2. TLR signaling may regulate the rate at which phagosomes mature into phagolysosomes.
3. TLR signaling may induce or inhibit production of proteins required at all stages of phagocytosis. Figure adapted and modified frorm (Underhill et al 2004).

in either TLR2/4 or MyD88 were presented with *E. coli, S. aureus,* or apoptotic cells. When compared to wild type macrophages, TLR2/4−/− and MyD88−/− macrophages exhibit normal rates of apoptotic cell clearance, but decreased rates of bacterial clearance within two hours of bacterial infection. Phagocytosis of bacteria seemed to be dependent on p38 MAPK activation downstream of TLR activation as this defect could be recapitulated by p38 MAPK inhibitors. Although this study indicated that TLR2 and TLR4 activation led to increased phagocytosis, the mechanisms underlying these interactions are yet to be defined.

There are many overlaps between the TRL signaling and the signaling pathways involved in phagosome formation. For example, Rac1, a Rho family GTPase, and PI3-kinase are both required for phagocytosis and are also utilized by TLRs (Underhill and Ozinsky, 2002). Arbibe et al. (2000), demonstrated that a dominant-negative Rac1 blocked TLR2-mediated NF-κB translocation in response to *Staphylococcus aureus*, and that both Rac1 and PI3-kinase are recruited to the intracellular domain of TLR2. Other work has implicated PI3-kinase in TLR4-dependent signaling as well (Li et al., 2003). It has been known for some time that LPS activates protein kinase C and phospholipase C, and these molecules are also both important in phagocytosis (Sweet and Hume, 1996).

TLRs regulate phagocytosis through transcriptional regulation

TLRs activate transcription of a large number of genes, including gene products known to participate in pathogen internalization (Kawai et al., 1999). In one survey, over 50 genes were identified whose protein products are likely to participate in phagocytosis (Hume et al., 2002). These gene products participate at all stages during phagocytosis, ranging from microbial recognition, actin cytoskeletal dynamics,

membrane trafficking, ion transport, proteolysis, and antigen presentation. Thus, there is no doubt that transcriptional responses to TLR activation modulate phagocytosis (Underhill and Gantner, 2004). Many receptors involved in phagocytosis are directly regulated by TLRs. For example, LPS stimulation of macrophages increases expression of scavenger receptor and FcgR. Cytokines that are produced in response to LPS favor expression of activating FcgRs on macrophages and reduce expression of inhibitory FcgR (Ravetch and Bolland, 2001). Literature shows that TLR ligands can activate a MyD88-independent pathway to increase phagocytosis; however, the exact mechanism of how this pathway enhances microbial engulfment needs to be clarified (Tricker and Cheng, 2008). Future studies examining the role of TLRs in modulating pathogen internalization will need to carefully discriminate between direct regulation of phagocytic machinery and the contribution of transcriptional responses in these processes (Underhill and Gantner, 2004).

C. TLR AND BACTERIAL KILLING

Since TLR activation increases rates of phagocytosis it is of importance to understand what happens to the phagosome following TLR activation. As the phagosome forms and matures, TLR receptors on the phagosome are capable of sensing phagosomal environment and identifying the internalized pathogen. Once the phagosome is formed it matures and fuses with lysosome resulting in effective bacterial clearance and sterilization of the phagosomal contents. During the phagocytosis of pathogens, two classes of innate immune receptors cooperate to mediate host defense: phagocytic receptors, such as the FcgR, signal particle internalization, and the TLRs on the phagosomal membrane sample the contents of the vacuole and trigger an

inflammatory response appropriate to defend against the specific organism (Underhill et al., 1999; Underhill and Gantner, 2004;Blander and Medzhitov, 2004).

TLR-induced signal comes from phagosomes containing cargo that engages TLRs. Literature shows that TLR activation induces phagosome maturation, while phagosomes containing apoptotic cells mature more slowly. Enhancement of phagosomal maturation is mediated through a MyD88-dependent activation of p38 MAPK (Blander and Medzhitov, 2004). This study showed that increased rate of phagosomal maturation was not influenced by the activation of TLR signaling within the same cell from a different phagosome that carried bacteria, or from the plasma membrane where the TLR was engaged by a nonparticulate ligand like LPS. This suggested that phagosome maturation was stimulated by a TLR signal that was spatially confined such that only phagosomes containing cargo that engaged TLRs were subject to inducible maturation.

In addition to enhancing phagosomal maturation, TLR activation leads to increase in NO and superoxide production (Ulevitch and Tobias, 1995). Macrophages activated by infection or elicited by injection of lipopolysaccharide (LPS), when stimulated with phorbol myristate acetate, release greater amounts of superoxide anion (O_2^-) than do normal resident macrophages. This enhanced production of O_2^- and of other oxygen metabolites derived from O_2^- is responsible, at least in part, for the enhanced microbicidal activity of these cells. These results suggest that the enhanced oxygen metabolite response of activated macrophages is due, in part, to modification of NADPH-oxidase (Sasada et al., 1983).

5. MORPHINE MODULATION OF IMMUNE SYSTEM

A. HISTORICAL PERSPECTIVE

Opioid mediated immune suppression has been observed and studied for centuries. As early as the 1800's Cantacuzene was the first to demonstrate that opium was capable of suppressing cellular immunity and lowering resistance of guinea pigs to bacterial infections. By 1940's opioid suppression of immune functions, as well as morphine's role in exacerbation of infectious diseases was recognized by several investigators (reviewed by Peterson et al., 1993). Decades later, as intravenous heroin use increased, epidemiological data surfaced indicating that heroin addicts had an increased incidence of infection (reviewed by Roy et al., 2006). These findings were initially attributed to the use of non-sterile or contaminated needles by drug addicts, impurities in street heroin as well as to the promiscuous behavior which comes with heroin abuse. However, more recently, morphine (the active metabolite of heroin) has gained even more significance after it has been implicated as a cofactor in development of opportunistic infections and HIV pathogenesis (McCarthy et al., 2001). With heroin and prescription drug abuse on the rise, the interest in morphine as a modulator of immune function has gained significant interest (Compton and Volkow, 2006).

B. MORPHINE INCREASES SUCEPTIBILITY TO INFECTION

Immune suppression in heroin abusers is displayed through increased incidence of various bacterial and viral infections. Several groups indicate that Intravenous drug abusers have a greater incidence of infection than non-abusers (Husey and Katz, 1950; Louria et al., 1967). Extensive studies in the area of morphine induced immune suppression noted that opioid addicts present with high prevalence of tuberculosis,

bacterial pneumonias, abscesses, CNS infections as well as viral hepatitis A, B, and C, and high rate of HIV infections (Louria et al., 1967; Reichman et al., 1979; Haverkos and Lange, 1990).

Seminal study by Tubaro et al. (1983), observed that following single daily injections of morphine given 24-72 h prior to iv injection of fungus *Candida albicans*, resulted in lethality from the organism. This study demonstrated that morphine was able to increase the number of viable *Candida albicans* in the kidney in a dose-dependent manner. More recent studies indicate similar results, where mice implanted with slow release morphine pellet presented with sepsis, which was manifested by increased bacterial loads in liver, spleen and peritoneal cavities (Hilburger et al., 1997). Additionally, studies from our laboratory show that *in vivo* chronic morphine treatment followed by intranasal inoculation with *Streptococcus pneumoniae* markedly delayed neutrophil recruitment, increased bacterial burden in the lung spleen and blood with a subsequent increase in mortality (Wang et al., 2005).

Morphine's immunosuppressive effects were first noted in its ability to increase susceptibility to infection, as well as accelerate the rate of their progression. Although many studies have explored this phenomenon, mechanisms elucidating how morphine inhibits immune function remain need further investigation.

C. MORPHINE'S MODULATION OF IMMUNE SYSTEM BY DIRECT AND INDIRECT MECHANISM

Morphine exerts effects through activation of opioid receptors. Opioid receptors are G-protein coupled receptors and they fall into three major classes μ, δ and κ. Originally it was thought that opioid receptors are expressed only in the central

nervous system (CNS) and therefore that the primary site of morphine action is in the CNS and that morphine's modulation of the immune system occurs through the activation of the hypothalamo-pituitary-adrenal axis (HPA) and the stress-responsive neuroendocrine pathway (Peterson et al., 1993).

HPA axis is a complex set of direct influences and feedback interactions among the hypothalamus, the pituitary gland, and the adrenal (or supradrenal) glands (**Figure 1.9**). The interactions among these organs constitute the HPA axis, a major part of the neuroendocrine system that controls reactions to stress, and through release of stress hormones exerts immunosuppressive effects. Morphine can activate HPA axis leading to release of stress hormones, corticosteroids (CORT) resulting in immune suppression. Therefore opioids can inhibit immune functions in an indirect manner by activating HPA axis to release immunomodualtory stress hormones.

Studies supporting a direct role of opioids on immune system emerged with the discovery of opioid receptors on the immune cells. The concept of direct and indirect morphine action was first introduced through work with morphine dependant rodents. Findings by our group and others indicate that morphine-induced immunosuppression is mediated by the μ–opioid receptor (MOR) and that although some functions are amplified in the presence of CORT or sympathetic activation, the inhibition of IFN-gamma synthesis and activation of macrophage-cytokine synthesis is CORT-independent and only partially dependent on sympathetic activation (Peterson et al., 1987; Bryant et al., 1991; Casellas et al., 1991; Perez-Castrillon et al., 1992). With discovery of opioid receptors on leukocytes, research focus has shifted to direct effects of opioids on immune cells, however when looking at *in vivo* models of drug abuse and immunomodulation it is important not to neglect the role of stress mechanisms mediated by the HPA axis.

FIGURE 1.9
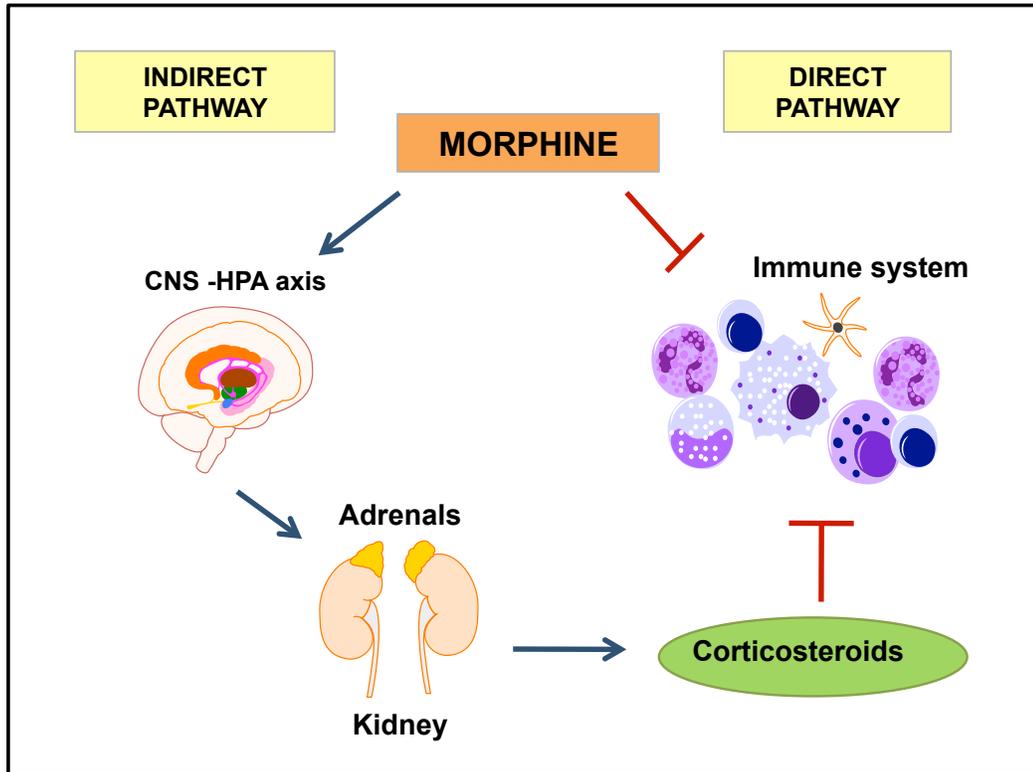
Figure 1.9. Pathways of opiate induced immune suppression.

Morphine can modulate immune system via direct and indirect pathway. Indirectly morphine acts on CNS and the hypothalamic-pituitary-adrenal (HPA) axis which leads to release of corticosteroids, immunosuppressive hormones which lead to suppression of the immune system. Direct interactions with immune cells involve opioid receptor expressed on the immune cells and through various interactions and crosstalk with receptors on immune cells, opioids are able to modulate immune cell function.

D. MORPHINE AND THE IMMUNE SYSTEM

Recently, as the prescription of opioid-based pain relievers began to rise, opportunistic infections have followed the same trend (Compton and Volkow, 2006; Wang et al., 2008). Prevalence of opioid use and abuse is undisputed, and has impacted a wide range of individuals in both the drug abuse populations as well as the patients in clinical setting. Research shows that chronic morphine can modulate both adaptive and innate immune systems.

Modulation of adaptive immune system has first been observed in morphine treated rodents where morphine treatment led to decreased splenic and thymic weight resulting in reduced function of T cells and their precursors (Bryant et al., 1988; Bryant et al., 1991). In addition, morphine treatment led to inhibition of IFN-γ , T-cell apoptosis, and through a naltrexone sensitive opioid receptor mechanism biases naive T-cells to a Th2 pathway (Roy et al., 2001). Contrasting T-cell research, work on morphine's effect on function of B-cells is limited. Morphine administration was found to inhibit IL-4 induced expression of MHC II RT1.B surface expression following antigen-induced cytokine production, as well as inhibit antibody response (Bussiere et al., 1993).

The modulation of innate immune system has been observed on several levels. Morphine treatment modulates leukocyte recruitment, cytokine secretion and bacterial clearance. By decreasing the proliferative capacity of macrophage progenitor cells and lymphocytes morphine treatment inhibits numbers of macrophages that are available to respond to the infection (Roy et al., 2006). In addition, morphine delays leukocyte migration, which affects the numbers of phagocytes recruited to the sight of infection and ultimately suppresses the capacity of macrophages to ingest opsonized pathogen (Casellas et al., 1991; Szabo et al., 1993; Tomei and Renaud, 1997). In the study by

Bussiere et al. authors conclude that since morphine mediated suppression of antibody response was reversible by addition of macrophage derived cytokine, immune suppression appears not to be directly due to the B-cell impairment but rather due to the reduced macrophage numbers or decreased release of cytokine which are needed for a normal antibody response. Collectively, these findings suggest that the macrophage is a key cellular target for the suppressive effects of morphine on the antibody response (Bussiere et al., 1993; Eisenstein et al., 1993). Although morphine modulates both innate and adaptive immune systems, defects in innate immunity seem to have broader consequences, with modulation of macrophage functions playing an essential role. Therefore examining morphine mediated modulation of macrophage processes will be the main focus of our discussion.

E. MODULATION OF MACROPHAGE FUNCTION BY MORPHINE

Macrophages form the first line of defense against pathogens, and play an essential role in innate immune defenses through their phagocytic and bactericidal roles as well as through their ability to recruit other cells to the site of infection. Therefore any defects in macrophage function can be detrimental for the host. Macrophage has been at the center of several studies for the significant role it plays in morphine mediated immune suppression.

Morphine modulation of macrophage phagocytosis

Initially, it was observed that morphine led to suppression of phagocytosis by peritoneal macrophages as well as inhibition of respiratory burst activity and chemotaxis (Perez-Castrillon et al., 1992). Due to inhibition of phagocytosis bacteria are inadequately removed and since respiratory burst was inhibited, morphine attenuated

bacterial killing which together with inhibited phagocytosis leads to increased bacteremia and bacterial escape from latency as shown by our group and others (Bhaskaran et al., 2001; Wang et al., 2005; Lugo-Chinchilla et al., 2006). Human studies and rodent models of drug abuse indicate that morphine impairs the ability to eradicate infection by inhibiting phagocytosis. *In vivo* studies of morphine abuse have shown that morphine inhibits phagocytosis by non-elicited and elicited macrophages in naltrexone reversible manner indicating involvement of classical opioid receptors (Rojavin et al., 1993a; Rojavin et al., 1993b). Subsequent *in vitro* studies indicate that morphine inhibits Fcγ receptor (FcgR) mediated phagocytosis essential for internalization of extracellular pathogens, and that inhibition of phagocytosis occurs through μ and δ opioid receptors (Szabo et al., 1993; Tomassini et al., 2004). Studies by our group confirmed that morphine mediated inhibition of phagocytosis was abolished in MORKO mice, adding further evidence for the role of μ opioid receptor in these functions (Roy et al., 1998a). Additionally, it was observed that *in vitro* administration of endogenous opioid peptides such as leu- and metenkephalin (delta receptor agonists) are able to inhibit phagocytosis of opsonized sheep red blood cells (Casellas et al., 1991). Role of endogenous opioids in immune suppression indicates an evolutionary mechanism by which the nervous system and immune system interact in order to regulate phagocytosis and maintain homeostasis.

Morphine modulation of bacterial killing by macrophages

Several studies support that morphine attenuates bacterial killing as evident by increased bacterial loads or sepsis (Wang et al., 2005; Hilburger et al., 1997). In mice, chronic morphine has been shown to modulate bacterial killing by inhibition of NO release (Fecho et al., 1994; Pacifici et al., 1995; Menzebach et al., 2004; Bhaskaran et

al., 2007). Our laboratory's previous data and other groups indicate that chronic morphine by inhibition of NO release increases the susceptibility to bacterial infection, resulting in bacteremia and bacterial invasion of CNS (Wang et al., 2005; Asakura et al., 2006; Bhaskaran et al., 2007). Recent study by Singh and Singal (2007), notes that morphine administration has a dose-dependent biphasic modulation in *Leishmania donovani* infected mice and peritoneal macrophages *in vitro*, via a NO-dependent mechanism. They show that morphine administration in nano molar range was protective against *L.donovani* infection, while morphine concentrations in micromolar range led to augmented intramacrophage parasite growth.

Furthermore, morphine has been implicated in the inhibition of superoxide production. Several groups studying morphine's effect on infection examined superoxide release as a mechanism of bacterial killing, where it was noticed that morphine inhibits superoxide production in neutrophils and macrophages (Sharp et al., 1985; Simpkins et al., 1986; Welters et al., 2000). In addition to exogenous opioids, endogenous opioids had similar inhibitory effect where pretreatment with endogenous opioid peptides leucine or methionine enkephalins reduced neutrophil's ability to generate superoxide production in response to the *Escherichia coli* product, N-formyl methionyl leucyl phenylalanine (FMLP) (Sharp et al., 1985; Simpkins et al., 1986). Morphine mediated suppression of superoxide production was reproduced in human peripheral mononuclear cells in studies done by Peterson et al., which examined respiratory burst activity in response to phorbol myristate acetate (PMA) (Peterson et al., 1987; Peterson et al., 1989).

Although morphine's inhibition of bacterial killing by macrophages has been studied by several groups, these studies have addressed some key mechanisms involved bactericidal process, leaving room for additional investigation of how these mechanisms are regulated by morphine.

F. MORPHINE MODULATION OF SIGNALING PATHWAYS INVOLVED IN MACROPHAGE PHAGOCYTOSIS

Role of cAMP in phagocytosis and morphine signaling

Several studies have examined morphine's effect on macrophage function, however a gap in knowledge still exists in understanding mechanisms of this modulation. Therefore in order to investigate potential mechanisms by which morphine modulates signaling pathways involved in pathogen internalization, we first identified key factors of opioid signaling, as well as signaling pathways involved in phagocytosis and bacterial killing.

Intracellular cyclic AMP (cAMP) regulation systems play an important role in the mechanisms of morphine dependence. Morphine mediated increase in cAMP has been shown to decrease the mean mouse survival time and increase bacterial burden in various tissues of infected mice (Feng et al., 2006). While acute morphine inhibits adenylyl cyclase (AC) activity, chronic morphine treatment has been known to increase cAMP levels by activation of G proteins, which then modulate the activity of ion channels, and intracellular levels of cAMP (Sharma et al., 1975; Childers, 1991). Chronic morphine leads to increased cAMP by super activation of AC in macrophages (Avidor-Reiss et al., 1997; Kelschenbach et al., 2008). Modulation of intracellular cAMP plays a crucial role in macrophage homeostasis. Literature shows that high levels of intracellular cAMP in macrophages inhibit phagocytosis, decrease iNOS activity, and inhibit nitric oxide production (Okado-Matsumoto et al., 2000). Although elevation of cAMP in lymphocytes leads to suppression of immune functions by a PKA-dependent mechanism, the effector mechanisms for cAMP regulation of immune functions of macrophages have not been fully elucidated (Bryn et al., 2006). Considering the broad

impact of cAMP signaling on phagocytic pathways, modulation of cAMP may be one of the mechanisms by which morphine inhibits immune function.

Role of MAPKs in phagocytosis and morphine signaling

In addition to cAMP, morphine has been implicated in modulation of mitogen-activated protein kinases (MAPKs), specifically extracellular signal-regulated kinase (ERK)-1/2 and p38 MAPK. ERK1 and ERK2 have 83% amino acid identity and are expressed to various extents in all tissues (reviewed by Chen et al., 2001). They are strongly activated by growth factors, serum, and phorbol esters and to a lesser extent by ligands of the heterotrimeric G protein-coupled receptors, cytokines, osmotic stress, and microtubule disorganization (Lewis et al., 1998). The signaling pathway from FcgR to the nucleus is not completely known, but it shares elements with the biochemical cascade used by other receptors known to activate gene transcription. In particular, ERK1/2 is required for FcgR-mediated activation of the nuclear factor NF-κB, as well as phagocytosis of IgG-opsonized particles (Sanchez-Mejorada and Rosales, 1998; Suchard et al., 1997; Mansfield et al., 2000).

p38 MAPK is critical for normal immune and inflammatory responses. p38 MAPK is activated in macrophages, neutrophils, and T cells by numerous extracellular mediators of inflammation, including chemo-attractants, cytokines, chemokines, and bacterial lipopolysaccharide. p38 MAPK participates in macrophage and neutrophil functional responses, including phagocytosis, respiratory burst activity (H_2O_2 production), chemotaxis, granular exocytosis, adherence, and apoptosis p38 MAPK can be activated by phagocytosis (Ono and Han, 2000; McLeish et al., 1998). Effectors of phagocytic signaling such as Rho family GTPases Rac1 and Cdc42 and the

cytoplasmic kinase Pak 1 have been implicated in the control of the p38 MAP kinase signaling pathway (Zhang et al., 1995).

Morphine's modulation of ERK1/2 and p38 MAPK has been investigated however literature seems to be controversial since results seem to vary greatly depending on dose and duration of incubation. Some studies indicate that morphine increases ERK1/2 and p38 MAPK phosphorylation, while others show an inhibition. In studies by Singhal et al. (2002), morphine at 10μM increased p38 MAPK phosphorylation and macrophage apoptosis, while lower, physiological plasma level concentrations of morphine 1 μM, led to inhibition of ERK1/2 and p38 MAPK phosphorylation as shown in studies by Wang et al., 2003. Therefore in concentrations in physiological range, morphine will inhibit MAPK activation. Although the role of morphine seems to have differential effects on ERK1/2 and p38 MAPK activation, one of the ways morphine can inhibit MAPK function is through cAMP. Morphine increases cAMP while increase in cAMP has been shown to inhibit ERK, and p38 activation induced by inflammatory stimuli (Feng et al., 2002; Zhu et al., 2008).

Morphine increases cAMP and inhibits ERK1/2 and p38 MAPK activation in concentrations similar to morphine plasma level concentrations of opioid users. cAMP and MAPKs play an essential role in phagocytosis, superoxide release and toll-like receptor activation, and therefore will be the main targets of our investigation of mechanistic studies by which morphine inhibits immune functions.

CHAPTER 2.
CHRONIC MORPHINE TREATMENT MODULATES MACROPHAGE PHAGOCYTOSIS

INTRODUCTION

Opioid use and abuse has seen a steady increase through the 1990s and has continued to rise during recent years (Nora D. Volkow, 2008)(Substance Abuse and Mental Health Services Administration, Department of Health and Human Services, 2007). The 2004 National Survey on Drug Use and Health (NSDUH), shows that between 1999 and 2001, annual incidence of opioid analgesic abuse increased from 628,000 initiates in 1990 to 2.4 million initiates in 2001 (Compton and Volkow, 2006). It is now well established that chronic opioid use or abuse results in severe immunosuppression and increased susceptibility to infection (Friedman and Eisenstein, 2004); (Roy et al., 1998c; Feng et al., 2006). Chronic morphine has been known to modulate innate immune system by decreasing the proliferative capacity of macrophage progenitor cells and lymphocytes, by inhibiting macrophage phagocytic (Tomei and Renaud, 1997; Tomassini et al., 2004) and migratory capabilities (Malik et al., 2002) and by leading to sepsis in mice (Roy et al., 1998c; Feng et al., 2006). Similar studies show that morphine's inhibition of certain cytokines can be restored by addition of untreated macrophages, suggesting that morphine-induced immunosuppression is due to a deficit in macrophage function (Eisenstein et al., 1993).

Despite these severe health consequences morphine and other opioid based pain relievers remain widely prescribed and abused around the world (Manchikanti and

Singh, 2008). There is clearly an urgent need to delineate the underlying cellular and molecular mechanisms by which chronic opiate use or abuse increases susceptibility to bacterial infection. Understanding these mechanism will allow for the development of novel approaches to treat and prevent bacterial infection in the opiate-abuse population and in patients that are on opioids for pain management.

Innate immunity plays a crucial role in clearance of bacterial infections. Phagocytes such as macrophages are essential as first line of defense against microbial pathogens. By utilizing their phagocytic abilities, macrophages are able to facilitate pathogen clearance and elimination. In order to internalize a wide variety of pathogens, macrophages have evolved a restricted number of phagocytic receptors, such as the mannose receptor, scavenger receptor, complement receptor and FcgRs. FcgRs belong to a class of macrophage receptors that are responsible for phagocytosis of opsonized extra-cellular bacteria. Cross linking of Fcγ activating receptors by immune complexes leads to tyrosine phosphorylation and signal transduction cascade ultimately leading to activation of Rho guanosine triphosphatases (Rho GTP-ases), molecular switches that control the organization of the actin cytoskeleton essential for phagocytosis, actin polymerization, membrane extension and pathogen engulfment (Bokoch, 2000). Interestingly, opioid abuse has been shown to increase the incidence of extracellular bacterial infections such as Streptococcus pneumoniae, and Enterococcus faecalis (Hilburger et al., 1997). We speculate that modulation of FcgR mediated phagocytosis by morphine may be a potential mechanism for the increased susceptibility to infections by extracellular pathogens (Tomei and Renaud, 1997; Tomassini et al., 2004). In this study the molecular mechanisms by which morphine modulates macrophage FcgR mediated phagocytosis was investigated. We examined signaling pathways involving cyclic adenosine monophosphate (cAMP), *protein kinase A* (PKA), and mitogen-

activated protein kinases (MAPK's) as potential mechanisms of morphine's action. Intracellular cAMP plays an important role in the mechanisms of morphine dependence. Chronic morphine has been known to increase cAMP levels by activation of G proteins which then modulate the activity of ion channels and levels of cAMP (Childers, 1991). In addition, literature shows that high levels of intracellular cAMP inhibit macrophage phagocytosis by inhibiting actin polymerization (Lang et al., 1996). Although elevation of cAMP in lymphocytes leads to immune suppression by a PKA-dependent mechanism, the mechanisms underlying morphine mediated cAMP regulation on immune functions in monocytes and macrophages are not fully understood (Bryn et al., 2006). Therefore we set out to examine if morphine mediated increase in cAMP could be a primary mechanism by which morphine inhibits phagocytosis.

In addition, recent studies show that p38 MAPK and the related extracellular signal-regulated kinase (ERK) MAPK's are activated during neutrophil phagocytosis (McLeish et al., 1998). Since morphine has been shown to inhibit p38 MAPK and ERK1/2 phosphorylation in T-cells, we hypothesized that similar mechanisms are at play in macrophages, and that morphine by modulating these kinases inhibits phagocytosis (Wang et al., 2003). Our results indicate that morphine utilizes several pathways involving cAMP, p38 MAPK, ERK1/2 and Rac1-GTPase in order to inhibit actin polymerization and opsonophagocytosis that follows.

METHODS

REAGENTS

Heat killed *E.coli* particles (E2861- *Escherichia coli* (K-12 strain) BioParticles, fluorescein conjugate (ex 494/ em 518), opsonizing reagent (E2870), dextran beads (yellow-green fluorescent Fluo-Spheres (F8852 Invitrogen; ex488nm/518nm), rhodamine phalloidin and DAPI (4,6diamidino-2-phenylindole) were obtained from Invitrogen (Crlsband, CA). Cytochalasin D, H89, forskolin, DB-cAMP, PD98059 and SB203580 were obtained from Sigma Aldrich (St. Louis, MO). cAMP detection kit from R&D Systems (R&D Systems), Rac-GTP GLISA kits (BK125) as well as the Rac-GTP pull-down kit (BK035) were purchased from Cytoskeleton (Denver, CO). Morphine-HCl powder as well as 75mg slow release pellets were a generous gift from NIDA. Macrophage cell line J774.1 was obtained from ATCC (Manassas, VA), and cultured in DMEM media supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), and 1% Penicillin/Streptomycin (GIBCO).

CELLS

Macrophage cell line J774.1 was obtained from ATCC, and cultured in DMEM media (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), and 1% Penicillin/Streptomycin (GIBCO).

ANIMALS

MORKO mice (C57BL/6 x 129/Ola genetic background) were produced as described previously by Loh and colleagues (Roy et al., 1998a). Briefly, a *Xho*I/*Xba*I fragment,

which spans the entire exons 2 and 3, was replaced with a Neo^r cassette followed by the ligation of a thymidine kinase expression cassette to the 3' end of this segment. WT mice (B6129PF1/J), 8 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME). Animal studies have been reviewed and approved by University of Minnesota Institutional Animal Care and Use Committee.

PRIMARY MACROPHAGES

Primary peritoneal macrophages were obtained from WT or MORKO male mice 6-8 weeks of age. Peritoneum was lavaged, cells were collected and plated in 96-well plates in serum-free media for 30 minutes, nonadherent cells were washed off with PBS and remaining adherent macrophages were maintained in enriched DMEM (see "Cells") with or without 1μM morphine, where appropriate, to avoid morphine withdrawal and used for further experimentation.

CHRONIC MORPHINE ADMINISTRATION

For all *in vitro* experiments 1μM morphine HCl was added overnight (18 hours). For studies involving morphine treatment *in vivo,* morphine mice were implanted with 75mg slow release morphine/placebo pellets for 72 hours. During the extraction of peritoneal cells from morphine treated WT or MORKO mice, 1μM morphine was maintained in all PBS and media used in the experiment in order to prevent withdrawal. Concentrations used in the *in vitro* paradigm were chosen to closely replicate morphine plasma levels (11ng/ml-1440ng/ml) which are present in patients undergoing morphine sulphate treatment (2.5mg-90mg every 4 hours), as well as mice following 72 hours implantation with 75mg morphine pellets (Aherne et al., 1979).

IN VIVO PHAGOCYTOSIS ASSAY

Mice were treated *in vivo* with morphine (as described above) and 30 minutes prior to sacrifice, they were injected with heat killed, *E. coli* Bioparticles. Macrophages were collected from peritoneal lavage (see above) and washed with 50% trypan blue in order to extinguish fluorescence of non internalized particles. Cells were plated in 96 well plates, left to adhere for 30 minutes in serum free DMEM media, washed, stained with DAPI and quantified using the fluorometric assay.

FLUOROMETRIC ASSAY

Cells were plated at 10,000/100ul of media per well of a 96-well plate, treated with morphine and cultured overnight in standard growing conditions ($37^{o}C$, 5% CO_2, 90% rh). The following day, opsonized dextran or heat killed *E. coli* was added (in 1:20, cell:bacteria/bead ratio). Opsonization was done with IgG opsonizing reagent (Invitrogen) for 1hour in 37 ^{o}C, according to the manufacturer's instructions. The reaction was stopped at different time points by addition of trypan blue which extinguishes fluorescence of non internalized particles. Cells were washed two times and stained with DAPI. Fluorescence was recorded using a fluorescence plate reader (BMG; FLUOstar Omega) at ex485/em520 (FITC), and ex355/em460 (DAPI). Data were quantified as Phagocytic Index=FITC (Relative Fluorescence Units (RFU)) / DAPI (RFU), indicative of particle fluorescence/cell. In actin polymerization experiments, cells were treated similar to above, washed, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton x100 and stained with rhodamine phalloidin (Invitrogen) according to manufacturer's protocol. After the rhodamine stain, cells were stained with DAPI and florescence was

measured using the fluorescence plate reader. Actin polymerization was quantified as rhodamine (RFU) (ex544/em590) / DAPI(RFU), indicative of actin polymerization per cell. The y-axis was normalized to fluorescence of the vehicle treatment with shortest bacterial incubation or to the vehicle control (depending on an experiment) and data was expressed as percentage of vehicle control (% control).

CONFOCAL MICROSCOPY

Following the same treatment as above, cells were stained with rhodamine phalloidin according to manufacturer's directions. Images were acquired using Nikon inverted confocal microscope (model: Ti-E eclipse 100) and Roper camera (model: Cool-snap HQQ) at 60x with additional digital magnification. Images shown are flattened sum of 15 cross sections.

PULL-DOWN AND GLISA ASSAYS

J774 cells were plated at 50,000 cells/ml of supplemented DMEM media (10cm Petri dish) and cultured for 2 days. On second day morphine was added, and on the third day cells were treated with OPDex beads (1:20 ratio) for 30 min. Cells were washed, collected, analyzed according to manufacturer's protocol. Briefly, cell lysates were incubated with PAK-PBD beads, and allowed to "pull-down" the PAK-PBD/GTP-Rac complex. The amount of activated Rac1 is determined by a Western blot using a Rac1 specific antibody. GTPγS is a non-hydrolysable GTP analog used for positive control and GDP is used as a negative control

PLASMID TRANSFECTION

Addgene plasmid 12981: pcDNA3-EGFP-Rac1-Q61L was obtained from Addgene database, donated by Dr. Gary Bokoch (Subauste et al., 2000). The plasmid was transfected using Effectine reagent (cat#301425; Qiagen-Valencia. CA) according to manufacturer's protocol to 10cm Petri dish, containing J774 cells. One day after the transfection cells were collected, plated and treated with 1μM morphine overnight and HKOP *E. coli* (Texas red) as described in "Flourometric assay".

RESULTS

CHRONIC MORPHINE INHIBITS MACROPHAGE PHAGOCYTOSIS IN VITRO AND IN VIVO

Macrophages play a crucial role in morphine induced immunosuppression (Eisenstein et al., 1993). In order to effectively study macrophage phagocytosis we utilized the model analyzing Fcγ receptor (FcgR) mediated phagocytosis, using IgG opsonized heat killed bacteria or dextran beads. Since opioid abuse models are susceptible to high variability, depending on the mode of administration, we investigated the effects of morphine administration on phagocytosis in primary macrophages *in vivo* and *ex vitro*.

In order to determine effect of chronic morphine treatment *in vivo* on primary macrophage FcgR mediated phagocytosis, mice were treated with 75mg slow releasing morphine or placebo pellets for 72 hours. After the morphine treatment, peritoneal macrophages from wild-type (WT) and mu opioid receptor knock out (MORKO) mice were isolated using standard protocols (see methods "Primary macrophages"). Macrophages were exposed to heat killed, IgG opsonized *E. coli* particles (HKO*E.coli*) in

continued presence of morphine and analyzed at different time points for phagocytosis using fluorometric analysis **(Figure 2.1)**. Time dependant increase in phagocytosis was observed in both morphine and placebo treatments, while morphine treatment significantly inhibited *ex vivo* internalization of HKO*E.coli* by primary peritoneal macrophages from WT mice following *in vivo* **(Figure 2.1A)** morphine treatments. In macrophages from MORKO mice treated with morphine *in vivo* (**Figure 2.1B**), morphine's inhibition of phagocytosis was abolished, indicating an essential role of mu-opioid receptor in this process. Phagocytic indexes from *in vitro* morphine treated primary macrophages **(Figure 2.2A)** correlated with those from *in vivo* morphine treated mice (**Figure 2.1A**), indicating that both are valid models for further study.

To determine the physiological effects of morphine treatment on macrophage's bacterial clearance *in vivo*, "*In vivo* phagocytosis assay" was conducted. Phagocytosis of HKO*E.coli* was inhibited in macrophages from morphine treated WT mice **(Figure 2.2Bi)** and remained unchanged in macrophages from morphine treated MORKO **(Figure 2.2Bii)** mice when compared to their respective placebo controls. In addition, phagocytosis of opsonized dextran bead (OPDex bead) was also inhibited in primary macrophages isolated from WT mice treated with morphine *in vitro* (**Figure 2.3A**), and this effect was abolished in macrophages from MORKO mice, treated with morphine *in vitro* (**Figure 2.3B**). In addition, phagocytosis of OPDex bead (**Figure 2.3A**) was greater and was further inhibited when compared to phagocytosis of HKO*E.coli* (**Figure 2.2A**). Together, these data indicate that the phagocytic ability of primary peritoneal macrophage is significantly reduced in mice treated with morphine *in vivo* and *in vitro* and that this effect is mediated by the mu opioid receptor (MOR) as shown by the MORKO studies.

To further establish the inhibitory effect of morphine on phagocytosis we validated the results we observed *in vivo,* in our *in vitro* model utilizing J774 macrophage cell line. Confocal microscopy illustrates morphine treated cells displaying significant inhibition of phagocytosis of live opsonized GFP tagged *E. coli* (**Figure 2.4**) by J774 when compared to the vehicle treated cells. Fluorometric analysis also confirms these findings and indicates a time dependant increase in J774 phagocytosis of HKO*E.coli* particles with steady inhibition of phagocytosis at each time point in morphine treated cells (**Figure 2.5A)**. Furthermore morphine mediated inhibition of HKOE.coli phagocytosis by J774 macrophages was abolished in cells pretreated with naltrexone **(Figure 2.5B),** indicating involvement of classical opioid receptors. These data indicate that morphine inhibits phagocytosis of J774 cells in a manner similar to previous *in vivo* studies.

Activation of Toll-like Receptor 4 (TLR-4) by Gram-negative bacteria has been implied in increasing the rate of phagocytosis (Anand et al., 2007). Therefore, in order to avoid confounding of the data through potential TLR-4 activation via LPS (lipopolysaccharide; TLR-4 ligand) from HKO*E.coli*, we utilized opsonized dextran (OPDex) beads in our further study of morphine's effect on phagocytosis. Chronic morphine led to a decrease in internalization of OPDex beads as demonstrated through a reduction in phagocytic index (PI) (**Figure 2.5C**). However macrophages internalizing OPDex beads displayed greater time dependant increase in PI, as well as greater inhibition of PI by chronic morphine. Morphine induced inhibition of phagocytosis was reversed by naltrexone (10 μM) pretreatment (**Figure 2.5B, and 2.5D**) which indicates involvement of classical opioid receptors in these processes in J774 cell line, consistent with results observed with primary macrophages (**Figure 2.1**). In order to rule out the possibility that morphine may be modulating FcgR surface expression, and in that

manner inhibit FcgR mediated phagocytosis, we conducted FACS analysis of morphine treated J774 macrophages (**Figure 2.6**). FACS analysis determined that expression of FcgR Ia and Ib (shown by CD64 antibody), was not altered in J774 cell line following morphine treatment (**Figure 2.6**), indicating that morphine modulation of FcgR was not a factor in morphine induced inhibition of phagocytosis. We speculate that crosstalk between of MOR and FcgR signaling pathways must be further downstream from the Fcg receptor.

Considering involvement of HPA axis in morphine mediated immune suppression we examined potential confounding effects of corticosterone mediated immunosuppression on morphine mediated inhibition of macrophage phagocytosis (Peterson et al., 1993). As expected corticosterone treatment had no effect on macrophage phagocytosis (**Figure 2.7**). This data indicates that although morphine may activate HPA axis, in our model, corticosterone and glucocorticoids do not play a role in morphine mediated inhibition of macrophage phagocytosis.

Considering some recent reports, which indicated that morphine induces cell apoptosis we wanted to verify if our findings were being confounded by changes in cell viability (Bhat et al., 2004) (Singhal et al., 2002). MTT assay was used to quantify cell viability following morphine treatment and HKO*E.coli* (**Figure 2.8A**) or OPDex bead exposure (**Figure 2.8B**). Data indicates that in this model there were no changes in cell viability with morphine, HKO*E.coli* or OPDex bead treatments.

Together these data show that morphine induced inhibition of phagocytosis *in vivo* can be replicated in a macrophage cell line J774, and that morphine's inhibitory effects on phagocytosis are not confounded by FcgR expression, involvement of gluococorticoids or changes in cell viability. Subsequently, all mechanistic studies were conducted on J774 and OPDex bead in order to avoid potential TLR-4 activation.

CHRONIC MORPHINE TREATMENT INHIBITS PHAGOCYTOSIS BY INHIBITING ACTIN POLYMERIZATION

Remodeling of the actin cytoskeleton is a prerequisite for FcgR mediated phagocytosis (Hall, 1998; Araki, 2006). Actin polymerization enables the formation of the phagocytic cup and it leads to the subsequent internalization of the phagosome and the phagocytic target (Araki, 2006).

In order to verify the role of actin polymerization in this model FcgR mediated phagocytosis, we utilized Cytochalasin D (Cyt D), a known inhibitor of actin polymerization. As expected, Cyt D (10μM) treatment, following morphine and vehicle treatments, led to abolished phagocytic capabilities of J774 cells (**Figure 2.9A**), indicating essential role of actin in FcgR mediated phagocytosis. In addition, confocal analysis of rhodamine phalloidin stained cells (**Figure 2.9B**), which have undergone treatments with morphine, and HKO*E.coli* (30 min) indicate that chronic morphine leads to defects in actin polymerization. Cells following chronic morphine treatment and phagocytosis, exhibit a reduction in membrane ruffling and formation of focal adhesions (as marked by arrows; **Figure 2.9B),** as well as an increase in number of filopodia (marked by arrowheads; **Figure 2.9B**) as compared to vehicle controls. In addition, filopodia of morphine treated cells exhibit a thicker, more elongated appearance compared to the vehicle control, indicating that morphine leads to inhibition of fiber retraction and phagosome formation. In order to further quantify the changes in actin polymerization, fluorometric analysis was conducted (see methods) after the primary macrophages (**Figure 2.10**) and J774 (**Figure 2.11**) were treated with morphine and undergone phagocytosis of OPDex beads. Morphine induced significant inhibition of actin polymerization in primary cells from WT mice treated with morphine *in vivo*, HKO*E.coli* (**Figure 2.10B**), and OPDex bead (**Figure 2.10A**) *in vitro*. As expected,

morphine modulation of actin polymerization was abolished in primary macrophages extracted from MORKO mice treated with morphine *in vivo*, and HKO*E.coli in vitro* (**Figure 2.10C**). Similar observations were noted in J774 cells, where naltrexone pretreatment (10μM), reversed morphine's inhibitory effect on actin polymerization following phagocytosis of OPDex beads (**Figure 2.11B**), further indicating crucial involvement of classical opioid receptors namely MOR in modulation of actin polymerization. Together these data show that morphine leads to inhibition of phagocytosis by inhibiting actin polymerization, and that classical opioid receptors, specifically MOR plays an important role in this process.

CHRONIC MORPHINE TREATMENT INDUCED INHIBITION OF PHAGOCYTOSIS IS MEDIATED BY cAMP AND PKA

We have previously shown that morphine up regulates cAMP levels in macrophages, and since elevated cAMP have been implicated in inhibition of phagocytosis, we investigated the up regulation of cAMP as a potential mechanism by which morphine modulates macrophage phagocytosis (Azzam et al., 2006; Makranz et al., 2006). Chronic morphine treatment of macrophage cell line J774 led to a significant increase in cAMP (**Figure 2.12A**). In order to directly observe the effects of elevated cAMP we used DB-cAMP, a cell permeable cAMP analog. Following 10 minute incubation with DB-cAMP (100μM) prior to addition of OPDex bead both phagocytosis (**Figure 2.13A**) of OPDex bead and actin polymerization (**Figure 2.13B**), were inhibited in vehicle and morphine treatments, further indicating that morphine is inhibiting FcgR phagocytosis via a cAMP mechanism.

In order to examine downstream targets of cAMP and their role in this process, we used H89, a known inhibitor of PKA. Pretreatment of J774 cells with H89 (1μM) prior

49

to phagocytosis of OPDex bead (**Figure 2.14**) led to loss of morphine's inhibitory effect on phagocytosis (**Figure 2.14A**) and actin polymerization (**Figure 2.14B**). Similar effects were observed with phagocytosis of HKO*E.coli* (**Figure 2.15).** Together these data indicate that cAMP, through PKA, mediates morphine induced inhibition of phagocytosis as well as actin polymerization.

CHRONIC MORPHINE TREATMENT INDUCED INHIBITION OF PHAGOCYTOSIS IS MEDIATED BY p38 AND ERK1/2 MAPKs

Phagocytosis has been implicated in activation of ERK1/2 and p38 MAPKs in human neutrophils (McLeish et al., 1998). Since opioids have been known to modulate MAPK and since they have been implied to play a role in neutrophil phagocytosis, studies were conducted to examine the role of p38 MAPK and ERK1/2 in morphine's inhibitory effects on macrophage function (McLeish et al., 1998).

ERK1/2 phosphorylation was inhibited by PD98059, a known ERK1/2 inhibitor. Macrophage pretreatment with 10μM PD98059 (prior to addition of morphine and OPDex beads) led to a dose dependant inhibition of HKO*E.coli* phagocytosis (**Figure 2.16A**). Western blot analysis **(Figure 2.16B**) showed that phosphorylation of ERK1/2 was enhanced with addition of OPDex bead. Morphine treatment inhibited ERK 1/2 phosphorylation similar to PD98059 treated cells. As expected inhibition of ERK1/2 phosphorylation by PD98059 led to inhibited phagocytosis of both vehicle and morphine treated macrophages (**Figure 2.17A**). Interestingly, 10μM PD98059 treatment led to inhibition of actin polymerization only in vehicle treated cells (**Figure 2.17B**). This can be explained by the fact that morphine already reached maximal inhibition of actin polymerization, and therefore the additional inhibition of polymerization by PD98059 was not seen in PD98059+morphine treated cells. Although morphine alone inhibits

50

phosphorylation of ERK1/2 (**Figure 2.16B**), addition of PD98059 further potentiates morphine's inhibition of phagocytosis, indicating a possible additional mechanism of action in morphine's inhibitory pathway.

Another MAPK known to play a role in phagocytosis is p38 (Hall, 2005). Given the role of p38 MAPK in phagocytosis and given that previous data indicates that morphine might be acting through additional pathways, we wanted to explore the role of p38 MAPK in morphine's inhibitory effects. Morphine's modulation of p38 MAPK has been investigated, however literature seems to be controversial since results seem to vary greatly depending on dose and duration of incubation (chronic vs. acute morphine) (Singhal et al., 2002; Wang et al., 2003). In this model, cells were pretreated with 10μM SB203580 (a p38 MAPK inhibitor) for four hours prior to morphine incubation, and treated with morphine overnight. After this incubation, cells were exposed to OPDex bead for 60 minutes. Consistent with current literature findings, western blot analysis **(Figure 2.18)** indicates that phagocytosis of OPDex beads increased p38 MAPK phosphorylation (Doyle et al., 2004). Morphine treatment significantly reduced, while SB203580 further decreased phosphorylation of p38 MAPK. When J774 cells were assayed for phagocytosis and actin polymerization, following the same treatment as above, SB203580 by inhibiting phosphorylation of p38 MAPK (**Figure 2.18**) led to inhibition of phagocytosis (**Figure 2.19A**) as well as actin polymerization (**Figure 2.19B**) in both vehicle and morphine treated J774 cells. Similar effects on phagocytosis and actin polymerization were observed in primary macrophages treated with morphine and SB203580 *in vitro* (**Figure 2.20A and 2.20B** respectively). Although morphine inhibited p38 MAPK activation, actin polymerization and phagocytosis, SB203580 further attenuated these responses. These results indicate that p38 MAPK plays an important role in phagocytosis and actin polymerization, and that in addition to ERK1/2, p38 MAPK

51

plays a part in the mechanism by which morphine reduces macrophage phagocytic ability.

CHRONIC MORPHINE INHIBITS MECHANISMS INVOLVED IN ACTIN POLYMERIZATION AND PHAGOCYTOSIS

Rac-GTP-ases play an essential role in actin polymerization, by modulating membrane ruffling and lamellipodial protrusions (Wells et al., 2004). Since morphine leads to defects in membrane ruffling, focal adhesions and filopodial formation as shown in figure (**Figure 2.9B**), we wanted to further examine its role in the activation Rac-GTPase. We note that activation of Rac1, Rac2, and Rac3 are inhibited with chronic morphine treatment in cells undergoing phagocytosis, as confirmed by the Rac-GLISA assay (**Figure 2.21A**). Since Rac1-GTP has been implicated in phagocytosis we focused specifically on Rac1 modulation. Chronic morphine inhibited activation of Rac1 in cells undergoing phagocytosis of OPDex beads, as shown in the pull-down assay (**Figure 2.21B**). Both pull-down assay and GLISA confirmed that morphine has inhibitory effects on Rac 1,2,3 and Rac1 activity. In order to verify these results on a functional level we utilized constitutively active Rac1-GTP plasmid (Addgene). J774 cells were transiently transfected with the constitutively active Rac1 plasmid, using effectine reagent (see "Plasmid transfection"). Transfected and non-transfected cells were visualized via confocal microscopy (**Figure 2.22A),** and quantified for phagocytosis (**Figure 2.22B**) and actin polymerization (**Figure 2.22C**) using fluorometric analysis. We were able to note distinct changes in cell morphology following Rac1-GTP transfection. In both vehicle and morphine treatments cells appeared to be larger, have more ruffled membranes and have more focal adhesions when compared to their respective controls (without transfection). These observations are in accordance with literature since Rac1

has been implicated in cell spreading as well as in formation of lamellipodia and focal adhesions (Wells et al., 2004). In addition, morphine's inhibitory effect was abolished in Rac1-GTP transfected cells, with respect to phagocytosis and actin polymerization, which was further confirmed by fluorometric analysis (**Figure 2.22 B and C** respectively). Since constitutive expression of Rac1-GTP was able to override morphine's inhibition of phagocytosis, these data indicate that morphine inhibits actin polymerization by modulating Rac1 activation, ultimately leading to inhibition of phagocytosis.

DISCUSSION

This study shows for the first time, that morphine induced inhibition of FcgR mediated phagocytosis occurs through modulation of actin polymerization by inhibition of Rac1-GTP-ase activity, and that modulation of actin occurs via cAMP, ERK1/2 and p38 MAPK dependant mechanism. Several lines of evidence support these findings. First, we show that chronic morphine treatment inhibits IgG opsonophagocytosis by inhibiting actin polymerization through Rac1-GTPase dependant mechanism. Second, that morphine modulates macrophage phagocytic ability and actin polymerization by increasing intracellular cAMP which through PKA exerts its inhibitory effects, and third line of support indicates that of ERK1/2 and p38 MAPKs play a role in these processes.

Prevalence of opioid use extends beyond the drug-abuse population, to the clinical setting. Morphine-mediated suppression of innate and adaptive immunity has been an established phenomenon and is often indicated by an increase in frequency in bacterial infections (Boschini et al., 1996);(Friedman and Eisenstein, 2004). Although morphine mediated immune suppression is well investigated, the mechanisms involved

in modulation of innate immunity have yet to be fully understood. Macrophages play an essential role in bacterial clearance and morphine has been implicated in inhibiting their function (Eisenstein and Hilburger, 1998; Eisenstein et al., 1998). Due to the important role that macrophages play in pathogen clearance, as well as significance of deleterious effects caused by disruption of their homeostasis in presence of opioids, we examined the mechanisms by which morphine modulates key macrophage functions such as phagocytosis.

Our results show that morphine treatment *in vitro* and *in vivo* inhibits FcgR mediated phagocytosis of primary peritoneal macrophages, as well as J774 macrophage cell line consistent with the literature (Rojavin et al., 1993a). To our knowledge this study is the first to show inhibition of phagocytosis *in vivo* following an *in vivo* morphine treatment. Furthermore, we show that morphine inhibits FcgR mediated phagocytosis and actin polymerization through the classical opioid receptors, specifically through μ opioid receptor (MOR). Morphine, through MOR inhibits phagocytosis without effecting FcgR expression, therefore indicating that the point of convergence between FcgR and MOR occurs further downstream in the signaling cascade. This point was further strengthened by our results, indicating that primary peritoneal macrophages from μ-receptor knock-out (MORKO) mice had overall higher rates of phagocytosis when compared to those from wild-type mice, indicating that due to the lack of MOR, endogenous opioids were unable to exert their inhibitory effects. Therefore, endogenous opioids might be playing a similar role in suppressing macrophage phagocytic ability, as would exogenous opioids such as morphine.

Our group and others have correlated chronic morphine mediated increase in intracellular cAMP to inhibition of phagocytosis (Tomei and Renaud, 1997; Tomassini et al., 2004) (Kelschenbach et al., 2008). Our research supports findings by Tomei &

Renaud, that cAMP plays an essential role in morphine withdrawal mediated modulation of phagocytosis (Tomei and Renaud, 1997; Tomassini et al., 2004). Our previously published work as well as in this study, show that cAMP levels remain elevated following 18hours of 1μM morphine treatment, and that this increase in cAMP leads to inhibition of phagocytosis (Kelschenbach et al., 2008). As expected, pharmacological increase in cAMP via DB-cAMP also lead to significant decrease in phagocytosis and actin polymerization, indicating importance of cAMP in these processes. Furthermore inhibition of PKA, via H89 was able to restore normal phagocytic rates indicating that cAMP acts through PKA in order to inhibit actin polymerization and phagocytosis.

In addition to elevating cAMP, morphine plays an important role in modulation of MAPKs. Three MAPK cascades have been identified in mammalian cells, the well-characterized MAPK cascade results in the activation of extracellular response kinases or ERKs (ERK1/2 also called p42, p44 MAPK). Opioids have been known to modulate MAPK and since ERK1/2 and p38 MAPKs have been implied to play a role in neutrophil phagocytosis, we examined their role in morphine's inhibitory effects on macrophage function (McLeish et al., 1998). Initially, we found that ERK1/2 is involved in FcgR mediated actin polymerization and phagocytic pathway. However, the role of ERK 1/2 in morphine mediated inhibition of phagocytosis seemed to be minor, since the inhibition of ERK1/2 by PD98059 only caused minor inhibition of actin polymerization.

On the other hand, results indicate that morphine inhibits p38 MAPK phosphorylation and that inhibition of p38 MAPK phosphorylation by SB203580 leads to reduction in phagocytosis. Inhibition of p38 MAPK activation had much more detrimental effect on macrophage phagocytosis than inhibition of ERK1/2 indicating that although both are involved, p38 MAPK plays a more significant role in FcgR mediated phagocytic mechanisms.

Since we have shown that morphine inhibits actin polymerization via cAMP, ERK1/2 and p38 MAPK we wanted to examine which step of actin polymerization is affected by chronic morphine treatment. FcgR mediated phagocytosis depends on actin polymerization and Rac-GTP-ases that lead to formation of lamellipodia and focal adhesions (Wells et al., 2004). There are three isoforms of Rac (1, 2 and 3) in mammals, but little is known about the relative contributions of each isoform to Rac-dependent responses. We chose to study Rac1, since it is the most extensively studied isoform, and it is known to play an important role in macrophage phagocytosis, whereas roles of Rac2 and Rac3 in phagocytosis are not well explored. Literature shows that Rac1 is essential in actin polymerization and dynamics, therefore any modulation of Rac1-GTPase will alter downstream functions such as actin polymerization and phagocytosis (Niedergang and Chavrier, 2005; Ridley, 2006). We note that in addition to previous mechanisms morphine exerts its inhibitory effects via inhibition of Rac1-GTPase. This was further confirmed following Rac1-GTP overexpression where constitutively activated Rac1 was able to override morphine's inhibitory effects. Our group is first to show morphine's modulation of actin polymerization via Rac1-GTPase. Although Rac1 plays an essential role in macrophage function and cytoskeletal reorganization it is also involved in many other mechanisms such as cell growth, vesicle trafficking, epithelial differentiation which can potentially be modulated by opioids in other model systems and lead to disruption in homeostasis (Ridley, 2006).

Morphine seems to have multiple pathways by which it inhibits phagocytosis. In the proposed diagram in f**igure 2.23**, we show the summary of the current literature and our findings. Through activation of cAMP and PKA or inhibition of ERK1/2, or p38 MAPK morphine is able to inhibit phagocytosis using three mechanisms. Literature implicates cAMP in inhibition of MAPK's such as p38 and ERK ½, therefore morphine may utilize

cAMP to inhibit MAPK phosphorylation   and thus inhibit phagocytosis (Feng et al., 2002; Zhu et al., 2008). Therefore the mechanisms of morphine action we are proposing must be acting in sequence. As morphine increases cAMP, cAMP inhibits ERK1/2 and p38 MAPK leading to inhibition of actin polymerization and phagocytosis. Additionally, our findings illustrate that morphine inhibits Rac1-GTP. Rac1-GTPase is known to lead to activation of p38 MAPK. Since morphine inhibits Rac1-GTP, morphine can also lead to inhibition of p38 MAPK indirectly via Rac1 (Mackay and Hall, 1998). Chronic morphine treatment alone and through inhibition of Rac1-GTP is able to inhibit p38 MAPK activation and lead to inhibition of phagocytosis and actin polymerization. Here we demonstrate that morphine seems to modulate several interlinked mechanisms in tandem. Morphine inhibits phagocytosis by inhibiting actin polymerization through inhibition of Rac1-GTPase and p38 MAPK, by increasing intracellular cAMP and acting through PKA, as well as by inhibition of ERK1/2 MAPK. The significance of these findings is not limited solely to macrophages, for similar modulations may be occurring in different cell types resulting in additional deleterious effects.

Our observations emphasize the broad scope of morphine's effects on modulation of diverse mechanisms significant to macrophage function. This study highlights several essential pathways of morphine's immunomodulation which signify importance and need for discoveries of new therapeutic agents used in pain management which would minimize these immunosuppressive effects.

FIGURE 2.1
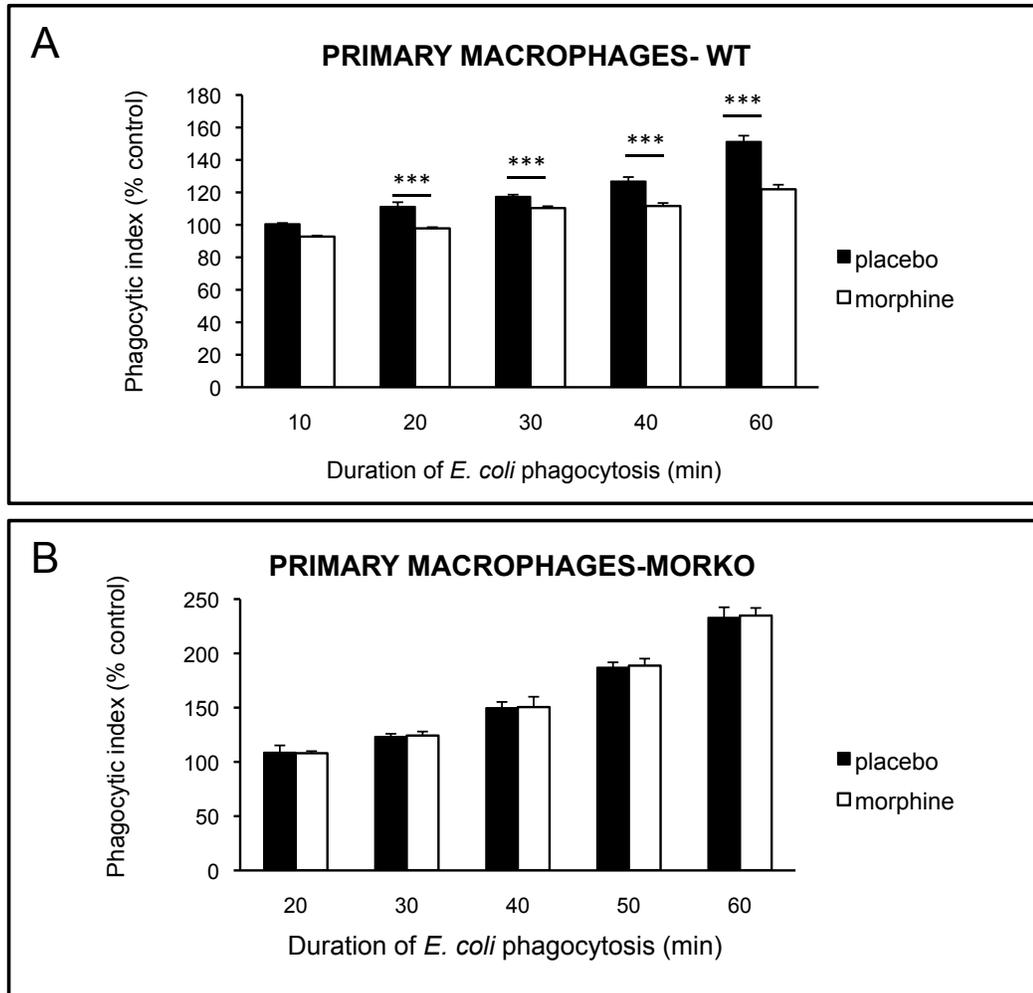
Figure 2.1. In vivo chronic morphine treatment inhibits phagocytic ability of primary peritoneal macrophage via MOR. Fluorometric analysis of PI of primary peritoneal macrophages treated *in vivo* with morphine Macrophages from *in vivo* treated mice **(A,B)** were extracted 72 hr following implantation of a slow releasing morphine/placebo (75mg) pellet. Cells were extracted and treated with FITC tagged HKO*E.coli* for varied periods of time. The data illustrates average phagocytic index for macrophages pooled from 3 mice in each treatment. Phagocytic index (%) = Phagocytic index % from the vehicle treatment of the shortest time point. Vertical bars represent mean (±SE).

FIGURE 2.2
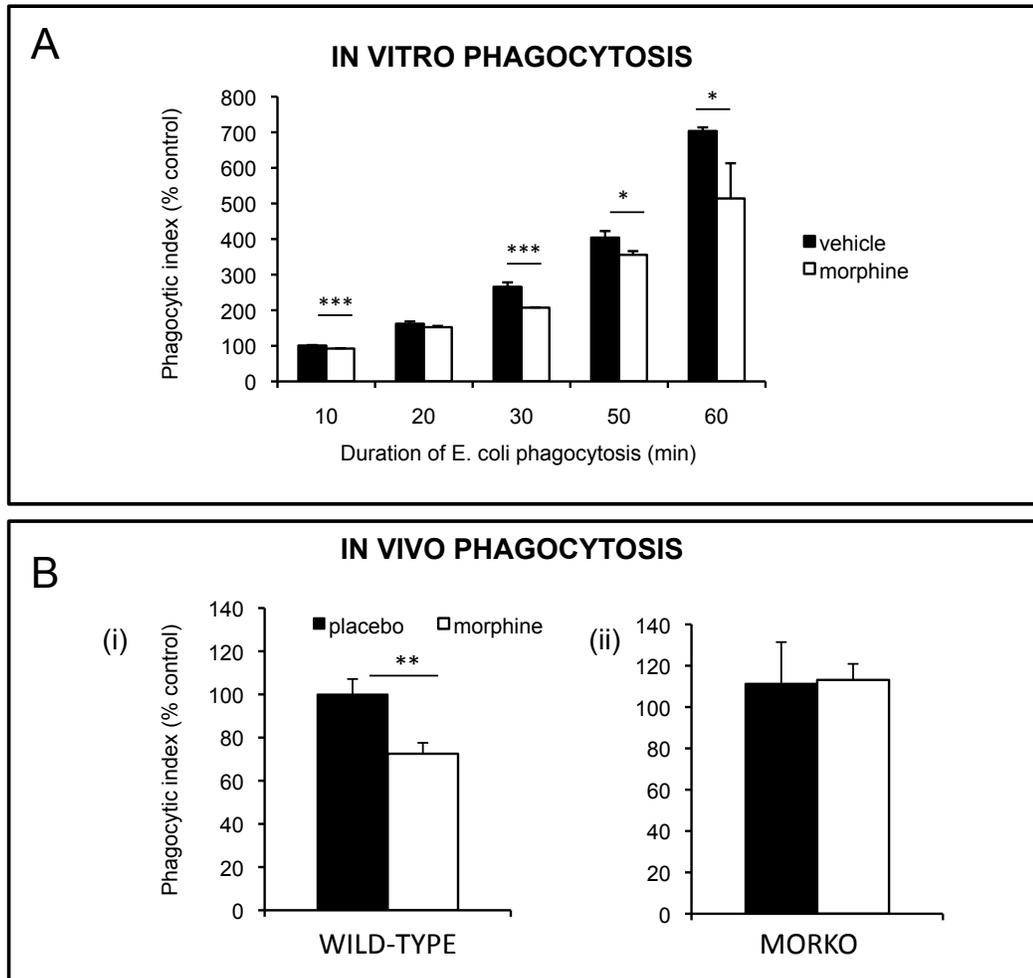
Figure 2.2. Chronic morphine treatment inhibits macrophage phagocytosis *in vivo* and *in vitro*. (A) primary macrophages extracted form wt mice and treated with morphine and HKOP E. coli *in vitro*. **(B)** Following 72hr of *in vivo* morphine treatment, *in vivo* phagocytosis was examined at 30 min following the IP injection of HKOP*E. coli* (WT **(Bi)** and MORKO **(Bii)** mice). The data illustrates average phagocytic index for 5 mice in each treatment. Phagocytic index (%) = Phagocytic index % from the vehicle treatment. Vertical bars represent mean ($\pm$SE). Significance determined using Student's T-test $p<0.05$*, $p<0.01$**, $p<0.001$***.

FIGURE 2.3
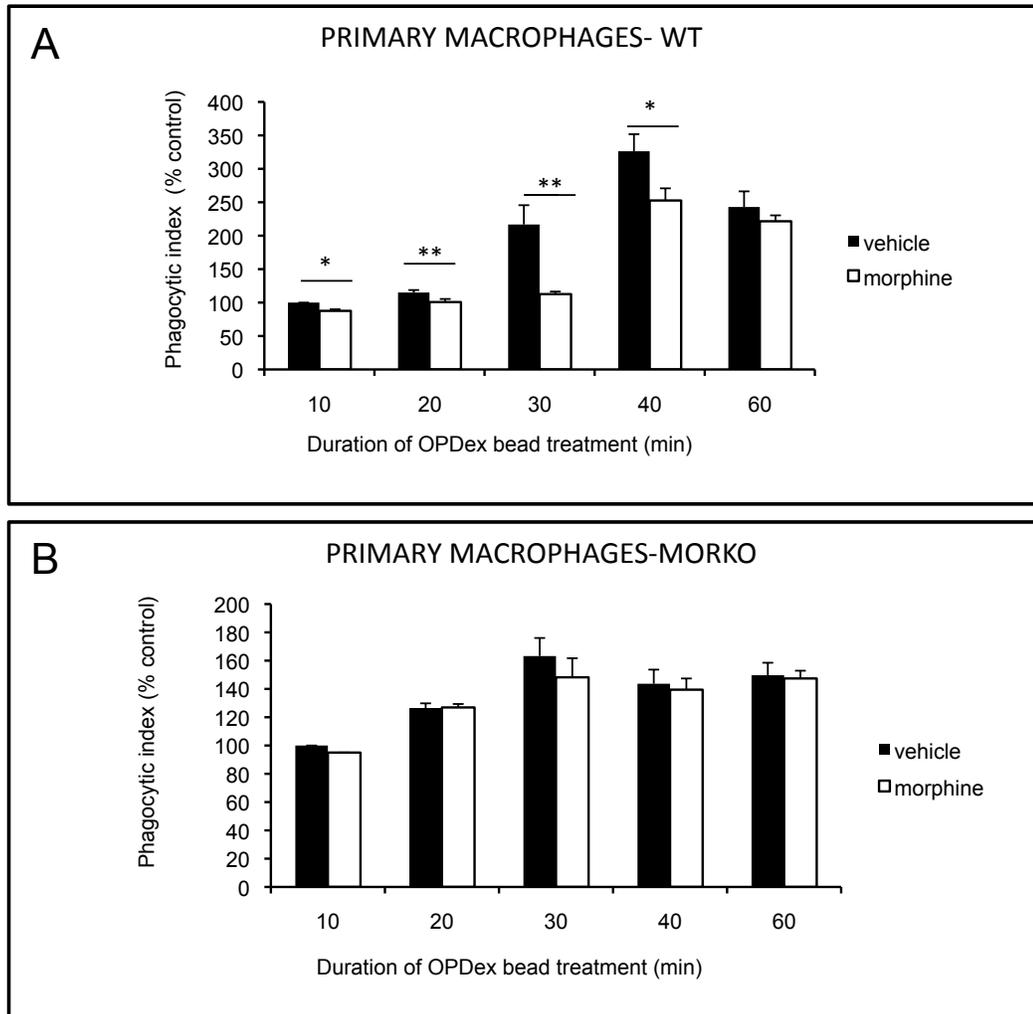
Figure 2.3. Chronic morphine treatment *in vivo* inhibits macrophage phagocytosis in primary macrophages. Primary macrophages were extracted and treated with overnightmorphine (1μM) and OPDex beads in 1:20 (cell:bead) ratio for varied durations of time. The data illustrates average phagocytic index. Phagocytic index (%) = Phagocytic index % from the vehicle treatment at shortest timepoint. Vertical bars represent mean (±SE). Significance determined using Student's T-test p<0.05*, p<0.01**.

FIGURE 2.4
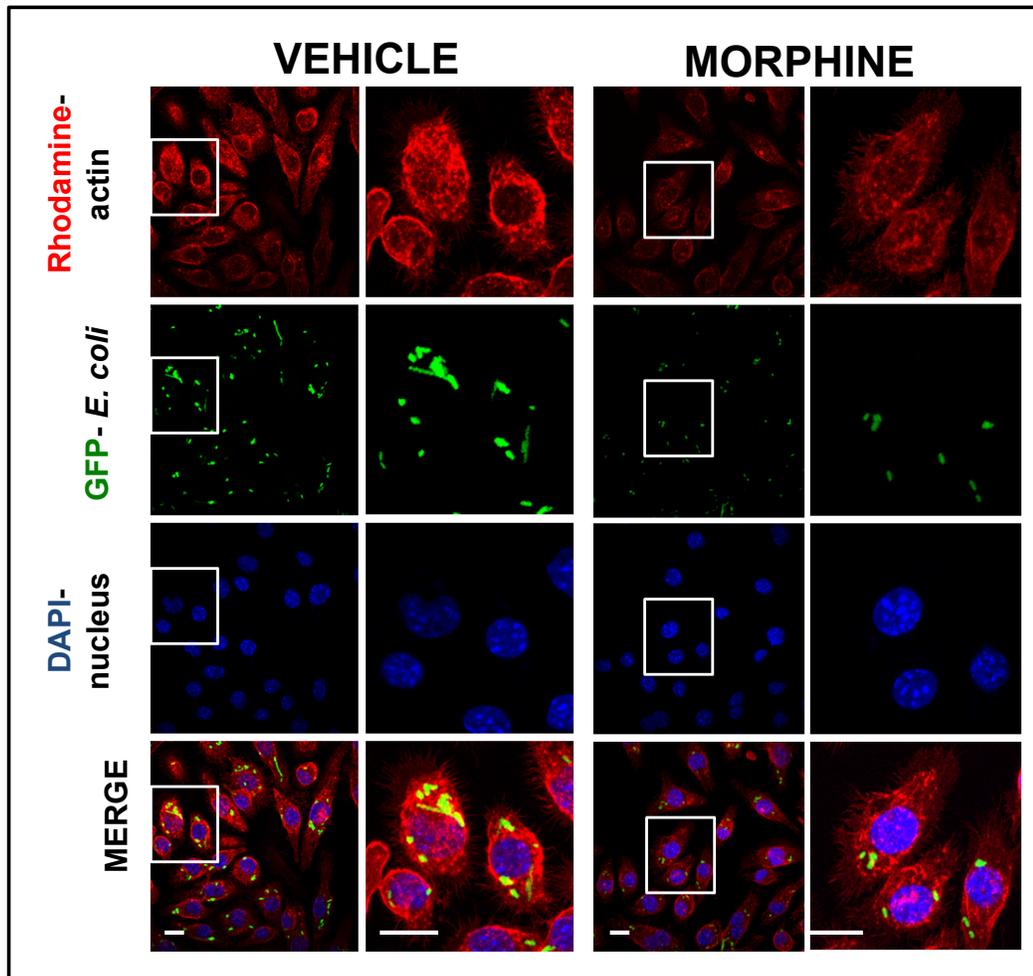
Figure 2.4. Chronic morphine leads to inhibition in FcgR mediated phagocytosis by J774 macrophage cell line. Cells were treated with chronic morphine treatment (1μM morphine overnight) and 30 minute incubation with live opsonized *E. coli.* Cells were washed, fixed with 4% paraformaldehyde, permealised with acetone, stained (DAPI- blue for nuclear staining, Rhodamine-red for actin and analyzed via confocal microscopy using Nikon EZ-C1 3.90 software (60x; white line indicates 20mm)) Right panel illustrates additional magnification from the left panel area marked by white box.

FIGURE 2.5

A

B
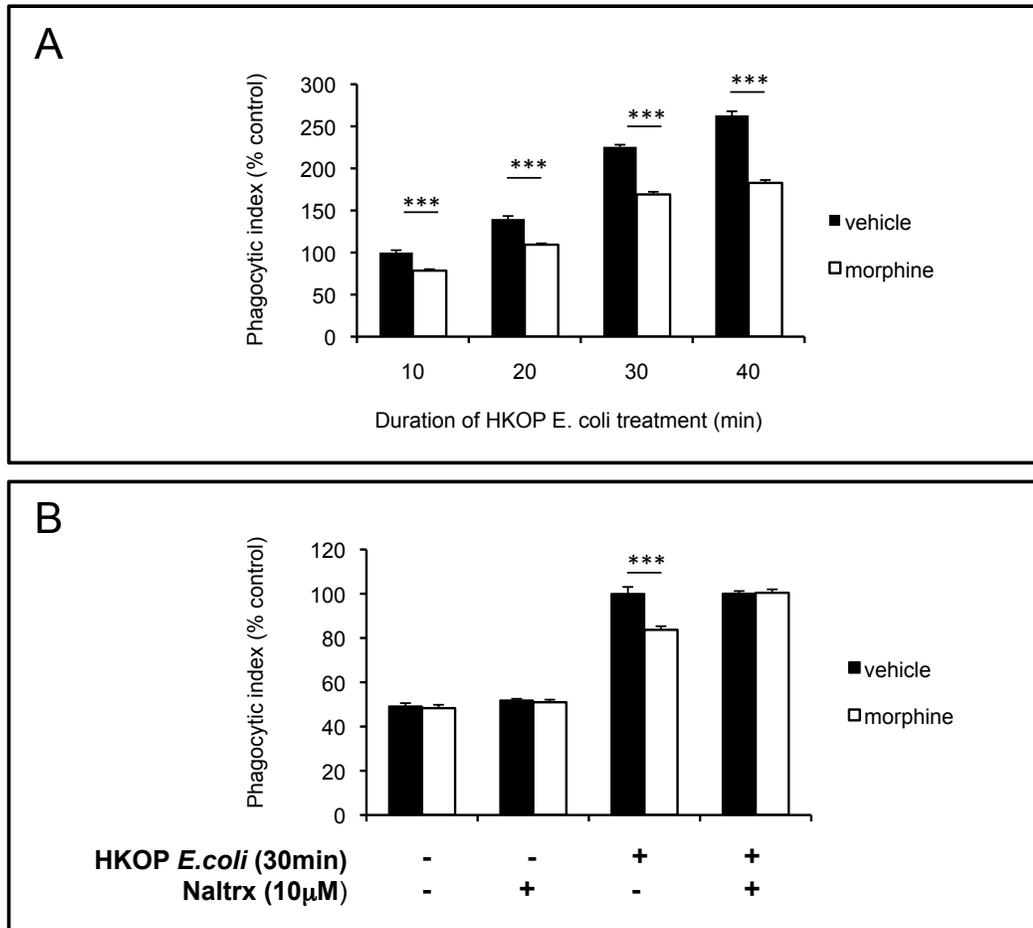
Figure 2.5. Chronic morphine mediated inhibition of FcgR mediated phagocytosis is Naltrexone reversible. Fluorometric analysis of phagocytosis HKOP *E. coli* **(A,B)** or OPDex bead **(C,D)** by J774 macrophage cell line following chronic morphine treatment.**(B,D)** Cells were pretreated with naltrexone (10μM) for 2 hours prior to overnight chronic morphine treatment (1μM) Cells were treated for 30 minutes with HKOP*E. coli* **(B)** or OPDex bead **(D)** and phagocytosis was measured using fluorometric analysis. Phagocytic index (%) = percent PI from the vehicle treatment at shortest time point. Vertical bars represent mean (±SE). Significance determined using Student's T-test p<0.05*, p<0.01**.

FIGURE 2.5
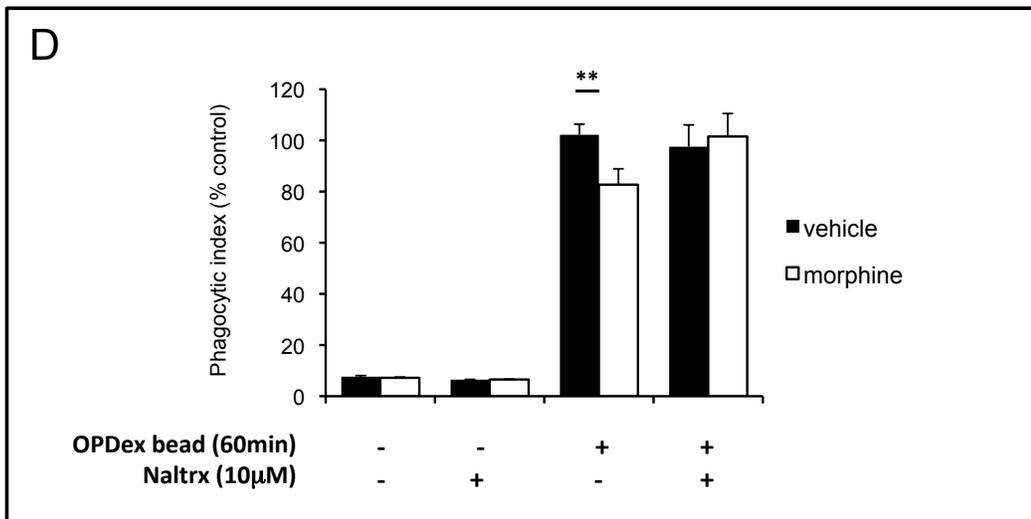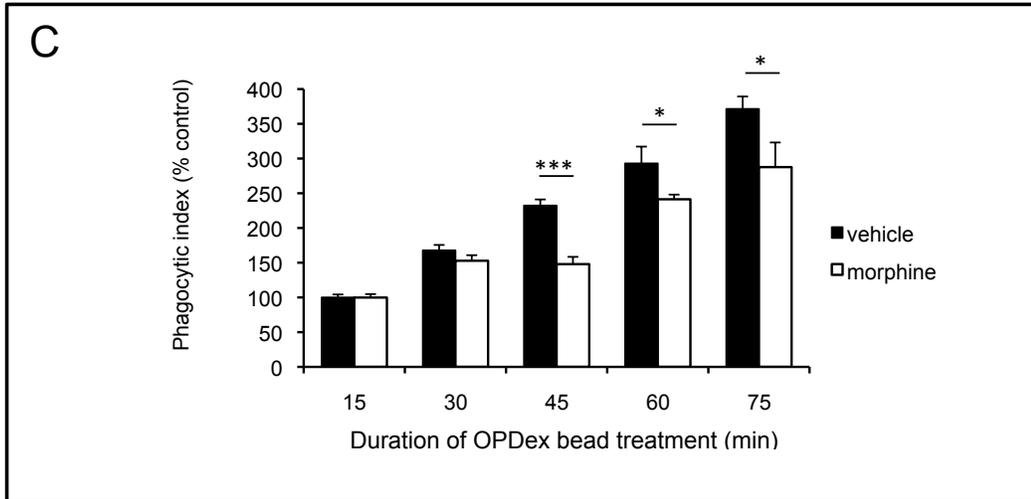
FIGURE 2.6
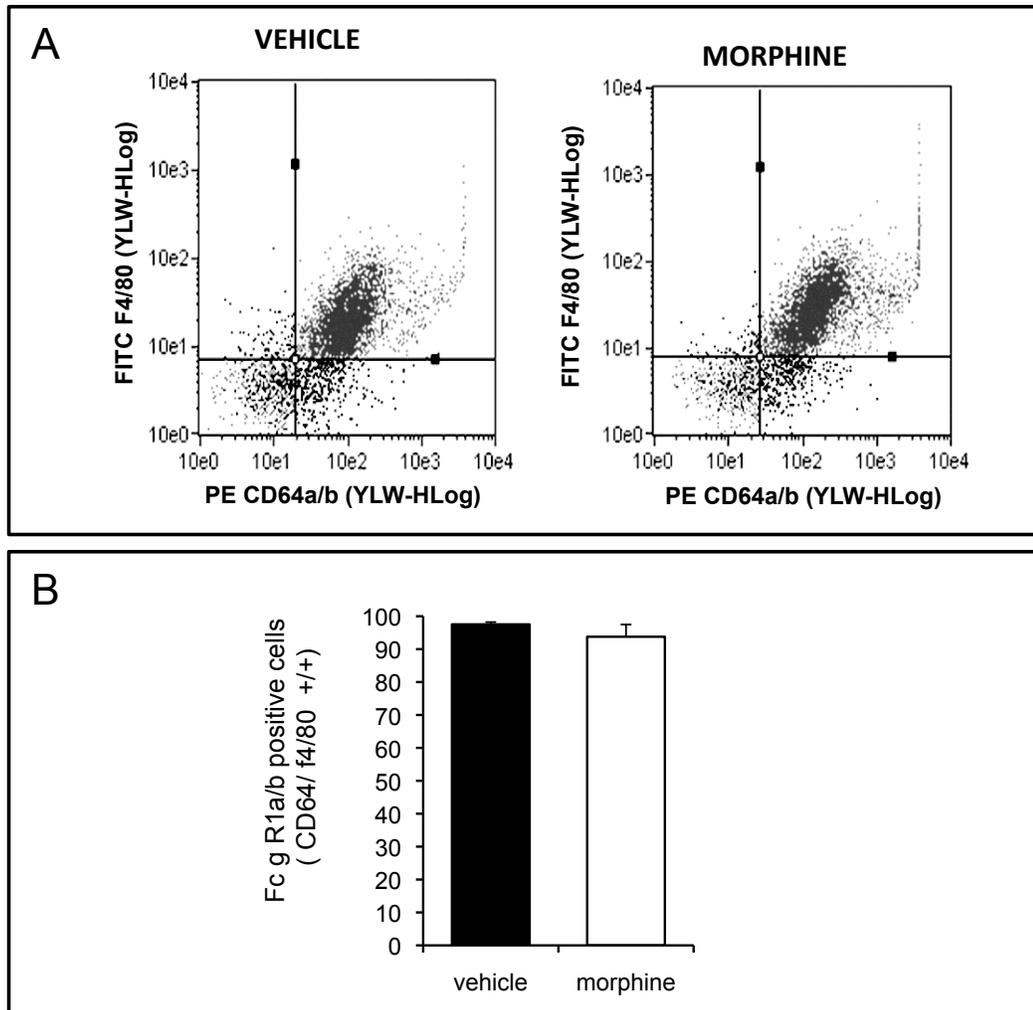
Figure 2.6. Morphine effects on FcgR expression. FACS analysis of FcgR 1a and b (CD64 a and b) surface expression. Antibodies CD64 a and b (PE; BD Pharmigen; cat#558455), and macrophage marker F4/80 - (FITC; eBioscience; cat#11-4801), data was collected using Guava EasyCyte and quantified using Guava cell plus software. Significance determined using Student's T-test $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$.

FIGURE 2.7
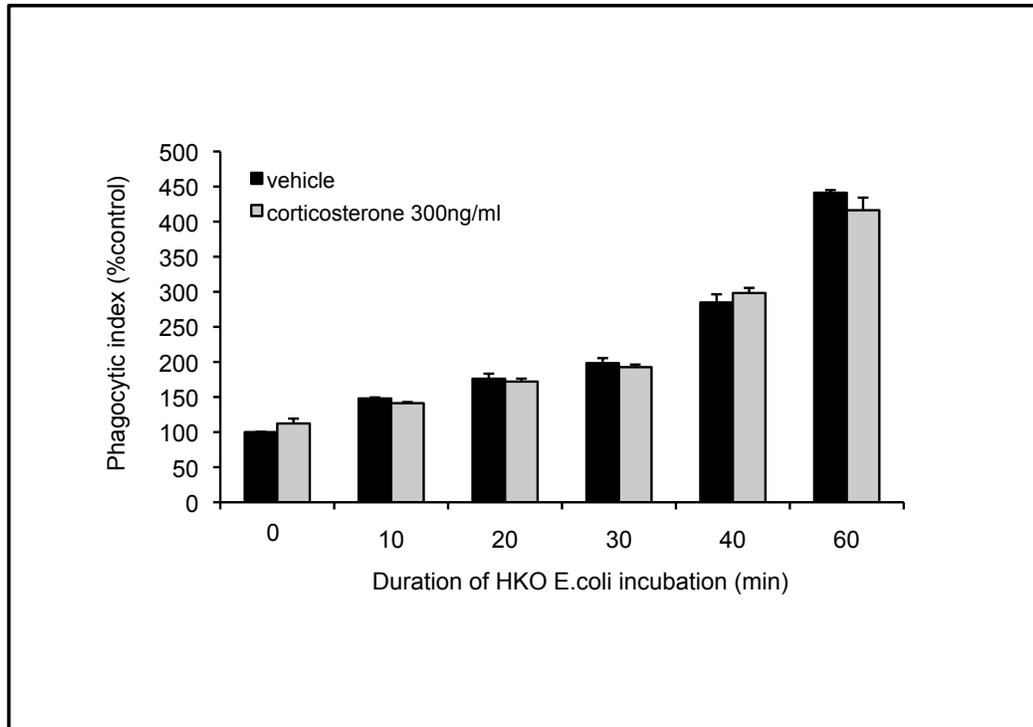
Figure 2.7. Cotricosterone has no effect on FcgR mediated macrophage phagocytosis. Cells were treated with 300ng/ml of corticosterone overnight and exposed to HKOE. Coli for varied amounts of time. Phagocytosis was quantified using fluorometric analysis, and data was displayed as % of control. Phagocytic index was quantified as described above.

FIGURE 2.8
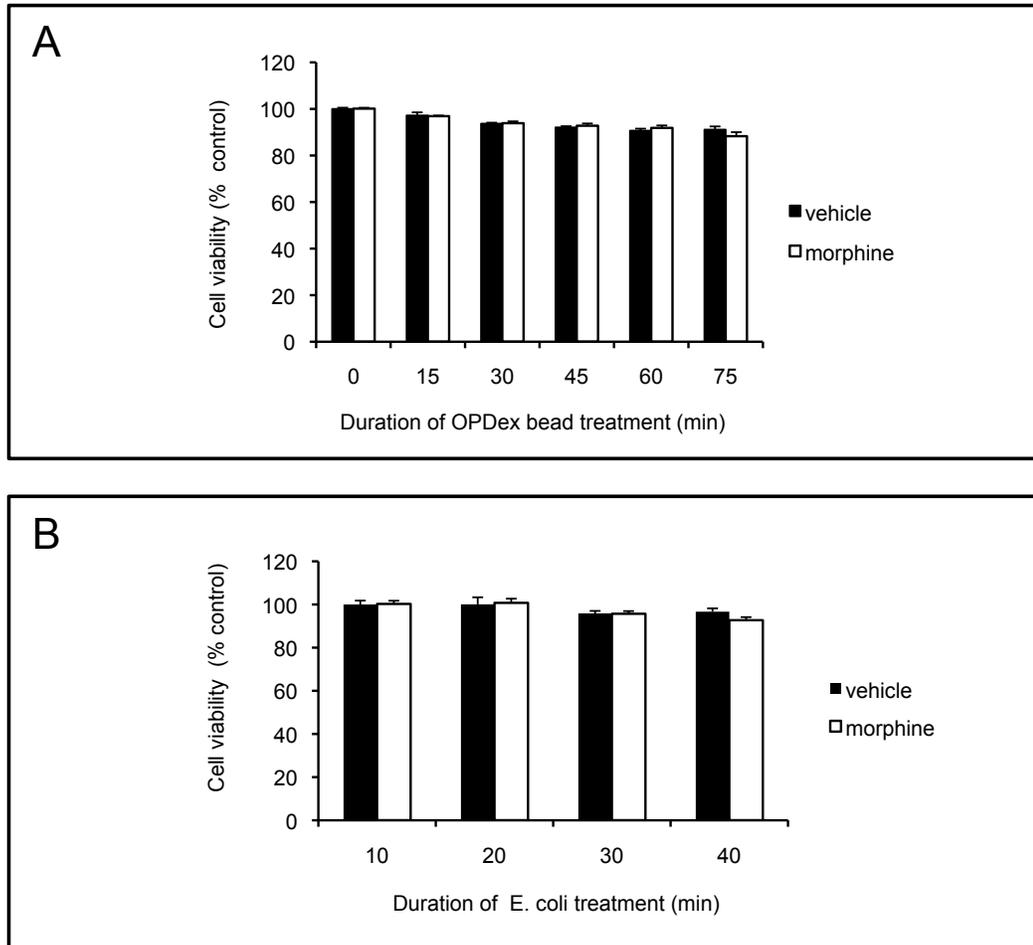
Figure 2.8. Chronic morphine treatment does not effect cell viability. J774 cells were treated with chronic morphine treatment (1μM overnight) and the next day exposed to phagocytosis of OPDex **(A)** or HKO*E. coli* **(B)** after which cell viability was determined via MTT assay according to standard protocol. Significance determined using Student's T-test $p<0.05^*$, $p<0.01^{**}$, $p<0.001^{***}$.

FIGURE 2.9
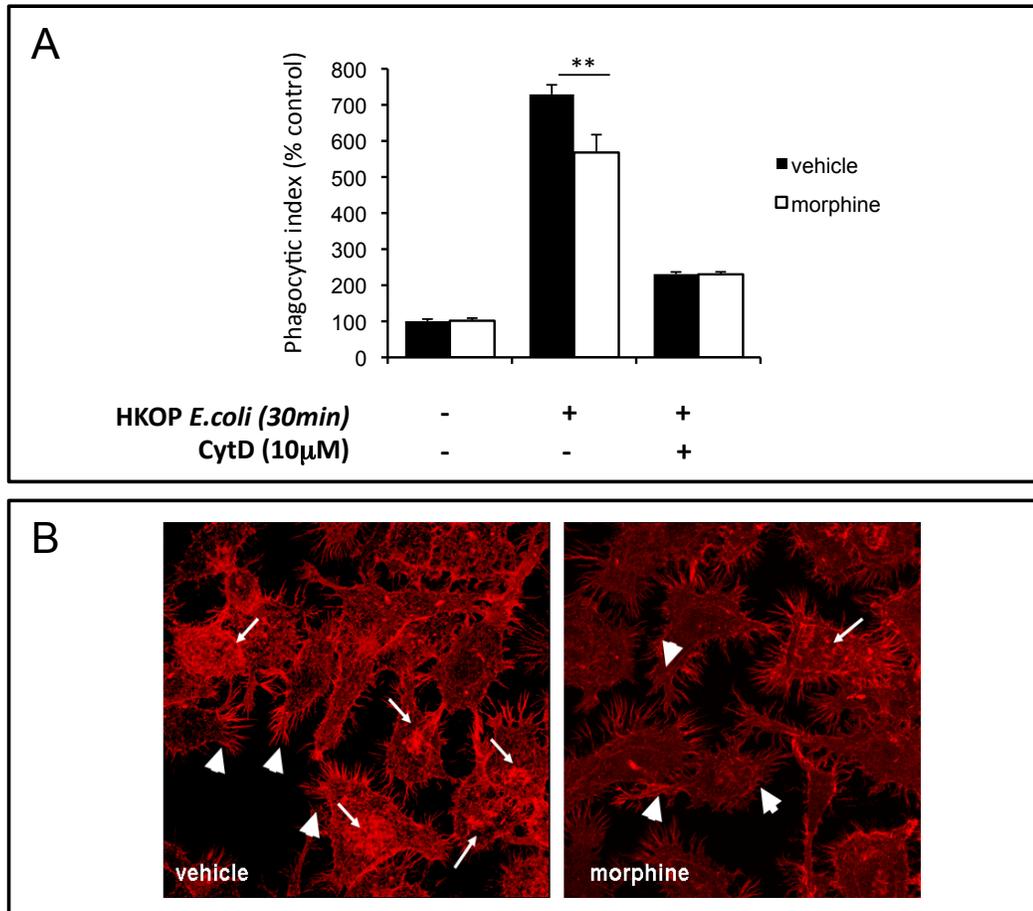
Figure 2.9. Actin polymerization is essential in morphine mediated inhbition of phagocytosis by macrophage cell line (J774). **(A)** Cells were treated overnight with 1μM morphine, and with Cytochalasin D (10μM) 10 minutes prior to addition of HKO*E.coli* particles. Phagocytosis was assayed using fluorometric analysis. **(B)** Confocal microscopic analysis was conducted morphine and HKOPE. Coli treatment followed by fixing (4% paraformaldehyde), permeabilizing (0.01% Triton x100) and staining with rhodamine phalloidin (Invitrogen). Arrowheads indicate changes in lamellipodia, and arrows indicate changes in focal adhesions Significance determined using Student's T-test. *p<0.01,**p<0.001, ***p<0.0001

FIGURE 2.10.
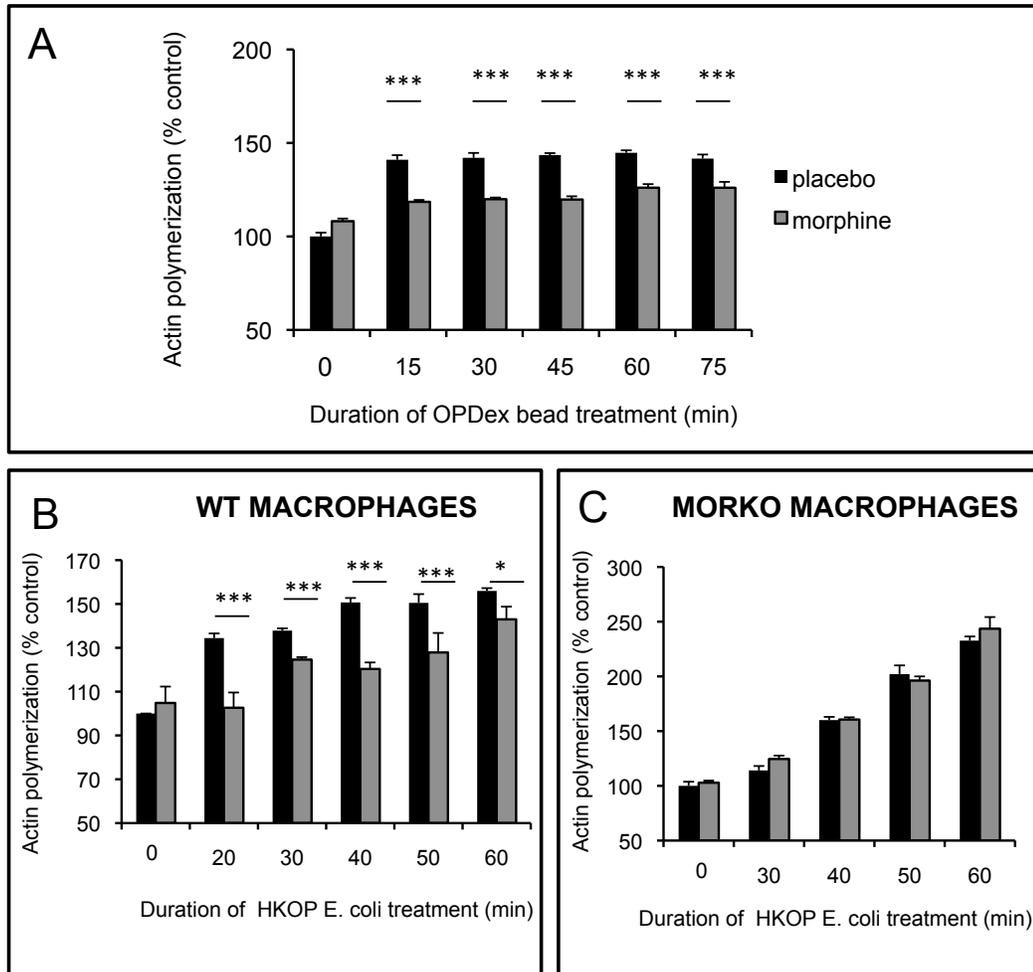
Figure 2.10. Morphine treatment *in vivo* inhibits actin polymerization in primary macrophages following FcgR mediated phagocytosis. Actin polymerization in primary macrophages was quantified following *in vivo* (72hr slow release pellet) morphine treatment of **(A,B)** WT or **(C)** MORKO mice after phagocytosis of OPDex bead **(A)** and HKO*E.coli* **(B,C).** API quantified via fluorometry and displayed as % from vehile control (actin polymerization %).Significance determined using Student's T-test. *p<0.01,**p<0.001, ***p<0.0001

FIGURE 2.11
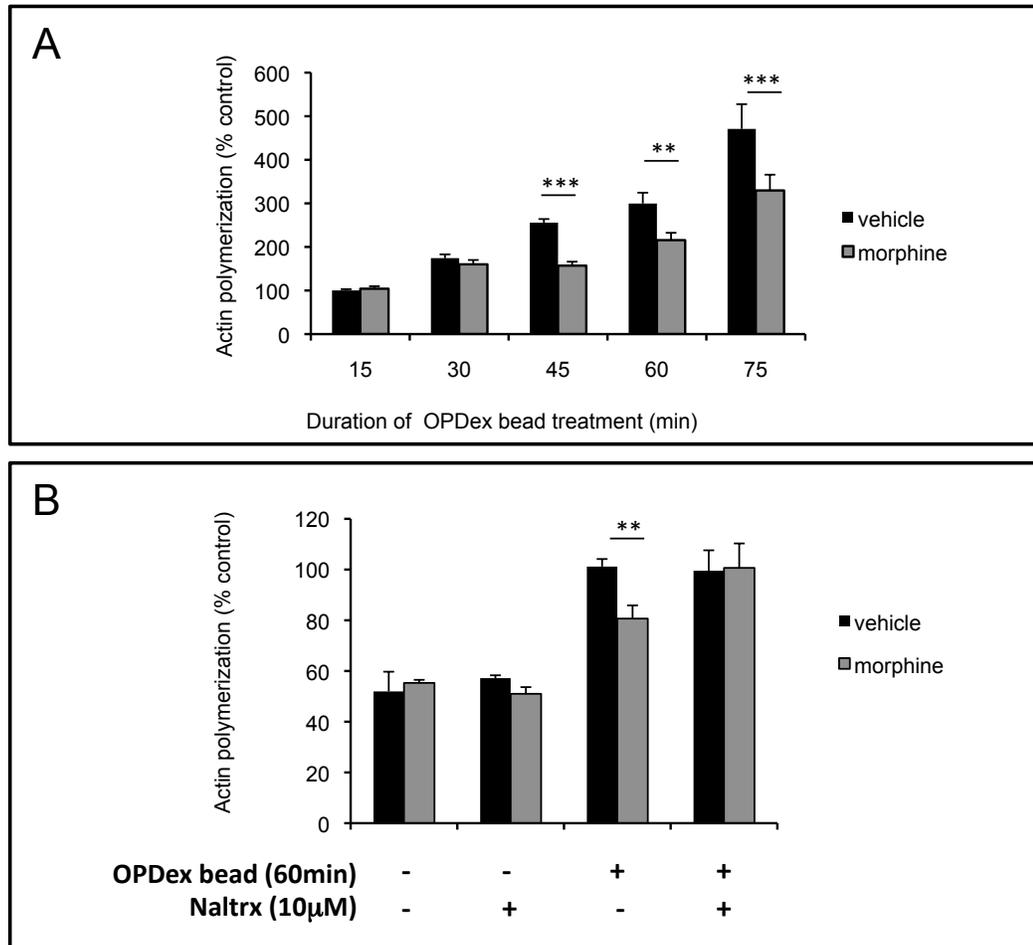
Figure 2.11. Morphine inhibits actin polymerization in a naltrexone reversible manner. (A) Following chronic morphine treatment J774 cell line cells were exposed to OPDex bead for a varied durations of time and assesed for actin polymerization using fluorometric assay. **(B)** J774 cells were pretreated with 10 μM naltrexone (Naltrx) 2 hr prior to addition of 1 μM morphine (overnight) and the next day cells were exposed to OPDex bead for 60 minutes,cells were stained and analyzed for actin polymerization. Actin polymerization index = rhodamine (RFU)/DAPI(RFU). Actin polymerization (%) – percent from vehicle control. Significance determined using Student's T-test. *p<0.01,**p<0.001, ***p<0.0001

FIGURE 2.12
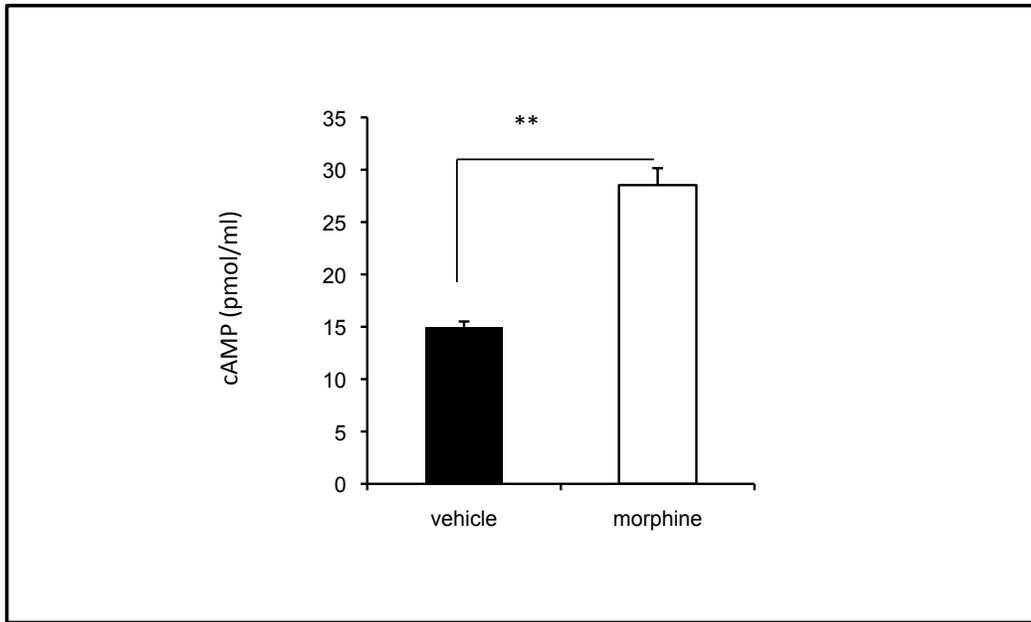
Figure 2.12. Morphine increases intracellular cAMP. J774 cells were treated overnight with morphine (1µM), washed in PBS, centifuged and resuspended at a final density of $1x10^7$ cells/ml,lysed in cell lysis buffer. The supernatant of lysed cells was collected and analyzed for cAMP production using cAMP detection kit (R&D systems, MN) (**p <0.01).

FIGURE 2.13
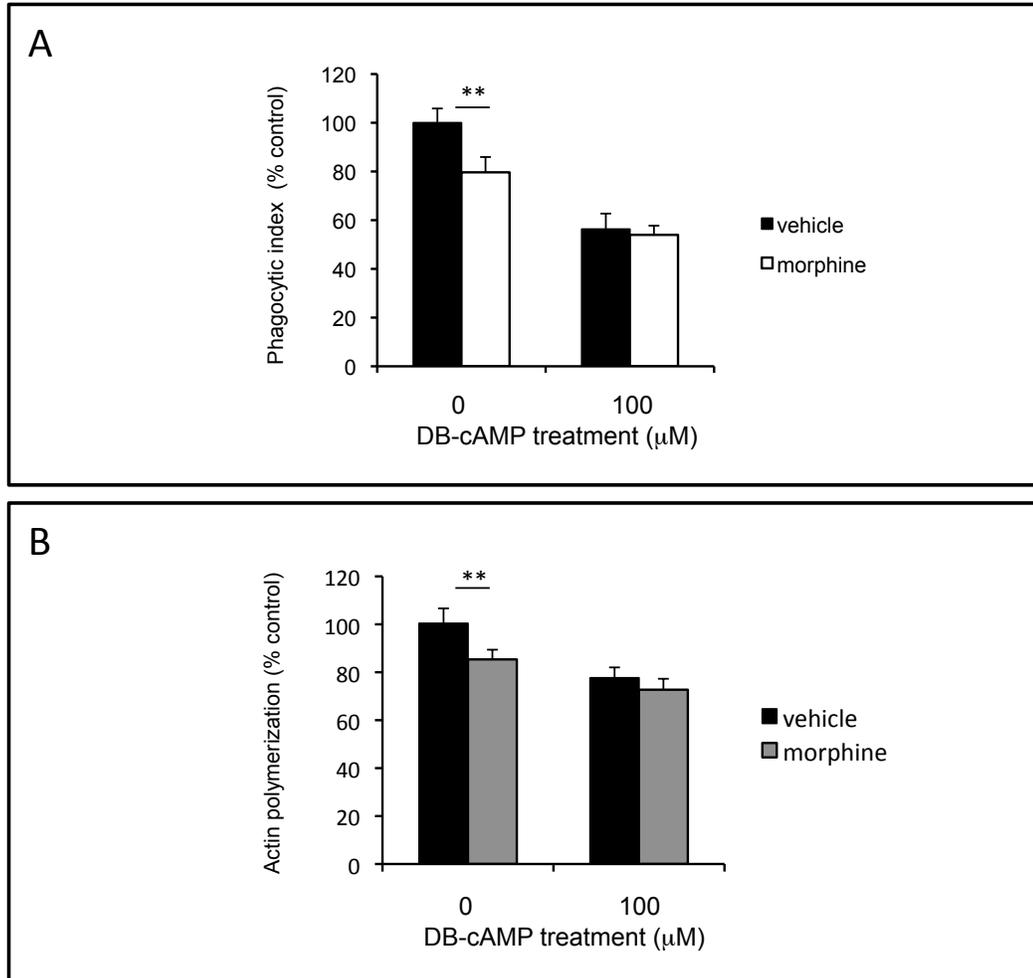
Figure 2.13. DB-cAMP inhibits FcgR mediated phagocytosis and actin polymerization. Cells were treated with 1μM morphine overnight and with DB-cAMP for 10 min prior to 60 min phagocytosis of OPDex bead. Cells were analyzed using fluorometric analysis for **(A)** phagocytosis and **(B)** actin polymerization. Data is expressed as PI or API percent from the vehicle control. Bars represent mean (±SE). Significance determined using Student's T-test p<0.01**.

FIGURE 2.14
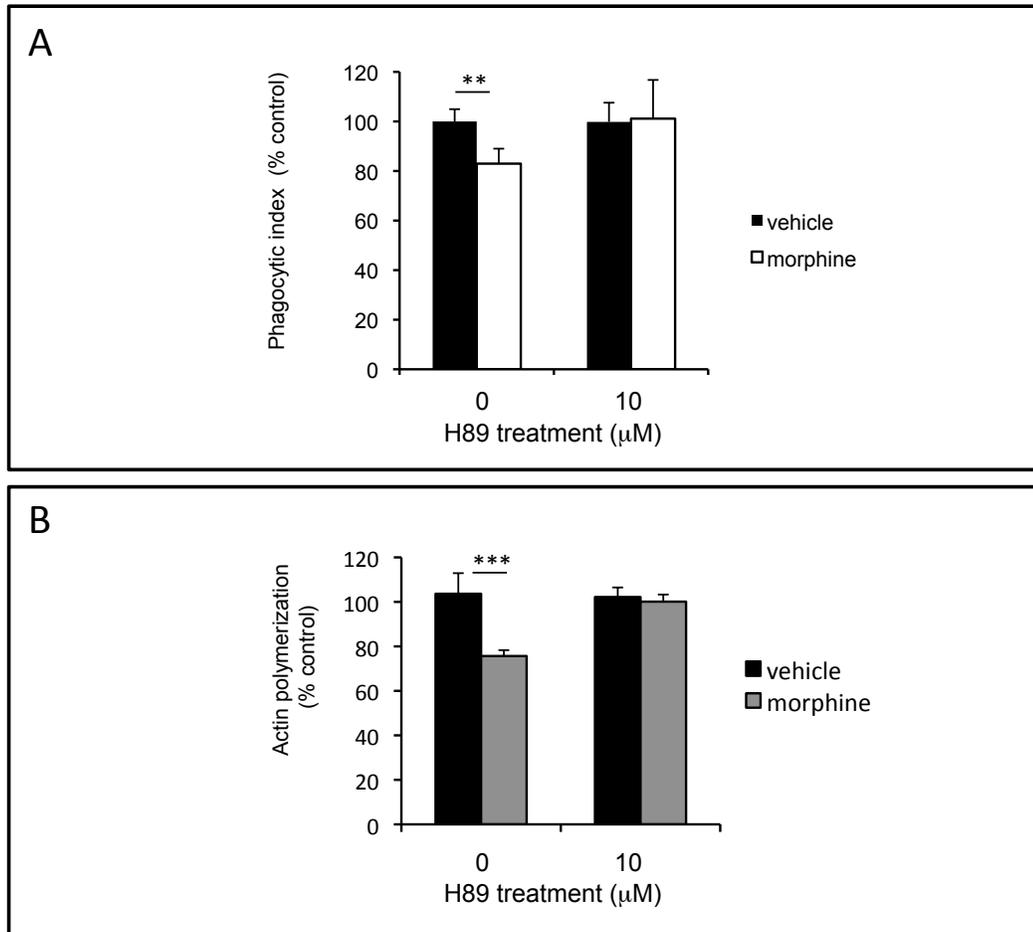
Figure 2.14. H89 reverses morphine's inhibitory effect on FcgR mediated phagocytosis and actin polymerization. Cells were treated with H89 (10 μM) for 2 hr prior to morphine (1μM)overnight treatment prior to 60 min phagocytosis of OPDex bead. Cells were analyzed using fluorometric analysis for **(A)** phagocytosis and **(B)** actin polymerization. Data is expressed as PI or API percent from the vehicle control. Bars represent mean (+SE). Significance determined using Student's T-test p<0.01**.

FIGURE 2.15
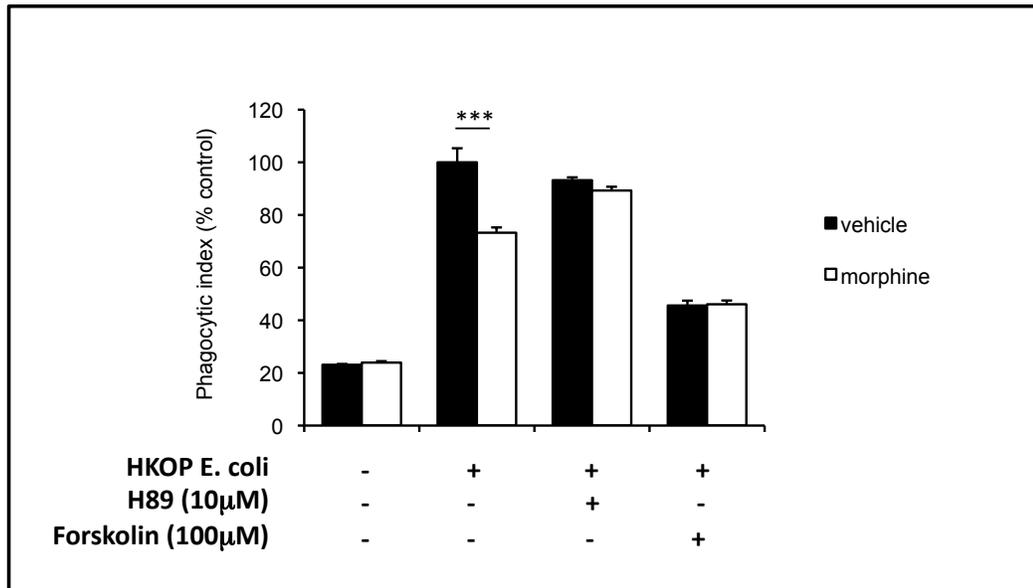
Figure 2.15. Morphine inhibits phagocytosis of HKOE. coli through a PKA
dependent mechanism. Fluorometric analysis of phagocytosis, following treatments
with morphine (overnigth), 10 min forskolin (100 μM) and 2 hr H89 (10 μM) treatment
(incubation prior to morphine treatment. Data expressed as percent from the vehicle
control. Bars represent mean (±SE). Significance determined using Student's T-test
p<0.05*, p<0.01**.

FIGURE 2.16
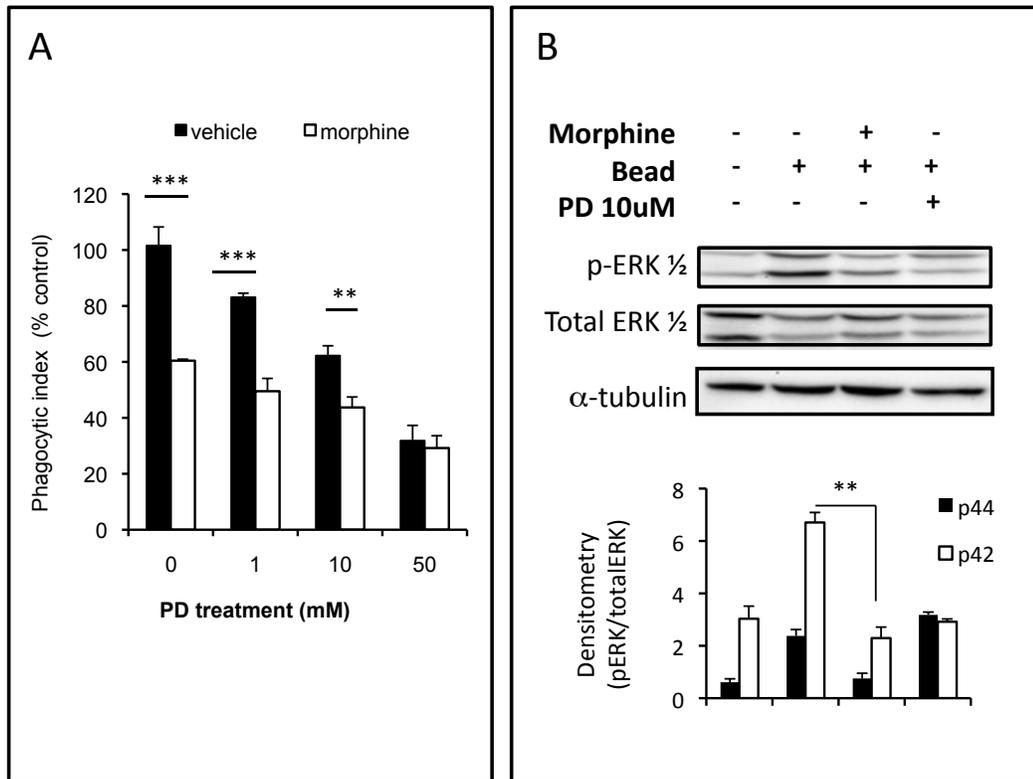
Figure 2.16. Morphine treatment inhibits ERK1/2 phosphorylation. J774 cells were treated with PD98059 for 4 hours prior to overnight morphine (1μM) treatment, phagocytosis of HKO*E.coli* **(A)** or OPDex bead **(B)** was conducted the next day and cells were assessed for phagocytosis via fluorometry **(A)** or ERK1/2 phosphorylation using western analysis **(B)**. Densitometry data generated using UltraLum software, comparing ratio of phospho-ERK1/2 (phospho-p44/p42) pixel density/ total ERK1/2 (p44/p42). Significance determined using Student's T-test. *p<0.01,**p<0.001, ***p<0.0001

FIGURE 2.17
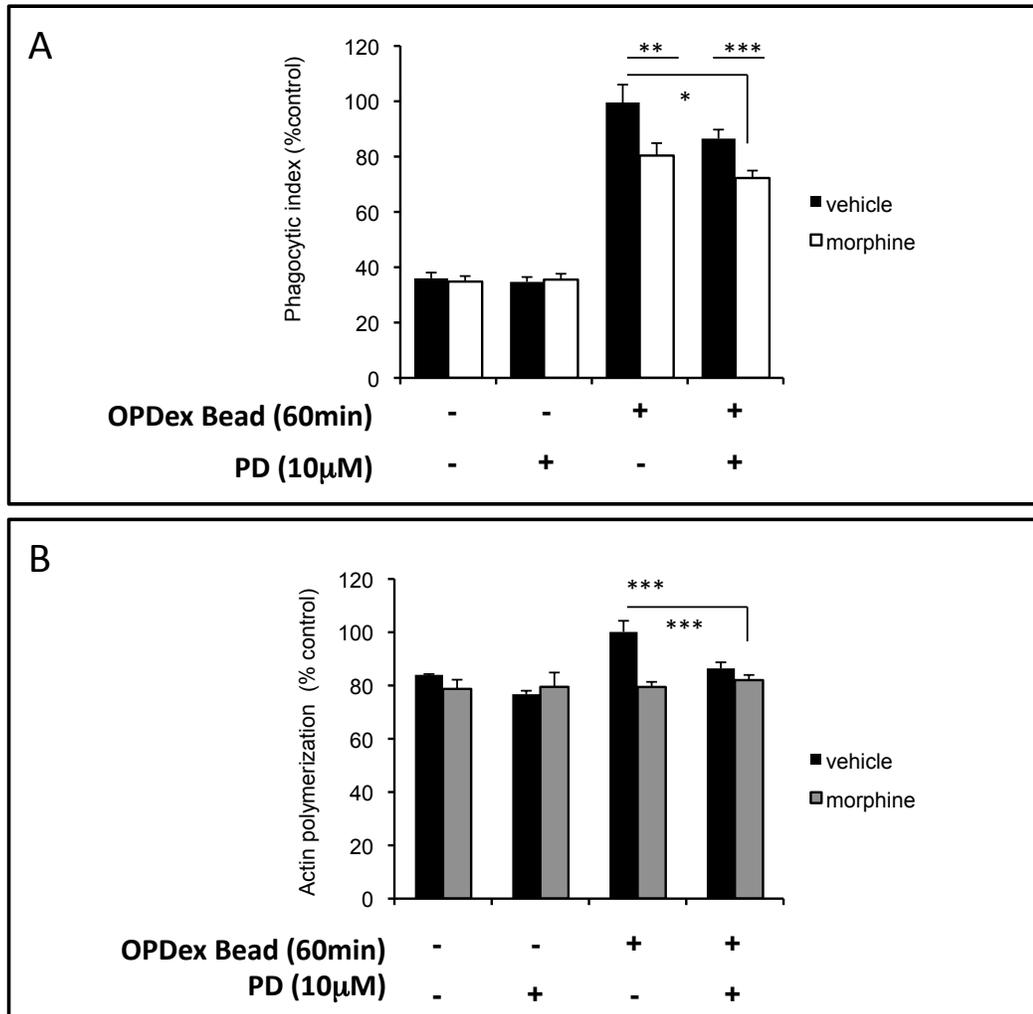
Figure 2.17. **Inhibition of ERK 1/2 inhibits phagocytosis and actin polymerization**. J774 cells were treated with PD98059 for 4 hours prior to overnight morphine (1μM) treatment, phagocytosis of OPDex bead was conducted the next day and cells were assessed for phagocytosis **(A)** or actin polymerization **(B)** via fluorometric analysis. Densitometry analysis represents average of three independent experiments. Significance determined using Student's T-test. *p<0.01,**p<0.001, ***p<0.0001

FIGURE 2.18
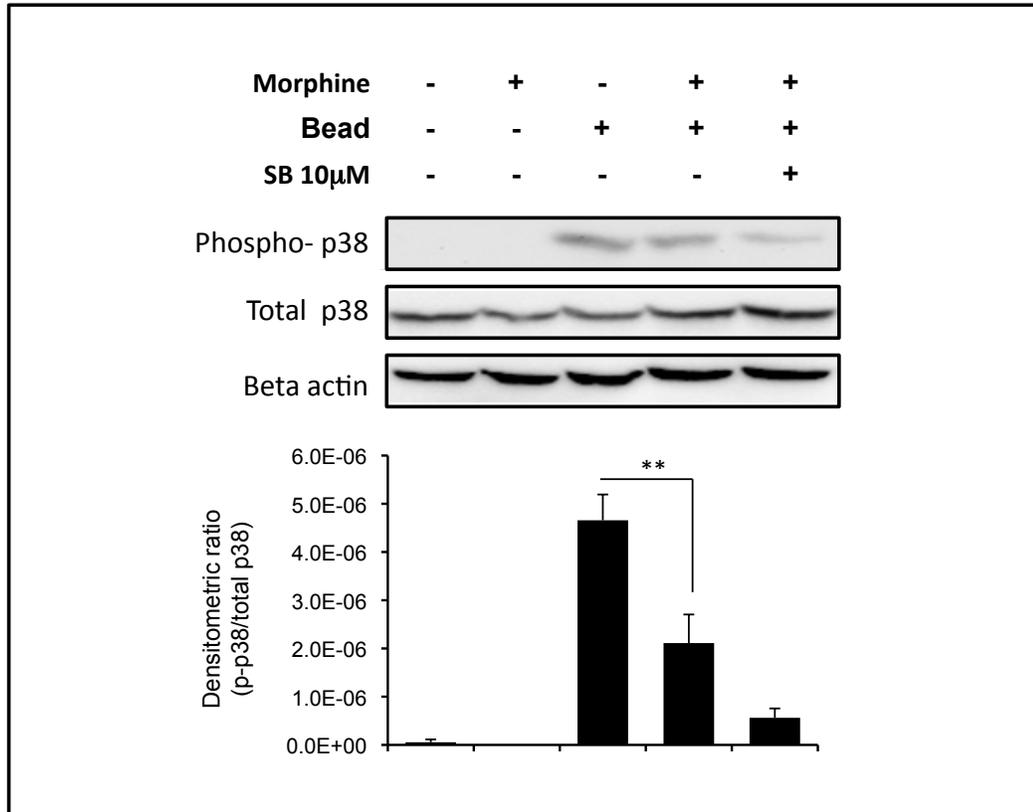
Figure 2.18. **Chronic morphine inhibits p38 MAPK phosphorylation.** Western blot analysis of p38 activation. Graph indicates densitometric analysis of the blot below comparing the ratio between phospho-p38 and total p-38. Densitometry analysis of the western blot represents average of three independent experiments. Significance determined using Student's T-test. **$p<0.01$,***$p<0.001$.

FIGURE 2.19
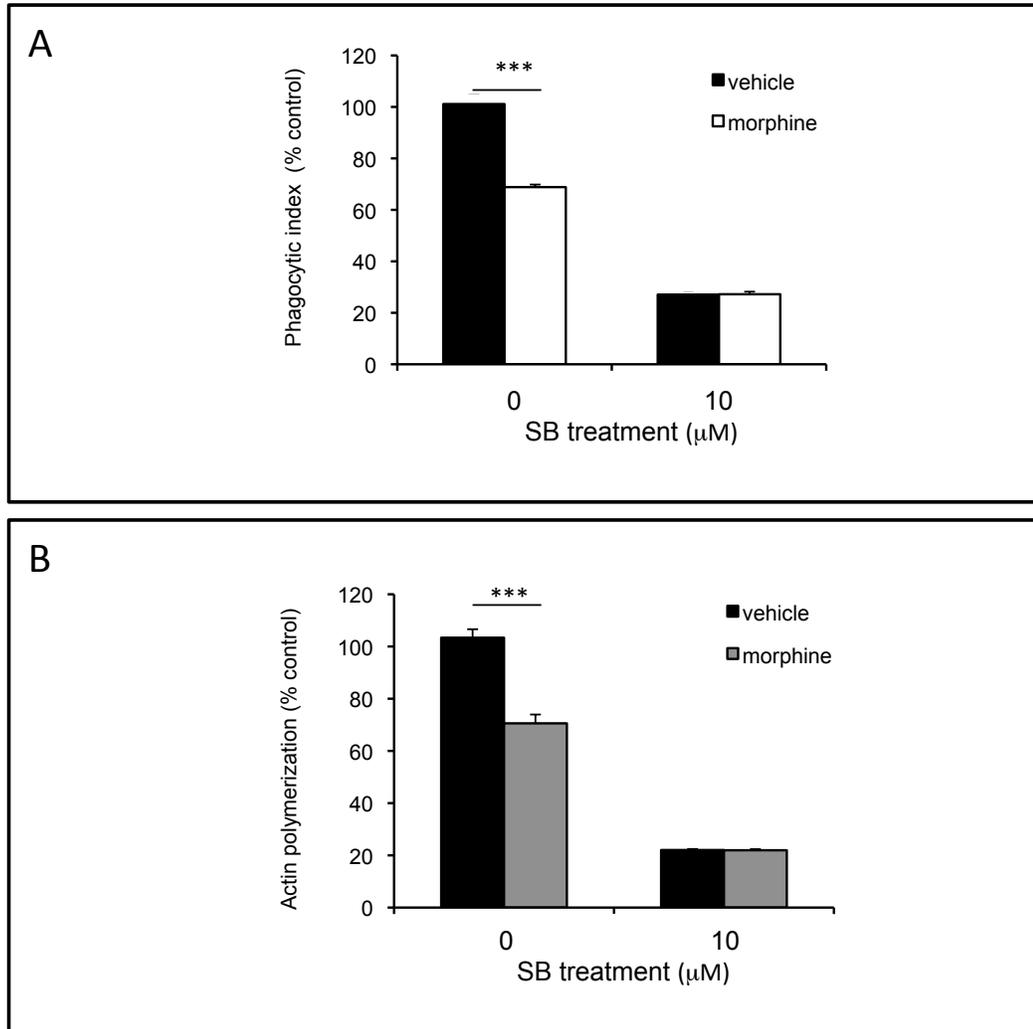
Figure 2.19. Inhibition of p38 MAPK activation leads to inhibition of phagocytosis by J774. Graphs indicate fluorometric analysis of OPDex bead phagocytosis **(A)** and actin polymerization **(B)** by morphine treated J774 macrophages in presence or absence of SB203580, a p38 inhibitor. In all experiments SB203580 was added 4 hours prior to morphine treatment *in vitro*. Significance determined using Student's T-test. *p<0.01,**p<0.001, ***p<0.0001

FIGURE 2.20
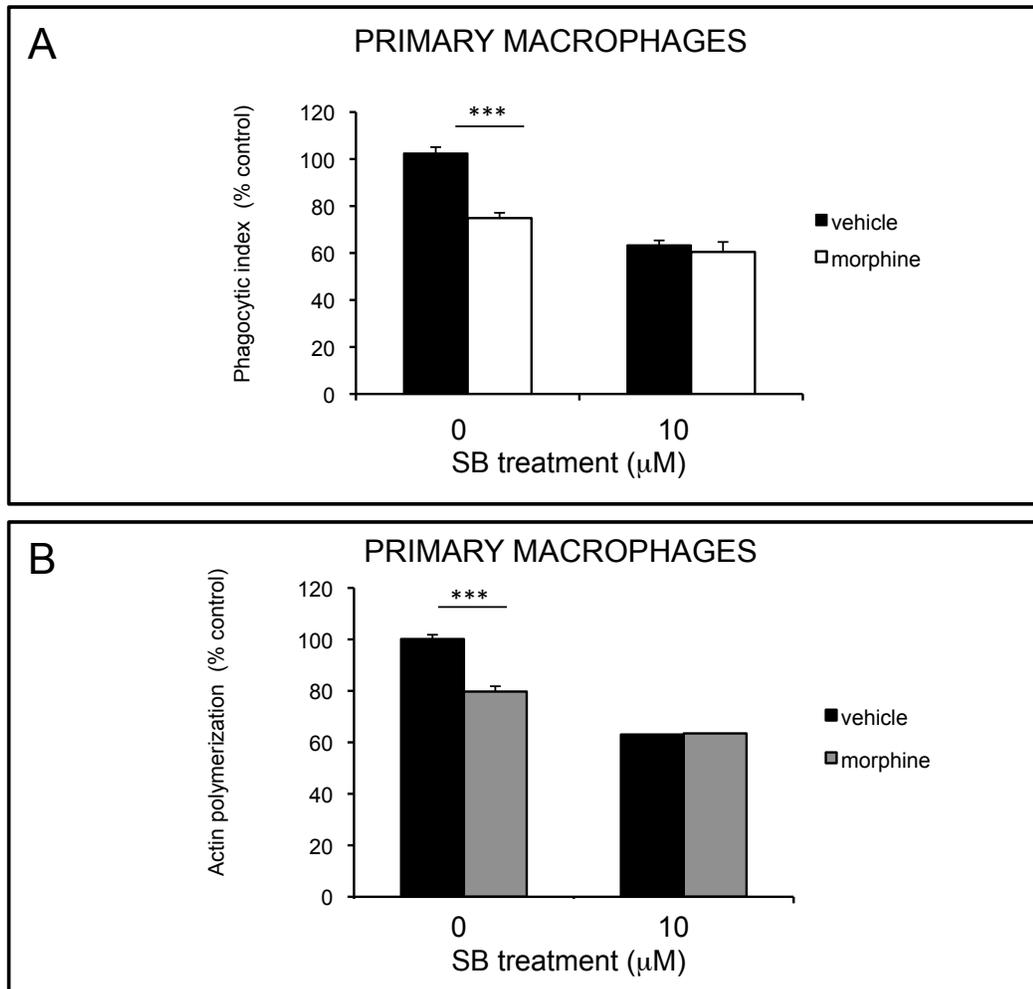
Figure 2.20. Inhibition of p38 MAPK activation leads to inhibition of phagocytosis by primary macrophages. Graphs indicate fluorometric analysis of OPDex bead phagocytosis **(A)** and actin polymerization **(B)** by morphine treated primary macrophages extracted from wt mice, following *in vitro* pre treatment SB203580 (4hr) and morphine (1mM overnight). Significance determined using Student's T-test. *p<0.01,**p<0.001, ***p<0.0001

FIGURE 2.21
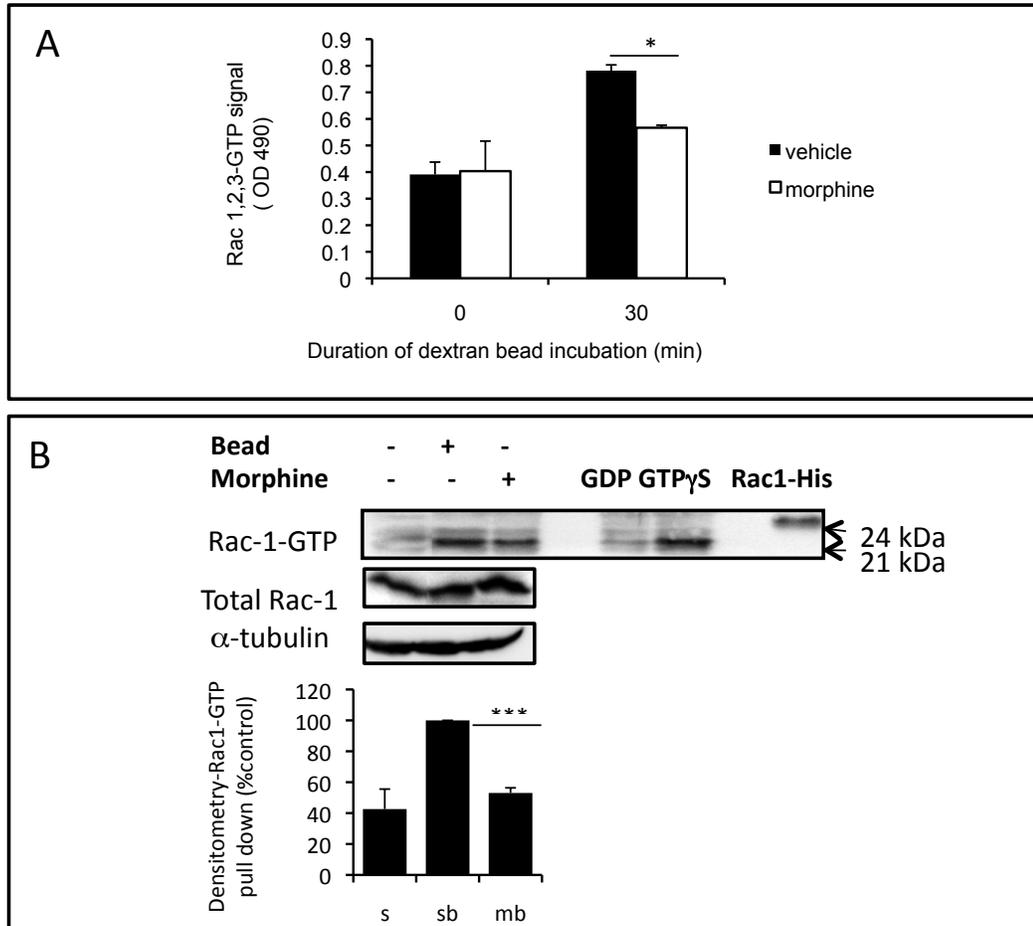
Figure 2.21. Chronic morphine treatment inhibits activation of Rac1-GTPase. J774 cells were grown for 3 days to confluence of 70%, treated with morphine and with OPDex bead for 30 min. Samples were collected, processed and assessed for Rac1 activation using **(A)** Rac1,2,3- GLISA and **(B)** Rac1-GTP pull-down assay. **(B)** shows the blots of the pull-down assay (top), and western blot probed with total Rac-1 antibody (middle), and beta actin for loading control (bottom); as well as densitometric analysis of the pull-down assay ratio of (Rac1-GTP) to western blot (total Rac1) as loading control (s-vehicle control; sb-vehicle+bead; mb-morphine +bead); Data expressed as % from vehicle control. Positive control (GTPγS) and Rac1-His (20mg), and negative control (GDP) were used to indicate antibody specificity**.**

FIGURE 2.22
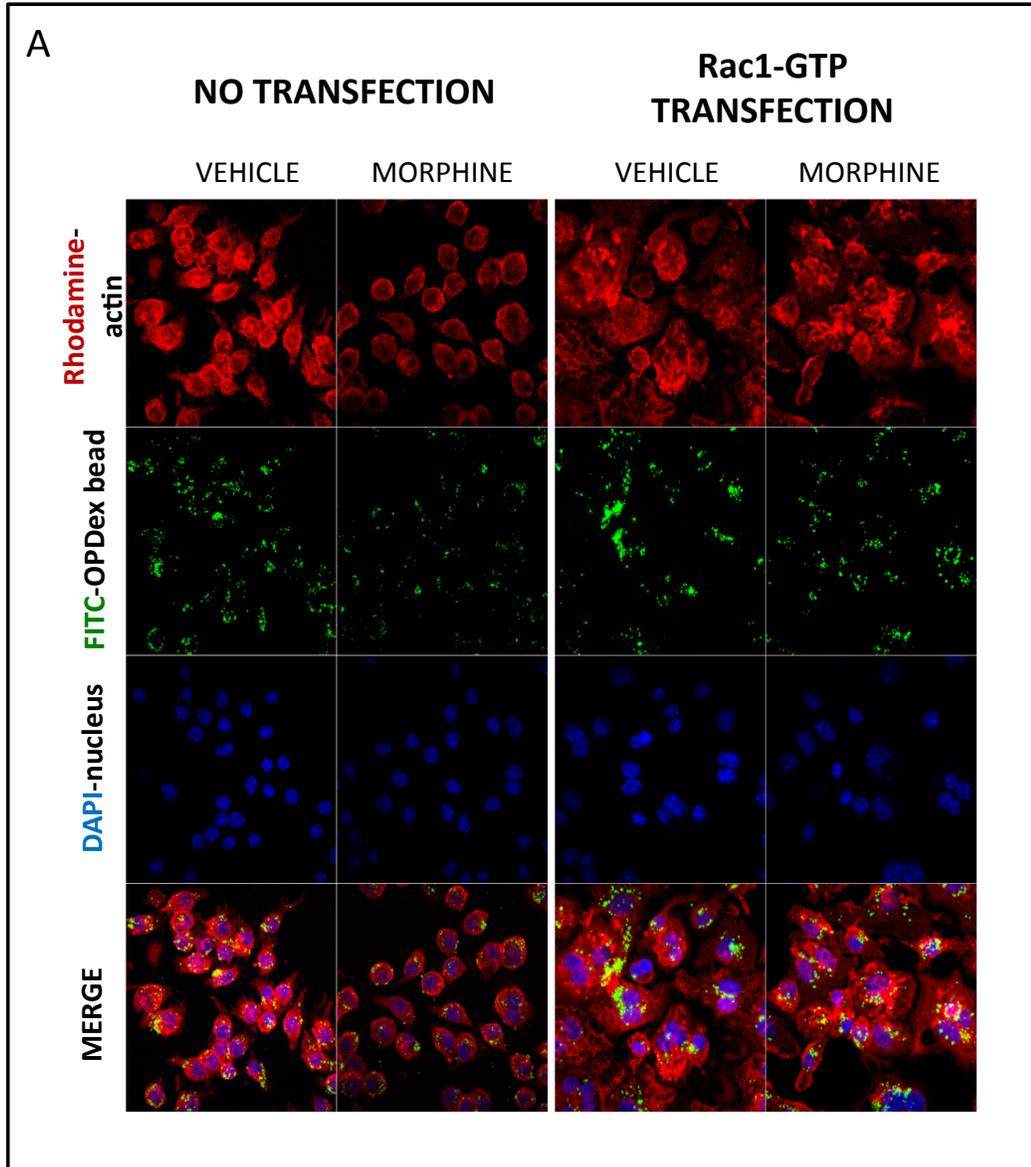
FIGURE 2.22
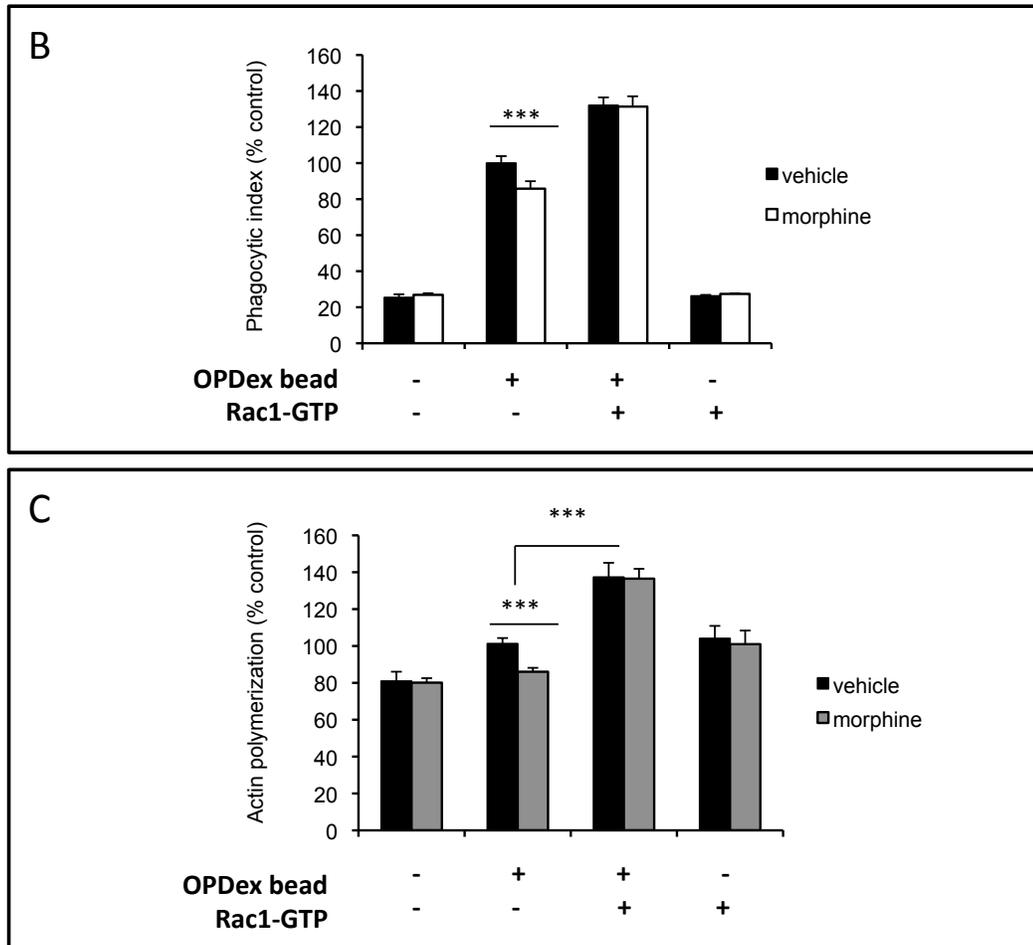
Figure 2.22. Rac-1 over-expression overrides morphine's inhibitory effect on phagocytosis and actin polymerization. Cells were transfected with Rac1-GTP plasmid (Adgene; see methods for detail). 24 hours after transfection cells were treated with morphine (1μM overnight) and the next day treated with HKO*E.coli* tagged with FITC **(A),** or Texas red **(B,C);** Molecular probes). Data was assessed for phagocytosis **(B)** and actin polymerization using fluorometric studies **(C). (A)** Confocal microscopy: red (rhodamine phalloidin), green -FITC tagged HKO*E.coli*, blue - DAPI-nucelus. Images taken at 60x. Significance determined using student's T-test. *p<0.01,***p<0.0001

FIGURE 2.23
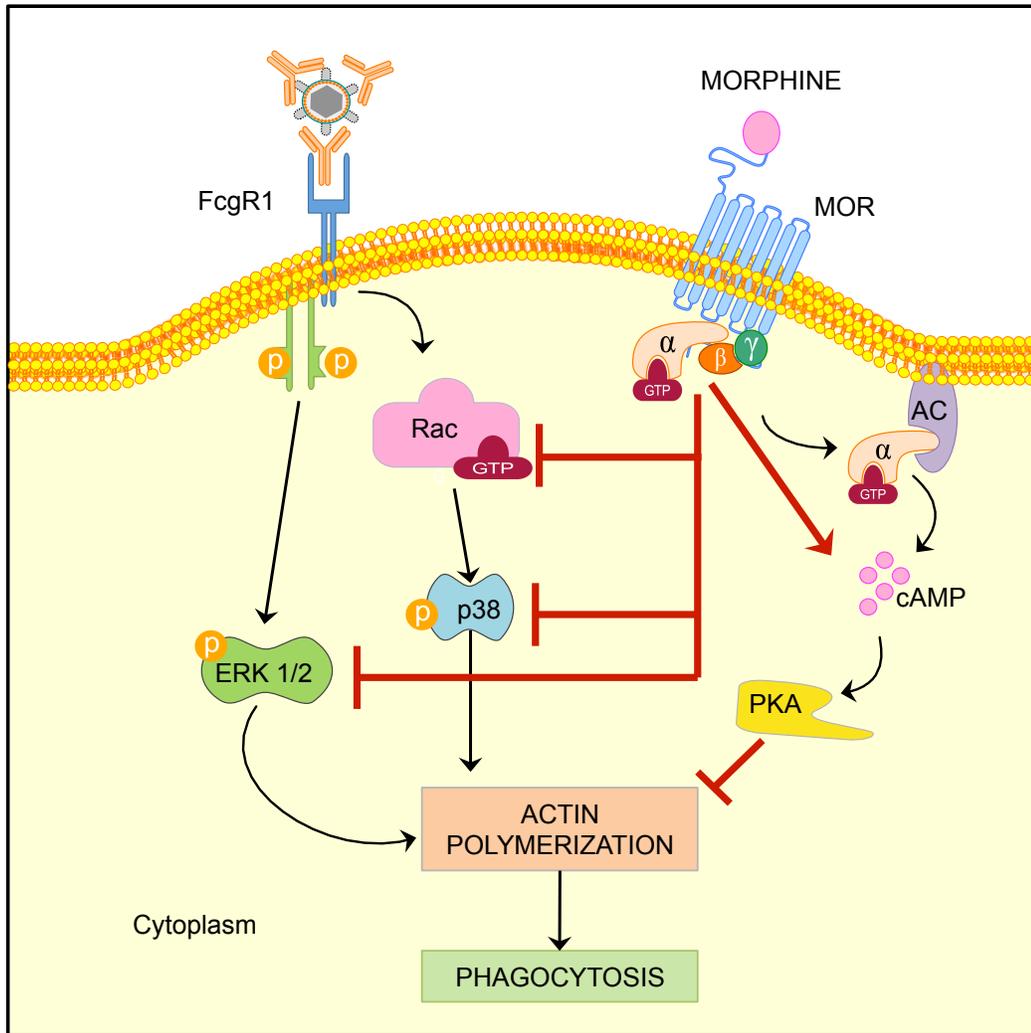
Figure 2.23. Mechanistic diagram of morphine mediated inhibition of phagocytosis. MOR activation during chronic morphine treatment leads to superactivation of adenylyl cyclase and elevation of cAMP which through PKA exerts it's effects and leads to inhibition of actin polymerization, thereby inhibiting phagocytosis, directly or as literature indicates, through inhibition of MAPKs , p38 and ERK 1/2. Following activation of FcgR, morphine, inhibits Rac1 which leads to inhibiton of actin as well as phagocytosis. Thin arrows indicate pathways downstream of FcgR, while thick lines or arrows show how morphine inhibits or activates these pathways.

CHAPTER 3.

DIFFERENTIAL MODULATION OF Fc-GAMMA RECEPTOR MEDIATED PHAGOCYTOSIS BY MORPHINE FOLLOWING TLR2 AND TLR4 ACTIVATION

<u>INTRODUCTION</u>

Morphine has been known to inhibit innate immune function. Deleterious effects of morphine's immune suppression have most frequently been observed in morphine users and abusers, where morphine use or abuse lead to increased susceptibility to infection as well as inhibition of bacterial clearance and increased dissemination of bacteria and sepsis (Hilburger et al., 1997)(Wang et al., 2005) One of the ways by which morphine increases bacterial dissemination is by modulating macrophage function, specifically inhibiting phagocytosis and modulating toll-like receptor mediated cytokine release (Bussiere et al., 1993; Roy et al., 1998a; Kelschenbach et al., 2008). Toll-like receptor (TLR) signaling and phagocytosis are hallmarks of macrophage-mediated innate immune responses to bacterial infection. However, the relationship between morphine and these two processes has not been well established.

Morphine has been known to inhibit immune function by modulating inflammatory pathways mediated by toll-like receptors (TLRs). Several groups including our own have reported that morphine inhibits the production of interlukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α (Tubaro et al., 1983; Eisenstein et al., 1993; Rojavin et al., 1993a; Roy et al., 1998a). Expression of these proinflammatory cytokines is dependent

on the activation of a transcription factor, NF kappa B (NF-κB) which is also inhibited by chronic morphine treatment (Roy et al., 1998b).

TLRs play an essential role in recognition of specific molecular patterns found in a broad range of microbial pathogens such as bacteria and viruses, triggering inflammatory and antiviral responses, which result in eradication of invading microbes (Akira and Takeda, 2004). TLRs are expressed on many different immune cells including T and B lymphocytes, dendritic cells, macrophages and epithelial cells. At least 10 TLRs have been identified, each having specificity to various bacterial, fungal and viral products. These pathogen-related products are often highly conserved structures that are critical for microbial survival (Tricker and Cheng, 2008). TLR2 and TLR4 distinguish bacterial products from Gram-positive bacteria such as lipoteichoic acid (LTA), and Gram-negative bacteria such as lipopolysaccharide (LPS) (respectively). Although primary role of TLRs is to initiate inflammatory pathways, they have also been implicated to play a role in macrophage phagocytosis.

Phagocytosis is an important step for host defense against microbial pathogens since it leads to degradation of the microbe as well as antigen presentation to the cells of the adaptive immune system. Fcγ receptor (FcgR) plays an essential role in internalization of extracellular pathogens. They mediate phagocytosis of IgG opsonized pathogens, by binding to the constant region of the IgG opsonin and initiating a signaling cascade which leads to actin polymerization, membrane extension and phagosome formation. As the phagosome is formed it goes on to mature by fusing with acidic lysosomal vacuoles which in accordance with reactive oxygen intermediates (superoxide etc.) as well as reactive nitrogen intermediates (NO etc.) lead to pathogen degradation. TLR activation is known to affect phagocytosis in three steps highlighted in **Figure 1.8**: 1) by modulation efficiency of phagosome formation, 2) by affecting phagosome

maturation and 3) by leading to transcriptional responses that affect genes in all steps of phagocytosis (Underhill and Gantner, 2004). Microarray analysis indicates that TLR stimulation upregulates the FcgR complement receptor and scavenger receptors. Upregulation of these genes is dependent upon p38 MAPK activation downstream of TLR signaling, and thus resulting in impaired phagocytosis (Garcia-Garcia and Rosales, 2002; Underhill and Ozinsky, 2002).

Although there are many different types of TLRs, their signaling pathways are highly homologous, but differ in that they can be dependent or independent of the myeloid differentiation primary response gene 88 (MyD88). Most TLR receptors including TLR2 signal through the MyD88 dependant pathways. After ligand binding, most (Takeuchi et al., 1999; Underhill and Gantner, 2004) TLRs sequentially recruit the adaptor molecules myeloid differentiation factor 88 (MyD88), IL-1 receptor–associated kinase (IRAK), and tumor necrosis factor receptor–associated factor 6 (TRAF6) (**Figure 1.7**). These adaptors in turn mediate the activation of the jun NH2-terminal kinase (JNK), nuclear factor (NF)-κB, p38 MAPK, extracellular signal–related kinase 1/2 (ERK 1/2), and phosphoinositide 3 kinase (PI3K) signaling pathways leading to activation of inflammatory target genes (Akira, 2006). On the other hand, TLR4 and TLR3 can signal through both MyD88 dependent and independent pathways (Alexopoulou et al., 2001). In the MyD88 independent pathway, TLR4 activates RIP/TRIF/TRAF complex, which leads to activation of Interferon regulatory factor 3, IRF3 leading to transcription of interferon alpha and beta. TLR4 and TLR2 signaling pathways also differ in that TLR4 homodimerizes while TLR2 heterodimerizes with either TLR1 or TLR6 depending on the ligand (Triantafilou et al., 2006). For example, TLR2/1 complex is activated by Pam3CSK4 (P3C), while TLR2/6 is activated by LTA (**Figure 1.7**).

Gaps still exist in our knowledge of the exact events that take place on the cell membrane following LPS binding (Triantafilou and Triantafilou, 2002). Morphine has been shown to synergize with LPS in enhancing LPS effect in T cells (Roy et al., 1999). Since morphine increases bacterial dissemination by inhibiting macrophage's phagocytic and inflammatory functions, the aim of this study was to examine the effects of morphine on FcgR mediated phagocytosis following activation of TLR2 and TLR4.

Our results indicate that morphine treatment leads to inhibition of phagocytosis following LTA (TLR2 ligand from Gram-positive bacteria), and increase in phagocytosis following LPS (TLR4 ligand from Gram-negative bacteria) treatment. We also note that LPS with morphine induced increase in phagocytosis through a MyD88 and p38 MAPK mediated mechanism.

Elucidation of mechanisms by which morphine modulates bacterial clearance following TLR activation will add to our understanding and treatment of infectious diseases in populations of opiate users and abusers.

<u>METHODS</u>

REAGENTS

Heat killed *E. coli* particles (E2861- *Escherichia coli* (K-12 strain) BioParticles, fluorescin conjugate (ex 494/em518), heat killed *S. aureus* particles (S-2851-*Staphylococcus aureus* (Wood strain without protein A) BioParticles®, fluorescein conjugate (ex 494/em518), respective opsonizing reagents (E2870 - *E. coli*, S-2860- *S. aureus*), dextran beads (yellow-green fluorescent Fluo-Spheres (F8852 Invitrogen; ex488nm/518nm) and DAPI were obtained from Invitrogen. MAPK inhibitors PD98059 and SB203580 were obtained from Sigma Aldrich. IL-6 detection kit obtained from R&D Systems. Morphine-HCl powder as well as 75mg slow release pellets were a generous

gift from NIDA. Lipopolysaccharides from *Escherichia coli* 055:B5 (cat# L2880) and Lipoteichoic acid from Staphylococcus aureus (cat# L2515) were obtained from Sigma Aldrich. MyD88 homodimerization inhibitory peptide set was obtained from Imgenex (cat# IMG-2005-1).

CELLS

Macrophage cell line J774.1 was obtained from ATCC, and cultured in DMEM media (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), and 1% Penicillin/Streptomycin (GIBCO).

ANIMALS

MORKO mice (C57BL/6 x 129/Ola genetic background) were produced as described previously by Loh and colleagues (Roy et al., 1998a). Briefly, a *Xho*I/*Xba*I fragment, which spans the entire exons 2 and 3, was replaced with a Neo^r cassette followed by the ligation of a thymidine kinase expression cassette to the 3' end of this segment. WT mice (B6129PF1/J), 8 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR2 KO and TLR4 KO C57BL/6 background mice were a generous gift from Dr. Olfest, and are commercially available through Jackson laboratories (strain # C.C3-*Tlr4^{Lps-d}*/J stock number 002930, and *Tlr2* - B6.129-*Tlr2^{tm1Kir}*/J stock number 004650) Animals studies have been reviewed and approved by University of Minnesota Institutional Animal Care and Use Committee.

CHRONIC MORPHINE ADMINISTRATION

For all *in vitro* experiments 1μM morphine HCl was added overnight (18 hours). For studies involving morphine treatment *in vivo,* morphine mice were implanted with 75mg slow release morphine/placebo pellets for 72 hours. During the extraction of peritoneal

cells from morphine treated WT or MORKO mice, 1μM morphine was maintained in all PBS and media used in the experiment in order to prevent withdrawal. Concentrations used in the *in vitro* paradigm were chosen to closely replicate morphine plasma levels (11ng/ml-1440ng/ml) which are present in patients undergoing morphine sulphate treatment (2.5mg-90mg every 4hours), as well as mice following 72hours implantation with 75mg morphine pellets (Aherne et al., 1979).

FLUOROMETRIC ASSAY

Cells were plated at 10,000/100ul of media per well of a 96-well plate, treated with morphine and cultured overnight in standard growing conditions (37°C, 5% CO_2, 60% rh). The following day, opsonized dextran or heat killed *E. coli* was added (in 1:20, cell:bacteria/bead ratio). Opsonization was done with IgG opsonizing reagent (Invitrogen) for 1hour in 37 ^{o}C, according to the manufacturer's instructions. The reaction was stopped at different time points by addition of trypan blue, which extinguishes fluorescence of non-internalized particles. Cells were washed two times and stained with DAPI. Fluorescence was recorded using a fluorescence plate reader (BMG; FLUOstar Omega) at ex485/em520 (FITC), and ex355/em460 (DAPI). Data were quantified as Phagocytic Index=FITC(Relative Fluorescence Units (RFU)) / DAPI(RFU), indicative of particle fluorescence/cell. In actin polymerization experiments, cells were treated similar to above, washed, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton x100 and stained with rhodamine phalloidin (Invitrogen) according to manufacturer's protocol. After the rhodamine stain, cells were stained with DAPI and florescence was measured using the fluorescence plate reader. Actin polymerization was quantified as rhodamine (RFU) (ex544/em590) / DAPI(RFU), indicative of actin polymerization per cell. The y-axis was normalized to fluorescence of the vehicle treatment with shortest

bacterial incubation or to the vehicle control (depending on an experiment) and data was expressed as percentage of vehicle control (% control).

FACS

Following treatment with morphine and/or LPS as described, cells were collected and stained in 0.1% sodium azide with PE conjugated TLR4 antibody (BD Pharmigen; cat# 558294). Data was analyzed using Guava EasyCyte and quantified using Guava cell plus software, and displayed in a bar graph.

ELISA

Cells were plated at 500,000 cell per well in a 6 well plate. Following treatment with morphine and/or LPS as described, cell supernant was collected and analyzed using a IL-6 duoset kit (R&D; cat# DY406). Absorbance was measured using BMG Omega Plate reader.

RESULTS

MORPHINE INHIBITS PHAGOCYTOSIS OF GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

Morphine has been known to modulate innate immunity, resulting in inhibition of clearance and increased dissemination of bacteria. Since infections in opioid users vary in their type and their Gram-positive and Gram-negative profile, we set out to examine morphine's effects on macrophage clearance of Gram-positive and Gram-negative bacteria.

Following chronic morphine treatment (1μM) macrophage cell line J774 was exposed to heat killed IgG opsonized *S.aureus* (HKO *S.aureus*) or heat killed IgG

opsonized *E. coli* (HKO *E.coli*) and analyzed using fluorometric assay. Initial fluorometric analysis indicated that morphine inhibits macrophage phagocytosis of Gram-positive HKO *S.aureus*, and Gram-negative HKO *E.coli* (**Figure 3.1**). Overall phagocytosis of HKO *S.aureus* was greater when compared to HKO *E.coli* however morphine's inhibition of phagocytic index was maintained with internalization of both pathogens (**Figure 3.1A**). Interestingly, morphine's inhibitory effect on phagocytosis was more significant with phagocytosis of HKO *S.aureus* (40% inhibition) than with phagocytosis of HKO *E. coli* (20% inhibition) (**Figure 3.1B**). Therefore greater inhibition of phagocytosis of Gram-positive bacteria by morphine treated macrophages, may lead to greater impairment of clearance causing more deleterious effects for the host than Gram-negative bacterial infection.

MORPHINE INCREASES PHAGOCYTOSIS IN PRESENCE OF LPS

In order to investigate mechanisms of morphine's differential inhibitory effect on Gram-positive and Gram-negative bacteria we examined the effects of lipopolysaccharide (LPS) (component of Gram-negative cell wall) a TLR4 ligand, and lipoteichoic acid (LTA) (component of Gram-positive bacteria) a TLR2 ligand, on phagocytosis. After undergoing chronic morphine treatment (1μM) overnight, J774 cells were treated with LPS (50 ng/ml) for 2, 4 and 6 hours prior to bacterial incubation. As expected, morphine alone inhibited, while LPS alone increased phagocytic index in a time dependant manner. Surprisingly J774 cells treated with morphine and LPS (morphine+LPS) potentiated the increase in phagocytosis compared to LPS alone or vehicle control (**Figure 3.2**). This effect was observed in cells exposed to HKO *E.coli* (**Figure 3.2A),** HKO *S.aureus* **(Figure 3.2B**), as well as opsonized dextran bead (OPDex bead) (**Figure 3.2C**).

LPS is a TLR4 ligand and as such leads to TLR4 activation. Activation of TLR4 has been implicated in enhancement of phagocytosis and bacterial clearance by upregulating Fc-gamma receptor (FcgR) (Garcia-Garcia and Rosales, 2002; Underhill and Ozinsky, 2002). Therefore we investigated morphine's effect on TLR4 expression in presence of morphine and LPS. FACS analysis indicates that morphine up regulates surface expression of TLR4, where morphine+LPS further potentiates this upregulation (**Figure 3.3**). These results were confirmed by our lab's unpublished findings indicating same results via PCR and Western blot analysis. We also note that morphine alone is not capable of activating TLR4 receptor, as indicated by ELISA analysis of IL-6 production (**Figure 3.4).** Together these data show that activation of TLR4 receptor by LPS leads to an increase in TLR4 expression and therefore an increase in phagocytosis. This effect is further exacerbated in presence of morphine since morphine+LPS increases TLR4 expression, thereby enhancing macrophage phagocytic ability.

ACTIVATION OF TLR2 LEADS TO INHIBITION OF PHAGOCYTOSIS

TLR2 receptors are activated by series of ligands from Gram-positive pathogens. Upon ligand binding TLR2 heterodimerizes with TLR1 or TLR6 leading to signal transduction. In order to study the effects of TLR2 signaling on phagocytosis we utilized lipoteichoic acid (LTA) and Pam3CSK4 (P3C). LTA is a major constituent of the cell wall of Gram-positive bacteria and it binds to TLR2/TLR6 heterodimer, while P3C, a tripalmitoylated lipopeptide which mimics the acylated amino terminus of bacterial lipoproteins, activates TLR2/TLR1.

Activation of TLR2 via LTA (10ng/μl) led to a time dependant inhibition of phagocytosis. In the presence of morphine, LTA maintained inhibitory effect without altering morphine's ability to inhibit phagocytosis of HKO *E. coli* (**Figure 3.5A)** or OPDex

bead **(Figure 3.5B)**. Contrasting LTA mediated TLR2 activation, P3C mediated TLR2 activation alone didn't affect phagocytosis while in presence of morphine it led to increase in phagocytosis **(Figure 3.5C)** similar to what was observed in LPS mediated TLR4 activation. Unpublished data generated by our lab indicated P3C, in addition to activating TLR2, leads to increased expression of TLR4, which explains why we see the same effect with P3C as we do with LPS mediated TLR4 activation.

Morphine+LPS treatment mediated increase in phagocytosis of OPDex beads, and morphine mediated decrease in phagocytosis were abolished in cells pretreated with naltrexone (**Figure 3.6A**), indicating that morphine exerts its effect on phagocytosis by J774 macrophages through a classical opioid receptor pathway. Although naltrexone reversed morphine's effect with morphine or morphine+LPS treated cells, LTA or morphine+LTA treated cells maintained their inhibition in presence of naltrexone **(Figure 3.6B**), suggesting that inhibition was due to LTA and not morphine treatment. Microscopic analysis of these cells (**Figure 3.6. C**) further confirmed the previous data indicating increased phagocytosis of OPDex beads and increased actin polymerization in presence of LPS, as well as morphine+LPS. LTA treated cells show an inhibition of phagocytosis, although their actin polymerization is greater than respective morphine and vehicle controls, but lower than LPS treated cells.

MORPHINE INCREASES PHAGOCYTOSIS FOLLOWING TLR4 ACTIVATION IN PRIMARY MACROPHAGES

To further elucidate effects of TLR on phagocytosis we utilized TLR2 and TLR4 knockout (KO) mice. Experiments with primary peritoneal macrophages from wild-type (WT) mice were affected by morphine in the same manner as the J774 cell line, where morphine treatment in presence of LPS increased phagocytosis and LTA treatment (with

and without morphine) reduced phagocytosis of OPDex bead (**Figure 3.7A**). In primary macrophages from TLR4 KO mice we observed that LPS had no effect (**Figure 3.7B**), due to the lack of TLR4 receptor. As expected LTA and morphine maintained same levels of inhibition of phagocytosis in TLR4 KO mice (**Figure 3.7B**) as seen in macrophages from WT mice (**Figure 3.7A**), since LTA and morphine do not require TLR4 for signaling. These data were also confirmed with the *in vivo* studies, following implantations with slow releasing morphine or placebo pellets (72 hours), and LPS injections (0.4mg/kg) 6 hours prior to sacrifice. Peritoneal macrophages were collected via peritoneal lavage and were treated with OPDex bead for 60 minutes. Similar to previous experiments, morphine treatment in presence of LPS results in increase in phagocytosis in WT mice (**Figure 3.8A**) while in TLR4 KO mice (**Figure 3.8B**) this effect was abolished.

Together these data indicate that morphine with LPS leads to an upregulation of TLR4 receptor which leads to additive increase in FcgR mediated phagocytosis in J774 cell line as well as in primary macrophages undergoing *in vivo* and *in vitro* treatments.

TLR2 ACTIVATION DECREASES PHAGOCYTOSIS IN PRIMARY MACROPHAGES

In order exert its effect LTA binds to a heterodimer of TLR2 and TLR1. Therefore we wanted to examine the role of TLR2 in LTA mediated inhibition of phagocytosis by using primary peritoneal macrophages from TLR2 KO mice treated *in vitro*.

As expected TLR2 activation, as well as morphine treatment led to inhibition of phagocytosis in primary macrophages from WT mice **(Figure 3.9A)**. In macrophages from TLR2 KO mice (**Figure 3.9B**), morphine mediated inhibition of phagocytosis was

sustained while LTA effect was abolished indicating that TLR2 is imperative in this process. In addition, inhibition of phagocytosis caused by morphine was abolished in cells pretreated with naltrexone indicating that morphine was acting through the classical opioid receptors.

Taken together these data indicate that morphine through activation of classical opioid receptors inhibits phagocytosis, while LTA through activation of TLR-2 also inhibits phagocytosis. Although both LTA and morphine lead inhibition of internalization it is difficult to say if they synergize in this process since individually they might be reaching maximal inhibition and causing the ceiling effect.

ROLE OF MOR IN TLR MODULATION OF PHAGOCYTOSIS

Previously mentioned data from our naltrexone studies (**Figures 3.6, 3.9B**) indicate that morphine regulates TLR modulation of FcgR mediated phagocytosis through classical opioid receptors. Therefore, since morphine has high affinity for μ opioid receptor (MOR), we wanted to examine the role of MOR in these processes. We extracted macrophages from MOR KO mice using previously mentioned procedures, and we treated primary peritoneal macrophages *in vitro* with morphine and TLR agonists. Absence of MOR abolished morphine's inhibitory effect on phagocytosis, as well as its stimulatory effect in presence of LPS, while LTA maintained the same inhibitory levels (**Figure 3.10**). Together these data indicate that morphine acts thorough the classical mu opioid receptor in order to regulate TLR4 mediated increase in phagocytosis.

MECHANISTIC STUDIES OF FcgR, TLR4 AND OPIOID RECEPTORS

TLR2 and TLR4 signaling differ in that TLR2 acts through a MyD88 dependant pathway while TLR4 can act through both MyD88 dependant and independent pathways (**Figure 1.7**). In order to examine differential signaling we wanted to see if MyD88 played a role in TLR modulation of FcgR phagocytosis and if these mechanisms were MyD88 dependent or independent.

MyD88 inhibitory peptide blocks MyD88 activation by inhibiting MyD88 homodimerization. MyD88 inhibitory peptide contains a protein transduction (PTD) sequence (DRQIKIWFQNRRMKWKK) (sequence used as the control peptide) derived from antennapedia which renders the peptide cell permeable. MyD88 inhibitor peptide (100μM), control peptide (100μM) and morphine (1μM) were added at the same time, and the J774 cells were incubated overnight. LPS (50ng/ml) was added the next day 4 hours prior to the OPDex bead incubation. MyD88 inhibitor abolished morphine+LPS induction of phagocytosis **(Figure 3.11)**. As expected, control peptide (PTD sequence) had no effect, and morphine mediated inhibition of phagocytosis was maintained in all the treatments where morphine was present (except morphine+LPS), indicating that morphine signaling was not affected by the MyD88 inhibitor peptide. These data show that morphine and LPS mediated induction of phagocytosis was mediated through a MyD88 dependent pathway.

MyD88 dependent pathway leads to ERK1/2 and p38 MAPK activation. Therefore we wanted to examine the role of these two MAPKs in the receptor crosstalk. ERK1/2 or p38 MAPK were inhibited 4 hours prior to morphine treatment. LPS and LTA were added 4 hours prior to addition of OPDex beads, cells were assayed for phagocytosis using fluorometric analysis. Inhibition of ERK1/2 via PD 98059, lead to inhibition of phagocytosis of both vehicle and morphine control in absence or presence

of LPS or LTA (**Figure 3.12**), indicating that ERK1/2, although important in phagocytosis does not play a role in TLR2, or TLR4 crosstalk with opioid receptors.

However inhibition of p38 MAPK via SB203580 prior to morphine and LPS treatment yielded interesting results. Inhibition of p38 MAPK abolished LPS effect on enhancement of phagocytosis in both vehicle and morphine treatments (**Figure 3.13**), indicating that p38 MAPK plays an important role not only in phagocytosis but in crosstalk between MOR, TLR4 and FcgR. Together these data indicate that opioid receptor activation in presence of TLR4 ligand enhances phagocytosis in a MyD88 dependant manner via a p38 MAPK signaling mechanism.

DISCUSSION

This study shows for the first time that morphine upregulates TLR4 expression thereby leading to increase in phagocytosis, through a Myd88 and p38 MAPK dependant pathway. Several lines of evidence support these findings. First that chronic morphine treatment alone inhibits, while in the presence of LPS, morphine potentiates FcgR mediated phagocytosis. Second, this effect is abolished in TLR4 KO but not in TLR2 KO mice, indicating that LPS is acting on the TLR4 receptor. Third, we show that morphine by upregulating TLR4 expression, in presence of LPS potentiates phagocytosis, and fourth that in presence of MyD88 and p38 MAPK antagonists this effect is abolished indicating the role of TLR4 activation of the MyD88 dependant pathway.

Morphine-mediated suppression of innate and adaptive immunity is manifested through high frequency of bacterial infections in opioid users and abusers. Macrophages play a key role in morphine mediated modulation of innate and adaptive immunity, and are essential in elimination of bacterial infections. Although many groups have researched morphine modulation of macrophage functions such as phagocytosis and

inflammation, to our knowledge there have been no studies examining morphine's effect on the crosstalk between the inflammatory TLR, phagocytic FcgR and opioid receptors. Due to the significant role that macrophages play in pathogen clearance as well as deleterious effects that occur as a result of disruption of macrophage function, we set out to explore this gap in knowledge and address mechanisms by which morphine modulates TLR signaling and FcgR mediated phagocytosis.

Our data shows that chronic morphine treatment alone inhibits FcgR mediated phagocytosis. However following LPS activation of TLR4, morphine leads to increased phagocytosis by J774 macrophages as well as by primary macrophages treated *in vitro* and *in vivo*. Morphine+LPS enhancement of phagocytosis is abolished in macrophages pretreated with naltrexone, as well as primary macrophages from TLR4 KO mice and MORKO mice. Data indicates that morphine+LPS increase in internalization is mediated by classical opioid receptors such as MOR as well as by the TLR4, and that expression and activation of both receptors is required for the enhancement of phagocytosis.

On the other hand, activation of TLR2 receptor by LTA leads to inhibition of phagocytosis bringing down the phagocytic index to same values as cells undergoing chronic morphine treatment. This was true for macrophage cell line J774 as well as *in vitro* treated macrophages from WT mice, while in primary macrophages from TLR2 KO mice as expected, this effect of LTA was abolished.

TLRs activate transcription of a large number of genes whose gene products are known to participate at all stages during phagocytosis, ranging from microbial recognition, actin cytoskeletal dynamics, membrane trafficking, ion transport, proteolysis, and antigen presentation (Underhill and Gantner, 2004; Hume et al., 2002). Our data shows that chronic morphine treatment by upregulating TLR4 surface expression, amplifies LPS mediated TLR4 activation when LPS is added in presence of morphine.

97

This was not surprising considering our group's early findings that morphine synergizes with LPS in a chronic endotoxemia model, where chronic morphine in presence of LPS enhanced mortality due to sepsis (Roy et al., 1999). Our group and others have explored LPS-induced inflammation and accelerated progression to septic shock seen with chronic morphine exposure. Morphine as been known to enhance LPS-induced leukocyte-endothelial adhesion, elevated IL-1beta, TNF-α, and IL-6 serum levels (Ocasio et al., 2004). Therefore it is not surprising that morphine amplifies TLR4 activation and through that mechanism enhances macrophage phagocytosis.

There has been some controversy in the literature as to LPS ability to activate both TLR2 and TLR4 (Kawai et al., 1999). Several groups report that LPS can activate TLR2, while others claim that LPS is a standard TLR4 ligand (Hirschfeld et al., 2000) Although, this controversy has not yet been fully resolved, our model confirms that LPS only activates TLR4. In our TLR-KO studies we note that in absence of TLR4 LPS and morphine+LPS amplification of phagocytic index is abolished while LTA and morphine maintained their inhibitory effect. In the absence of TLR2, morphine+LPS increase in phagocytosis was as robust as in WT, indicating that morphine+LPS had no effect on TLR2 activation.

Study by Doyle et al (2004) shows that TLR-mediated induction of phagocytic scavenger receptors occurs through MyD88, IRAK4, and p38 MAPK, and that activation of this pathway is essential for TLR promotion of phagocytosis. They suggest that this mechanism is evolutionarily conserved between mice and humans, suggesting that this pathway is a critical mediator of bacterial clearance by mammals after infection. Therefore we wanted to look at the role of MyD88, p38 MAPK and ERK1/2 MAPK in TLR4 and MOR mediated modulation of phagocytosis. Data shows that in presence of MyD88 homodimerization inhibitor peptide morphine+LPS increase in phagocytosis was

abolished and brought down to same levels of inhibition as morphine treatment alone. This indicated that MyD88 dependant pathway of TLR4 activation is essential in transducing signals necessary for amplification of phagocytosis. Same was true for p38 MAPK. In presence of p38 MAPK inhibitor SB203580, LPS mediated enhancement of phagocytosis was abolished, and the same was true for morphine+LPS. Our group's unpublished work (Dhas et al) offers further support for role of p38 MAPK in this process. Unpublished data shows with western blot analysis that p38 MAPK phosphorylation is increased in presence of LPS, and further enhanced in J774 macrophages treated with morphine+LPS. These findings correlate with our hypothesis that morphine by upregulating TLR4 in presence of LPS, through a MyD88 dependant pathway potentiates p38 MAPK phosphorylation which when inhibited abolishes LPS effect (summarized in **Figure 3.14**).

We also investigated the role of ERK1/2 MAPK as a potential effector in this process. Although ERK1/2 inhibition by PD 98059 was important for phagocytosis it did not play a role in LPS modulation of phagocytosis. Together these data indicate that chronic morphine treatment and TLR4 activation lead to enhancement of pathogen internalization by morphine mediated increase in TLR4 expression, MyD88, and p38 MAPK dependent signaling pathways.

Although chronic morphine treatment inhibits phagocytosis of Gram-positive and Gram-negative bacteria, morphine treatment leads to a greater inhibition of phagocytosis of Gram-positive bacteria. This is in part due to the fact that LPS, a component of Gram negative bacterial cell wall and a TLR4 ligand leads to increased phagocytosis in presence of morphine thereby increasing the internalization of the pathogen by activation of TLR4 which has been upregulated by morphine. On the other hand LTA, a component of Gram-positive bacteria, activates TLR2 leading to inhibition of

phagocytosis and maintaining morphine's inhibitory effect on pathogen internalization. In our studies comparing phagocytosis of heat killed, opsonized, Gram-positive and Gram-negative bacteria, since we claim that TLR4 activation in presence of morphine has a dramatic increase of phagocytosis, one would expect to see an increase of phagocytosis of Gram-negative bacteria and their high LPS content. However, in presence of morphine we see an inhibition of phagocytosis. This finding can be explained by the brief incubation time of cell and bacteria. Macrophages are exposed to HKO *E. coli* for maximum of 30 minutes which insufficient amount of time to induce the same effect as the 4 hour pretreatment with LPS. Physiologically one would expect to see an increase in phagocytosis since during an infection bacterial growth and macrophage phagocytosis are long-term processes which would resemble the effects we see in 4 hour pre incubation with LPS.

Although our study testifies to immune activation in presence of morphine and LPS, end result of this interaction would be increased sepsis and bacterial dissemination by macrophages which become Trojan horses overloaded with bacteria causing further dissemination through different tissues as seen in the study by (Hilburger et al., 1997).(Wang et al., 2005) On the other hand, interactions of morphine and LTA would cause inadequate clearance of gram positive bacteria and bacteremias as seen in our group's work (Wang et al., 2005).

Morphine mediated immunosuppression is a complex and important process. As opioid prescription pain relievers continue to be prescribed as the standard therapies for pain management, and as the opioid abuse continues to rise, it is important to further understand these mechanisms in order to identify targets necessary for development of better, less immunosuppressive pain management strategies.

FIGURE 3.1
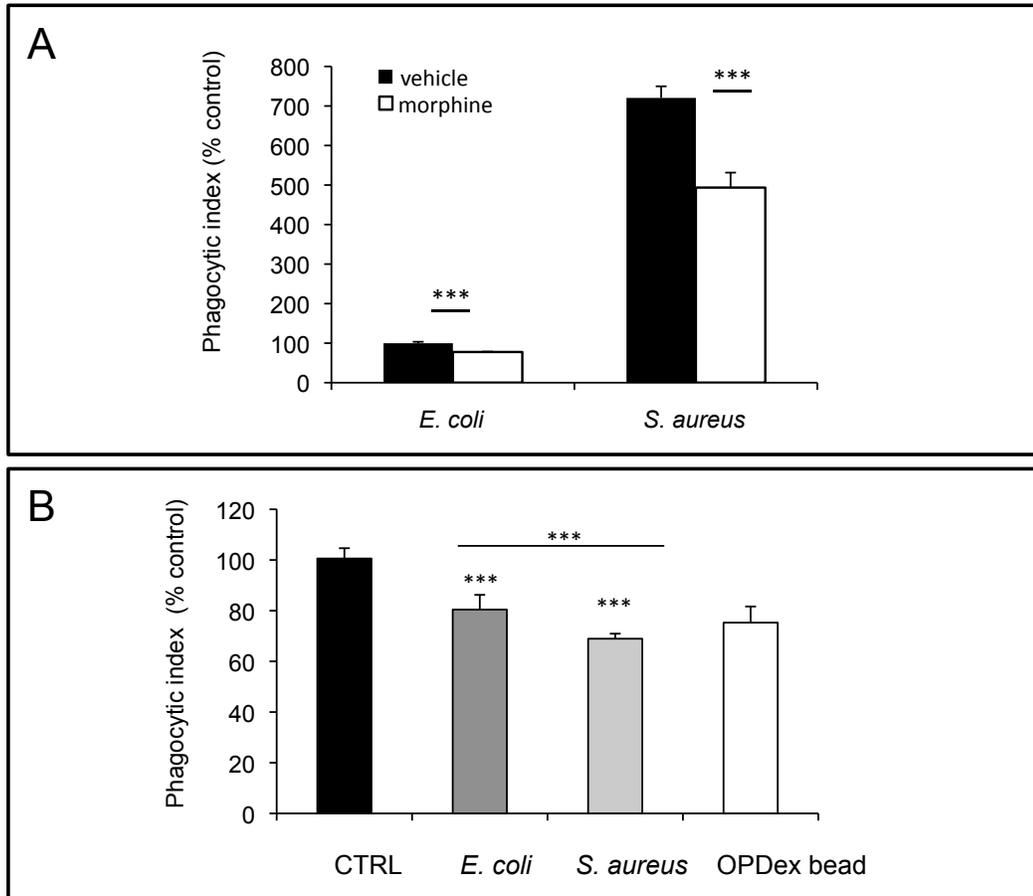
Figure 3.1. Morphine's differential effects on phagocytosis of Gram-positive and Gram-negative bacteria. (A) Phagocytosis of HKOP *E. coli* and HKOP *S. aureus* following chronic morphine treatment. Phagocytic index expressed as ratio of FITC/DAPI compared to vehicle control (E. coli). **(B)** Phagocytosis of opsonized HKOP E. coli, HKOP *S. aureus*, and OPDex bead following chronic morphine treatment. Phagocytic index illustrates phagocytosis by morphine treated macrophages compared to relative vehicle controls. Bars illustrate mean of three independent experiments $\pm$ SE (student's t-test ***p<0.001-significance to respective vehicle control)

FIGURE 3.2
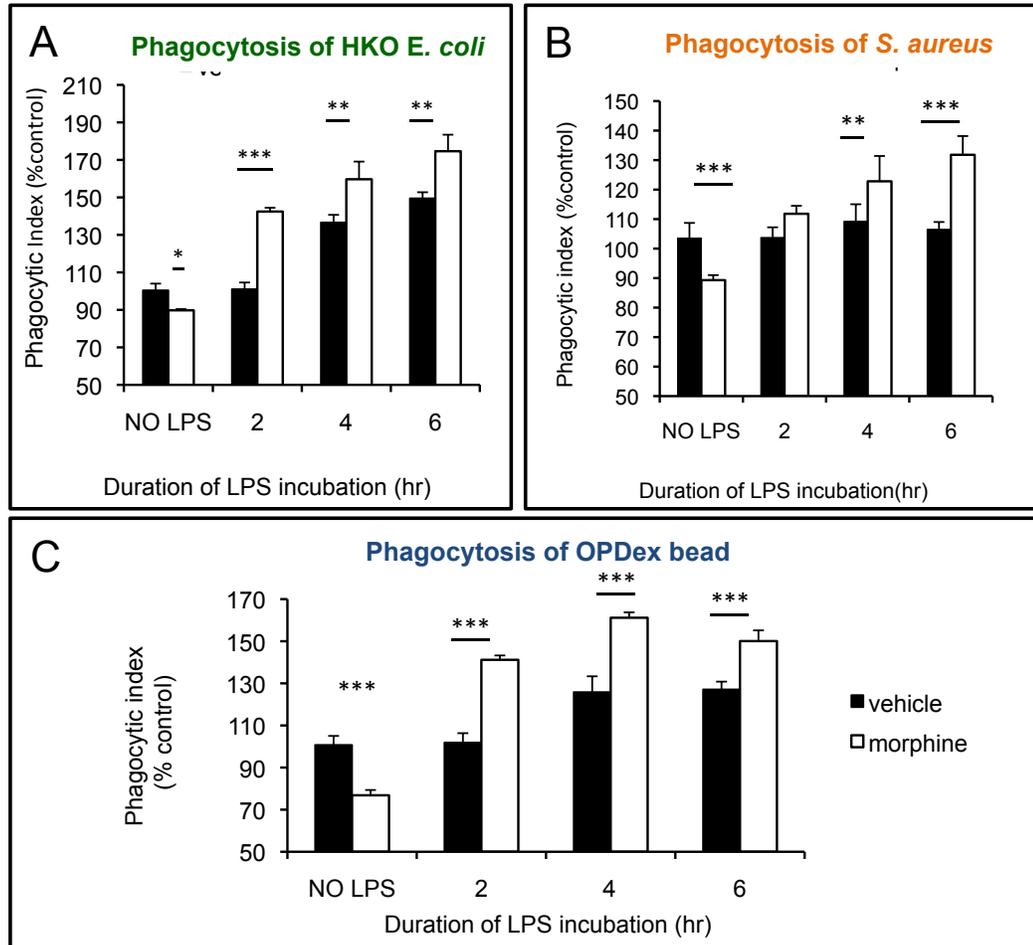
Figure 3.2. Morphine treatment increases phagocytosis following TLR-4 activation. After undergoing chronic morphine (1μM) treatment overnight Cells were treated with LPS (50 ng/ml) for 2,4 and 6 hours. Phagocytosis of HKOP *E. coli* **(A),** HKOP *S. aureus* **(B)** and OPDex bead **(C)** were conducted for 30 min. Macrophage phagocytioc ability was assesed using fluorometric analysis, and data was represented as Phagocytic index (RFU FITC/ RFU DAPI) % from vehicle control.

FIGURE 3.3
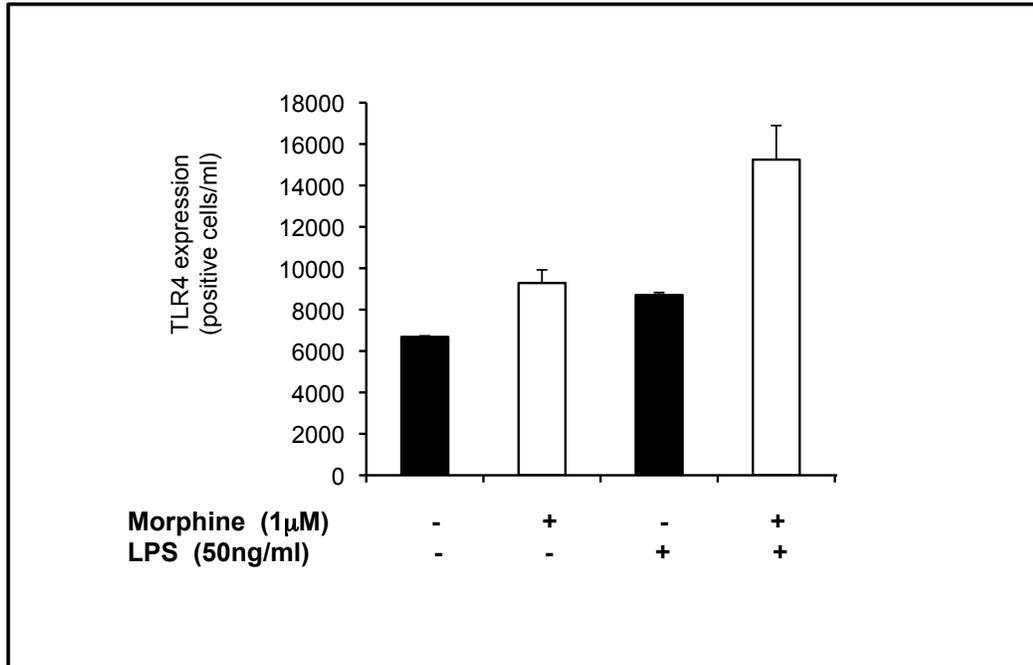
Figure 3.3. Morphine increase in TLR-4 expression is exacerbated by TLR-4 activation. After undergoing chronic morphine (1μM) treatment overnight cells were treated with LPS (50 ng/ml) 4 hours. Cells were collected, fixed and stained with PE conjugated TLR-4 antibody (BD Pharmigen; cat# 558294) and analyzed using FACS analysis. Data was collected using Guava EasyCyte and quantified using Guava cell plus software, and displayed in a bar graph.

FIGURE 3.4
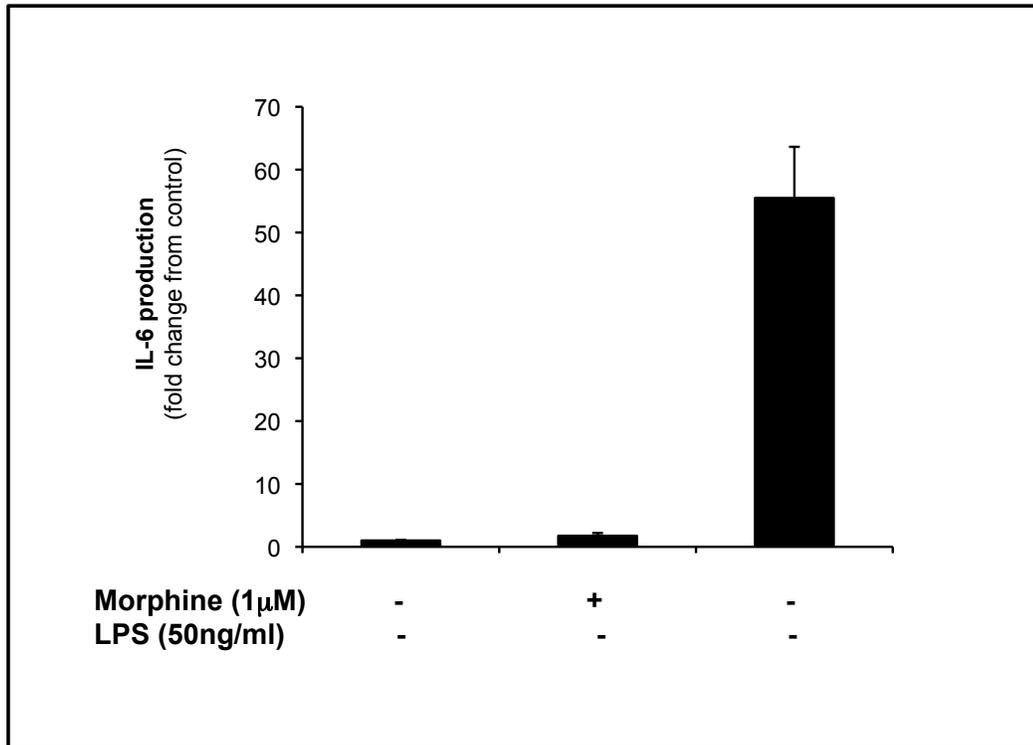
Figure 3.4. Morphine alone can not induce TLR4 activation. After undergoing chronic morphine (1μM) treatment overnight or 4 hour LPS (50 ng/ml). Supernant was collected, and analyzed via ELISA for IL-6 production.

FIGURE 3.5
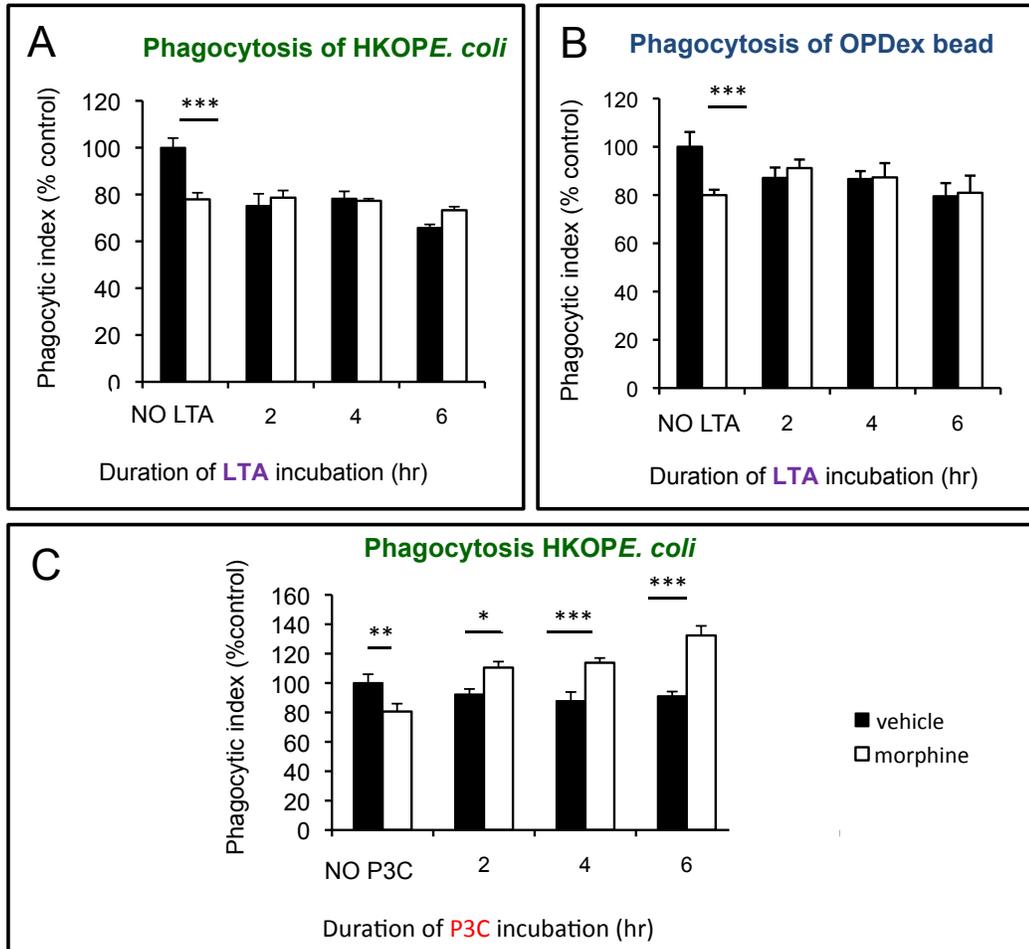
Figure 3.5. TLR-2 activation inhibits phagocytosis of gram positive bacteria more than gram negative. Figures **(A-C)** indicate fluorometric analysis of phagocytosis following overnight treatment with 1μM morphine, and 4hr incubation with LTA (10ng/μl) **(A, B)** or Pam3CSK4 (1μg/ml) **(C)** prior to addition of HKOP *E. coli* particles for 30 min **(A, C)** or OPDex for 60 min **(B)**. Significance was calculated using Student's t-test comparing significance of morphine to vehicle at a given treatment, or as indicated by the bar (*p<0.05, **p<0.01, ***p<0.0001)

FIGURE 3.6
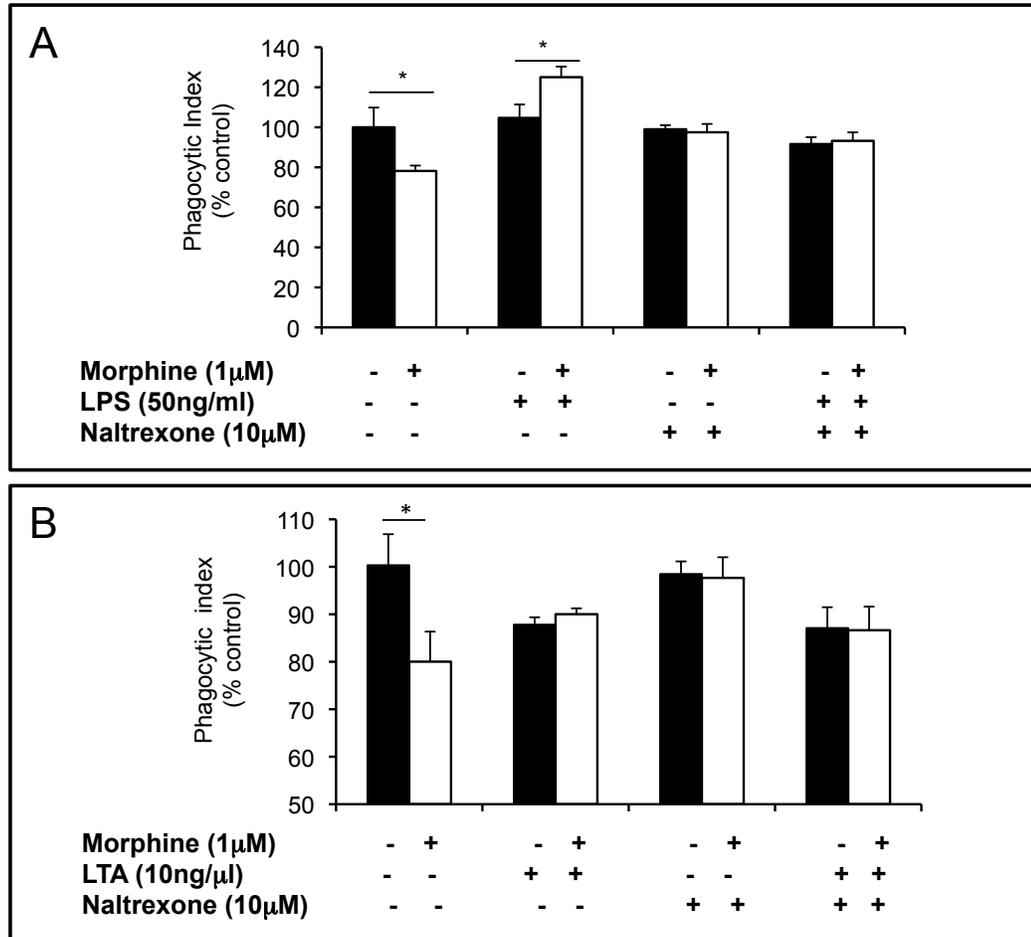
Figure 3.6. Modulation of phagocytosis following activation of TLR 4 and TLR 2 in morphine treated primary macrophages is naltrexone reversible. (A,B) shows fluorometric quantification of phagocytosis. Naltrexone (10μM) was added 2 hr prior to overnight morphine (1μM) incubation of primary macrophages form WT mice. LPS (50ng/ml) (A) and LTA (10ng/μl) (B) were added 4 hr prior to addition of OPDex beads. Phagocytosis was allowed for 60 min and cells were analyzed via fluorometric analysis and confocal microscopy (C). Phagocytic index was quantified as before. Data quantified as % from vehicle control, mean ± SE(***p<0.0001, *p<0.05)

FIGURE 3.6-C
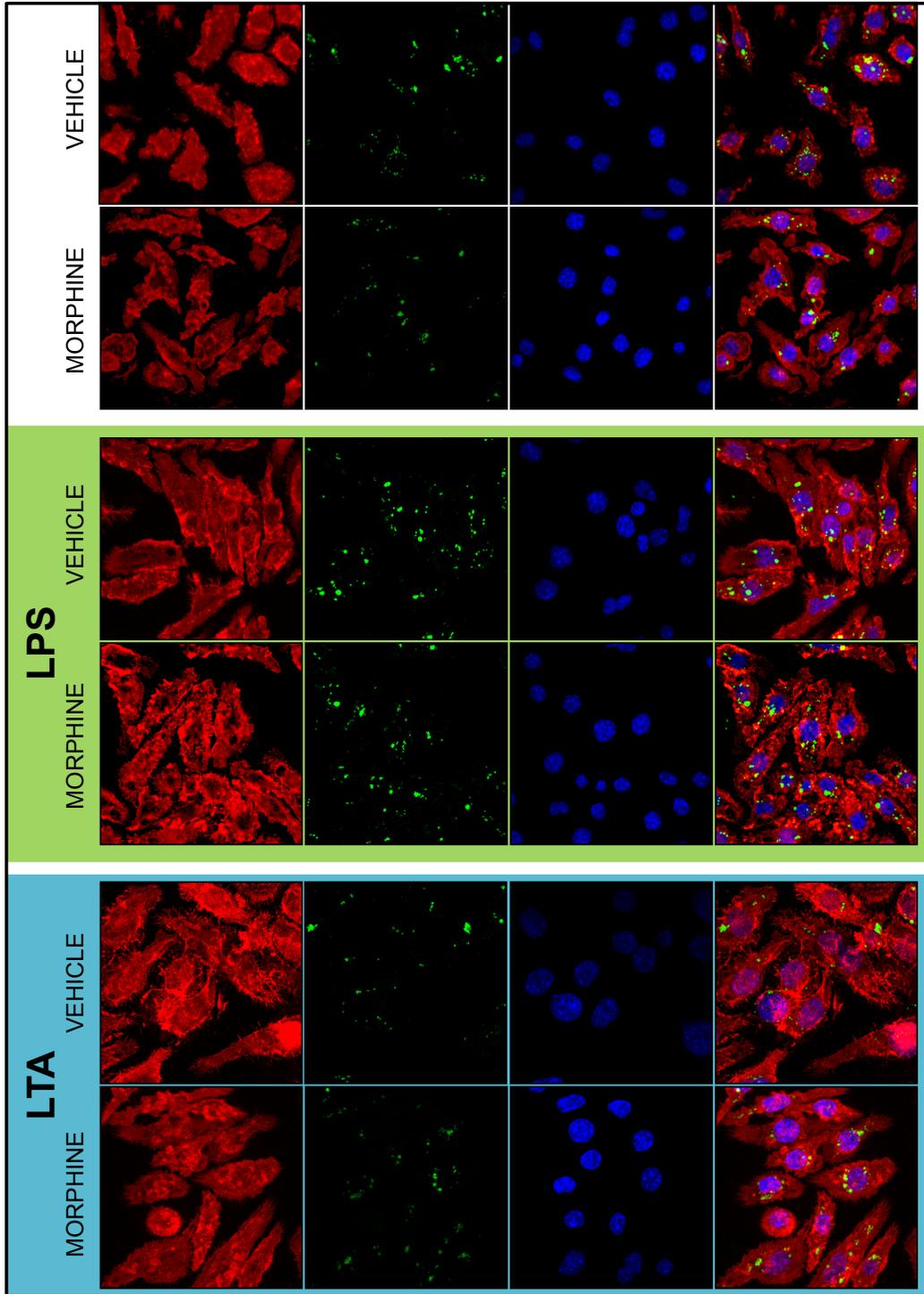
FIGURE 3.7
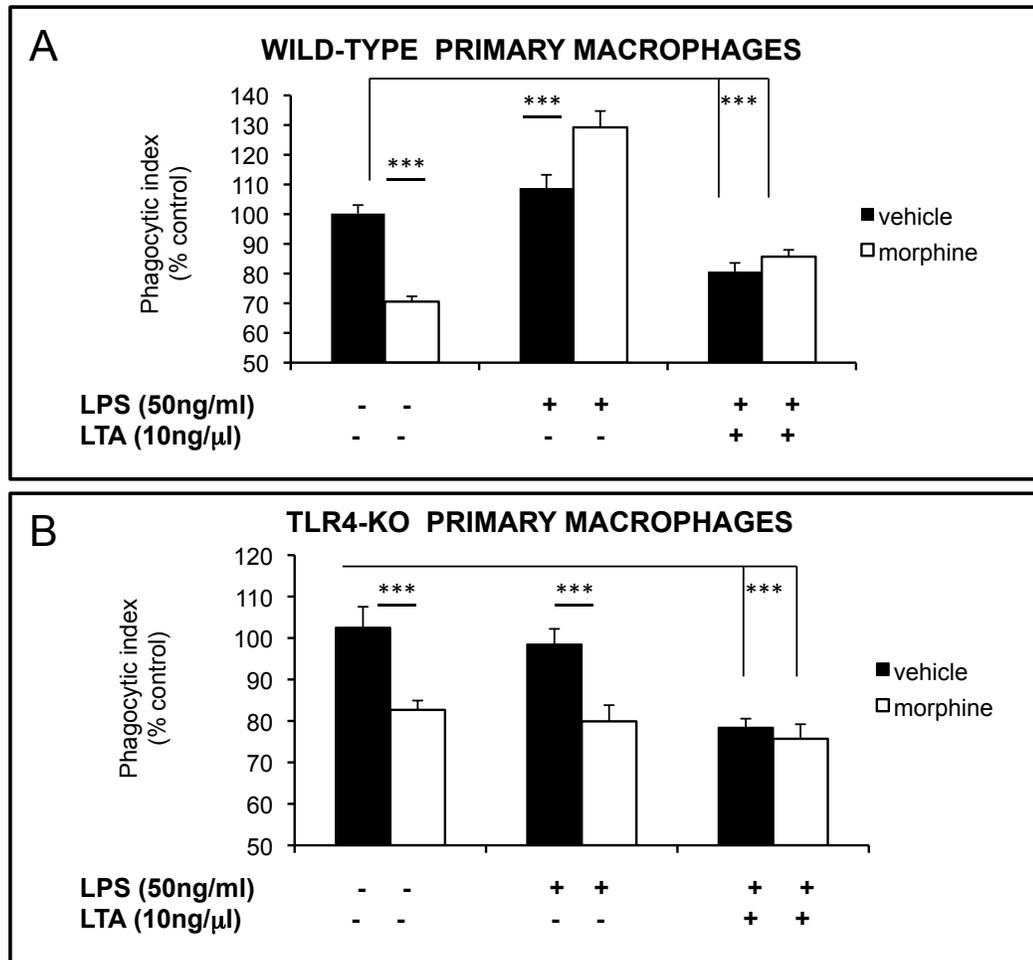
Figure 3.7. Effects of chronic morphine, and TLR4 and TLR2 ligands administered in vitro on phagocytosis are observed in primary peritoneal macrophages form WT mice but not in those of TLR4 KO mice. Fluorometric analysis of primary peritoneal macrophages extracted from wild type **(A)** and TLR4 KO **(B)**. Peritoneal cells were treated overnight with 1mM morphine, and with dextran particles for 60 min. Phagocytic index was quantified (RFU FITC/RFU DAPI) and expressed as % from vehicle control. Bars represent mean $\pm$ SE (*p<0.01,***p<0.0001).

FIGURE 3.8
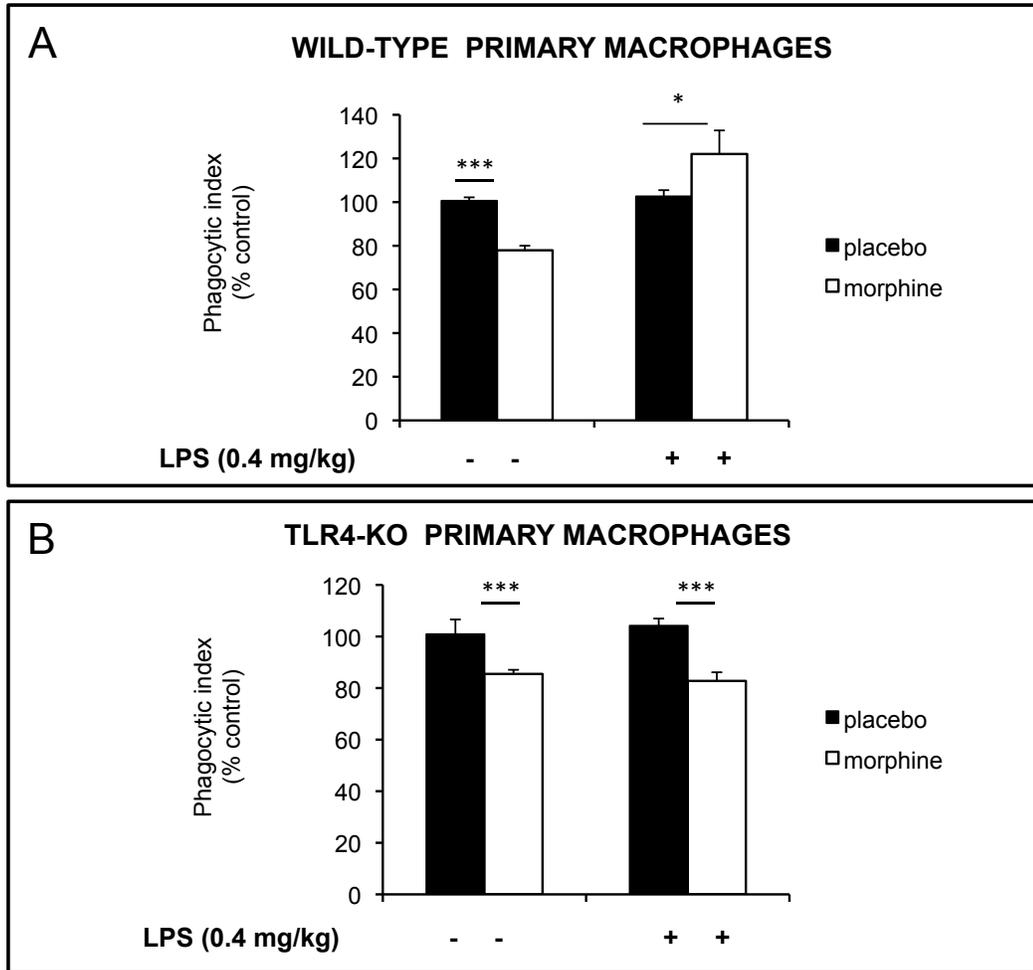
Figure 3.8. Effects of *in vivo* chronic morphine, and LPS treatment on phagocytosis are observed in primary peritoneal macrophages from WT mice but not in those of TLR4 KO mice. Fluorometric analysis of primary peritoneal macrophages extracted from wild type **(A)** and TLR4 KO **(B)** following three day morphine treatment and 6 hr LPS treatment in vivo. Cells were collected, and allowed to phagocytose OPDex for 60 min. PI was quantified (RFU FITC/RFU DAPI) and expressed as % from vehicle control. Bars represent mean $\pm$ SE (*p<0.01,***p<0.0001).

FIGURE 3.9
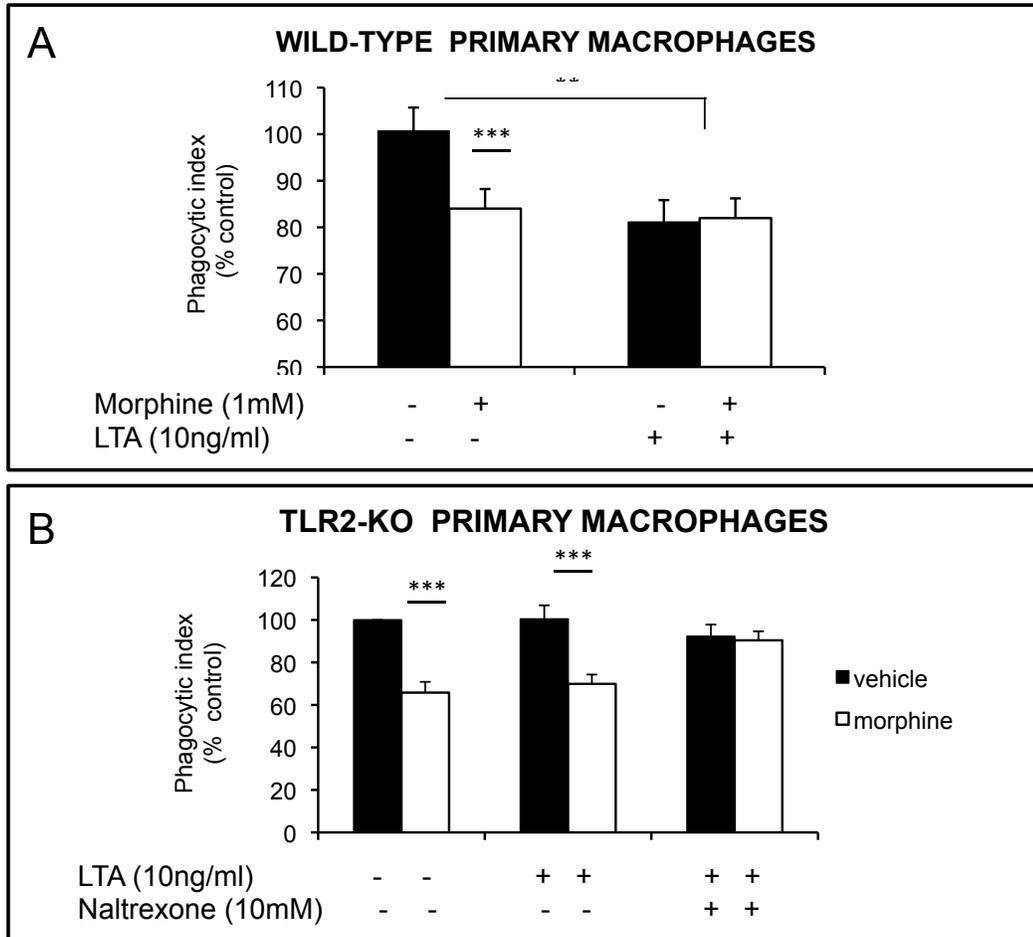
Figure 3.9 Effects of *in vivo* chronic morphine, and LTA treatment on phagocytosis are observed in primary peritoneal macrophages form WT mice but not in those of TLR2-KO mice. Fluorometric analysis of primary peritoneal macrophages extracted from wild type **(A)** and TLR2-KO **(B).** Peritoneal cells were treated overnight with 1μM morphine, and with OPDex particles for 60 min. Phagocytic index was quantified (RFU FITC/RFU DAPI) and expressed as % from vehicle control. Bars represent mean ± SE (*p<0.01,***p<0.0001).

110

FIGURE 3.10

MORKO PRIMARY MACROPHAGES

Morphine	-	+	-	+	-	+
LPS (50ng/ml)	-	-	+	+	+	+
LTA (10ng/µl)	-	-	-	-	+	+
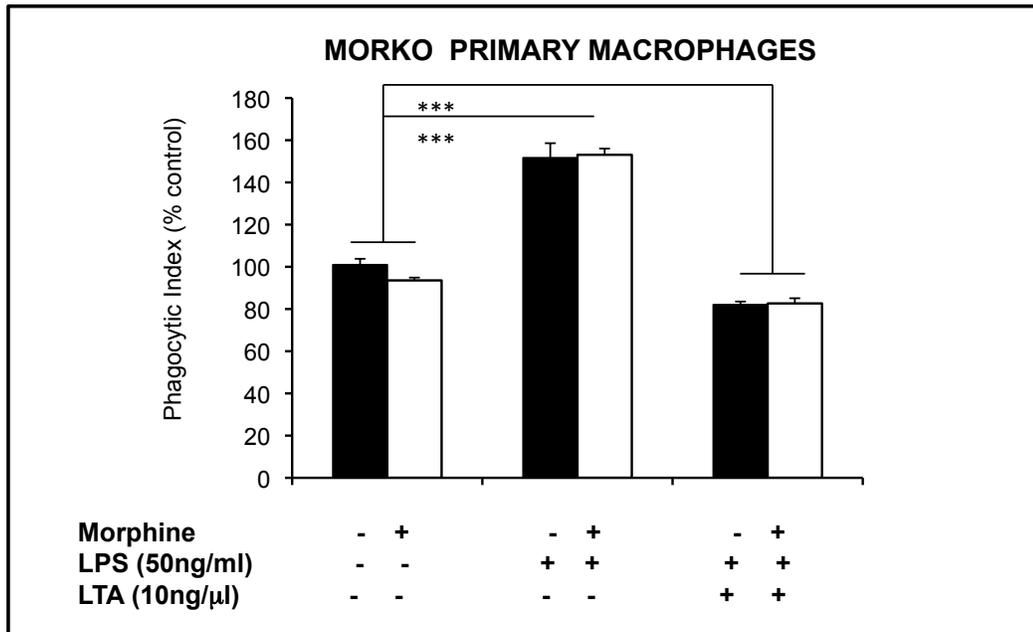
Figure 3.10. Effects of chronic morphine is abolished in primary peritoneal macrophages form MORKO mice while TLR4 and TLR2 ligands maintain their effect. Fluorometric analysis of primary peritoneal macrophages extracted from MORKO mice. Peritoneal cells were treated overnight with 1µM morphine, LPS an LTA (for 4 hours prior to dextran) and with OPDex beads for 60 min. Phagocytic index was quantified (RFU FITC/RFU DAPI) and expressed as % from vehicle control. Bars represent mean ± SE (*p<0.01,***p<0.0001).

FIGURE 3.11
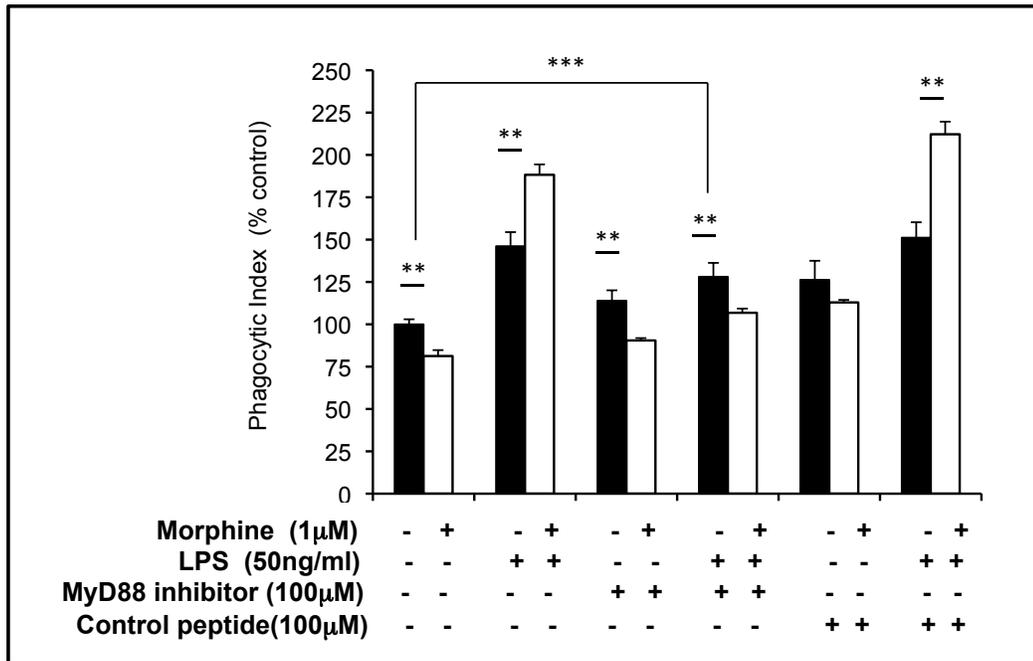
Figure 3.11. Morphine signaling crosstalk with TLR4 receptors is mediated through a MyD88 dependant pathway. Prior to undergoing chronic morphine (1μM) treatment overnight, cells were treated for 4 hours with PD 98059 (a ERK1/2 inhibitor). After morphine treatment cells were treated with LPS (50 ng/ml) and LTA (10 ng/μl) for 4 hours. Cells were collected, and analyzed for phagocytosis using fluorometric analysis.

FIGURE 3.12
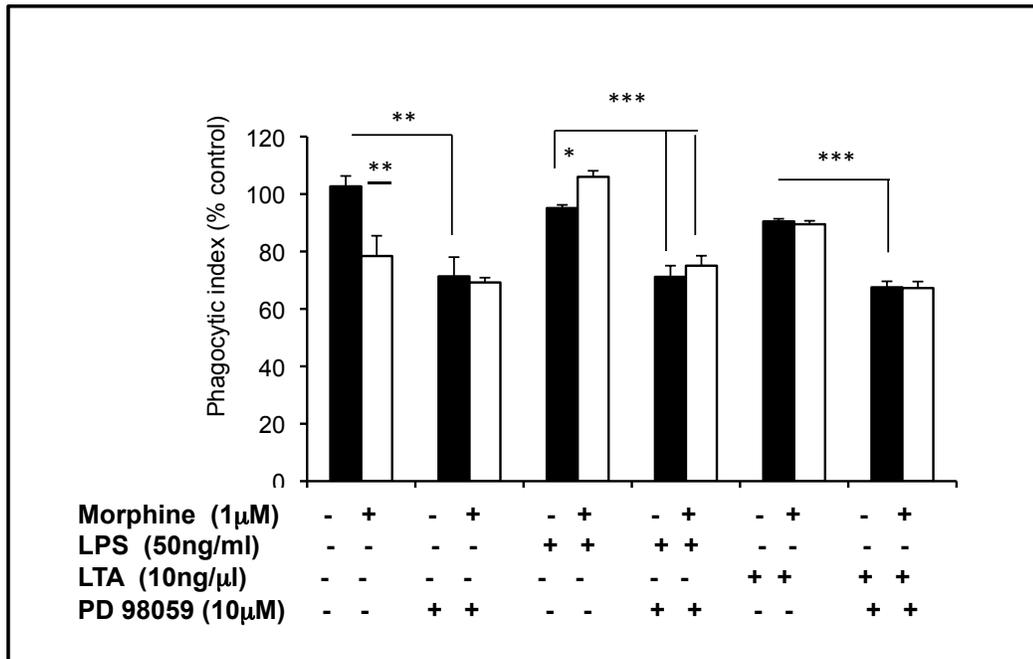
Figure 3.12. Morphine signaling crosstalk with TLR4 and TLR2 receptors is not mediated through ERK1/2 MAPK. Prior to undergoing chronic morphine (1µM) treatment overnight, cells were treated for 4 hours with PD 98059 (a ERK1/2 inhibitor). After morphine treatment cells were treated with LPS (50 ng/ml) and LTA (10 ng/µl) for 4 hours. Cells were collected, and analyzed for phagocytosis using fluorometric analysis.

FIGURE 3.13
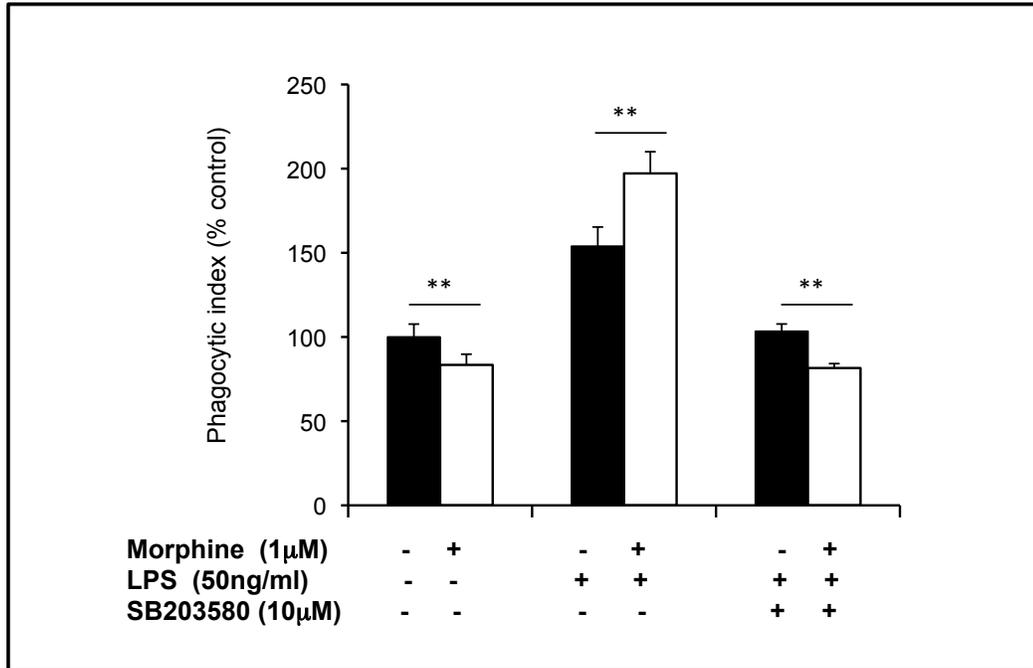
Figure 3.13. Morphine signaling crosstalk with TLR4 receptors is mediated through p38 MAPK. Prior to undergoing chronic morphine (1μM) treatment overnight, cells were treated for 4 hours with SB203580 (a p38 MAPK inhibitor). After morphine treatment cells were treated with LPS (50 ng/ml) and LTA (10 ng/μl) for 4 hours. Cells were collected, and analyzed for phagocytosis using fluorometric analysis.

FIGURE 3.14.
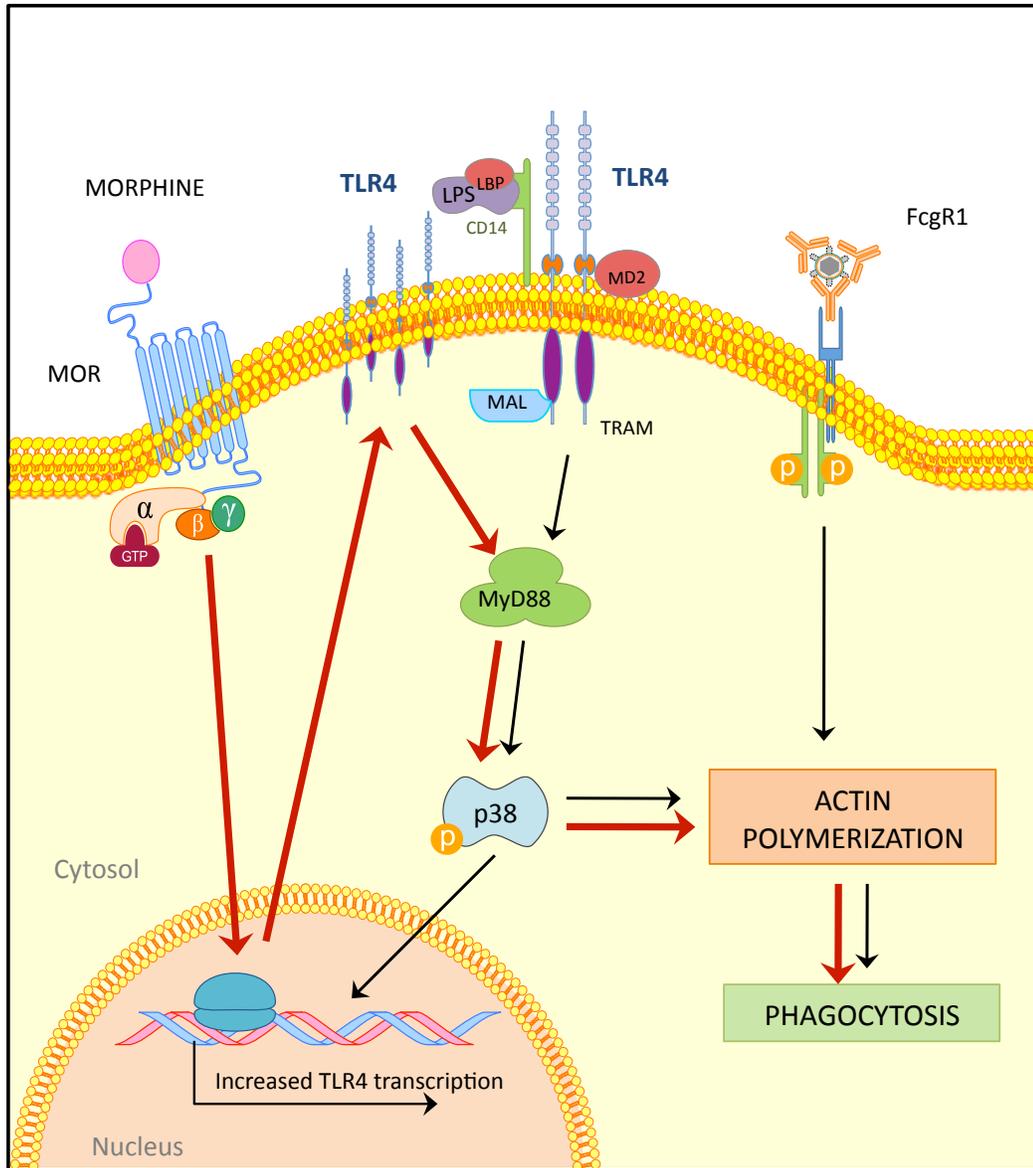
Figure 3.14. Mechanisms of TLR modulation of phagocytosis.

TLR4 can act through p38 to modulate phagocytosis. Morphine increases TLR4 expression, LPS mediated TLR4 activation increases p38 phosphorylation thereby enhancing actin polymerization and phagocytosis. Red arrow indicates morphine action in presence of LPS.

CHAPTER 4.

MORPHINE INHIBITS MACROPHAGE BACTERICIDAL MECHANISMS

INTRODUCTION

Opiate use and abuse has been known to increase frequency and severity of bacterial infections (Boschini et al., 1996). Prolonged opioid use increases risk of respiratory tract infections such as pneumococcal pneumoniae (Boschini et al., 1996). Bacterial pneumonia and pneumococcal disease are strongly associated with HIV infection and drug abuse, where opioid users are at 10 fold greater risk than the non abusers (Tumbarello et al., 1998; Feldman, 2005; Klugman et al., 2007). In addition to increasing the risk of infection, chronic morphine administration in animal models has been shown to cause bacterial sepsis and increased bacterial loads in liver, spleen and peritoneal cavities (Hilburger et al., 1997). Studies from our lab clearly demonstrate morphine treatment leading to markedly increased bacterial burden in the lung spleen and blood of mice following infection with *Streptococcus pneumoniae* resulting in subsequent increase in mortality (Wang et al., 2005). Morphine has been implicated in immune suppression causing inadequate immune defenses and exacerbating bacterial and viral infections. Studies have shown that chronic morphine impairs a number of innate immune functions, including the expression of chemokine and proinflammatory cytokines, leukocyte migration, phagocytosis and bacterial killing (Rojavin et al., 1993b; Szabo et al., 1993; Holan et al., 2003).

Since macrophages play an important role as the first line of defense against microbial invasion through their phagocytic, cytotoxic, and inflammatory activities, we decided to focus our studies on mechanisms underlying macrophage's bactericidal ability. Macrophage's effectiveness in killing microbes hinges on its capability to internalize or phagocytose. Our group and others implicates morphine in inhibition of phagocytosis, however increased bacterial loads and dissemination indicate that morphine potentially might be inhibiting macrophage bactericidal mechanisms as well. Therefore we set out to examine if and how morphine modulates macrophage bactericidal mechanism.

Phago-lysosomal fusion is one of essential mechanisms involved in bacterial killing. As the pathogen is internalized, and as the phagosome matures it binds to a highly acidic lysosome. Lysosomes are used for digestion of materials internalized through phagocytosis, endocytosis or autophagy (Zurier et al., 1973). During the phago-lysosomal formation lysosome empties it's acidic contents along with lysosomal enzymes into the phagosome leading to bacterial killing (**Figure 1.5**). Lysosomal enzymes within the phagosome function at acidic pH of 4.5, however with the increase in pH in alkaline environment of the cytosol, their function is impaired. This protective mechanism is often utilized by bacteria to evade killing by lysosomal enzymes (Tapper and Sundler, 1990), and therefore could be one of mechanisms used by morphine to inhibit bacterial killing.

In addition to lysosomal fusion, phagosomal compartment generates reactive oxygen intermediates (ROI) such hydrogen peroxide (H_2O_2), superoxide anion (O_2-), and hydroxyl radical (OH) (Gamaley et al., 1994; Gamaley et al., 1994; Caron and Hall, 1998; Bokoch, 2000; Segal et al., 2000). These molecules are essential to macrophage bactericidal ability and they are produced by nicotinamide adenine dinucleotide

phosphate (NADPH)-oxidase complex. It is multimolecular complex, which assembles at the plasma membrane upon stimulation by bacterial pathogens or other signals. NADPH oxidase consists of two integral membrane proteins, gp91phox and p21phox, and two cytosolic proteins, p67phox and p47phox, as well as Rho-GTPases Rac 1 and Rac 2. Upon stimulation of phagocytic cells Rac 2 recruits the complex to the membrane while Rac 1 interacts with p67phox leading to activation of NADPH-oxidase (Diekmann et al., 1994; Knaus et al., 1991). NADPH-oxidase is an electron transport chain found in the wall of endocytic and phagocytic vacuoles, which leads to reduction of NADPH to NADP and production of ROIs.

In addition to ROI, phagosomes are capable of generating reactive nitrogen intermediates (RNIs) such as NO. Although NO is a short-lived, gaseous radical it has an essential role in microbial killing (Bogdan, 2001). NO can damage DNA and several chemical moieties on which microbe's propagation and protection depend. Inducible nitric oxide synthase (iNOS) utilizes production of superoxide (O_2^-) and nitric oxide (NO) in nearly equimolar amounts and thus can be a prolific generator of their joint and particularly destructive product, peroxynitrite ($OONO^-$)(Nathan and Shiloh, 2000).

Morphine has been implicated in inhibition of superoxide production and NO production. First studies of morphine's effect on infection examined superoxide release where it was noticed by several groups that morphine inhibits superoxide production in neutrophils and macrophages (Sharp et al., 1985; Simpkins et al., 1986; Welters et al., 2000). Early studies found that pretreatment with endogenous opioid peptides leucine or methionine enkephalins reduce neutrophil's ability to generate superoxide production in response to *Escherichia coli* product, N-formyl methionyl leucyl phenylalanine (FMLP) (Sharp et al., 1985; Simpkins et al., 1986). Morphine mediated suppression of superoxide production was further confirmed in human peripheral mononuclear cells by

studies such as those by Peterson et al which examined respiratory burst activity in response to phorbol myristate acetate (PMA) (Peterson et al., 1987; Peterson et al., 1989). In addition, morphine concentrations in micromolar range led to augmented intramacrophage parasite growth (Singh and Singal, 2007). In mice, chronic morphine has been shown to modulate bacterial killing by inhibition of NO release *in vivo* and *in vitro* (Menzebach et al., 2004; Bhaskaran et al., 2007; Fecho et al., 1994; Pacifici et al., 1995)

Our previous data and literature indicate that morphine abuse leads to inhibition of phagocytosis and that following TLR4 and TLR2 activation morphine differentially modulates internalization of pathogens. Morphine has been shown to inhibit macrophage phagocytosis and oxidative burst activity; however no studies thus far have examined the mechanisms underlying morphine modulation of macrophage bactericidal ability (Menzebach et al., 2004; Tomassini et al., 2004). Since morphine inhibits phagocytosis and since bacterial clearance requires internalization as well as pathogen elimination, the aim of this study was to examine effects of chronic morphine treatment on macrophage bacterial killing of Gram-positive and Gram-negative bacteria, as well as mechanisms underlying this modulation. Specifically, studies were designed to investigate how morphine modulates bactericidal ability by looking at key mechanisms involved in this process such as phago-lysosomal fusion, formation of reactive oxygen intermediates (ROI), as well as formation of reactive nitrogen intermediates (RNI). Overall, our results reveal for the first time that morphine modulates mechanisms involved in bacterial killing of Gram-positive *S. pneumoniae* more dramatically than Gram-negative *E. coli*. Morphine leads to greater inhibition of phago-lysosomal fusion and maturation as well as superoxide production following Gram-positive bacterial infection.

METHODS

BACTERICIDAL ASSAY

J774 macrophage cells were plated in 6 well plates at 200,000 cells per well, in supplemented (antibiotic free) DMEM media. Cells were pretreated with naltrexone (10 μM) for two hours prior to overnight morphine (1μM) treatment. Second day cells were washed and media was replaced with antibiotic free, supplemented DMEM media. Bacteria were grown to log phase and added in 1:20 cell : bacteria ratio. After 60 minutes of bacterial incubation cells were washed in PBS and supplemented DMEM containing 1% penicillin/streptomycin, was added to the cells, which were then incubated overnight to resume with killing of internalized bacteria. Third day, cells were washed, scraped and plated on bacterial growth plates (LB for *E. coli*, and Blood agar plates for *S. pneumoniae*)

NO ASSAY

Cells were plated in 6 well plates at 200,000 cells per well, in supplemented (antibiotic free) DMEM media, treated with naltrexone (10μM) for 2hr followed by overnight morphine treatment (1μM). Next day, bacteria in log growth phase were added (1:20; cell : bacteria ratio) for 60 minutes, cells were washed and 1ml of fresh media (containing corresponding treatments) was added. Cells were allowed to incubate for 6 hours, after which supernant was collected and analyzed for nitrite release using Greiss reagent. Reagent was added in 1:1 ratio (50ul of sample, 50ul of reagent), incubated for 15 minutes and absorbance was measured using a BMG fluostar omega plate reader (OD 540).

ROI ASSAY AND PHAGO-LYSOSOMAL ASSAY

Cells were plated in chamber slides at 50,000cells/ chamber, and treated as mentioned above. Before the bacterial treatment cells were incubated with CFDA (10μM) or Lysotracker-red (60nM) for 1 hour. Bacteria were added for 30 and 45 minutes after which, cells were washed, fixed with 4% paraformaldehyde, and stained with DAPI. Data was collected via fluorometric of microscopic analysis

MICROSCOPIC ANALYSIS AND COLOCALIZATION

Images were acquired using Nikon inverted confocal microscope (model: Ti-E eclipse 100) and Roper camera (model: Cool-snap HQQ) at 60x with additional digital magnification. Fluorescent images were analyzed for pixel density using image J or for fluorescence colocalization using EZ-C1 3.90 Nikon software. Pearson's quotient was used as measure of colocalization, and was generated as a numerical value indicating overlay between the green (bacteria) and red (lysosome) laser channels.

FLUOROMETRIC ANALYSIS

Cells were plated in 96-well plates at 10,000/well and treated as above (see ROI assay). After bacterial incubation cells were washed, fixed with 4% paraformaldehyde and stained with DAPI. Fluorescence was recorded using a fluorescence plate reader (BMG; FLUOstar Omega) at ex485/em520 (FITC), and ex355/em460 (DAPI). ROI production was quantified as FITC (Relative Fluorescence Units (RFU)) / DAPI(RFU), indicative of ROI released /cell. The y-axis was normalized to phagocytic index of the control (defined as vehicle treatment at 30 minutes bacterial incubation) and data was expressed as percentage of vehicle control.

NADPH OXIDASE ACTIVITY

NADPH-oxidase activity was assayed using Fluorescent NADP/NADPH Detection Kit (Cell Technology cat# 100-2), according to manufacturer's protocol. Briefly NADPH is oxidized to NADP by NADPH-oxidase in order to generate ROI. Fluoro NADP/NADPH detection kit utilizes a non-fluorescent detection reagent, which is oxidized in presence of NADPH to produce its fluorescent analog NADP. NADP is further converted to NADPH via an enzyme-coupled reaction. The enzyme reaction specifically reacts with NADP/NADPH. Two sets of samples are prepared, one examining NADP+NADPH content, and another examined NADPH content. NADP values are obtained by the subtracting NADPH values from NADP/NADPH values, indicating concentration of NADP and therefore activity of NADPH oxidase.

<u>RESULTS</u>

MORPHINE INHIBITS BACTERIAL KILLING OF E. COLI AND S. PNEUMONIAE

Morphine use leads to increased susceptibility to infections, as well as impaired ability to fight of such infections, which is manifested, by bacteremia and sepsis in opioid users. Previous studies done by our group indicate that morphine differentially modulates phagocytosis in presence of Gram-positive or Gram-negative ligands. Since morphine together with LPS (component Gram–negative bacteria) a TLR4 ligand, increased phagocytosis, while morphine and LTA (TLR2 ligand from Gram-positive bacteria) decreased phagocytosis, we set out to examine if morphine would have

differential effects on bacterial killing of Gram-positive *S. pneumoniae*, and Gram-negative *E. coli*.

In order to examine morphine's effect on overall macrophage bactericidal ability we utilized "Bactericidal assay" (see methods for details). Briefly, J774 macrophage cells were pretreated with naltrexone 10μM, two hours before undergoing overnight chronic morphine treatment (1μM). Next day, macrophages were allowed to phagocytose for 60 min either *E. coli* or *S. pneumonia* (in 1:20, cell : bacteria ratio), and were allowed to kill internalized bacteria for 24 hours. Cells were collected and quantified for bacterial loads, after being cultured on Petri Dishes containing bacteria nutrient agar (Lauria Broth–*E. coli*; Blood agar plates–*S. pneumoniae*). Following 24 hours of incubation bacterial colonies were counted and data was expressed as colony forming units (CFU/ml). Our results indicate that chronic morphine treatment inhibits bacterial killing as illustrated by increased bacterial loads of *E. coli* **(Figure 4.1A)** and *S. pneumoniae* **(Figure 4.1B).** There was a slight although significant increase in bacterial loads of morphine treated cells incubated with *S. pneumonia* compared to those with *E. coil*, indicating that morphine has greater inhibitory effects on bacterial killing of Gram-positive bacteria. Naltrexone pretreatment was able to fully abolish morphine's inhibitory effect **(Figure 4.1)** therefore indicating that inhibition of bacterial killing by morphine is mediated by the classic opioid receptors. Additionally, when cells were pretreated with LPS and LTA (TLR4 or TLR2 ligands respectively) for 4 hours prior to bacterial exposure, inhibition of macrophage bactericidal ability was maintained in presence of LTA while it was abolished in presence of LPS (**Figure 4.2**).

Together these data show that morphine inhibits bacterial killing of Gram-positive and Gram-negative bacteria. Morphine also leads to differential levels of inhibition of bacterial killing in presence of TLR ligands. As TLR4 activation by LPS following chronic

123

morphine treatment decreases bacterial loads by enhancing bactericidal ability, TLR2 activation by LTA in presence of morphine maintains high bacterial loads and inhibition of killing.

MORPHINE INHIBITS NITRIC OXIDE RELEASE

In order to further investigate mechanisms of morphine's inhibition of macrophage bactericidal ability we studied release of reactive nitrogen intermediates (RNI). Studies by several groups noted that morphine inhibits NO production by *in vitro* treated macrophages as well as *in vivo* morphine treated mice (Fecho et al., 1994; Pacifici et al., 1995). RNI are produced following induction of inducible nitric oxide synthase (iNOS), leading to nitric oxide (NO) production. We examined NO release using Greiss reagent **(Figure 4.3),** a standard technique monitoring NO production by measuring nitrite (NO_2^-), which is one of two primary stable and nonvolatile breakdown products of nitric oxide. Data show that morphine inhibits nitrite production indicative of inhibition of NO production by J774 macrophages (**Figure 4.3**). Naltrexone pretreatment abolished inhibition of NO by chronic morphine treatment (**Figure 4.3**) indicating involvement of classical opioid receptors in this process. In addition, there was no observed difference in NO release following *S. pneumoniae* versus *E. coli* incubation.

These data suggest that although morphine inhibits NO release via activation of the classical opioid receptors, morphine has no differential effect on production of NO following exposure to Gram-positive and Gram-negative bacteria.

MORPHINE INHIBITS RELEASE OF REACTIVE OXYGEN INTERMEDIATES

In order to further study morphine's effect on differential modulation of mechanisms involved in bacterial killing we examined morphine's effect on release of

reactive oxygen intermediates (ROIs). ROIs have an essential role in bacterial clearance. As the phagosome forms, NADPH-oxidase complex is recruited and activated, resulting in production of reactive oxygen intermediates such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH), which area part of a potent system capable of killing both bacteria and viruses.

We utilized CFDA in order to monitor ROI production by J774 macrophages. Following previously described protocol, cells were treated with naltrexone and morphine, while CFDA was added 1hour prior to bacterial treatment (see "ROI assay"). Cells were fixed and analyzed via fluorescence microscopy or fluorometric analysis. As expected, microscopic analysis indicated that ROI production (shown by CFDA green fluorescence), increased with time in the vehicle control but remained inhibited in morphine treated cells following *S. pneumoniae* (**Figure 4.4A**) and *E. coli* exposure (**Figure 4.4B**). In addition, ROI release was greater in cells exposed to *S. pneumoniae*, when compared to cells exposed to *E. coli*, while in both cases morphine effect was abolished in presence of naltrexone. These results were further confirmed following quantification of acquired images by pixel density of green fluorescence (via Image J software). Pixel density confirms that morphine inhibits ROI production following exposure to *S. pneumoniae* **(Figure 4.4C)** and *E. coli* **(Figure 4.4D)** in a naltrexone reversible manner. Same was observed in cells quantified using fluorometric analysis (**Figure 4.5**). CFDA – FITC fluorescence was quantified and compared to DAPI fluorescence indicative of ROI production (ROI/cell).

NADPH-oxidase is the major generator of ROIs in macrophages. By oxidizing NDAPH to NADP, this enzyme generates oxygen intermediates. Therefore, we examined morphine's effect on NADPH oxidase activity as measured by NADP production. After J774 macrophages were exposed to previously described treatments,

cells were collected and analyzed using NADP/NADPH detection kit (**Figure 4.6**). We note that morphine treatment decreased production of NADP. Low NADP levels indicate low NADPH-oxidase function, thereby illustrating morphine's inhibition of NADPH-oxidase. Furthermore, morphine mediated inhibition of NADPH was abolished in presence of naltrexone, indicating involvement of classical opioid receptors.

Taken together, these data indicate that morphine inhibits release of reactive oxygen intermediates in a naltrexone reversible manner, by inhibiting NADPH-oxidase function. Furthermore morphine mediated inhibition of ROIs was more exacerbated in cells incubated with Gram-positive *S. pneumonia*e, when compared to cells incubated with Gram-negative *E. coli* as confirmed by microscopic and fluorometric analysis.

MORPHINE INHIBITS PHAGO-LYSOSMAL MATURATION

Another mechanism by which macrophages kill bacteria and prevent further dissemination is phago-lysosomal fusion. As phagocytosis takes place phagosome forms and further matures by fusing with a highly acidic lysosomal compartment, thereby changing the phagosomal acidity and leading to destruction of the pathogen. In order to study this process we utilized Lysotracker-red, a red fluorescent dye which stains acidic lysosomal compartments.

Following naltrexone pretreatment and overnight morphine treatment, cells were treated with Lysotracker-red (60 nM) or 1 hour prior to addition of bacteria. After 30 and 45 minutes bacterial treatment cells were fixed and analyzed by microscopic analysis. We note that number of acidic vacuoles increases over time in both vehicle and morphine treated cells. However, morphine leads to reduction in number and intensity of acidic vesicles when compared to vehicle control **(Figure 4.7).** This decrease of acidic vacuoles is indicative of inhibition of phago-lysosomal fusion. Cells exposed to *S.*

pneumoniae (**Figure 4.7 A)** had a greater increase in phago-lysosomal formation as well as greater inhibition by morphine than those exposed to E. coli (**Figure 4.7 B)**. This was further confirmed by analysis of pixel density where we see that morphine's inhibition of acidic vesicles was obvious in cells treated with *S. pneumoniae* (**Figure 4.7C**) and *E. coli* (**Figure 4.7D**). As expected morphine's inhibitory effects were abolished in presence of naltrexone **(Figure 4.7 A,C)**, indicating that morphine acts through a classical opioid receptor pathway to exert its inhibitory effect on phago-lysosomal fusion.

Further evidence of morphine's inhibition of phago-lysosomal formation is provided by the colocalization studies. Here we show that in morphine treated cells GFP tagged *E. coli* (green) and lysosomal compartment (red) are not colocalizing (**Figure 4.8A**), indicating that in presence of morphine lysosomal vesicles remain vacant and that bacteria remains in the phagosome. Colocalization of red lysosome and green *E. coli* was quantified (**Figure 4.8 B,C**) using Pearson's quotient generated by the microscope software (EZ-C1 3.9 for Nikon). The program was able to quantify yellow coloration per slide frame, which resulted from colocalization of GFP-tagged bacteria and lysotracker-red lysosome. Therefore chronic morphine treatment reduces phago-lysosomal fusion as indicated by colocalization of bacteria with lysosome. Taken together these data indicate that morphine not only reduces numbers of lysosomal vesicles but also inhibits phago-lysosomal fusion thereby hindering macrophage's ability to eliminate bacterial pathogens.

DISCUSSION

This study is the first to show that morphine differentially inhibits mechanisms involved in macrophage killing of Gram-positive and Gram-negative bacteria. Morphine inhibits production of RNI, ROI as well as phagosomal maturation (**Figure 4.11**). However morphine exerts more robust inhibition of ROI release and phago-lysosomal fusion in presence of Gram-positive bacteria, such as *S. pneumonia* when compared to Gram-negative *E. coli.*

First step to adequate bacterial clearance is through effective pathogen internalization and elimination. Previously, we have shown that morphine inhibits phagocytosis and now we observe that morphine also leads to inhibition of bacterial killing of Gram-positive and Gram-negative bacteria. In addition, activation of TLR2 in presence of morphine maintained morphine's inhibitory effect on macrophage bactericidal ability, while activation of TLR4 abolished morphine's inhibitory effect. Therefore these data indicate that Gram-positive bacterial infections would have more detriment for opioid users than Gram-negative. This data further explains morphine mediated inhibition of macrophage functions and further supports increased susceptibility to infection in opioid users.

Essential part of bacterial killing is phagosomal maturation and fusion with the lysosomal compartment. To our knowledge, this study is the first to demonstrate that morphine inhibits phagosomal maturation. Cells treated with morphine display fewer lysosomal vacuoles as well as reduced colocalization of the lysosomes with phagosomes containing bacteria. Furthermore, macrophages internalizing *S. pneumoniae* display a much stronger inhibition of phagosomal maturation in presence of

morphine than *E. coli*, indicating that Gram-positive bacteria are affected more dramatically in this process.

In addition to phago-lysosomal fusion, morphine inhibits ROI formation by modulating NADPH-oxidase activity. Inhibition of ROI production was more exacerbated in cells incubated with Gram-positive *S. pneumonia*e, when compared to cells incubated with Gram-negative *E. coli* as confirmed by microscopic and fluorometric analysis. Morphine modulation of these key pathways involved in bacterial killing, explains how it impairs macrophage functions to lead to bacteremia and sepsis.

Some of these differences seen in morphine inhibition of bacterial killing can be explained by our laboratory's previous findings that morphine increases intracellular cAMP, inhibits actin polymerization and Rac-GTPase and upregulates TLR4 expression.

Release of lysosomal enzymes is inhibited in presence of cAMP (Sasada et al., 1983), since morphine leads to superactivation of AC and increased intracellular levels of cAMP, it may be utilizing this mechanism to inhibit release of lysosomal enzymes and inhibit phago-lysosomal fusion (Ulevitch and Tobias, 1995; Blander and Medzhitov, 2004). In addition, lysosomes require intact microtubule function in order to fuse with other vesicles (Weissmann et al., 1971; Goldstein et al., 1973). Previously we have shown that MS inhibits actin polymerization. By hindering actin polymerization, morphine inhibits mechanisms involved in microtubule formation, and in this way may lead to reduced recruitment of NADPH-oxidase complex and inhibition of lysosomal fusion. Previously we have shown that morphine modulates actin polymerization and activation Rac-GTPases which are important in recruitment (Rac2) and activation (Rac1) of NADPH-oxidase (Sharma et al., 1975; Childers, 1991; Avidor-Reiss et al., 1997; Kelschenbach et al., 2008). By modulating Rac-GTP morphine can inhibit NADPH-oxidase recruitment and activation therefore inhibiting ROI formation. Differential effects

of morphine on ROI production in presence of Gram-positive versus Gram-negative bacteria can be explained by TLR mediated activation of Rac. TLR4 upon LPS stimulation induces Rac/Cdc42 activation, which can not only increase phagocytosis but also NADPH recruitment (Zurier et al., 1973). Since morphine increases TLR4 expression, LPS activation of TLR4 increases Rac, leading to increased NADPH-oxidase and increased killing; however this is not the case for TLR2 activation.

Although we have previously shown that TLR4 activation by morphine increased phagocytosis by upregulating TLR4 expression, we also note that bacterial killing is further increased potentially by the same mechanism. As TLR4 activation is implicated in enhancing phagocytosis and bactericidal mechanisms, it is to be expected that morphine by upregulation of TLR4 receptors and in presence of TLR4 ligands such as LPS would lead to greater phagocytosis and probably greater bacterial killing (Blander and Medzhitov, 2004). Our previous studies show that this is the case, and in this study we note that activation of TLR4 following chronic morphine treatment leads to enhanced bacterial killing of *E. coli* by macrophage cell line J774. TLR activation has been known to increase NO production, NADPH activity and phagosome maturation (Blander and Medzhitov, 2004). Consequently morphine might be exerting its stimulatory effects on bacterial killing of *E. coli* through TLR4 upregulation.

In conclusion, these findings add to the understanding of morphine mediated immune suppression. By inhibiting phagocytosis and bacterial killing, morphine increases susceptibility to infection. Morphine's differential effects on TLR activation following activation by Gram-positive and Gram-negative ligands highlight the significance and detriment that morphine abuse has on bacterial infections.

CONCLUSION

Morphine mediated immune suppression remains a growing concern in our society today. Although immunosuppressive effects of morphine have been well established, a gap in understanding of the mechanisms that underlie this phenomenon still exists. The goal of these studies was to address this gap in knowledge by examining mechanisms involved in morphine modulation of macrophage phagocytic and bactericidal functions.

Initially, we observed that morphine treatment leads to inhibition of Fc-gamma receptor mediated phagocytosis through modulation of actin polymerization and Rac-GTPases. Through super activation of adenylyl cyclase, increase in intracellular cAMP, and PKA activation, morphine leads to inhibition of actin polymerization and decreased pathogen internalization. Furthermore, MAPKs such as ERK1/2 and p38 MAPK are inhibited by morphine and are key effectors in mediating inhibition of the phagocytic process. Additional studies revealed that morphine had differential effects on phagocytosis of Gram-positive and Gram-negative bacteria. The same was true for phagocytosis by macrophages undergoing chronic morphine treatment with concurrent toll-like receptor (TLR) activation. Morphine, by up regulating TLR4 enhances phagocytosis leading to increased internalization and bacterial clearance. Interestingly this was not the case with activation of TLR2 receptors. TLR2 activation does not significantly change morphine's inhibitory effect on bacterial phagocytosis. Therefore our findings were the first to show morphine's differential effect on phagocytosis in the presence of Gram-positive and Gram-negative TLR ligands, such as LTA and LPS.

In addition to modulation of phagocytosis, morphine leads to inhibition of bacterial killing. Morphine inhibits macrophage bactericidal mechanisms, such as

143

formation of ROI and RNI, as well as phago-lysosomal fusion. In addition morphine had a differential effect on bacterial killing in presence of Gram-positive and Gram-negative TLR2 and TLR4 ligands. Following TLR4 activation morphine increases bacterial killing while activation of TLR2 maintains morphine's inhibitory effect. These differences in morphine's modulation were illustrated by the greater inhibition in ROI formation and phago-lysosomal fusion in macrophages exposed to Gram-positive bacteria. We show that TLR2 activation (by ligands from Gram-positive bacteria) in presence of morphine leads to significant inhibition of phagocytosis and bacterial killing compared to TLR4 activation by Gram-negative ligands. This indicates that in presence of morphine, clearance of Gram-positive pathogens will be significantly more impaired than clearance of Gram-negative pathogens. Although no specific epidemiological studies have been conducted in order to examine effect of morphine on dissemination of Gram-positive versus dissemination of Gram-negative bacteria, our group has observed significant mortality in mice undergoing chronic morphine treatment following infection with *S. pneumoniae*, a Gram-positive pathogen.

Morphine inhibits essential mechanisms involved in bacterial clearance. By blocking phagocytic and bactericidal mechanisms morphine ultimately leads to higher bacterial loads and prolonged pathogen survival within the cell. By allowing intracellular bacterial growth, morphine facilitates further bacterial dissemination leading to sepsis and bacteremia. Taken together, these findings support the view that morphine acts on multiple mechanisms to inhibit innate immune system. Studies presented herein have added knowledge and expanded our current understanding of mechanisms underlying morphine mediated immune suppression.

Our findings have added new insights in this underrepresented field of study, however with some limitations. First, limitation we encountered was that use of cell lines

144

was necessary in majority of our studies examining signaling mechanisms in order to avoid potential confounding factors. Although cell line provides a uniform system with few confounding effects it also possesses several caveats. Cell lines do not always behave like primary cells, which often poses problems and risks. In order to provide proof that the cell line used in these studies was mimicking effects seen in primary cells, we utilized primary macrophages treated with morphine *in vivo* and *ex vivo* and verified that morphine mediated inhibition was observed in both. In this case, it was concluded that the cell line chosen was adequate and representative of the physiological environment we set out to investigate. Second limitation was that fluorometry as a technique, was not a faithful representation of morphine's dramatic inhibition of phagocytosis seen in microscopic analysis. Morphine's inhibition of phagocytosis and actin polymerization observed using confocal microscopy was dramatic, while the fluorometric data indicated a significant but marginal inhibition. This limitation was overcome with use of several techniques such as confocal microscopy and densitometric analysis. Although fluorometry did not fully highlight the extent of morphine's inhibition it was the optimal method for showing the overall trends in phagocytic changes and was a good choice in elucidating modulation of phagocytosis in presence or absence of kinase inhibitors. Third limitation of this study was that we did not examine mechanisms by which morphine administered *in vivo* can lead to inhibition of phagocytosis *in vivo*. Although we have shown that activation of HPA axis and release of corticosterone does not play a role in modulation of macrophage phagocytosis there are other mechanisms by which morphine could be modulating bacterial clearance *in vivo*. Through inhibition of recruitment and by modulating cytokine and chemokine release morphine can directly or indirectly (through activation of HPA axis) modulate macrophage phagocytic and bactericidal functions. These mechanisms weren't explored

in this study due to the time constraints, but remain an interesting aim for future studies.

These findings have identified a potential signaling pathway that morphine utilizes in order to disrupt signal transduction, and have shed some light on mechanisms involved in morphine mediated immune suppression. Future studies stemming from this work should explore if or how clinically prescribed opioids such as oxycodone may lead to inhibition of phagocytosis by human macrophages. This work would add a translational perspective to our studies and would add clinical relevance to our findings. Although we have shown that activation of TLRs by Gram-positive and Gram-negative ligands has differential effects on morphine mediated inhibition of phagocytosis it will be interesting to examine how viral pathogens such as HIV modulate morphine's inhibition of phagocytosis. High prevalence of opioid use and abuse is observed in AIDS patients. Opioids are frequently prescribed for chronic pain management in the late stages of AIDS and due to the immunosuppressive nature of this disease, AIDS patients are often susceptible to different bacterial pathogens. Therefore studying how morphine modulates bacterial clearance in presence of HIV, or HIV proteins would fill a gap in knowledge as well as add translational significance to this work. Ultimately additional research is required to further elucidate mechanisms of morphine mediated immune dysfunction, and it is only then that we can hope to find solutions to the problems of opioid use and susceptibility to infection.

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