EFFECTS OF CHRONIC MORPHINE ADMINISTRATION ON CYTOKINE AND CHEMOKINE PROTEIN AND GENE EXPRESSION

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ABSTRACT

Title: Effects of Chronic Morphine Administration on Cytokine & Chemokine Protein and Gene Expression

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Chemokine and chemokine receptors play a major role in HIV-1 infectivity, and their expression can be modulated by opioid drugs of abuse, further implicating a role for these drugs in altering HIV-1 susceptibility. Several of the opioid agonists including morphine and heroin impair resistance to a variety of infectious agents including HIV-1 by modulating both innate and acquired immune responses. The aim of my thesis is to understand whether chronic morphine administration alters the expression of pro-inflammatory cytokines and chemokines. Since there are limited reports in the literature describing the effects of chronic opioid administration on immune competence, a macaque model was devised to analyze the immune system following chronic morphine administration. My results show that animals receiving morphine exhibit enhanced proinflammatory CXCL8 protein expression in response to stimulation with various Toll Receptor (TLR) ligands. This result was observed in responses to either the combination of LPS and IFNγ, or with the TLR ligand peptidoglycan. These results suggest that chronic morphine administration increases

immune system responsiveness. We extended these studies on opioid-induced signaling and gene expression in human subjects and observed that opioid treatment induces the expression of CXCL10, TLR4, and the aryl hydrocarbon receptor (AHR) in leukocytes early in response to treatment. In sum, I have shown that opioid agonists modulate important immune-response genes, and these genes are important for the generation of antimicrobial immunity.

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LIST OF ABBREVIATIONS

AHR	Aryl hydrocarbon receptor
AIDS	Acquired Immune Deficiency Syndrome
CDC	Center for Disease Control
DNA	Deoxyribonucleic Acid
DOR	Delta Opioid Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular-signal-regulated kinases
GPCR	G-protein coupled Receptor
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon gamma
IL	Inter Leukin
IVDU	Intravenous Drug User
JNK	Jun N-terminal kinases
KOR	Kappa Opioid Receptor
LPS	Lipopolysaccharide
LTR	Long Terminal Repeat
MAPK	Mitogen-activated protein kinase
MOR	Mu Opioid Receptor
PAGE	Polyacrylamide Gel Electrophoresis
PBMC	Peripheral Blood Mononuclear Cells
PHA	Phytohemagglutin
PMA	Phorbol 12-myristatel 3-acetate
RNA	Ribonucleic Acid
SIV	Simian immunodeficiency virus
TLR	Toll-Like Receptor

TNF-α	Tumor necrosis Factor alpha

Chapter 1

INTRODUCTION

1.1 Opioids and their effects

Opium and its derivatives have historically been used as pain-killers (analgesic) and a vast literature can be found describing their psycho-stimulatory, euphoric, and analgesia-inducing properties. The Opium poppy was first grown in the western Mediterranean, in the region of southern France and Italy. Preparations of Opium poppy *Papaver sominferum* have been used for centuries primarily to relieve pain. Most opiates give a sense of euphoria and a degree of sedation. These side effects make them popular as drugs of abuse.

Opioid drugs of abuse alter resistance to a variety of infectious agents by modulating both innate and acquired immune responses. One-third of Human Immunodeficiency Virus (HIV)-infected individuals in the United States have been identified as intravenous drug users (IVDUs) (CDC, 1996) and intravenous drug abuse is a prominent risk factor for HIV infection in the world.

1.2 Opioids and Opioid Receptors

Opioid receptors belong to the seven trans-membrane G- protein coupled receptor (GPCR) superfamily. These receptors have a hydrophobic trans-membrane domain, a short intracellular domain and an extracellular domain. The amino termini of GPCRs are on the extracellular side of the plasma membrane and usually contain sites for N-linked glycosylation (Asn-X-Ser/Thr), while the carboxyl-termini are on the cytoplasmic side of the membrane. Opioid receptors can be classified under three major categories designated as: μ, κ and δ (Evans et al., 1992; Chen et al., 1993; Meng et al., 1993). Each type of receptor is encoded by a different gene and the coding regions having distinct, although highly homologous sequences (~60%) (Satoh and Minami, 1995). Higher identities are found in the trans-membrane regions and intracellular regions, the extracellular regions are more divergent (Minami and Satoh, 1995).

The opioid receptors and endogenous opioid peptides are expressed in both the central and the peripheral nervous systems and are expressed by neurons, astrocytes and microglial cells (Ruzicka et al., 1995; Bodnar, 2007). Additionally, pharmacological and molecular evidence has been reported for the expression of opioid receptors by cells of the immune system. Wybran (Wybran et al., 1979) observed that addition of morphine to the human T cells in culture would affect their ability to rosette with the sheep red blood cells (SRBCs). Addition of the exogenous opioid met-enkephalin increased the T cells rosetting and the actions of both the opioids were blocked by naloxone, an antagonist for all types of opioid receptors, confirming a role for the opioid receptors for these effects. Radiolabeled agonist and antagonist binding analyses confirmed that opioid receptors were expressed on cells of the immune system (Bidlack et al., 1992; Carr et al., 1989; Sibinga

& Goldstein, 1988). Moreover, μ-, κ-, and δ-opioid receptors have been cloned by RT-PCR from mRNA isolated from lymphocytes and macrophages and the sequences of these transcripts are essentially identical to receptors in the CNS (Belkowski et al., 1995b; Chuang et al., 1994; Sedqi et al., 1995). Additional studies have shown that the opioid receptors are expressed at lower levels on immune cells, but the level of expression is still sufficient to mediate biological effects including mitogen-activated protein kinase (MAP kinase) activation (Bohn et al., 2000; Gutstein et al., 1997) and chemotaxis (Ruff et al., 1985).

The opioid receptors are members of the GPCR superfamily, and these receptors specifically are coupled to G_i/G_o proteins, inhibit adenylyl cyclase, activate second messenger pathways, augment potassium conductance, decrease voltage-dependent calcium channels, and regulate mitogen-activated protein (MAP) kinases (Childers, 1991; Jordan & Iyengar, 1998; Law et al., 2000). The inhibition of adenylyl cyclase activation and the modulation of both potassium and calcium conductance are commonly used as measures of opioid receptor activation for neuronal cells. Signaling pathways mediated by opioid receptors expressed by leucocytes are much less well understood.

Endogenous opioids

Endogenous opioids are expressed in the brain and they act as agonists for the opioid receptors (Hughes et al., 1975; Terenius & Wahlstrom, 1975). There are four major endogenous opioid peptide families: endorphins, enkephalins, dynorphins, and endomorphins. Each family is derived from a distinct precursor protein. The precursors- prepro-opiomelanocortin (POMC), preproenkephalin and preprodynorphin are encoded by three corresponding genes, which code for the endorphins, enkephalins and dynorphins respectively. Each precursor protein undergoes complex cleavages and post-translational modifications that result in the synthesis of active peptides (Koneru et al., 2009). The precursor protein for the fourth family member- the endomorphins has yet to be identified.

The opioid receptor binding selectivity is well documented for each of the endogenous opioid peptides and some of these peptides bind to more than one receptor type. β-Endorphin has been shown to bind both μ- and δ-opioid receptors (Goldstein, 1984). Radiolabeled binding analysis shows that β-endorphin has similar affinities for both μ and δ binding sites, but a much lower affinity for κ–opioid receptors (Zadina et al., 1997). Met-enkephalin and leu-enkephalin both pentapeptides, have high affinities for δ-opioid receptors, ten-fold lower affinities for μ-opioid receptors, and a very low affinity for κ-opioid receptors (Hughes et al., 1975). Dynorphin binds with high affinity to the κ-opioid receptor, with little detectable binding to the δ- or μ-opioid receptors (Goldstein & Ghazarossian, 1980). Based, in part, on an initial synthetic peptide screen for μ- opioid receptor binding, Zadina et al., (1997) identified endomorphin-1 and endomorphin-2 as having high selectivity and affinity for the μ-opioid receptor. Subsequently, these peptides were identified in brain tissue. Endomorphin-1 and endomorphin-2 are amidated tetra peptides structurally unrelated to other endogenous peptides. These endogenous opioids are termed as ‘natural pain relievers’ due to their ability to increase dopamine efflux in the nucleus accumbens via μ- opioid receptors (Okutsu et al., 2006).

Exogenous Opioids

A large battery of exogenous synthetic agonists has been generated which possess high binding affinity and opioid receptor subtype selectivity for the μ-, δ-, or κ-opioid receptors. For example, the exogenous μ-opioid receptor agonist [D-ala^2, (Me) Phe4, Gly-ol^5] enkephalin (DAMGO) binds to the μ-opioid receptor with affinity similar to that of endomorphin-1, and a selectivity ratio greater than 500 for the μ-opioid receptors compared with δ-opioid receptors, and greater than 3000 for μ-opioid receptors when compared to the κ-opioid receptors (Zadina et al., 1997). A selective exogenous agonist for the δ-opioid receptor is the peptide deltorphin, which was shown to exhibit a high affinity and selectivity for the δ-opioid receptor (Kreil et al., 1989). Similarly, Mosberg et al., (1983) synthesized an enkephalin analog, [D-Penicillamine2, D-Penicillamine^5]enkephalin (DPDPE), which also exhibited high affinity for the δ-opioid receptor. Whereas, the alkyloid U50 488H, and the related compound U69 593, have a 1000-fold higher affinity for κ-opioid binding sites than for μ- or δ-opioid binding sites (Goldstein & Naidu, 1989). There is a partial list of endogenous and exogenous agonists and antagonist for each opioid receptor on the next page.

Receptor Type	Endogenous Agonist	Exogenous Agonist	Antagonist
κ	Dynorphin	U50,488H U69,593 Morphine	Nor-BNI Naloxone
δ	Met-enkephalin Leu-enkephalin β-endorphin	Deltorphin DPDPE Morphine	Naloxone Naltrindole
μ	Endomorphin-1 Endomorphin-2 β-endorphin	DAMGO Morphine	CTAP β-FNA Naloxone

1.3 Effects of μ- Opioids on the Immune System

Effects on Innate Immunity

Natural Killer Cell Function: Opioid agonists exert effects on cells of the immune system and can modulate a number of immune functions (Eisenstein and

Hilburger, 1998; Carr et al., 1996; McCarthy et al., 2001; Sharp et al., 1998; Roy and Loh, 1996; Mellon and Bayer, 1998). For example, opioid drugs of abuse alter the innate immune responses by altering the Natural Killer (NK) cell activity. NK cells are the very first responders to inflammation or infection. Acute morphine treatment of either experimental animals or human subjects results in suppressed NK cell activity (Shavit et al., 1987; Weber and Pert el al., 1989; Yeager et al., 1995; Gaveriaux-Ruff et al., 1998).

Monocyte/Macrophage function is also inhibited by morphine treatment, based on work with both murine peritoneal macrophages and human PBMCs (Rojavin et al., 1993; Peterson et al., 1987a). Moreover, morphine also inhibits the ability of phagocytic cells to exhibit an oxidative burst response and produces superoxide or peroxide (Peterson et al., 1987a). These reactive oxygen intermediates contribute to the microbicidal activity of macrophages and the inability to elicit an oxidative burst would substantially impair antimicrobial immune responses. Peterson and colleagues (Chao et al., 1992) have also shown that the inhibitory effects of morphine on the phagocytic activity of the macrophages are due to the production of TGF-beta. Morphine is known not only to affect the effector pathways of macrophages but also interfere with the mobilization of macrophages from bone marrow. Moreover, morphine administration either in vivo or in vitro suppressed macrophage colony formation from bone marrow cells in culture (Roy et al., 1991).

Effects on Adaptive Immunity

Antibody Responses: Early studies have shown that in vivo morphine treatment results in a reduced ability of murine B cells to respond to bacterial lipopolysaccharide (LPS) (Bryant et al., 1987). Based on both ex vivo and in vivo studies, morphine has been shown to block the capacity of murine spleen cells to generate an antibody response (Bussiere et al., 1992). This effect was blocked by the administration of naltrexone suggesting that immunosuppression was induced via the classical opioid receptors ((Gavériaux-Ruff et al., 1998). The immunosuppressive effects of morphine are mediated in part through effects on T cells and macrophages (Bussiere et al., 1993; Guan et al., 1994), typically required for the generation of an antibody response.

Cytokine and Chemokine Expression

Pro-inflammatory and anti-inflammatory cytokines and chemokines play a critical role in mediating innate or acquired immunity, including the febrile response (IL-1), organ damage (TNF-α), migration of phagocytic cells (CXCL8, CCL2) and B cell maturation /antibody production (IL-5). Morphine and other opioids have been shown to modulate the production of a number of cytokines and chemokines. Studies carried out by Peterson et al (1987b) showed that morphine treatment suppressed IFN-γ production in human PBMCs. Studies by Jessop & Taplits (1991) and Roy et al. (1997) have shown that morphine induces dose-dependent suppression of IL-2 expression in murine splenocytes and thymocytes. Furthermore, Lysle et al. (1993) reported that in vivo morphine treatment in rats lead to a naltrexone-reversible, dose dependent suppression of both IFN-γ AND IL-2 in splenic lymphocytes. Also, Chao et al. (1992) previously demonstrated a naloxone-reversible increase in TGF-β production following morphine treatment in stimulated PBMCs; it is possible that the immunosuppressive action of TGF-β contributed to the morphine-induced inhibition of IL-2 and IFN-γ. However, in contrast, the expression of some pro-inflammatory cytokines including IL-6 and TNF-α are up regulated in response to low-doses of morphine in peritoneal macrophages (Roy et al., 1998a), and IL-12 and TNF-α are up-regulated in response to high–doses of morphine (Peng et al; 2000). Hence, evidence suggests that morphine administration in vivo biases the immune response towards a Th2 phenotype, based on the up-regulation of IL-4 and down regulation of IFN-γ (Roy et al., 2001).

Chemokine and chemokine receptors play a major role in HIV-1 infectivity (Bleul et al., 1996; Alkhatib et al., 1997; Dragic et al., 1996; Feng et al., 1996; Oberlin et al., 1996), and their expression can be modulated by opioid drugs of abuse, further implicating a role for these drugs in altering HIV-1 susceptibility. Astrocytes treated with morphine exhibit reduced expression of CXCL8, CCL4 and CCL2 in a dose-dependent manner (Mahajan et al., 2002; Mahajan et al., 2005). Morphine treatment of microglial cells resulted in a reduced production of CCL5, (Hu et al., 2000). These morphine-mediated effects in both cases were reversed by the addition of the μ-opioid receptor antagonist β-funaltrexamine (β-FNA), suggesting a role for the μ- opioid receptor. As far as their role in HIV-1 susceptibility, it has been previously reported that the decrease in

CCL4 could negatively affect the ability of this chemokine to protect against HIV-1 in the CNS (Mahajan et al., 2002). These results are in contrast with data that show that treatment of PBMC's with DAMGO increases the expression of pro-inflammatory chemokines such as CCL2, CCL5 and CXCL10 (Wetzel et al., 2000). It has been suggested that the induction of these chemokines may promote the replication of HIV by attracting uninfected cells to the site of opioid receptor expression and chemokine induction (Wetzel et al., 2000). Recent studies have also shown that activation of the μ-opioid receptor increases the expression of the chemokine receptors CXCR4 and CCR5, which interestingly are the major (HIV)-1 co-receptors. The opioid-induced up-regulation of co-receptor expression correlates with increases in monocyte and T-cell virus uptake, leading to an elevation in HIV-1 replication (Steele et al., 2003).

1.4 Effects of Opioid Drugs of abuse on HIV/SIV

As mentioned earlier, about one-third of HIV-infected individuals in the United States are intravenous drug abusers (CDC 1996). Several of the opioid agonists reviewed above including morphine and heroin impair resistance to a variety of infectious agents by modulating both innate and acquired immune responses. Even though it could be implied that an elevated incidence of HIV infection in intravenous drug users (IVDUs) might simply be due to the more commune exposure to the virus since contaminated needles and high-risk sexual behavior are the main modes of HIV transmission in the drug abusing population (Chaisson et al., 1987; Des Jarlais et al., 1988; Friedland et al., 1985; McCoy et al., 1998). However, there are other possible explanations, which may suggest the greater susceptibility to various infections within the opioid drug abuse population (Donahoe & Falek, 1988). There are mixed observations from epidemiological studies concerning the relationship between opioid drug abuse and susceptibility to HIV infection and disease. On one hand, there are reports, which show a faster progression to AIDS in IVDUs (Selwyn et al., 1992; Rothenberg et al., 1986; Miller et al., 1984), and others, which find no statistical relationship (Margolick et al., 1994; Margolick et al., 1992). And yet there are others which show drug use slows the progression to AIDS (Spijkerman et al., 1995; Spijkerman et al., 1996). It's very important to recognize that epidemiological studies may have a number of potential limitations. For instance, IVDUs may carry multiple infections (Louria et al., 1967) including hepatitis B and C (Garfein et al., 1996), causing repeated immune stimulation and recurrent HIV activation. There is also considerable variability in the demographic and lifestyle factors that might complicate analysis of the epidemiological data. IVDUs also use many different drugs that might have possible interactive effects (Crum et al., 1996), which can further confound epidemiological analyses. The complexity of the epidemiologic reports makes laboratory studies testing the effects of drugs of abuse on HIV progression very desirable, but the lack of an adequate small animal model has limited research on this topic. Peterson and colleagues (Peterson et al.,1990) reported that morphine treatment promoted the growth of HIV in vitro. They showed that peripheral blood mononuclear cells (PBMCs) from normal humans, when co-cultured with morphine in the presence of HIV-infected lymphocytes, resulted in an increase in HIV-1 infectivity. Also, it was previously shown by the same group that a co-culture of infected promonocytic cell line U1 along with fetal human brain cells, enhanced HIV replication, and morphine pre-treatment of these fetal brain cells further augmented the capacity of the brain-cells to induce HIV replication by inducing the production of the pro-inflammatory cytokines TNFα and IL-6 (Peterson et al.,1994). Other studies have shown that morphine stimulates HIV replication in human Kupffer cells (Schweitzer et al., 1991). More recent work has shown that morphine induces an increase in HIV replication in fetal brain microglia and in human monocyte-derived macrophages (Li et al., 2000). Morphine up regulates CCR5 (co-receptor for HIV-1) expression in these macrophages, and this is likely to be responsible for the increase in HIV replication. Morphine treatment also down regulates Interferon-γ (IFN-γ), a potent antiviral cytokine produced in part by CD8+ T cells in response to HIV infection (Wang et al., 2005). Finally, morphine and other μ-opioids increase the expression of both major HIV-1 co-receptors (CXCR4 and CCR5) in PBMC's, and substantially increase susceptibility to both X4 and R5 HIV-1 infection (Steele et al., 2003).

Studies to assess the effects of morphine on resistance to SIV infection in macaques have produced conflicting results. Donahoe and colleagues (Donahoe et al., 1993; Donahoe et al., 2009) have reported that macaques treated with morphine show slower SIV disease progression and a slower decline in CD4 counts. In contrast, Chuang et al., 1997 have shown that chronic morphine administration results in more rapid SIV progression, and more rapid evolution of HIV mutants, which are resistant to neutralizing antisera. Additionally, Kumar and colleagues (Kumar et al., 2004) showed that both morphine-treated and control animals showed rapid T cell depletion in response to SIV infection, but the morphine-treated animals exhibited a reduced tendency to recover normal levels of $CD4^+$ T-cells. The inconsistent results in these studies may be due to the fact that the studies were conducted with very diverse SIV strains with uncertain virulence. Further investigation is hence warranted and necessary in this regard.

1.5 Effects of Opioids on Gene and Protein Expression

Morphine treatment has been shown to modulate the function of immune cells both at the transcriptional and translational levels. For example, it has been previously established that morphine pretreatment of macrophages followed by LPS stimulation resulted in elevated activation of NFκB (Roy et al., 1998). This NFκB activation led to an elevation in TNF-α and IL-6 expression (Roy et al., 1998). NFκB is present as an inducible transcription factor in the brain (Bakalkin et al., 1993). Acute (2 or 4 hours) or long term (72 hours) treatment of cultured cortical neurons with morphine increased the DNA binding activity of NFκB, in a naloxone-sensitive manner. Together these data suggest that μ-opioids, such as morphine and DAMGO, exert direct effects on cells of the immune system and neurons at the transcriptional level. It has also been established that morphine modulates the expression of downstream signaling molecules, Fos and Jun (Weiss et al., 1993). It should be noted that Fos and Jun proteins influence the transcriptional regulation of HIV-1 through the AP-1 *cis*-regulatory sequence within the HIV-1 LTR (reviewed in Weiss, 1993). Squinto et al., 1990 showed that morphine administration led to a 2.5-fold elevation in intracellular levels of both Fos and Jun by utilizing an HIV-1/LTR-Cat (HIV-1-long terminal repeat-chloramphenical acetyltransferase) fusion gene reporter assay. These data suggest that elevated levels of Fos and Jun lead to an activation of latent HIV-1.

Opioid receptors are G_i/G_o-coupled receptors that upon activation dissociate from the α and $\beta\gamma$ subunits of the receptor. The $G\beta\gamma$ subunits have been shown to mediate signal transduction pathways generated by G_i-coupled receptors and activate the mitogen-activated protein (MAP) kinase signaling cascade (Koch et al., 1994). There are three main MAPK activation pathways, the extracellular-signal-regulated kinases (ERKs), the Jun N-terminal kinases (JNKs) and the p38 kinases. Stimulation of ERK1 and ERK2 by opioids was first demonstrated with MOR in transfected CHO cells (Li and Chang, 1996). An inhibitor of the upstream mitogen-activated protein kinase kinase (MEK1/2) can completely prevent MOR from inducing ERK1/2 phorphorylation (Tso and Wong, 2001). There are other reports that have demonstrated that the opioid receptors can stimulate both JNK and p38 MAPK pathways in neuronal cells (Chan and Wong, 2000; Zhang et al., 1999) as well. However, the capacity of MOR to induce MAP Kinase activation in hematopoietic cells has not been fully addressed.

•<u>CXCL10</u>

It has been previously reported by our laboratory that the MOR-selective agonist DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin (synthetic μ-opioid) treatment) can induce CXCL10 production in nonactivated and PHA-stimulated peripheral blood mononuclear cells (PBMCs) at both the mRNA and protein levels, and, this effect is blocked by administration of the MOR-selective antagonist H-D-Phe –Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP) indicating that this effect is mediated through MOR (Wetzel et al., 2000). In addition, a major increase in CXCL10 expression was induced in T-tropic HIV-1 infected cells indicating that CXCL10 chemo attracts activated T-lymphocytes during HIV-1 infection (Foley et al., 2005) rendering its evaluation significant.

•<u>TLR4</u>

TLR4 is constitutively expressed on macrophages, monocyte-derived dendritic cells and B cells. The TLR4 receptor, is activated by certain toll-receptor agonists such as LPS, and can form a dimer with CD14 further evoking a pro-inflammatory cytokine response. An increased expression of TLR2 and TLR4 has been observed in myeloid dendritic cells of HIV-1 patients co-infected with opportunistic infections. In addition, TLR4 is increased in plasmacytoid dendritic cells, compared to both HIV-1 infection without opportunistic infections and healthy subjects (Hernandez et al., 2011)

•<u>AHR</u>

The aryl hydrocarbon receptor (AhR) is activated by certain hydrocarbon agents including the halogenated polycyclic aromatic hydrocarbon, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), an environmental toxin (Okey et al., 2007). AhR was found to have a basic helix-loop-helix structure and functions as a ligand-activated transcription factor. Located in the cytoplasm of most cells, AhR forms a receptor complex with several proteins including the chaperone protein hsp90 (a 90kDa heat shock protein). AHR ligands caused the induction of T_H17 cells and altered the expression of proinflammatory cytokines, such as TNF-α, IL-6 (Kimura et al., 2009). It is also known to be involved in the induction of gene expression from HIV-1 LTR (Ohata et al., 2003)

MOR Regulation of Chemokine Expression

Chemokines and cytokines are a vital component of the immune response as well as host defense and opioids are known to regulate their expression. MOR activation has been shown to modulate cytokine and chemokine production that is important for generating an inflammatory response (Wetzel et al., 2000; Mahajan et al., 2002; McCarthy et al., 2001). As previously reported endogenous endorphins and enkephalins increase the production of IL-1, IL-2 and IFN-γ (Brown and Van Epps, 1986; Mandler et al., 1986; Bessler et al., 1990). Our lab has reported that the MOR-selective agonist DAMGO can elevate CCL2, CXCL10, and CCL5 production in non-activated and PHA-stimulated peripheral blood mononuclear cells (PBMCs) both at the mRNA and protein level, and, this effect is blocked by administration of the MOR-selective antagonist H-D-Phe –Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP) confirming that this effect is mediated primarily through MOR (Wetzel et al., 2000). Rock *et al.* (Rock et al., 2006) showed that morphine stimulates CCL2 production at both the mRNA and protein levels in neurons. In addition the MOR antagonist, β-funaltrexamine (β-FNA) was used to block the morphine-mediated increase in CCL2, demonstrating that this effect is mediated through the MOR (Rock et al., 2006). Caco-2 cells are an intestinal epithelial cell line constitutively expressing MOR and KOR which, upon activation with the endogenous μ tetrapeptide, endomorphin-1, demonstrated a significant increase in CXCL8 secretion. The effect was reversible by pre-treatment with the MOR antagonist, β-FNA (Neudeck and Loeb, 2002).

•<u>CCL2</u>

CCL2 is a 76-amino acid peptide, and a member of the C-C chemokine family that induces chemotaxis of monocytes/macrophages, memory T lymphocytes, and NK cells to the areas of infection and inflammation (Baggiolini et al., 1997; Luster, 2002). The level of expression of CCL2 is fairly low prior to induction until induced by a number of stimuli of several cell types including fibroblasts, endothelial cells, monocytes/macrophages, neutrophils and activated lymphocytes (Leonard and Yoshimura, 1990).

CCL2 plays a major role in cellular immune reactions and acute tissue injury (Leonard and Yoshimura, 1990). The main role of CCL2 is to recruit monocytes to the sites of inflammation (Leonard and Yoshimura, 1990). CCL2 has also been shown to play a role in monocyte recruitment to the CNS during HIV-1 infection (Conant et al., 1998; Weiss et al., 1999). CCL2 can elicit a number of responses including chemotaxis, calcium flux, increased IL-1 and IL-6 secretion, and the induction of leukocyte adhesion molecules (Viedt et al., 2002). The receptor for CCL2 is CCR2, and due to the expression of CCR2 by mononuclear cells, this chemokine is an important regulator of inflammatory disease. Increased expression of CCL2 has been associated with diseases such as rheumatoid arthritis, cardiovascular disease, and HIV infection (Sozzani et al., 1997; Romagnani, 2002; Gu et al., 1998). Additionally, as described previously the MOR selective agonist, DAMGO, has the ability to increase the expression of the pro-inflammatory chemokines, CCL2, CCL5 and CXCL10 in peripheral blood mononuclear cells (Wetzel et al., 2000). NF-κB, a transcription factor that plays a pivotal role in inflammation and the immune response has a role in this MOR-induced activation with NF-κB inhibitors able to prevent the MOR-induced activation of CCL2 and CCL5 completely. According to Happel et al., 2011 *in vivo*, DAMGO administration induces the p65 (NF-κB subunit) binding to the enhancer region of the CCL2 promoter. Furthermore, the paper demonstrated how PKCζ is phosphorylated following DAMGO-induced MOR activation and, is essential for NF-κB activity as well as CCL2 expression and transcriptional activity. In conclusion, these data suggest that activation of MOR favors a pro-inflammatory response involving NF-κB activation and PKCζ as a regulator of pro-inflammatory chemokines.

•<u>CCL22</u>

CCL22 belongs to the CC chemokine family and was isolated from clones of monocyte-derived macrophages. At the amino acid sequence level, MDC shows less than 35% identity to other CC chemokine family members. Human MDC is expressed by dendritic cells, macrophages and activated monocytes. MDC displays a chemotactic activity for monocytes, dendritic cells, and natural killer cells as well as the chronically activated T lymphocytes (Andrew et al., 1998). It also displays a mild activity for primary activated T lymphocytes and has no chemoattractant activity for neutrophils, eosinophils and resting T lymphocytes. Both CCL22 and CCL17 bind and activate chemokine receptor CCR4. The primary functions of CCL22 are trafficking of activated T lymphocytes to sites of inflammation and maintaining normal T lymphocyte physiology.

Recent studies show that Hepatitis C virus induces the expression of CCL17 and CCL22 and these chemokines attract regulatory T cells to the site of infection (Boj JI et al., 2011). Further experiments have shown that up-regulation of these chemokines enhance Treg migration (Boj JI et al., 2011). Additionally, recent reports suggest that both CCL17 and CCL22 are produced as a result of NF-κB activation in IFN-γ induced keratinocytes (Qi et al., 2011). Since it has previously been established that μ-opioids, such as morphine and DAMGO, can induce NF-κB, it is possible that CCL22 may be induced following MOR activation.

•CXCL8

CXCL8 belongs to the CXC family of chemokines and is produced by various cells including epithelial and endothelial cells. Interestingly, endothelial cells store CXCL8 in vesicles called Weibel-Palade bodies. CXCL8 activates both CXCR1 and CXCR2, but binds to CXCR1 with a higher affinity. CXCL8 is a chemo-attractant for neutrophils and granulocytes towards the site of the infection. A major function of CXCL8 is to recruit neutrophils to the site of infection. Upon activation with, endomorphin-1, Neudeck and Loeb (Neudeck and Loeb, 2002) demonstrated a significant increase in CXCL8 secretion. The effect was reversible by pre-treatment with the MOR antagonist, β-FNA (Neudeck and Loeb, 2002).

Neutrophils are also known to produce the peptides such as Met-enkephalin and β-endorphin (Vindrola et al., 1990; Mousa et al., 2004). In an inflamed subcutaneous tissue these opioid peptides can be released, bind to MOR and DOR expressed by peripheral sensory nerves, and locally inhibit pain (reviewed in Stein et al., 2003). Human neutrophils express the chemokine receptors CXCR1 and 2, while murine and rat neutrophils only express CXCR2. It has been demonstrated that CXCR1/2 ligands (i.e. human CXCL8) induce opioid peptide release from human and rat neutrophils in vitro and inhibit inflammatory pain in vivo (Rittner et al., 2006a). These results are very interesting and may point out towards an autocrine loop where opioids induce CXCL8 production and CXCL8 induces opioid release.

1.6 Significance of the Study

Opioids exert a wide range of effects on the immune responses by directly altering the function of immune cells. Based on the studies showing the capacity of μ-opioids to alter cytokine/chemokine expression as well as HIV-replication, we extended these studies to investigate the following:

1. To determine whether opioid administration alters the expression of certain

chemokines and cytokines, in response to TLR activation.

2. To determine the opioid –induced effects on specific gene expression and a possible signaling mechanism for opioid induction.

3. To determine association of the opioid-induced effects on specific chemokine expression on disease progression

To our knowledge there are limited reports in the literature describing the effects of opioids on simian immunodeficiency virus (SIV) progression in macaques. Our project will help to obtain more complete analysis of the immune response to the viral infection in the presence of morphine. We are using the SIV macaque model of human HIV infection, which is currently the best experimental animal model system for studying this host-parasite interaction in vivo. The macaque model allows for analysis of the activities of opioids administered over an extended period of time, by which it mirrors (more closely) the human drug abuse condition. In addition, the macaque model also offers the opportunity to evaluate neurological consequences of the viral infection to a greater degree than other alternative animal model systems. In conducting our experimental studies we also used a moderately virulent SIV strain which has the capacity to elicit the full range of pathological consequences of the SIV infection.

Chapter 2

MATERIALS AND METHODS

Drugs

The μ-opioid selective agonist [D-ala^2, N-Me-Phe^4-Gly-ol^5] enkephalin (DAMGO) (Multiple Peptide Systems, San Diego, CA) and the μ-opioid selective antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP) (Multiple Peptide Systems). The κ-opioid selective agonist (U50, 488 H) and the κ-opioid selective antagonist nor-binaltorphimine (NORBNI) were suspended in Dulbecco's Phosphate Buffered Saline (PBS: Mediatech, Inc), aliquoted, and stored at -80°C prior to use. Reconstituted DAMGO was stored for no more than 2 weeks at -80°C.

Chemicals

Ficoll-Paque Plus (Pharmacia Biotech), TRIzol® (Invitrogen), 40% paraformaldehyde (methanol and Rnase free) (Fisher Scientific), Tween-20 (Fisher Scientific), 95% Ethanol (molecular biology grade) (Fisher Scientific), Isopropanol 99.5% (Fisher Scientific), 10% SDS (Invitrogen).

Reagents

Phytohemogglutinin-M (PHA; Boehringer Mannheim Biochemical) PHA, Interleukin 4 (Sigma Aldrich), Interleukin 13 (Sigma-Alderich), CpGa (Invivogen), CpGb (Invivogen), R848 (Invivogen), Peptidoglycan (Invivogen), Lipotechoic Acid (Invivogen), IFNg (Sigma-Aldrich), LPS (Invivogen) were suspended in PBS (sterile filtered) and added to the peripheral blood mononuclear cell (PBMC) cultures.

Antibodies

Antibodies used for techniques including western blot analysis, flow cytometry, and ELISA, were ordered from a number of companies including Cell Signaling Technology, Santa Cruz Biotechnology Inc. (Santa Cruz, CA), Sigma-Aldrich (St. Louis, MO), Invitrogen (Carlsbad, CA) and R&D Systems (Minneapolis, MN). Antibodies, including company, catalog number and application, are listed in Table 1.

Table 1. List of Antibodies

Antibody	Company	Catalog #	Source	Application
anti-IL8	R&D Systems	DY208	Mouse	ELISA
anti-CCL22	R&D Systems	DY336	Mouse	ELISA
anti-MCP-1	R&D Systems	DY279	Mouse	ELISA
anti-phosphor Erk 1&2	Sigma	M5189	Mouse	Western Blot
anti-Erk1&2	Sigma	M3807	Mouse	Western Blot

2.1 Animals and Morphine Treatment Conditions

The nonhuman primates used in these studies were Macaca mulatta (Rhesus macaque). These Indian rhesus macaques were employed to assess the effect of morphine on the immune system. Animals were housed at BIOQUAL, Inc animal housing facilities in Rockville, MD. Care and husbandry of all non-human primates were provided in compliance with federal laws and guidelines as well as in accordance with recommendations provided in the NIH guide and other accepted standards of laboratory animal care and use. BIOQUAL is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, (AAALAC file #624) and holds an Assurance on file with the National Institute of Health, Office for Protection of Research Risks as required by the US Public Health

Service Policy on Humane Care and Use of Laboratory Animals. Morphine treatment of these animals was initiated (3mg/kg three times daily) with a morphine dose acceleration to 5mg/kg during the following 2 weeks. The dosage of morphine was continued at 5mg/kg three times daily) for the rest of the duration of study.

2.2 Isolation of Peripheral Blood Mononuclear Cells in Macaques and Humans

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood of normal donors and macaques under aseptic conditions. For humans, a volume of up to 200 ml of blood was collected into sterile 50 ml polystyrene conical tubes each containing 12.5 ml of the anti-coagulant acid sodium citrate (ACD) (Fisher Scientific) for a total of 50 ml of the blood and ACD mixture. For the macaques, the typical blood draw was about 15 ml. The isolation of PBMCs for both macaques and humans was carried out using density gradient centrifugation. Whole blood was layered over Ficoll-Paque Plus (Pharmacia Biotech) and centrifuged at 1300 rpm for 30 minutes at room temperature. Following the centrifugation, the upper plasma layer was removed leaving the mononuclear layer undisturbed at the interface. PBMCs are then recovered as carefully as possible to prevent contamination with granulocytes or platelets and placed in a separate conical tube. PBMCs were washed with PBS and centrifuged at 1300 rpm for 10 minutes at room temperature. PBMCs were then resuspended in 5 ml of 1X red blood cell lysis solution (15 M ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA, and water up to 1 liter) and allowed to incubate on ice for 10 minutes. PBMCs were then centrifuged and resuspended in PBS, counted, and finally resuspended into RPMI media (Life Technologies) with 10% fetal bovine serum (FBS; Hyclone, Inc.) and 10 μg/ml gentamicin (R10) cell culture media. PBMC cultures were maintained in R10 and cell cultures were maintained at 37^{o}C, 5% CO_2.

2.3 Cell Culture

Primary human peripheral blood mononuclear cells (PBMCs) were cultured in complete R10 media, which consists of RPMI 1640 medium supplemented with 10% heat-inactivated (56^{o}C, 30 min) endotoxin-free FBS and 10 μg/ml gentamicin (Life Technologies). The cells were seeded in 384-well polystyrene plates at two seeding densities- 5x 10^4 and 1.25 x 10^4 cells per well.

Human osteosarcoma cells (HOS) which expressed CD4/CCR5 (kindly provided by Dr. Ji Ming Wang, National Cancer Institute, Frederick, MD; Shen et al., 2000a) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies Inc.) supplemented with 10% low endotoxin FBS, 500 ug/mL puromycin (Sigma), and 2 mg/ml geneticin (Life Technologies, Inc.). The PBS-washed adherent cells were removed from the polystyrene T25 tissue culture flask using 2 ml of 1X trypsin EDTA (Sigma) and passed every 3 days at a 1 to 10 dilution.

2.4 Macaque PBMCs Functional Assay

The assay was defined in three steps: Treatment set-up, PBMC isolation, and supernatant collection. A. Treatment Set-up. Prior to the arrival of blood, 18 plate maps were constructed for 35 macaques (designations 4663-4697) at 2-cell density conditions. However, the focus of the present study was limited to 5 animals that were treated with a regimen of chronic morphine over a 3-month period of time. Various treatments using the RPMI media (without the 10% FBS and 10 μg/ml gentamicin) and the various compounds (μ- opioid receptor agonists, κ- opioid receptor agonists as well as Toll-Receptor agonists) were generated following the treatment table. Sterile basins were used to transfer a final volume of 50μL (per treatment; per well) to the eighteen 384-well plates by pipetting on the backside of each well. The plates were set-up at both high cell density (5x 10^4 cells/per well) and low cell density (1.25 x 10^4 cells per well). After all the treatments were added, plates carefully wrapped with parafilm and stored at 4°C until the next day when the PBMCs were available.

Table 2. A sample 384-well plate map showing the various treatment groups

PHA				**DAMGO 100pM + PHA**			
LPS				**DAMGO 1μM + LPS**			
IFNg/LPS				**DAMGO 10nM + LPS**			
PEPTIDO				**DAMGO 100pM + LPS**			
LIPO				**DAMGO 1μM + IFNg/LPS**			
IL4				**DAMGO 10nM + IFNg/LPS**			
IL13				**DAMGO 100pM + IFNg/LPS**			
IL4/IL13				**DAMGO 1μM + R848**			
CpGA				**DAMGO 10nM + R848**			
CpGB				**DAMGO 100pM + R848**			
R848				**MCP-1 10nG/mL**			
DAMGO 1μM				**MCP-1 50nG/mL**			
DAMGO 10nM				**MCP-1 100nG/mL**			
DAMGO 100pM				**Untrx**			
DAMGO 1μM + PHA				**Untrx**			
DAMGO 10nM + PHA				**Untrx**			
Animal #4663	Animal #4664	Animal #4665	Animal #4666	Animal #4663	Animal #4664	Animal #4665	Animal #4666

B. PBMC purification. Appropriate volumes of cells (adequate for every treatment condition) were brought to a concentration of $1x10^6$ cells/mL (High density) and $2.5x10^5$ cells/mL (Low density) in two different conical tubes for each animal. Using sterile basins, 50μL of cells (in R10 medium) from the low-density tube for each animal was transferred to the designated well of the low-density plate. Similarly, 50ul of cells from the high-density tube for each animal was transferred to the designated well of the high-density plate. This was repeated for all 35 animals. After the addition of the cells to the respective treatment plates (based on the plate map) they were transferred to the 37°C incubator for a 48 hr incubation.

C. Supernatant Collection. Supernatants were collected and stored in 384-well polypropylene plates. 60uL of sups were collected for each well without going all the way to the bottom of the well. For all the plates, this 60uL was split into three identical plates with 20uL in each. This not only allows for backups in case an ELISA or Luminex doesn't work but also prevents constant freezing and thawing cycles. The plates were labeled with the following connotation: Date, Plate#, Density (High vs. Low) - *Example: 06/13/2010 SC 1A H*. Plates are labeled 1A, 1B, 2A, 2B, etc according to the plate map file (see sample plate map file). Plates were then sealed with plate seals and stored at -80°C for further analysis.

2.5 Drug Treatments

A. Cell Treatments. In order to study the immune response under chronic opioid administration (especially cytokine production) a number of stimulants were utilized for treating the PBMCs including: Phytohemogglutinin-M (PHA; Boehringer Mannheim Biochemical), Interleukin 4 (Sigma Aldrich), Interleukin 13 (Sigma-Alderich), CpGa (Invivogen), CpGb (Invivogen), R848 (Invivogen), Peptidoglycan (Invivogen), Lipotechoic Acid (Invivogen), IFNg (Sigma-Aldrich), LPS (Invivogen) and SIV peptides Gag, Pol and Env (kindly provided by Dr. Michele Kutzler, Drexel University College of Medicine, Philadelphia, PA). PBMCs were treated with the various stimulants individually and in combination with DAMGO or U50, 488H at the concentrations described below. PBMCs were pretreated with PHA (2μg/ml), IL4 (20ng/ml), IL13 (20ng/ml), CpGa (1μM), CpGb (1μM), R848 (2μg/ml), Peptidoglycan (10μg/ml), Lipotechoic Acid (10μg/ml), IFNg (20 ng/ml), LPS (4ng/ml) for 48 hours and the supernatants were removed for analyzing chemokine levels using ELISA and Luminex assays.

B. Treatment of PBMCs with μ-Opioid Agonist, κ-Agonist and Antagonist. In the macaque functional assay, freshly isolated PBMCs were diluted to a final concentration of 5 x 10^4 cells and 1.25 x 10^4 cells per well in a 384-well plate in R10 medium. In designated wells from the plates, μ-opioid receptor agonist DAMGO (at concentrations ranging from 100nM to 1nM) was added. In other selected experiments, μ-opioid receptor antagonist CTAP (at a fixed concentration of 1μM) was added to the PBMCs. Additionally, in other experiments both DAMGO and CTAP were added in combination with other agonists at concentrations of 100nM, 10nM and 1nM (DAMGO) and 1μM (CTAP). The κ-opioid receptor agonist U50, 488H was added to the PBMCs at concentrations of 1μM, 10nM and 100pM, while the κ-opioid receptor antagonist NORBNI was added at a fixed concentration of 1μM. In other designated plates U50, 488 H and NORBNI were added in conjunction with other defined agonists at the above-mentioned concentrations.

To determine the effect of DAMGO on downstream signaling pathways in human PBMCs, freshly isolated PBMCs were diluted to a final concentration of 0.5x 10^6 cells/ml in R10 medium and 2 ml of cells were seeded into each well of a 6 well polystyrene tissue culture plate. Cells were cultured for 24 hours in the absence of serum. After the initial 24-hour culture, these PBMCs were treated with DAMGO at a concentration of 100nM. Total protein was extracted from 2 “pooled” wells of PBMCs after 0, 5, 10, 15 minutes of DAMGO administration, and downstream phosphor-Erk activation was determined by western blot analysis.

To determine the effect of DAMGO on cytokine transcript expression in PBMCs, freshly isolated PBMCs were diluted to a final concentration of 0.5x 10^6 cells/ml in R10 medium and 2 ml of cells were seeded into each well of a 6 well polystyrene tissue culture plate. Cells were treated with DAMGO and RNA was extracted from 6 "pooled" wells of PBMCs after 6 and 24 hrs of DAMGO administration. Supernatants and/or cells were harvested at the designated time points after DAMGO treatment and chemokine protein and mRNA expression was determined by ELISA or quantitative PCR and microarray analysis.

2.6 Measurement of Chemokine Protein Levels by ELISA

The concentration of chemokine proteins present in the various culture supernatants was determined by ELISA, using matched mouse monoclonal or polyclonal capture and detection antibodies in a sandwich ELISA. The optimal concentration of anti-chemokine "capture" antibodies (R & D Systems) used in these experiments were: 4 μg/ml monoclonal anti-human IL-8 (DY 208), 1μg/ml monoclonal anti-human MCP-1 (DY279), and 2μg/ml monoclonal anti-human MDC/CCL22 (DY336). The capture antibody stocks (720μg/ml (IL-8), 180μg/ml (MCP-1) and 360μg/ml (MDC)) were diluted in PBS, and 100 μl were coated onto plastic 96 well, flat bottom maxisorb microwell plates (Nunc), sealed with parafilm and aluminum foil overnight at 4 C. The next day, the plates and solutions were brought to room temperature, and capture antibody solution was removed by tapping plates on a stack of paper towels. Non-specific binding was blocked with 200 µl of blocking buffer (1% BSA; Life Technologies) in PBS at room temperature for 1 hour. The plates were washed 3 times with 100 µl of PBS/Tween 20 (0.5 ml of Tween 20 into 1 L of PBS) using an 8-well vacuum manifold plate washer (Nunc). The plates are blotted on paper towels in between washes. One hundred µL of the IL-8, MCP-1, and MDC standards (R &D Systems) and samples diluted in blocking buffer/Tween 20 were added to each well. To obtain linear standard curves, human IL-8 ranging from 2000 to 15.625 pg/ml, human MCP-1 ranging from 1000 to 7.81 pg/ml, and human MDC ranging from 500 to 3.90 pg/ml were used. After the standards and samples were added, the plates were sealed and incubated at room temperature for 2 hours. The plates were washed 4 times with 100 µl of PBS/Tween 20, blotted, and detector antibodies were added to the wells. Optimal concentrations of biotin-conjugated monoclonal anti-human IL-8 (DY208) (20ng/ml), monoclonal anti-human MCP-1 (DY279) (100ng/ml), and monoclonal anti-human MDC (DY336)(50ng/ml) were diluted in 100 µl of blocking buffer/Tween 20 and added to the appropriate wells, sealed, and incubated for 1 hour at room temperature. The plates were washed 4 times with 100 µl of PBS/Tween 20, blotted, and 100 µl of 1: 20 of HRP-linked streptavidin (R &D Systems) diluted in blocking buffer/Tween 20 was added to each well, sealed, and incubated at room temperature for 30 minutes. Before addition of the substrate, the Solution A &B (described below) substrate solution was thawed within 20 minutes of use. After the HRP-streptavidin incubation, the plates were washed 4 times with

100 μl of PBS/Tween 20, blotted, and 100 μl of the substrate solution (1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems Catalog # DY999) is added to each well. The plates were sealed, and incubated at room temperature for 25 minutes for color development. The color reaction was stopped by adding 50 μl of stop solution (2 N H2SO4(R&D Systems Catalog # DY994)). The level of colored product was measured spectrophotometrically (Molecular Devices) at 405 nm. For plotting purposes, stimulation Index was calculated for each treatment group for chemokines- IL-8, MCP-1 and MDC. The Mean stimulation index (+SD) of all 5 animals was then plotted for each treatment group. Mann-Whitney and t-test were used for Statistical analysis.

Luminex 5-plex/ 28-plex assay

The concentration of chemokine proteins present in the various culture supernatants was also determined by using multi-analyte (28-plex, and a 5-plex) kits (Invitrogen (Catalog #: LPC0003M) and EMD Millipore (Catalog# PRCYTOMAG-40k-05) respectively). All analytes are in the form of magnetic beads, that are sonicated, and vortexed for about a minute before adding 60μl of each analyte to the mixing bottle. 10X Wash buffer that needs to be diluted to a 1X, and a standard was prepared using five-fold dilutions ranging from 10000pg/ml to 0.64pg/ml. The assay plate was wetted by adding 200μl Assay Buffer for 10 minutes. Assay Buffer is then removed using a vacuum manifold. 25μl of standard and quality Controls were added to the designated wells on the plate map. An additional, 25µl of R10 media was added to the standard and control wells. 25µl of assay buffer then added to the sample wells, followed by the 25µl of sample to the appropriate wells as designated on the plate map. The mixing bottle containing the beads was vortexed and about 25µl was added to each well. The plate is then sealed and incubated on a plate shaker overnight at 4°C. The fluid was then removed using the vaccum manifold, the plate was washed, and the buffer removed by vacuum. 25µl of the detection antibodies was added to each well. The plate was sealed and incubated on a plate shaker at room temperature for 1 hour. This is followed by the addition of 25µl phycoerythin (PE) to each well. The plate was again incubated for about 30 minutes on the plate shaker following the removal of buffers by vacuum. The plate was washed twice with 200µl per well 1X wash buffer, and finally, 150 µl of sheath fluid was added to all the wells. Beads are resuspended on a plate shaker for 5 minutes before being read on the Luminex 200.

2.7 Mammalian Cell Transfection

Human osteosarcoma cells (HOS), which expressed CD4/CCR5, were transfected with the vector construct pCDNA3.1 containing hMOR-FLAG using Lipofectamine 2000 (Invitrogen). Briefly, the day before transfection, cells were seeded at 3 x 10^5 cells per well into each well of a 24-well plate. For each transfection 0.8 µg of vector construct was diluted in 100 µl of Opti-MEM®

(Invitrogen). 2 µl of Lipofectamine 2000 was also diluted into 100 µl of Opti-MEM® (Invitrogen) and allowed to incubate for 10 minutes at room temperature. The diluted DNA and Lipofectamine are then mixed and set aside for 25 minutes at room temperature to allow for the formation of DNA: Lipofectamine 2000 complexes. Following this incubation, 100 µl of complexes were added to each well containing cells and gently mixed. Transfected cells were then allowed to recover at 37°C/5% CO_2 for 24-48 hours prior to assay.

2.8 Western Blot Analysis

To determine phospho-Erk protein content, cell lysates were analyzed using western blot analysis. Phorbol 12-myristate 13-acetate (PMA) -treated extracts were used as a positive control as they are shown to give a positive Erk response for all DAMGO responsive western blot experiments. For all western blots, the total protein concentration of samples was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Optical density (O.D.) was measured at 595 nm and concentration was determined based on a standard curve generated using known concentrations of BSA (Pierce). Whole cell lysates were prepared using RIPA buffer (150mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50mM TRIS). Protease inhibitor cocktail set II (Calbiochem) and phosphotase inhibitor cocktail (Sigma), containing cantharidin, which inhibits protein phosphatase 2A, bromotetramisole, which inhibits alkaline phosphatases, and microcystin LR, which inhibits protein phosphatases 1 and 2A, are added to RIPA buffer immediately prior to use. Samples are then collected and supernatants are removed. RIPA buffer is added (approximately 1 ml of buffer per $2x10^{-6}$ PBMCs) and cells are placed on a rotating wheel at 4°C for 30 minutes. Samples are then centrifuged at 13000 rpm for 15 minutes at 4°C. The supernatants that contain the cellular proteins are then removed and stored at -80°C. 15-30 μg of lysates were then mixed with 4X loading dye and heated to 95°C for 5 minutes. Samples were then cooled on ice for 1 minute and loaded onto a NUPAGE bis-tris 4-12% gel (Invitrogen). NUPAGE gels were run with 1X SDS MOPS running buffer (Invitrogen) at 150 V for 1 hour and 30 minutes on ice in the X-Cell Blot System. The gel is then transferred onto a nitrocellulose (.45μm) membrane (Pierce) in X transfer buffer (Invitrogen) and at a constant 35 V for 2 hrs on ice. The membrane was stained with Ponceau S to ensure equal loading. Ponceau S was then removed with 1X TBST washes and membranes were blocked for 30 minutes in 1X TBST containing 5% BSA or 5% w/v dry non-fat milk at room temperature. Primary antibody was added and incubated overnight at 4°C with mild agitation. The following day, membranes were placed at room temperature and washed for 30 minutes in 1X TBST at room temperature with shaking. Secondary antibody conjugated with horseradish peroxidase (HRP) was diluted (1:5000) in 5% milk in 1X TBST and incubated with membranes for 1 hour at room temperature with shaking. Membranes are then washed for 30 minutes as previously described and developed with ECL Plus (Amersham) according to manufacturer's protocol. The chemiluminescent signal was recorded with autoradiography film (Lab Scientific) and developed.

2.9 RNA Isolation and Quantitation

PBMCs were harvested and the cell pellet was resuspended in TRIzol® (Invitrogen) in 1.5ml RNase-free microfuge tubes (Ambion). 200µl of BCP phase separation reagent (Fisher Scientific) was then added to each tube. The samples were vigorously shaken and then centrifuged at 13000 rpm for 15 minutes at 4°C. The upper aqueous phase was then transferred to a new tube and mixed with 0.5ml of isopropanol. Samples were allowed to incubate for 10 minutes at room temperature followed by centrifugation at 12000 rpm for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol followed by centrifugation for 5 min at 8000 rpm. The RNA is then allowed to dry at room temperature for approximately 5 minutes and dissolved in 20-30µl of nuclease-free water.

The concentration and purity was assessed by optical density (OD). Two µl of RNA was diluted in 998 µl of water and the optical density at both 260 nm and 280 nm was determined using a Spectronic Instruments spectrophotometer (Fisher Scientific). RNA concentration was determined using the formula:

Unknown mass (mg) = (sample OD_{260})(40 μg/ml) X dilution factor

The ratio of the OD_{260} to OD_{280} was used as an indication of the RNA purity, and these values were usually between 1.70 and 1.95. Occasionally diethyl pyrocarbonate (DEPC) gels were run as an additional means of verifying the integrity of the RNA. These gels were prepared with 1X TAE solution (40 mM Tris-acetate and 1 mM EDTA pH 8.0) containing sterile DEPC-treated water and 1.2% agarose. The RNA generated was submitted to Penn Microarray Core Facility (UPEN) for microarray analysis (~1μg) and was later used for quantitative RT-PCR analysis in the lab (see below).

2.10 Reverse Transcriptase Reaction

RNA was subjected to DNase treatment using Turbo DNA-free (Ambion, Austin, TX) and 1-3 μg was used for cDNA synthesis using Invitrogen SuperScript III first-strand synthesis kit. The manufacturer's protocol for first-strand synthesis was followed using 100-200 ng of random hexamers. The cDNA product was diluted 1:200 into water and the OD was measured at both 260 nm and 280 nm using a Spectronic Instruments spectrophotometer (Fisher Scientific, Pittsburgh, PA) for quality and concentration.

Quantitation of cDNA following RNA isolation and the reverse transcriptase reaction was completed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems). 50-500 ng of cDNA was used for each real time PCR reaction. Real time PCR was performed with Taqman gene expression kit. PCR primers were used as follows:

Table 3. Real Time Primers

Genes	Amplicon length	Catalog#
AHR	105	**Hs00169233_m1**
TLR4	89	**Hs00152939_m1**
CXCL10	98	**Hs00171042_m1**
GAPDH	122	**Hs99999905_m1**

Primers were used at a concentration of 1 μM and real time reactions were generated using the Taqman Universal PCR Master Mix (Catalog# 4304437). PCR conditions were 95°C for 10 min, 50°C for 30 sec, followed by 40 cycles of 95°C for 15sec, 60°C for 5 sec and cooling at 40°C for 30 sec. Data analysis was carried out using ABI StepOnePlus™ software. Target gene cDNA levels were determined by comparing the ΔCt values with the housekeeping gene GAPDH. Data are represented as the results from five donors.

Chapter 3

RESULTS

3.1 The effect of chronic morphine administration on leukocytes population dynamics in non-human primates

Our laboratory has previously reported results, which show that acute administration of morphine or other μ-opioids, up-regulate the expression of CCL2, CCL5 and CXCL10 in human PBMCs (Wetzel et al., 2000). However, very little is known about the effect of chronic morphine (or heroin) administration in vivo, particularly in humans or non-human primates. In an effort to extend our understanding of the effect of morphine on cytokine expression, a macaque model was devised to analyze the responses to morphine either with or without SIV infection. The studies described here focus on the effect of chronic morphine administration.

The morphine study animal group consisted of 5 non-infected SIV animals that were followed for 21 weeks and during this time blood samples were collected at 4-week intervals so that leukocyte population dynamics could stabilize. A blood sample was collected again at week 25, and the following day morphine administration was initiated (Figure 1). The morphine administration protocol consisted of measuring the general immune parameters for the macaques including total WBC, monocyte and CD4 and CD8 counts by FLOW Cytometry at BIOQUAL, INC.

We conducted this analysis because of the significance of these cell populations for resistance to infectious agents, particularly SIV. Reductions in these populations are primary indicators of the overall level of immune suppression or damage done by the virus. Moreover, monocytes and macrophages are important targets of HIV/SIV infection and a critical link between innate and adaptive immunity (Hasegawa et al.; 2009). For these reasons we wished to determine whether morphine induces changes in these cell populations. The results show (Figure 2, Panel A) that there is no significant changes in the WBC count following morphine administration compared with non-morphine treated groups.

The results from analysis of the CD4/CD8 ratio (Figure 2, Panel B) show that morphine administration has no significant effect on this characteristic of T cell populations. Further analysis also shows that the absolute numbers of CD4 and CD8 cells are not altered by the administration of morphine (data not shown). Finally, further analysis of the blood shows that morphine treatment does not alter the numbers of circulating monocytes (Figure 2, Panel C).

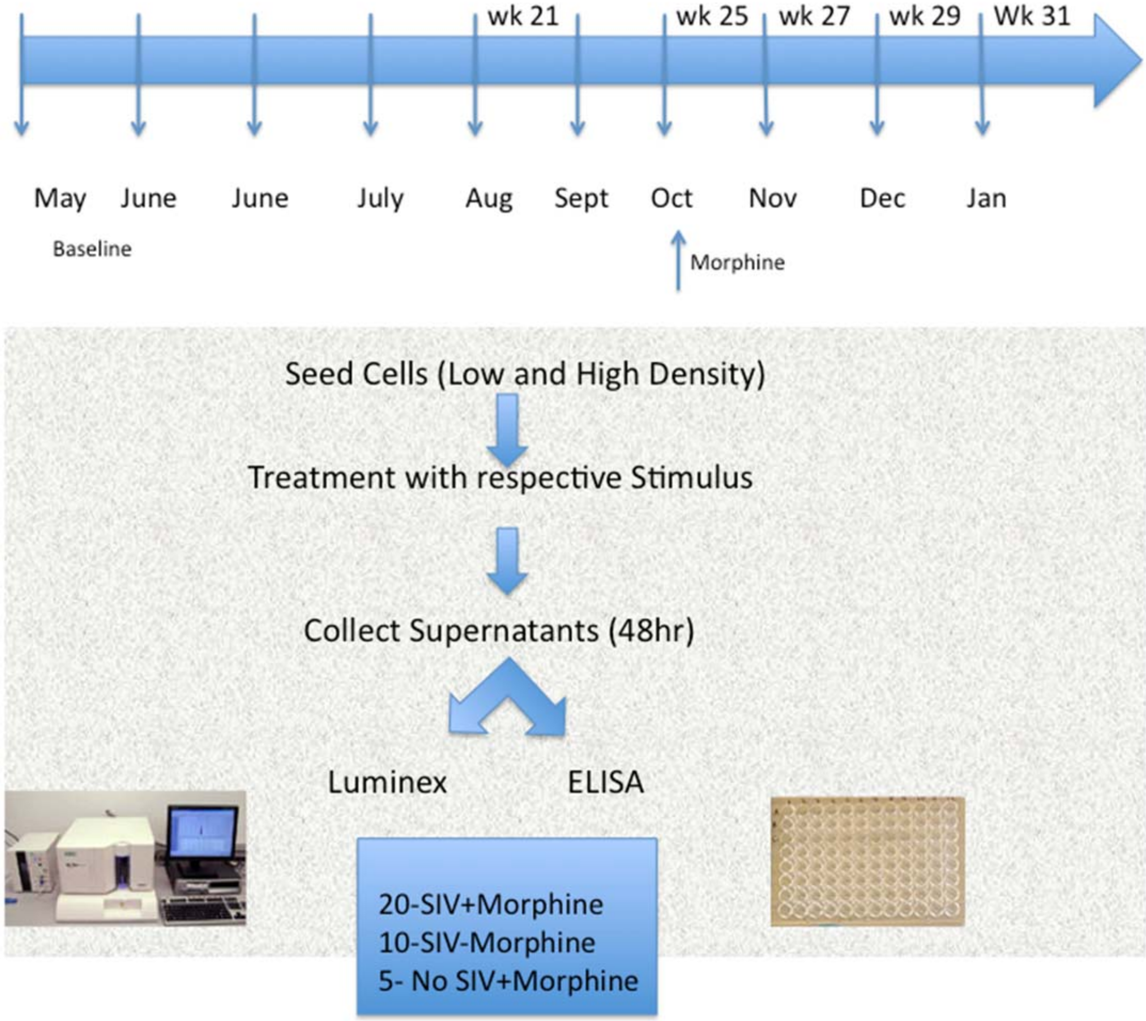
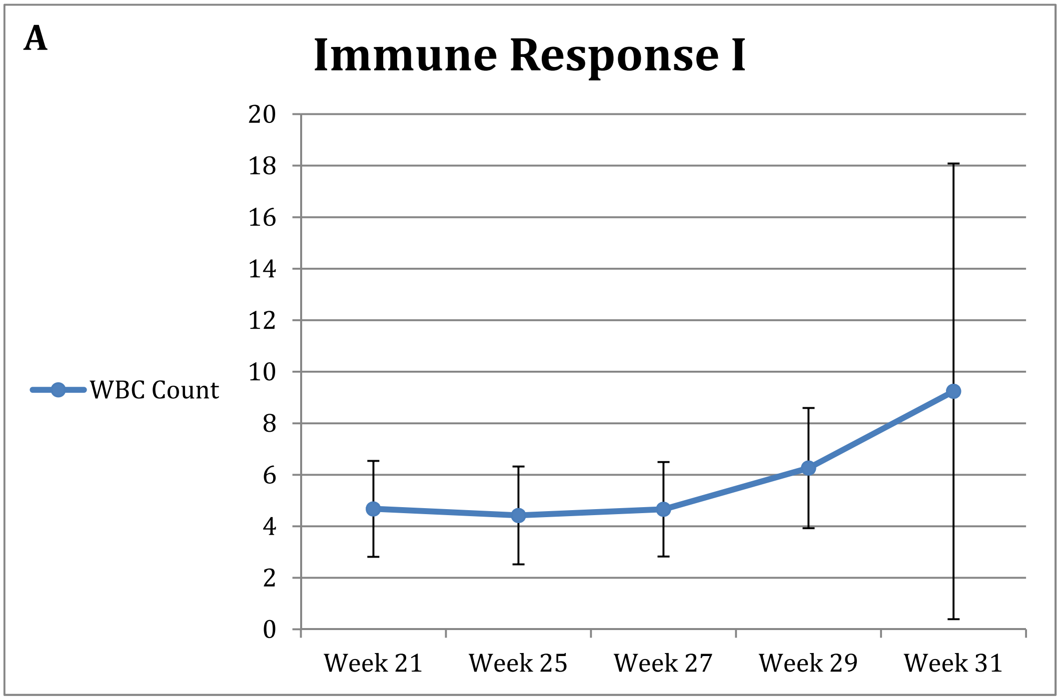
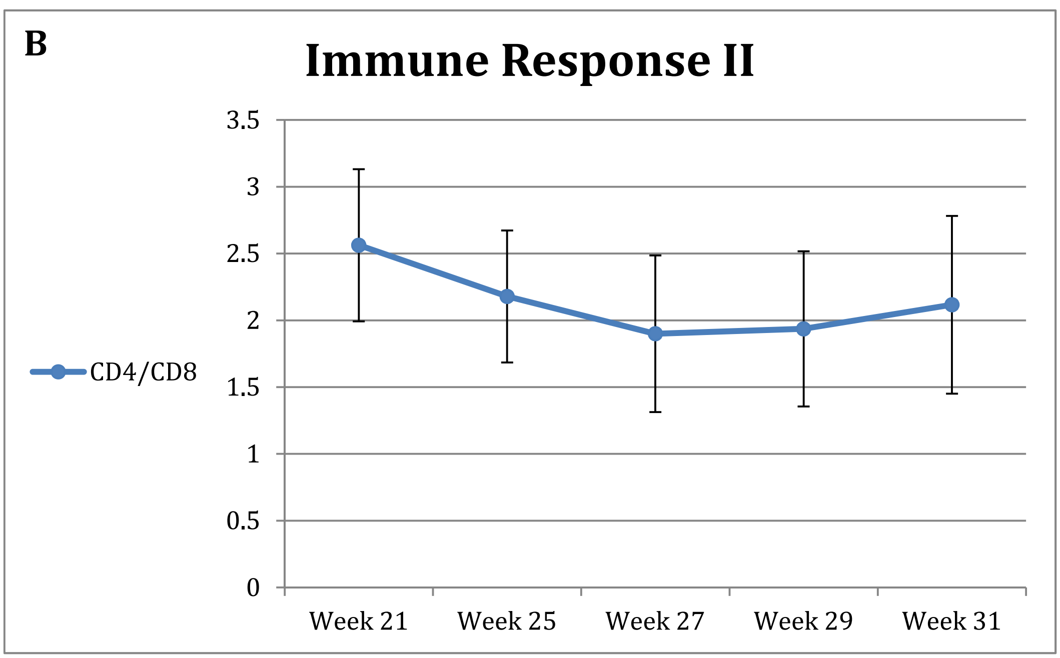


Figure 1. Brief schematic of the research strategy used in the macaque study. The first flowchart depicts the timeline (through-months) of the study; downward arrows represent the drawing of the blood each month; upward arrows denote the beginning of the corresponding treatments. The second flowchart defines the layout of the experiments post Macaque PBMC Isolation. A total number of 35 non-human primates were used in these studies, which were divided into three groups; the first group of 20 was given SIV and morphine; the second group of 10 was given SIV but no morphine, while the third group of 5 was given only morphine. The focus of my study will be on these 5 morphine only animals.


A
Immune Response I
WBC Count
20
18
16
14
12
10
8
6
4
2
0
Week 21
Week 25
Week 27
Week 29
Week 31


B
Immune Response II
CD4/CD8
3.5
3
2.5
2
1.5
1
0.5
0
Week 21
Week 25
Week 27
Week 29
Week 31

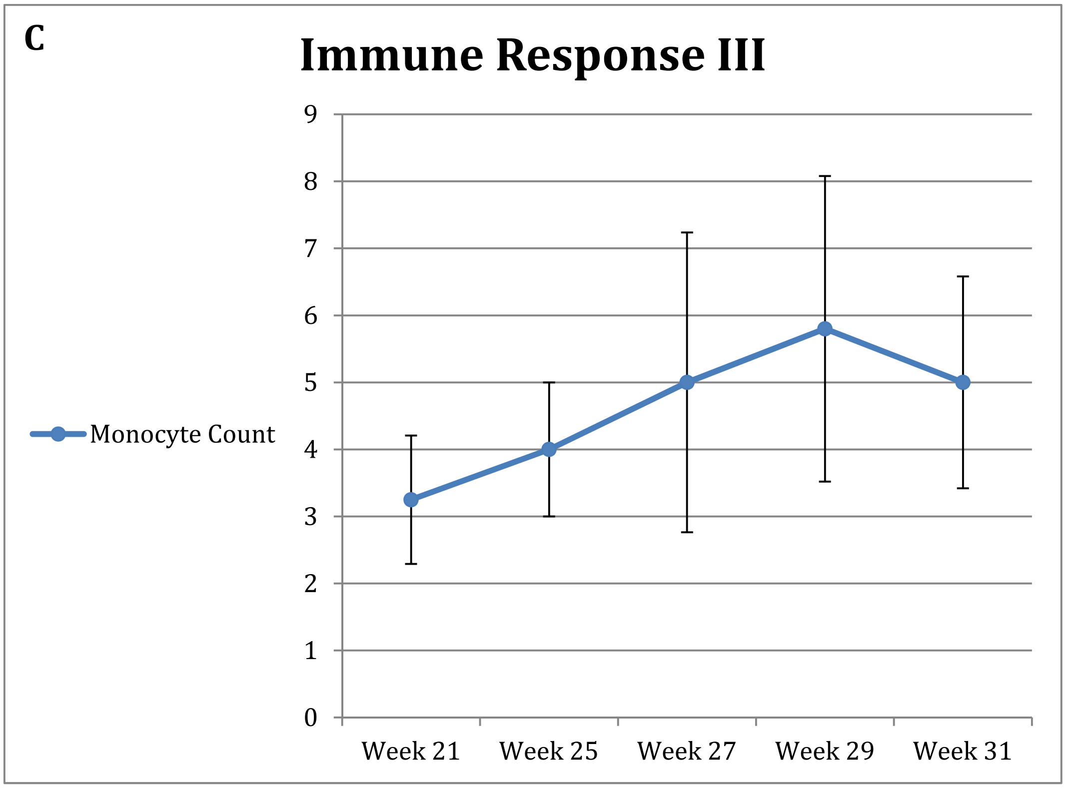


Figure 2. Macaque (Non-infected/Non-treated) Immune response to chronic Morphine Panel A show changes in the total white blood cell (WBC) count. Panel B show changes in the CD4/CD8 ratio & Panel C show changes in the total monocyte count. At week 25, morphine was administered chronically to the animals. The results are presented as the Mean (±SD) of 5 animals at each time point

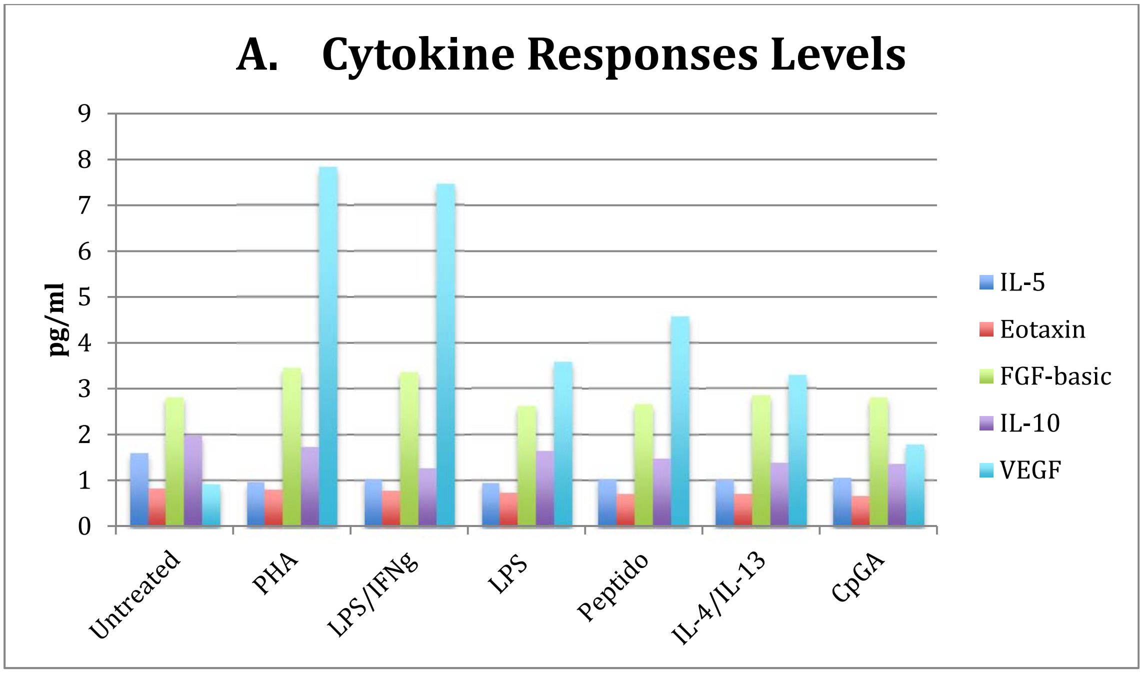
3.2 The effect of Chronic Morphine Administration on Cytokine Expression in Macaques

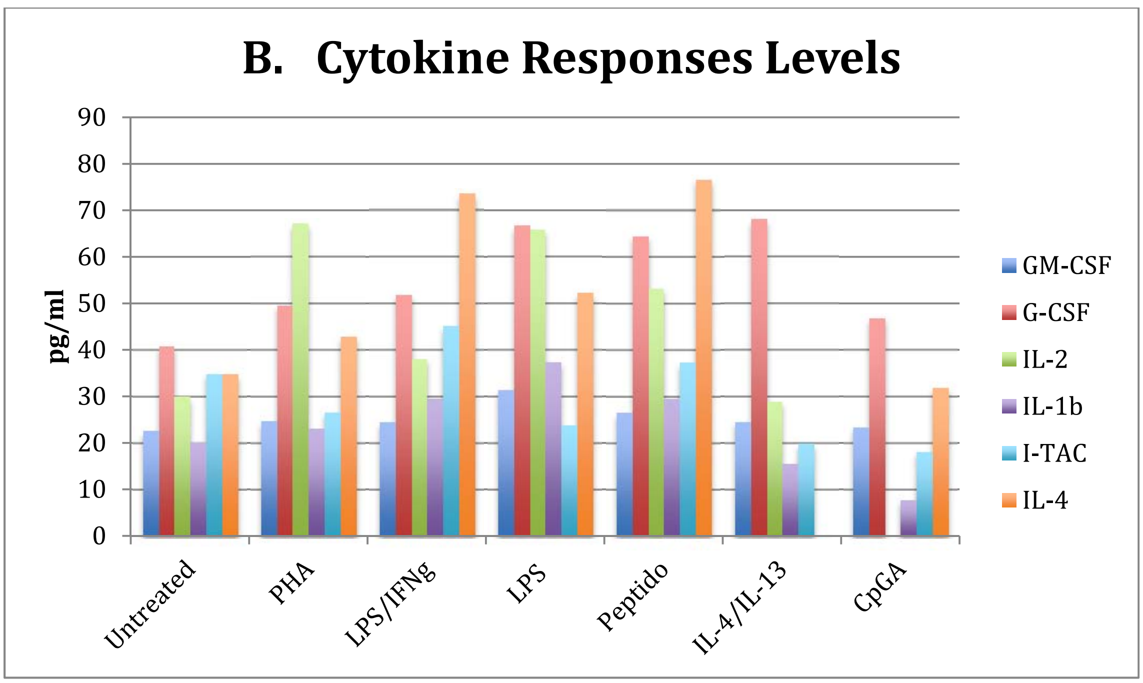
We wanted to examine the status of the immune system following morphine administration in these macaques. To analyze the immune competence of these macaques we first needed to establish an effective experimental system where we could study and evaluate the expression of certain cytokines and chemokines in response to various activators of leukocyte function. We employed a variety of leukocyte activators for these studies, including; PHA (mitogen to trigger cell division in T-cells), LPS (TLR2 Stimulant), IFNγ/LPS (activates T cells and monocytes), IL-4 (humoral and adaptive immune response, class switching), and IL-13 (involved in allergic inflammation, induces m2 macrophage activation, activates STAT6). Additionally, toll-like Receptor responses were examined using activators like CpGA (activates TLR9), CpGB (activates TLR9 in dendritic cells), R848 (activates TLR 7/8) Peptidoglycan (activates TLR2) and Lipoteichoic acid (activates CD14 and TLR2)

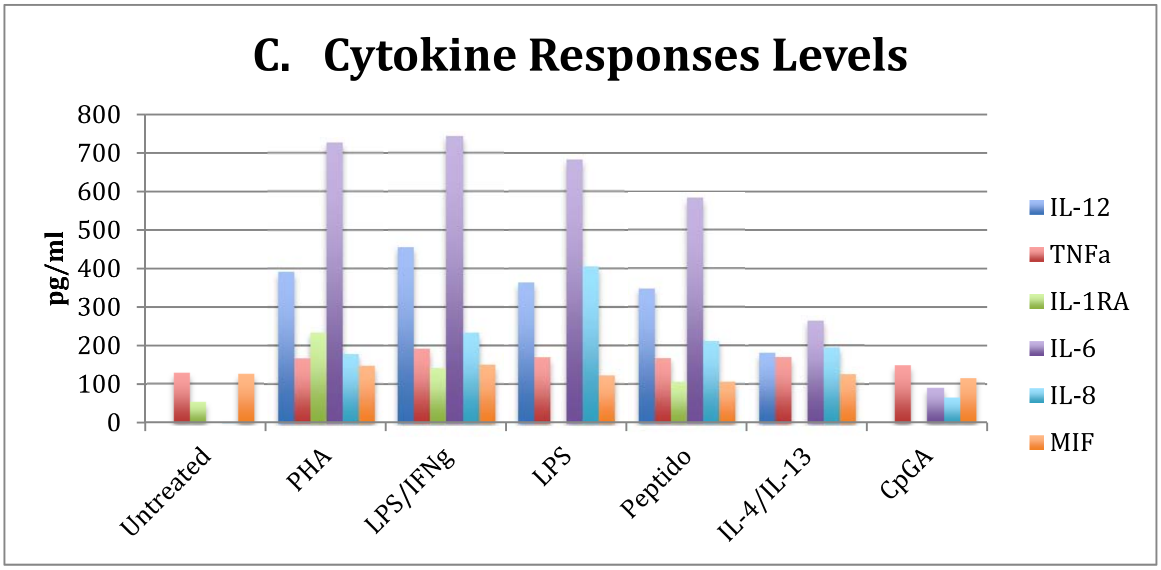
A PBMC sample from a baseline month (Week 0) non-infected macaque (Animal #4684) was treated with PHA (2μg/ml), IL-4 (20ng/ml), IL-13 (20ng/ml), CpGa (1μM), Peptidoglycan (10μg/ml), IFNγ (20 ng/ml) or LPS (4ng/ml). Supernatants were harvested after 48 hours, and the levels of a number of analytes were determined. The analytes tested include-IFNγ, TNF-A, CXCL8, CCL2, CCL22, IL-12, IL-6, IL-5, IL-2, Eotaxin, GM-CSF, FGF, IL-10, EGF, HGF, IL-15, IL-17, MIG, VEGF, G-CSF,

IL-2, IL-4, I-TAC, IL-1β, MIP1α, MIP1β, RANTES, IL-1RA and MIF. The purpose of this analysis was to determine which cytokines these activating agents in non-human primates induce. We also wished to verify that the luminex materials would successfully detect the various analytes.

The results were divided into four categories; Figure 3 Panel A shows the cytokines that were produced in the range of 1-10pg/ml. VEGF was secreted in response to all the activators with production as high as 7.5pg/ml with PHA-stimulation. No major changes were observed for other cytokines like IL-10, IL-5, Eotaxin and FGF-Basic. Figure 3 Panel B shows the cytokines that were expressed in the range of 1-90pg/ml. I-TAC, GM-CSF and IL-1β showed no alterations when compared to the control. On the other hand, IL-4 was up regulated in response to LPS/IFNγ and Peptidoglycan, IL-2 showed an increase under PHA, LPS and Peptidoglycan-stimulation, and G-CSF was induced in response to LPS, IL-4/13 and Peptidoglycan. Figure 3 Panel C shows the cytokines that were produced in the range of 1-800pg/ml. MIF, IL-1-RA and TNF-A showed no significant changes when compared to the control. However, IL-12, IL-6 and CXCL8 were significantly up regulated in response to PHA, LPS, LPS/IFNγ, Peptidoglycan and IL-4/13. Finally, Figure 3 Panel D shows the cytokines that were expressed in the range of 10-10000pg/ml. Both CCL22 and CCL2 were produced in response to all the activators with production as high as 10,000pg/ml of CCL22 and 4000pg/ml of CCL2 following being produced under IL-4/13 or LPS/IFNγ stimulation, respectively. Finally, Table 4 shows the cytokines that were produced at very low levels or were undetectable with the exception of IFNg, which was produced in response to stimulants PHA or LPS/IFNg.


A. Cytokine Responses Levels
pg/ml
0
1
2
3
4
5
6
7
8
9
Untreated
PHA
LPS/IFNg
LPS
Peptido
IL-4/IL-13
CpGA
IL-5
Eotaxin
FGF-basic
IL-10
VEGF


B. Cytokine Responses Levels
pg/ml
0
10
20
30
40
50
60
70
80
90
Untreated
PHA
LPS/IFNg
LPS
Peptido
IL-4/IL-13
CpGA
GM-CSF
G-CSF
IL-2
IL-1b
I-TAC
IL-4



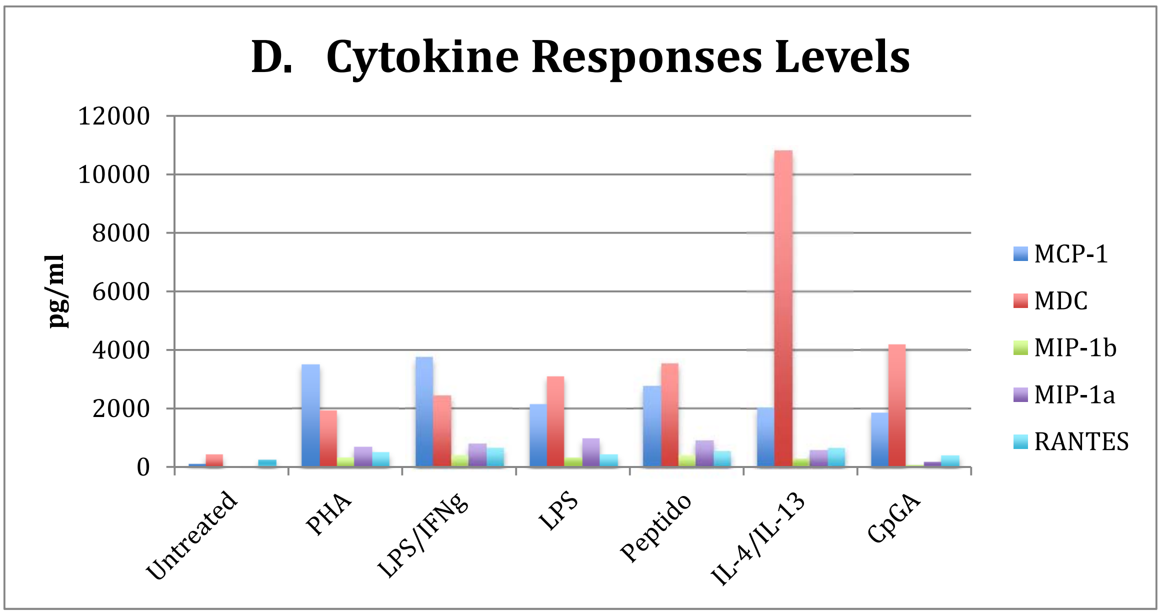


Figure 3. Cytokine and Chemokine Immune response in Macaque # 4684 (Week 0, Non-Treated) using the 28-plex-macaque multianalyte kit. Panel A & B represent distinct changes in multiple analytes that were expressed in low amounts Panel C & D represent distinct changes in multiple analytes that were expressed in higher amounts. PBMCs were pretreated with PHA (2μg/ml), IL-4 (20ng/ml), IL-13 (20ng/ml), CpGa (1μM), Peptidoglycan (10μg/ml), IFNγ (20 ng/ml), LPS (4ng/ml). Supernatants were harvested after 48 hours, and Luminex Analysis determined the levels of different analytes.

Table 4. Cytokines that were produced at very low levels or were undetectable

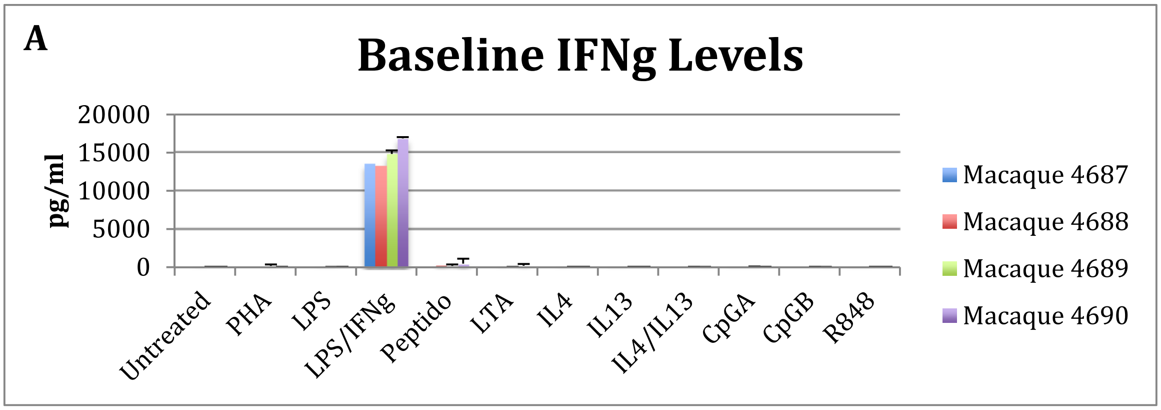
Animal	Treatment	EGF	HGF	IFNg	IL-15	IL-17	MIG
4684	Untreated	6.4	13.4	12.4	16.4	8.9	5.3
	PHA	19.4	27	10506.4	16.4	8.9	4.9
	LPS/IFNg	4.9	13.4	11640.7	16.4	8.9	4.9
	LPS	3.3	13.4	7.8	16.4	8.9	4.9
	Peptido	8.4	24	7.8	16.4	8.9	4.9
	IL-4/IL-13	2.7	13.4	7.8	16.4	8.9	4.9
	CpGA	2.7	20.2	7.8	16.4	8.9	4.9

Next we wanted to look in greater detail at specific cytokines that are significant in terms of disease progression and are produced in larger amounts in response to the various stimulants in the absence of morphine. We focussed on five cytokines (IFNγ ,CCL2, TNF-A, IL-12p40 and CXCL8) for this study and employed four different animals (#4687,#4688,#4689,#4690) to generate the results. PBMC samples from a baseline month (week 0) were treated with PHA (2μg/ml), IL-4 (20ng/ml), IL-13 (20ng/ml), CpGa (1μM), CpGb (1μM), R848 (2μg/ml), Peptidoglycan (10μg/ml), Lipoteichoic Acid (10μg/ml), IFNγ (20 ng/ml), LPS (4ng/ml), and U50 (1μM). Supernatants were then harvested after 48 hours, and the levels of 5 cytokines were determined.

Animal 4687 showed an 8 fold increase, 4689 showed a 5-6 fold increase, and 4690 showed a 4-5 fold increase in CXCL8 expression in response to PHA, LPS and LPS/IFNγ. Animal 4688 showed no significant changes in the CXCL8 levels in response to PHA, LPS and LPS/IFNγ when compared to the untreated samples. Animal 4687 showed a 9-12 fold increase, 4688 showed a 2.5 fold increase, 4689 showed a 6-7 fold increase and 4690 showed a 6-10 fold increase in the CXCL8 expression in response to peptidoglycan and LTA as compared to the control. Animal 4687 showed a 6-9 fold increase, 4688 showed a 2 -3 fold increase, 4689 showed a 6-7 fold increase and 4690 showed a 6-7 fold increase in CXCL8 levels in response to IL-4, IL-13 and IL-4/13 as compared to the untreated samples. Animal 4687, 4688 and 4689 showed a 2 fold increase in CXCL8-levels in response to CpGa and CpGb. However animal 4690 showed no change in the CXCL8 levels in response to both CpGa and CpGb. Animal 4687 showed a 6-fold increase in CXCL8 levels as compared to a 3-fold increase in animals 4688,4689 and 4690 in response to R848 (Figure 4 Panel B). Macaques 4687, 4688 showed a ~30-fold increase and 4689 showed a ~10-fold increase in the CCL2 levels in response to PHA, LPS and LPS/IFNγ as compared to the untreated samples. Animals 4687, 4688 and 4689 showed a 4-6 fold increase in the CCL2 levels in response to Petidoglycan and a ~30, ~80, ~15-fold increase in response to LTA as compared to the untreated samples. Additionally, animals 4687 and 4688 showed a ~15 and ~50 fold increase compared to a 6-8 fold increase in animal 4689 in the CCL2 levels in response to IL-4, IL-13 and IL-4/13. Animal 4690 consistently showed no change in the CCL2 levels in response to the various activators except a 5-fold increase in response to R848 (Figure 4 Panel C) . Animals 4687 and 4689 showed a ~12 fold increase and 4688 showed a~2-fold increase in TNF-A levels in response to PHA, LPS and LPS/IFNγ.

Animals 4687and 4688 showed a~5 fold increase, while animals 4689 and 4690 showed a ~12 fold increase in TNF-α levels in response to IL-4/13 (Figure 4 Panel D). IL-12p40 levels were significantly low with animal 4687, 4688, 4689 and 4690 showing a ~2-4 fold increase in IL-12p40 in response to PHA, LPS and LPS-IFNg, LTA, IL-4/13 and R848 (Figure 4 Panel E).

Finally, no macaques produced detectable amounts of IFNγ in response to the various stimulants except possibly the IFNγ/LPS group, which also served as an internal control. It is not possible to be certain that these macaque cells failed to exhibit an IFNγ response to any of these activating agents (Figure 4 Panel A). It is also possible that the luminex antibodies failed to detect low levels of indian rhesus macaque IFNγ.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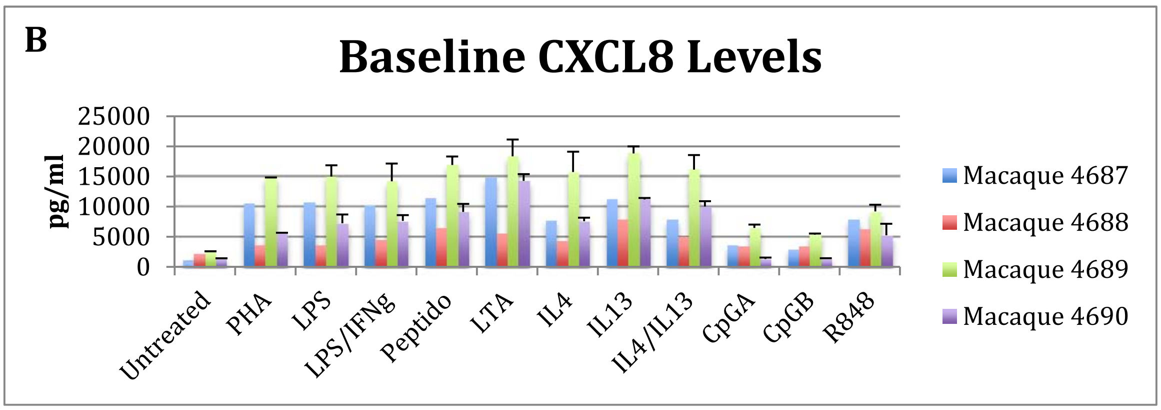
Baseline IFNg Levels
20000
15000
10000
5000
0
pg/ml
Untreated
PHA
LPS
LPS/IFNg
Peptido
LTA
IL4
IL13
IL4/IL13
CpGA
CpGB
R848
Macaque 4687
Macaque 4688
Macaque 4689
Macaque 4690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Baseline CXCL8 Levels
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20000
15000
10000
5000
0
pg/ml
Untreated
PHA
LPS
LPS/IFNg
Peptido
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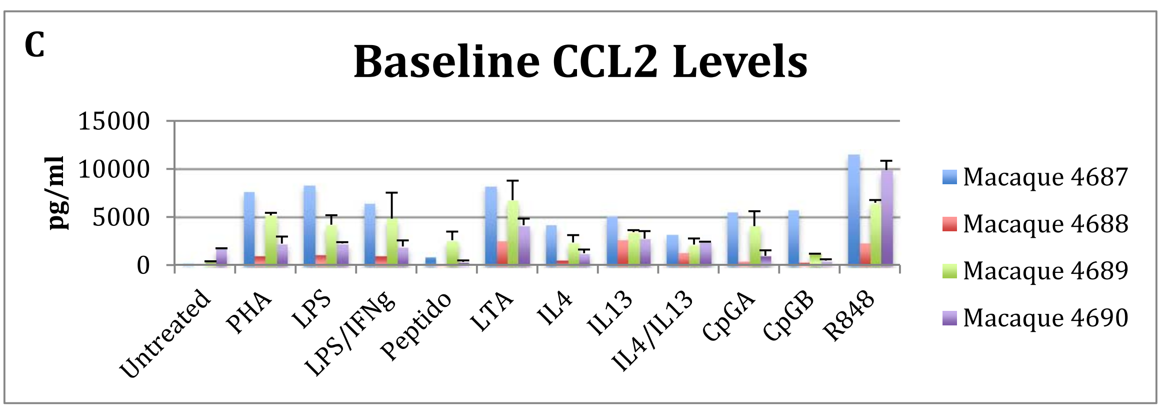
Macaque 4689
Macaque 4690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Baseline CCL2 Levels
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10000
5000
0
pg/ml
Untreated
PHA
LPS
LPS/IFNg
Peptido
LTA
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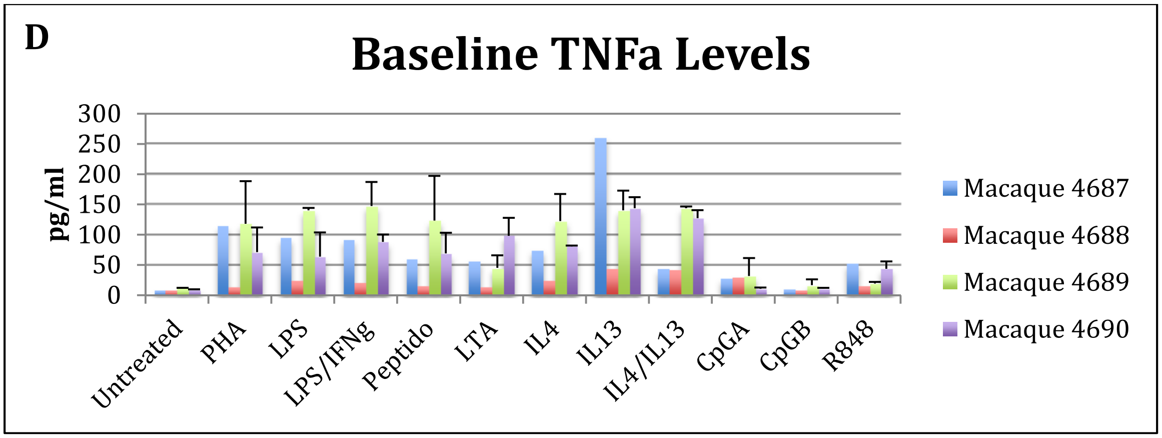
Macaque 4689
Macaque 4690



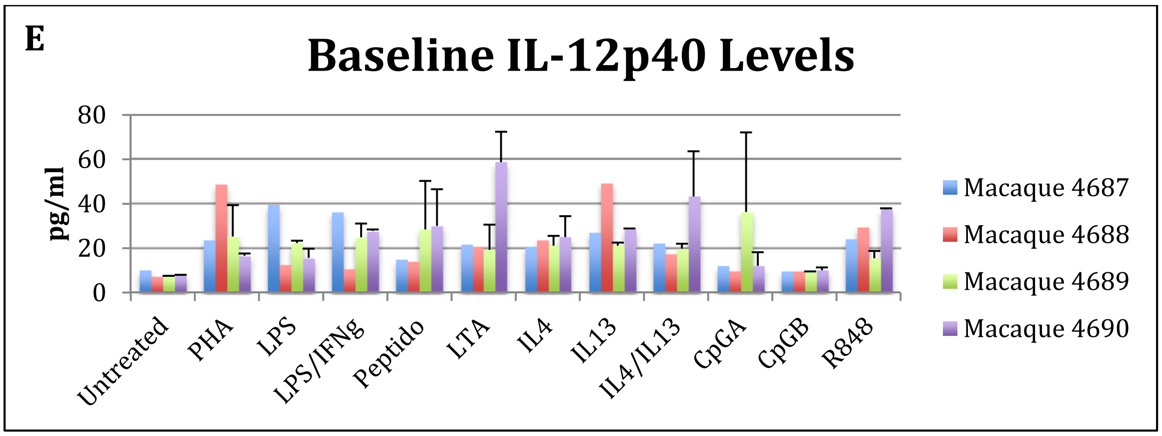


Figure 4C. IFNγ, CXCL8, CCL2, TNF-α, IL-12p40 cytokine responses in macaques (non-infected). Freshly isolated PBMCs were diluted to a final concentration of 1x10^6 cells/mL and 50μl of cells were plated into each well of a 384-well tissue culture plate. Cells were pretreated with PHA (2μg/ml), IL-4 (20ng/ml), IL-13 (20ng/ml), CpGa (1μM), CpGb (1μM), R848 (2μg/ml), Peptidoglycan (10μg/ml), Lipotechoic Acid (10μg/ml), IFNγ (20 ng/ml), LPS (4ng/ml), and the κ-opioid U50, 488H (1μM). Supernatants were harvested after an additional 48 hours, and Luminex Analysis determined the levels of different analytes.

We wanted to determine the effect of chronic morphine on the expression of three critical pro-inflammatory chemokines CCL2, CCL22 and CXCL8 in each of the five macaques (#4684, #4689, #4690, #4695, #4697). Since all three of these critical chemokines have an established role in antimicrobial immunity and exhibited strong responses in the earlier baseline experiments we wanted to analyze them in greater detail. The pre-morphine PBMC samples were obtained during week 23 of the study prior to the initiation of morphine administration, while the post-morphine PBMC samples were obtained from blood samples obtained at 90 days post-morphine administration. Freshly isolated PBMCs were pretreated with PHA (2μg/ml), IL-4 (20ng/ml), IL-13 (20ng/ml), CpGa (1μM), CpGb (1μM), R848 (2μg/ml), Peptidoglycan (10μg/ml), Lipoteichoic Acid (10μg/ml), IFNγ (20 ng/ml), and LPS (4ng/ml) and the supernatants were collected at 48 hours, the levels of CXCL8, CCL2 and CCL22 were determined using ELISA.

The post-morphine levels of CXCL8 were compared with the pre-morphine levels of CXCL8 for the various treatment groups. When the average (for the five macaques) stimulation index for each treatment group was analyzed, there was an increase in CXCL8 for all the groups except CpGa and CpGb, with groups LPS/IFNγ and Peptidoglycan showing a statistically significant increase.

The changes in CCL2 responses following morphine treatment follow a dissimilar pattern in comparison to CXCL8. When the average (for the five macaques) stimulation index for each treatment group was analyzed, there was a decrease in

CCL2 for all the groups, with none of them exhibiting a statistically significant decrease.

Finally, when comparing post-morphine levels of CCL22 with pre-morphine levels, the differences in the average stimulation index for each treatment group shows a decrease in CCL22, with groups Peptidoglycan and CpGb showing a statistically significant decrease.

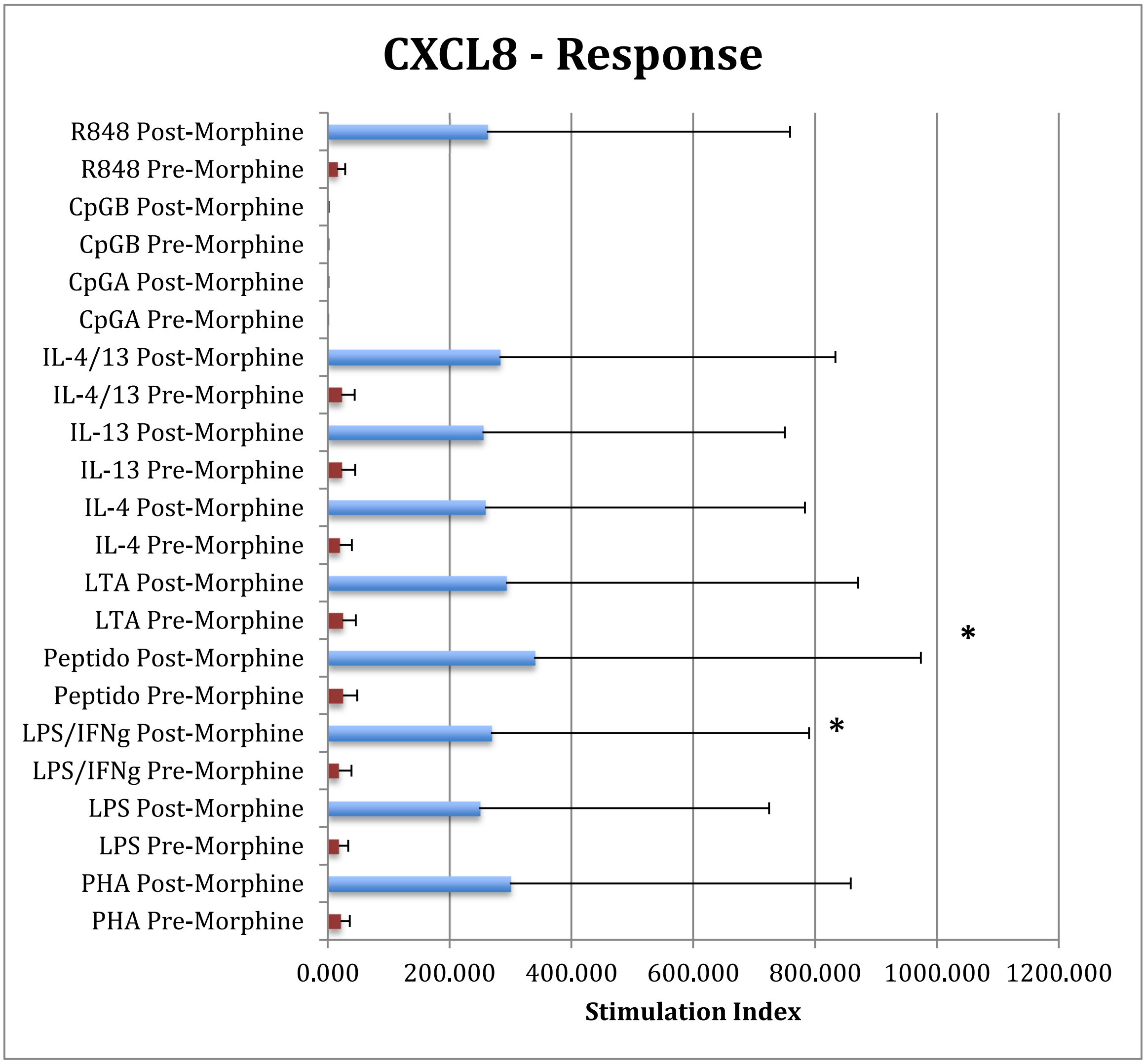


Figure 5. Effect of Morphine administration on CXCL8 chemokine protein levels expressed in treated-PBMCs of macaques by ELISA. Freshly isolated PBMCs were diluted to a final concentration of $1x10^6$ cells/mL and 50µl of cells were plated into each well of a 384-well tissue culture plate. Cells were pretreated with PHA (2µg/ml), IL-4 (20ng/ml), IL-13 (20ng/ml), CpGa (1µM), CpGb (1µM), R848 (2µg/ml), Peptidoglycan (10µg/ml), Lipotechoic Acid (10µg/ml), IFNg (20 ng/ml), and LPS (4ng/ml). Supernatants were harvested after 48 hours, and the levels of CXCL8 were determined. Values represent the average stimulation index (+SD) of five animals for all groups.

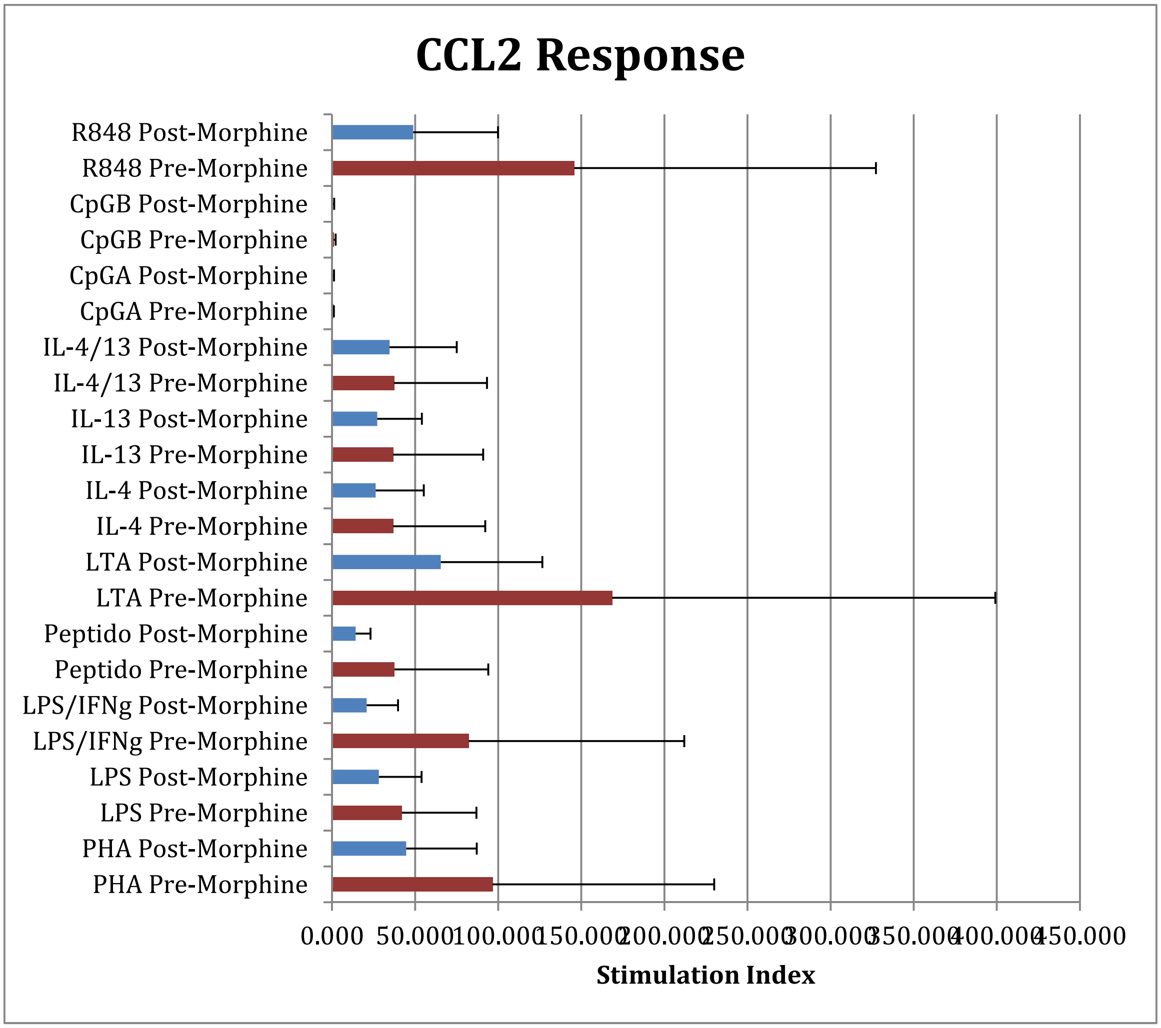


Figure 6. Effect of Morphine administration on CCL2 chemokine protein levels expressed in treated-PBMCs of macaques by ELISA. Freshly isolated PBMCs were diluted to a final concentration of 1x10^6 cells/mL and 50µl of cells were plated into each well of a 384-well tissue culture plate. Cells were pretreated with PHA (2µg/ml), IL-4 (20ng/ml), IL-13 (20ng/ml), CpGa (1µM), CpGb (1µM), R848 (2µg/ml), Peptidoglycan (10µg/ml), Lipotechoic Acid (10µg/ml), IFNγ (20 ng/ml), and LPS (4ng/ml). Supernatants were harvested after 48 hours, and the levels of CCL2 were determined. Values represent the average stimulation index (+SD) of five animals for all groups.

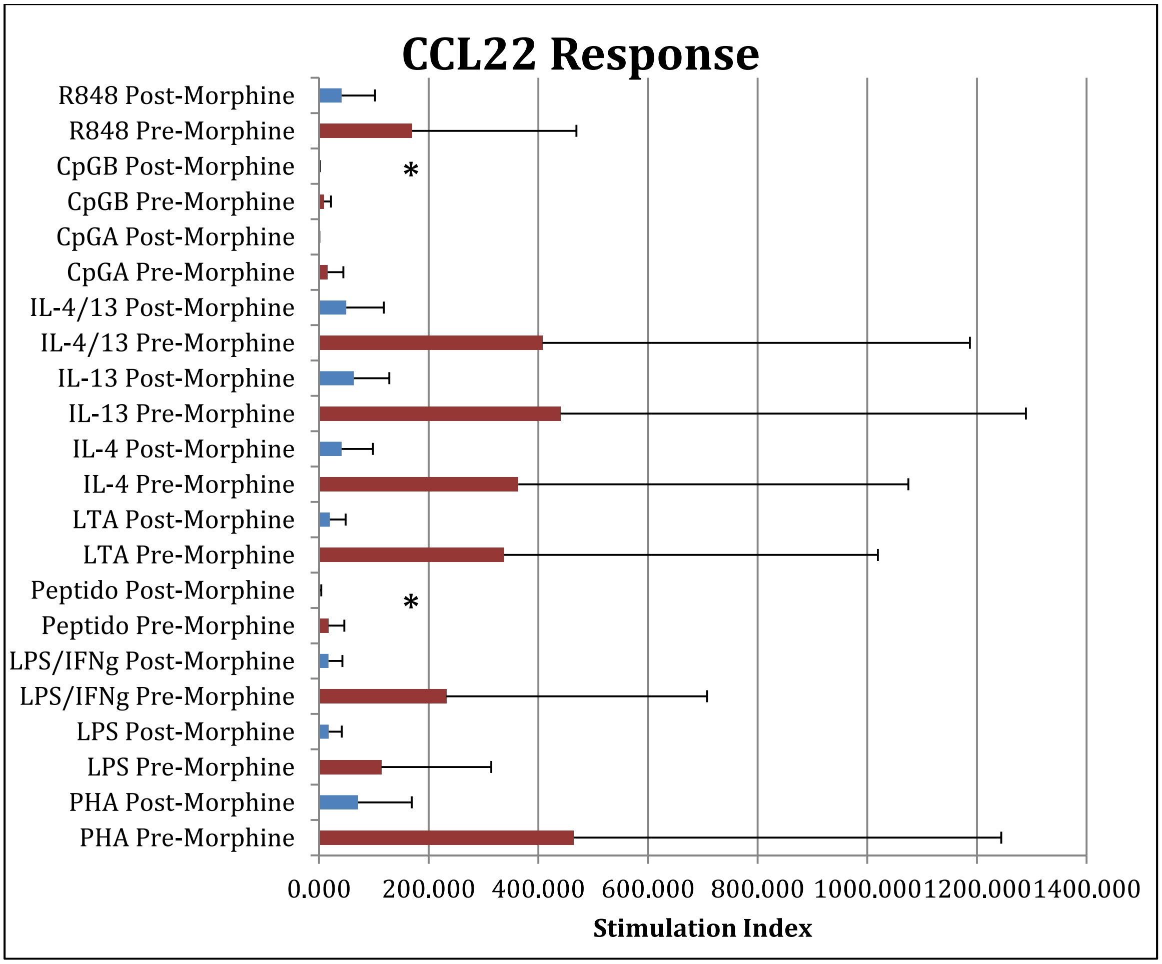
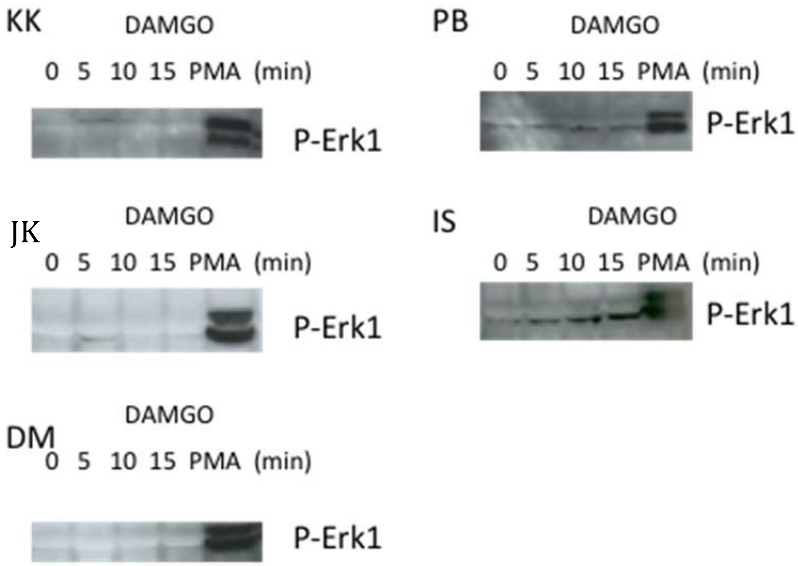


Figure 7. Effect of Morphine administration on CCL22 chemokine protein levels expressed in treated-PBMCs of macaques by ELISA. Freshly isolated PBMCs were diluted to a final concentration of $1x10^6$ cells/mL and 50µl of cells were plated into each well of a 384-well tissue culture plate. Cells were pretreated with PHA (2µg/ml), IL-4 (20ng/ml), IL-13 (20ng/ml), CpGa (1µM), CpGb (1µM), R848 (2µg/ml), Peptidoglycan (10µg/ml), Lipotechoic Acid (10µg/ml), IFNγ (20 ng/ml), and LPS (4ng/ml). Supernatants were harvested after 48 hours, and the levels of CCL22 were determined. Values represent the average stimulation index (+SD) of five animals for all groups.

3.3 Effect of μ-opioid administration on gene expression

Morphine treatment has been shown to modulate the function of leukocytes at both the protein and transcriptional levels. Some of the effects of opioid agonists on neuronal cells are thought to be mediated in part by the activation of the MAP kinase cascade (Williams et al., 2001). A number of signal transduction events following opioid receptor activation eventually may be responsible for their diverse cellular functions. Using phospho-Erk activation as a measure of responsiveness to the highly selective μ-opioid agonist DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin (synthetic μ-opioid) treatment), we identified genes that were regulated by DAMGO using microarray analysis in DAMGO-responsive subjects. We isolated five sets of human PBMCs from normal donors (designated Donor KK, JK, DM, PB and IS). And we tested DAMGO responsiveness of these cell preparations by analyzing their ability to induce phospho-ERK activation using western blot analysis. Next, in the subjects who showed strong phospho-Erk activation we wanted to identify genes that were regulated by the DAMGO treatment. As a result, part of the isolated PBMC cell preparations were used to carry out the western blot analysis, and a second part was used for RNA isolation to study gene expression. Isolated RNA samples from the DAMGO-responsive subjects were analyzed using microarray analysis, and quantitative RT-PCR. As seen in Figure 8 the amount of Erk-phosphorylation was measured after 0, 5, 10, and 15 minutes of DAMGO treatment. Phorbol 12-myristate 13-acetate (PMA) is known to illicit strong phospho-Erk activation and hence was used as a positive control. As seen on the respective blots in figure 8, donor KK, JK and DM exhibited no detectable DAMGO-response based on the absence of evidence for activation (phosphorylation of ERK) at any of the incubation times, while donors PB and IS exhibited a more obvious DAMGO response based on the appearance of pospho-Erk at 5, 10 and 15 minutes. Donor IS appeared to exhibit the strongest response. In a separate experiment we used the second half of the PBMC cell preparations and treated them with DAMGO for 0hrs, 6 hrs, and 24 hrs. mRNA preparations were isolated from the treatment groups, and the RNA samples extracted at 0 and 6 hrs for donor IS were sent for microarray analysis. Figure 9 is a graphic representation of the number of genes that display a fold change (up regulated vs. down regulated) as characterized from the microarray results. Table 5 is a partial tabulated list that was taken from the full-list of genes that were regulated in-response to DAMGO. The list includes significant genes that were up regulated as a result of DAMGO treatment. CXCL9, CXCL10, TLR8 and CCR2 show about a ~5 fold increase in their expression post-DAMGO treatment. While IL-13RA1, TLR7, TLR4, AHR and CD14 show a ~2-3 fold increase in their expression post-DAMGO treatment. This experiment provides a large panel of DAMGO-responsive genes whose regulation can be further analyzed.



Response	DAMGO	PMA
Donor KK	-	+++
Donor JK	-	+++
Donor DM	-	+++
Donor PB	+	+++
Donor IS	+	+++

Figure 8. The effect of DAMGO administration on phospho-ERK activation in human PBMCs. In a separate experiment freshly isolated PBMCs were diluted to a final concentration of 0.5x 10^6 cells/ml in R10 medium and 2 ml of cells were seeded into each well of a 6 well polystyrene tissue culture plate. Cells were cultured for 24 hours in the absence of PHA. After the initial 24-hour culture, non PHA-activated PBMCs were treated with DAMGO at a concentration of 100nM for 0, 5, 10, 15 minutes, and downstream phosphor-Erk activation was determined by Western Blot analysis.

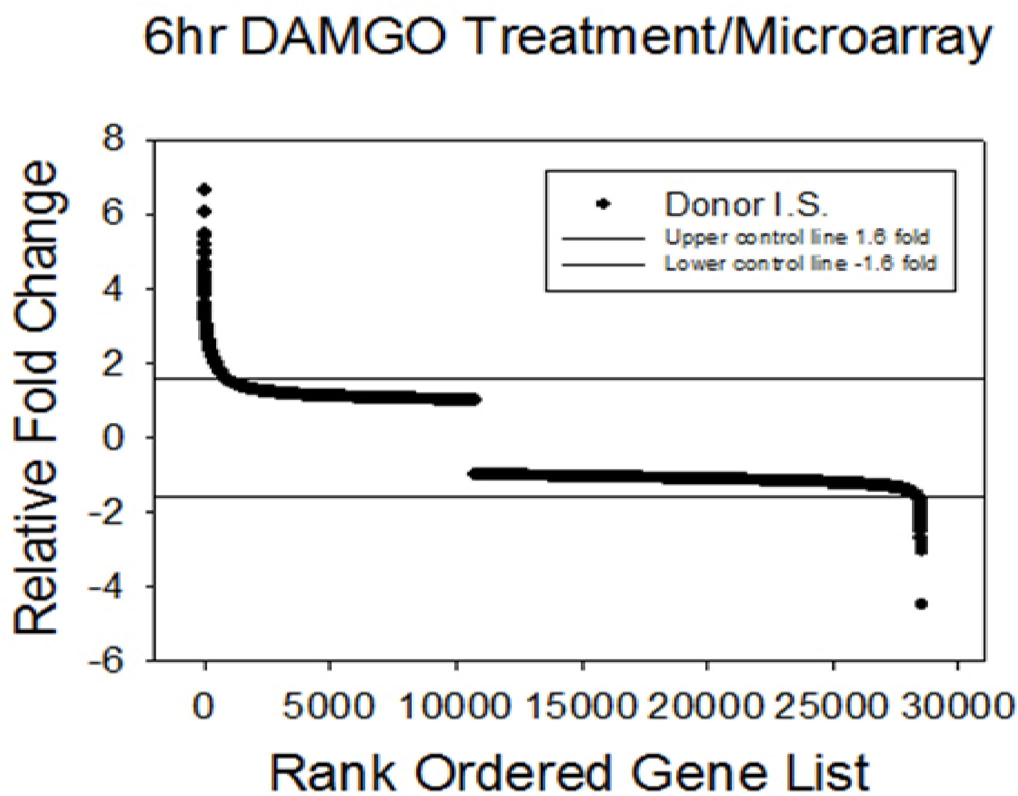


Figure 9. The effect of DAMGO on cytokine transcript expression in human PBMCs using micro-array analysis. Freshly isolated PBMCs were diluted to a final concentration of 0.5x 10^6 cells/ml in R10 medium and 2 ml of cells were seeded into each well of a 6 well polystyrene tissue culture plate. Cells were treated with DAMGO and RNA was extracted after 0 and 6 hrs of DAMGO administration. Following treatment, PBMCs were harvested and the total RNA was isolated. The RNA isolated was used for microarray analysis (~1μg) giving us a list of genes, which were both positively and negatively modulated at the mRNA level

Table 5. The effect of DAMGO treatment on selected responsive genes. CXCL9, CXCL10, CCR2 and TLR8 expression was increased 5-fold. IL-13RA1, TLR7 and TLR4 expression was increased ~3-fold. AHR and CD14 expression was increased ~2-fold.

Fold Change	Gene Symbol	Description	NO DAMGO	DAMGO
5.456182875	CXCL9	Homo sapiens chemokine (C-X-C motif) ligand 9 (CXCL9), mRNA.	72.15987828	393.7174921
4.964797928	CCR2	Homo sapiens chemokine (C-C motif) receptor 2 (CCR2), transcript variant A, mRNA.	189.4550781	940.6061795
4.692572387	CXCL10	Homo sapiens chemokine (C-X-C motif) ligand 10 (CXCL10), mRNA.	580.7973256	2725.433493
4.543861937	TLR8	Homo sapiens toll-like receptor 8 (TLR8), mRNA.	101.2472561	460.0535533
3.553503204	IL13RA1	Homo sapiens interleukin 13 receptor, alpha 1 (IL13RA1), mRNA.	416.8278896	1481.199241
3.519654948	TLR7	Homo sapiens toll-like receptor 7 (TLR7), mRNA.	198.5950701	698.9861212
3.104547489	TLR4	Homo sapiens toll-like receptor 4 (TLR4), transcript variant 3, transcribed RNA.	631.2234533	1959.663187
2.312093915	CD14	Homo sapiens CD14 molecule (CD14), transcript variant 1, mRNA.	2676.700486	6188.782907
2.070845612	AHR	Homo sapiens aryl hydrocarbon receptor (AHR), mRNA.	1298.931828	2689.887275

Next, we selected three significant responsive genes, CXCL10, TLR4 and AHR that were up-regulated in donor IS post DAMGO-treatment (as determined by the microarray results) and we analyzed the changes in the expression of these three genes using quantitative PCR in both responsive (IS) and non-responsive (KK, JK, and DM) donors to confirm the latter results. The results (Figure 10) show that donor IS exhibit similar-fold changes in the DAMGO-treatment group for the three genes as detected in the micro-array experiment (see Table 5). On the other hand donors KK, DM and JK showed no changes between the DAMGO-treatment and control groups. These results suggest that phospho-Erk activation as a reliable system to study DAMGO-responsiveness in some human subjects.

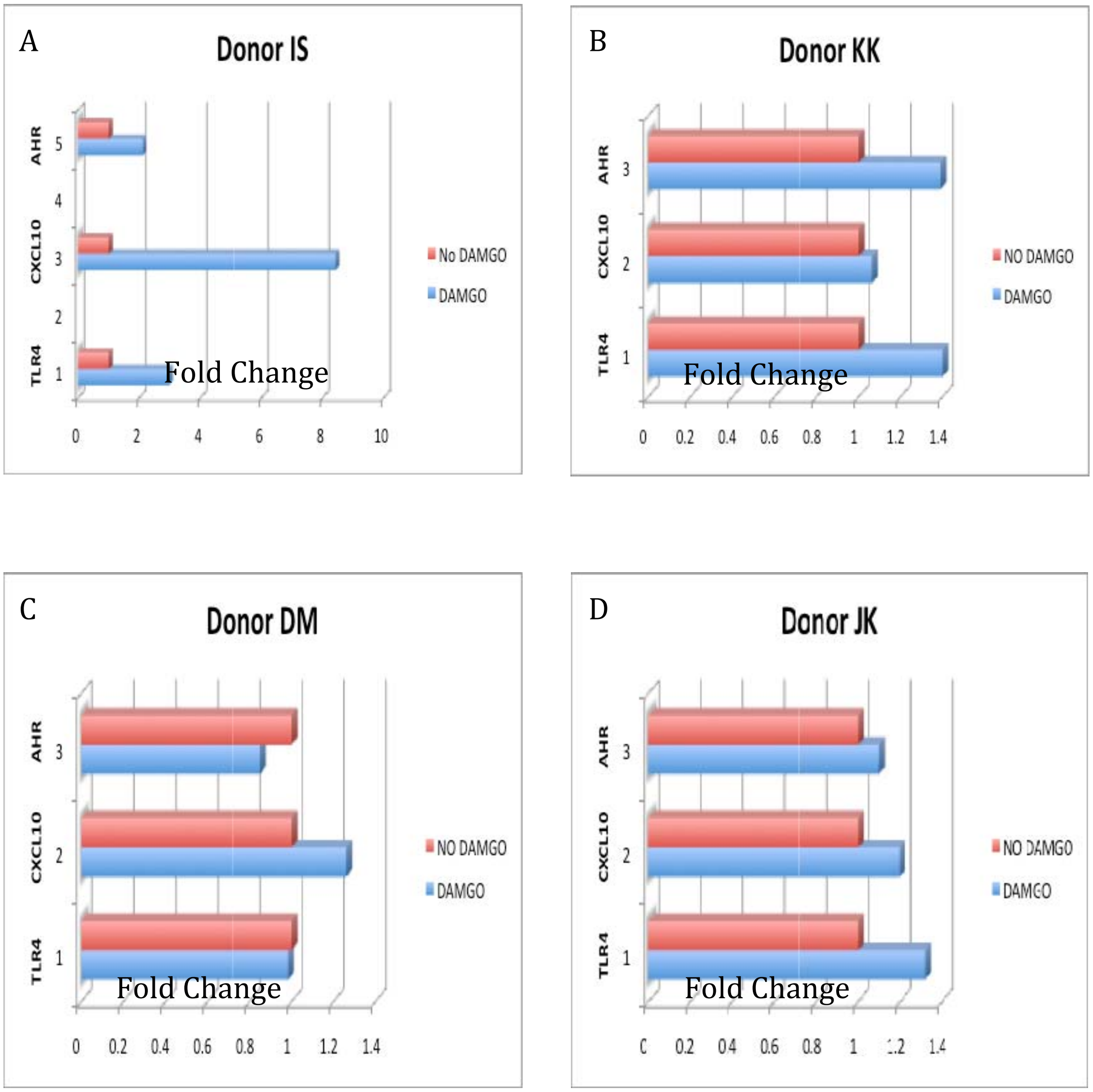
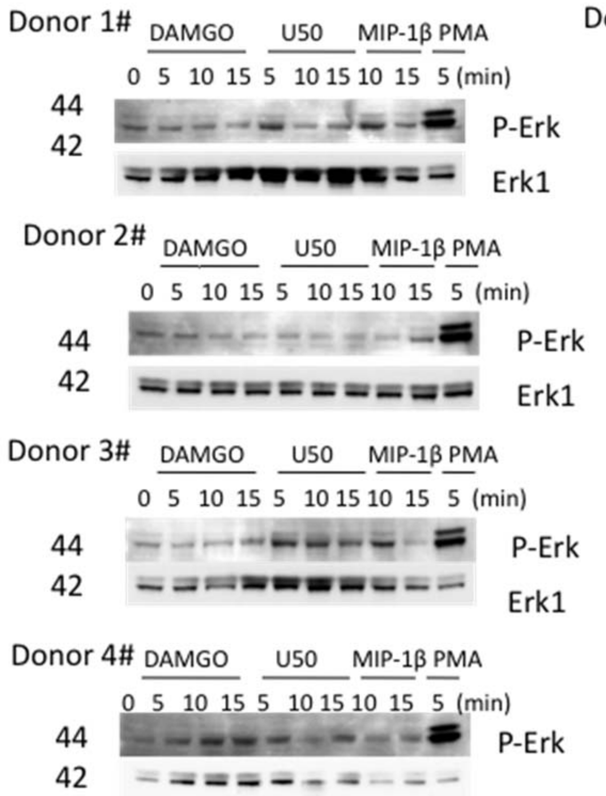
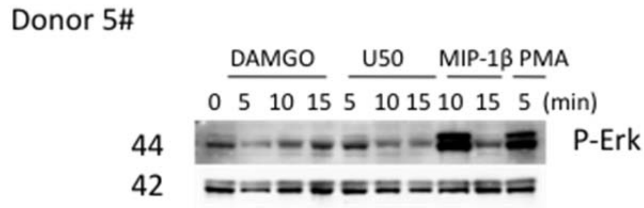


Figure 10. The effect of DAMGO on cytokine transcript expression in human PBMCs using quantitative RT PCR. Freshly isolated PBMCs from the same donors as the phosphor-Erk experiment (figure 8) were diluted to a final concentration of 0.5x 10^6 cells/ml and 2 ml of cells were seeded into each well of a 6 well polystyrene tissue culture plate. Cells were treated with DAMGO and RNA was extracted after 6 hrs of DAMGO administration. Following treatment, PBMCs were harvested and the total RNA was isolated. The RNA isolated was reverse transcribed into cDNA that is quantified using the Taqman probes (Applied Biosystems, StepOnePlus™ Real-Time PCR System)

In an effort to extend the Phospho-ERK analysis, we obtained PBMCs from 5 additional subjects and we conducted a similar analysis of the response to DAMGO. We tested the capacity of DAMGO, the κ-opioid U50, 488H, and the CCR5 agonist CCL4 to induce ERK activation. Each set of PBMCs was treated with DAMGO, U50, 488H and CCL4 for 5, 10 and 15 minutes, and the level of phospho-ERK was determined. The results show (Figure 12) that Donors 1 and 3 fail to exhibit a response to DAMGO, but these donors do exhibit a response to U50, 488H and CCL5 at 5 and 10 minutes, respectively. Donor 2 shows no response to DAMGO or U50488H, but a weak response to CCL5 is apparent at 15 min. Donors 4 and 5 show a response to DAMGO, U50488H, and CCL5, with the Donor 5 response to CCL5 being very strong. These results demonstrate that the ERK activation analysis detects responsiveness in two out of five subjects, at least based on these experimental conditions.





Response	DAMGO	U50	MIP-1β	PMA
Donor 1#	-	+	+	+++
Donor 2#	-	-	+	+++
Donor 3#	-	+	+	+++
Donor 4#	+	+	+	+++
Donor 5#	+	+	+++	+++

Figure 11. The effect of DAMGO and U50 administration on phosphor-ERK activation in human PBMCs. Freshly isolated PBMCs were diluted to a final concentration of 0.5x 10^6 cells/ml in R10 medium and 2 ml of cells were seeded into each well of a 6 well polystyrene tissue culture plate. Cells were cultured for 24 hours in the absence of PHA. After the initial 24-hour culture, non PHA-activated PBMCs were treated with DAMGO at a concentration of 100nM and U50 at a concentration of 1μm for 0, 5, 10, 15 minutes, and downstream phosphor-Erk activation was determined by Western Blot analysis. Data is representative of representative of 5 different donors.

Chapter 4

DISCUSSION

In this thesis we wanted to study the effects of opioids on the development of HIV encephalitis by monitoring the changes in the expression of critical chemokines and cytokines. Reports from both our as well as other labs have implicated the role of opioid drugs in the trafficking process that leads to migration of infected cells to the brain (Reddy et al., 2012).

The degree of HIV-1 disease progression depends on the balance between two major factors, proinflammatory cytokines that stimulate cellular activation, and viral products that may slow down disease progression. As described previously many of the immunomodulatory activities of the opioids are due to the alterations in critical cytokine expression. We have previously hypothesized that morphine administration promotes the development of a pro-inflammatory cellular phenotype in which there is an increased capacity to produce and respond to pro-inflammatory chemokines, and by virtue of greater trafficking capacity, an accelerated transit of virally infected cells into the brain (Rogers and Peterson, 2003). In this project, we have tried to examine the effect of opioid administration on development of a proinflammatory functional phenotype, and understand the basis for μ opioid-induced effects on the expression of the critical pro-inflammatory chemokines like CCL2, CXCL8 and CCL22.

Effects of opioids on chemokine and cytokine expression

In order to study the effects of opioids on cytokine and chemokine expression macaque PBMCs were activated using a battery of toll receptor agonists that include exogenous microbial components such as bacterial lipoteichoic acid (TLR2), LPS (TLR4), ss viral RNA (TLR7/8) and CpG-DNA (TLR9). These toll receptor agonists bind to specific toll-like receptors that are expressed on leukocytes. Toll-like receptors function as pattern-recognition receptors that recognize pathogen-associated molecular pattern (PAMPs) shared by various pathogens. The cells involved in innate and adaptive immune responses, even though activated by different ligands, are typically activated through one or more of the TLRs as a part of the immune response. In order to evaluate the competence of the cells of the immune system in animals receiving chronic morphine administration, we determined the response to several TLR ligands ex vivo.

When toll-like receptors recognize specific ligands/TLR agonists a series of protein-protein interactions follow which further promote the activation of transcription factors and the release of inflammatory mediators that play a central role in the immune responses of the body. The type and the amount of inflammatory mediators secreted are then capable of determining the CD4+ T-cell differentiation status (Th1, Th2, or regulatory phenotype). To further understand pathogen-host interactions both in the presence and absence of morphine, we observed the induction of several cytokines including IFN-γ and IL-12 (Th1 cytokines), IL-4 and 1L-13 (Th2 cytokines), TNF-α, IL-1β, IL-6, and IL-2 (pro-inflammatory cytokines), IL-10 (anti-inflammatory cytokine), CCL2, CCL3, CCL4, CXCL8, CCL22, and CCL5 (pro-inflammatory chemokines). Moreover, our data show (Table 1) that LPS/IFN-γ and the TCR agonist (PHA) were the only two stimuli that induced a robust IFN-γ response, in contrast to IL-4/IL13, TLR2 and TLR4 agonist groups which all induced significant IL-12p40 expression (Figure 3, Panel D). Additionally, TLR2, TLR4, and TCR agonist (PHA) stimulation induces a low level of IL-4 (Figure 3, Panel B). Together these data suggest that macaque PBMCs exhibit a cytokine expression profile, which is biased toward a Th1 phenotype.

We also observed that TLR2, TLR4 and the TCR ligand were the most potent inducers of the pro-inflammatory cytokine IL-6, but were weak to only moderate inducers of the pro-inflammatory cytokine TNF-α (Figure 3, Panel D). Moreover, TLR2, TLR 4 and TCR ligands induced fairly low but measurable anti-inflammatory cytokine IL-10 (Figure 3, Panel A). The difference in the secretion levels of these pro- and anti-inflammatory cytokines induced by the various TLR ligands is important in evaluating the capacity of the immune system to mount a response. Furthermore, all TLR agonists along with the TCR agonist and the IL4/IL13 group robustly induced CC-chemokines CCL2, CCL4, CCL22 and CXCL8 (Figure 3, Panel C&D respectively), which suggests that macaque PBMCs, are capable of expressing a broad pro-inflammatory chemokine response to these stimuli.

After generating baseline preliminary data with macaque PBMCs, we chose to focus our studies on the expression of five cytokines (IFN-γ, TNF-α, CCL2, CXCL8 and IL-12 p40) in greater detail. These cytokines and chemokines were selected because of their ability to contribute to the inflammation associated with HIV (or SIV) infection. IFN-γ has a broad range of functions, and perhaps the most important of these is to the promote phagocytosis and up-regulate of microbial killing (Boehm et al., 1997). TNF-α increases the endothelial cell expression of adhesion molecules specific to monocytes and T cells, and promotes the expression of chemokines like CCL2 and CXCL8 that specifically attract mononuclear cells and are particularly important in HIV neuropathogenesis (Cuff et al., 1998). Lastly, IL-12 p40 is a crucial cytokine that is important in the generation of a cellular immune function. It promotes the differentiation of naive CD4+ T cells to the Th1 phenotype, enhances the generation and activity of cytotoxic T lymphocytes, induces IFN-γ production, and influences T cell trafficking (Hamza et al., 2010).

Our experiments to assess immune competence showed that LPS/IFN-γ was the only stimulation that induced a robust IFN-γ response in all four macaques (Figure 4, Panel A). TLR2, TLR4, TLR7/8 and TCR ligands were moderate inducers of the pro-inflammatory cytokine TNF-α with IL-13 being the most potent inducer (Figure 4, Panel D). TCR, TLR2, TLR4 agonists and IL4/13 induced significant CCL2 with TLR7/8 agonist having the largest effect on the CCL2 levels in all four macaques (Figure 4, Panel C). Unlike CCL2, all agonists induced a <u>high level of CXCL8, with the TCR agonist (Figure 4, Panel B) inducing a maximal response</u>.

Finally, all TCR and TLR agonists seem to induce very low (10-40 pg/ml) levels of IL-12p40. Since IL12p40 is required for the synthesis of IFN-γ, very low levels of IL-12p40 could be the reason for a weak IFN-γ response. Also, TLR agonists, with the exception of TLR9, induced higher levels of chemokines and cytokines suggesting a more anti-inflammatory role of TLR9 as compared to TL2, TLR4 and TLR 7/8.

Since TLR agonists induced strong chemokine responses, and because critical chemokines like CCL2, CXCL8 and CCL22 already have an established role in antimicrobial immunity, we wanted to study the effect of morphine on these chemokines in greater detail. For example, opioid-induced up-regulation and down-regulation of specific chemokines can either amplify HIV replication by the virtue of increased pool of available infectable target cells, or reduce HIV replication by means of increased migration of potentially protective neutrophils to the site of infection. Little is known about the effect of chronic morphine administration on cytokine expression, or the capacity of leukocytes in chronic opioid abusers to respond to various TLR ligands.

In regard to chemokines, it has been previously established that exposure of PBMCs to DAMGO (synthetic peptide selective for the μ-opioid receptor) or morphine has shown to increase the expression of pro-inflammatory chemokines CCL2, CCL5, and CXCL10 (Wetzel et al., 2000), and the chemokine receptors CXCR4 and CCR5, the major HIV co-receptors (Steele et al., 2003), which directly leads to increased replication of HIV. It has also been reported that morphine suppresses the synthesis and secretion of CXCL8 in U87 and NHA cell-lines (Mahajan et al., 2002). In our experiments, we wanted to analyze the effects of chronic morphine on CCL2, CXCL8 and CCL22 chemokine expression in non-SIV infected macaques. The results show that chronic morphine administration enhances CXCL8 expression in response to various activators. A statistically significant increase in CXCL8 expression is observed in response to either LPS/IFNγ and peptidoglycan stimulation. It is highly interesting to observe that three months of chronic morphine administration produced significant expression of pro-inflammatory CXCL8 protein. The situation mirrors patients that abuse heroin, contrary to immune-suppression that is associated with chronic drug abuse, chronic administration of morphine increased immune responsiveness for LPS/IFNγ and Peptidoglycan groups that further augments macrophage activation (Held et al., 1999).

Our results also show that animals receiving chronic morphine administration exhibit reduced CCL2 and CCL22 expression in response to most of the stimulants. In fact, a statistically significant decrease in CCL22 is observed in response to either peptidoglycan or CpGb. However, both of these TLR ligands induced very low levels of chemokine, and it is unlikely that the morphine effect on the expression of these chemokines has physiological significance.

Studies investigating the influence of opioids on the development of immunodeficiency diseases by means of altering cytokine production have received greater attention in the literature recently. Since CXCL8 and CCL22 can act as HIV-1 suppressive factors by binding to HIV-1 specific receptor CXCR2 (Reddy et al., 2012) and exhibiting anti-CXCR4 activity respectively (Abdelwahab et al., 2003;Lambert et al., 2007), while CCL2 can propagate the virus to different organs, including the lungs, skin, and lymph nodes, as well as the brain by infiltrating the site of infection with increased number of monocytes (Wetzel et al., 2000), it will be interesting to monitor how the changes in protein expression of CCL2, CXCL8 and CCL22 would effect disease progression in the SIV –infected macaque PBMC samples. Cytokines including IL-2, IFN-γ and IL12p-40 have been implicated in controlling the virus and a host of opportunistic pathogens during disease progression especially in SIV-infected macaques (Sun et al., 2005;Villinger et al., 2010). It would be interesting to investigate IL-2, IFN-γ and IL12p-40 expression by leukocytes from morphine-treated macaques that are infected with SIV.

Effects of opioids on gene expression

It is apparent that responses to opioids are highly variable among human subjects, and the factors responsible for this variability could be environmental, physiological, and/or genetic. Allelic variants in the genes involving the opioid systems- UDP-glucuronosyltransferase ((UGT) 2B7) gene, ATP-binding cassette, sub-family B, number 1 (ABCB1) gene, and μ-opioid receptor 1 (OPRM1) gene and non-opioid systems - Catechol O-methyltransferase (COMT) gene may affect the efficacy of morphine, or drug metabolizing enzymes may play a significant role in opioid metabolism, which could influence individual responsiveness (Smith, 2008). Since opioid agonists activate several intracellular pathways including MAPK/ERK pathway via specific G-protein coupled receptors (Ligeza et al., 2008), we decided to utilize the activation of ERK (based on phosphorylation status) as a cellular marker to analyze opioid responsiveness in our cohort of human subjects. Our results (Figure 11) show that a detectable Erk response was observed in 40% of subjects in experiments in which we employed the μ-opioid agonist DAMGO. This was in contrast to the results in which we observed a response in 80% of subjects in response to KOR activation. These results suggest that Erk activation may be a convenient method to screen subjects for opioid responsiveness, possibly as a first step in the study of the opioid-induced signaling pathways. The results from these experiments shed some light on inter-subject variability in reported opioid-induced immunomodulatory activity.

We extended these studies on opioid-induced signaling and gene expression and we conducted micro-array analysis using RNA extracted from a DAMGO-responsive donor. The results (Table 5) show that a number of immune response genes were up regulated in response to DAMGO. We selected three specific genes for confirmation of the microarray results, CXCL10, TLR4 and AHR (Figure 10). As mentioned earlier it has been previously reported by our lab that the MOR-selective agonist DAMGO can elevate CXCL10 production in nonactivated and PHA-stimulated peripheral blood mononuclear cells (PBMCs) both at the mRNA and protein level. However, an increased TLR4 and AHR gene expression as early as 6 hours post-DAMGO treatment is novel. It would be interesting to analyze changes in these genes in HIV-infected PBMC samples since all 3 genes have a possible role in disease progression. Elevated CXCL10 expression can promote the infection by attracting noninfected monocytes and T cells to the site of infection, while elevated TLR4 expression can promote increased opportunistic infections during HIV infection (without HAART) and finally AHR can affect disease progression by altering the expression of pro-inflammatory cytokines like TNF-α and IL-6.

In closing, our data provides important information regarding certain cytokines that are affected in response to opioids, changes in which can further affect HIV-1 infection, and HIV pathogenesis. This information may also yield useful targets for the development of therapeutic strategies for the treatment of inflammatory and infectious diseases.

REFERENCES

1. Abdelwahab S, Cocchi F, Bagley KC, Kamin-Lewis R, Gallo R, DeVico A, and Lewis G (2003). HIV-1-suppressive factors are secreted by CD4+T cells during primary immune responses. Proc. Natl. Acad. Sci. U. S. A, 100, 15006–10

2. Alkhatib, G., Ahuja, S. S., Light, D., Mummidi, S., Berger, E. A., & Ahuja, S. K (1997). CC chemokine receptor 5-mediated signaling and HIV-1 co-receptor activity share common structural determinants. J Biol, Chem, 272, 19771–76.

3. Andrew DP, Chang M, McNinch J, Wathen ST, Rihanek M, Tseng J, Spellberg JP and Elias III CG(1998).STCP-1 (MDC) CC Chemokine Acts Specifically on Chronically Activated Th2 Lymphocytes and Is Produced by Monocytes on Stimulation with Th2 Cytokines IL-4 and IL-13. J Immunol, 161, 5027-5038

4. Baggiolini,M., Dewald,B., and Moser,B. (1997). Human chemokines: an update. [Review] [181 refs]. Ann. Rev. Immunol. 15, 675-705.

5. Bakalkin, G., Yakovleva, T., & Terenius, L. (1993). NF-kappa B-like factors in the murine brain. Developmentally-regulated and tissue-specific expression. Brain Res Mol Brain Res 20, 137-146.

6. Belkowski, S. M., Zhu, J., Liu-Chen, L. Y., Eisenstein, T. K., Adler, M. W., & Rogers, T. J. (1995b). Sequence of kappa-opioid receptor cDNA in the R1.1 thymoma cell line. J Neuroimmunol 62, 113-117.

7. Bessler, H., Sztein, M. B., & Serrate, S. A. (1990). Beta-endorphin modulation of IL-1-induced IL-2 production. Immunopharmacology 19, 5-14.

8. Bidlack, J. M., Saripalli, L. D., & Lawrence, D. M. (1992). kappa-Opioid binding sites on a murine lymphoma cell line. Eur J Pharmacol 227, 257-265.

9. Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., et al. (1996). The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature, 382, 829–33.

10. Bodnar,R.J. (2007). Endogenous opiates and behavior: 2006. Peptides 28, 2435-2513.

11. Bohn,L.M., Belcheva,M.M., and Coscia,C.J. (2000). Mitogenic signaling via endogenous kappa-opioid receptors in C6 glioma cells: evidence for the involvement of protein kinase C and the mitogen-activated protein kinase. J Neurochem. 74, 564–573.

12. Brown, S.L., & Van Epps, D.E. (1986). Opioid peptides modulate production of interferon gamma by human mononuclear cells. Cell Immunol 103, 19-26.

13. Bryant, H. U., Bernton, E. W., & Holaday, J. W. (1987). Immunosuppressive effects of chronic morphine treatment in mice. Life Sci 41, 1731-1738.

14. Bryant, H. U., Bernton, E. W., & Holaday, J. W. (1988a). Morphine pellet-induced immunomodulation in mice: temporal relationships. J Pharmacol Exp Ther 245, 913-920.

15. Bussiere, J. L., Adler, M. W., Rogers, T. J., & Eisenstein, T. K. (1992). Differential effects of morphine and naltrexone on the antibody response in various mouse strains. Immunopharmacol Immunotoxicol, 14, 657–73.

16. Bussiere, J. L., Adler, M. W., Rogers, T. J., & Eisenstein, T. K. (1993). Cytokine reversal of morphine-induced suppression of the antibody response. J Pharmacol Exp Ther, 264, 591–97.

17. Carr, D. J., DeCosta, B. R., Kim, C. H., Jacobson, A. E., Guarcello, V., Rice, K. C., & Blalock, J. E. (1989). Opioid receptors on cells of the immune system: evidence for delta- and kappa-classes. J Endocrinol 122, 161-168.

18. Carr,D.J., Rogers,T.J., and Weber,R.J. (1996). The relevance of opioids and opioid receptors on immunocompetence and immune homeostasis. [Review] [97 refs]. Proceedings of the Society for Experimental Biology & Medicine 213, 248-257

19. Centers for Disease Control. (1996). HIV/AIDS surveillance report. 7 ed. Atlanta: US Department of Health and Human Services.

20. Chaisson, R. E., Moss, A. R., Onishi, R., Osmond, D., & Carlson, J. R. (1987). Human immunodeficiency virus infection in heterosexual intravenous drug users in San Francisco. Am J Pub Health, 77, 169–72

21. Chan AS and Wong,Y.H. (2000). Regulation of c-Jun N-terminal kinase by the ORL(1) receptor through multiple G proteins. J. Pharmacol. Exp. Ther. 295, 1094-1100.

22. Chao, C. C., Hu, S., Molitor, T. W., Zhou, Y., Murtaugh, M. P., Tsang, M., & Peterson, P. K. (1992). Morphine potentiates transforming growth factor-beta release from human peripheral blood mononuclear cell cultures. J Pharmacol Exp Ther 262, 19-24.

23. Childers, S. R. (1991). Opioid receptor-coupled second messenger systems. Life Sci 48, 1991-2003.

24. Chen, Y., Mestek, A., Liu, J., & Yu, L. (1993). Molecular cloning of a rat kappa opioid receptor reveals sequence similarities to the mu and delta opioid receptors. Biochem J 295, 625-628.

25. Chuang L.F., Chuang T.K., Killam K.F., Chuang A.J., Kung H.F., Yu L., & Chuang R.Y. (1994). Delta opioid receptor gene expression in lymphocytes. Biochem Biophys Res Commun 202, 1291-1299.

26. Chuang, L.F., Killam, K.F., Jr., and Chuang,R.Y. (1997). Induction and activation of mitogen-activated protein kinases of human lymphocytes as one of the signaling pathways of the immunomodulatory effects of morphine sulfate. J. Biol. Chem. 272, 26815-26817.

27. Conant,K., Garzino-Demo,A., Nath,A., McArthur,J.C., Halliday,W., Power,C., Gallo,R.C., and Major,E.O. (1998). Induction of monocyte chemoattractant protein-1 in HIV-1 Tat-stimulated astrocytes and elevation in AIDS dementia. Proc. Natl. Acad. Sci. U. S. A 95, 3117-3121.

28. Crum, R. M., Galai, N., Cohn, S., Celentano, D. D., & Vlahov, D. (1996). Alcohol use and T-lymphocyte subsets among injection drug users with HIV-1 infection: A prospective analysis. Alcohol Clin Exp Res, 20, 364–71.

29. Cuff CA, Schwartz J, Bergman CM, Russel KS, Bender JR & Ruddle NH (1998). Lymphotoxin α3 Induces Chemokines and Adhesion Molecules: Insight into the Role of LTα in Inflammation and Lymphoid Organ Development . J ImmunoL, 161,6853-6860

30. Des Jarlais, D. C., Friedman, S. R., & Stoneburner, R. L. (1988). HIV infection and intravenous drug use: Critical issues in transmission dynamics, infection outcomes, and prevention. Rev Infect Dis, 10, 151–58.

31. Donahoe, R. M., Byrd, L. D., McClure, H. M., Fultz, P., Brantley, M., Marsteller, F., et al. (1993). Consequences of opiate-dependency in a monkey model of AIDS. Adv Exp Med Biol, 335, 21–28.

32. Donahoe, R. M. & Falek, A. (1988). Neuroimmunomodulation by opiates and other drugs of abuse: Relationship to HIV infection and AIDS. In T.P. Bridge (Ed.). Psychological, neuropsychiatric, and substance abuse aspects of AIDS (pp 145–58). New York: Raven Press.

33. Donahoe, R. M., O'Neil, S. P., Marsteller, F. A., Novembre, F. J., Anderson, D. C., Lankford-Turner, P., et al. (2009). Probable deceleration of progression of simian AIDS affected by opiate dependency: Studies with a rhesus macaque/SIVsmm9 model. J AIDS, 50, 241–49.

34. Donahoe, R. M. & Vlahov, D. (1998). Opiates as potential cofactors in progression of HIV-1 infections to AIDS. J Neuroimmunol, 83, 77–87.

35. Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., et al. (1996). HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature, 381, 667–73.

36. Eisenstein,T.K. and Hilburger,M.E. (1998). Opioid modulation of immune responses: effects on phagocyte and lymphoid cell populations. [Review] [100 refs]. J. Neuroimmunol. 83, 36-44.

37. Evans, C. J., Keith, D. E., Jr., Morrison, H., Magendzo, K., & Edwards, R. H. (1992). Cloning of a delta opioid receptor by functional expression. Science 258, 1952-1955.

38. Feng, Y., Broder, C. C., Kennedy, P. E., & Berger, E. A. (1996). HIV-1 entry cofactor: Functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science, 272, 872–77.

39. Foley JF, Yu Cheng-Rong, Solow Rikki , Yacobucci M, Peden K and Farber JM (2005).Roles for CXC Chemokine Ligands 10 and 11 in Recruiting CD4+ T

Cells to HIV-1-Infected Monocyte-Derived Macrophages, Dendritic Cells, and Lymph Nodes. J Immunol, 174, 4892-4900

40. Friedland, G. H., Harris, C., Butkus-Small, C., Shine, D., Moll, B., Darrow, W., et al. (1985). Intravenous drug abusers and the acquired immunodeficiency syndrome (AIDS). Demographic, drug use, and needle-sharing patterns. Arch Intern Med, 145, 1413–17.

41. Garfein, R. S., Vlahov, D., Galai, N., Doherty, M. C., & Nelson, K. E. (1996). Viral infections in short-term injection drug users: The prevalence of the hepatitis C, hepatitis B, human immunodeficiency, and human T-lymphotropic viruses. Am J Pub Health, 86, 655–61.

42. Gavériaux-Ruff, C., Matthes, H. W. D., Peluso, J., & Kieffer, B. L. (1998). Abolition of morphine-immunosuppression in mice lacking the mu-opioid receptor gene. Proc Natl Acad Sci USA, 95, 6326–30.

43. Ghosh T, Mickelson DJ, Fink J, Solberg JC, IngleWeld JR, Hook D, Gupta SK, Gibson S, and Alkan SS (2006). Toll-like receptor (TLR) 2–9 agonists-induced cytokines and chemokines: I. Comparison with T cell receptor-induced responses. Cellular Immunology, 243,48–57

44. Goldstein, M. (1984). Introduction to the symposium on endorphins and neurotransmitters. J Am Osteopath Assoc 84, 96-97.

45. Goldstein, A., & Ghazarossian, V. E. (1980). Immunoreactive dynorphin in pituitary and brain. Proc Natl Acad Sci U S A 77, 6207-6210.

46. Goldstein, A., & Naidu, A. (1989). Multiple opioid receptors: ligand selectivity profiles and binding site signatures. Mol Pharmacol 36, 265-272.

47. Gu,L., Okada,Y., Clinton,S.K., Gerard,C., Sukhova,G.K., Libby,P., and Rollins,B.J. (1998). Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low-density lipoprotein receptor-deficient mice. Mol. Cell 2, 275-281.

48. Guan, L., Eisenstein, T. K., Adler, M. W., & Rogers, T. J(1997). Inhibition of T cell superantigen responses following treatment with the mu-opioid U50,488H. J Neuroimmunol, 75, 163–68.

49. Guan, L., Townsend, R., Eisenstein, T. K., Adler, M. W., & Rogers, T. J(1994). Both T cells and macrophages are targets of mu-opioid-induced immunosuppression. Brain Behav Immun, 8, 229–40.

50. Gutstein,H.B., Rubie,E.A., Mansour,A., Akil,H., and Woodgett,J.R. (1997). Opioid effects on mitogen-activated protein kinase signaling cascades. Anesthesiology 87, 1118-1126.

51. Hamza T, Barnett J and Li B (2010). Interleukin 12 A Key Immunoregulatory Cytokine in Infection Applications. Int. J. Mol. Sci, 11, 789-806

52. Happel C,,Kutzler M, & Rogers TJ (2001).Opioid-induced chemokine expression requires NF-kB activity: the role of PKCz. J Leukoc Biol,, 89, 302-09

53. Hasegawa A, Liu H, Ling B, Borda JT, Alvarez X, Sugimoto C, Vinet-Oliphant H, Kim W, Williams KC, Ribeiro RM, Lackner AA, Veazey RS & Kuroda MJ (2009). The level of monocyte turnover predicts disease progression in the macaque model of AIDS.Blood, 114, 2917-25

54. Hedin, K. E., Bell, M. P., Kalli, K. R., Huntoon, C. J., Sharp, B. M., & McKean, D. J. (1997). Mu-opioid receptors expressed by Jurkat T cells enhance IL-2 secretion by increasing AP-1 complexes and activity of the NF-AT/AP-1-binding promoter element. J Immunol, 159, 5431–40.

55. Held TK, Weihua X, Yuan L, Kalvakolanu DV, & Cross AS (1999). Gamma interferon augments macrophage activation by lipopolysaccharide by two distinct mechanisms, at the signal transduction level and via an autocrine mechanism involving tumor necrosis factor alpha and interleukin-1. Infect Immun,67,206-12.

56. Hernández JC, Arteaga J, Paul S, Kumar A, Latz E, & Urcuqui-Inchima S. (2011). Up-regulation of TLR2 and TLR4 in dendritic cells in response to HIV type 1 and coinfection with opportunistic pathogens. AIDS Res Hum Retroviruses. 10, 1099-109

57. Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., & Morris, H. R. (1975). Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature 258, 577-580.

58. Jessop, J. J., & Taplits, M. S. (1991). Effect of high doses of morphine on Con-A induced lymphokine production in vitro. Immunopharmacology 22, 175-184.

59. Jordan, J. D., & Iyengar, R. (1998). Modes of interactions between signaling pathways. Biochem Pharmacol 55, 1347-1352.

60. Koch,W.J., Hawes, B.E., Allen,L.F., and Lefkowitz,R.J. (1994). Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by G beta gamma activation of p21ras. Proc. Natl. Acad. Sci. U. S. A 91, 12706-12710.

61. Kimura A, Naka T, Nakahama T, Chinen I, Masuda K, Nohara K, Fujii-Kuriyama Y, and Kishimoto T (2009).Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses.J Exp Med, 206, 2027–2035

62. Koneru A, Satyanarayana S, and Rizwan S (2009). Endogenous Opioids: Their Physiological Role and Receptors.Global Journal of Pharmacology, 3, 149-153

63. Kreil, G., Barra, D., Simmaco, M., Erspamer, V., Erspamer, G. F., Negri, L., Severini, C., Corsi, R., & Melchiorri, P. (1989). Deltorphin, a novel amphibian

skin peptide with high selectivity and affinity for delta opioid receptors. Eur J Pharmacol 162, 123-128.

64. Kumar, R., Torres, C., Yamamura, Y., Rodriguez, I., Martinez, M., Staprans, S., et al. (2004). Modulation by morphine of viral set point in rhesus macaques infected with simian immunodeficiency virus and simian-human immunodeficiency virus. J Virol, 78, 11425–28

65. Lambert J, Machado E, Watson D, Sill AM, Lim JK, Charurat M, Cunha SM, Afonso AM, Oliviera RH, Tanuri A, and DeVico AL (2007). Production of the HIV-Suppressive Chemokines CCL3/MIP-1a and CCL22/MDC Is Associated with More Effective Antiretroviral Therapy in HIV-Infected Children. Pediatr Infect Dis J, 26, 935–944

66. Law, P.Y., Wong, Y.H., & Loh, H.H. (2000). Molecular mechanisms and regulation of opioid receptor signaling. Annu Rev Pharmacol Toxixol 40, 389-430.

67. Leonard,E.J. and Yoshimura,T. (1990). Human monocyte chemoattractant protein-1 (MCP-1). [Review]. Immunology Today 11, 97-101.

68. Li,L.Y. and Chang,K.J. (1996). The stimulatory effect of opioids on mitogen-activated protein kinase in Chinese hamster ovary cells transfected to express mu-opioid receptors. Mol. Pharmacol. 50, 599-602

69. Ligeza, A, Wawrzczak-Bargiela A, Kaminska D, Korostynski M and Przewlocki R (2008). Regulation of ERK1/2 phosphorylation by acute and chronic morphine – implications for the role of cAMP-responsive element binding factor (CREB)-dependent and Ets-like protein-1 (Elk-1)-dependent transcription; small interfering RNA-based strategy FEBS Journal 275,3836–3849

70. Luster,A.D. (2002). The role of chemokines in linking innate and adaptive immunity. [Review] [30 refs]. Curr. Opinion Immunol. 14, 129-135.

71. Lysle, D. T., Coussons, M. E., Watts, V. J., Bennett, E. H., & Dykstra, L. A. (1993). Morphine-induced alterations of immune status: Dose dependency, compartment specificity and antagonism by naltrexone. J Pharmacol Exp Ther, 265, 1071–78

72. Mahajan, S. D., Schwartz, S. A., Aalinkeel, R., Chawda, R. P., Sykes, D. E., Nair, M. P. N. (2005). Morphine modulates chemokine gene regulation in normal human astrocytes. Clin Immunol, 115, 323–32.

73. Mahajan S, Schwartz S, Shanahan T, Chawda R, and Nair M (2002). Morphine Regulates Gene Expression of a- and b-Chemokines and Their Receptors on Astroglial Cells Via the Opioid m Receptor. J Immunol 2002; 169,3589-3599

74. Mandler,R.N., Biddison,W.E., Mandler,R., and Serrate,S.A. (1986). beta-Endorphin augments the cytolytic activity and interferon production of natural killer cells. J. Immunol. 136, 934-939.

75. Margolick, J. B., Muñoz, A., Vlahov, D., Astemborski, J., Solomon, L., He, X-Y., et al. (1994). Direct comparison of the relationship between clinical outcome and change in CD4+ lymphocytes in human immunodeficiency virus-positive homosexual men and injecting drug users. Arch Intern Med, 154, 869–75.

76. Margolick, J. B., Muñoz, A., Vlahov, D., Solomon, L., Astemborski, J., Cohn, S., et al. (1992). Changes in T-lymphocyte subsets in intravenous drug users with HIV-1 infection. JAMA, 267, 1631–36.

77. McCarthy,L., Wetzel,M., Sliker,J.K., Eisenstein,T.K., and Rogers,T.J. (2001). Opioids, opioid receptors, and the immune response. [Review] [128 refs]. Drug & Alcohol Dependence 62, 111-123.

78. McCoy, C. B., Metsch, L. R., Chitwood, D. D., Shapshak, P., & Comerford, S. T. (1998). Parental transmission of HIV among injection drug users: Assessing the frequency of multiperson use of needles, syringes, cookers, cotton, and water. JAIDS, 18 Suppl 1 S25–S29.

79. Mellon,R.D. and Bayer,B.M. (1998). Role of central opioid receptor subtypes in morphine-induced alterations in peripheral lymphocyte activity. Brain Res. 789, 56-67.

80. Meng,F., Xie,G.X., Thompson,R.C., Mansour,A., Goldstein,A., Watson,S.J., and Akil,H. (1993). Cloning and pharmacological characterization of a rat kappa opioid receptor. Proc. Natl. Acad. Sci. (USA) 90, 9954-9958.

81. Miller, G. C., Murgo, A. J., & Plotnikoff, N. P. (1984). Enkephalins—enhancement of active T-cell rosettes from normal volunteers. Clin Immunol Immunopathol, 31, 132–37.

82. Minami,M. and Satoh,M. (1995). Molecular biology of the opioid receptors: structures, functions and distributions. [Review] [164 refs]. Neuroscience Research 23, 121-145.

83. Neudeck,B.L. and Loeb,J.M. (2002). Endomorphin-1 alters interleukin-8 secretion in Caco-2 cells via a receptor mediated process. Immunol. Lett. 84, 217-221.

84. Oberlin, E., Amara, A., Bachelerie, F., Bessia, C., Virelizier, J. L., Arenzana-Seisdedos, F., et al. (1996). The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. Nature, 382, 833–35.

85. Okey AB (2007). An aryl hydrocarbon receptor odyssey to the shores of toxicology: the Deichmann Lecture, International Congress of Toxicology-XI. Toxicol Sci; 98:5–38

86. Okutsu H, Watanabe S, Takahashi I, Aono Y, Saigusa T, Koshikawa N and Cools AR (2006). Endomorphin-2 and Endomorphin-1 Promote the Extracellular Amount of Accumbal Dopamine via Nonopioid and Mu-Opioid Receptors, Respectively.Neuropsychopharmacology 31, 375–383

87. Ohata H, Tetsuka T, Hayashi H, Onozaki K, and Okamoto T (2003).3-methylcholanthrene activates human immunodeficiency virus type 1 replication via aryl hydrocarbon receptor. Microbiol Immunol, 47,363-70

88. Peng, X., Mosser, D. M., Adler, M. W., Rogers, T. J., Meissler, J. J. Jr., & Eisenstein, T. K. (2000). Morphine enhances interleukin-12 and the production of other pro-inflammatory cytokines in mouse peritoneal macrophages. J Leukoc Biol, 68, 723–28.

89. Peterson, P. K., Sharp, B., Gekker, G., Brummitt, C., & Keane, W. F. (1987a). Opioid-mediated suppression of cultured peripheral blood mononuclear cell respiratory burst activity. J Immunol 138, 3907-3912.

90. Peterson, P. K., Sharp, B., Gekker, G., Brummitt, C., & Keane, W. F. (1987b). Opioid-mediated suppression of interferon-α production by cultured peripheral blood mononuclear cells. J Clin Invest, 80, 824–31.

91. Peterson, P. K., Sharp, B. M., Gekker, G., Portoghese, P. S., Sannerud, K., & Balfour, H. H. Jr. (1990). Morphine promotes the growth of HIV-1 in human peripheral blood mononuclear cell cocultures. AIDS, 4, 869–73.

92. Peterson, P. K., Gekker, G., Hu, S., Anderson, W. R., Kravitz, F., Portoghese, P. S., et al. (1994). Morphine amplifies HIV-1 expression in chronically infected promonocytes cocultured with human brain cells. J Neuroimmunol, 50, 167–75

93. Qi XF, Teng YC, Yoon YS, Kim DH, Cai DQ, and Lee KJ (2011).Reactive oxygen species are involved in the IFN-γ-stimulated production of Th2 chemokines in HaCaT keratinocytes. J Cell Physiol., 226,58-65.

94. Reddy P, Pilakka-Kanthikeel S, Saxena S, Saiyed Z, and Nair M (2012). Interactive Effects of Morphine on HIV Infection: Role in HIV-Associated Neurocognitive Disorder. [Review] AIDS Research and Treatment Volume 2012

95. Riezu-Boj J, Larrea E, Aldabe R, Guembe L, Casares N, Galeano E, Echeverria I, Sarobe P, Herrero I, Sangro B, Prieto J and Lasarte J (2011). Hepatitis C virus induces the expression of CCL17 and CCL22 chemokines that attract regulatory T cells to the site of infection. Journal of Hepatology, 54, 422–431

96. Rogers, T. J. & Peterson, P. K. (2003). Opioid G protein-coupled receptors: Signals at the crossroads of inflammation. Trends Immunol, 24, 116–21.

97. Rojavin, M., Szabo, I., Bussiere, J. L., Rogers, T. J., Adler, M. W., & Eisenstein, T. K. (1993). Morphine treatment in vitro or in vivo decreases phagocytic functions of murine macrophages. Life Sci, 53, 997–1006.

98. Romagnani,S. (2002). Cytokines and chemoattractants in allergic inflammation. Mol. Immunol. 38, 881-885.

99. Rothenberg, R., Woelfel, M., Stoneburner, R., Milberg, J., Parker, R., & Truman, B. (1986). Survival with the acquired immunodeficiency syndrome. Experience with 5833 cases in New York City. N Eng J Med, 317, 1297–302.

100. Roy, S., Ramakrishnan, S., Loh, H. H., & Lee, N. M. (1991). Chronic morphine treatment selectively suppresses macrophage colony formation in bone marrow. Eur J Pharmacol 195, 359-363.

101. Roy,S., Balasubramanian,S., Sumandeep,S., Charboneau,R., Wang,J., Melnyk,D., Beilman,G.J., Vatassery,R., and Barke,R.A. (2001). Morphine directs T cells toward T(H2) differentiation. Surgery 130, 304-309

102. Roy, S., Barke, R. A., & Loh, H. H. (1998a). Mu-opioid receptor-knockout mice: Role of μ-opioid receptor in morphine-mediated immune functions. Mol Brain Res, 61, 190–194.

103. Rock,R.B., Hu,S., Sheng,W.S., and Peterson,P.K. (2006). Morphine stimulates CCL2 production by human neurons. J. Neuroinflammation. 3, 32.

104. Roy, S., Chapin, R. B., Cain, K. J., Charboneau, R. G., Ramakrishnan, S., & Barke, R. A. (1997). Morphine inhibits transcriptional activation of IL-2 in mouse thymocytes. Cell Immunol, 179, 1–9.

105. Roy,S. and Loh,H.H. (1996). Effects of opioids on the immune system. [Review] [71 refs]. Neurochem. Res. 21, 1375-1386.

106. Ruff,M.R., Wahl,S.M., Mergenhagen,S., and Pert,C.B. (1985). Opiate receptor-mediated chemotaxis of human monocytes. Neuropeptides 5, 363-366.

107. Ruzicka,B.B., Fox,C.A., Thompson,R.C., Meng,F., Watson,S.J., and Akil,H. (1995). Primary astroglial cultures derived from several rat brain regions differentially express mu, delta and kappa opioid receptor mRNA. Brain Res. Molecular Brain Research. 34, 209-220.

108. Satoh,M. and Minami,M. (1995). Molecular pharmacology of the opioid receptors. [Review] [158 refs]. Pharmacology & Therapeutics 68, 343-364.

109. Schweitzer, C., Keller, F., Schmitt, M. P., Jaeck, D., Adloff, M., Schmitt, C. et al. (1991). Morphine stimulates HIV replication in primary cultures of human Kupffer cells. Res Virol, 142, 189–95.

110. Sedqi, M., Roy, S., Ramakrishnan, S., Elde, R., & Loh, H. H. (1995). Complementary DNA cloning of a mu-opioid receptor from rat peritoneal macrophages. Biochem Biophys Res Commun 209, 563-574.

111. Sharp,B.M., Roy,S., and Bidlack,J.M. (1998). Evidence for opioid receptors on cells involved in host defense and the immune system. [Review] [104 refs]. J. Neuroimmunol. 83, 45-56.

112. Shavit, Y., Martin, F. C., Yirmiya, R., Ben-Eliyahu, S., Terman, G. W., Weiner, H., et al. (1987). Effects of a single administration of morphine or footshock stress on natural killer cell cytotoxicity. Brain Behav Immun, 1, 318–28.

113. Sibinga, N. E., & Goldstein, A. (1988). Opioid peptides and opioid receptors in cells of the immune system. Annu Rev Immunol 6, 219-249.

114. Simon,M.I., Strathmann,M.P., and Gautam,N. (1991). Diversity of G proteins in signal transduction. Science 252, 802-808.

115. Smith H (2008). Variations in Opioid Responsiveness Pain Physician, 11, 237-248

116. Sozzani,S., Introna,M., Bernasconi,S., Polentarutti,N., Cinque,P., Poli,G., Sica,A., and Mantovani,A. (1997). MCP-1 and CCR2 in HIV infection: regulation of agonist and receptor expression. [Review] [23 refs]. J. Leuk. Biol. 62, 30-33.

117. Spijkerman, I. J., Koot, M., Keet, I. P., van den Hoek, A. J., Miedema, F., Coutinho, R. A. (1995). Lower prevalence and incidence of HIV-1 syncytium-inducing phenotype among injecting drug users compared with homosexual men. AIDS, 9, 1085–92.

118. Spijkerman, I. J., Langendam, M. W., Veugelers, P. J., van Ameijden, E. J., Keet, I. P., Geskus, R. B., et al. (1996). Differences in progression to AIDS between injection drug users and homosexual men with documented dates of seroconversion. Epidemiology, 7, 571–77.

119. Squinto SP, Mondal D, Block AL, Prakash O. (1990). Morphine-induced transactivation of HIV-1 LTR in human neuroblastoma cells. AIDS Res Hum Retroviruses.,6(10),1163-8.

120. Steele, A. D., Henderson, E. E., & Rogers, T. J. (2003). μ-Opioid modulation of HIV-1 coreceptor expression and HIV-1 replication. Virol, 309, 99–107.

121. Steele, A. D., Szabo, I., Bednar, F., & Rogers, T. J. (2002). Interactions between opioid and chemokine receptors: Heterologous desensitization. Cytok.Growth Factor Rev, 13, 209–22.

122. Stein C, Schäfer M, and Machelska H (2003). Attacking pain at its source: new perspectives on opioids. Nat Med, 9, 1003-8.

123. Szabo, I., Rojavin, M., Bussiere, J. L., Eisenstein, T. K., Adler, M. W., & Rogers, T. J. (1993). Suppression of peritoneal macrophage phagocytosis of Candida albicans by opioids. J Pharmacol Exp Ther, 267, 703–6.

124. Szabo, I., M. Wetzel, A., Zhang, N., Steele, A. D., Kaminsky, D. E., Chen, C., et al. (2003). Selective inactivation of CCR5 and decreased infectivity of R5 HIV-1 strains mediated by opioid-induced heterologous desensitization. J Leukoc Biol, 74, 1074–82.

125. Terenius, L., & Wahlstrom, A. (1975). Search for an endogenous ligand for the opiate receptor. Acta Physiol Scand 94, 74-81.

126. Tso,P.H. and Wong,Y.H. (2001). Role of extracellular signal-regulated kinases in opioid-induced adenylyl cyclase superactivation in human embryonic kidney 293 cells. Neurosci. Lett. 316, 13-16.

127. Viedt,C., Vogel,J., Athanasiou,T., Shen,W., Orth,S.R., Kubler,W., and Kreuzer,J. (2002). Monocyte chemoattractant protein-1 induces proliferation and interleukin-6 production in human smooth muscle cells by differential activation of nuclear factor-kappaB and activator protein-1. Arterioscler. Thromb. Vasc. Biol. 22, 914-920.

128. Villinger F and Ansari AA (2010). Role of IL-12 in HIV infection and vaccine. Eur. Cytokine Netw, Vol. 21 n° 3, September 2010, 215-8

129. Wang, X., Tan, N., Douglas, D., Zhang, T., Wang, Y. J., & Ho, W. Z. (2005). Morphine inhibits CD8+ cell-mediated, noncytolytic, anti-HIV activity in latently infeted immue cells. J Leukoc Biol, 78, 772–76.

130. Weber, R. J. & Pert, A. (1989). The periaqueductal gray matter mediates opiate-induced immunosuppression. Science, 245, 188–90.

131. Weiss,J.M., Nath,A., Major,E.O., and Berman,J.W. (1999). HIV-1 Tat induces monocyte chemoattractant protein-1-mediated monocyte transmigration across a model of the human blood-brain barrier and up-regulates CCR5 expression on human monocytes. J. Immunol. 163, 2953-2959.

132. Weiss, R. A. (1993). How does HIV cause AIDS? Science 260, 1273-1279.

133. Wetzel, M. A., Steele, A. D., Eisenstein, T. K., Adler, M. W., Henderson, E. E., & Rogers, T. J. (2000). μ-opioid induction of monocyte chemoattractant protein-

1, RANTES, and IFN-α-inducible protein-10 expression in human peripheral blood mononuclear cells. J Immunol, 165, 6519–24

134. Williams JT, Christie MJ, & Manzoni O (2001). Cellular and Synaptic Adaptations Mediating Opioid Dependence.Physiol Rev,8,:299-343

135. Wybran, J., Appelboom, T., Famaey, J. P., & Govaerts, A. (1979). Suggestive evidence for receptors for morphine and methionine- enkephalin on normal human blood T lymphocytes. J Immunol 123, 1068-1070.

136. Yeager, M. P., Colacchio, T. A., Yu, C. T., Hildebrandt, L., Howell, A. L., Weiss, J., et al. (1995). Morphine inhibits spontaneous and cytokine-enhanced natural killer cell cytotoxicity in volunteers. Anesthesiology, 83, 500–508.

137. Zadina, J. E., Hackler, L., Ge, L. J., & Kastin, A. J. (1997). A potent and selective endogenous agonist for the mu-opiate receptor. Nature 386, 499-502.

138. Zhang,Z., Xin,S.M., Wu,G.X., Zhang,W.B., Ma,L., and Pei,G. (1999). Endogenous delta-opioid and ORL1 receptors couple to phosphorylation and activation of p38 MAPK in NG108-15 cells and this is regulated by protein kinase A and protein kinase C. J. Neurochem. 73, 1502-1509.

139. Zlotnik,A. and Yoshie,O. (2000). Chemokines: a new classification system and their role in immunity. Immunity. 12, 121-127.