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Methylenenedioxymethamphetamine (MDMA; "Ecstasy") suppresses the innate immune system: A critical role for the anti-inflammatory cytokine interleukin-10.

by

Noreen Boyle

Thesis submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College

Thesis submitted June 2007

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I Declaration

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II Abstract

3,4-methylenedioxymethamphetamine (MDMA; "Ecstasy") is a ring-substituted phenylisopropylamine and globally abused drug that has been implicated to have immunosuppressive effects in animals and humans. Here MDMA was shown to suppress production of the pro-inflammatory and Th_1 promoting cytokine IFN-γ in response to an *in vivo* lipopolysaccharide (LPS) challenge in mice. Impaired IFN-γ production was accompanied by reduced expression of IL-12 and IL-15; two cytokines involved in IFN-γ induction. MDMA also resulted in impaired IFN-γ signalling, indicated by reduced STAT1 phosphorylation and reduced expression of several IFN-γ inducible molecules, namely the chemokine interferon inducible protein 10 (IP-10), the co-stimulatory molecule CD40, and the enzyme responsible for nitric oxide synthesis, iNOS. Furthermore, MDMA down-regulated cell surface expression of MHC class II and the co-stimulatory molecules CD40, ICAM-1 (CD54) and B7.2 (CD86) on dendritic cells and macrophages; these molecules are required for effective antigen presentation, and T-cell activation. Consistent with its ability to interfere with antigen presentation, MDMA suppressed T-cell activation in the mixed lymphocyte reaction (MLR) signifying that the proliferative capacity of stimulator cells was abolished. In addition, MDMA suppressed antigen-specific cytokine responses to the protein antigen KLH.

The results presented here demonstrate that *in vitro* exposure to MDMA does not mimic the suppression of innate IFN-γ production observed *in vivo*, indicating that observed suppression is most likely due to the release of endogenous immunomodulatory substances following drug administration. In this regard, previous studies indicate that MDMA increases production of the anti-inflammatory cytokine IL-10 *in vivo*, an event that is mediated by activation of β-adrenoceptors on immune cells. Consequently, the possibility that IL-10 could mediate the suppressive effect of MDMA on the innate IFN-γ response was examined. In support of this hypothesis, *in vitro* exposure of spleen cells to IL-10 mimicked the suppressive effect of MDMA on IL-12 and INF-γ production. Furthermore, by pre-treating mice with an anti-IL-10 receptor antibody it was demonstrated that IL-10 is a critical mediator of MDMA-induced suppression of IFN-γ production and signaling, and MHC class II and co-stimulatory molecule expression. Similarly, pre-treatment with the β-adrenoceptor antagonist nadolol completely blocked MDMA-induced increase in IL-10 production, and also inhibited the suppressive action of MDMA on the innate IFN-γ response and MHC class II expression. Overall these results indicate that MDMA suppresses the innate IFN-γ and antigen presenting capability by increasing production of the anti-inflammatory cytokine IL-10. These findings further support the notion the MDMA has immunosuppressive properties, and that a major target of its immunosuppressive actions is the cytokine network.

III Acknowledgements

I would like to take this opportunity to sincerely thank everyone who has helped me over the last three years to get me to this point of completing my Ph.D.

First, I would like to acknowledge my supervisor, Dr. Tom Connor, for giving me the opportunity to do this Ph.D, for his continuous help, guidance and never-ending support during the years, I really appreciate it. You were a fantastic supervisor, with great drive and enthusiasm, thanks a million!

I would like to thank Professor Chris Bell and Dr. Veronica Campbell for allowing me to do my Ph.D in the Physiology Department.

I am extremely grateful to all the lovely technical staff in Physiology for all their help and assistance throughout my Ph.D. I would like to thank everyone in the TCIN also. Thanks to Dr.Andrew Harkin for his help over the last few years.

To everyone in the Connor Lab, where would I be with y'all? To Joan, Eoin and Niamh, thank you for all the practical help and of course, the great laughs, funny moments and wonderful memories over the last 3 years. Thanks to all the new recruits to the lab, Karen and Dana and the Harkin crew, Sarah, Lorna and Natasha for making our lab such a lovely and friendly place to work. I know you will all be very successful whichever path in life you choose.

To all the PIs, post grads and post docs in Mal, VAC, Kelly and Anwyl labs for helping out in every way possible. Thanks a million for welcoming me into your department and all the nights out over the last few years. Not only have I gained a wealth of knowledge over the last 3 years but I've made such great friends too.

I would like to say thanks to Steve Todryk and all the staff in Department of Biochemistry and Immunology for all their help and assistance and to everyone in the Bioresources unit.

I would like to say a massive thank you to all my relatives and fantastic friends for their love, support and words of encouragement.

To my family, my dad, mum, sister, two brothers, in-laws, niece and nephew, you all mean the world to me. I couldn't have done this without all your love, support & encouragement, go raibh maith agaibh.

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VII Abbreviations

5HT	Serotonin
AIDS	Acquired immunodeficiency syndrome
AP-1	Activating protein
APC	Antigen presenting cell
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Ca2+	Calcium
CAMP	3'5'-cyclic adenosine monophosphate
CC	Cysteine-cysteine
CD	Cluster designation
CRF	Corticotrophin releasing factor
CIITA	MHC class II transactivator
CNS	Central nervous system
COMT	catechol-O-methyltransferase
Con A	Concanavalin A
CSIF	Cytokine synthesis inhibitory factor
CXC	Cysteine-x amino acid-cysteine
DA	Dopamine
DBH	Dopamine β– hydroxylase
DC	Dendritic cell
DEA	Drug enforcement agency
EAE	Experimental autoimmune encephalomyelitis
FCS	Fetal calf serum
FDC	Follicular dentritic cell
GAS	Gamma-activated sequence elements
H_2O_2	Hydrogen peroxide
HVB	Hepatitis virus B
HIV	Human immunodeficiency virus
HPA	Hypothalamic pituitary adrenal axis
HZO	Herpes zoster opthalmicus
Ikβ	Inhibitor of kβ
ICAM	Intracellular cell adhesion molecule
IFN-γ	Interferon gamma

IFNGR	Interferon gamma receptor
IGIF	Interferon gamma inducing factor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP-10	Interferon protein of 10kDa
IRAK	Interleukin 1 receptor associated kinase
JAK	Janus Kinase
KLH	Keyhole Limpet Haemocyanin
LBP	Lipid binding protein
LFA	Lymphocyte function antigen-1
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
Mal	MyD88 adapter like protein
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-Methyldioxymethamphetamine
MHC class II	Major histocompatibility complex class II
MLR	Mixed lymphocyte reaction
MMM	Marginal metallophilic macrophage
MZM	Marginal zone macrophage
MyD88	Myeloid differentiation factor 88
NA	Noradrenaline
NFkβ	Nuclear factor-kβ
NK	Natural Killer
NO	Nitric oxide
O2-	Superoxide anion
OH	Hydroxyl radical
PALS	Periarteriolar lymphoid sheath
pDC	Plasmacytoid dendritic cells
PRR	Pattern-recognition receptor
PKA	Protein kinase A
ROS	Reactive oxygen species
RNI	Reactive oxygen intermediate
SERT	Serotonin transporter
SH2	Src-homology 2
SNS	Sympathetetic nervous system

STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
TGF-β	Transforming growth factor-beta
TIR	Toll-Interleukin IL-1 receptor
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRAF	Tumor necrois factor-associated factor
TRAM	TIR-related adapter molecule
VCAM	Vascular cell adhesion molecule

Chapter 1: Introduction

1.1 The Immune System

The immune system is a complex network comprised of organs, cells and effector molecules expressed in various tissues and the peripheral circulation. The primary function of the immune system is in maintaining homeostasis, and protection against invading organisms that enter the body. It infers such protection by immuno-surveillance, detection of foreign or non-host cells, and induction of appropriate responses to eradicate hazardous infectious agents. The immune system also plays an important anti-neoplastic role, in that it detects and eradicates transformed (cancerous) cells from the body. In fact the immune system has been described as a "sensory organ" as it possesses the "molecular machinery" to detect infectious agents such as viruses and bacteria upon entry to the body (Blalock *et al.*, 1984). From an anatomical perspective the immune system is diffuse, in that it is spread throughout various tissues of the body. Therefore in the event of an infectious agent entering the body at any point there are vast numbers of immune cells available that can be recruited, and have the potential to clear the infection with minimal damage to the host tissue. The activities of the immune system can be broadly categorised into one of two phases, namely the innate immune response, and the adaptive immune response.

1.2 Organs of the immune system

The organs of the immune system are collectively referred to as the lymphoid system. It is arranged into either separately enclosed organs or diffuse areas of tissue. The lymphatic organs are those in which lymphocyte maturation, differentiation and proliferation take place. These organs can thus be divided into two further categories: primary and secondary lymphoid organs. The primary lymphoid organs are the thymus and bone marrow, microenvironments in which the maturation (antigen independent differentiation) of T- and B-lymphocytes into antigen-recognizing lymphocytes occurs. The secondary lymphoid organs and tissues consist of lymph nodes, spleen, peyers patches.

1.2.1 Primary Lymph Organs: Thymus and bone marrow

It is in the primary lymph organs that an individual acquires their diverse collection of specific antigen receptors in order to cope with antigenic challenge. Immature

lymphocytes must reside here to undergo structural and functional maturation. Cells presenting auto-antigen receptors are also eliminated here.

The thymus gland facilitates the differentiation of progenitor cells into T lymphocytes. The human thymus is a bi-lobed elongated organ weighing about 40 g at puberty but then begins to involute. It is located overlying the heart near the throat, resting on the pericardium behind the breastbone. Each lobe is organised into lobules by and within each lobule the lymphoid cells are arranged into an outer cortex and inner medulla. The cortex contains high levels of immature proliferating thymocytes, whereas the medulla contains higher levels of mature thymocytes. The release of lymphocytes from the thymus is slow, only 5-10% of all maturing lymphocytes survive and eventually leave the thymus.

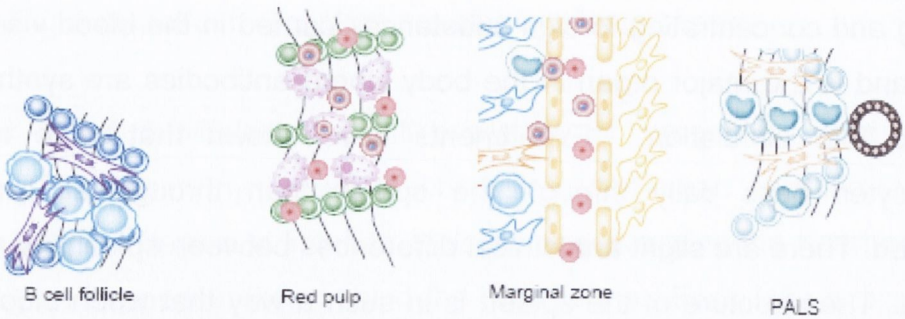
The bone marrow contains pluripotent hematopoietic stem cells which are the common ancestral cells for all blood cells. These cells have the ability to proliferate and then to differential into all of the blood cell lineages. Specific discreet organs for B-cell development do not exist in mammals, these cells develop from lymphoid stem cells in the haemopoietic tissue of the fetal liver. Later the production of these cells moves to the bone marrow, where it continues for adult life. The bone marrow is the B-cell factory in mammals, and is the birthplace for all immune cells.

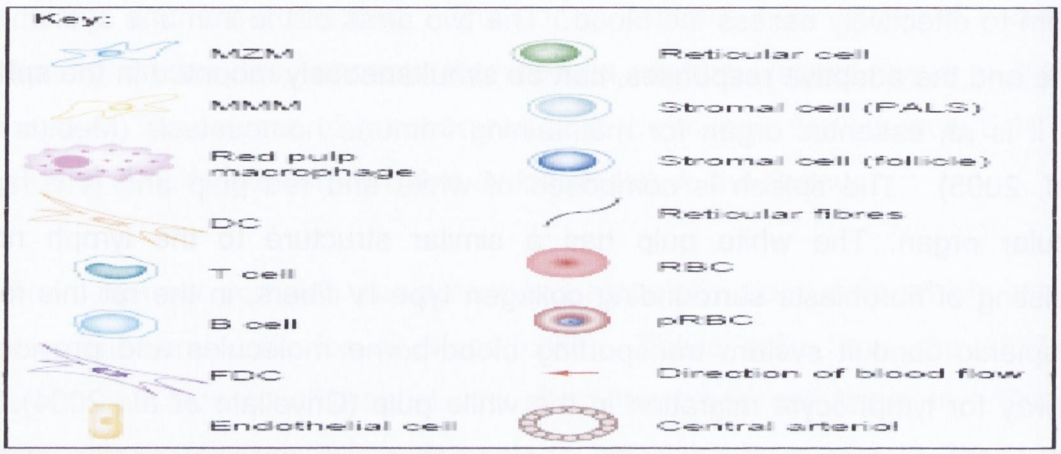
1.2.2 Secondary Lymph Organs: Spleen and lymph node

Immune cells travel trough the body via the circulatory and lymphatic systems. The lymphatic system is the drainage system of the body and serves to recover materials lost from the blood capillary network and antigens that enter the tissue spaces and return them to the blood. The secondary lymph organs have two major functions: they are highly efficient in trapping and concentrating foreign substances, and they are the major sites of production of antibodies and the induction of antigen specific T lymphocytes. As many of the immune parameters evaluated in this thesis were measured in spleen tissue, the spleen will receive most of the focus in this section.

The spleen is the largest of the secondary lymphoid organs, and its main function is a rapid response to blood born antigens. It is located at the upper left quadrant

of the abdomen cavity behind the stomach. The spleen is highly efficient in trapping and concentrating foreign substances carried in the blood via the splenic artery, and is the major organ in the body where antibodies are synthesized and released into circulation. Experiments have shown that more recirculating lymphocytes pass daily through the spleen than through all lymph nodes combined. There are slight anatomical differences between spleens in rodents and humans. The structure of the spleen is in such a way that when blood is filtered and passed through the marginal zone to the white pulp, it allows the immune system to effectively assess the blood. The two arms of the immune system, the innate and the adaptive responses, can be simultaneously mounted in the spleen, thus it is an essential organ for maintaining immune homeostasis (Mebius and Kraal, 2005). The spleen is composed of white and red pulp and is a highly vascular organ. The white pulp has a similar structure to the lymph node, consisting of fibroblasts surrounding collagen type IV fibers, in the rat this forms the splenic conduit system transporting blood-borne molecules and provides a pathway for lymphocyte migration in the while pulp (Crivellato *et al.*, 2004). The functional aspect of the white pulp is associated with antigen-specific immune responses. It is divided into 3 regions (Figure 1.1), the periarteriolar lymphoid sheaths (PALS), lymph follicles and a marginal zone that separates the white pulp from the red pulp. The PALS compartment is a T-cell dependent area whereas B-cells are predominantly occupied in the lymph follicles and marginal zone. Contained within the lymph follicles are interdigitating dendritic cells and follicular DCs (FDCs).





MZM- Marginal zone macrophage

MMM-Marginal metalophillic macrophage

Figure 1.1: Various regions of spleen indicating the expression of each immune cell type (Adapted from Engwerda *et al.*, 2005).

The functions of the PALS and the lymph follicles are associated with a homing mechanism for T and B cells, respectively (Crivellato *et al.*, 2004). The function of the marginal zone is to allow the entrance to the white pulp for blood-borne antigens, antigen presenting cells and lymphocytes. This area contains B-cells, NK cells and two populations of highly specialised macrophages, marginal metallophilic macrophages (MMM) and marginal zone macrophages (MZM) (Mebius and Kraal, 2005). Blood enters via the afferent splenic artery, where it passes through the white pulp in central arterioles that terminate in cords of the red pulp. From here, blood flows into venous sinuses and will be eventually collected into the efferent collecting splenic vein (Mebius and Kraal, 2005). Once bacteria are detected within the circulatory system, blood dendritic cells (DCs) are involved in the immediate response with uptake of such pathogens and transport

to the spleen. When these DCs enter the spleen, they induce B cells to differentiate to antibody-producing B cells (Balazs *et al*, 2002).

The spleen is richly innervated by the sympathetic nervous system (Felten, 1987 and Nance *et al.*, 1989) and has been the focus of neuro-immune research over the last number of years. Mouse spleen cells consist of mixed populations of T-cells and monocytic cells and studies have indicated that sympathetic nerve endings contact splenic T-cells and macrophages in a synaptic-like manner, thus forming a neuro-immune junction (Straub *et al*, 1998). Therefore the spleen can be used as a model organ for analysing brain-immune communication (Crivellato *et al*, 2004).

The lymph nodes consist of a network which antigens from the interstitial fluid and lymph during its passage from the periphery to the major collecting ducts. Clusters of nodes are found at various points around the body: neck, axillae, groin, mediastinum and abdominal cavity. The nodes protect the skin and mucosal surfaces of the respiratory, digestive and genitourinary tracts. Nodes are very efficient at trapping antigens entering via afferent lymphatic vessel.

1.3 Cells of the Immune System

The immune response is mediated through a variety of cells and by the different molecules and factors they secrete. Carried within the blood and lymph are a group of white blood cells (leukocytes) which play a pivotal role in the immune response (Figure 1.2). The functions of the various categories of cells involved in the immune response are outlined below.

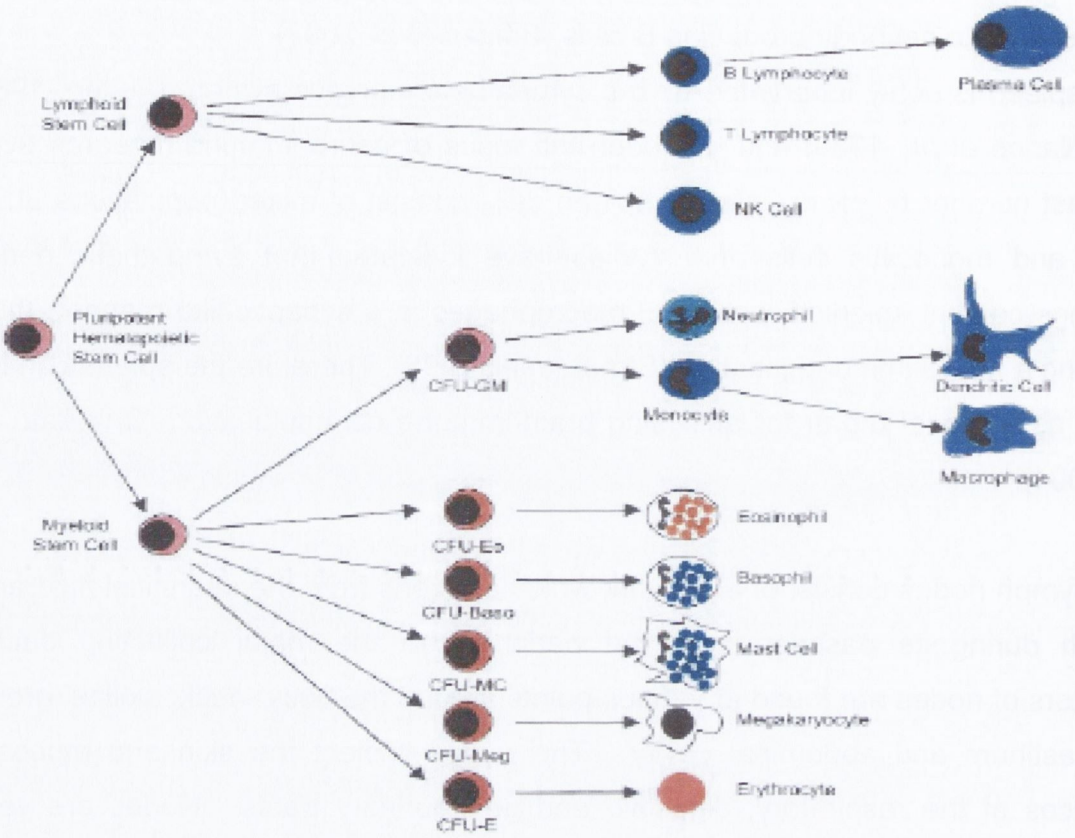


Figure 1.2: Immune cell lineages derived from pluripotent hematopoiesis stem cells (Chaplin, 1996).

1.3.1 Monocytes

Monocytes (mononuclear phagocytes) are a group of cells whose function is to engulf, internalise and destroy foreign particles. During times of infection these cells migrate from the blood and develop into tissue macrophages. There are two major functions for these types of cells: to remove particulate antigens and process and present antigenic peptides to T-cells. They encounter an antigen, internalise it by phagocytosis or receptor-mediated endocytosis and degrade it through lysosomal proteolysis. The ability of macrophages to secrete pro- and anti- inflammatory mediators has been established and over 100 different products have been identified which are secreted by these cells: hydrolytic enzymes, complement components, oxygen metabolites, nitric oxide, nucleosides and bioactive lipids. Upon activation, macrophages become proficient antigen presenting cells (APC's), expressing higher levels of MHC class II molecules.

1.3.2 Dendritic Cells

Dendritic cells (DCs) are classified as being professional and the most efficient antigen presenting cells during innate immune responses (Steinman et al., 1978). Dendritic cells that are immature are located in the periphery where they are distinguished by their ability to capture antigen, the presence of high levels of intercellular MHC class II molecules and the low expression of co-stimulatory molecules including CD80 and CD86. Thus, immature DCs functions are associated with antigen capture in the periphery. At the site of antigen deposition, stimulation of DCs by pathogens triggers the onset of DC maturity after which they migrate to lymphoid organs in a matter of hours. The capacity to capture antigen is reduced in these mature DCs while co-stimulatory molecules are up-regulated and MHC class II molecules are expressed from the cytoplasm to the cell surface. Hence, mature DCs are specialized for effective presentation of antigens to T-lymphocytes in lymphoid tissues (Mellman *et al.*, 1998 and Banchereau *et al.*, 1998). Very low concentrations of DCs are expressed in the blood compared to that of other leucocytes. Blood derived DCs express very low levels of CD40, CD86, CD80 and MHC class II molecules are concentrated in endosomal compartments rather than on plasma membrane. DCs are found throughout the body in various organs including lymph nodes, spleen, gut, skin and lungs. There are seven distinct populations of dendritic cells that have been characterized in mouse spleen and lymph nodes so far (Shortman *et al.*, 2002). One of these populations known as plasmacytoid dendritic cells (pDC) is easily distinguished by the expression of CD45RA and intermediate levels of CD11c. Since DC numbers are so low, very few specific markers exist, however CD11c marker is used in mouse (Metlay *et al.*, 1990). It is well established that dendritic cells are originally derived from the hematopoietic cell line, however in humans, they have myeloid and plasmacytoid origin while in mice they have lymphoid and myeloid origin. Subsets of dendritic cells in the mouse spleen are located in two areas, the marginal zone, and others are expressed in T-cell areas (Banchereau *et al.*, 1998).

1.3.3 Neutrophils

Neutrophils (Polymorphonuclear granulocytes) constitute the majority of blood leukocytes, and like monocytes migrate into inflamed tissues engulfing material destroying it and then die themselves. They roll along endothelial cells to the site of infection whereby they attack invading organisms by phagocytosis. Neutrophils

have a huge arsenal of antibiotic proteins stored in granules within the cells. The primary granules are lysosymes containing acid hydrolases, myeloperoxidase and muramidase, while the secondary granules contain lactoferrin and lysozyme. In addition the granules contain antibiotic proteins: defensins, seprocidins, cathelicidins and bacterial permeability inducing protein. Respiratory burst activity occurs within the granules and is an oxygen dependent mechanism involving NAPDH oxidase. This results in the formation of reactive oxygen species (ROIs) such as hydrogen peroxide and hydroxyl radicals that are extremely toxic to infectious agents (Ward and Lentsch, 1999).

1.3.4 Lymphocytes

There are two major classes of lymphocytes responsible for the recognition of antigens: B-cells and T-cells. Whereas all lymphocytes are derived from the bone marrow, T-cells differentiate in the thymus and B-cells develop in the bone marrow. T-cells are required for the full expression of immunity. Following maturation, lymphocytes migrate via the circulation and populate in secondary lymphoid tissue (Parkin and Cohen, 2001). Lymphocytes express surface molecules on their surface which can be recognised by monoclonal antibodies. A group or cluster of antibodies to a specific molecule is defined as cluster designation (CD). The CD surface molecules are divided into different families depending on their structure, for instance, surface molecules including CD4, CD8, MHC class I and II are designated to the immunoglobulin family.

1.3.4.1 T-cells

T cells have several different subsets with diverse functions. Each T-cell recognises antigens presented on the surface of other cells by MHC molecules. T-cells recognise protein antigens that have been processed and presented bound to MHC molecules displayed on a host cell. Upon engagement with a self-MHC molecule, the T-cell becomes activated and performs several different tasks. Cellular immunity is mediated through the production of cytokines, promoting proliferation and differentiation of other T-cells and components of the immune system. Lymphocytes can also directly interact with pathogen infected or abnormal cells: viruses, foreign tissue and tumour cells. T-cells can be further differentiated into two mature kinds of cell: Helper T-cells and Cytotoxic T-cells. Helper T-cells help other T-and B-cells multiply into large clones and activate the

immune response. Cytotoxic T cells are directly responsible for killing tumour cells, virus infected cells and foreign tissue.

1.3.4.2 B-cells

B cells have receptors on their surface for a specific antigen, and each B-cell contains the genetics to produce antibodies of unique antigen specificity, which is expressed via membrane receptors. Upon recognition of this antigen, the cell differentiates into a plasma cell and begins secreting large amounts of the receptor in soluble form known as an antibody. Mature B-cells do not secrete antibody until they differentiate into anti-body secreting cells during inflammation. Antibodies secreted from B-cells facilitate innate immunity by activating complement, opsonising parasites prior to phagocytosis, preventing infectious agents attaching to mucosal surfaces and inducing antibody dependent cytotoxic attack on tumour and infected cells (Parkin and Cohen, 2001 and Nathan, 2006).

1.3.5 NK-cells

This group of cells has the ability to recognize tumour cells and virally infected cells, and like macrophages, have the ability to destroy the cells. NK cells kill infected targets coated with IgG antibodies due to the expression of the receptor for IgG (FcγIII:CD16) on the cell surface. This mechanism is known as antibody-dependent cellular cytotoxicity (ADCC). NK cells recognise host cells by the expression of MHC class I molecule, so will not lyse these cells whereas tumor cells or viruses can down-regulate these receptor molecules. NK cells use their intracellular granules as toxic agents to kill invading parasites. They adhere to non-host cells while releasing molecules from the granules including perforins, which form pores on the cell surface and granzymes that are injected in through pores on the membrane (Tosi, 2001). Once inside the infected cell, these granzymes induce cell apoptosis. In addition, they secrete cytokines such as IFN-γ and chemokines, which are involved with initiating the adaptive response and controlling pathogens and tumour growth.

1.4 Soluble effector molecules: Cytokines and chemokines

Cytokines may be defined as small molecular weight proteins acting as messengers and are secreted by immune cells to change the activity of other cells. There is a multitude of diverse and complex roles played by these messengers: growth, differentiation, effector function, and the survival of cells. These pluripotent proteins convey their chemical message to cells via receptors on the target, which act as molecular switches. Each cytokine exerts numerous effects on several cells, which can also be modulated in a synergistic or inhibitory fashion by other cytokines. The cytokines comprise a large family of similar immunologically active molecules including interleukins, growth factors, interferons, chemokines and lymphokines. The term interleukin (IL) was defined as a molecule that transmitted chemical signals from leucocytes, monokines as products of monocytes and lymphokines as products of lymphocytes.

The extracellular complexity of cytokine signalling cannot be understated, as most cytokines have diversely shared roles in the biological system. As knockout studies have shown, very few individual cytokines seem to be essential for survival. The genetic loss of one cytokine is usually compensated by another cytokine acting via a similar pathway. Since cytokines act locally, the *in vivo* activity of these cells is restricted to the immediate vicinity of their release and to cells presenting specific receptors for that cytokine. Their signals are transmitted by binding to receptors on the surface of specific cells (Parkin and Cohen 2001). Cytokines produced by the innate immune system may influence the differentiation of naïve Th_0 cells towards Th_1 and Th_2 cytokine profile (Fearon *et al*, 1996). Depending on which type of cytokine that is produced, whether it is pro-inflammatory or anti-inflammatory determines the phenotype of the immune response. Upon stimulation of innate immune system inflammatory cytokines are released from macrophages/monocytes and dendritic cells and perform cell-cell communication roles within the immune system (Rouveix *et al.*, 1997; Boraschi *et al.*, 1998). Cytokines that are studied in this thesis are discussed below.

1.4.1 Interleukin 12p40

The production of Interleukin 12 (IL-12) plays an important role as it bridges the gap between the innate and acquired immunity (Trinchieri *et al.*, 1995). IL-12 is mainly derived from monocytes/ macrophages but also secreted from dendritic

cells (Cella *et al.*, 1996), B cells, Langerhans cells, polymorphonuclear neutrophils and mast cells. It is a heterodimeric cytokine of 70kDa (p70) consisting of two covalently linked subunits, a 40kDa heavy chain (p40) and 35kDa light chain (p35) (Kobayashi et al., 1989). To ensure that the production of heterodimeric IL-12 is adequate, both p40 and p35 chains must be expressed correctly. The two genes encoding p40 and p35 are unrelated and located on separate chromosomes (5q 31-33 & 3p12-q13.2 in humans and chromosome 11 & 6 in mice) (Trinchieri, 1998**).** The cells that primarily secrete the IL-12 heterodimer express the p40 gene while the p35 is constitutively expressed in low levels in most cell types (Aste-Amezaga *et al*, 1998). Studies have shown that IL-12p35 gene is thought to be expressed in all cells, however, IL-12p40 transcripts is tightly controlled and is detected only in cell types with biological activity of IL-12. It is vital that the genes, p40 and p35, encoding IL-12 are expressed collectively in the same cells so that a heterodimer with biologically activity is produced (Wolf *et al.*, 1991). Yet in the absence of p35, p40 is secreted as a monomer or a homodimer whereas p35 can be secreted only when associated with p40 (Ma *et al.*, 1998). Studies have shown that mice lacking in the p40 chain of IL-12 produce minimal levels of IFN-γ. In addition, it was noted that the $CD8^+$ T-cell response was greatly reduced in p40 knockout mice compared to p35 deficient mice and similarly for resistance to infection with mycobacteria (Cooper *et al.*, 2002).

The IL-12 receptor consists of β1 and β2 subunits (Presky *et al.*, 1996) and mainly expressed NK cells, T cells and dendritic cell (Grohmann *et al.*, 1998). It is clear that expression of both β1 and β2 subunits are required for IL-12 to bind to the receptor. IL-12p40 interacts with β1 while p35 binds to β2 subunit. Intracellular enzymes, Tyk2 and JAK2 are associated with β1 and β2 subunits of IL-12R respectively (Zou *et al.*, 1997).

Infections, such as mycobacterium and salmonella in humans, occur as a consequence of mutated genes for IL-12p40 and IL-12Rβ1 (de Jong *et al.*, 1998). Following interaction of IL-12 with the IL-12Rβ chain on the cell surface, activation the Jak/Stat signaling pathway occurs resulting in the nuclear translocation of STAT 3 or STAT 4, the transcription factors involved in regulating IFN-γ production (Jacobson *et al.*, 1995). However, most studies have documented that STAT4

molecule is primarily associated with IL-12 signaling (Bacon *et al.*, 1995). Moreover, STAT4 knockout mice present deficits in IFN-γ production and a reduced capacity to induce a Th1 immune response (Kaplan *et al.*, 1996).

The functions of IL-12 are activation, proliferation (Bertagnolli *et al.*, 1992) and cytotoxicity of pre-activated Natural Killer (NK cells) and antigen-specific T-helper cells. It is a potent inducer of IFN-γ production from these cells (Trinchieri, 2003). In fact, the original term for IL-12 was the Natural Killer stimulatory factor (Kobayashi *et al.*, 1989). It interacts with the IL-12R and induces NK cells to produce IFN-γ resulting in antimicrobial activity to inhibit pathogen growth. It has been shown that IL-12 induces IFN-γ in the mouse following large doses of LPS (Wysocka *et al.*, 1995). IL-12 is an important cytokine involved in the development of cell-mediated immunity against intracellular pathogens such as *Mycobacterium tuberculosis*, *Listeria monocytegenes* and HIV (Trinchieri 2001, 1997, Biron 1995 and Cooper 2002).

1.4.2 Interleukin 15 and Interleukin 18

Interleukin-15 is a 15kDa cytokine and is a member of the 4α-helix family. IL-15 and IL-2 share a common receptor, the IL-2/IL-15Rβ, in addition, it is associated to another subunit, known as γc, which is common to other cytokines including, IL-4, IL-7, IL-9 and IL-21 (Ohteki, 2002) However IL-2R and IL-15R contain α subunit that is unique to these receptors. LPS-TLR4 signalling IL-15 up-regulates the synchronised expression of IL-15 and IL-15Rα on the cell surface of macrophage and dendritic cells. The IL-15R complex is recycled in endosomal vesicles within the cell. Once re-expressed on the cell surface, IL-15R delivers IL-15 in trans to T-cells and NK cells that express IL-2/IL-15Bγ2. Once IL-15α interacts with the receptor, it induces the phosphorylation of intracellular Jak1 and Jak3 therefore enhancing the formation of STAT5 homodimers which translocate to the nucleus (Waldmann, 2006). IL-15 plays a crucial role for development, proliferation of NK cells, NKT cells, memory $CD8^+$ T-cells and also for the maturation of dendritic cells and macrophages. IL-15 production acts in concerted fashion with IL-12 to induce IFN-γ secretion (Fehniger *et al.*, 2002). Studies have shown that dendritic cells and macrophages from IL-15 knockout mice lose their ability to produce IFN-γ in response to IL-12. This is partly due to the IL-12Rβ1 receptor which is not properly

expressed on the cell surface. Therefore it is thought that IL-15 induces IL-12Rβ on immature NK cells and following stimulation with IL-12, produces maximal concentrations of IFN-γ (Ohteki *et al.*, 2000). Monocytes release IL-15 and IL-12 with similar kinetics.

Interleukin 18 was originally coined as IFN-γ inducing factor (IGIF) due to its' ability to stimulate large amounts of IFN-γ from NK and T cells. However, it is now recognized as a member of the T helper (Th) type I cell along with other cytokines, including IL-12, IL-15 and IFN-γ. It has been well considered that IL-12 is required to up-regulate the IL-18 receptor. IL-18 synergises with IL-12 to induce the production of IFN-γ from bone marrow derived macrophages, since as an individual cytokine, it is only capable of inducing low levels of IFN-γ (Munder *et al.*, 1998). The IFN-γ promoter is activated by IL-18 via binding of c-jun to AP-1 (activating protein-1) and by IL-12 via binding to the STAT4 site (Dinarello, 1999). Stimuli, such as IL-18, associated with potentiating the effects of IL-12, activate the NF-kβ family of transcription factors (Matsumoto *et al.*, 1993). It has been shown that NF-kβ binding site in the IFN-γ promoter region, hence it is increased levels of IFN-γ production (Sica *et al.*, 1992).

1.4.3 Interferon-γ

IFN-γ is one of the three members of the IFN family. The nomenclature was derived from the ability of the molecules to "interfere" with viral activity, where IFN-γ activity is very slight compared to the others, IFN-α and IFN-β. It is produced from T-helper lymphocytes, NK cells and NKT cells. IFN-γ plays an important role in orchestrating cell-mediated immunity (Farrar *et al.*, 1993). IFN-γ stimulates macrophage functions including phagocytosis, respiratory burst activity, antigen presentation and cytokine secretion in order to facilitate an antimicrobial immune response (Boehm, 1997). Other functions include secretion of IgG_{2a} antibodies from B cells, promoting cytotoxic T cell maturation, neutrophil, activation and importantly stimulating Th_1 cell differentiation (Boehm, 1997). Studies have shown that IFN-γ can also be produced by macrophages and dendritic cells (Munder *et al.*, 1998 and Ohteki *et al.*, 1999), but these cells are regarded as minor sources of IFN-γ in comparison to NK-cells and T-cells.

It is well documented that Natural Killer (NK) cells are known to be the most potent inducers of IFN-γ during the early stages of viral, parasitic or bacterial infection which provoke the cytolytic activity of these cells. NK cells are the classical set due the non-existent expression of TCR, hence is not a T-cell and a key player in immune surveillance. IL-12 binds to the IL-12R on the cell surface, inducing downstream signaling that results in the production of IFN-γ. This in turn, activates antimicrobial responses involved with the prevention of pathogen growth. In addition to IL-12, previous in vitro studies suggest that IL-18 stimulates IFN-γ production from NK cells (Micallef *et al.*, 1996).

The second group of NK cells is known as natural killer T-cells since they express TCR and NK cell markers. NKT cells activate NK cells thus are involved with regulating the innate immune response. Moreover, NKT cells produce IFN-γ, which in turn activates antigen presenting cells such as dendritic and macrophage cells to secrete IL-12 (Kitamura *et al.*, 1999), and up-regulate MHC class II, CD40 and iNOS expression. Moreover, secreted IL-12 from activated dendritic cells and macrophages stimulates IFN-γ from NK cells and $CD4^{+}$T-cells, depicting an autocrine loop of IFN-γ production (Figure 1.3a). However, several investigators have recently ascertained that IFN-γ is also derived from antigen presenting cells (Golab *et al.*, 2000 and Munder *et al.*, 2001). It has been shown that stimuli including LPS, IFN-γ, *Listeria monocytogenes*, IL-12 and IL-18 and *Mycobacterium tuberculosis* produce IFN-γ from macrophages in mouse and human. This suggests that IFN-γ secreted from macrophages depicts an autocrine loop for IFN-γ production, therefore potentiaing a Th_1 phenotype (Munder *et al*, 1998). Moreover, dendritic cells are also thought to release IFN-γ (Figure 1.3b) in response to the synergism of both IL-12 and IL-18 (Stober *et al.*, 2001). It has been demonstrated that IFN-γ is secreted from dendritic cells in a IL-12 dependent manner, since the IL-12 receptor is expressed on mouse DCs. Therefore, like macrophages, it is possible that IL-12 derived from DCs is associated with autocrine activation between IL-12 and IFN-γ (Grohmann *et al.*, 1998 and Ohteki *et al.*, 1999).

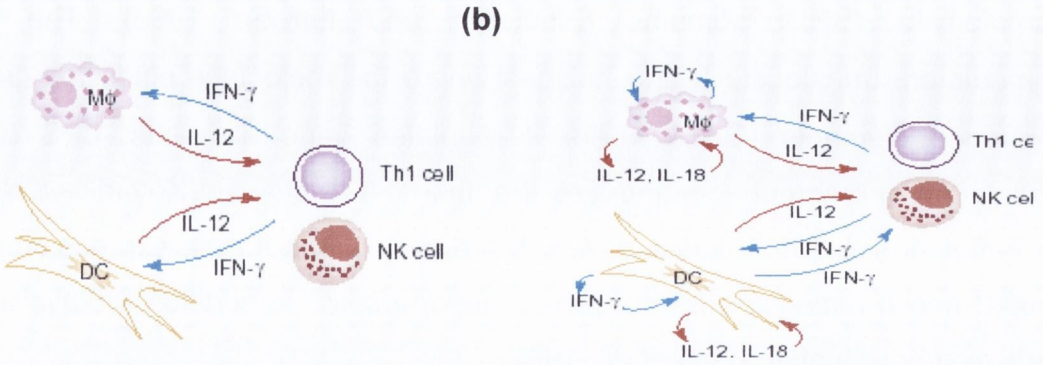


Figure 1.3: Mechanisms of IFN-γ secretion (a) the "classical model" of IFN-γ production form T and NK cells (b) the new "jump start model" of IFN-γ secretion from APCs including macrophages and dendritic cells. (Frucht *et al.*, 2001). Antigen presenting cells such as dendritic cells and macrophages secrete IL-12, this induces IFN-γ production from NK cells and Th_1 cells. In turn, the IFN-γ feeds back to and activates DCs and macrophages to up-regulate phagocytotic and antigen presentating mechanisms. However, the production of IL-12 by DCs and macrophages induces their autocrine activation, hence production of IFN-γ.

The biological responses of IFN-γ are regulated through the JAK-STAT pathway (Figure 1.4). It was the first signalling pathway deduced as being activated by the interferon family of cytokines (Schlindler *et al.*, 1992; Fu *et al.*, 1993; Silvennoinen *et al.*, 1993). The JAK family consist of four members, JAK 1, JAK2, JAK 3 and tyrosine kinase 2 (TYK2) (Shuai *et al*, 2003). The JAK1 and JAK2 kinases are associated with the IFN-γ receptor where they are constitutively expressed. The cytoplasmic transcription factors coupled to this signalling pathway are the STAT molecules, (signal transducer and activator of transcription) (Darnell *et al.*, 1994; Kerr *et al.*, 2003) of which there are seven members, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 (Plantias, 2005). Of these, STAT1 is the most crucial transcription factor during IFN-γ signalling. The IFN-γ receptor is composed of 2 subunits, IFNGR1 and IFNGR2 that are expressed on the surfaces of most cell types. Extracellular IFN-γ binds to the receptor and induces the receptor to oligomerize leading to the phosphorylation of JAK 1 and JAK 2. The intracellular side of the receptor becomes phosphorylated by the activated JAK kinases, thus exposing a site to enable the attachment of STAT1. The phophorylation of STAT1 occurs on tyrosine 701 and its' dimerization is via Src-

homology–2 (SH2) domains (Finbloom and Larner, 1995). The STAT1 homodimers translocate to the nucleus where the molecules binds to γ-activated sequence elements (GAS) where it regulates gene elements. LPS or IFN-γ stimulation of macrophages induces the Jak-STAT pathway (Kovarik *et al.*, 1998) and effector signalling molecules which are all required for the expression of MHC class I and II molecules on antigen-presenting cells, in addition it plays a role in nitric oxide production (Gao *et al.*, 1997).

In vivo studies have indicated that mice deficient in IFN-γ, IFN-γ R1, IFN-γ R2 or STAT1 have dysfunctional immune responses shown by the enhanced susceptibility to some viruses and microbial pathogens (Huang *et al.*, 1993; Dalton *et al.*, 1993; Durbin *et al.*, 1996 and Kamijo *et al.*, 1993). Moreover, mutations in the human IFN-γ receptor-signaling pathway results in severely impaired immune responses to bacterial infections. This can be so severe that it can be potentially fatal early in childhood due to the host not being able to control mycobacterial infections (Newport *et al.*, 1996; Jouanguy *et al.*, 1996 and Pierre-Audigier *et al.*, 1997). The classical model of IFN-γ production elucidates IL-12 induced activity of NK cells secrete IFN-γ at an early stage to control intracellular pathogens and is followed by further production of IFN-γ from CD4+T-lymphocytes. However, it has been demonstrated that in fact, myeloid cells produce high levels of IFN-γ. The first reports indicated that IFN-γ was produced in both lymphokine-activated splenic macrophages and tumor-activated peritoneal macrophages.

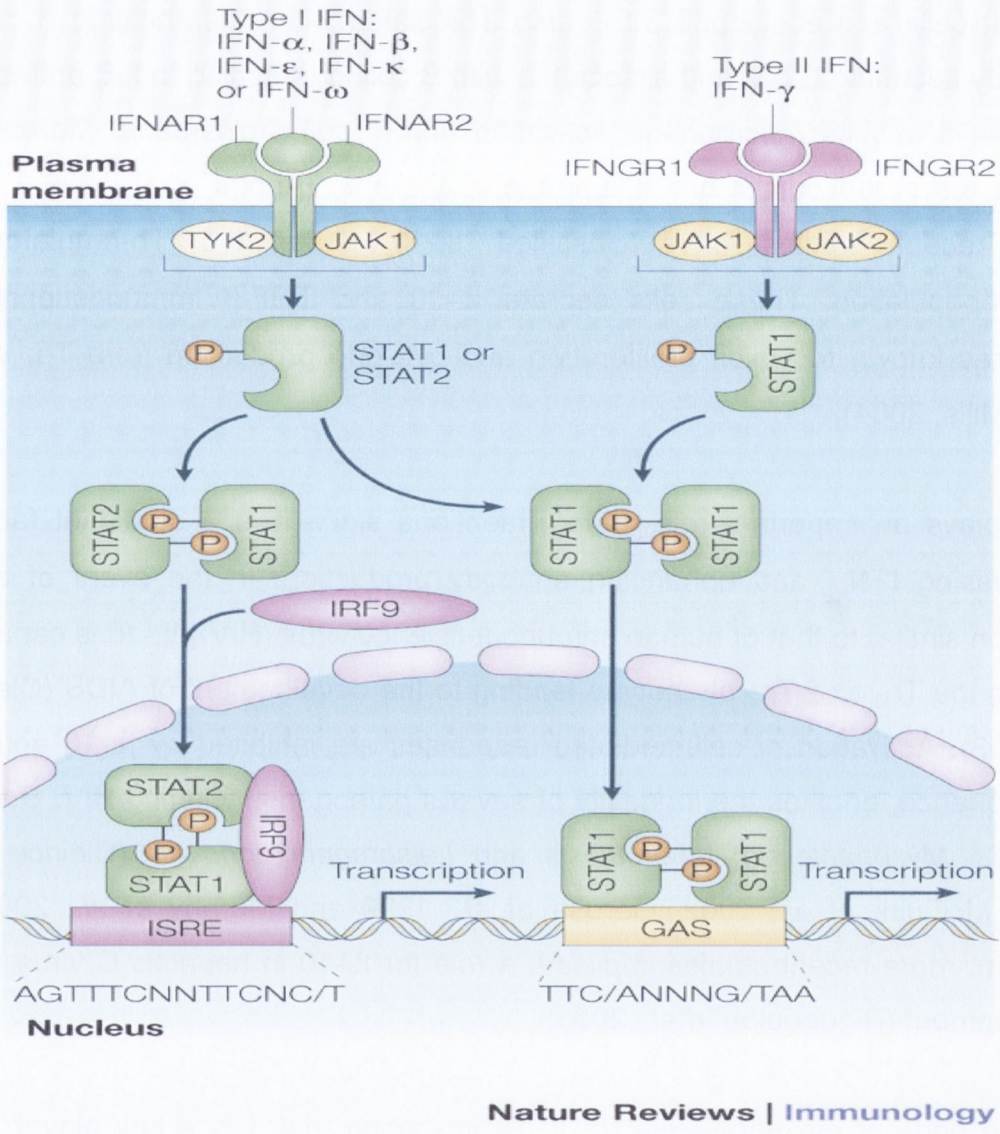


Figure 1.4: Activation of the JAK-STAT signalling pathways by the interferon family (Platanias, 2005).

1.4.4 Interleukin 10

Interleukin 10 was originally described as cytokine synthesis inhibitory factor (CSIF), when it was first discovered in the late 1980's (Fiorentino *et al.*, 1989) and is a 35 kDa protein. It was classified as a Th_2 member of the subgroup of T-helper populations, as well as being produced from macrophages and B cells (Mac Neil *et al*, 1990). Studies have indicated that murine IL-10 inhibits the production of IL-2, IL-3, TNF-α, IL-1β from macrophages and IFN-γ from NK and Th_1 cells by acting on antigen presenting cells (Fiorentino et al., 1991; de Waal Malefyt et al., 1991; Gruber et al., 1994 and D'Andrea et al., 1993).

IL-10 is primarily involved in promoting humoral immunity by activation and growth of mast cells and eosinophils, it is also associated with differentiating B cells to antibody secreting B cell and associated with B cell Ig switching to IgE antibody. In addition, it inhibits macrophage activation and T cell proliferation. Moreover, it inhibits the production of pro-inflammatory cytokines (Abbas *et al*, 1996). In fact more recent studies have classified IL-10 under the Th-regulatory or Th_3.subpopulation. These cells secrete IL-10 and TGF-β immunosuppressive cytokines known to inhibit proliferation and cytokine production from Th_1 or Th_2 cells (Mills, 2004).

IL-10 plays an important role during infections since it is a dominant factor in suppressing IFN-γ and enhancing antibody production. In the event of a viral infection similar to that of human immunodeficiency virus (HIV), IL-10 is capable of shifting the Th_1 to a Th_2 phenotype leading to the development of AIDS (Clerici *et al*, 1993). Activation of cell-mediated responses are inhibited by IL-10 and as a consequence, enables the longevity of several pathogens including HIV, *Bordtella pertusis*, *Mycobacterium tuberculosis* and *Leishmania donovani* (Cleirici *et al.*, 1994, McGuirk *et al.*, 2002, Gerosa *et al.*, 1999 and Murphy *et al.*, 2001). In addition, more recent studies implicate a role for IL-10 in hepatitis C virus (HCV) development (Rigopoulou *et al.*, 2005).

A large body of evidence exits for IL-10 indicating that it is a key player as an immunosuppressive cytokine. IL-10 is thought to be a functional antagonist of the pro-inflammatory cytokine IL-12 where it inhibits its expression in antigen presenting cells including macrophages (Uyemura *et al.*, 1996). Previous studies have also demonstrated that IL-10 suppressed IL-12 production by dendritic cells (Koch *et al.*, 1996). Moreover studies have documented that IL-10 acts directly on splenic antigen presenting cells to reduce IFN-γ production. Studies have also demonstrated that IL-10 reduces MHC class II expression and APC function (de Waal Malefyt *et al.*, 1991). IL-10 has also been shown to inhibit co-stimulatory molecule expression, for instance IL-10 reduced B7.1 expression on Langherans cells (Chang *et al.*, 1995), and decreased expression of B7.2 on blood DC's (Buelens *et al.*, 1995). The mechanism whereby IL-10 inhibits the expression of MHC class II molecule has been suggested to depend upon inhibiting the transport of the molecule to the cell membrane (Koppelman *et al.*, 1997).

The most established signaling pathway for IL-10 is the Jak-STAT pathway. The intracellular proteins, Jak and Tyk are associated with the receptor subunits, the IL-10R1 and IL-10 R2 on the cell membrane (Moore *et al.*, 1993). Interaction of IL-10 with its receptor stimulates tyrosine phosphorylation and induction of transcription factors such as STAT3 (Weber-Nordt *et al.*, 1996).

It has been well established that IL-10 inhibits antigen presenting function and inhibiting a Th1 cell-mediated response, but the mechanism of action of how it produces these effects remains to be elucidated. While several investigators suggest various roles for different signaling cascades, there is still considerable research to be carried out to understand this phenomenon.

A primary function of IL-10 is to deactivate macrophage pro-inflammatory cytokine production IL-2, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-1, IL-12, IL-8 and granulocyte colony-stimulating factor (Clarke *et al.*, 1998 and Brandtzaeg *et al.*, 1996). Soluble IL-10 circulates as a homodimer and binds with high affinity to its IL-10R, cellular receptor. The IL-10R consists of two subunits IL-10Ra/IL-10R1 which ligand binds to a CRF2 accessory subunit. These subunits are members of the IFN receptor family (Liu *et al.*, 1994). IL-10 down-regulates LPS-induced nuclear translocation of NF- κB and stimulates mRNA breakdown for pro-inflammatory mediators (Opal *et al.*, 1998). Indeed several investigators believe that inhibition of NF-κB, is the molecular target by which IL-10 induces its anti-inflammatory actions (Wang *et al.*, 1995), while others believe that IL-10s actions occur via MAPK molecules (Lim *et al.*, 2002 and Haddad *et al.*, 2003). It is thought that infection of macrophages by intracellular pathogens actually "hijack and exploit" IL-10 by inducing its production to prevent clearance of pathogen by dampening potential inflammatory response (Redpath *et al.*, 2001). Similarly, tumor cells are proficient in secreting IL-10, hence escaping cell-mediated immunity (Williams *et al.*, 2004). Increased levels of systemic IL-10 with a concomitant suppression of TNF-α concentrations have been linked with increased fatality from meningococcemia (Lehmann *et al.*, 1995). IL-10 not only suppresses pro-inflammatory responses but enhances anti-inflammatory molecules by stimulating the release of IL-1 receptor antagonist (IL-1RA) and soluble TNF receptors (Moore *et al.*, 2001). Studies in several animal models of inflammation have been conducted demonstrating the anti-inflmmatory effects of IL-10 in sepsis, experimental autoimmune encephalomyelitis (EAE), inflammatory

bowel disease and collagen induced arthritis (Gerard *et al.*, 1993, Cua *et al.*, 1999, Lindsay *et al.*, 2003 and Walmsley *et al.*, 1996). In contrast, studies have found a strong correlation between IL-10 and progression of human infectious diseases including malaria, visceral leishemaniasis, leprosy and tuberculosis (Moore *et al.*, 2001).

1.5 Chemokines

Chemokines are a large family of low-weight chemotactic cytokines that are involved in regulating leucocyte trafficking and maintain homeostasis of circulating leucocytes in various tissues (Oppenheim, 1991). Chemokine secretion is augmented during an inflammatory response and so results in recruitment of specific leucocytes to the inflamed tissue. These chemotactic cytokines are classified small proteins with a molecular mass of about 7-10 kDa and are divided into two subfamilies, α and β chemokines that contain 4 cysteines, due to the positions of the 2 pairs of conserved cysteines. In the first subfamily the α-chemokines, known as CXC, the first two cysteine residues are separated by an amino acid (cysteine-x-amino acid). In the second subfamily, β-chemokines, described as CC, the two cysteine residues are adjacent to each other without an amino acid between (Charo and Ransohoff, 2006).

In the α-chemokines, the subfamily containing the glutamic-acid-leucine-arginine sequence near the N-terminal are chemotactic for neutrophils while those that do not contain this sequence are chemotactic for lymphocytes. Human and murine Interferon-inducible protein of 10kd (IP-10) was among the first chemokines to be discovered (Ohmori *et al.*, 1990). IP-10 belongs to the α-chemokines subfamily associated with attracting activated T-cells (Loetscher *et al.*, 1996). Some of the biological functions of IP-10 include cell development, division, differentiation, chemotaxis, adhesion, cytolysis, angiogenesis and anti-tumor activity (Neville *et al.*, 1997). Chemokines bind to and activate cell surface receptors that are coupled to G proteins (Premack *et al.*, 1996). The receptors for chemokines are expressed on various types of leucocytes. For instance, CXCR3 is the chemo-receptor associated with the IP-10 ligand and its expression is restricted to activated T-lymphocytes of Th_1 clones and NK cells, thus it is involved with potentiating a cell mediated Th_{-1} type immune response (Luster, 1998). Stimulated cells secrete chemokines in response to early pro-inflammatory cytokines such as IL-1 and

TNF-α, lipopolysaccharide and viral infections (Luster, 1998). During viral meningitis, lymphocytes and monocytes recruitment is increased as a result of elevated chemotractant chemokines including IP-10 and MCP-1 expression, on leucocytes in the CSF (Lahrtz *et al.*, 1997), thus indicating over-expression of these molecules is implicated in disease states.

1.6. Innate immune response

Initially, when the host comes in contact with any infectious agent or foreign protein in the body, the innate immune response is rapidly activated within minutes hence is classified as being the first line of defense (Dempsey *et al.*, 2003). At this stage, the most abundant leucocytes to be recruited to the site of infection are neutrophils. These cells have intrinsic cytotoxic activity achieved by producing reactive oxygen species that kill microbes (Gregory and Wing, 2002). In addition, macrophages are considered pivotal players during an innate reaction since they phagocytose microbes, synthesise substances such as nitric oxide (NO), which are toxic to pathogens, and produce large quantities of pro-inflammatory cytokines such as IL-12, IL-1β and TNF-α. Other cells that play a crucial role in the innate immune response include natural killer (NK) cells, whose cytolytic activity is necessary for viral clearance and for clearance of transformed cells, and dendritic cells (DCs) which are primarily involved in presenting antigens to T-cells (Dempsey *et al.*, 2003). Monocytes are derived from hematopoietic precursors and they are the most prevalent of the antigen presenting cells (APCs). They circulate in the body where they pass through various differentiation programs to form functionally and morphologically distinct macrophages. The monocytes will differentiate into and activate macrophages only in the presence of specific cytokines and effector molecules depending on the environment whether it be *in vivo* or *in vitro*. Macrophages can be divided into 2 categories, the classically activated, those induced by pro-inflammatory cytokines such as IFN-γ and the alternatively activated, are stimulated by anti-inflammatory cytokines, IL-10, IL-4 and TGF-β (Takahashi *et al.*, 1996; Goerdt *et al.*, 1999). Acute inflammation is characterised by vasodilation, which is induced by prostaglandins and nitric oxide, originally called endothelial derived relaxing factor and is recognisable by heat and redness, in order to facilitate the delivery of inflammatory cells and soluble mediators to inflammatory site. Other inflammatory processes that occur include increased vascular permeability, adhesion marker expression and complement

activation. Macrophage and dendritic cells are very important effector mediators for microbial recognition and cytokine production during innate immunity (Aderem *et al.*, 2001; Granucci *et al.*, 2003). To have the ability to distinguish pathogens from host cells is a vital and essential requirement within this immune network of effector cells and mediators. Macrophages have innate receptors expressed on their surface known as pattern-recognition receptors (PPRs). Expression of such receptors enables the recognition of unique structures known as pathogen-recognition molecular patterns present on microbes for instance lipopolysaccharide on the gram negative cell wall (Beutler *et al.*, 2003).

1.7 Adaptive Immune response

The second branch of immunity is the adaptive immune response. It is associated with the activation and proliferation of specific cell types for a specific pathogen and improves with each successive encounter with the same pathogen. Although, this phase of immunity takes longer (days to weeks) to develop than the primary innate response, the adaptive response is advantageous for overall immunity as it provides two key features, specificity and memory. The primary mediators of the adaptive immune system include T- and B-lymphocytes (Alam, 2003). B- and T-cells originate from pluripotent stem cells in bone marrow and undergo differentiation. The T cells migrate to the thymus while the B-cells end in peripheral lymphoid tissue. Lymphocytes leaving the thymus and bone marrow are naïve, since they have not encountered a specific antigen within an immune response. Secondary lymphoid tissues such as the spleen and lymph nodes are where these cells populate. They provide an essential microenvironment containing necessary antigen presenting cells, cytokines and adhesion molecules for when naïve cells encounter their specific antigen. Lymphocytes express antigen-specific receptors (T-cell receptors, TCR and B-cell receptors) on their cell surface to allow recognition between self and non-self antigens during the process of differentiation. T-lymphocytes are the main mediators of cellular immune responses, however it is antigen-presenting cells that regulate their function while B-cells are associated with antibody production. T-lymphocytes express $CD4^+$ and $CD8^+$ molecules on their cell surface (Figure 2). The $CD4^+$ T cells are subdivided into two populations and differentiated by the cytokines they produce (Mosmann *et al.*, 1989). Naïve cells (Th_0) are stimulated and differentiated to Th_1 or Th_2 cells. Th_1 cells secrete IL-2 and IFN-γ. whose primary functions are to induce T-cell

proliferation and macrophage activation, respectively, thereby stimulating a cell-mediated response (Swain *et al.*, 1991). In contrast, Th_2 cells produce IL-4, IL-5 and IL-10 that promote antibody production, thus promoting a humoral mediated response (Williams *et al.*, 1991). Ehrlich and Metchnicoff elucidated the two phases of adaptive immunity as cellular and humoral responses (Elenkov, 2000). The $CD8^+$ T-cells are associated with cytotoxicity and are also known as cytotoxic T-lymphocytes (CTLs) and have anti-tumor activity. In addition, more recently another subset of T-cells have been established, Th_3 or T_{reg} contain regulatory or suppressor cells that have the ability to dampen down the Th1 and Th_2 response if either T-helper cell response is in an uncontrollable state. Regulatory cells are characterised as $CD4^+CD25^+$ cells that secrete immunoregulatory cytokines TGF-β and IL-10 (Shevach *et al.*, 2002).

The two phases, innate and adaptive responses, act together in a concerted fashion essentially allowing an intact and complete immune response to be mounted for protection against invading infectious agents. In this regard antigen presentation is a critical process that facilitates activation of the adaptive immune response.

1.7.1 Antigen Presentation

During an effective immune response, antigen-presenting cells such as macrophages and dendritic cells must present the antigen associated with the major histocompatibility class II molecule (MHC class II) to the T-cell receptor (TCR) on the naïve T-cells. APC's engulf pathogens that they encounter in the extracellular matrix by endocytosis. The pathogens are then degraded into small peptides upon contact with lysosomal proteases. Finally, the endosome fuses with MHC class II molecule-containing vesicles, the vesicle translocates to the cell surface by exocytosis which facilitates loading of the peptides onto the MHC class II molecule for presentation to CD4+ T-cells. (Alam *et al*, 2003). APCs expressing MHC class II molecules (Figure 1.5) recognise T-lymphocytes with $CD4^+$ only on the cell surface. Following this, activation and clonal expansion of the T-cells occur. It is essential that T cells have TCR signalling to enable the cells to survive and proliferate. Activation of T-cells is analogous to a synapse between two neurons. In this instance, there is an interaction between the TCR and MHC class II molecules and this is stabilised by CD4. In addition, interaction between the co-

stimulatory molecules, B7.1 and B7.2 (CD80 and CD86), CD40 and ICAM-1(CD54) and their counterparts on T-cells is crucial for an immunological synapse. The B7.1/B7.2: CD28 pathway is considered to be the dominant co-stimulatory pathway and plays an essential role in the regulation of T-cell viability, cytokine production, clonal expansion and effector function (Boussiotis *et al.*, 1994). B.7 was originally thought to activate B-cells, is now also expressed on macrophages (Freedman *et al.*1991) and dendritic cells (Larsen *et al.*, 1992).

1.7.2 MHC class II and co-stimulatory molecules

Antigen presenting cells (APCs) engulf antigens inside endosomes produced from the endocytic reticulum and golgi apparatus inside the cell. These endosomes contain proteases and high levels of major histocompatibility complex (MHC) class II molecules. The proteases digest the antigen into smaller peptides that bind to the MHC class II molecules and are loaded onto the class II dimers at the surface of the APC (Robinson and Delvig, 2002). The genes for MHC class II molecule encoding heterodimeric (α and β chains) cell surface molecules and class II protein are constitutively expressed on several types of cells such as dendritic cells, macrophages and B-lymphocytes. Following this phagocytic process, MHC class II molecules bind T-cell receptors of specific $CD4^+$T-helper cells. MHC class I molecules are involved in activating $CD8^+$ T-cytotoxic cells. The MHC class II transactivator (CIITA) is the master regulatory factor involved in the expression of MHC class II genes and is induced by IFN-γ (Steimle *et al.*, 1994). More importantly, the expression of class II transactivator (CIITA) genes is indispensable for the transcription of class II genes (Ting and Trowsdale, 2002; Reith *et al.*, 2005). Reports have indicated that viruses prevent the expression of MHC II genes by inhibiting IFN-γ signal transduction cascade hence suppressing CIITA expression (Hegde *et al.*, 2003). It has been well established that the interaction with the T-cell receptor (TCR) is the primary stimulus for T-cell activation and proliferation. However, for antigen-specific clonal expansion of naïve T-cells to occur another signal must also be delivered. It is now thought that co-stimulatory molecules expressed on antigen presenting cells provide the major signal for T-cell activity. In light of this, recent research has postulated that the most important co-stimulatory molecules are the B7 family of glycoproteins, CD80 and CD86 (Lenschow and Walunas, 1996) since they provide a major signal secondary to that of the MHC/TCR signal. When B7 family molecules ligate CD28

on T-cells the secretion of IL-2 is elevated thus enhancing clonal expansion of naïve T-cells. The original B7.1 molecule is now known as CD80 and the B7.2 molecule is CD86. B7 molecules and CD28 receptors are expressed on numerous cells including dendritic cells, macrophages, B- and T-cells (Robey and Allison, 1995). It is thought that CD86 is largely expressed on dendritic cells more so than that of CD80. In addition, it appears that CD86 is up-regulated at a faster rate when it ligates with CD28, than that of CD80 following activation by certain stimuli (Bhatia *et al.*, 2006). Their binding affinities for the relevant receptors, CD28 and CTLA-4 on T-cells are quite similar. Although in relation to receptor affinity, there is a slower dissociation rate for CD80 than CD86 binding to CTLA-4 (Robey *et al.*, 1995). In addition, data from studies suggest that CD86/CD80 ligation with CTLA-4 co-receptor induces inhibitory actions on the TCR expressed on T-cells by reducing IL-2 release therefore dampening down subsequent signaling pathway resulting in anergy (Walunas *et al.*, 1994 and Perrez *et al.*, 1997). Studies have documented that B7.1/B7.2 interaction with CD28 up-regulates the expression of anti-apoptotic gene, $bcl\text{-}_{xl}$, and cytokine expression thus increasing proliferation and preventing cell death (Sperling *et al.*, 1996). Moreover such co-stimulation is implicated in regulating the susceptibility of $CD4^{+}$T-cells to HIV infection (Carroll *et al.*, 1998).

Another important cell surface molecule expressed on macrophages and dendritic cells is CD40, and the interaction of it with receptor CD40L (CD154) on T-cells (Schriever at al., 1989) is associated with sustaining the co-stimulatory signal and is central for T-cell stimulation and activation (Peng *et al.*,1996 and Cayabyab *et al.*, 1994). The CD40/CD40L signal is necessary for regulating pro-inflammatory cytokines and chemokines for instance, TNF-α, IL-12, MIP-1α and IL-8 production from dendritic cells (Caux *et al.*, 1996; Cella *et al.*, 1996 and Koch *et al.*, 1996). These pro-inflammatory effector molecules are fundamental for inducing inflammatory response but more specifically IL-12 since it is the primary cytokine associated with a cell-mediated response due to its differentiating functions and proliferative capacity on naïve T-cells (Armitage *et al.*, 1993). Studies have provided evidence that the ligation of CD40 with CD40L is required for generating a protective response against infection with Leishmania major (Kamanaka *et al.*, 1996) as well as other bacterial and viral pathogens (Grewal and Flavell, 1998).

In addition, the intercellular adhesion molecules (ICAM-1) are a family of surface-bound molecules that a play a significant role in cell-cell interactions and assists with antigen presentation (Aplin *et al.*, 1999 and Lebedeva *et al.*, 2005). They bind to their corresponding receptor lymphocyte function associated antigen-1 (LFA-1) on T-lymphocytes (Dustin and Springer, 1989) and ligation of the adhesion molecule stimulates a signal transduction cascade that promotes cellular activation and cytokine production. IFN-γ induction of ICAM-1 genetic expression is dependent on the interaction of STAT1 and the transcription factor, Sp1 and can only be up-regulated in the presence of these proteins when they bind nucleic DNA (Walter *et al.*, 1997). In addition, ICAM-1 expression is enhanced in macrophages in the presence of IL-18 (Kohka *et al.*, 1998). Studies have indeed deduced that induction of ICAM-1 and V-CAM-1 (vascular adhesion molecules) occurs via signaling cascades that up-regulates NF-kβ and AP-1, the transcription factors associated with pro-inflammatory cytokines (Schreck *et al.*, 1992 and Abate *et al.*, 1990). More importantly, recent studies have elucidated an elemental role for ICAM/LFA-1 interaction for promotion of anti-tumor responses (Lefor *et al.*, 1998 and Blank *et al.*, 2005) due to its stimulatory effects on antigen-specific $CD8^+$ T-cells (Chen *et al.*, 1999).

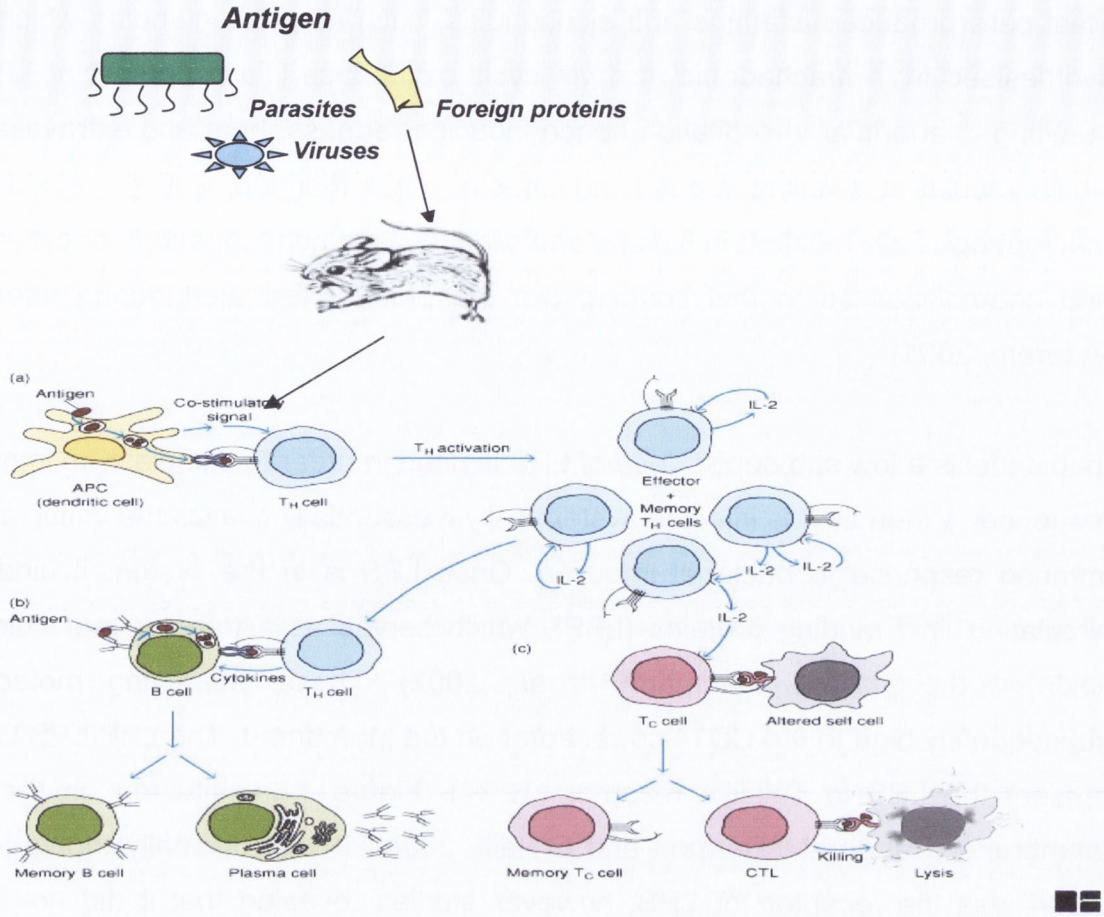


Figure 1.5: Activation of immune responses, innate (a) and adaptive (Th_1 cell-mediated and Th_2 humoral (b)) responses that occur in mouse following infection such as parasite, virues, fungi, bacteria or foreign proteins that are capable of stimulating immune activity (Duane, 1997).

1.8 Stimulating the immune system with LPS

Endotoxicity induced by gram-negative bacteria is due to the lipopolysaccharide (LPS) component of the bacterial wall (Westphal *et al.*, 1965). The physiological changes that occur due to the toxicity include hypotension, fever, tissue necrosis and death. In the event of infection, the host cells orchestrate immediate signals so that systemic responses will become activated to control the traumatic insult. Initially fever sets in as an index of neuroendocrine activity with production of neuropeptides while some behavioural effects include anorexia, somnolence and lethargy. The symptoms of acute inflammation were originally coined by Celcus as "rubor, calor, dolor and tumor" meaning redness, heat, pain and swelling. Mast cells are one of the primary cells to respond to the site of infection, since they are

resident in tissues while most leucocytes have to be recruited from the circulation. Mast cells produce histamine and eicosanoids, such as prostaglandins which are synthesised from arachadonic acid via cyclo-oxygenase (Tabernero *et al.*, 2003) resulting in arteriolar vasodilation hence inducing redness, heat and extravasation which results in swelling around the infected area (Nathan, 2002). In addition, macrophages are resident in tissues and produce vast concentrations of cytokines and chemokines so in this context, are the primary red alert during infection (Aderem, 2001).

In this thesis a low sub-septic dose of LPS is used in order to stimulate an immune response. When LPS is injected systemically it essentially mimics the initial innate immune response to bacterial infection. Once LPS is in the system it binds to circulating lipid binding proteins (LBP), which serves as a transfer molecule for lipids in the plasma (Heumann *et al.*, 2002). These interacting molecules subsequently bind to the CD14 co-receptor on the membrane. The LBP/LPS/CD14 present the LPS to Toll-like receptor (TLR4) (Figure 1.6a) situated on the cell membrane (Palsson-McDermott and O'Neill, 2004). It was originally thought that CD14 was the receptor for LPS, however studies revealed that it did not have transmembrane domain and therefore, was unable to conduct downstream signaling. The family of Toll like receptors consist of type 1 transmembrane receptor made up of extracellular leucine rich repeat (LRR) domain and intracellular Toll/interleukin IL-1 receptor (TIR) domain (Medzhitov, 2001). For the stimulated LPS-TLR4 intracellular signaling to continue it requires the presence of certain molecules known as adapter proteins. Attached to TLR4 are two molecules of the MD2 adapter protein which are essential components of the receptor complex. The LPS-induced TLR4 signaling can occur in two stages (1) an early response dependent on MyD88 (myeloid differentiation factor 88) and (2) a delayed response independent of MyD88 (Figure 1.6b). During the early stage, the LPS-TLR4 complex homodimerise and recruits MyD88 and another adapter protein called MyD88 adapter-like protein (Mal/TIRAP) to the receptor complex. Following activation, the complex induces the activity of the signaling molecules interleukin 1 receptor-associated kinase (IRAK) and tumour necrosis factor receptor-associated factor (TRAF6) resulting in activation of inhibitor of κB (IκB). As a result, the protein degrades and releases cytoplasmic nuclear factor-κB (NF-kβ). Once activated, NF-κB translocates to the nucleus where it binds to promoter

regions of pro-inflammatory genes and regulates the expression of such mediators. During the delayed or late response, MyD88 adapter protein is not required however other adapter proteins are associated with this response such as TIR-containing adapter molecule (TRIF) and TIR- related adapter molecule (TRAM) enhance the activity of TRAF6 and TBK1 which in turn induce the activity of NF-kβ. This event is not as potent as that of the early phase in the presence of MyD88.

It is now considered that LPS binds to the CD14/TLR4/MD2 receptor complex previously described, on cells of the innate immune system such as monocytes, macrophages and dendritic cells (Latz *et al.*, 2002 and Akira *et al.*, 2003), and stimulates this cascade of signaling events resulting in the activation of NF-kβ and ultimate production of pro-inflammatory cytokines such as IL-1, TNF-α, IL-12. Moreover, stimulation of TLR4 promotes up-regulation of MHC proteins and co-stimulatory molecules (CD80 and CD86) (Akira *et al.*, 2003). TLR-induced signals activate various genes associated with unique functions. In addition, TLR activation mediates important inflammatory responses during innate immunity including antigen presentation, phagocytosis and apoptosis. Futhermore, it plays a major role in up-regulating cell trafficking and recruitment via stimulating the expression of pleiotropic cytokines and chemokines.

Following initiation of the immune response, the recruited circulating leukocytes are directed by the up-regulation of specific cell surface molecules on surrounding vascular endothelium to allow binding on leukocyte ligands. These surface molecules are known as intracellular adhesion molecule-1 (ICAM-1) (Wang *et al.*, 2002). If the immune system is not stimulated with LPS, basal levels of cytokines are below detectable concentrations. Hence, it is essential to challenge the immune system in order to stimulate cytokine production.

(a) (b)

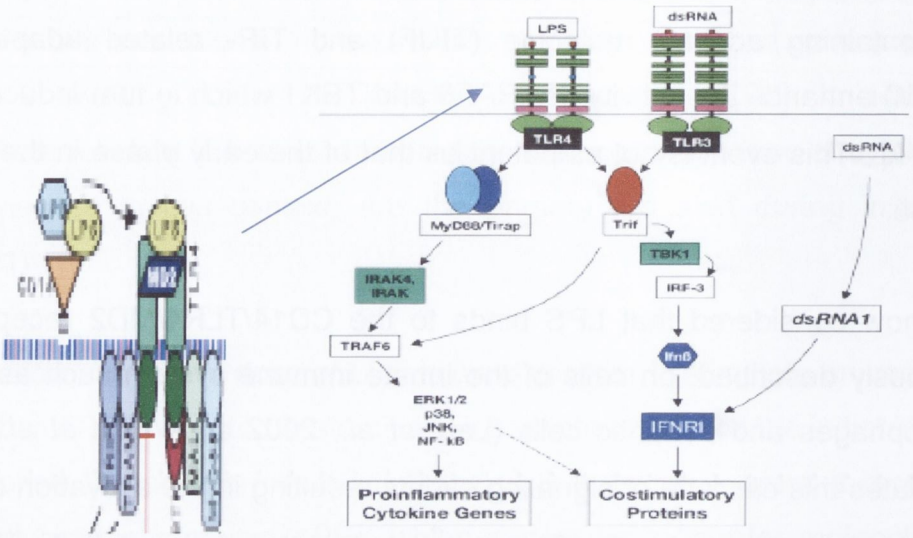


Figure1.6: Lipopolysaccharide signaling via TLR4

(a) LPS/LBP/CD14 receptor complex presenting LPS to TLR4 on cell surface membrane (Palsonn-Mc Dermott and O'Neill, 2004) (b) MyD88 dependent and independent signaling required for genetic expression of pro-inflammatory mediators (Beutler *et al.*, 2004).

1.9 Neuro-immune interaction

The first evidence that demonstrated a link between the nervous system and immune system dates back to the 1800's where it was first observed that nerves entering the lymph nodes were independent of blood vessels (Tonkoff, 1899). In the early 20th century, an interaction between the immune, the endocrine and the nervous system was demonstrated by work in rats that underwent stress tests resulting in an enlargement of the adrenal gland and reduction of the thymus gland (Seyle *et al*, 1936). Most research occurring during the 1970-80's proposed that bi-directional communication existed between the brain and the immune system and that this crosstalk was functionally relevant (Besedovsky *et al*, 1979). This was due to the detection of soluble factors (now known as cytokines) that when released from immune cells could enter the central nervous system (CNS) and stimulate the hypothalamus and brainstem (Besedovsky *et al.*, 1986). Moreover, studies using histofluorescence also indicated that the lymphoid organs just like blood vessels were predominantly supplied by sympathetic/noradrenergic innervation (Dahlstrom & Zetterstrom, 1965; Reilly *et al*, 1976; Felten *et al.*, 1985).

A substantial number of reports have now established that cross-talk exists between the central nervous system and the immune system (Elenkov *et al.*, 2000; Kohm and Sanders, 2002 and 2001; Haddad *et al.*, 2002; Freidman and Irwin, 1997), and each system has a bi-directional mode of action by activating the opposite system (Figure 1.7). Hence, the CNS activates two anti-inflammatory pathways, the hypothalamic pituitary adrenal axis (HPA axis) and the sympathetic nervous system (SNS) to regulate the immune responses and maintain homeostasis (Elenkov *et al.*, 2000). Neurotransmission from the CNS to the periphery is via projections extending from the paraventricular nucleus of the hypothalamus, rostral ventrolateral medulla, ventromedial medulla and raphe nucleus to preganglionic neurons in the spinal cord (Sawchenko *et al.*, 1982). The catecholamine noradrenaline is the main neurotransmitter released into the periphery once the SNS is activated. The effects of noradrenaline and adrenaline are mediated by activation of two principal types of receptors known as alpha (α) and beta (β) adrenergic receptors, where noradrenaline has greatest affinity for α-adrenergic receptors while adrenaline binds with greater affinity to β-adrenergic receptors. The subtypes of alpha-adrenergic receptors include α_1 and α_2 while those of the beta-adrenergic receptors are β1, β2 and β3. β-adrenoceptor expression has been detected on most lymphoid cell types, except the Th_2 cells (Kohm and Sanders, 2001). Studies have indicated that β_2AR expression is retained on clones and newly generated Th_1 cells, however in contrast, β_2AR expression is repressed on clones and newly generated Th_2 cells (Sanders *et al.*, 1997).

The signalling pathway following noradrenergic stimulation is conducted via guanine nucleotide-binding proteins (G-proteins) from the β-adrenoceptors that in turn activate the intracellular enzyme, adenylate cyclase (Elenkov et al., 2001). Second messenger cyclic adenosine 5'-monophosphate (cAMP) production is increased and this in turn induces an increase in protein kinase A (PKA) activity (Meinkoth *et al.*, 1993). It has been shown that following stimulation of β_2AR on immune cells, cAMP levels are increased (Fedyk *et al.*, 1996 and Yang *et al*, 2000). Considering the numerous studies indicating that increased intracellular levels of cAMP promote an anti-inflammatory cytokine phenotype (Suberville *et al.*, 1996, van der Poll *et al.*, 1996 and Platzer *et al.*, 2000), it is not surprising that noradrenaline and β_2-AR agonists are also anti-inflammatory in nature. For

instance, it has been demonstrated that exposure of LPS-activated monocytes to noradrenaline reduces production of the pro-inflammaotry cytokine IL-12, in parallel with increasing production of the anti-inflammatory cytokine IL-10 (Elenkov *et al.*, 1996). In addition, dendritic cells stimulated with LPS for 6 hours, noradrenaline down-regulates IL-12 production with a concomitant increase of IL-10 release (Sallusto *et al.*, 1999). Hence, the results from these studies indicate that β_2AR activation on antigen presenting cells may favour the development of Th_2 cells by inhibiting IL-12 production from APCs that is necessary for Th_1 differentiation. Reports have hypothesised that a suppression of the Th_1 cytokine response could potentially diminish cell-mediated immunity, thus augmenting the chances for susceptibility to viruses, fungi and bacteria (Romani *et al*, 1994).

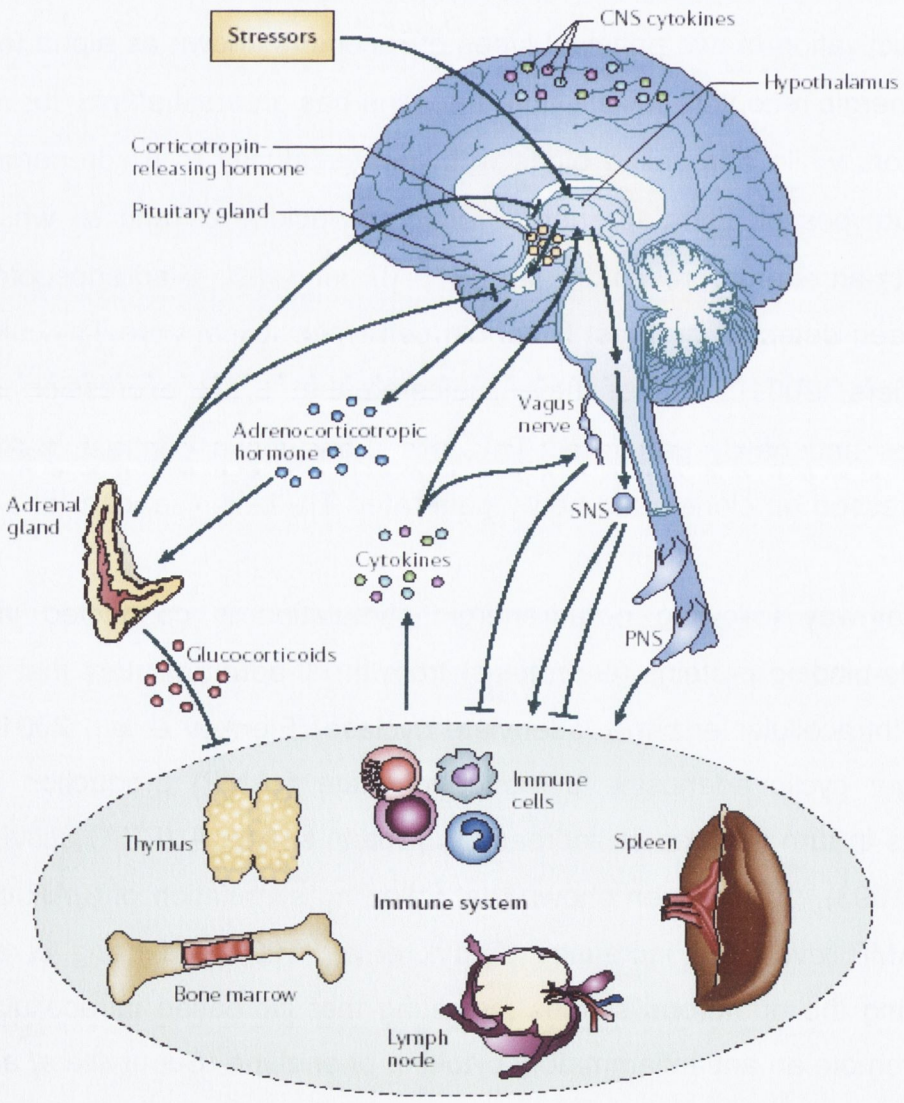


Figure 1.7: Neuro-immune communication indicating the signalling pathways involved (Sternberg, 2006).

1.10 History and Properties of MDMA

History

3,4-Methyldioxymethamphetamine (MDMA,"Ecstasy") is a ring-substituted phenylisopropolamine, amphetamine derivative and is structurally related to stimulants and hallucinogens. It is a widely abused psychoactive recreational drug and has attracted a lot of attention due to its abuse among the young generation. MDMA falls under the entactogen category of drugs, these are substances that can produce (gen) an inner (en) touching (tact). The amphetamine derivative was first discovered by a German company Merck Pharmaceuticals in 1912 (Mc Kenna *et al.*, 1990). Previous reports in literature have documented that the drug was patented in 1914 as a potential appetite suppressant, however there is no evidence to suggest this, since original documents fail to state these facts. Recent investigations have sourced that MDMA was in fact used as a precursor, known as 'Methylsafrylamin', required for the production of haemostatic agents (Freudenmann et al., 2006). In the 1950's, the US army sponsored confidential studies to test MDMA in animals for the first time, these results were not released until the 1970's. Several decades passed before the drug was re-discovered by Dr. Alexander Shulgin who carried out further research on its' therapeutic potential. Due to its psychoactive properties he introduced and promoted its' use as an adjunct to psychotherapy during counselling sessions (Downing, 1986). Moreover, it evolved as a popular drug of abuse in the late 70's-early 80's where it was particularly associated with rave and techno club cultures (Ghodse *et al.*, 1997). However, in 1985, the US government succeeded in making it a controlled substance in 1985 by the Drug Enforcement Agency (DEA) as a Schedule I drug and a Class A drug in the UK. This was as a result of reports indicating long-term neurotoxic effects on serotonin neurotransmission (Ricaurte *et al.*, 1985, Steele *et al.*, 1994, McCann *et al.*, 1996), but despite the increased awareness, it is still a popular recreational drug of abuse especially among young people attending rave nightclubs.

Mechanism of action

MDMA is a lipophilic chemical and crosses the blood-brain barrier with ease (Farre *et al*, 2004). It acts as an indirect serotonin agonist on pre-synaptic neurons where it is a potent inducer of serotonin (5-HT) release from neuronal endings and also inhibits its reuptake by its action on the pre-synaptic serotonin transporter (SERT).

Initial studies carried out on nonhuman primates showed that it acted as a potent neurotoxin and induced degeneration of serotonerigic neuronal fibers (Ricuarte *et al.*, 1988a; Ricaurte *et al.*, 1988c). In addition to its action on the serotonergic system, MDMA also releases the catecholamines dopamine (DA) and to a lesser extent, noradrenaline (NA) (Yamamoto *et al.*, 1988, Schmidt *et al.*, 1987, Nichols *et al.*, 1982). MDMA binds to all 3 neurotransmitter transporters but has highest affinity for SERT. In fact, it also binds to other receptors including α_2-adrenergic; M_1 muscarinic and H_1 histamine receptor with high affinity, and has a lower affinity for M_2 muscarinic; α_1 and β-adrenergic and $5HT_1$ receptors. The psychological properties of MDMA include euphoria, decreased inhibitions, increased self-awareness, self-esteem and empathy, and evidence suggests that the psychoactive effects of MDMA are largely due to it actions on the serotonergic and dopaminergic systems (White et al., 1999 and Liechti *et al.*, 2001).

Physiological effects and adverse effects of MDMA

Some of the physiological effects that occur after MDMA ingestion include increased blood pressure and heart rate, hot and cold flushes, insomnia, nausea, sweating and jaw clenching (McCann *et al.*, 1996). Following administration of MDMA, levels of cortisol, ACTH, prolactin and anti-diuretic hormone are elevated in the blood (Farre *et al.*, 2004). One of the most dangerous symptoms that occurs as a consequence of MDMA ingestion is hyperthermia, and has been implicated to be the major cause of death related incidents associated with MDMA consumption. Increased body temperatures produce a breakdown in skeletal muscle, also known as rhabdomyolysis, and also induce kidney failure (Schifano, 2004).

As a result of the stimulatory action of MDMA on central monoamine neurotransmitters, corticotrophin releasing factor (CRF) is released from the median eminence, thus activating the sympathetic nervous system (SNS) and hypothalamic-pituitary-axis (HPA) (Mas *et al.*, 1999). Human subjects also experience physiological side effects such as jaw clenching, pupil dilation deep tendon reflexes and gait instability as a result of stimulating somatic motor tone (Steele *et al.*, 1994). Other physiological side effects of MDMA have been described, for instance, hyperthermia, increased locomotor hyperactivity, myoclonus, hyperreflexia, shivering, tremor and these side effects are sometimes

referred to as "serotonin syndrome", due to altered serotonergic function (Spanos *et al.*, 1989; Parrot, 2002; Burgess *et al.*, 2000 and Green *et al.*, 2003). Studies have implicated a role for MDMA metabolites as the underlying mechanism for MDMA-induced neurotoxicity (Miller, 1995) and hepatotoxicity (Carvalho *et al*, 2001) and nephrotoxicity (Carvalho *et al*, 2002).

Metabolism of MDMA

The recreational use of Ecstasy tablets can involve the consumption of a dose between 75-150mg (Steele et al., 1994), and it is often the case the multiple tablets will be taken over a number of hours. MDMA concentrations detected in human serum are maximal 2hrs post ingestion, however, levels are detectable as soon as 15 minutes (Vereby *et al.*, 1988 and Farre *et al.*, 2000). Furthermore, studies using HPLC analysis have demonstrated that the half-life for MDMA is 6.6hrs (Garrett *et al.*, 1991). The metabolism of MDMA is regulated by enzymes, CYP2D6 and COMT, hence the differences in activity of these enzymes could account for the interspecies differences in relation to the toxicity of MDMA. It is thought that the adverse effects of MDMA may be due to its metabolic products, of which two pathways have been described. The metabolism of MDMA occurs via two pathways. The first pathway involves the O-demethylenation followed by COMT catalysed methylation, glucuronide or sulphate conjugation. The second pathway is associated with the N-dealkylation, deamination and oxidation followed by conjugation with glycine. In humans, following consumption of MDMA, the main plasma metabolite is N-methyl-alpha-methyl dopamine and MDA is a minor metabolite (Segura *et al.*, 2001). While in the rat and mouse, the major metabolite is MDA (Cho *et al.*, 1990). MDMA is cleared by hepatocytes (de la Torre, 2004) where it is N-demethylated to form MDA and O-demethylenated to form HHMA. When MDMA is administered to mice, MDMA is the main chemical species observed in plasma and brain. It has been reported that the LD_{50} of MDMA in mice is between 80-115mg/kg (Steele *et al.*, 1994). In the mouse, administration of MDMA produces dopamine neurotoxicity as opposed to the serotonergic neurotoxicity that is observed in rats. The ability of MDMA to produce dopamine neurotoxicity in rats is thought to be due to the variability in MDMA metabolism in mouse and rat (Logan *et al.*, 1988 and Sanchez *et al.*, 2003).

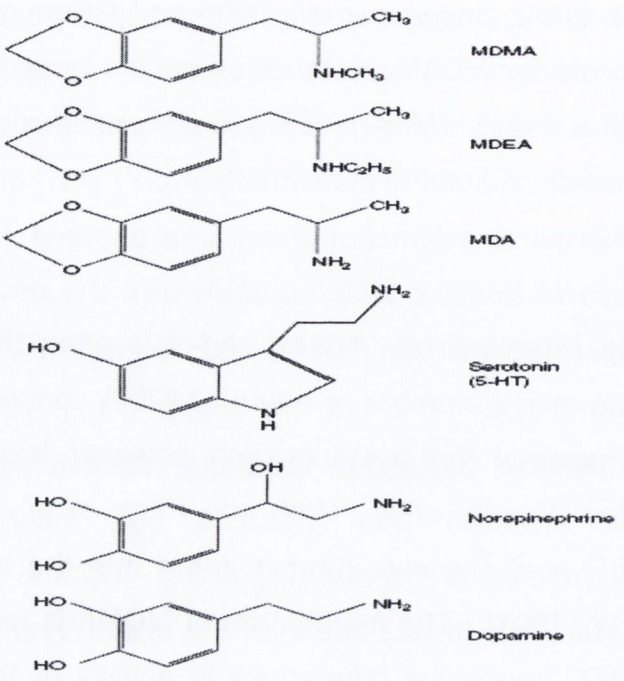


Figure 1.8: The chemical structure of MDMA "Ecstasy", "E" or "X-TC" and its similarity to other stimulants (MDA and MDEA), and to monoamine neurotransmitters (Hall *et al*, 2006).

1.11 MDMA as an immunosuppressive drug of abuse

For several years now, numerous studies have been carried out to investigate the effects of MDMA *in vivo* using rodents (see Connor, 2004), summarised in Table 1. Moreover, it is thought that such immunosuppression may diminish host resistance, and therefore increase susceptibility to infection. Medical complications associated with drug abuse have been well established but research is now focusing on the immune system as a potential target (Pillai *et al.*, 1990). The first study to demonstrate an immunosuppressive action of MDMA showed that MDMA administered at 20mg/kg i.p to rats resulted in a rapid suppression of Con A-induced lymphocyte proliferation, a reduction in total leucocyte count which lasted for at least 6hrs following injection (Connor *et al.*, 1998). In later studies it was reported that MDMA decreased the production of pro-inflammatory cytokines, TNF-α and IL-1β following an in vivo LPS challenge and also increased production of the anti-inflammatory cytokine IL-10 (Connor *et al.*, 2000, 2005). In terms of the adaptive immune response, MDMA suppressed the antigen specific immune response to the protein antigen KLH by inhibiting antibody class switching from

IgM to IgG. The results of this study suggested that MDMA altered the ability of IgM to switch to IgG_{2a} production by suppressing antigen-specific IFN-γ production (Connor *et al.*, 2001).

Human studies have also been conducted to examine the ability of MDMA to alter immune function (Pacifici *et al*, 2002). The studies demonstrate that acute MDMA administration produced a time-dependent immune dysfunction, characterised by a significant decrease in $CD4^+/CD8^+$ T-cell ratio. Drug treatment also produced an increase in levels of the immunosuppressive cytokines IL-10 and TGF-β. A switch from a Th1 (IL-2 & IFN-γ) to a Th2 (IL-4) cytokine phenotype was observed (Pacifici *et al*, 2001). It may be postulated that the disproportional increase in IL-10 and TGF-β levels could compromise T-cell functions by the direct inhibitory effect on T-cell proliferation and also by promoting the expansion of T-helper 2-type immune response versus T-helper 1 cell activation. Results from these studies are in accordance with other studies Baldwin (1998) and Stanulis (1997) that noted a disruption of Th_1/Th_2 balance with a shift towards a Th_2-type cytokine phenotype following cocaine abuse.

Table 1.1: A summary of the immunosuppressive actions of MDMA in humans and animals (from Connor, 2004).

Parameter	Source	Stimulant	MDMA Conc./dose	Effect	Reference
Neutrophil phagocytosis	Rat blood	Zymosan	*In vivo:* 10 mg/kg (i.p.)/ *in vitro* (10 μg/ml)	Decrease	[Connor *et al.*, 2003]
IL-1β production	Rat blood	LPS	*In vivo*: 10–20 mg/kg (i.p.)	Decrease	[Connor *et al.*, 2000; 2001]
	Rat blood	LPS	*In vitro*: 1–10 μg/ml	No change	[Connor *et al.*, 2000]
	Human blood	PHA	*In vivo*: 100 mg (p.o.)	Decrease	[Pacifici *et al.*, 2001]
TNF-α production	Rat blood	LPS	*In vivo*: 5–20 mg/kg (i.p.)	Decrease	[Connor *et al.*, 2000; 2001]
	Rat blood	LPS	*In vitro*: 1–10 μg/ml	No change	[Connor *et al.*, 2000]
	Mouse macrophages	LPS	*In vitro*: 0·0001–100 μm	No change	[House *et al.*, 1995]
IL-6 production	Mouse macrophages	LPS	*In vitro*: 0·0001–100 μm	No change	[House *et al.*, 1995]
	Human blood	PHA	*In vivo*: 100 mg (p.o.)	Decrease	[Pacifici *et al.*, 2001]
IL-10 production	Rat blood	LPS	*In vivo*: 1·25–10 mg/kg (i.p.)	Increase	[Connor *et al.*, 2003]
	Rat blood	LPS	*In vitro*: 1–10 μg/ml	No change	[Connor *et al.*, 2003]
	Rat blood	Con A	*In vivo*: 7·5 mg/kg (i.p.)	Decrease	[Connor *et al.*, 2003]

Con A, concanavalin A; CTL, cytotoxic T lymphocyte; IFN-γ, interferon-γ; IL, interleukin; i.p., intraperitoneal; KLH, keyhole limpet haemocyanin; LPS, lipopolysaccharide; NK, natural killer; PHA, phytohaemagglutinin; p.o., *per os*; TGF-β1, transforming growth factor-β1; TNF-α, tumour necrosis factor-α.

There are major concerns that if indeed drugs of abuse are immunosuppressive, people who abuse these drugs will be prone to infectious diseases and as a consequence will develop increased mortality to infections. Cases have been reported where MDMA abuse was closely followed by the development of meningococcal meningitis (Prasad *et al.*, 1994). More recently, another report documented the case of a 24yr old male who was diagnosed with herpes zoster opthalmicus (HZO), and it was concluded that chronic ecstasy consumption over a 3 day period led to the development of HZO in this young male; a condition that is

rarely found in adults under 50 years of age (Zwick *et al.*, 2005). In addition, d-amphetamine has been shown to reduce host resistance to infection by influenza A virus, and to the bacterial pathogen Listeria monocytogenes (Freire-Garabal *et al*, 1991 and Nunez *et al*, 1993). It is also noteworthy that several epidemiological studies have indicated that there is a higher incidence of HIV infection in drug users (Estrada *et al.*, 2002; Bell *et al.*, 2002).

1.11.1 Mechanism(s) underlying the immunosuppressive action of MDMA

The first study to examine the effect of MDMA on immune function was conducted by House *et al.* (1995). In this study the effect of *in vitro* exposure to MDMA on a number of measures of macrophage and lymphocyte function were examined. They concluded from their results that MDMA had the potential to attenuate some immune function, but it depended on the concentration of drug used and the immune parameter tested cells which promote cell mediated immunity and humoral immunity respectively. Lymphocyte function was examined by measuring B-cell proliferation, important for antibody production and antigen presentation. But no significant effect of MDMA was observed. In addition, T-cell regulatory function was investigating by assessing production of the Th_1 cytokines IFN-γ and IL-2 and the Th_2 cytokines IL-4 and IL-10. Results indicated that IL-2 was increased at very low levels of MDMA while it was suppressed at high concentrations (100μM), while MDMA didn't alter IL-4 production. In addition to these results, they investigated the effect of MDMA on macrophage function by measuring production of IL-6 and TNF-α. Again, no significant effect on cytokine production was detected. All in all, the *in vitro* studies conducted by House *et al.*, indicate the MDMA has a very limited direct modulatory effect of immune cells. Other laboratories have reported similar findings, where in vitro exposure to MDMA did not alter cytokine production (Connor *et al.*, 2000, 2005). Since previous reports have documented that the effect of MDMA is not as a direct action upon immune cells we must ask the question of how MDMA is mediating this immunotoxicity or immune dysfunction.

In this regard, MDMA is a potent releaser of neurotransmitters such as serotonin, dopamine and noradrenaline in the central nervous system, and thus stimulate the activation of peripheral pathways such as the sympathetic nervous system (SNS) and hypothalamic-pituitary axis (HPA) (Sprague *et al.*, 2003; Gerra *et al.*, 2003). Upon stimulation of the SNS and HPA, there is an induction of catecholamine and

glucocorticoid release of which these are the endogenous products of both pathways. Both glucocorticoids and catecholamines have potent immunomodulatory actions as indicated by the table below:

Table 1.2: A summary of the immunosuppressive effects of catecholamines and glucocorticoids.

Parameter	Cytokine	Catechcholamines	Glucocorticoids	Reference
Cellular components		Inhibits neutrophil phagocytosis Inhibits T-lymphocyte proliferation	Suppresses neutrophil function Anti-proliferative	Elenkov *et al.*, 2000, 2004 Almawi *et al.*, 1999
Pro-inflammatory cytokines	IL-12	Decrease	Decrease	Elenkov *et al.*, 2000, 2004
	IFN-γ	Decrease	Decrease	Elenkov *et al.*, 2000, 2004
	TNF-α	Decrease	Decrease	Elenkov *et al.*, 2000 Almawi *et al.*, 1999
Anti-inflammatory cytokines	IL-10	Increase	Increase	Elenkov *et al.*, 2000, 2004
	TGF-β	Increase	Increase	Elenkov *et al.*, 2000, 2004
Antigen presention		Suppresses MHC class II expression	Suppresses MHC class II expression	Elenkov *et al.*, 2000 Almawi *et al.*, 1999

As it is well established that both adrenergic receptors and glucocorticoid receptors are expressed on the majority of immune cells, thus it has been suggested that release of supraphysiological concentrations of catecholamines and glucocorticoids by MDMA *in vivo* could mediate the immunosuppressive actions of these drugs (see Connor, 2004).

In this regard, the hypothesis being explored in this thesis is that MDMA can mediate immunosuppressive actions via activation of the sympathetic nervous system, and subsequent β-adrenoceptor activation on antigen presenting cells by noradrenaline (Figure 1.8). It has previously been demonstrated that MDMA increases production of the anti-inflammatory cytokine via β-adrenoceptor activation (Connor *et al.*, 2005), hence IL-10 is suggested as a likely mediator of the immunosuppressive effect of MDMA.

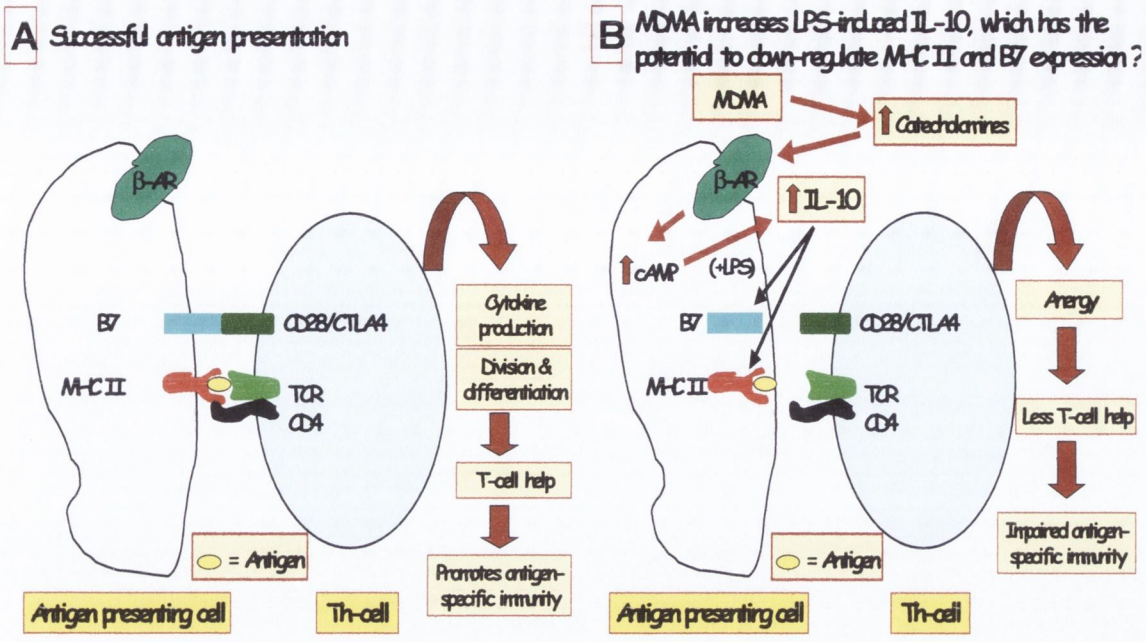


Figure 1.8: A schematic diagram demonstrating (a) normal antigen presentation and (b) a hypothesis regarding the ability of MDMA to interfere with antigen presentation and consequently antigen-specific immunity (from Connor, 2004).

1.12 Objectives of this thesis

From reported evidence, it is well established that MDMA has immunosuppressive actions in both animals and humans. The overall aim of this thesis is to further investigate the immunosuppressive effects of MDMA using a mouse model.

The specific aims of the thesis are:

1. To determine if MDMA suppresses the innate IFN-γ response.
2. To determine if MDMA can reduce expression of MHC class II and co-stimulatory molecules on antigen presenting cells.
3. To determine if MDMA suppresses antigen presentation, and development of an antigen specific immune response.
4. To explore the role of the anti-inflammatory cytokine IL-10 in the immunosuppressive effects of MDMA.

Chapter 2: Materials and Methods

2.1 Materials

Animals

CD1 mice	Bioresources, TCD
C57Bl/5 mice	Harlan Labs, UK
Lab mouse diet	Red Mills

Treatments (*In vivo* and *In vitro*)

Escherichia coli Lipopolysaccharide serotype 0111:B4	Sigma
Keyhole Limpet Haemocyanin	Calbiochem
MDMA	NIDA USA
Nadolol	Sigma
(-)-Norepinephrine (+) bitartrate salt hydrate (99%)	Sigma
Recombinant mouse IL-10	R & D Systems

Cell Culture

Acrodisc syringe filter (0.2μM)	Pall Corporation
Cell strainers (40μM)	BD Falcon
Fetal Calf Serum	Gibco
Haemocytometer	VWR International
RPMI 1640 Culture Medium	Gibco
Penicillin-streptomycin	Gibco

ELISA

Mouse IL-5 ELISA	BD Biosciences
Mouse IL-10 ELISA Kit	Biosource
Mouse IL-12 ELISA Kit	Biosource
Mouse IFN-γ ELISA Kit	Biosource
96 well maxisorp immunoplates	Nunc

Flow Cytometry

APC-Conjugated Hamster Anti-Mouse CD11c Monoclonal Ab	BD Biosciences
APC-Conjugated Rat Anti-Mouse CD11b Monoclonal Ab	BD Biosciences

FITC-Conjugated Hamster Anti-Mouse CD54 Monoclonal Ab	BD Biosciences
FITC-Conjugated Hamster Anti-Mouse CD80 Monoclonal Ab	BD Biosciences
PE-Conjugated Rat Anti-Mouse CD40 Monoclonal Ab	BD Biosciences
PE-Conjugated Rat Anti-Mouse CD86 Monoclonal Ab	BD Biosciences
FITC -MHC Class II	MACs

Western Blotting

Anti-mouse-β-Actin antibody	Sigma-Aldrich
Anti-mouse iNOS antibody	Transduction Labs
Anti-mouse IgG Horseradish Peroxidase antibody	Sigma
Anti-rabbit IgG Horseradish Peroxidase antibody	Amersham
Broad-range molecular weight standard	Biorad
Filter paper	Whatman
Hyper-film	Amersham
Nitrocellulose membrane	Amersham
Phospho-Stat1 (Tyr701) antibody	Cell Signaling
Restore™ Western Blot stripping buffer	Pierce
STAT1 antibody	Cell Signaling
Supersignal® West Dura extended substrate solution	Pierce
Standard grade No.3 filter paper	Whatman

Chemicals

Acrylamide	Sigma
N'N' Bis Acrylamide	Sigma
Ammonium chloride	Sigma
Ammonium Persulphate	Sigma
Bicinchonic acid (BCA) protein assay	Pierce
Bovine serum albumin (BSA)	Sigma
Bromophenol blue	Sigma
β-Mercaptoethanol	Sigma
Diethyl Pyrocarbonate (DEPC)	Sigma
DL-Dithiothreitol (DTT)	Sigma

Dulbeccos PBS	Sigma
Glycerol	Sigma
Glycine	Sigma
Hydrochloric acid (HCl)	BDH
Methanol (MeOH)	BDH
Protease inhibitor cocktail	Sigma
Potassium Chloride (KCl)	Merck
Potassium dihydrogen orthophosphate (KH_2PO_4)	Sigma
2-propanol	Sigma
Sodium azide	Sigma
Sodium carbonate (Na_2HCO_3)	BDH
Sodium bicarbonate($NaHCO_3$)	BDH
Sodium phosphate monobasic monohydrate (NaH_2PO_4)	Sigma
Sodium chloride (NaCl)	Sigma
Sodium dodecyl sulfate (SDS) 99%	Sigma
Sodium phosphate (biphasic)	BDH
Sulphuric acid (H_2SO_4) 98%	Sigma
N,N,N',N'-Tetramethylethylene-diamine (TEMED)	Sigma
Tetramethylbenzidine	Dakocytmation
Tween-20	Sigma
Tris-base	Sigma
Trypan Blue	Sigma
Thymidine (^3H)	New Dupont ISIS

Molecular Reagents

Absolute ethanol	Sigma
Agarose	Condra
Biosphere filter tips (1000, 200 and 100 μl)	Sarstedt
Diethyl pyrocarbonate	Sigma
Ethidium bromide	Sigma
High capacity cDNA archive kit	Applied Biosystems
Loading dye (6X)	Promega
Molecular grade water	Sigma
Optical adhesive covers	Applied Biosystems
PCR tubes	Sarstedt

RNA*later*™	Ambion
RNase away	Invitrogen
RNase-free 1.5ml and 2ml microfuge tubes	Ambion
RNAse Zap wipes	Ambion
Total RNA isolation kit	Macherney-Nagel
TaqMan gene expression assays	Applied Biosystems
TaqMan universal PCR master mix	Applied Biosystems
10X TBE buffer	Invitrogen
96-well optical reaction plates	Applied Biosystems

Laboratory Plastics

Sterile falcon tubes (15 & 50ml)	Sarstedt Inc
Sterile petri dishes	Sarstedt Inc
Sterile 48 well plates	Sarstedt Inc
Sterile 96 well plates	Sarstedt Inc
Serological Pipettes (10ml & 25ml)	Sarstedt Inc
Pipettes tips (1ml & 500μl)	Sarstedt Inc
Flat bottom 96 well plates	Sarstedt Inc
Sterile Round bottom 96 well plates	Sarstedt Inc
Plastic syringes (20ml & 1ml)	Becton Dickenson
Polystyrene round bottomed tubes (5ml)	Becton Dickenson
Microtubes (1.5ml & 0.5ml)	Sarstedt Inc

2.2 Methods

2.3. Animals

Female CD1 mice weighing approximately 25-30g were obtained from the Bioresources breeding colony TCD, and housed 8 per cage. The mice were maintained on a 12 hour/12hour (light/dark) cycle, lights were on at 08:00h and off at 20:00h in a temperature controlled room (21-22°C). Food and water were available *ad libitum*. The experimental protocols were in compliance with the European Communities Council directive (86/609/EEC).

2.4. Drug Administration: *In vivo* studies

2.4.1. 3,4-Methylenedioxymethamphetamine (MDMA) administration

MDMA obtained from NIDA (Research Triangle Park, NC, USA) was dissolved in 0.89% NaCl solution to give final concentrations of 0.125 - 2 mg/ml. 0.89% saline was used as the vehicle for the control group. In all experiments MDMA or vehicle was injected at a volume of 10ml/kg via the intra-peritoneal route, resulting in a dose range of 1.25-20 mg/kg for MDMA.

2.4.2. Lipopolysaccharide (LPS) administration

Lipopolysaccharide from *Escherichia coli* serotype 0111:B4 (Sigma-Aldrich, Ireland) was dissolved in 0.89% NaCl solution to give a concentration of 10μg/ml. Animals were injected intra-peritoneally at an injection volume of 10ml/kg, thereby delivering a final dose of 100 μg/kg for LPS.

2.4.3. Keyhole Limpet Haemocyanin (KLH) administration

Keyhole Limpet Haemocyanin (Calbiochem) was dissolved in 0.89% NaCl solution to give a concentration of 0.5mg/ml and injected 100μg/mouse at an injection volume of 0.2ml.

2.4.4. Nadolol administration

Nadolol (Sigma-Aldrich, Ireland) was dissolved in 0.89% saline solution to give final concentrations of 0.03mg/ml or 0.1mg/ml. Animals were injected intra-

peritoneally at an injection volume of 10ml/kg resulting in a dose of 0.3 or 1 mg/kg for nadolol.

2.4.5. Anti-IL-10 Receptor antibody

An antibody against the mouse IL-10 receptor or a rat IgG_1 isotype control antibody (BD Bioscience) was injected to mice at a concentration of 0.5mg/mouse. The antibodies were administered intra-peritoneally at an injection volume of 0.2ml.

2.5. Drug Treatments: In vitro studies

2.5.1. LPS

LPS was diluted in complete RPMI medium to give a stock concentration of 10μg/ml. 50μl LPS was added to a total volume of 500μl, yielding a final working concentration of 1μg/ml. LPS was filter-sterilised before it was added to splenocyte cultures.

2.5.2. MDMA

MDMA was dissolved in complete RPMI medium to give a stock concentration of 250μM, 50μM, 25μM and 5μM. 100μl of MDMA was added to wells to get final working concentrations of 50μM, 10μM, 5μM and 1μM. MDMA was filter-sterilised before it was added to splenocyte cultures.

2.5.3. Recombinant mouse IL-10

Mouse IL-10 was diluted in complete RPMI medium to give a stock concentration of 50ng/ml, 15ng/ml and 5ng/ml. 100μl of mouse IL-10 was added to wells to get final working concentrations of 10ng, 3ng and 1ng/ml. Mouse IL-10 was filter-sterilised before it was added to splenocyte cultures.

2.5.4. Noradrenaline

Noradrenaline was diluted in complete RPMI medium to give a stock concentration of 50μM, 5μM and 0.5μM. 100μl noradrenaline was added to wells to get a final working concentration of 10μM, 1μM and 0.1μM. Noradrenaline was filter-sterilised before it was added to splenocyte cultures.

2.5.5. KLH

KLH was diluted in complete RPMI medium to give a working concentration of 2.5mg/ml; 250μg/ml and 25μg/ml. 100μl KLH was added to wells to get a final concentration of 500μg/ml; 50μg/ml and 5μg/ml. KLH was filter-sterilised before it was added to splenocyte cultures.

2.6. Experimental Design

2.6.1. Effect of MDMA on LPS-induced IL-12p40 and IFN-γ production in serum, expression of splenic mRNA (IL-12p40; IL-12p35; IL-15, IL-18 & IFN-γ) and IFN-γ signalling: A dose-response study.

MDMA (5-20mg/kg) or 0.89% NaCl as vehicle was co-administered with LPS (100μg/kg) to CD1 mice, and mice were sacrificed 8 hours post drug administration. This time-point was chosen as an optimal time-point to measure pro-inflammatory cytokines, IFN-γ and IL-12 production. Previous studies have demonstrated that IFN-γ is maximally produced 7-9 hours (Fukuda *et al.*, 2005) following a lipopolysaccharide challenge. Following sacrifice, trunk blood was collected, centrifuged at 2000rpm for 15 minutes and serum was stored at –80°C for cytokine analysis. A section of spleen tissue was harvested and stored immediately in RNA later, to prevent degradation of RNA. The rest of spleen was stored at –80°C for protein analysis by ELISA and western immunoblotting.

2.6.2. Effect of MDMA on LPS-induced IL-10 production *in vivo*: A time-course study.

CD1 mice received LPS (100μg/kg) co-administered with MDMA (10mg/kg) or vehicle (0.89% NaCl), via the intra-peritoneal route. Mice were sacrificed 60, 120 or 240 minutes following drug administration by decapitation, and blood and spleen samples were stored for cytokine analysis.

2.6.3. Effect of MDMA on LPS-induced IL-10 production in vivo: A dose-response study.

MDMA (5-20mg/kg) or 0.89% NaCl as vehicle was co-administered with LPS (100μg/kg) to CD1 mice and sacrificed 120 minutes post drug administration. This

time-point was chosen as the results from the previous time-course study indicated this was the optimal time for maximal increase in IL-10 production.

2.6.4. *In vitro* effect of (i) MDMA on IL-10 production (ii) MDMA on IL-12p40 and IFN-γ production from LPS-stimulated splenocyte cultures.

Cultured splenocytes were used in this study to examine the direct effect of MDMA on immune cells. Single cells suspensions were obtained following spleen dissection from non-treated mice. 400μl cells were added to plates at a final concentration of 2 x 10^6ml. Added to each well was 100μl of complete RPMI as control, or MDMA were added to each well yielding a final working concentration of 1, 5, 10 or 50μM. After a 1hr incubation period with MDMA, 50μl of LPS was added to each well yielding a final working concentration of 1μg/ml. Plated cells were incubated at 37°C in a 5% CO_2 atmosphere for 24hours in order to measure IL-10 concentrations, or 72hr to measure IL-12 and IFN-γ concentrations. Supernatants were harvested, centrifuged and stored at –80°C for cytokine analysis.

2.6.5. *In vitro* effect of recombinant mouse IL-10 on IL-12p40 and IFN-γ from LPS-stimulated splenocyte cultures.

Single cells suspensions were obtained following spleen dissection from non-treated mice. 400μl cells were added to plates at a final concentration of 2 x 10^6ml. 100μl of recombinant mouse IL-10 was added to each well to yield a final working concentration of 10, 3 or 1ng/ml, and 100μl of RPMI 1640 was added to control wells as a vehicle. Splenocyte cultures were incubated with recombinant mouse IL-10 for 1hour prior to stimulation with LPS at a final working concentration of 1μg/ml. Cells were incubated at 37°C in a 5% CO_2 atmosphere for 96 hours. Supernatants were harvested, centrifuged and stored at –80°C for cytokine analysis.

2.6.6. Effect of pre-treating with anti-IL-10 receptor antibody on MDMA induced immunosuppression.

CD1 mice were pre-treated with anti-IL-10 receptor antibody or IgG1 isotype control 2 hours prior to co-administration of MDMA (20mg/kg) and LPS (100μg/kg). All injections were via the intra-peritoneal route. Animals were sacrificed 8 hours

post MDMA/LPS administration. Following sacrifice, serum was harvested for cytokine analysis. Spleens were harvested for mRNA analysis, cytokine measurement and western immunoblotting.

2.6.7. Effect of the β-adrencoeptor antagonist nadolol on the ability of MDMA to augment LPS-induced IL-10 production.

CD1 mice were pre-treated with the β-adrenoceptor antagonist, nadolol at a concentration of 0.3mg/kg or 1.0mg/kg 30 minutes prior to co-administration of MDMA (10mg/kg or 20mg/kg) and LPS (100μg/kg). All injections were via the intra-peritoneal route, and animals were sacrificed 2 hours post drug administration. Nadolol was chosen as it is a peripherally acting β-adrenoceptor antagonist..

2.6.8. Effect of the β-adrenoceptor antagonist, nadolol, on the suppressive effect of MDMA on LPS-induced IL-12p40 and IFN-γ production and IFN-γ signalling.

CD 1 mice were pre-treated with the peripherally acting β-adrenoceptor antagonist, nadolol at a concentration of 1.0mg/kg 30 minutes prior to co-administration of MDMA (20mg/kg) and LPS (100μg/kg). All injections were via the intra-peritoneal route, and animals were sacrificed 8 hours following MDMA/LPS administration. Following sacrifice serum was harvested for cytokine analysis. Spleen tissue was harvested and used for mRNA analysis, cytokine measurement and western immunoblotting.

2.6.9. *In vitro* effect of noradrenaline on IL-12p40, IFN-γ and IL-10 from LPS-induced splenocytes

Single cells suspensions were obtained following spleen dissection from non-treated mice. 400μl cells were added to plates at a final concentration of 2 x 10^6ml. 100μl of noradrenaline was added to wells to yield final working concentrations of 0.5, 5 or 50μM, and 100μl of RPMI 1640 was added to control wells as a vehicle. Splenocyte cultures were incubated with noradrenaline for 1 hour prior to stimulation with LPS at a final working concentration of 1μg/ml. Cells were incubated at 37°C in a 5% CO_2 atmosphere for 96 hours. Supernatants were harvested, centrifuged and stored at -80°C for cytokine analysis.

2.6.10. Effect of MDMA on MHC class II and co-stimulatory molecule (CD40, CD54, CD86 and CD80) expression on dendritic cells ($CD11c^+$ cells) and macrophages ($CD11b^+$ cells).

MDMA (5-20mg/kg) or 0.89% NaCl as vehicle was co-administered with LPS (100μg/kg) to CD1 mice, and mice were sacrificed 8 hours later. Spleens were harvested, and single cell suspensions were prepared for flow cytometric analysis.

2.6.11. Effect of pre-treatment with the anti-IL-10 receptor antibody on MDMA induced suppression of MHC class II and co-stimulatory molecule expression on dendritic cells and macrophages.

CD1 mice were pre-treated with anti-IL-10 receptor antibody 2 hours prior to co-administering MDMA (20mg/kg) and LPS (100μg/kg). All injections were via the intraperitoneal route, and animals were sacrificed 8 hours post MDMA/LPS administration. Spleens were harvested, and single cell suspensions were prepared for flow cytometric analysis.

2.6.12. Effect of pre-treatment with β-adrenoceptor antagonist nadolol on MDMA induced downregulation of MHC class II expression on dendritic cells and macrophages.

CD 1 mice were pre-treated with the peripherally acting β-adrenoceptor antagonist, Nadolol at a concentration of 1.0mg/kg 30 minutes prior to co-administration of MDMA (20mg/kg) and LPS (100μg/kg). All injections were via the intraperitoneal route, and animals were sacrificed 8 hours following drug injections. Spleens were harvested and single cell suspensions were prepared for flow cytometric analysis.

2.6.13. Effect of MDMA on antigenic-stimulation on cytokine production

Cultured splenocytes were used in this study to examine the effect of MDMA on KLH stimulated cytokine production. On Day 1, CD1 mice were administered with KLH (100μg/mouse), LPS (100μg/kg) and MDMA (10mg/kg) simultaneously. Animals received either vehicle only; vehicle and LPS; vehicle and MDMA or LPS and MDMA. On Day 4, animals were sacrificed by decapitation and spleens dissected. All preparations from this point on were carried out in a sterile laminar flow hood. Added to each well was 100μl of complete RPMI as control, or KLH at a

final concentration of 5, 50 and 500μM that was dissolved in complete RPMI 1640. Plated cells were incubated at 37°C in a 5% CO_2 atmosphere for 72 hours. Supernatants were harvested, centrifuged and stored at –80°C for cytokine analysis.

2.6.14. Effect of MDMA on T-cell activation

CD1 mice received MDMA (10mg/kg) or vehicle co-administered ± LPS (100μg/kg) and sacrificed 6 hours post drug administration, spleens were dissected and were used as stimulator cells. C57BL/6 mice were not administered drug treatment, spleens were dissected and used as responder cells in the mixed lymphocyte reaction.

2.7. Blood sampling and serum preparation

Mouse trunk blood was obtained by decapitation and exsanguination using a plastic boat and collecting microtube. Following collection of trunk blood, it was allowed to clot and centrifuged at 2000rpm for 15-20 minutes. Serum was carefully decanted from the remaining pellet, ensuring not to disturb the pelleted red blood cells, after which serum was aliquoted and stored at –80°C until further analysis.

2.8. Preparation of splenocytes

2.8.1. Spleen dissection

CD1 mice sacrificed by cervical dislocation and placed on a glass plate with its abdomen facing upwards. The skin was swabbed with 70% EtOH and an incision (using a dissection kit that has been sterilised) to the left of the midline was performed from the bottom to the top of the abdomen. The spleen is located on the left side just below the diaphragm and ribcage, it is easily distinguished by its dark red colour and flat, elongated shape. The spleen was removed and any excess peritoneal tissue attached to the spleen was trimmed off.

2.8.2. Splenocyte Preparation

Spleens dissected from mice were stored in 5ml falcon tubes containing 2ml RPMI 1690 medium. All work from this point onwards was carried out in a sterile environment using a laminar flow hood. Spleens were placed in a nylon mesh sieve (pore size 40 micron) and placed in a petri dish containing of RPMI 1640

medium. The spleens were mashed through the nylon sieve where it was collected in the petri dish, while non-splenic tissue remained inside the nylon mesh. A single cell suspension was achieved by titrating the solution with 10 ml sterile pipettes until all tissue clumps were removed. The splenocyte suspension was placed in a sterile 15ml falcon tube and centrifuged @1300rpm for 5 minutes. Supernatant was aspirated off. Red blood cells were lysed by addition of 0.89% ammonium chloride and incubating for 2 minutes. Samples were centrifuged at 1300rpm and pellet re-suspended with 10mls complete RMPI 1640 (supplemented with 10% foetal calf serum and 1% penicillin/streptomycin). This wash step was carried out twice to remove any ammonium chloride. White cell counts were determined using a haematology counter (abc ^{TM} Vet) and adjusted to the required final concentration. Splenocyte cells were plated for in vitro re-stimulation, mixed lymphocyte reaction studies or prepared for flow cytometry.

2.9. Western Immunoblotting

Western Blotting is a technique used to identify specific proteins in a complex mixture of proteins and is therefore capable of determining the distribution and expression of a specific immunoreactive protein in tissue. Electrophoresis is the process in which charged molecules will migrate when an electric field is applied. Proteins migrate through the gel and are separated from each other by charge or size.

2.9.1. Tissue Preparation

Spleen samples were homogenized using a potter homogeniser at 800rpm for 10-15 strokes in a lysis buffer (50mM Tris Base; 150mM NaCl; 2mM EDTA; 1%NP-400; 25% Nadeoxycholate; 0.1% SDS) and left to incubate on ice for 10 minutes before centrifuging at 13000 x g for 15 minutes. The supernatant was removed from the remaining pellet and protein concentration was determined using the BCA assay. All samples containing the extracted proteins were equalized and sample buffer added to each and boiled at 90°C for 5 minutes to denature proteins. Samples were aliquoted and stored at –80°C until further analysis.

2.9.2. Gel Preparation and Electrophoresis

Sodium dodecyl sulfate-Polyacrylamide (SDS-PAGE) gels of varying concentrations (7.5% and 10%) were prepared as the separating gels while a 4%

gel was used as the stacking gel for loading the samples. The separating gels were carefully poured using plastic pasteur pipettes between two glass plates, previously wiped with 70% EtOH, placed in a Biorad casting frame and were held firmly on a casting stand. The gels were allowed to set for 40 minutes and a layer of isopropanolol poured on gel to prevent it evaporating while it set. This layer was removed and gel was rinsed with distilled water before applying the stacking gel on top and placing a 10-well comb into the gel to allow wells to form. Once the gels were completely set, the casting frames were placed in a Biorad Electrode Assembly and inserted into a clamping frame. This assembly was placed into a gel rig (BioRad Mini-PROTEAN 3), the middle chamber and surrounding rig was filled with 1x electrode running buffer, enough to allow sufficient migration of proteins through the mesh-like network within the gel. The combs were removed from the gels and equalised samples were loaded at a concentration of approx 25μg per well (10μl/well), with a different treatment group per lane. Molecular weight marker (5μl: Biorad) was loaded in to the first lane of each gel, this acts as an indicator for successful transfer of proteins and produces a ladder of bands with various molecular weights. The samples were electrophoresed using a Biometra-Standard power pack 25 at 55mV for 40-45 minutes or until the blue dye of the sample buffer had run to the bottom of the gel.

2.9.3. Semi Dry Transfer

Following electrophoresis, gels were removed and placed on a layer of nitrocellulose membrane (Amersham) soaked in distilled water and filter paper soaked in transfer buffer. One layer of filter paper was placed on the plate, on top of which the nitrocellulose membrane was placed, then the gel and a second sheet of filter paper (Fig 2.1). This sandwich was arranged carefully on the plate of a semi-dry transfer apparatus and arranged so that the proteins migrated from the cathode (-) end to the anode (+) end of the apparatus. The samples were transferred for 1hour 30minutes @ 225mA.

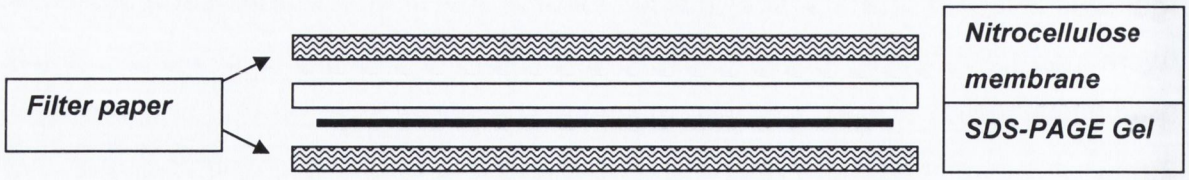


Figure 2.1: Diagram of sandwich preparation for semi-dry transfer of protein from SDS gel to nitrocellulose membrane.

2.9.4. Immunoblotting

Following the transfer of proteins, nitrocellulose membranes were removed and blocked to for non-specific binding 1-2 hours in 5% BSA-TBST blocking buffer. The membranes were washed for 3 x 15 minutes washes. The membrane was then incubated in 5% BSA-TBS Tween (0.1%) with the appropriate primary antibody overnight @ 4°C with gentle shaking on a rock and roller to allow an even distribution of the antibody over the nitrocellulose membrane. The next day, a washing step was applied to the membranes (1 x 5 minutes and 3 x 15 minutes washes) to ensure the primary antibody was completely washed off.

The following step involved the application of the secondary antibody, which is a horseradish peroxidase-linked antibody that binds to the primary antibody because it has been raised against the animal the primary antibody was produced in. Another washing step was applied to the membranes to ensure sufficient removal of any residual antibody. Super signal west dura chemiluminescence solution was added to membrane in order to detect bands (1ml per blot) for up to 1-2 minutes, before draining and placing in a cassette. The membranes were exposed to photographic hyperfilm and developed using an automated developer (Fuji Processor). Band density on developed blots was quantified by densitometry using or the Gel Doc It Imaging System (UVP) and analysed using LabWorks software package.

2.9.4.1. STAT1 phosphorylation

To examine the phosphorylation of STAT1 molecules, the nitrocellulose membranes were blocked in 20mls of 5%TBS-Tween with 5% BSA and allowed to incubate for 1 hour at room temperature. The membranes were washed with TBS-

T wash buffer for 3 x 15 minute incubation periods. The primary antibody, in this case phospho-STAT1 (Cell Signalling Technologies, USA), which detects endogenous levels of STAT1 only when phoshorylated at tyrosine 701 (91 and 84 kDa) raised in rabbit and cross reactive with human, mouse and rat species, was diluted to a concentration of 1:1000 in 5% BSA-TBS-T solution.

The antibody solution (10mls) was added to the membranes and allowed to incubate overnight at 4°C with gentle shaking. The following day, antibody was removed from the membrane and a wash step was applied to the membranes (3 x 15 minute washes). The secondary antibody, anti-rabbit IgG, Horseradish Peroxidase-Linked Species-Specific whole antibody from donkey was diluted to a concentration of 1:2000 in 5% BSA-TBST solution and added to membranes (10mls) and allowed to incubate for 1hour at room temperature. The secondary antibody was removed from membranes and washes applied (3 x 15 minutes). 1 ml of supersignal west dura chemilluminescence was added to the membranes for 1-2 minutes, drained and placed in a cassette prior to exposure to hyperfilm in a dark room and developed using an automated developer.

2.9.4.2. STAT1 expression

The nitrocellulose membranes were washed overnight with TBS-T wash buffer and to ensure that phospho-STAT1 primary antibody was removed, membranes were placed in 10-15mls of stripping solution and incubated for 15 minutes. In order to analyse the expression of STAT1, which detects endogenous levels of total STAT1 protein, STAT1 antibody was diluted to a concentration of 1:1000 in 5% BSA-TBS-T solution. The antibody solution (10mls) was added to the membranes and allowed to incubate overnight at 4°C with gentle shaking. The following day, antibody was removed from the membrane and a wash step was applied to the membranes (3 x 15 minute washes). The secondary antibody, anti-rabbit IgG, Horseradish Peroxidase-Linked Species-Specific whole antibody from donkey was diluted to a concentration of 1:2000 in 5% BSA-TBS-T solution and added to membranes (10mls) and allowed to incubate for 1hour at room temperature. The secondary antibody was removed from membranes and washes applied (3 x 15 minutes). 1 ml of supersignal west dura chemilluminescence was added to the membranes for 1-2 minutes, drained and placed in a cassette prior to exposure to hyper-film in a dark room and developed using an automated developer.

2.7.4.3. Inducible Nitric Oxide Synthase (iNOS) expression

To examine the expression of iNOS, nitrocellulose membranes were blocked in 20mls of 5%TBS-Tween with 5% BSA and allowed to incubate for 1-2 hours at room temperature. The membranes were washed with TBS-T wash buffer for 3 x 15 minute incubation periods. The primary antibody, in this case iNOS/ NOS type III (BD Bioscience, UK) with a molecular weight of 130kDa, raised in rabbit and cross reactive with human, mouse and rat species, was diluted to a concentration of 1:1000 in 5% BSA-TBS-T solution. The antibody solution, a volume of 10mls, was added to the membranes and allowed to incubate overnight at 4°C with gentle shaking. The following day, antibody was removed from the membrane and a wash step was applied to the membranes (3 x 15 minute washes). The secondary antibody, anti-rabbit IgG, Horseradish Peroxidase-Linked Species-Specific whole antibody from donkey (Amersham) was diluted to a concentration of 1:2000 in 5% BSA-TBS-T solution in a volume of10mls, added to membranes, and allowed to incubate for 1 hour at room temperature. The secondary antibody was removed from membranes and washes applied (3 x 15 minutes). 1ml of supersignal west dura chemilluminescence was added to the membranes for 1-2 minutes, drained and placed in a cassette prior to exposure to hyperfilm in a dark room and developed using an automated developer.

2.7.4.4. β-actin expression

Nitrocellulose membranes were soaked in TBS-T wash buffer overnight and to ensure that iNOS primary antibody was removed, membranes were placed in 10-15mls of re-blot stripping solution and incubated for 15 minutes. To determine the expression of β-actin, the housekeeping protein that is constitutively expressed in all tissues with a molecular weight of 45kDa, monoclonal anti-β-actin (Santa-Cruz Biotechnology, USA) was diluted at a concentration of 1:2000 in 5% BSA-TBST solution added to membranes (10mls) and allowed to incubate overnight at 4°C or for 2 hours at room temperature. Primary antibody was removed from membrane and wash steps were applied (3 x 15 minutes). The secondary antibody used was anti-mouse IgG Horseradish Peroxidase (HRP) and was diluted to a concentration of 1:3000 in 5% BSA-TBS-T solution, in a volume of 10mls, added to membranes and allowed to incubate for 1 hour at room temperature. The secondary antibody was removed from membranes and washes applied (3 x 15 minutes). 1 ml of super-signal west dura chemilluminescence was added to the membranes for 1-2

minutes, drained and placed in a cassette prior to exposure to hyper-film in a dark room and developed using an automated developer.

2.10. Cytokine analysis by ELISA

2.10.1. Mouse IL-10 ELISA

The concentrations of IL-10 in serum, spleen supernatants or culture supernatants were determined using a commercially available mouse ELISA kit (Biosource, Belgium). The coating antibody (rat anti-mouse serum) was diluted to a working concentration of 1.25μg/ml with bicarbonate coating buffer. Flat-bottomed 96 well maxisorp immunoplates were coated with 100μl of the coating antibody solution and incubated overnight at 4°C. The next morning, 16-18 hours later, the plates were washed with PBS-T wash buffer using the Tecan automated-washer and blotted dry on paper towels to remove any excess wash buffer. Following this, plates were blocked with 300μl of blocking buffer (5% BSA PBS-T) per well for 2 hours at room temperature. The samples and standards were prepared in standard diluent assay buffer (SDAB).

Samples: Serum or supernatant samples were removed from the –80°C freezer and allowed to thaw on ice. Samples were vortexed well before application on the plate.

Standards: Stock IL-10 (recombinant mouse IL-10) was reconstituted in 1.6ml of reagent diluent to give a concentrated reconstituted standard of 10,000pg/ml. The concentrated stock solution was diluted to a working concentration of 2000pg/ml and used as the top standard, after which, 1:2 serial dilutions were carried out using the standard diluent assay buffer or RPMI medium.

A further wash step (4x washes) was carried out on the plates to remove samples and standards from the plates. The detection antibody (rat anti-mouse IL-12 biotin) was diluted in SDAB to a working concentration of 0.125μg/ml and 100μl of solution added to each well and incubated for 1hour at room temperature. Another wash step was carried out on the plates. Streptavidin-Horse Radish Peroxide was diluted in SDAB and 100μl was added to each well and incubated for 45minutes. Plates were washed again as before. 100μl of Tetramethylbenzidine (TMB) was added to each well and 100μl of sulphuric acid (stop solution) was added to stop

the enzyme-substrate reaction. Following this, absorbance was measured using a microtitre plate reader at 450nm wavelength.
Calculations were determined by subtracting the blank readings from all the sample readings and concentrations measured using a software analysis package Graph Pad Prism.

2.10.2. Mouse IL-12 ELISA

IL-12 concentrations were measured in serum and spleen supernatant samples using a commercially available mouse ELISA kit (Biosource, Belgium).
The coating antibody (rat anti-mouse IL-12 serum) was diluted to a working concentration of 1.25μg/ml with bicarbonate coating buffer and 100μl of solution added to flat-bottomed (96well) maxisorp immunoplates. Plates were incubated overnight at 4°C, the following day, plates were washed 4 times using the Tecan auto-washer and blotted on dry paper towels. The plates were blocked with blocking buffer (5%BSA-PBS-T) at room temperature for 2 hours. Subsequently, plates were washed as mentioned previously.
Samples: Serum or supernatant aliquots were thawed on ice and vortexed. 100μl of sample or standard was added to each well and the plates incubated for 1 hour 30 minutes at room temperature.
Standards: Stock IL-12 (recombinant mouse IL-12) was reconstituted in 2.5ml of reagent diluent to give a concentrated reconstituted standard of 500pg/ml and used as the top standard, after which, 1:2 serial dilutions were carried out using the standard diluent assay buffer or RPMI medium.

A further wash step (4x washes) was carried out on the plates to remove samples and standards from the plates. The detection antibody (rat anti-mouse IL-12 biotin) was diluted in SDAB to a working concentration of 0.125μg/ml and 100μl of solution added to each well and incubated for 1hour at room temperature. Another wash step was carried out on the plates. Streptavidin-Horse Radish Peroxide was diluted in SDAB and 100μl was added to each well and incubated for 45minutes. Plates were washed again as before. 100μl of Tetramethylbenzidine (TMB) was added to each well and 100μl of sulphuric acid (stop solution) was added to stop the enzyme-substrate reaction. Following this, absorbance was measured using a microtitre plate reader at 450nm wavelength.

Calculations were determined by subtracting the blank readings from all the sample readings and concentrations measured using a software analysis package Graph Pad Prism.

2.10.3. Mouse IFN-γ ELISA

The concentrations of IFN-γ in serum, spleen supernatants and culture supernatants were determined using a commercially available mouse ELISA kit (Biosource, Belgoum). The coating antibody (rabbit anti-mouse/rat serum) was diluted to a working concentration of 1.25μg/ml with bicarbonate coating buffer. Flat-bottomed 96 well maxisorp immunoplates were coated with 100μl of the coating antibody and incubated overnight at 4°C. The plates were washed with PBS-T wash buffer using the Tecan auto-washer and blotted dry on paper towels. Following this, plates were blocked with 300μl of blocking buffer per well for 2 hours at room temperature. The samples and standards were prepared in standard diluent assay buffer (SDAB).

Samples: Serum or supernatant aliquots were thawed on ice and vortexed. 100μl of sample or standard was added to each well and the plates incubated for 1hour 30 minutes at room temperature.

Standards: Stock IFN-γ (recombinant mouse IFN-γ) was reconstituted in 0.79ml of reagent diluent to give a concentrated reconstituted standard of 10,000pg/ml. A further dilution of stock concentration to a working concentration for the standard curve is 1000pg/ml and used as the top standard, after which, 1:2 serial dilutions were carried out using the standard diluent assay buffer or RPMI medium.

Another wash step (4x washes) was carried out on the plates to remove samples and standards from the plates. The detection antibody (rat anti-mouse IFNγ biotin) was diluted in SDAB to a working concentration of 0.125μg/ml and 100μl of solution added to each well and incubated for 1hour at room temperature. Another wash step was carried out on the plates. Streptavidin-Horse Radish Peroxide was diluted in SDAB and 100μl was added to each well and incubated for 45minutes. Plates were washed again as before. 100μl of TMB was added to each well and 100μl of stop solution (sulphuric acid) was added to stop the enzyme-substrate reaction. Following this, absorbance was measured using a microtitre plate reader at 450nm wavelength. Calculations were determined by subtracting the blank

readings from all the sample readings and concentrations measured using a software analysis package Graph Pad Prism.

2.10.4. Mouse IL-5 ELISA

The concentrations of IL-5 in culture supernatants were determined using a commercially available mouse ELISA kit (BD Biosciences, UK). The coating antibody (anti-mouse IL-5) was diluted to a working concentration of 4μg/ml with bicarbonate coating buffer. Flat-bottomed 96 well maxisorp immunoplates were coated with 100μl of the coating antibody and incubated overnight at 4°C. The plates were washed with wash buffer using the Tecan auto-washer and blotted dry on paper towels. Following this, plates were blocked with 200μl of assay diluent per well for 1hour at room temperature. The samples and standards were prepared in assay diluent.

Samples: Supernatant aliquots were thawed on ice and vortexed. 100μl of sample or standard was added to each well and the plates incubated for 2 hours at room temperature.

Standards: Stock IL-5 (recombinant mouse IL-5) was reconstituted in 1.0ml of assay diluent to give a concentrated reconstituted standard of 80,000pg/ml. A further dilution of stock concentration to a working concentration for the standard curve is 1000pg/ml, after which 1:2 serial dilutions were carried using RPMI medium.

A wash step (4x washes) was carried out on the plates to remove samples and standards from the plates. The detection antibody (biotinylated Anti- mouse IL-5) was diluted in SDAB to a working concentration of 4μg/ml. 100μl of working detector (detection antibody and avidin-HRP reagent) was added to each well and incubated for 1 hour at RT. Another wash step was carried out on the plate as previously described.100μl of TMB was added to each well and 100 μl of stop solution (sulphuric acid) was added to stop the enzyme-substrate reaction. Following this, absorbance was measured using a microtitre plate reader at 450nm wavelength. Calculations were determined by subtracting the blank readings from all the sample readings and concentrations measured using a software analysis package Graph Pad Prism.

2.11. Protein Analysis

Protein concentrations were determined using the BCA assay (Pierce) (Smith *et al.*, 1985). Standards were prepared from a stock solution of 2.0mg/ml bovine serum albumin (BSA). 25μl of sample or standard was added into a 96 well microplate in duplicate. To this, 200μl of working reagent (50 parts of BCA reagent A with 1 part of BCA reagent B) was added and mixed by gentle tapping. The plate was covered and incubated at 37°C for 30 minutes. Following this, absorbance was measured using a microtitre plate reader at 562nm wavelength. Calculations were determined by subtracting the blank readings from all the sample readings and concentrations measured using a software analysis package Graph Pad Prism.

2.12. Mixed Lymphocyte Reaction (MLR)

The mixed lymphocyte reaction is an assay used to determine the functional ability of antigen presenting cells (Steinmen and Witmer, 1978). The MLR is a mixture of lymphocytes from two genetically different animal strains, and is a useful assay to assess antigen presenting capacity which is an important process in normal immune function.

Spleens from female CD1 mice were used as stimulators in MLR experiments, while those spleens obtained from C57BL/6 male mice were used as a source of responder cells in these studies. Single cell suspensions for splenocytes were prepared as described previously. Responder cells were plated in sterile 96-well round bottom plates, run in triplicate at a concentration of $2x10^6$ cells/ml and 100μl per well. The stimulator cells were irradiated with 3500rads to destroy the proliferative capacity of lymphocytes in culture (Sellins and Cohen, 1987). These cells were plated in triplicate at a concentration of $1x\ 10^6$cells/ml at 100μl per well. RMPI 1640 medium was added in triplicate to responder cells to assess non-specific or background proliferation. Plates were incubated at 37°C for 72hours. The cells were pulsed with tritiated thymidine (^3H) at 0.5μCi/well for 18-24 hours before the end of culture time. Plates were removed at the end of incubation period and placed in the freezer at –20°C until further analysis. When the experiment was finished all plates were harvested using a cell harvester where

cells were collected onto a fibreglass-backed filter paper. The plates were washed several times to ensure all cells were removed from the wells. The filter paper was allowed to air-dry at room temperature for several hours or alternatively, placed in a microwave for 1-2 minutes to dry. Filter paper mats were placed in a plastic envelope and 5mls of scintillation cocktail applied before sealing and removal of any air bubbles. Radioactivity was measured in counts per minute (CPM) of the incorporated thymidine in responder cells, to determine the proliferation of co-cultured cells in the mixed lymphocyte reaction.

2.13. Immunophenotyping by flow cytometry

Flow cytometry is a useful technique due to the fact that live individual cells can be monitored, providing sensitive and specific information about each single cell. In relation to the optics of flow cytometry, when the light source hits a cell, the amount of light scattered to the side is detected in the side scatter (SS) (Fig 2.2). The information provided from side scatter describes the size and shape of the cell. Flow cytometers use lasers as their source to excite cells. The excitation from the lasers must be equivalent to the absorption wavelengths of the fluorochromes used (Robinson, 2004). The argon laser is the most commonly used since it produces several lines in the UV, and can excite fluorscein, which is a common fluorchrome. The other parameter detected is forward scatter (FS) and it provides information about the surface properties, complexity of the cell and can determine how granulated the cells are. Various populations of cells can be distinguished from the information provided by side and forward scatter following aquisiton of samples. In addition, antibodies have fluorescent labels attached enabling the surface expression of specific cell markers.

(a) (b)

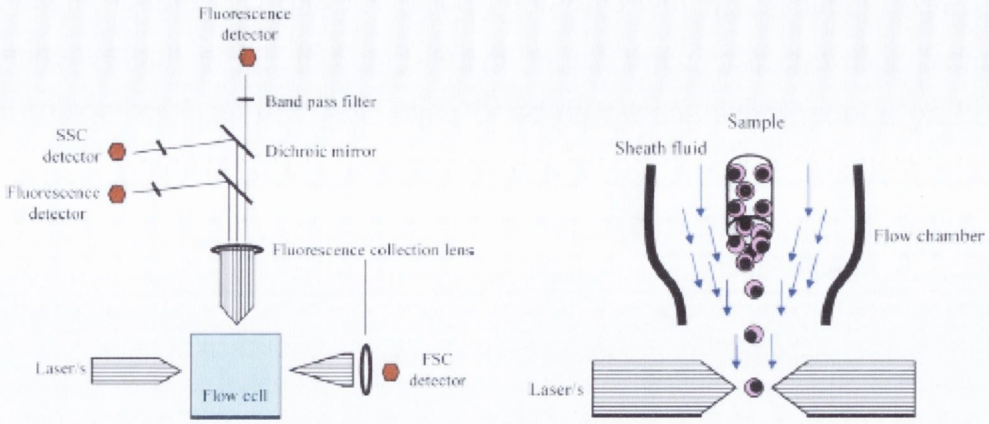


Figure 2.2: Diagrams to illustrate the optics of flow cytometry (a) and how single cells suspended in sheath fluid are focused between the light-source (b) (Tarrant, 2005).

2.13.1 Preparation and staining of splenocytes

Single cell suspensions of splenocytes were obtained at a concentration of $10x10^6$/ml as previously described. Samples were centrifuged at 250 x g for 5 minutes, supernatant decanted and pellets re-suspended. Cells were blocked in 50% fetal calf serum and incubated on ice for 30 minutes. Samples were centrifuged again and two washes were applied using fluorescence-activated cell sorting (FACs) buffer following blocking. Cells were finally re-suspended in 3mls of FACs buffer. Splenocytes were stained with APC-conjugated antibodies for $CD11c^+$ or $CD11b^+$ in order to identify dendritic cells and macrophages respectively. Cells were also stained with PE or FITC labelled antibodies against MHC class II, and the co-stimulatory molecules CD40, CD54, CD80 (B7.1) and CD86 (B7.2) (Table 2.1).

Relevant antibodies (2μl) and appropriate isotype controls and 100μl of cells were added to wells of a 96well plate, the plate was incubated for 45 minutes at 4°C. Unstained cells from each animal were processed through all of the same steps as the stained cells. Plates were centrifuged and two wash steps applied. Cells were re-suspended in 200μl of FACs buffer, and made up to a final volume of 500μl in 5ml FACs tubes. Tubes were stored on ice and covered with tinfoil at all times to prevent decay of flourochromes and cell clumping, respectively.

2.13.2 Acquisition

Samples were acquired by flow cytometry using a Dako Cytomation Cyan ADP, and Summit software was used for analysis (Fig 2.3). The fluorescence was measured on a logarithmic scale with band pass filter 520 for fluorescein (FITC), 576 for phyoerythrin (PE) and 660 for allophycocyanin (APC) (Table 2.2).

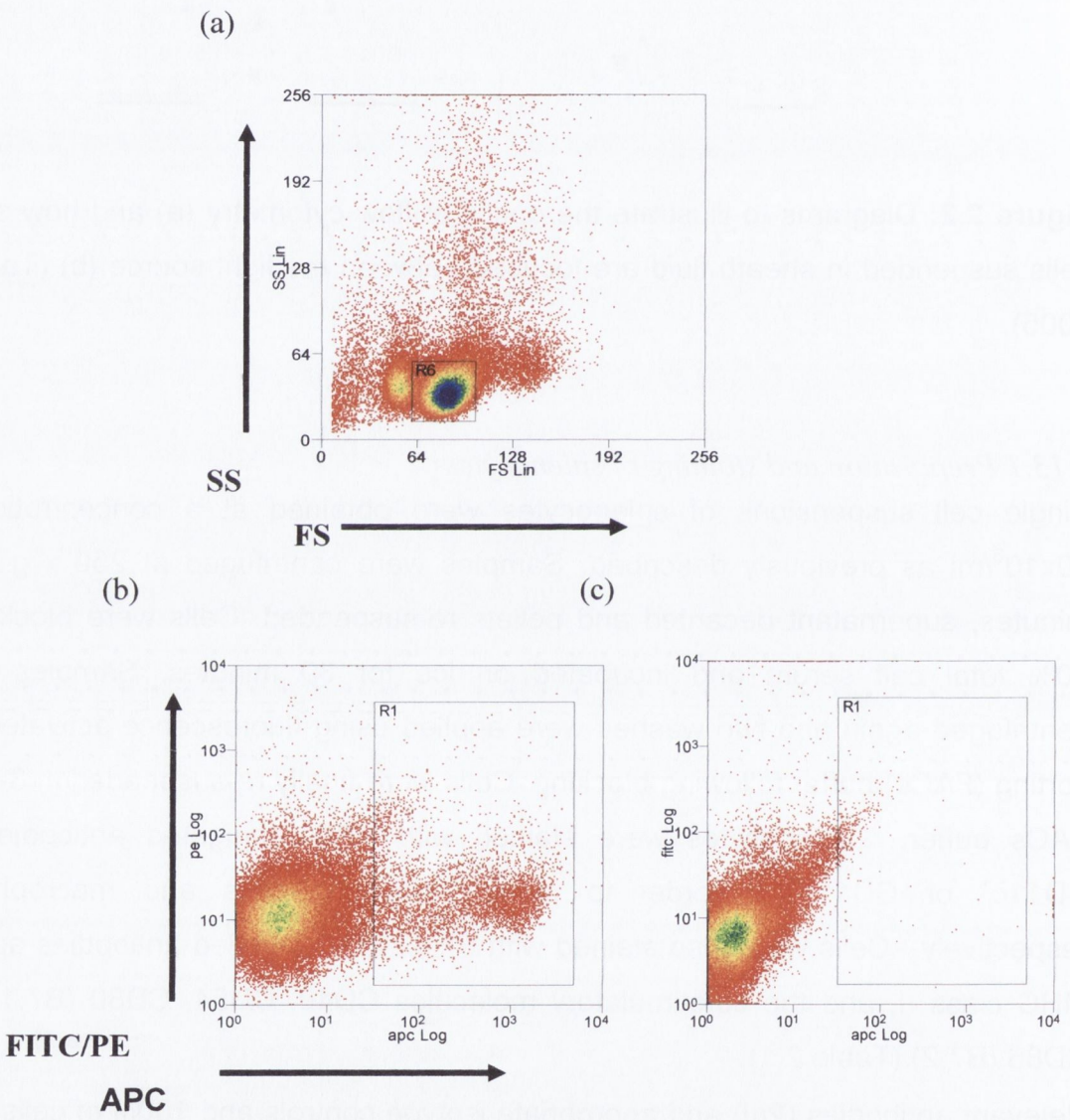


Figure 2.3: Sample dot plots indicating forward scatter (FS) versus side scatter (SS) of acquired spleen cells (a), gated region showing APC-stained $CD11c^+$ cells (dendritic cells) (b), and isotype controls for APC, FITC and PE together (c).

Table 2.1: List of primary antibodies and their relevant fluorescent tags used for staining splenic cells.

Cell marker	Primary monoclonal antibodies	Conjugated to fluorescent tags
Dendritic cells	Hamster anti-mouse CD11c	APC
Macrophages	Rat anti-mouse CD11b	APC
MHC class II	Rat Anti-mouse MHC II	FITC
CD40 antigen	Rat Anti-mouse CD40	PE
ICAM	Hamster Anti-mouse ICAM	FITC
B7.1	Hamster Anti-mouse CD80	FITC
B7.2	Rat Anti-mouse CD86	PE

Table 2.2: List of fluorochromes used for immunophenotyping

Fluorochrome	Excitation Peak	*Emission Peak*	Laser Wavelength
FITC	495	520	488
PE	565	575	488,514,568
APC	650	660	633,635,647

2.14. Real Time Polymerase Chain Reaction (RT-PCR)

Real Time PCR (RT-PCR) is a technique used to quantitate messenger RNA (mRNA) from minimal volumes of tissue or cultured cells. It is able to detect the accumulation of product during the reaction where it is measured at the exponential phase of the PCR technique.

2.14.1 Total RNA extraction

Isolation of total RNA from mouse spleens was carried out using Nucleospin RNA II kits (Macherey-Nagel) as per manufacturers protocol. Tissue dissected was placed in RNA later and stored at 4°C to allow the tissue sample to be fixed and disable any potential Rnases present. All instruments used were wiped with Rnase Away and rinsed with DEPC water before using. About 30mg of tissue was homogenized in lysis buffer (350µl RA1 buffer and 3.5µl β-mercaptoethanol) using a Polytron for several minutes until all splenic tissue was chopped up. However, due to the elasticity of spleen tissue, chopping the spleen into several pieces was necessary prior to polytroning. Following this, the lysate was filtered through Nucleospin Filter units and centrifuged for 1 minute at 11,000g. 350µl of 70% ethanol was applied to the lysate and pipetted several times until dissolution occurred. The total lysate was loaded to a nucleospin II column and centrifuged for 30 seconds at 11,000 x g. This column now contains the RNA so therefore every stage is important from this step onwards. The next step involved desalting the membrane of the column by applying 350µl membrane desalting buffer and centrifuging at 11,000 x g for 1minute. Following this, any potential DNA contamination present was digested by adding 95µl Dnase reaction mixture directly onto the center of the silica membrane of the column and incubated at room temperature for 15 minutes. The additional steps involved washing. 200µl of RA2 buffer (inactivates Dnase) was added and centrifuged for 30 seconds at 11,000 x g and column was placed into a new collecting tube. A second wash was carried out and involved adding 600µl buffer RA3 to the column and centrifuging for 30s at 11,000 x g and the final wash was with 250µl RA3 and centrifuged for 2 minutes at 11,000 x g to allow the membrane to dry completely. The column was placed in a nuclease-free micro-centrifuge tube and RNA was eluted with 60µl Rnase-free H_2O and finally centrifuged for 1 minute at 11,000 x g. RNA was stored at –80°C.

2.14.2 RNA quantification

The next stage following RNA extraction was to assess the integrity of the RNA and this was determined by electrophoresisng a sample of RNA on agarose gels. A small volume of RNA (~3µl) was diluted in 2µl Rnase free H_2O and loading dye. 4µl of diluted RNA was loaded onto a 1% agarose gel and electrophoresed at

90mV. When the gel is observed under UV light, 2 ribosomal RNA (rRNA) bands (Figure 2.4) can be seen, a dense 28S band on top and a lighter 18S band below as shown in figure. Total RNA was quantified using a spectrophotometer in which the purity was demonstrated by the ratio of $A_{260/280}$ and concentration measurements taken of RNA present in each sample. RNA was equalized to the lowest concentration of RNA detected.

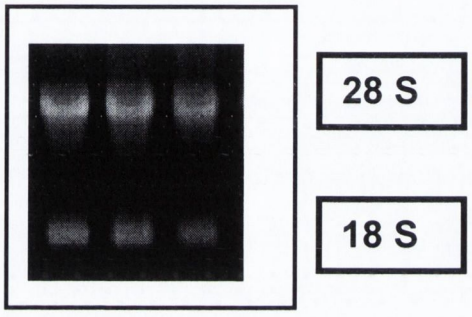


Figure 2.4: Sample blot demonstrating the integrity of extracted RNA from spleen (ribosomal 28S and 18S bands).

2.14.3 cDNA preparation

Following RNA equalization, samples were assembled for cDNA production using the ABI High Capacity cDNA kit (Applied Biosystems) since it is more sensitive and synthesises a larger volume of cDNA. A master-mix solution containing reverse transcription buffer, dNTPs, random primers, multiscribe reverse transcriptase and Rnase free H_20 was prepared and stored on ice. RNA was diluted to a range between 0.02-0.2 μg/μl. Equal volumes of the master mix were added to an equivalent volume of diluted RNA, eg 25μl of master mix was added to 25μl RNA in PCR tubes. The tubes containing samples were placed in a thermocycler and program was set according to protocol as follows, step 1 was set for 10 minutes at 25°C and step 2 set for 120 minutes at 37°C. When the thermocycler was finished with performing the amplification stage, samples were removed and stored at –20°C or used immediately for real time PCR.

2.14.4 Real-time PCR

Real-time PCR was performed using Taqman Gene Expression Assay's (Applied Biosystems), which contain forward and reverse primers, and a FAM-labeled MGB Taqman probe to each gene of interest. The assay IDs for the genes examined are listed in Table 2.3. A 1 in 5 dilution of cDNA was prepared with sigma water. All real-time PCR was conducted using an ABI Prism 7300 instrument (Applied Biosystems). A 20µl volume was added to each well (9µl of diluted cDNA, 1µl of primer and 10µl of Taqman® Universal PCR Master Mix). Electronic pipettes (EDP3 20-200µl, 2-20µl and 10-100µl) were used to ensure pipetting accuracy. Samples were assayed in duplicate in one run (40 cycles), which composed of 3 stages, 95°C for 10 minutes, 95°C for 15 seconds for each cycle (denaturation) and finally the transcription step at 60°C for 1 minute. 18S rRNA or β-actin was used as an endogenous control to normalize gene expression data, and 18s rRNA or β-actin expression was measured using gene expression assays containing forward and reverse primers (primer limited) and a VIC-labeled MGB Taqman probe (Applied Biosystems). Gene expression was calculated relative to the endogenous control samples and to the control sample giving an RQ value (2^{-DDCt}, where CT is the threshold cycle).

In relation to the various stages carried out during the PCR reaction, initially samples are heated to 95°C for 15 seconds, which allows the double stranded cDNA to denature. Hereafter, the temperature of the reaction decreases to allow annealing and extension of the cDNA, however the target probe must anneal to the single-stranded cDNA since it has a higher melting temperature than that of the target primers (Applied Biosystems). This probe contains a FAM/VIC dye and a proprietary non-fluorescent quencher (NFQ) dye, this quencher blocks the dye from emitting a fluorescent signal by fluorescence resonance energy transfer (FRET) technology (Applied Biosystems). Once the reaction temperature reaches 60°C the primers anneal to the strand of cDNA and it is extended by 5' nuclease activity of the Taq polymerase. This induces the release of the FAM/VIC-labelled probe causing the FRET between the dye and quencher to be broken, and the generation of a fluorescent signal. Due to the specificity of the probe and primers for the cDNA sequence, one fluorescent signal is produced for each new cDNA copy and measured during the annealing stage of the PCR cycle (60°C).

Table 2.3: List of genes used in single RT-PCR studies with the gene expression assay and genbank ref sequence numbers.

Gene Symbol	Gene Name	Taqman Gene Expression Assay ID	GenBank accession no
IFN-γ	Interferon gamma	Mm00801778_m1	NM_008337.1
IL-12a	Interleukin 12p35	Mm00434165_m1	NM_008351.1
IL-12b	Interleukin 12p40	Mm00434170_m1	NM_008352.1
IL-15	Interleukin 15	Mm00434210_m1	NM_008357.1
IL-18	Interleukin 18	Mm00434225_m1	NM_008360.1
CXCl10	IP-10	Mm00445235_m1	NM_021274.1
CD40	CD40 antigen	Mm00441891_m1	NM_170702.2

http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi

2.14.5 Real-time PCR analysis

The ΔΔCT method (Applied Biosystems RQ software, Applied Biosystems, UK) was used to assess gene expression for all real-time PCR analysis. This method is used to assess relative gene expression by comparing gene expression of treated/experimental samples to a normal or untreated sample (control), rather than quantifying the exact copy number of the target gene. In this manner the fold-difference (increase or decrease) can be assessed between treated and control samples. The fold-difference is assessed using the cycle number (CT) difference between samples. Briefly, a threshold for fluorescence is set, against which CT is measured. To accurately assess differences between gene expression the threshold is set when the PCR reaction is in the exponential phase, when the PCR reaction is optimal or 100% efficient. Thus, samples with low CT readings demonstrate high fluorescence, indicating greater amplification and hence, greater gene expression. When a PCR is 100% efficient a one-cycle difference between samples means a 2-fold difference in copy number (2^2), similarly a 5-fold difference is a 32-fold difference (2^5).

To measure this fold-difference relative to control, the CT of the endogenous control (β-actin or S18) is subtracted from the CT of the target gene for each

sample, thus accounting for any difference in cDNA quantity that may exist. This normalised CT value is called the (ΔCT). The CT difference (ΔCT) of the control is subtracted from itself to give 0, and subtracted from all other samples, this is the ΔΔCT value. The ΔΔCT (cycle difference corrected for β-actin or S18) is then converted into a fold-difference. As a one-cycle difference corresponds to a two-fold increase or decrease relative to control, 2 to the power of the ΔΔCT (difference in control and sample CT corrected for actin) gives the fold-difference in gene expression between the control and treated samples. The control sample always has a ΔΔCT value of 0, thus 0^{-2} gives a $2^{\Delta\Delta CT}$ of 1, against which all other samples are referenced

Table 2.4: Basal and LPS-induced levels of pro-inflammatory gene expression in mouse spleen.

	Ct for S18		Ct for target gene		LPS-induced change	
Target	**Control**	**LPS**	**Control**	**LPS**	**ΔCT (Target-S18)**	**Fold Increase**
IL-12p40	12.77	12.69	26.8	25.8	0.92	1.89
IL-12p35	12.77	12.69	36.7	35.6	1.02	2.03
IL-15	12.77	12.69	27.3	25.1	2.12	4.34
IL-18	12.77	12.69	23.3	22.5	0.72	1.64
IFN-γ	12.77	12.69	29.0	22.5	6.42	85.6
IP-10	12.77	12.69	23.3	17.1	6.12	69.5
CD40	12.77	12.69	22.3	20.7	1.52	2.86

Ct = cycle number

ΔCT = Difference in CT between Control and LPS treated animals corrected for S18

2.15. Statistical analysis of Data

Data was analysed using a by performing one-or two-way analysis of variance (ANOVA) while repeated 2-way ANOVA was performed on ex vivo studies where cells were re-stimulated. If any significant changes were detected, post hoc comparisons were performed using Newman-Keuls test. In the anti-IL-10 receptor antibody study, vehicle and MDMA groups treated with the IgG_1 isotype control were isolated, and analysed using a Student's t-test. This was necessary, as the robust pro-inflammatory action of the anti-IL-10 antibody precluded detection of differences between vehicle and MDMA groups treated with the IgG_1 isotype control antibody. Data were deemed significant when $P < 0.05$., and expressed as group means with standard errors.

Chapter 3: Results

3. Results

3.1: MDMA suppresses production of the pro-inflammatory cytokine IL-12 following an *in vivo* LPS challenge.

The aim of the present study was to investigate the ability of MDMA to alter production of IL-12, a pivotal pro-inflammatory cytokine required to induce a Th_1 phenotype by driving IFN-γ production. MDMA (5-20mg/kg) was co-administered with LPS (100μg/kg; i.p.) to mice, and mice were sacrificed 8hrs post injection. Concentrations of IL-12p40 were measured in serum and spleen tissue. In order to investigate whether MDMA regulated IL-12 production at the transcriptional level, IL-12p40 and IL-12p35 mRNA expression was assessed in spleen tissue. In addition, mRNA expression for IL-15 and IL-18, two other cytokines that are known to induce IFN-γ production were also examined in spleen tissue following MDMA administration.

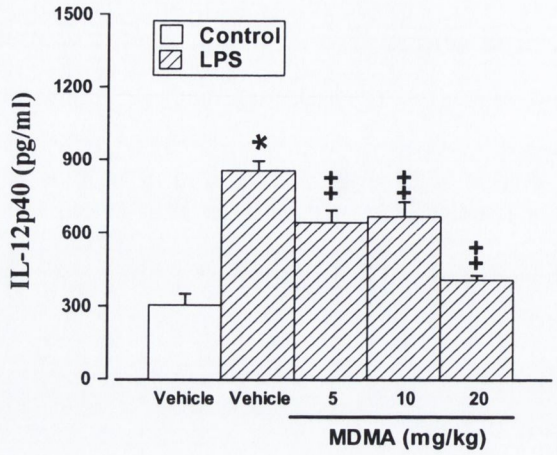
3.1.1: Serum and spleen IL-12p40 concentrations

LPS induced a significant increase in IL-12p40 concentrations in serum ($P<0.05$) and spleen ($P<0.01$) compared to vehicle treated animals. Circulating concentrations of IL-12p40 were significantly reduced ($P<0.01$) by all doses of MDMA (5-20mg/kg) (Figure 3.1.1a). In addition, IL-12p40 concentrations in spleen were suppressed following administration of MDMA (20 mg/kg) ($P<0.01$) (Figure 3.1.1b).

3.1.2: Splenic IL-12p40, IL-12p35, IL-15 and IL-18 mRNA expression

LPS administration induced a significant increase in expression of splenic IL-12p40, IL-12p35, IL-15 and IL-18 mRNA. MDMA also significantly decreased IL-12p40 ($P<0.05$) mRNA expression (Figure 3.1.2a) in response to an LPS challenge. In addition, MDMA significantly reduced IL-15 mRNA expression in spleen ($P<0.01$) (Figure 3.1.3a), but failed to alter LPS-induced IL-12p35 (Figure 3.1.2b) or IL-18 mRNA (Figure 3.1.3b) expression.

(a)



(b)

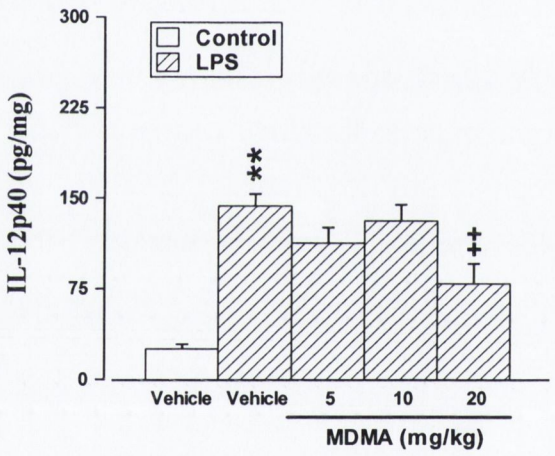
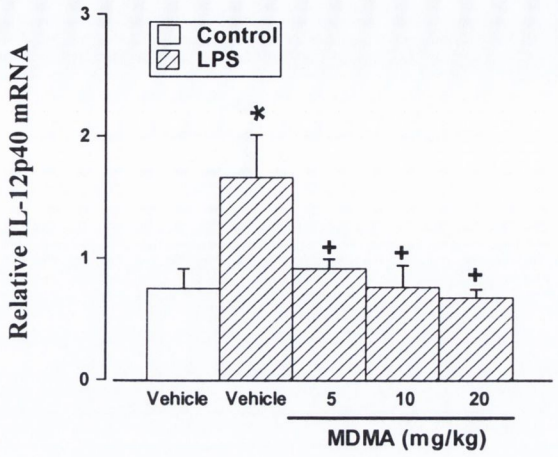


Figure 3.1.1: MDMA suppresses IL-12p40 concentrations in serum (a) and spleen (b) following an *in vivo* LPS challenge.

ANOVA demonstrated a significant effect of treatment on IL-12p40 concentrations in serum [$F(4,31) = 19.69$, $P < 0.0001$] and in spleen [$F(4,41) = 16.01$, $P < 0.0001$].

Data expressed as means ± SEM (n = 8-10). * $P<0.05$, ** $P<0.01$ vs. Vehicle; $^{++}$ $P < 0.01$ vs. Vehicle & LPS (Newman-Keul's test).

(a)



(b)

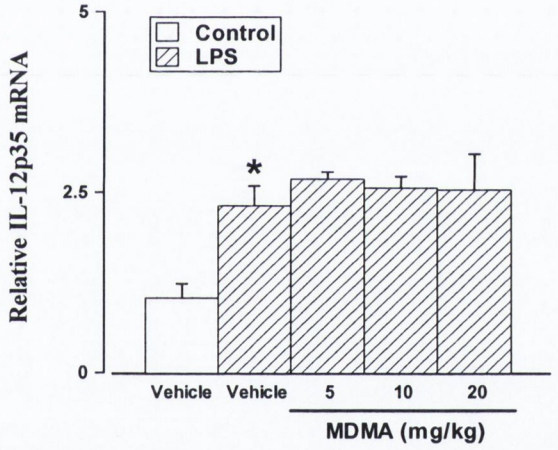
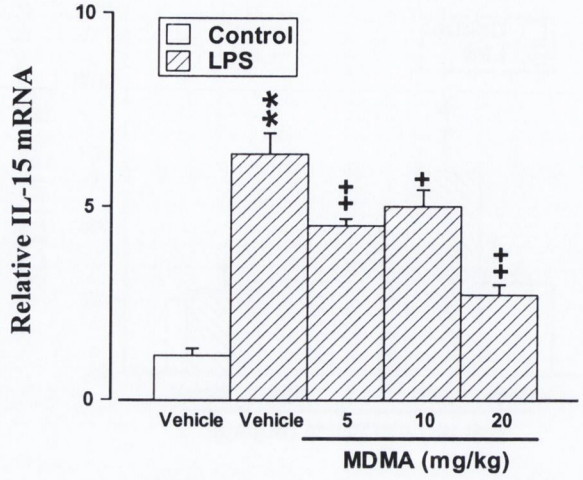


Figure 3.1.2: MDMA reduces splenic IL-12p40 mRNA expression (a) but not IL-12p35 mRNA expression (b) following an *in vivo* LPS challenge.

ANOVA demonstrated a significant suppression of treatment on IL-12p40 [$F(4,19)= 4.06$, $P <0.01$] expression in spleen. Data expressed as means ± SEM (n = 4-5). * $P < 0.05$ vs. Vehicle; $^{+}$ $P < 0.05$ vs. Vehicle & LPS (Newman-Keul's test).

(a)



(b)

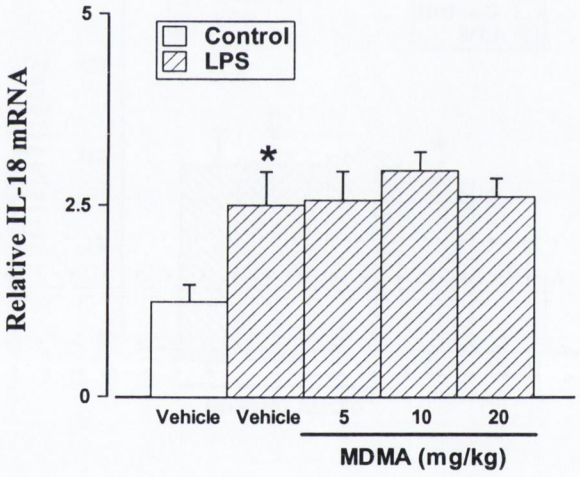


Figure 3.1.3: MDMA reduces splenic IL-15 mRNA expression (a) but not IL-18 mRNA expression (b) following an *in vivo* LPS challenge.

ANOVA demonstrated a significant suppression of treatment on IL-15 mRNA [$F(4,19) = 11.02$, $P < 0.0001$] expression in spleen. Data expressed as means ± SEM (n = 4-5). * $P < 0.05$, ** $P < 0.01$ vs. Vehicle; $^{++}$ $P < 0.01$ vs. Vehicle & LPS (Newman-Keul's test).

3.2: MDMA suppresses innate IFN-γ production in response to an *in vivo* LPS challenge.

Following the observation that MDMA induced a suppressive effect on IL-12 production, we examined whether MDMA altered IFN-γ production, an essential cytokine necessary during the innate immune response. MDMA (5-20mg/kg) was co-administered with LPS (100μg/kg; i.p.) to mice, and mice were sacrificed 8hrs post injection. Circulating concentrations of IFN-γ were measured in serum, and IFN-γ mRNA expression was assessed in spleen tissue.

3.2.1: Serum IFN-γ concentrations

LPS administration induced a significant increase in circulating IFN-γ concentrations compared to vehicle treated animal. *Post-hoc* analysis revealed that MDMA (20 mg/kg) significantly reduced LPS-induced IFN-γ production 8hrs post administration ($P<0.01$) (Figure 3.2.1a).

3.2.2: Splenic IFN-γ mRNA expression

LPS administration induced a significant increase in IFN-γ mRNA expression in spleen tissue, and MDMA (5-20mg/kg) significantly decreased IFN-γ mRNA expression following an LPS challenge ($P<0.05$) (Figure 3.2.1b).

3.3: MDMA suppresses IFN-γ signaling in response to an *in vivo* LPS challenge.

The JAK-STAT pathway is primarily associated with downstream signalling of IFN-γ receptor activation, thus the ability of MDMA to alter LPS-induced phosphorylation of STAT1 protein was examined in spleen tissue. Since STAT1 is a critical transcription factor required for the activation of IFN-γ inducible genes, we examined the expression of IFN-γ inducible targets including iNOS, IP-10 and CD40 in spleen. MDMA (5-20mg/kg) was co-administered with LPS (100μg/kg) to mice, and mice were sacrificed 8hrs post injection.

3.3.1: Splenic STAT 1 phosphorylation

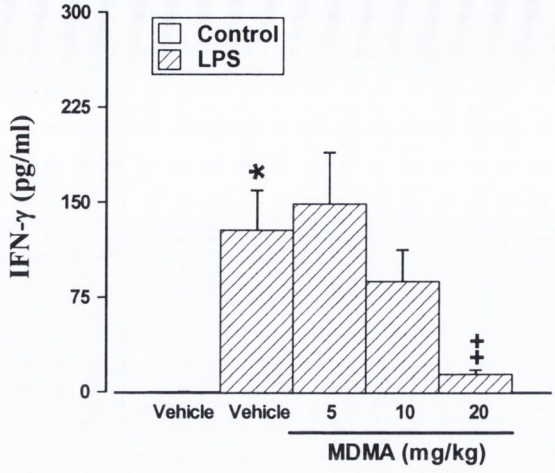
LPS administration induced a significant increase in pSTAT1 expression compared to vehicle treated animals (n=5). MDMA significantly decreased phosphorylation of STAT1 (91 & 84 kDa) on tyrosine 701 in mouse spleen in response to an LPS challenge. There was a dose-dependent suppression of STAT1 phosphorylation with a significant suppression observed with MDMA (10mg/kg) ($P<0.05$) and MDMA (20mg/kg) ($P<0.01$). Sample immunoblots (n=4) indicate changes induced following drug administration (Figure 3.3.1).

3.3.2: Splenic iNOS, IP-10 and CD40 expression

iNOS protein expression: LPS administration induced a significant increase in iNOS protein expression compared to vehicle treated animals. MDMA significantly reduced iNOS expression in mouse spleen in response to LPS, with a significant reduction in iNOS induced by MDMA (20mg/kg) ($P<0.05$). Sample immunoblots (n=4) indicate changes induced following drug administration (Figure 3.3.2).

IP-10 and CD40 mRNA expression: Following LPS administration, there was a significant increase in splenic IP10 and CD40 mRNA expression compared to vehicle treated animals, and MDMA (5-20mg/kg) significantly suppressed LPS-induced IP10 and CD40 mRNA expression (Figure 3.3.3).

(a)



(b)

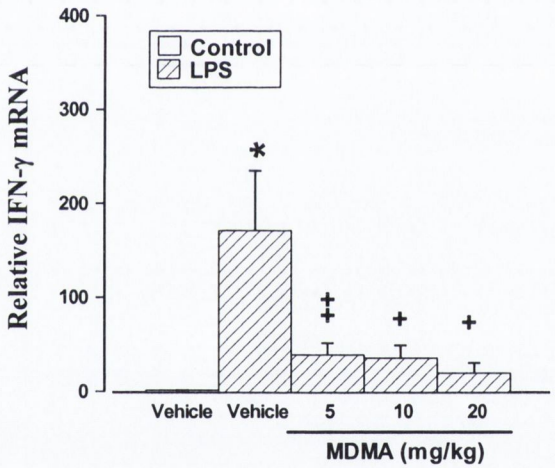
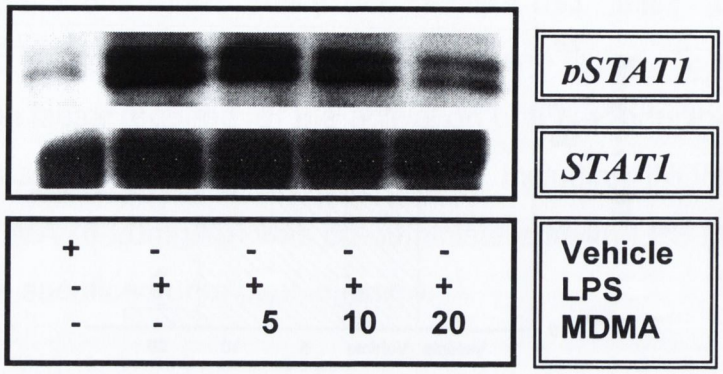


Figure 3.2.1: MDMA decreases circulating IFN-γ concentrations in serum (a) and IFN-γ mRNA expression in spleen following an *in vivo* LPS challenge.

ANOVA demonstrated a significant effect of treatment on serum IFN-γ concentrations [$F(4,35) = 5.54$, $P < 0.001$], and on splenic IFN-γ mRNA expression [$F(4,22) = 4.35$, $P < 0.01$]. Data expressed as means ± SEM (n = 4-8). * $P < 0.05$ vs. Vehicle; $^{+}P < 0.05$, $^{++}P < 0.01$ vs Vehicle & LPS (Newman-Keuls test).

(a)



(b)

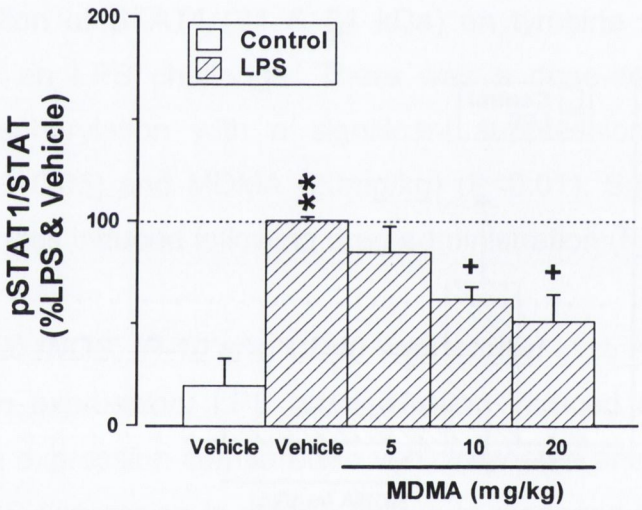
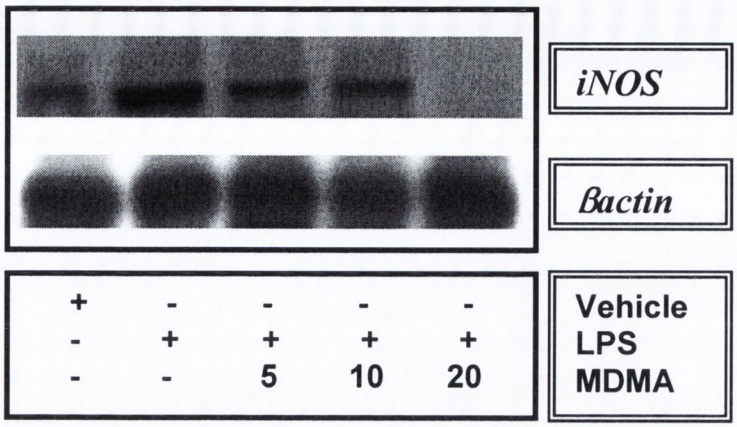


Figure 3.3.1: MDMA decreases STAT1 phosphorylation following an *in vivo* LPS challenge.

ANOVA demonstrated a significant effect of treatment on pSTAT1 expression [$F(4,20) = 8.85$, $P < 0.0003$]. Sample immunoblot (a) and densometric analysis (b) are displayed. Data expressed as means ± SEM (n = 5). ** $P < 0.01$ vs. Vehicle; $^{+}$ $P < 0.05$ vs. Vehicle & LPS (Newman-Keuls test).

(a)



(b)

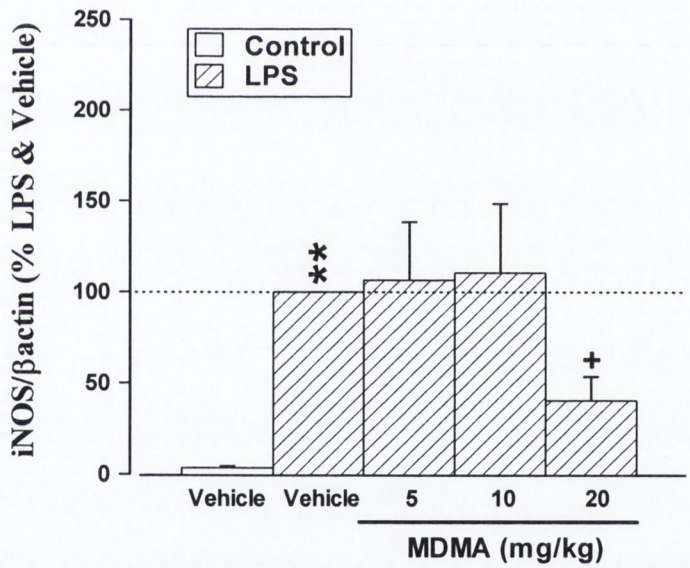
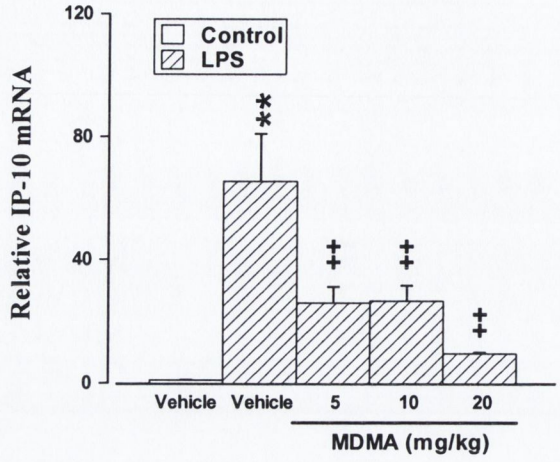


Figure 3.3.2: MDMA decreases splenic iNOS expression following an *in vivo* LPS challenge.

ANOVA demonstrated a significant effect of treatment on iNOS expression in spleen tissue [$F(4,26) = 6.94$, $P < 0.0009$]. Sample immunoblot (a) and densometric analysis (b) are displayed. Data expressed as means ± SEM (n = 4-7). ** $P < 0.01$ vs. Vehicle; $^{+}$ $P < 0.05$, $^{++}$ $P < 0.01$ vs. Vehicle & LPS (Newman-Keuls test).

(a)



(b)

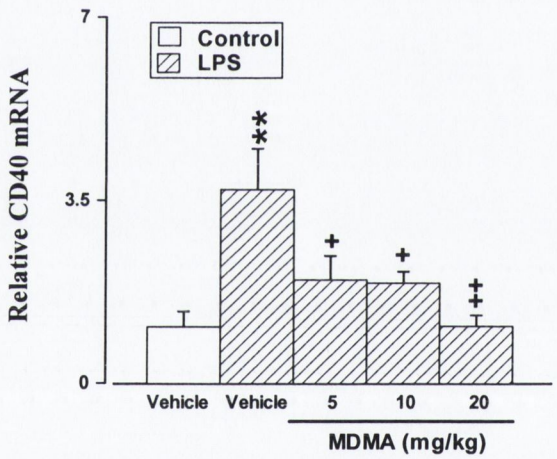


Figure 3.3.3: MDMA suppresses IP10 mRNA expression (a) and CD40 mRNA expression (b) in spleen following an *in vivo* LPS challenge.

ANOVA demonstrated a significant effect of treatment on splenic IP10 mRNA expression [$F(4,22) = 8.7$, $P < 0.0004$] and on CD40 mRNA expression [$F(4,22) = 5.9$, $P < 0.003$]. Data expressed as means ± SEM (n = 4-5). ** $P < 0.01$ vs. Vehicle; $^{+}$ $P < 0.05$, $^{++}$ $P < 0.01$ vs. Vehicle & LPS (Newman-Keuls test).

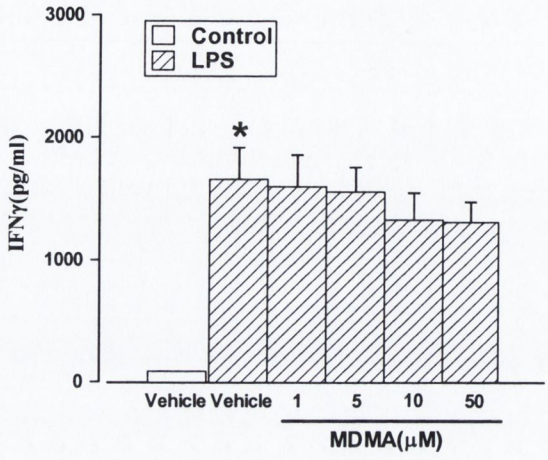
3.4: Effect of MDMA on LPS-induced IFN-γ and IL-12p40 from splenocytes *in vitro.*

To determine if the MDMA-induced suppression on pro-inflammatory cytokines *in vivo* was due to a direct action on immune cells, we investigated the effect of exposing spleen cells to MDMA *in vitro.* Splenocyte cultures were pre-incubated with MDMA (1-50μM) for 1hr before stimulating with LPS (1μg/ml). Cell culture supernatants were harvested after 72hr for cytokine measurement.

3.4.1: IFN-γ and IL-12p40 concentrations

Exposure of splenocytes to LPS induced marked production of IL-12p40 and IFN-γ compared to unstimulated samples ($P<0.05$). *In vitro* exposure to MDMA failed to significantly alter LPS-induced IFN-γ or IL-12p40 production from splenocytes. These results are in contrast to the profound suppression of pro-inflammatory cytokines induced by MDMA *in vivo* (Figure 3.4.1).

(a)



(b)

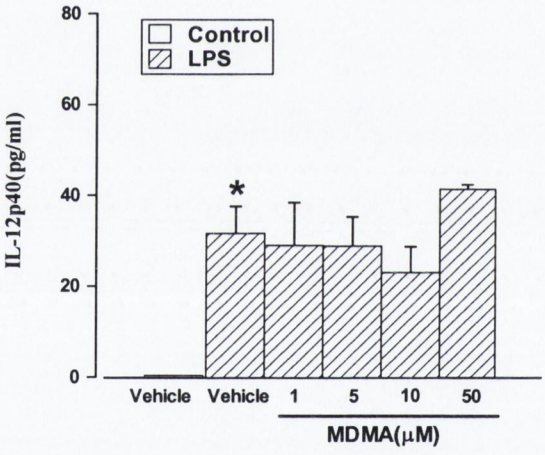


Figure 3.4.1: *In vitro* exposure of mouse splenocytes to MDMA fails to alter LPS-induced (a) IFN-γ or (b) IL-12p40 production.

There was no significant effect of MDMA on IFN-γ or IL-12p40 at any concentration used in the study. Data expressed as means ± SEM (n = 7-8).

3.5: MDMA augments production of the anti-inflammatory cytokine IL-10 in response to an *in vivo* LPS challenge: A kinetic analysis.

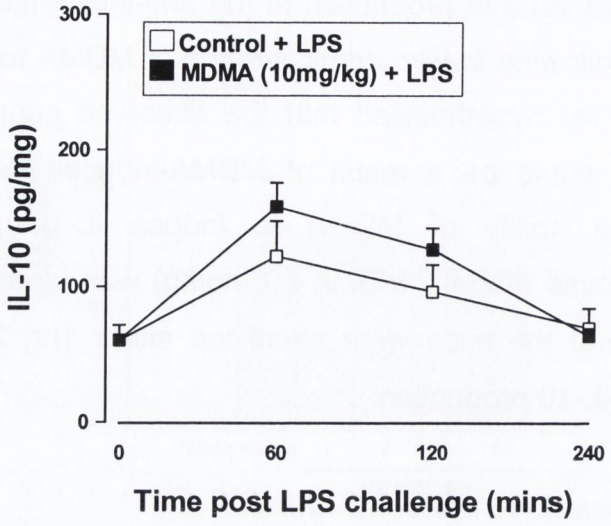
It was previously established that production of the anti-inflammatory cytokine IL-10 is potently induced following *in vivo* administration of MDMA to rats (Connor *et al.*, 2005). Therefore, we hypothesised that the observed suppression of pro-inflammatory cytokines could be a result of MDMA-induced increase of IL-10 production. Here the ability of MDMA to induce IL-10 production was characterized in our mouse model. MDMA (10mg/kg) was co-administered with LPS (100μg/kg; i.p.), and the mice were sacrificed either 1hr, 2hr, or 4hr post injection for analysis of IL-10 production.

3.5.1: Serum and Spleen IL-10 concentrations

Administration of MDMA significantly increased LPS-induced IL-10 concentrations in serum in a time-dependent manner. IL-10 concentrations were significantly increased 1hr post MDMA ($P<0.05$), with a maximal increase observed 2hrs following drug treatment ($P<0.01$). 4hrs following MDMA administration circulating IL-10 concentrations had returned towards baseline, and was not significantly different from control.

In contrast to the observation in serum, MDMA produced a modest increase in LPS-induced IL-10 concentrations in spleen tissue, but it did not reach statistical significance. However, the slight increase in IL-10 appeared 1hr post MDMA administration and decreased thereafter, suggesting that this early increase in IL-10 from spleen may contribute to the increased levels observed at a later time-point observed in serum. (Figure 3.5.1)

(a)



(b)

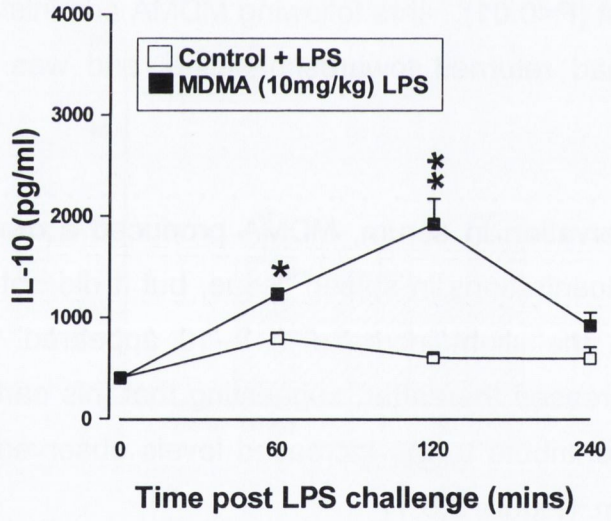


Figure 3.5.1: Effect of MDMA on LPS-induced IL-10 production in serum (a) and spleen (b).

ANOVA demonstrated a significant effect of treatment on serum IL-10 concentrations [$F(5,50) = 15.49$, $P < 0.0001$]. Data expressed as means ± SEM (n = 8-10). * $P < 0.05$, ** $P < 0.01$ vs. Vehicle & LPS (Newman-Keuls test).

3.6: Dose related effects of MDMA on LPS-induced IL-10 production in response to an *in vivo* LPS challenge.

The current study investigated the doses at which MDMA induces IL-10 production following an LPS challenge. MDMA (5-20mg/kg) was co-administered with LPS (100μg/kg; i.p.) to mice, and mice were sacrificed 2hrs post injection. Concentrations of IL-10 production were analysed in serum and spleen.

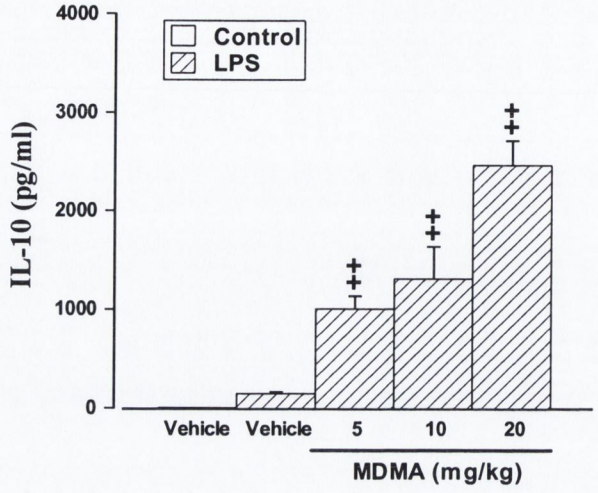
3.6.1: Serum IL-10 concentrations

MDMA (5-20mg/kg) significantly increased LPS-induced IL-10 concentrations ($P<0.01$) in serum in a dose-dependent manner following LPS challenge (Figure 3.6.1a).

3.6.2: Spleen IL-10 concentrations

MDMA induced a modest but statistically insignificant increase in LPS-induced IL-10 production in the spleen (Figure 3.6.1b).

(a)



(b)

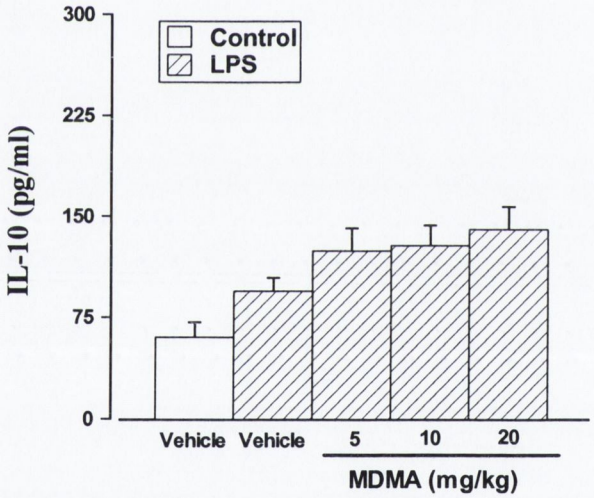


Figure 3.6.1: Dose-dependent effects of MDMA on serum (a) and spleen (b) IL-10 production following an *in vivo* LPS challenge.

ANOVA demonstrated a significant effect of treatment on serum IL-10 concentrations [$F(4,41) = 32.45$, $P < 0.0001$], but failed to significantly alter splenic IL-10 concentrations. Data expressed as means ± SEM (n = 8-10). $^{++}$ $P < 0.01$ vs. Vehicle & LPS (Newman-Keuls test).

3.7: Effect of MDMA on LPS induced IL-10 in splenocytes *in vitro.*

In order to determine if the MDMA-induced increase in IL-10 production was a result of direct action on immune cells, we examined the effect of exposing MDMA to spleen cells *in vitro*. Splenocyte cultures were pre-incubated with MDMA (1-50μM) for 1hr before stimulating with LPS (1μg/ml). Cell culture supernatants were harvested after 24hrs for cytokine measurement.

3.7.1: IL-10 concentrations

Cultured splenocytes pre-incubated with MDMA failed to alter LPS-induced IL-10 production at any concentration examined in the study as shown in Figure 3.7.1. These results are in contrast to the MDMA-induced increase in IL-10 production previously observed from *in vivo* studies.

(a)

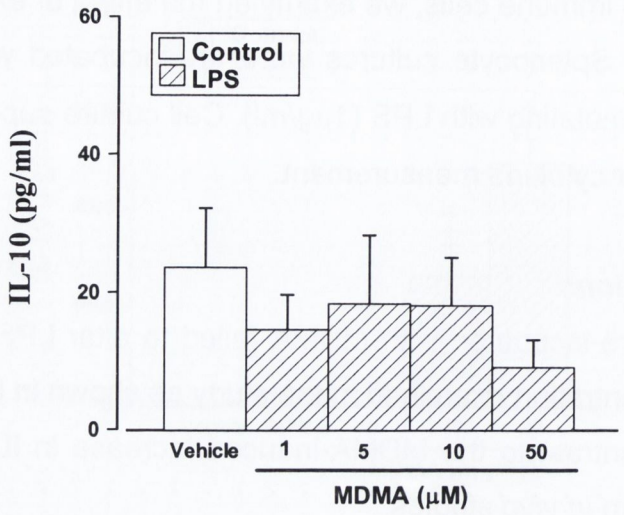


Figure 3.7.1: *In vitro* exposure of mouse splenocytes to MDMA fails to alter LPS-induced IL-10 production.

There was no significant effect of MDMA on LPS-induced IL-10 production at any concentration used in the study. Data expressed as means ± SEM (n = 7-8).

3.8: Effect of IL-10 on LPS-induced IL-12p40 and IFN-γ production from splenocytes *in vitro.*

The current study examined the effect of IL-10 on pro-inflammatory cytokine production from splenocytes, in order to determine if IL-10 had the ability to suppress IL-12p40 and IFN-γ production *in vitro*. Cells were cultured for 96hrs in the presence or absence of recombinant mouse IL-10 (1-10ng/ml). Results indicate that recombinant mouse IL-10 exposure suppressed LPS-induced IL-12p40 and IFN-γ production from splenocytes *in vitro*.

3.8.1: IFN-γ and IL-12p40 production

In vitro exposure to recombinant mouse IL-10 (1-10ng/ml) significantly suppressed LPS-induced IFN-γ and IL-12p40 production ($P<0.01$) (Figure 3.8.1).

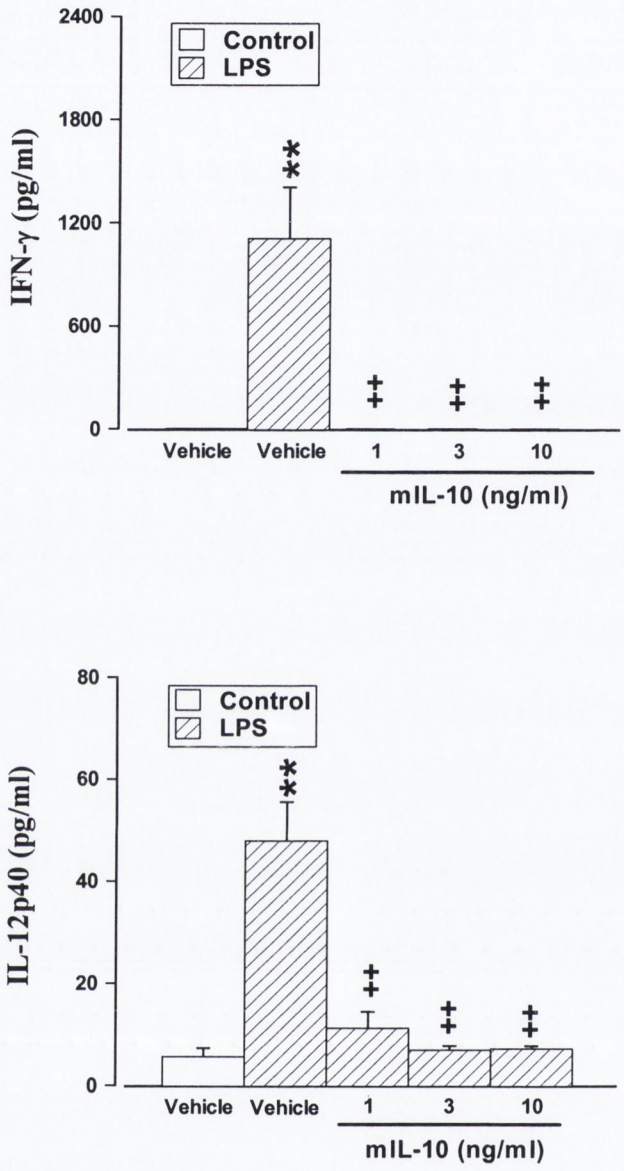


Figure 3.8.1: *In vitro* exposure of mouse IL-10 to splenocytes suppressed LPS-stimulated IL-12p40 and IFN-γ.

ANOVA demonstrated a significant effect of treatment on IFN-γ [$F(5,18) = 14.22$, $P < 0.0001$] and IL-12 [$F(5,18) = 21.7$, $P < 0.0001$] production from splenocytes *in vitro.*

Data expressed as means ± SEM (n = 8-10). ** $P < 0.01$ vs. Vehicle; $^{++}$ $P < 0.01$ vs. Vehicle & LPS (Newman-Keuls test).

3.9: Pre-treatment with a neutralising antibody against the IL-10 receptor inhibits the ability of MDMA to suppress production of the pro-inflammatory cytokines IL-12p40 and IL-15 *in vivo*.

To test the hypothesis that the increase in IL-10 production induced by MDMA mediates it ability to suppress IL-12p40 and IL-15 production, the ability of pre-treatment with an anti-IL-10 receptor antibody to prevent the suppressive effect of MDMA on IL-12p40 and IL15 production was examined. This IL-10 antibody binds to the IL-10 receptor therefore preventing IL-10 from binding to its receptor and activating downstream signalling. Mice were pre-treated with anti-IL-10 receptor antibody (0.5mg/mouse) or rat IgG_1 control 2hrs prior to administration of MDMA (20mg/kg) and LPS (100μg/kg). Following this, mice were sacrificed 8hrs post drug injection. Circulating levels of IL-12p40 were measured in serum, while IL-12p40 and IL-15 mRNA expression was assessed in spleen tissue.

3.9.1 Serum IL-12p40 and Splenic mRNA

MDMA significantly reduced LPS-induced IL-12p40 concentrations in serum ($P<0.05$) in animals treated with the IgG_1 control antibody. However, pre-treatment with the anti-IL-10 receptor antibody completely blocked the ability of MDMA to suppress of IL-12p40 concentrations in serum (Figure 3.9.1). Similarly, MDMA significantly decreased splenic IL-12p40 and IL-15 mRNA expression ($P<0.05$), and pre- treatment with the anti-IL-10 receptor antibody inhibited these immunosuppressive effects of MDMA (Figure 3.9.2). The results indicate that the observed augmentation of IL-10 production induced by MDMA has an important role in the transcription and translation of pro-inflammatory cytokines. It is also noteworthy that administration of the anti-IL-10 receptor antibody induced a profound increase in IL-12p40 protein and mRNA expression in its own right.

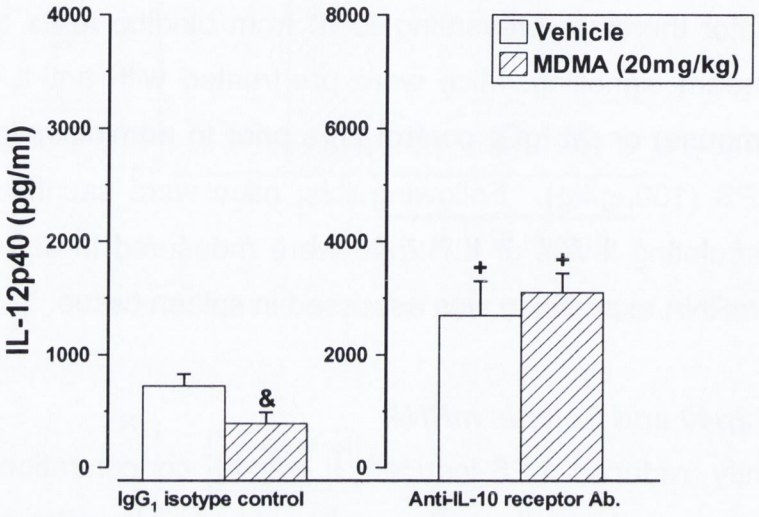
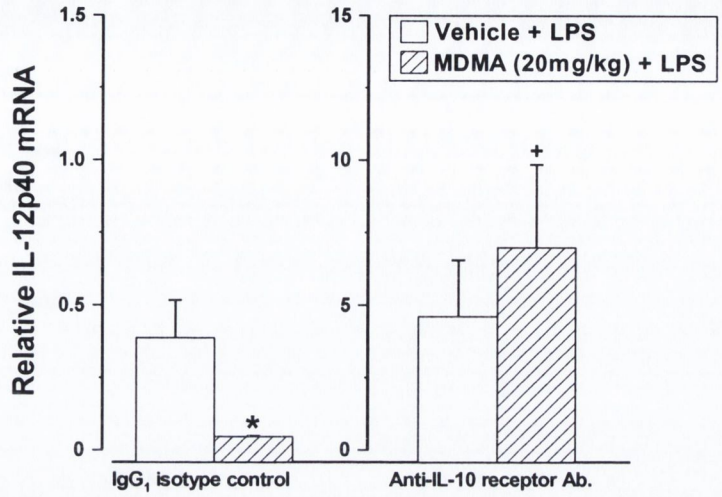


Figure 3.9.1: Pre-treatment with anti- IL-10 receptor antibody blocks the ability of MDMA to suppress circulating levels of IL-12p40.

MDMA had a significant effect on IL-12 production ($P<0.05$) while there was also a significant effect of anti IL-10 treatment [$F(1,17)=54.17$, $P<0.0001$] on IL-12p40 production. Data expressed as means ± SEM (n = 5-6). $^{\&}$ $P < 0.05$ vs. Vehicle (Student t-test), +$P<0.01$ vs MDMA & Vehicle (Newman Keuls test).

(a)



(b)

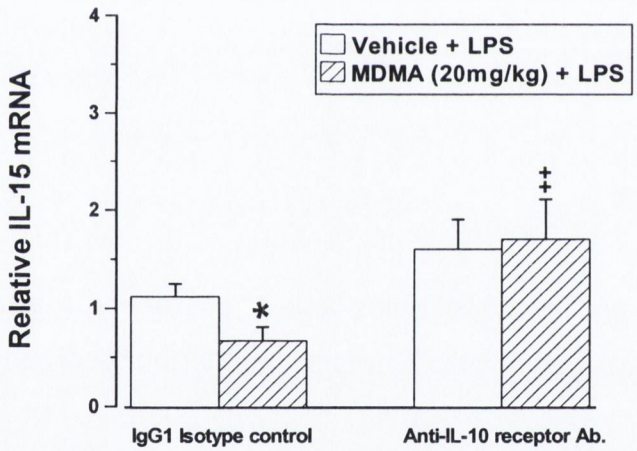


Figure 3.9.2: Pre-treatment with anti-IL-10 receptor attenuates the suppressive effect of MDMA on the expression of (a) IL-12p40 and (b) IL-15 mRNA in spleen effects.

ANOVA demonstrated a significant effect of anti-IL-10 receptor antibody on IL-12p40 [$F(1,20)=10.44$, $P<0.04$] and IL-15 [$F(1,18)=12.67$, $P<0.002$]. Data expressed as means ± SEM (n =6). $^{\&}$ $P < 0.05$ vs. Vehicle (Students t-test), $^{+}$ $P<0.05$, $^{++}P<0.01$ vs MDMA & Vehicle (Newman Keuls test).

3.10: Pre-treatment with a neutralising antibody against the IL-10 receptor inhibits the ability of MDMA to suppress IFN-γ production and signalling.

Here the ability of pre-treatment with the anti-IL-10 receptor antibody to block the suppressive effect of MDMA on IFN-γ production and signalling was examined. Mice were pre-treated with anti-IL-10 receptor antibody (0.5mg/mouse) or rat IgG_1 control 2hrs prior to administering MDMA (20mg/kg) and LPS (100μg/kg) and were sacrificed 8hrs post drug injection. IFN-γ concentrations were measured in serum, while IFN-γ, IP10 and CD40 mRNA expression and STAT1 phosphorylation was assessed in spleen.

3.10.1: Serum IFN-γ and IFN-γ mRNA

MDMA significantly reduced LPS-induced splenic IFN-γ mRNA ($P<0.05$) in animals treated with the IgG1 control antibody. Following pre-treatment with anti-IL-10 receptor antibody the MDMA-induced suppression of IFN-γ was abrogated (Figure 3.10.1). IFN-γ concentrations were undetectable in serum from animals treated with the IgG_1 isotype control. However, there was a significant augmentation of circulating IFN-γ in animals treated with the anti-IL-10 receptor antibody.

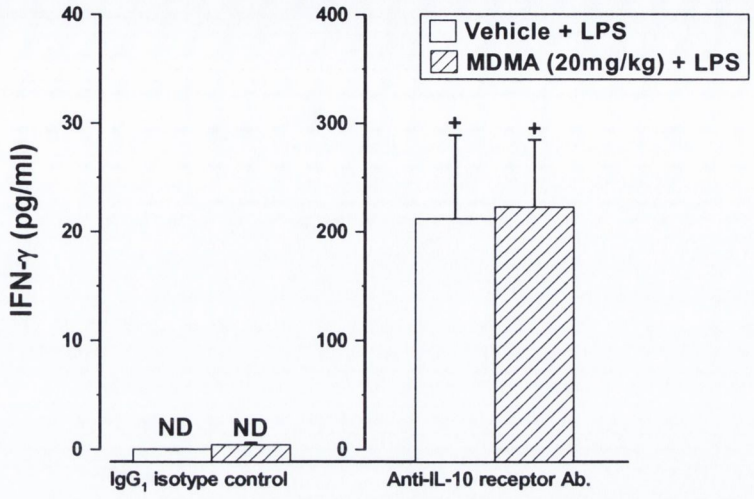
3.10.2: IFN-γ signalling

STAT 1: MDMA significantly decreased LPS-induced phosphorylation of STAT1 ($P<0.05$), however pre-treatment with anti-IL-10 receptor antibody completely blocked this suppressive effect. Sample immunoblots clearly demonstrate a decrease in pSTAT1 expression that has been attenuated by pre-treating with anti-IL-10 receptor antibody (Figure 3.10.2).

CD40 and IP-10: MDMA reduced LPS-induced IP-10 ($P<0.05$) and CD40 mRNA expression in spleen. Following pre-treatment with anti-IL-10 receptor antibody, the MDMA-induced suppression was abrogated (Figure 3.10.3).

It is noteworthy that in this experiment the anti-IL-10 receptor antibody induced a profound increase in LPS-induced IFN-γ expression and IFN-γ signalling in its own right.

(a)



(b)

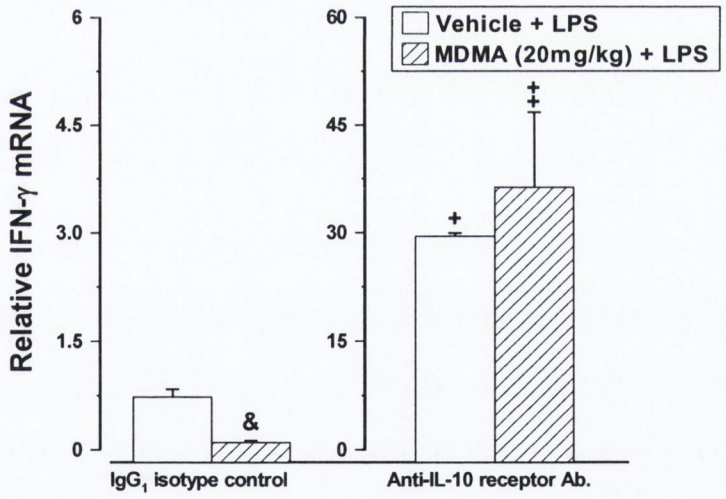
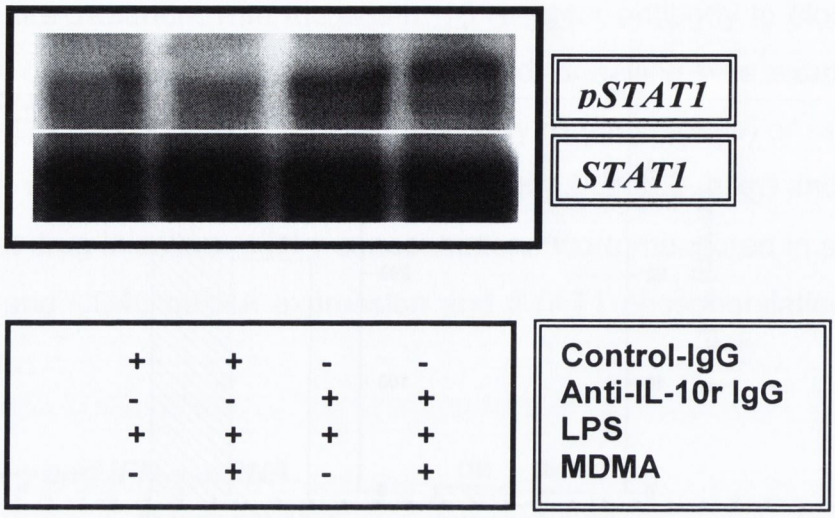


Figure 3.10.1: Pre-treatment with anti-IL-10 receptor abrogates the suppressive effects of MDMA on LPS-induced IFN-γ production.

ANOVA demonstrated a significant effect of anti-IL-10 receptor antibody on (a) serum IFN-γ concentrations [$F(1,19)=20.14$, $P<0.0003$] and on (b) splenic IFN-γ mRNA expression [$F(1,19)=21.8$, $P<0.0002$]. Data expressed as means ± SEM (n=5-6). * $P < 0.05$ vs. Vehicle (Student t-test), $^{+}$ $P<0.05$ vs MDMA & Vehicle (Newman Keuls test).



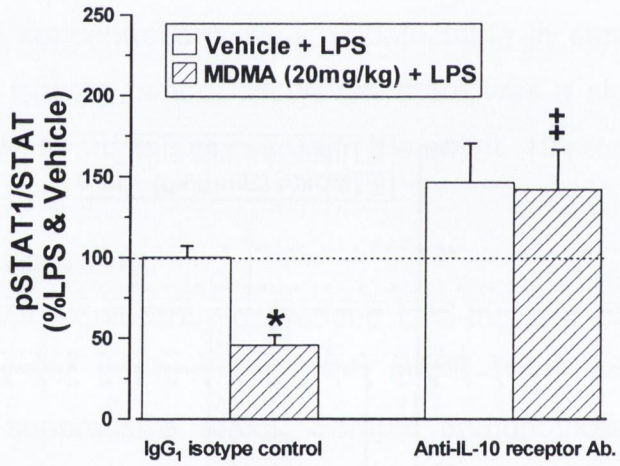
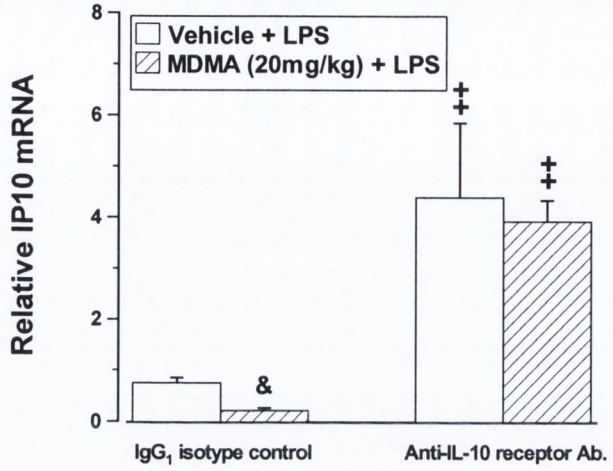


Figure 3.10.2: Pre-treatment with anti-IL-10 receptor antibody inhibits the ability of MDMA to suppress the phosphorylation of STAT1 following an *in vivo* LPS challenge.

ANOVA demonstrated a significant effect of anti-IL-10 receptor antibody on phosphorylation of pSTAT1 [$F(1,16)=16.08$, $P<0.001$]. Sample immunoblots (n=4) (a) and densometric analysis (b) are displayed. * $P < 0.05$ vs. Vehicle, $^{++}$ $P<0.05$ vs MDMA & Vehicle (Neuman-Keuls test). Data expressed means ± SEM (n=5).

(a)



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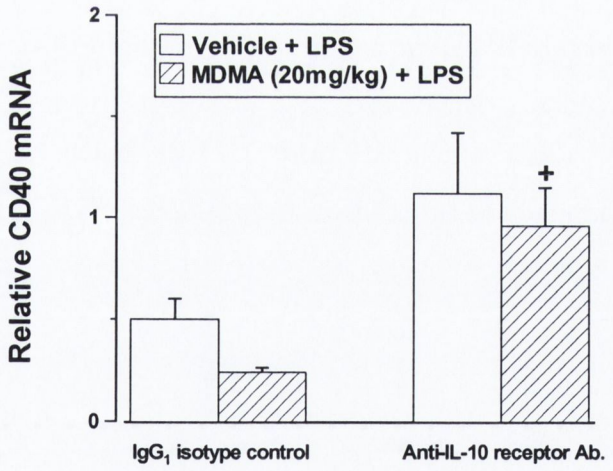


Figure 3.10.3: Effect of anti-IL-10 receptor antibody on IP10 (a) and CD40 (b) mRNA expression in the spleen.

There was a significant effect of anti IL-10 receptor blockade on CD40 [$F(1,20)=23.54$, $P<0.0001$] and IP10 [$F(1,20)= 12.8$ $P<0.002$] mRNA expression. Data expressed as means ± SEM (n=6). $^{\&}$ $P < 0.05$ vs. Vehicle (Student t-test), + *P<0.05,* ++P<0.01 vs MDMA & Vehicle (Newman Keuls test).

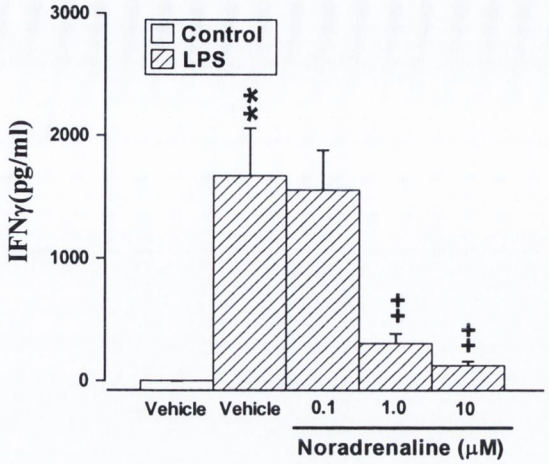
3.11 Noradrenaline decreases LPS-induced IL-12p40 and IFN-γ from splenocytes *in vitro.*

The aim of this experiment was to investigate the effect of noradrenaline on cytokine production from splenocytes *in vitro*. It is well established that catecholamines including, adrenaline and noradrenaline suppress pro-inflammatory cytokine production, IL-12 & TNF-α while conversely enhance IL-10 secretion from antigen presenting cells (Elenkov *et al.*, 1996). Splenocyte cultures were pre-incubated with noradrenaline (0.1, 1, 10 μM) for 1hr before stimulating with LPS (10μg/ml). Cell culture supernatants were harvested after 96hrs for IFN-γ and IL-12 production and 48hrs for IL-10 production. Results indicate that noradrenaline reduces IFN-γ and IL-12p40 but without any significant alteration in IL-10 production.

3.11.1 IFN-γ and IL-12p40

There was a significant increase in production of IFN-γ and IL-12p40 following LPS stimulation. Following exposure to noradrenaline, 1 and 10 μM, there was a significant decrease of LPS-induced IFN-γ and IL-12p40 production form splenocytes (Figure 3.11.1). There was a marked increase in IL-10 production in response to LPS, whilst exposure to noradrenaline slightly increased concentrations of IL-10 in splenocytes (Figure 3.11.2), but it was not statistically significant. Results indicate that noradrenaline reduces IFN-γ and IL-12p40 but does not alter IL-10 production.

(a)



(b)

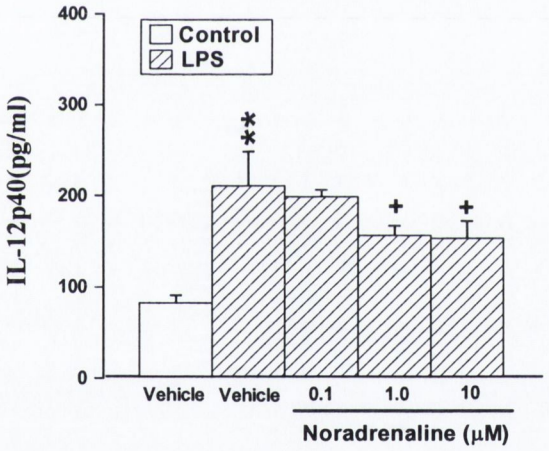


Figure 3.11.1: Exposure of splenocytes to noradrenaline reduces LPS-induced IFN-γ and IL-12p40 production.

ANOVA demonstrated a significant effect of treatment on IFN-γ [$F(4,35)= 12.4$, $P<0.0001$] and IL-12p40 [$F(4,34)= 9.03$, $P<0.0001$] production. Data expressed as means ± SEM (n = 7-8). **P<0.01 vs. Vehicle (Newman-Keuls test), $^{+}$ $P < 0.05$ and $^{++}P< 0.01$ vs Vehicle and Noradrenaline.

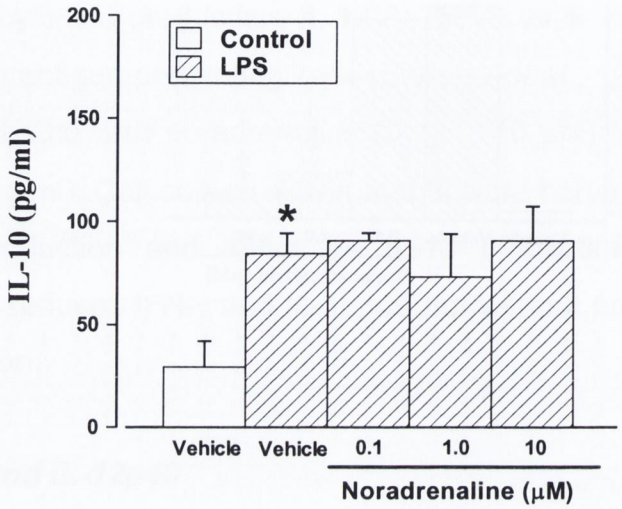


Figure 3.11.2: Effect of noradrenaline exposure on LPS-induced IL-10 production from splenocytes, *in vitro*.

ANOVA demonstrated a significant effect of LPS treatment on IL-10 [$F(4,22)= 3.83$, $P<0.01$] There was no significant effect of noradrenaline on IL-10 production from splenocytes. Data expressed as means ± SEM (n = 4-6). * $P < 0.05$ vs. Vehicle (Newman-Keuls test).

3.12: Pre-treatment with β-adrenoceptor antagonist nadolol blocks the ability of MDMA to increase IL-10 production, and also blocks the suppressive effects of MDMA on LPS-induced IL-12p40 and IL-15.

The aim of this experiment was to assess the role of endogenous catecholamines in mediating MDMA-induced increase in IL-10 production while conversely suppress pro-inflammatory cytokines by examining β-adrenoceptor activity. Mice were pre-treated with a peripheral β-adrenoceptor antagonist nadolol (1.0mg/kg) 30 minutes prior to administering MDMA (20mg/kg) and LPS (100μg/kg). Mice were sacrificed 2hrs post-LPS for the IL-10 study and 8hrs for the IL-12p40 study. Circulating levels of IL-10 and IL-12p40 were measured in serum while IL-12p40 and IL-15 mRNA was assessed in spleen.

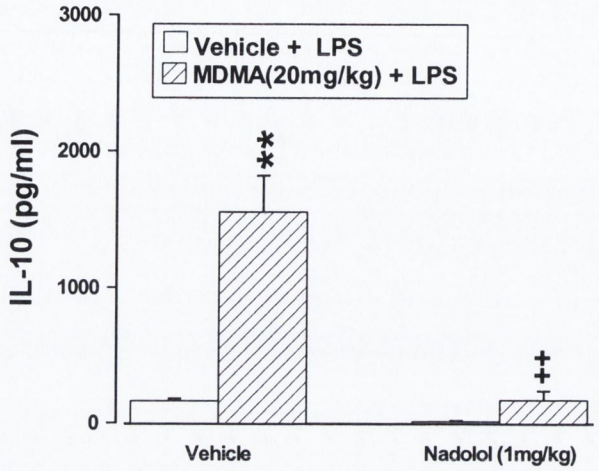
3.12.1 Serum IL-10

MDMA (10/20mg/kg) significantly increased circulating LPS-induced levels of IL-10 production. Pre-treatment with nadolol with either dose (0.3/1.0mg/kg) completely inhibited the ability of MDMA (10/20mg/kg), respectively, to increase LPS-induced IL-10 production (Figure 3.12.1). The results indicate that β-adrenoceptor activity is associated with the MDMA-induced increase in IL-10 production.

3.12.2 Serum IL-12p40 and Splenic IL-12p40 mRNA

MDMA significantly reduced LPS-induced levels of IL-12p40 production in serum and spleen. However, pre-treatment with nadolol blocked the MDMA-induced suppression of IL-12p40 production (Figure 3.12.2). MDMA decreased expression of splenic IL-12p40, but following pre-treatment with nadolol, the MDMA-induced suppression of IL-12p40 was attenuated (Figure 3.12.3). The results indicate that the immunosuppressive actions of MDMA are mediated by the β-adrenoceptor.

(a)



(b)

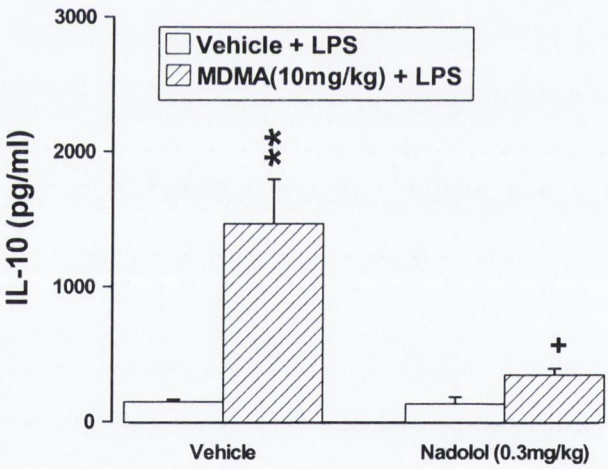
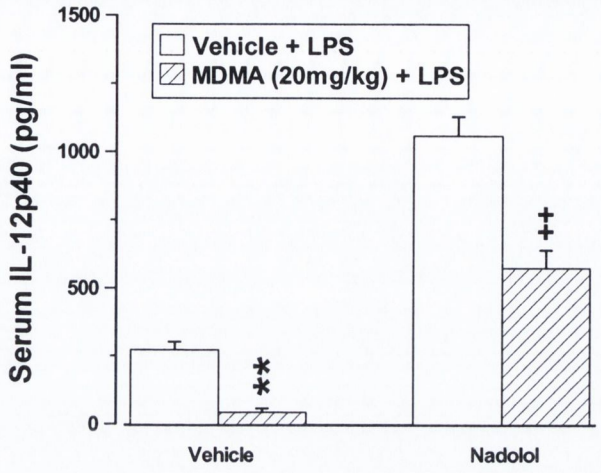


Figure 3.12.1: β-adrenoceptor blockade antagonizes MDMA-induced increase in IL-10 production following an *in vivo* LPS challenge.

ANOVA demonstrated a significant Nadolol (1.0mg/kg) x MDMA interaction for circulating levels of IL-10 concentrations (a) [$F(1,22)= 23.14$, $P<0.0001$] or (b) [$F(1,24)=10.80$, $P<0.003$] production. Data expressed as means ± SEM (n = 6-9). ** $P < 0.01$ vs. Vehicle, $^{++}$ $P < 0.01$, $^{+}$ $P < 0.05$ vs. Vehicle + MDMA (Newman-Keuls test).

(a)



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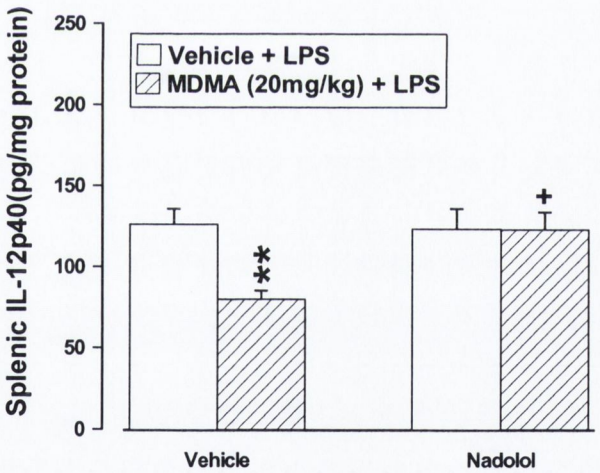


Figure 3.12.2: β-adrenoceptor blockade antagonizes MDMA-induced decrease in IL-12 production in serum and spleen following an *in vivo* LPS challenge.

ANOVA demonstrated a significant Nadolol (1.0mg/kg) x MDMA interaction for circulating levels of IL-12p40 concentrations in serum (a) [$F(1,35)= 7.01$, $P<0.01$]. There was a significant effect of MDMA on IL-12p40 production in the spleen (b) [$F(1,30)= 5.77$, $P<0.02$]. Data expressed as means ± SEM (n = 8-10). ** $P < 0.01$ vs. Vehicle, $^{+}P<0.05$ or $^{++}$ $P < 0.01$ vs. Vehicle + MDMA (Newman-Keuls test).

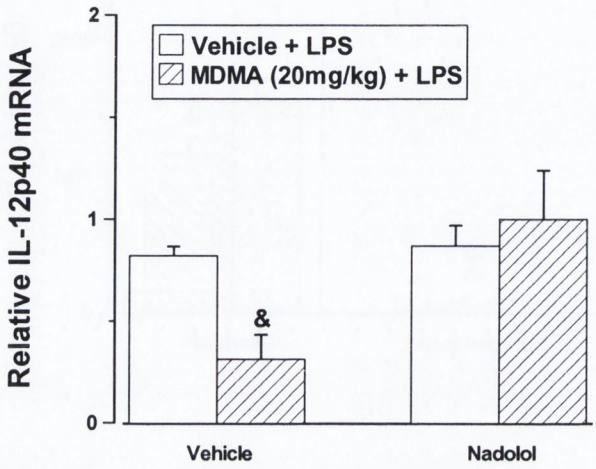


Figure: 3.12.3: Effect of β-adrenoceptor blockade on the MDMA-induced suppressive effects on IL-12p40 mRNA expression in spleen.

ANOVA demonstrated a significant interaction between MDMA and nadolol on splenic IL-12p40 mRNA [$F(1,12)=6.37$ $P<0.02$] expression. Data expressed as means ± SEM (n = 3-5). $^{\&}P < 0.05$ vs. Vehicle (Student t-test).

3.13: Pre-treatment with β-adrenoceptor antagonist nadolol blocks the suppressive effects of MDMA on LPS-induced IFN-γ production and signalling.

As nadolol blocked the suppressive effect of MDMA on IL-12 production, we examined the effect of pre-treatment with nadolol on IFN-γ production and signalling. Mice were pre-treated with nadolol (1.0mg/kg) 30 mins prior to administering MDMA (20mg/kg) and LPS (100μg/kg) and sacrificed 8hrs post drug injection. Circulating levels of IFN-γ were measured in serum, while IFN-γ mRNA was assessed in spleen. pSTAT1 expression, and IP10 and CD40 mRNA expression was examined in spleen.

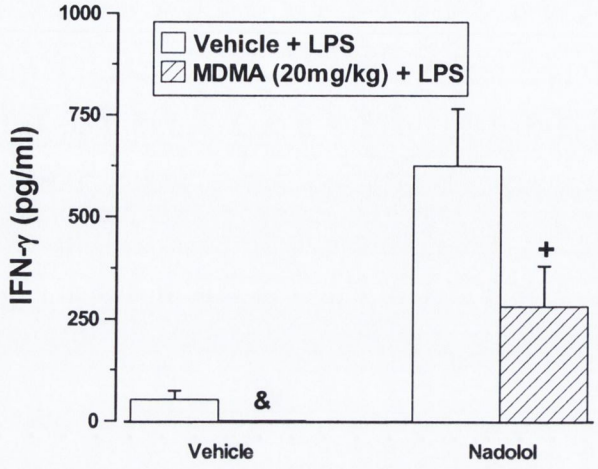
3.13.1 IFN-γ production

MDMA decreased circulating levels of IFN-γ, while pre-treatment with nadolol attenuated the MDMA-induced suppression of IFN-γ production in serum and IFN-γ mRNA expression in spleen (Figure 3.13.1). The reduced concentrations of IFN-γ production observed in serum were reflected in splenic mRNA but this was not statistically significant. However, it is clearly shown that nadolol attenuated IFN-γ mRNA expression similar to IFN-γ protein.

3.13.2 IFN-γ signalling

MDMA significantly decreased pSTAT1 expression in spleen, and nadolol pre-treatment completely blocked the ability of MDMA to suppress STAT1 phosphorylation. A sample immunoblot clearly demonstrates the MDMA-induced suppression of pSTAT1 was attenuated by nadolol (Figure 3.13.2). MDMA also reduced expression of IFN-γ inducible genes, IP10 and CD40, and this suppressive effect of MDMA was blocked by pre-treatment with nadolol (Figure 3.13.3). Results suggest a role for β-adrenoceptor activation as a potential mechanism for MDMA to down-regulate pro-inflammatory expression.

(a)



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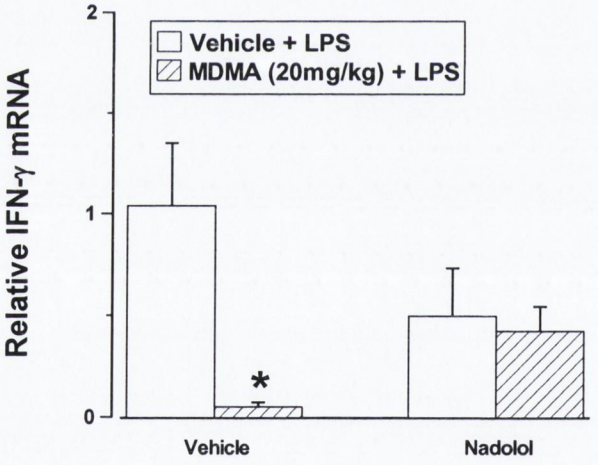
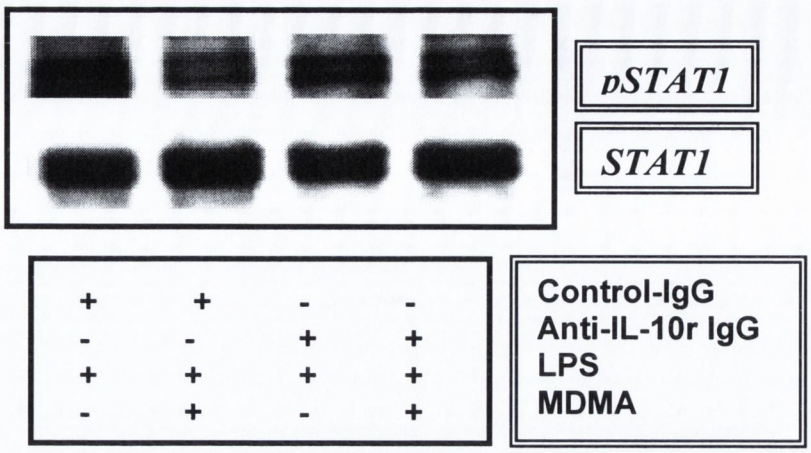


Figure: 3.13.1: β-adrenoceptor antagonism abrogates the MDMA-induced suppressive effects on IFN-γ production.

ANOVA demonstrated a significant interaction between MDMA and nadolol on circulating levels of IFN-γ [$F(1,35)=21.6$, $P<0.0001$] production and splenic IFN-γ mRNA [$F(1,12)=4.54$, $P<0.05$] expression. Data expressed as means ± SEM (n = 3-8). $^{\&}P < 0.05$ vs. Vehicle (Student t-test), $^{*}P<0.05$ vs. Vehicle and $^{+}P<0.05$ vs. Vehicle + MDMA (Newman-Keuls test).

(a)



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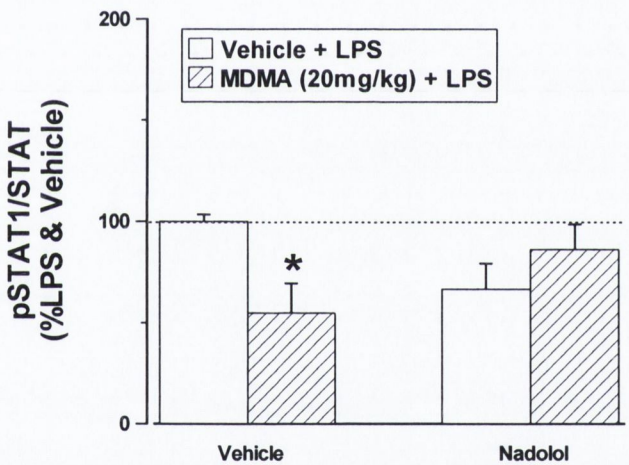
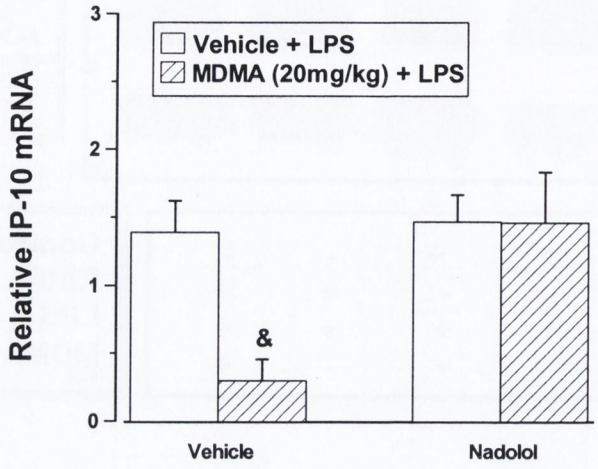


Figure 3.13.2: β-adrenoceptor blockade inhibits MDMA-induced suppression of pSTAT1 phosphorylation.

ANOVA demonstrated a significant Nadolol x MDMA interaction on STAT1 phosphorylation [$F(1,16)=8.87$, $P<0.0089$]. Sample immunoblot (n=4) (a) and densometric analysis (b) are displayed. Data expressed as means ± SEM (n= 4-6). * $P < 0.05$ vs. Vehicle (Newman-Keuls test).

(a)



(b)

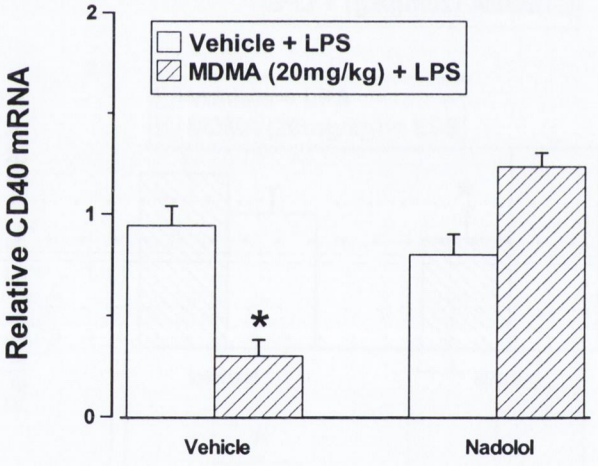


Figure 3.13.3: β-adrenoceptor blockade antagonizes MDMA-induced reduction in splenic IP10 and CD40 mRNA.

ANOVA demonstrated a significant Nadolol x MDMA interaction on mRNA expression of CD40 [$F(1,14) = 7.83$, $P < 0.01$]. Data expressed as means ± SEM (n = 4-5).

* $P < 0.05$ vs. Vehicle (Newman-Keuls test) and $^{\&}P < 0.05$ vs Vehicle (Student t-test).

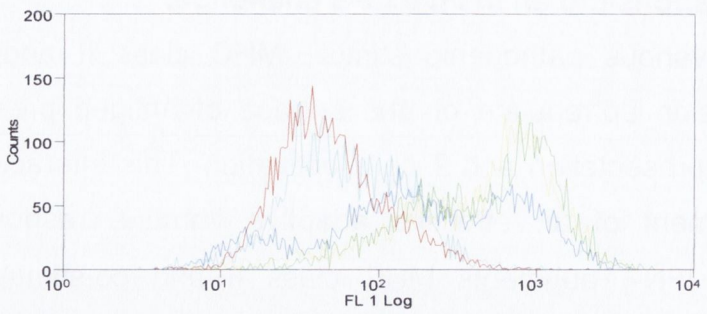
3.14: MDMA suppresses MHC class II and co-stimulatory molecule expression in response to an *in vivo* LPS challenge.

In response to various pathogenic stimuli, MHC class II and co-stimulatory molecule expression up-regulate on the surface of antigen presenting cells to facilitate antigen presentation and T-cells activation. This interaction is essential for the development of an effective adaptive immune response. It is well established that IFN-γ augments MHC class II and co-stimulatory molecule expression (Steimle *et al.*, 1994), so the aim of this study was to investigate the effect of MDMA on expression of these molecules. MDMA (5-20mg/kg) was co-administered with LPS (100μg/kg) to mice and animals were sacrificed 8hrs post drug injection and spleens dissected. The surface expression of MHC class II antigen and co-stimulatory molecules were assessed by flow cytometry.

3.14.1: MHC class II, CD40, CD86, CD80 and ICAM-1 (CD54) expression

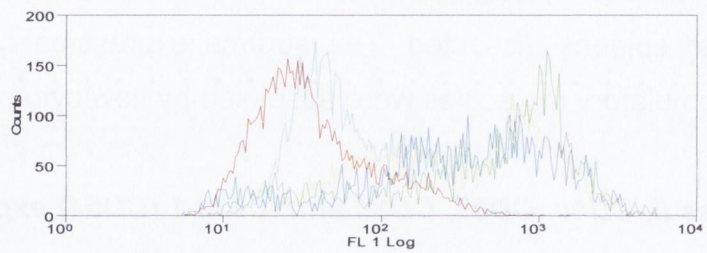
LPS administration produced a marked increase in MHC class II antigen and co-stimulatory molecule (CD40, CD54, CD80 & CD86) expression on the surface of dendritic cells and macrophages, compared to saline controls. MDMA suppressed the LPS-induced MHC class II (Figure 3.14.1), CD40 (Figure 3.14.2), CD86 (Figure 3.14.3) and ICAM-1 (Figure 3.14.4) expression on CD11c+ cells (dendritic cells) and CD11b+ cells (macrophages). However, MDMA did not alter CD80 (Figure 3.14.5) expression on dendritic cells, even though there was a reduction of CD80 on macrophages.

(a)



CD11b$^+$ MHC class II (%)

(b)



CD11c$^+$ MHC class II (%)

Treatment	CD11c-MHC II		CD11b-MHC II	
	Mean	SEM	Mean	SEM
Vehicle/Vehicle	29.3	8.2	33.65	5.17
Vehicle/LPS	61.17	8.87	62.71	6.7
MDMA 5/LPS	*25.76	8.72	52.9	3.57
MDMA 10/LPS	*25.12	8.27	32.63	11.57
MDMA 20/LPS	*20.89	5.55	*32.8	6.07

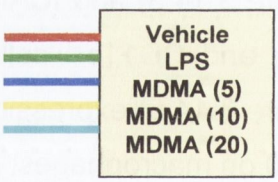
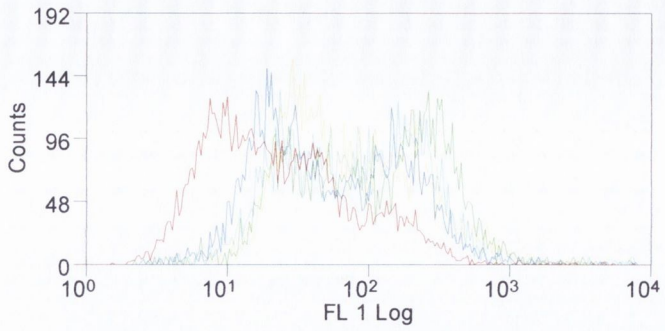


Figure 3.14.1: Dose dependent effects of MDMA on expression of MHC class II antigen on dendritic cells (CD11c+ cells) (a) and macrophages (CD11b+ cells) (b). ANOVA demonstrated a significant of MDMA on LPS induced MHC class II expression on splenic dendritic cells [$F(4,13) = 3.75$, $P < 0.03$] and macrophages [$F(4,15) = 3.82$, $P < 0.02$]. Histograms display percentage positive cells and are representative of 3-4 independent experiments.

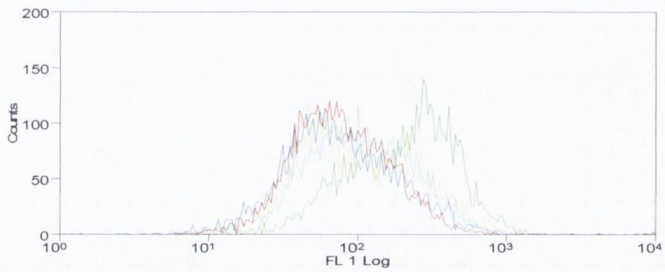
Data expressed as means ± SEM (n = 4-5). * $P < 0.05$ vs. Vehicle (Newman-Keuls test).

(a)



$CD11b^+CD40$ (%)

(b)



$CD11c^+$ CD40 (%)

Treatment	CD11c-CD40		CD11b-CD40	
	Mean	SEM	Mean	SEM
Vehicle/Vehicle	37.5	2.22	44.93	2.57
Vehicle/LPS	65.08	6.43	50.6	4.05
MDMA 5/LPS	**33.53	4.25	37.3	0.71
MDMA 10/LPS	*40.07	4.45	38.39	3.17
MDMA 20/LPS	50.6	6.5	41.86	4.45

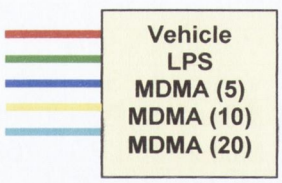
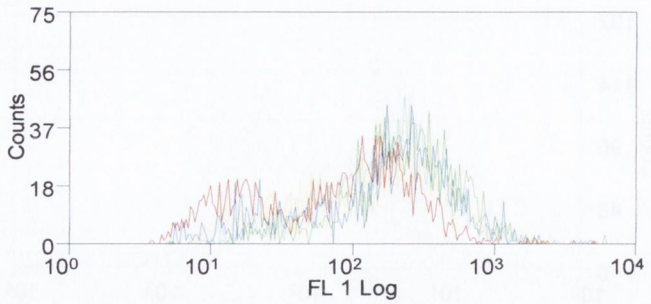


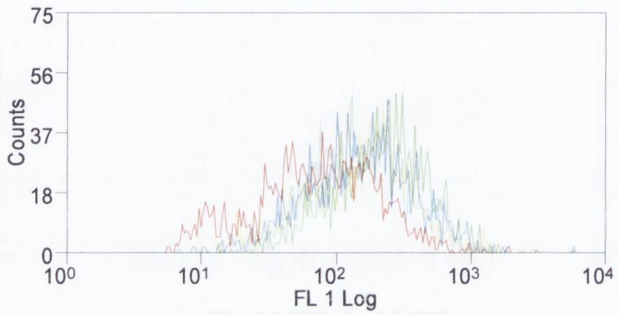
Figure 3.14.2: Dose dependent effects of MDMA on expression of the co-stimulatory molecule CD40 on dendritic cells (CD11c+) (a) and macrophages (CD11b+ cells) (b). ANOVA demonstrated a significant of MDMA on LPS induced CD40 expression on splenic dendritic cells [$F(4,12) = 6.86$, $P < 0.004$], while a reduction in CD40 expression on macrophages was observed, however it was not significant. Histograms display percentage positive cells and are representative of 3-4 independent experiments. Data expressed as means ± SEM (n = 3-4). **P < 0.01, * P < 0.05 vs. Vehicle (Newman-Keuls test).

(a)



CD11b⁺ ICAM (%)

(b)



CD11c⁺ ICAM (%)

Treatment	CD11c-ICAM		CD11b-ICAM	
	Mean	SEM	Mean	SEM
Vehicle/Vehicle	37.80	2.07	44.2	5.18
Vehicle/LPS	77.35	3.02	63.3	7.58
MDMA 5/LPS	**48.14	6.12	*33.27	4.53
MDMA 10/LPS	*55.08	4.67	*34.44	4.5
MDMA 20/LPS	63.10	7.74	*41.26	4.08

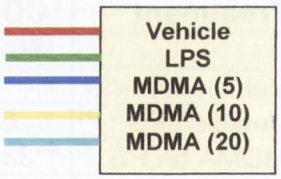
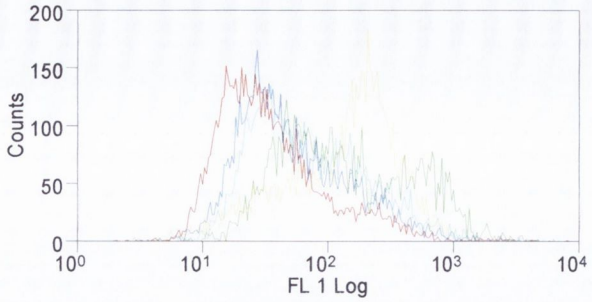


Figure 3.14.3: Dose dependent effects of MDMA on expression of the co-stimulatory molecule ICAM-1 on dendritic cells (CD11c+ cells) (a) and macrophages (CD11b+ cells) (b).

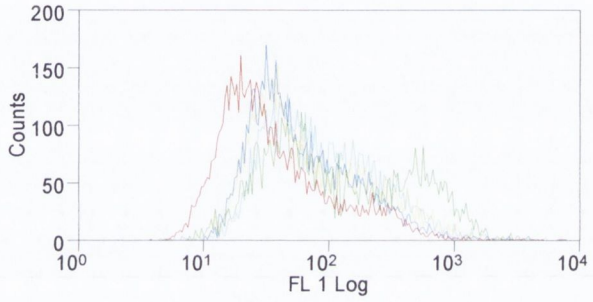
ANOVA demonstrated a significant of MDMA on LPS induced ICAM expression on splenic dendritic cells [$F(4,15) = 8.45$, $P < 0.001$] and macrophages [$F(4,12) = 4.72$, $P < 0.02$]. Histograms display percentage positive cells and are representative of 3-4 independent experiments. Data expressed as means ± SEM (n = 3-4). **P < 0.01, * P < 0.05 vs. Vehicle (Newman-Keuls test).

(a)



CD11b$^+$ CD86 (%)

(b)



CD11c$^+$ CD86 (%)

Treatment	CD11c-CD86		CD11b-CD86	
	Mean	SEM	Mean	SEM
Vehicle/Vehicle	28.35	4.17	19.87	4.04
Vehicle/LPS	65.3	5.74	54.63	11.22
MDMA 5/LPS	*41.03	10.75	36.48	3.83
MDMA 10/LPS	46.05	8.1	34.98	2.06
MDMA 20/LPS	53.4	6.67	44.85	8.37

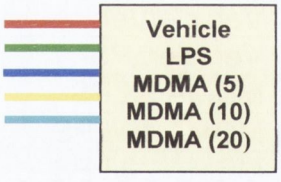
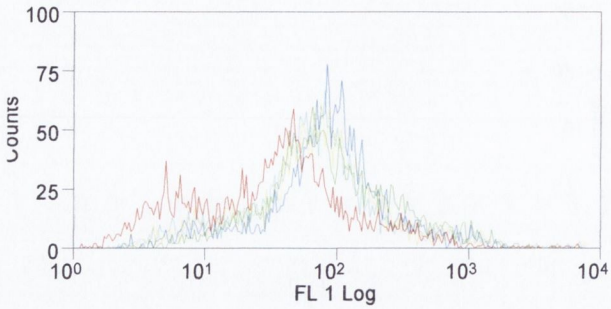


Figure 3.14.4: Dose dependent effects of MDMA on expression of the co-stimulatory molecule CD86 on dendritic cells (CD11c+ cells) (a) and macrophages (CD11b+ cells) (b).

ANOVA demonstrated a significant of MDMA on LPS induced CD86 expression on splenic dendritic cells [$F(4,12) = 4.52$, $P < 0.018$], while a reduction in CD86 expression on macrophages was observed, however it was not significant

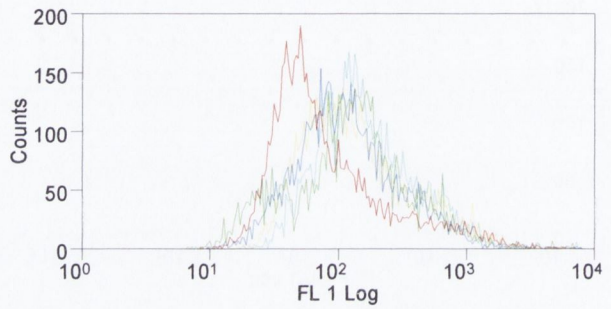
Histograms display percentage positive cells and are representative of 3-4 independent experiments. Data expressed as means ± SEM (n = 3-4). * $P < 0.05$ vs. Vehicle (Fishers Test).

(a)



CD11b$^+$ CD80 (%)

(b)



CD11c$^+$ CD80 (%)

Treatment	CD11c-CD80		CD11b-CD80	
	Mean	SEM	Mean	SEM
Vehicle/Vehicle	40.06	2.22	29.51	3.56
Vehicle/LPS	64.65	1.48	62.13	9.36
MDMA 5/LPS	60.49	1.71	52.81	7.15
MDMA 10/LPS	63.03	4.69	50.89	7.36
MDMA 20/LPS	61.22	4.94	56.24	10.78

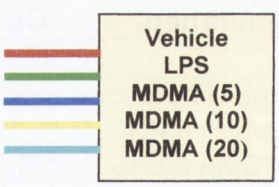


Figure 3.14.5: Dose dependent effects of MDMA on expression of the co-stimulatory molecule CD80 on dendritic cells (CD11c+ cells) and macrophages (CD11b+ cells). MDMA (5-20mg/kg) decreases LPS-induced CD80 expression on splenic macrophages, however it is not significant, but does not effect CD80 expression on dendritic cells. Histograms display percentage positive cells and are representative of 3-4 independent experiments. Data expressed as means ± SEM (n = 3-4).

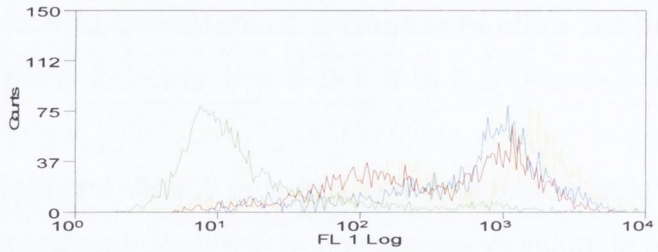
3.15: Pre-treatment with neutralising anti-IL-10 receptor antibody attenuates the ability of MDMA to suppress MHC class II and co-stimulatory molecule expression on dendritic cells in response to an *in vivo* LPS challenge.

From the previous observations that anti-IL-10 receptor inhibited the suppressive effects of MDMA on pro-inflammatory cytokine production, we examined whether neutralising IL-10 could potentially attenuate the MDMA-induced suppression of MHC class II and co-stimulatory expression, since IL-10 has been shown to down-regulate MHC class II and co-stimulatory expression (de Waal Malefyt *et al.*, 1991 and Ding *et al.*, 1993). The results suggest that MDMA induces an increase in IL-10 production and therefore enhanced levels of IL-10 inhibit the up-regulation of MHC class II and co-stimulatory molecules, which are required for an effective immune response.

3.15.1: MHC class II, CD40, CD86 and CD54 molecules

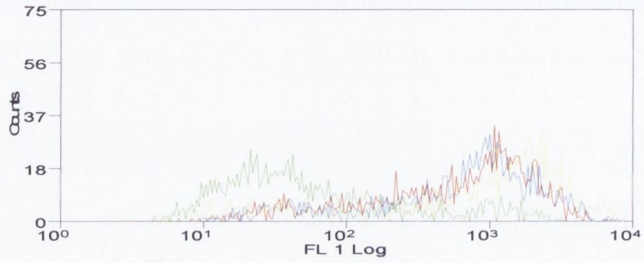
MDMA (20mg/kg) administration down-regulated MHC class II expression on dendritic cells ($CD11c^+$ cells) and macrophages ($CD11b^+$ cells) (Figure 3.15.1), and expression of the co-stimulatory molecules ICAM-1, CD86 (Figure 3.15.2) and CD40 (Figure 3.15.3) on the surface of dendritic cells. However, pre-treatment with anti-IL-10 receptor antibody attenuated the suppressive effect of MDMA on MHC class II and co-stimulatory molecule expression on antigen presenting cells.

(a)



CD11b$^+$ MHC II (%)

(b)



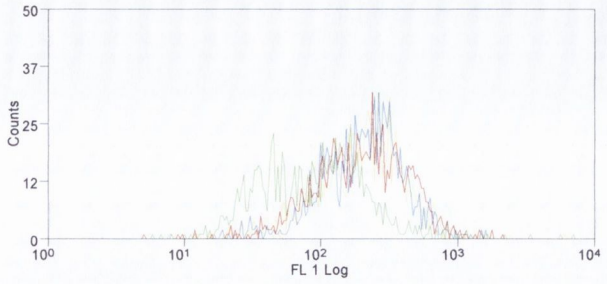
CD11c$^+$ MHC II (%)

Treatment	CD11c-MHC II		CD11b-MHC II	
	Mean	SEM	Mean	SEM
Control IgG/Vehicle	64.78	3.12	66.38	9.75
Control IgG/MDMA	**36.6	9.62	*42.83	6.98
Anti IL-10r/Vehicle	67.55	4.39	68.26	5.59
Anti IL-10r/MDMA	&&62.9	4.36	68.4	3.15

Figure 3.15.1: Pre-treatment with an anti-IL-10 receptor antibody attenuated the suppressive of MDMA on the expression of MHC class II molecules on (a) macrophages (CD11b+ cells) and (b) dendritic cells (CD11c+).

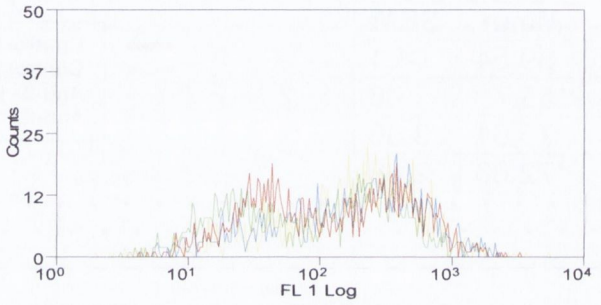
MDMA (20mg/kg) reduces expression of MHC class II molecules on macrophages and dendritic cells, and this effect was abrogated by pre-treatment with the anti-IL-10 receptor antibody. ANOVA demonstrated a significant anti-IL-10 receptor antibody x MDMA interaction on MHC II expression on dendritic cells [$F(1,10)=5.68$, $P<0.03$]. Data expressed as means ± SEM (n=3-4). Histograms display percentage positive cells and are representative of 3-4 independent experiments

(a)



CD11c$^+$ ICAM (%)

(b)



CD11c$^+$ CD86 (%)

Treatment	CD11c-ICAM		CD11c-CD86	
	Mean	SEM	Mean	SEM
Control IgG/Vehicle	91.59	1.59	71.04	4.35
Control IgG/MDMA	*65.07	10.13	**40.33	1.73
Anti IL-10r/Vehicle	93.05	4.52	78.06	6.14
Anti IL-10r/MDMA	&&90.87	2.61	&& 68.49	7.71

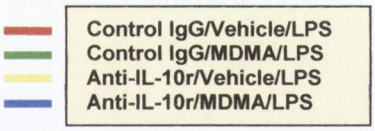
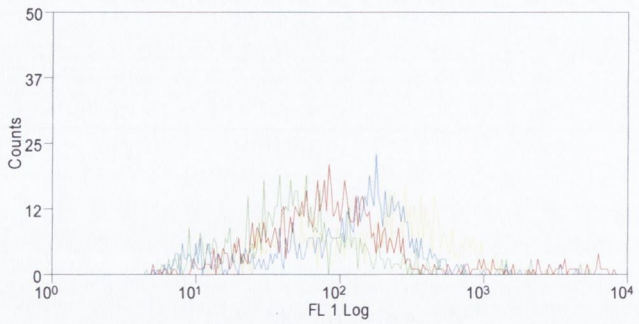


Figure 3.15.2: Pre-treatment with an anti-IL-10 receptor antibody attenuates the suppressive of MDMA on the expression of the co-stimulatory molecules (a) ICAM-1 and (b) CD86 on dendritic cells (CD11c+ cells).

MDMA reduces expression of ICAM-1 and CD86 on dendritic cells, and this effect was abrogated by pre-treatment with the anti-IL-10 receptor antibody.

ANOVA demonstrated a significant anti-IL-10 receptor antibody x MDMA interaction on CD86 [$F(1,9)=6.58$, $P<0.03$] and ICAM [$F(1,8)=5.16$, $P<0.05$] expression on dendritic cells. Data expressed as means ± SEM (n=3-4). Histograms display percentage positive cells and are representative of 3-4 independent experiments



$CD11c^+$ CD40 (%)

Treatment	CD11c-CD40	
	Mean	SEM
Control IgG/Vehicle	69.54	4.1
Control IgG/MDMA	*55.7	3.8
Anti IL-10r/Vehicle	75.61	3.66
Anti IL-10r/MDMA	&72.63	2.69

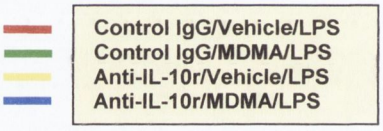


Figure 3.15.3: Pre-treatment with anti-IL-10 receptor attenuates the suppressive of MDMA on the expression of the co-stimulatory molecule CD40 on dendritic cells (CD11c+ cells).

MDMA reduces expression of CD40 on dendritic cells, and this effect was abrogated by pre-treatment with the anti-IL-10 receptor antibody.

ANOVA demonstrated a significant anti-IL-10 receptor antibody x MDMA interaction on CD40 [$F(1,10)=6.19$, $P<0.03$] expression on dendritic cells. Data expressed as means ± SEM (n=3-4). Histograms display percentage positive cells and are representative of 3-4 independent experiments

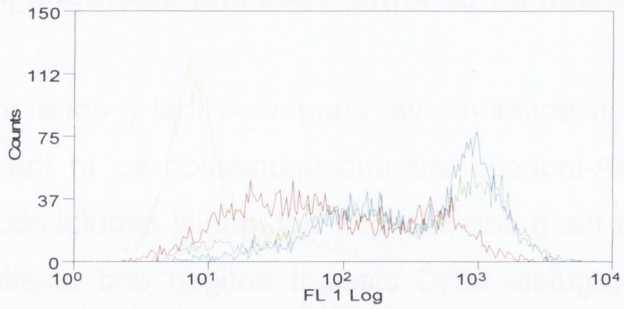
3.16: β-adrenoceptor blockade prevents the MDMA-induced suppression of MHC class II expression on dendritic cells and macrophages in response to an LPS challenge.

From our previous observations, we suggested that β-adrenoceptor activity was mediating the MDMA-induced immunosuppression so in the current study we investigated whether the β-adrenoceptor antagonist nadolol could block the ability of MDMA to down-regulate MHC class II antigen and co-stimulatory molecule expression on antigen presenting cells. Mice were pre-treated with nadolol (1.0mg/kg) 30 mins prior to administering MDMA (20mg/kg) and LPS (100μg/kg). Animals were sacrificed 8hrs post drug administration, spleens dissected and the cell surface expression of MHC class II and co-stimulatory molecules were assessed by flow cytometry. Results indicate that pre-treatment with nadolol attenuated the MDMA-induced suppression of MHC class II and co-stimulatory molecule expression on dendritic cells.

3.16.1: MHC class II expression

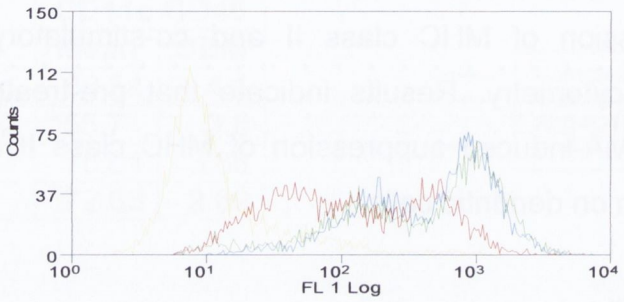
MDMA (20mg/kg) administration produced a modest decrease in MHC class II molecules on the surface of dendritic cells (CD11c+) and macrophages (CD11c+) compared to control treatment. However, pre-treatment with nadolol attenuated the MDMA-induced suppression of MHC class II molecule expression.

(a)



CD11b$^+$ MHC II (%)

(b)



CD11c$^+$ MHC II (%)

Treatment	CD11b-MHC II		CD11c-MHC II	
	Mean	SEM	Mean	SEM
Vehicle/ Vehicle	65.96	8.7	61.6	9.3
Vehicle/MDMA	40.72	17.48	48.6	20.9
Nadolol/Vehicle	36.27	8.7	31.76	11.9
Nadolol/MDMA	43.24	19.4	21.53	13.8

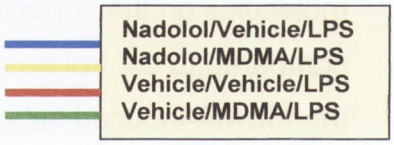


Figure 3.16.1: β-adrenoceptor blockade antagonises MDMA-induced suppression of MHC class II expression on dendritic cells (CD11c+ cells) and macrophages (CD11b+ cells).

MDMA (20mg/kg) reduced MHC class II expression on antigen presenting cells however pre-treatment with β-adrenoceptor antagonist abrogated the suppressive effects of MHC class II expressed on macrophages and dendritic cells, induced by MDMA. Histograms display percentage positive cells and are representative of 3 independent experiments. Data expressed as means ± SEM (n = 3).

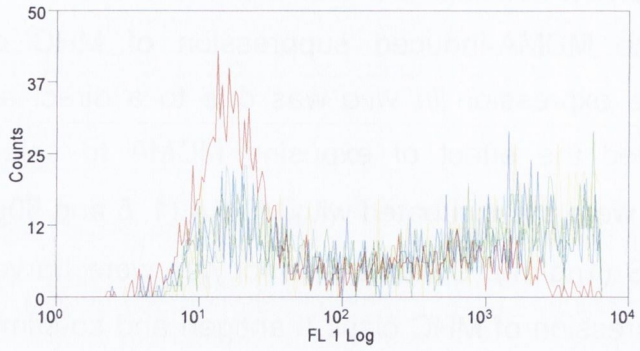
3.17: Effect of MDMA on LPS-induced MHC class II and co-stimulatory molecule expression on dendritic cells *in vitro.*

To determine if the MDMA-induced suppression of MHC class II and co-stimulatory molecule expression *in vivo* was due to a direct action on immune cells, we investigated the effect of exposing MDMA to spleen cells *in vitro.* Splenocyte cultures were pre-incubated with MDMA (1, 5 and 50μM) for 1hr before stimulating with LPS (1μg/ml). Cultured splenocytes were harvested after 72hrs, and the surface expression of MHC class II antigen and co-stimulatory molecules on dendritic cells were assessed by flow cytometry.

3.17.1: MHC class II and co-stimulatory molecule expression

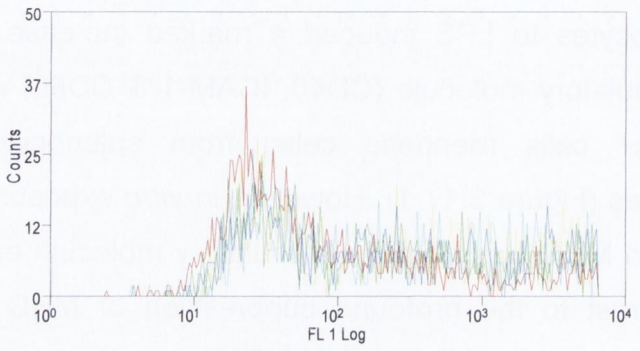
Exposure of splenocytes to LPS induced a marked increase in MHC class II antigen and co-stimulatory molecule (CD40, ICAM-1 & CD86) expression on the surface of CD11c+ cells (dendritic cells) from splenocytes compared to unstimulated samples (Figure 3.17.1). However, *in vitro* exposure to MDMA failed to alter LPS-induced MHC class II or co-stimulatory molecule expression. These results are in contrast to the profound suppression of MHC class II and co-stimulatory molecule expression induced by MDMA *in vivo*.

(a)



CD11c$^+$ MHC II (%)

(b)



CD11c$^+$ CD40 (%)

Treatment	CD11c-MHC II		CD11c-CD40	
	Mean	SEM	Mean	SEM
Vehicle/Vehicle	37.4	8.5	47.9	7.8
Vehicle/LPS	45.4	4.5	59	3.9
MDMA 5/LPS	59.1	7	59	3.5
MDMA 10/LPS	53.2	1.4	59.7	2.1
MDMA 20/LPS	56	5.2	61.8	3.3

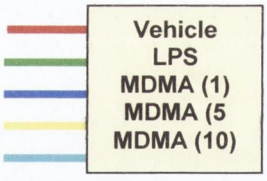
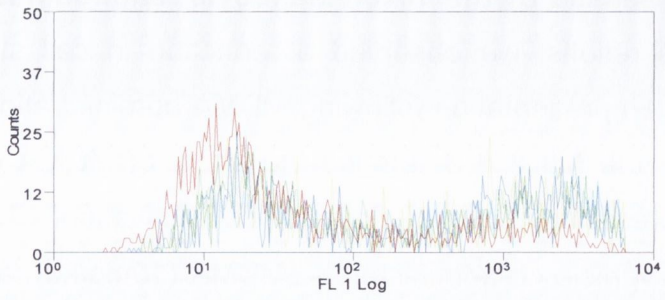


Figure 3.17.1: *In vitro* exposure of mouse splenocytes to MDMA fails to alter LPS-induced (a) MHC class II molecule or (b) CD40 expression.

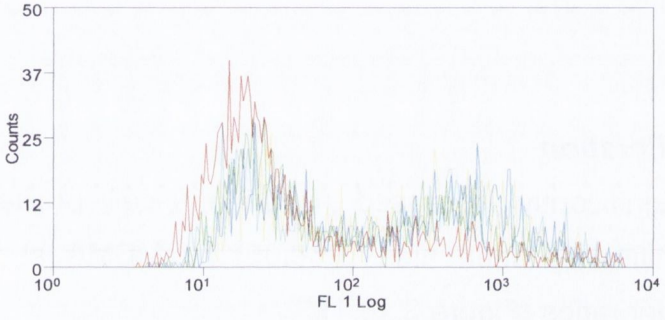
MDMA (1-50μM) did not decrease LPS-induced MHC class II or CD40 expression from splenic CD11c$^+$ cells (dendritic cells). Histograms display percentage positive cells and are representative of 3 independent experiments. Data expressed as means ± SEM (n = 3).

(a)



CD11c$^+$ CD86 (%)

(b)



CD11c$^+$ ICAM (%)

Treatment	CD11c-CD86		CD11c-ICAM	
	Mean	SEM	Mean	SEM
Vehicle/Vehicle	35.8	9.1	37.4	8.5
Vehicle/LPS	49.4	3.1	45.4	4.5
MDMA 5/LPS	53.6	2.1	59.1	7
MDMA 10/LPS	52.9	0.6	53.2	1.4
MDMA 20/LPS	53.6	3.1	56	5.2

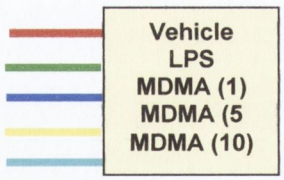


Figure 3.17.2: *In vitro* exposure of mouse splenocytes to MDMA fails to alter LPS-induced (a) CD86 or (b) ICAM expression.

MDMA (1-50μM) did not decrease LPS-induced CD86 or ICAM expression from splenic CD11c$^+$ cells (dendritic cells). Histograms display percentage positive cells and are representative of 3 independent experiments. Data expressed as means ± SEM (n = 3).

3.18: MDMA attenuates antigen presenting cell (APC) function in mouse splenocytes as assessed by the mixed leukocyte reaction (MLR).

From the previous results, we observed a reduction in cell surface molecules necessary for antigen presentation following MDMA administration. Therefore, the current study used the MLR to determine the effect of MDMA on the functional ability of antigen presenting cells to induce T-cell activation. CD1 mice received LPS (100μg/kg) or vehicle co-administered ±MDMA (10mg/kg), and animals were sacrificed 6hrs post injection. Single cell suspensions were prepared from CD1 mouse spleens, were irradiated and cultured for 72hr with responder spleen cells harvested from untreated C57BL/6 and cell proliferation assessed by uptake of tritiated thymidine.

3.18.1: T-cell proliferation

LPS challenge significantly increased ($P<0.01$) T-cell proliferation, and this increase was significantly attenuated ($P<0.05$) by MDMA at the 1:2 and 1:4 stimulator: responder ratios (Figure 3.18.1).

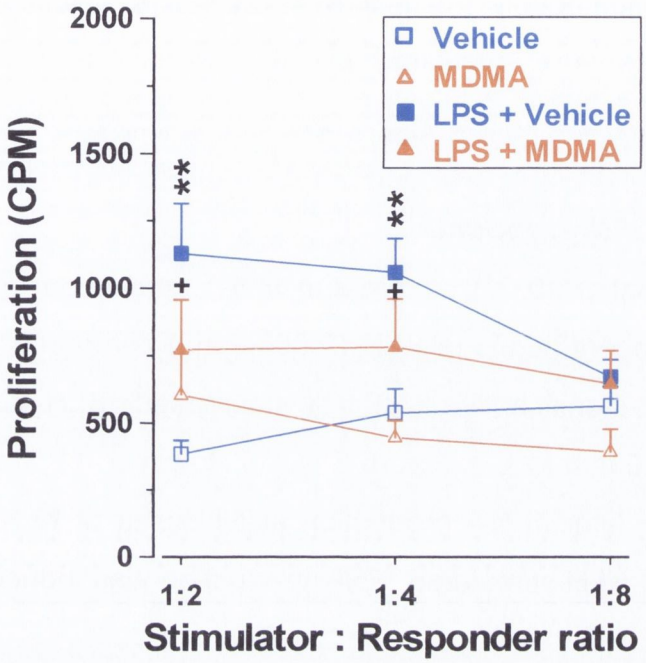


Figure 3.18.1: Effect of MDMA on T-cell activation using the Mixed Lymphocyte Reaction (MLR).

ANOVA demonstrated there was a significant effect of Stimulator: Responder ratio [$F(2,64)=13.04$, $P<0.0001$] on T-cell proliferation. Data expressed as means ± SEM (n=4-5). ** $P<0.01$ vs. Vehicle, $^{+}P < 0.05$ vs. LPS and Vehicle (Newman-Keul's test)

3.19: Effect of MDMA on antigen-specific cytokine production

The aim of the current study was to determine whether MDMA alone or when administered with LPS could alter antigen-specific immunity to the protein antigen KLH. Animals were administered with KLH (100μg/mouse), MDMA (10mg/kg) and LPS (100μg/kg) simultaneously; sacrificed 4 days post injection and spleens harvested. Splenocytes were stimulated with KLH at various concentrations and cultured for 72hrs.

3.19.1: KLH-specific IFN-γ production

There was a significant ($P<0.01$) increase in IFN-γ production from splenocytes stimulated with all concentrations of KLH (5-500μg/ml) compared to unstimulated splenocytes. MDMA significantly ($P<0.01$) decreased KLH-stimulated IFN–γ production at 50 & 500μg/ml KLH (Figure 3.19.1). However, LPS also significantly suppressed KLH-stimulated IFN-γ production at all doses of KLH. Therefore, no further suppression of KLH-stimulated IFN-γ production was induced by MDMA in LPS treated mice.

3.19.2: KLH-specific IL-5 production

Concentrations of IL-5 were significantly ($P<0.01$) increased from splenocytes incubated with 50μg/ml KLH compared to unstimulated splenocytes. KLH-stimulated IL-5 production was significantly ($P<0.01$) decreased in MDMA treated animals at KLH (50μg/ml) (Figure 3.19.2a). LPS treatment also resulted in a significant ($P<0.01$) reduction in KLH-stimulated IL-5 production at 50μg/ml dose of KLH, and no further suppression of KLH-stimulated IL-5 production was induced by MDMA in LPS treated mice.

3.19.3: KLH-specific IL-10 production

KLH-stimulated IL-10 production from splenocytes was significantly ($P<0.01$) increased at all doses of KLH used in this study. MDMA significantly ($P<0.01$) reduced KLH-stimulated IL-10 production at KLH (5-500μg/ml) (Figure 3.19.2b). LPS treatment also significantly suppressed KLH-stimulated IL-10 production at 5 & 500μg/ml ($P<0.05$) and 50μg/ml ($P<0.01$) doses of KLH. No further suppression of KLH-stimulated IL-10 was induced by MDMA in LPS treated mice.

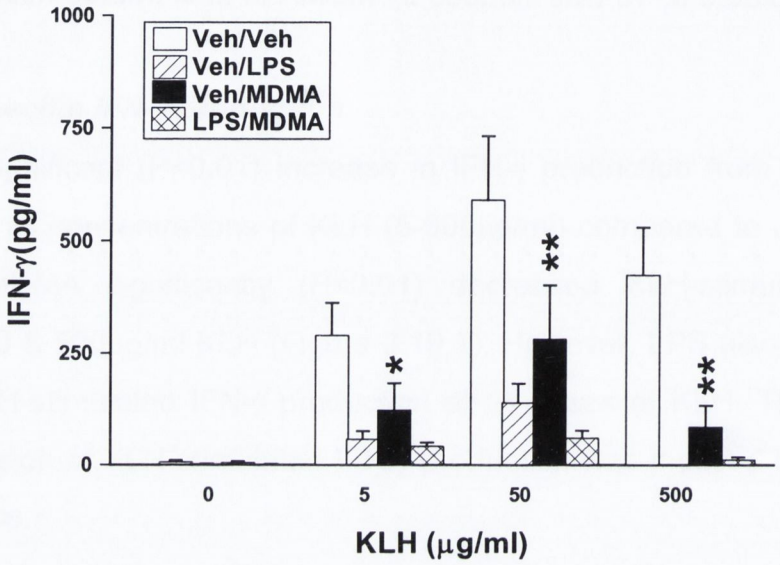
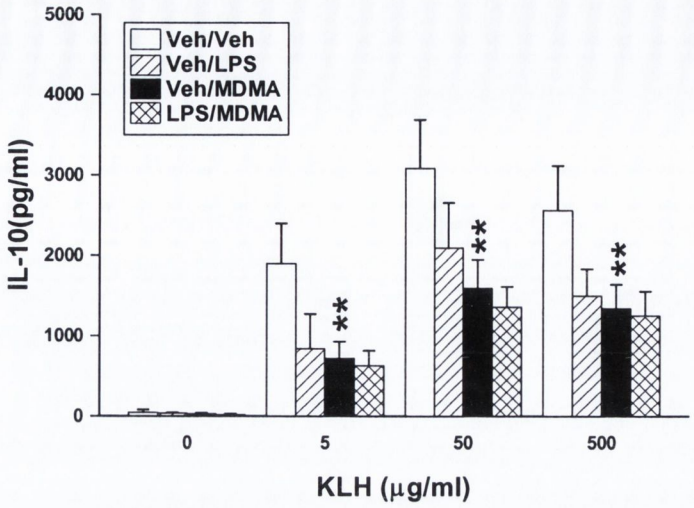


Figure 3.19.1: Effect of MDMA administration alone or in combination with LPS on KLH specific IFN-γ production in cultured splenocytes 4 days post KLH challenge. ANOVA demonstrated a significant *in vitro* KLH x MDMA treatment interaction [$F(9, 84)= 5.53$ $P<0.0001$]. Data expressed as means ± SEM (n=8).

(a)



(b)

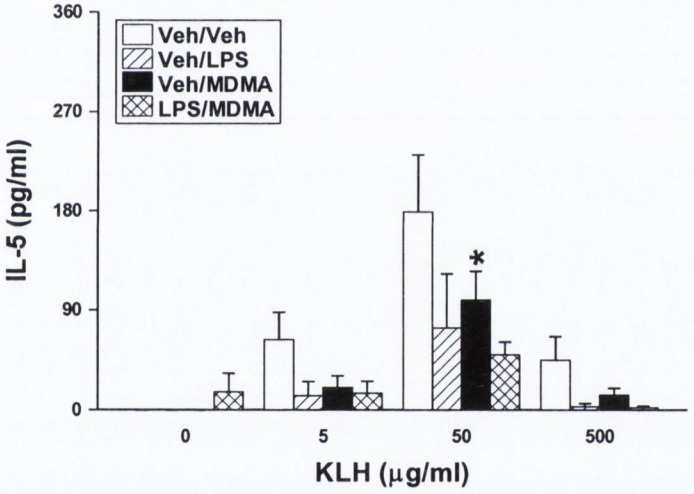


Figure 3.19.2: Effect of MDMA administration alone or in combination with LPS on KLH specific IL-5 (a) and IL-10 (b) production in cultured splenocytes 4 days post KLH challenge.

ANOVA demonstrated a significant effect of *in vitro* KLH stimulation on IL-5 [$F(3,84)=22.59$ $P<0.0001$] and IL-10 [$F(3,84)=62.98$ $P<0.0001$] production. Data expressed as means ± SEM (n=8).

3.20: Effect of pre-treatment with β-adrenoceptor antagonist on MDMA induced suppression of antigen-specific cytokine production.

In order to elucidate if β-adrenoceptor activation was involved with the suppressive effects of MDMA on antigen-specific cytokine production, animals were pre-treated with the β-adrenoceptor antagonist nadolol, and the ability of MDMA to suppress IFN-γ (Th1), IL-5 (Th2) and IL-10 (Th2/3) cytokine production was examined. Animals were pre-treated with nadolol (1.0mg/kg) 30mins prior to administering KLH (0.5mg/mouse) and MDMA (10mg/kg) simultaneously, and animals were sacrificed 4 days post injection and spleens harvested. Splenocytes were stimulated with KLH at 5μg/ml and cultured for 72hrs.

3.20.1: KLH-specific IFN-γ production

MDMA significantly reduced KLH-stimulated IFN-γ production from splenocytes, and pre-treatment with nadolol attenuated this MDMA-induced suppression of IFN-γ production (Figure 3.20.1).

3.20.2: KLH-specific IL-5 production

MDMA significantly reduced KLH-stimulated IL-5 production from splenocytes, however pre-treatment with nadolol failed to attenuate this MDMA-induced suppression of IL-5 production (Figure 3.20.2a).

3.20.3: KLH-specific IL-10 production

MDMA significantly decreased KLH-stimulated IL-10 production from splenocytes, and pre-treatment with nadolol failed to attenuate the ability of MDMA to suppress KLH-stimulated IL-10 production (Figure 3.20.2b).

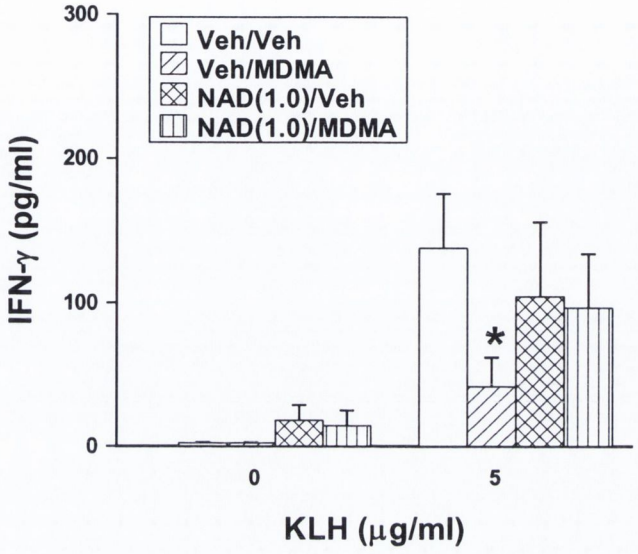
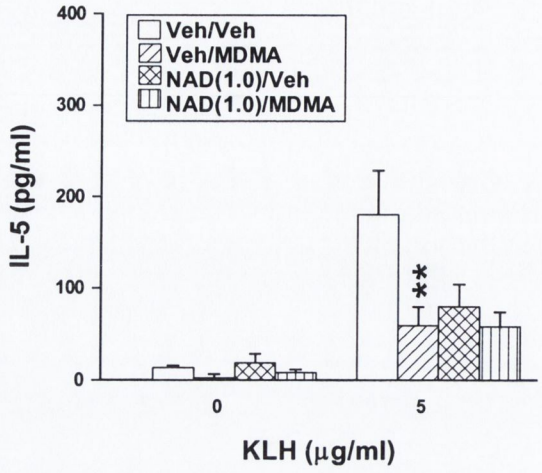


Figure 3.20.1: β-adrenoceptor antagonism blocks the ability of MDMA to suppress KLH-stimulated IFN-γ production from splenocytes.

ANOVA demonstrated a significant effect of MDMA treatment [$F(1,19)=22.65$ ($P<0.0001$)] on KLH stimulated IFN-γ production. Data expressed as means ± SEM (n=4-8).

(a)



(b)

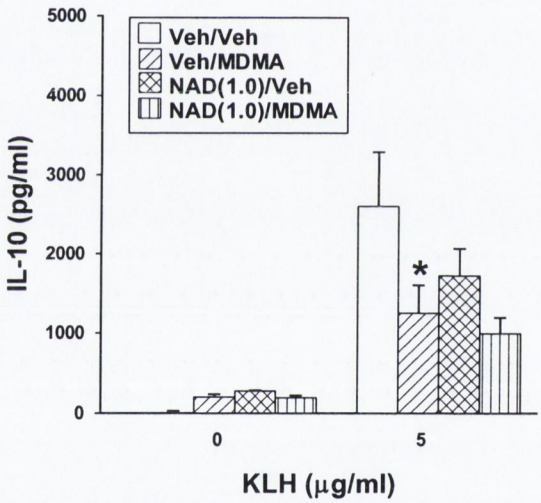


Figure 3.20.2: β-adrenoceptor blockade inhibits MDMA induced suppression of KLH-stimulated (a) IL-5 and (b) IL-10 production from splenocytes.

ANOVA demonstrated a significant effect of MDMA treatment on KLH-stimulated IL-5 [$F(1,20)=21.43$ ($P<0.0002$)] and IL-10 [$F(1,24)=36.95$ ($P<0.0001$)] production. Data expressed as means ± SEM (n=5-8).

Chapter 4: Discussion

Discussion

The studies presented in this thesis examined the immunosuppressive effects of MDMA following an LPS challenge in mice, a paradigm that essentially mimics the initial stages of a bacterial infection *in vivo*. A mouse model was used in the present investigation due to the fact that knowledge of the mouse immune system has been studied much more extensively than the rat, and consequently a more extensive repertoire of specialised reagents are available to study immunology in the mouse. Specifically, the present studies focused on the ability of MDMA to suppress the innate IFN-γ response. Innate IFN-γ production is vital at the onset of infection, and serves as an essential link between innate and adaptive immunity, any disruption or deficit of innate IFN-γ production can be perilous for the host defense (Rossouw *et al.,* 2003; Jouanguy *et al.*, 1996; Karnigo *et al.*, 1993). It is noteworthy that the dose-range of MDMA used in the present series of experiments (5-20mg/kg) is of relevance to doses of Ecstasy ingested by recreational users. For instance, reports indicate that a wide range of doses (from 1 to 10 mg/kg) are ingested by humans since the concentrations of MDMA detected in serum in rats are similar to that reported in humans (see Connor *et al.*, 2000; Schifano, 1995). Although the doses employed in many of the studies presented here are on the high end of what would be ingested by humans, it is known that smaller animals exhibit higher drug elimination rates than larger mammals, which results in drugs being eliminated from their systems more rapidly. Consequently, higher drug doses on a mg/kg basis are required in smaller animals in order to elicit equivalent pharmacodynamic effects to those observed in humans (Green *et al.*, 1995).

4.1 MDMA suppresses production of the IFN-γ inducers IL-12 and IL-15

Initially, the ability of MDMA to impact upon production of the pro-inflammatory cytokines IL-12, IL-15 and IL-18 was examined in response to an *in vivo* LPS challenge. These three cytokines are well established IFN-γ inducing factors (Kublin *et al.*, 1994; Chan *et al.*, 1991; Dinarello, 2000; Fehinger *et al.*, 1999). MDMA suppressed LPS-induced IL-12p40 concentrations in serum in a dose-dependent manner (5-20mg/kg), and similarly MDMA reduced IL-12p40 production in spleen but was only significant at 20mg/kg. These data are in line with previous *in vivo* studies where MDMA was found to suppress production other LPS-induced

pro-inflammatory cytokines, namely TNF-α and IL-1β (Connor *et al.*, 2000; Connor *et al.*, 2005). The results also demonstrate that MDMA suppressed mRNA expression for IL-12p40 in spleen, and these results are reflective of the suppressive effect of MDMA on circulating IL-12p40 protein concentration. In contrast, MDMA failed to alter LPS-induced expression of IL-12p35 mRNA. IL-12p35 is constitutively expressed in mouse spleen (Varma *et al.*, 2002), while the p40 subunit is the inducible subunit of IL-12. IL-12p40 gene expression is tightly controlled, and largely governs the biological activity of IL-12 (Trinchieri *et al.*, 2003). As IL-12 is a pivotal cytokine in inducing IFN-γ production and in driving a Th_1 cell-mediated immune response (Trinchieri *et al.*, 2000), and in host resistance to infectious disease (Trinchieri *et al.*, 1995; Magram *et al.*, 1996), the ability of MDMA to suppress IL-12 production represents a key mechanism by which it can interfere with the development of an effective immune response. In this regard, other drugs of abuse such as morphine and tetrahydrocannabinol (THC) have also been shown to suppress IL-12 production (Lu *et al.*, 2006, Martucci *et al.*, 2007; Newton *et al.*, 2004), and in the case of THC it was demonstrated that the deficiency in IL-12 production resulted in impaired IFN-γ production. In addition, a recent report suggests that the stressful experience of opiate withdrawal can also suppress production of this cytokine and the suppression of IL-12 is associated with increased sensitivity to Salmonella infection (Feng *et al*, 2005).

IL-15 and IL-18 both synergise with IL-12, to enhance IFN-γ production from NK and T-cells (Fehniger *et al.*, 1999, Ohteki, 2002). So here, the effect of MDMA on splenic IL-15 and IL-18 mRNA expression following LPS challenge was examined. The results demonstrate that MDMA suppresses expression of IL-15 mRNA in spleen in a dose-dependent manner, while conversely, splenic IL-18 mRNA was not altered. Similar to IL-12p35, IL-18 is constitutively expressed in mononuclear cells (Dinarello, 1999), and the results indicate that these genes are less sensitive to modulation by pharmacological agents than inducible genes such as IL-12p40 and IL-15. In agreement with this hypothesis, a recent study using chlorophyllin, a drug with anti-carcinogenic and anti-oxidant properties elicited a suppressive effect on IFN-γ and IL-12p40 mRNA, but failed to alter expression of the IL-12 p35 transcript or IL-18 expression in mouse splenocytes (Yun *et al.*, 2005). In the context of the results presented here where IL-18 production was unaltered by

MDMA, it is clear that IL-18 could not compensate or substitute for the reduction in IL-12 and IL-15 induced by MDMA, thereby resulting in impaired IFN-γ production.

4.2 MDMA induced suppression of IFN-γ production and signalling

Considering the ability of MDMA to suppress IL-12p40 and IL-15 production, it was not surprising to observe that MDMA also suppressed IFN-γ production in response to LPS. Specifically, circulating IFN-γ concentrations were significantly decreased at 20mg/kg of MDMA, and splenic IFN-γ mRNA expression was significantly reduced at all doses of MDMA (5-20mg/kg). Recent reports have indicated that IFN-γ production that occurs in response to LPS *in vivo* is predominantly derived from NK cells (60%) and NKT cells (20-25%), with T-cells representing a minor contributor (5%) (Varma *et al.*, 2002; Fukuda *et al.*, 2005). Therefore, as NK cells do not express TLR 4 the LPS-induced IFN-γ response is most likely to occur secondary to inducing factors such as IL-12 and IL-15 produced by accessory cells such as macrophages and dendritic cells. This would also explain why it takes some time (8hr) before the peak IFN-γ response to LPS is observed *in vivo*, whereas circulating concentrations of IL-12 and related pro-inflammatory cytokines are observed much earlier (see Fukuda *et al.*, 2005).

In order to determine if the reduction in IFN-γ production induced by MDMA resulted in impaired IFN-γ signalling, the ability of MDMA to alter LPS-induced phosphorylation of signal transducer and activator of transcription 1 (STAT1) was examined. The Janus Kinase (JAK)-STAT signalling cascade is a key pathway in converting cytokine receptor activation into changes in gene expression that control the behaviour of immune cells (Pfitzner *et al.*, 2004). IFN-γ receptor activation results in recruitment of JAK1 and activated JAK2, and these molecules phosphorylate STAT1. Upon phosphorylation, two STAT1 molecules dimerise, and this dimer translocates to the nucleus where it binds to DNA resulting in altered gene expression (Dinarello, 1999). In this study, MDMA dose-dependently suppressed LPS-induced pSTAT1 expression with significant effects observed at doses of 10 and 20mg/kg of MDMA. The JAK-STAT signalling pathway is associated with transcription of IFN-γ inducible genes such as inducible nitric oxide synthase (iNOS), the co-stimulatory molecule CD40, and the chemokine IP-10 (Narumi *et al.*, 1992; Zhang *et al.*, 2005; Luster *et al.*, 1987; Xie *et al.*, 1993; Boss

et al., 1997). In this study it was demonstrated that in tandem with suppressing LPS-induced pSTAT1 expression, MDMA also suppressed LPS-induced iNOS protein expression, and CD40 and IP-10 mRNA expression in spleen. The suppressive effect of MDMA on iNOS expression only reached statistical significance at the 20mg/kg dose of MDMA, whereas IP-10 and CD40 mRNA expression were suppressed by all three doses of MDMA, suggesting that these IFN-γ-inducible genes are more susceptible to suppression by MDMA than iNOS. It is of interest that previous studies have also demonstrated that iNOS expression is suppressed by other drugs of abuse. For instance, the opiate heroin suppresses LPS-induced iNOS mRNA expression *in vivo*, in rat spleen, liver and lungs, and induces a parallel suppression of plasma nitrite/nitrate levels (Lysle *et al.*, 2000). Similarly, evidence suggests that cannabinoids prevent gene expression of iNOS in LPS-stimulated macrophages (Jeon *et al.*, 1996). The induction of iNOS plays an important role in innate immunity (Hibbs *et al.*, 1987) due to its ability to synthesise large amounts of nitric oxide in macrophages (Palmer *et al.*, 1988; Bogdan, 2001). In this regard, iNOS induced by IFN-γ in mouse macrophages prevents the growth of ectromelia, vaccinia and HSV-1 viruses (Karupiah *et al.*, 1993). Furthermore, studies have shown that infection with bacteria such as *Salmonella enterica* stimulates a robust inflammatory response by up-regulating activity of iNOS in mice (Khan *et al.*, 2001). However, in contrast, iNOS deficient mice do not survive following infection due to proliferation of salmonella (Mastroeni *et al.*, 2000). Similarly, infection with the *Trypanosoma cruzi* parasite stimulates both innate and adaptive responses of which IFN-γ and iNOS are critical mediators. Specifically, IFN-γ R-/- mice and iNOS-/- mice are highly susceptible to *T.cruzi* infection, and the rate of fatality is enhanced as a consequence of vigorous disease progression (Holscher *et al.*, 1998). Thus the ability of MDMA to suppress iNOS expression is likely to have a significant negative impact on host defence to infectious disease.

IP-10 is a chemokine that functions as a primary mediator in attracting effector T-cells to the site of the infection (Taub et al, 1993, Loetscher et al., 1996). In support of this, it has been shown that in mice infected with *T.gondii*, *in vivo* neutralization of IP-10 results in a deficit in antigen-specific T-cells responses in the spleen (Khan *et al.*, 2000). Chemokines such as IP-10 are known to function as chemoattractants for DCs, and more specifically are involved in attracting DCs from the periphery to lymph nodes in response to an antigen challenge (Peters *et*

al., 2001; Sato *et al.*, 2000). Since IP-10 is constitutively expressed in lymph nodes and spleen, it is involved in guiding DCs loaded with antigen into lymphoid tissue. Therefore, IP-10 potentiates antigen presentation and T-cell activation, and thereby plays an important role in generating effector T-cells (Patterson *et al.*, 2000). In this regard, more recent studies report that murine bone marrow derived macrophages stimulated with IP-10 has the ability to decrease parasitic infection (*L. amazonesis*). In addition, injection of IP-10 deferred the progression of disease in susceptible mice, and this occurred subsequent to the observed increases in IFN-γ, IL-12 and NO production (Vasquez *et al.*, 2006). Moreover, several investigators have proposed that IP-10 may have important functions during tumor surveillance as studies have indicated it is involved with the production of tumor-protective CD8+ T-cell mediated responses (Pertl *et al.*, 2001; Guo *et al.*, 2004). Consequently the ability of MDMA to suppress IP-10 production is likely to have a negative impact on immune cell trafficking and ultimately on host defense to infections and/or cancer.

4.3 Effect of MDMA on LPS-induced MHC class II and co-stimulatory molecule expression on antigen presenting cells, and on an antigen specific immunity.

During an innate immune response, IFN-γ induces expression of MHC class II molecules on APC's such as dendritic cells and macrophages and thereby facilitates antigen presentation to T-cells ,augmenting proliferation and clonal expansion of a Th_1-cell mediated response (Alam, 2003). Moreover, when MHC class II molecules are not expressed on the surface of APCs, effective antigenic presentation cannot occur. Activation of STAT1 by IFN-γ induces the transcription of CIITA and hence results in the activation of MHC class II molecules (Williams, 1990; Ramana *et al.*, 2000). MHC class II molecules are transcribed following activation of CIITA which interacts with DNA binding proteins on MHC class II promoters (Loh *et al.*, 1992 and Steimle *et al.*, 1994). In fact, in humans, a disease known as bare-lymphocyte syndrome has been recognized by a deficit in CIITA. Lymphocytes from these patients are not capable of expressing MHC class II proteins even when exposed to IFN-γ (Krawczyk and Reith, 2006). Therefore considering the ability of MDMA to suppress IFN-γ production in response to LPS *in vivo*, it was not surprising to observe that MDMA (5-20mg/kg) also induced a

marked down-regulation of MHC class II expression on dendritic cells ($CD11c^+$ cells) and macrophages ($CD11b^+$ cells) in response to LPS.
Other drugs of abuse such as morphine and cannabinoids have also been found to suppress MHC class II molecule expression (Beagles *et al.*, 2004; Gongora *et al.,* 2004)

In addition to the interaction between MHC class II and the T-cell receptor, additional activation signals transmitted via the co-stimulatory molecules CD80 (B7.1), CD86 (B7.2), CD40 and CD54 (ICAM-1) are required for effective antigen presentation and T-cell activation (Fujii *et al.*, 2004). The results of the studies presented here indicate that in addition to suppressing MHC class II expression, MDMA also suppresses expression of the co-stimulatory molecules CD40, CD54 and CD86 on dendritic cells and macrophages. The suppressive effect of MDMA on co-stimulatory molecule expression was not as profound as observed for MHC class II, for instance, the mean fluorescent intensities (MFI's) for the co-stimulatory molecules remained in the third log decade, while in the case of MHC class II expression MFIs were suppressed to a lower intensity in the first and second log decade by MDMA. The ability of MDMA to suppress co-stimulatory molecule expression is a significant finding, as these molecules are essential for effective T-cell activation. For instance, when expression of the adhesion molecule ICAM-1 is down-regulated on antigen presenting cells, a dysfunctional immune response to infection results (Zuckerman *et al.*, 1998). In addition, the interaction between ICAM-1 and LFA-1 on T-cells is thought to play an important role in augmenting anti-tumor activity (Lefor *et al.*, 1998; Kikuchi *et al.*, 1999). CD40 expression on macrophages functions as an inducer of various cytokines including IL-12 (Kennedy *et al.*, 1996), and more specifically stimulates the expression of the p40 transcript (Kato *et al.*, 1996). *In vitro* studies have shown that in mixed DC and T-cell cultures, blockade of the CD40/CD40L interaction suppresses T-cell proliferation (Mc Lellan *et al.*, 1996). In addition, CD40L -/- mice are susceptible to Leishmania infection possibly due to the suppression of IL-12 production by APCs and therefore an impaired Th_1 response (Kamanaka *et al.*, 1996; Campbell *et al.*, 1996). Thus CD40 is regarded as a quintessential molecule during innate immune responses as it induces DC activation (Cella *et al.*, 1996), the up-regulation of CD86/CD80 co-stimulatory molecules (Caux *et al.*, 2004) and IL-12 production (Cella *et al.*, 1996). In this study it was demonstrated that MDMA suppressed

mRNA expression for CD40 in spleen, thus inhibition of gene transcription represents a mechanism by which MDMA can suppress CD40 cell surface expression on APC's.

In contrast to the suppressive action of MDMA on CD86, CD54 and CD40 expression, MDMA failed to alter expression of CD80 in DCs, which, in contrast, was suppressed on macrophages. Previous studies have demonstrated that abrupt withdrawal from morphine attenuated levels of B7.2 (CD86) in total spleen cells and macrophages cells but did not alter B7.1 (CD80) expression on either type of cell (Rahim *et al.*, 2003), suggesting that CD80 (B7.1) may be less susceptible to down-regulation than its sister co-stimulatory molecule CD86 (B7.2).

4.4 MDMA increases production of the anti-inflammatory cytokine IL-10: A key mediator in suppressing the innate IFN-γ response and MHC class II and co-stimulatory molecule expression

In the present investigation it was observed that MDMA elicited a rapid (within 2 hours), and dose-dependant increase in circulating concentrations of the anti-inflammatory cytokine IL-10 in response to an *in vivo* LPS challenge. Whilst a modest increase in splenic IL-10 concentrations was observed in MDMA-treated mice, it did not reach statistical significance. It is likely that MDMA enhanced splenic IL-10 production, but that any increase in splenic IL-10 spilled over into the circulation following its production. This may account for the lack of a significant increase in splenic IL-10 concentrations following MDMA treatment. The finding that MDMA increases circulating IL-10 concentrations is consistent with previous research where MDMA was found to increase IL-10 production in a rat model (Connor *et al.*, 2005).

IL-10 is an anti-inflammatory cytokine which has a broad spectrum of immunosuppressive/anti-inflammatory actions (Moore *et al.*, 2001), and consistent with a previous report (D'Andrea *et al.*, 1993) the results of the current study clearly demonstrate that IL-10 suppresses IL-12 and IFN-γ production from LPS-stimulated spleen cells. It is known that IL-10 down-regulates expression of MHC class II, and co-stimulatory molecule expression on APCs, thereby inhibiting antigen presentation (Ding *et al.*, 1993; Gerard *et al.*, 1993). In contrast, other reports suggest that IL-10 reduces MHC class II expression by interfering with the

exocytosis and recycling of the protein (Moore *et al.*, 1993; Koppelman *et al.*, 1997). In addition, it is known that IFN-γ is a potent inducer of MHC class II expression.

Considering that increased IL-10 production precedes suppression of the IFN-γ response induced by MDMA, and also considering that IL-10 potently inhibits IL-12 and IFN-γ production and expression of MHC class II and co-stimulatory molecules, it was hypothesised that IL-10 could mediate the aforementioned immunosuppressive effects of MDMA. By pre-treating mice with an anti-IL-10 receptor antibody it is demonstrated that IL-10 is a critical mediator of MDMA-induced suppression of IFN-γ production. This conclusion is largely based on the IFN-γ mRNA data in this study, as serum IFN-γ concentrations could not be detected in the control (IgG1 treated group). In this regard, it is likely that the high concentration of IgG1 antibody administered to control mice in this study interfered with the ELISA. It is suggested that the increase in IL-10 production induced by MDMA reduces IFN-γ synthesis by inhibiting production of IFN-γ-inducing factors such as IL-12 and IL-15 from macrophages/dendritic cells, as opposed to having a direct inhibitory action on IFN-γ production from NK-cells and other minor producers of innate IFN-γ such as NK/T-cells. In this regard, it was previously demonstrated that IL-10 suppresses LPS-induced IFN-γ production in tandem with reducing IL-12 production, but in contrast increases innate IFN-γ production induced by IFN–γ inducing cytokines such as IL-12/IL-18 (Shibata *et al.*, 1998). Similarly, it was observed that the inhibition of STAT1 phosphorylation, and IP-10 mRNA and CD40 mRNA expression induced by MDMA was attenuated following pre-treatment with anti-IL-10 receptor antibody. It is of interest to note that *in vitro* studies have indicated that IL-10 inhibits the ability of IFN-γ to activate STAT1 (Ito *et al.*, 1999), and to induce iNOS expression in macrophages (Crespo *et al.*, 2002; Baetz *et al.* 2004; Qasimi *et al.*, 2005) by inducing the suppressor of cytokine signalling family of proteins (SOCS-3). Similarly, studies have also shown that IL-10 down-regulates IP-10 mRNA expression, and mRNA expression for related chemokines (Kopydlowski *et al.*, 1999; Tebo *et al.*, 1998), and it has been demonstrated that the ability of IL-10 to suppress IP-10 production is due to its ability to suppress IFN-γ production as opposed to a direct effect of IFN-γ signaling *per se* (Tebo *et al.*, 1998). Thus, considering the potent suppressive effect of

MDMA on IFN-γ production, it is likely that the reduction in STAT-1 and IP-10 expression induced by MDMA is mediated via the ability of IL-10 to suppress IFN-γ production, either directly or secondary to a reduction in IL-12 production.

The fact that treatment with the anti-IL-10 antibody increased the innate IFN-γ response in its own right is most likely due to its ability to inhibit the actions of basal IL-10, and is consistent with previous reports indicating that IL-10 deficiency increases IFN-γ and IL-12 production (Varma *et al.*, 2002; Berg *et al.*, 1995).). More importantly, the pro-inflammatory effect of the anti-IL-10 antibody observed here clearly demonstrates the efficacy of our treatment regime in blocking the IL-10 receptor.

The results of the present investigation indicate that IL-10 is also a key mediator of the suppressive effects of MDMA on MHC class II and co-stimulatory molecule expression, in that the suppressive effects of MDMA could be blocked by pre-treatment with the anti-IL-10 receptor antibody. These findings are in line with previous observations demonstrating that IL-10 down–regulates expression of B7, ICAM and CD40 on antigen presenting cells (Ding *et al.*, 1993; Willems *et al*, 1994, Mottonen *et al.*, 1998). Specifically, studies have provided substantial evidence that IL-10 inhibits IFN-γ induced ICAM expression on the surface of human monocytes and this effect is prevented by addition of an anti-IL-10 antibody. In addition, it was demonstrated that IL-10 down-regulated CD40 expression in mouse DCs (Shurin *et al.*, 2002). Moreover, disrupting the interaction between CD40-CD154 and CD80/CD86-CD28 using antibodies during a mixed lymphocyte reaction with human PBMCs induced an anergic response, and this was correlated with a marked increase in IL-10 production (Van Gool *et al.*, 1999).

It is now considered that IL-10 down-regulates MHC class II molecules via an increase in cAMP production. Studies have shown IL-10 enhances cAMP increasing the activity of PKA and this in turn induces the phosphorylation of CIITA. The resulting increase in CIITA phosphorylation by PKA reduces the rate of transcription of MHC class II (Li *et al.*, 2001 and Sisk *et al.*, 2003) and similar reports have also found that the expression of MHC class II molecules is controlled at the level of transcription (Boss, 1997).

It is noteworthy that other drugs of abuse have been shown to increase IL-10 production *in vivo.* For instance, cocaine increases production of IL-10 via sigma receptors (Zhu *et al.*, 2003), an event that mediates that ability of cocaine to augment of tumour growth in a mouse model (Gardner *et al.*, 2004). Morphine has also been shown to increase IL-10 production (Martucci *et al.*, 2007), as has THC (Smith *et al.*, 2000; Zhu *et al.*, 2000;). Moreover, it was demonstrated using an *in vivo* immunoneutralization study that IL-10 is a significant contributor to the tumor-promoting actions of THC in mice (Zhu *et al.*, 2000). Therefore, based on the findings in the present investigation and on the published literature, it appears that increased IL-10 production represents a common mechanism by which a number of drugs of abuse can elicit immunosuppressive actions. Hence, consumption of MDMA with other drugs of abuse may well have a synergistic action in disrupting the normal immune response.

Overall, the present findings in mice are consistent with Pacifici *et al.*(2001b) who found that MDMA administration to humans induced a shift from a Th_1-type response (IFN-γ and IL-2 production) to a $Th_{2/3}$-type cytokine response characterized by increased IL-10, IL-4 and TGF-β production. Therefore, the studies conduced in laboratory animals appear to be a good predictor of the immunological consequences of MDMA abuse in humans.

4.5 A role for β-adrenoceptor activation in mediating MDMA-induced immunosuppression

It was previously demonstrated that the ability of MDMA to increase LPS-induced IL-10 production, and suppress LPS-induced IL-1β and TNF-α production *in vivo* could not be mimicked by *in vitro* exposure to the drug (Connor *et al.*, 2000; 2005). Similarly in this study *in vitro* exposure to MDMA did not alter LPS-induced IL-10, IL-12 or IFN-γ production. These data indicate that the ability of MDMA to induce immunosuppression is not due to a direct action on immune cells per se, and is likely to be due to the release of endogenous immunomodulatory substances. In this regard, MDMA activates the sympathetic nervous system and hypothalamic pituitary adrenal axis (Grob *et al.*, 1996; Nash *et al.*, 1988), and the end products of these pathways (catecholamines and glucocorticoids) have previously been implicated as mediators of MDMA-induced immunosuppression (see Connor,

2004). Furthermore, a large body of evidence now exists that the SNS innervates primary and secondary lymphoid organs (Felten *et al.*, 1985). For instance, Connor *et al.*, (2005) implicated β-adrenoceptor activation in mediating the increase in IL-10 induced by MDMA, although the precise source of the catecholamines involved was not determined.

Considering the evidence that IL-10 is a key mediator of the immunosuppressive actions of MDMA, and the evidence that MDMA increases IL-10 production via β-adrencoeptor activation (Connor *et al.*, 2005), in this study the ability of the β-adrenoceptor antagonist nadolol to prevent the immunosuppressive actions of MDMA was evaluated. Similar to the previous study conducted in rats (Connor *et al.*, 2005), in this study it was observed that the peripherally acting β-adrenoceptor antagonist nadolol inhibited the ability of MDMA to increase LPS-induced IL-10 production *in vivo*. These data indicate that the ability of MDMA to increase IL-10 production is also a β-adrenoceptor-mediated event in the mouse. The results presented here also demonstrate that the suppressive effect of MDMA on LPS-induced IFN-γ and IL-12 production, STAT-1 phosphorylation, IP-10 and CD40 mRNA expression and cell surface MHC class II expression was attenuated by pre-treatment with nadolol. Therefore, the present studies indicate that β-adrenoceptor activation is a key event in mediating the immunosuppressive actions of MDMA, via its ability to increase LPS-induced IL-10 production. In this regard, numerous studies have demonstrated that the immunosuppressive effects of noradrenaline are mediated by activation of β-adrenoceptors present on a range of immune cells including antigen presenting cells (Kohm and Sanders, 2001). In addition, the spleen and receives rich noradrenergic sympathetic innervation (Klein *et al.*, 1982; Felten *et al.*, 1988), and noradrenaline is released in the splenic regions, in particular the white pulp and PALS (Elenkov *et al.*, 2000) associated with antigen presentation.

Investigators have postulated that the presence of noradrenaline accelerates the transition of dendritic cell from an active, IL-12 producing state, to the exhausted IL-10 producing condition (Langenkamp *et al.*, 2000). In addition, noradrenaline has been shown to inhibit production of IL-12 from human monocytes (Elenkov *et al.*, 1996) and dendritic cells *in vitro* and *in vivo* (Panina-Bordignon *et al.*, 1997), and so implicate a role for catecholamines in the Th_1/Th_2 imbalance, since

noradrenaline potentiated the production of IL-10 from human monocytes. In line with these findings, in this study it was observed that noradrenaline suppressed LPS-induced IL-12 and IFN-γ production, however it failed to increase IL-10 production at the timepoint examined. However it is likely that noradrenaline would have increased LPS-induced IL-10 production at an earlier timepoint (24hrs approximately) based on previous published data (Maestroni, 2002).

The fact that nadolol itself increased LPS-induced IL-12 and IFÑ-γ production suggests that catecholamines play a critical tonic role in controlling inflammatory processes *in vivo.* In line with this suggestion a previous report demonstrated that β-adrenoceptor blockade augmented production of the pro-inflammatory cytokine TNF-α following LPS administration in mice (Bencsics *et al.*, 1997).

The ability of drugs of abuse to mediate immunomodulatory actions via catecholaminergic mechanisms has previously been documented. For instance, the suppressive effect of morphine on splenic lymphocytes is mediated via the β-adrenoceptors (Fecho *et al*, 1996; Fecho *et al*, 1993). Similarly, activation of the sympathetic nervous system has been shown to mediate immunosuppressive effects of other drugs of abuse such as morphine and d-amphetamine (Bencsics *et al.*, 1997; Pezzone *et al.*, 1992; Heilig *et al.*, 1993; Hall *et al.*, 1998).

4.6 Effect of MDMA antigen presentation and antigen specific immunity

Based on the flow cytometry data demonstrating reduced MHC class II following MDMA administration it was hypothesised that MDMA could down-regulate antigen-presentation and subsequent T-cell activation. To this end, the ability of splenocytes from MDMA treated mice to act as stimulator cells in the mixed lymphocyte reaction was evaluated. The MLR has served as a useful test for assessing the functional ability of antigen presentation (Steinmann *et al*, 1978; Van Voorhis *et al.*, 1983). Here it was observed that the LPS-induced MLR was abolished following MDMA administration. Similar findings have been reported, following morphine injection to C57BL/6 mice, the functional status of T lymphocytes is profoundly decreased, as indicated by the reduced mixed lymphocyte reaction using popliteal lymph nodes (Maity *et al.*, 1995).

Although MDMA promoted a shift from a Th_1 (IL-12/IFN-γ) to a Th_2/T_{reg} (IL-10) cytokine profile *in vivo* in response to LPS, a different response was observed following antigenic challenge with KLH. Specifically, MDMA induced a broad spectrum of immunosuppressive actions, resulting in suppression of the Th_1 cytokine IFN-γ, the Th_2 cytokine IL-5 and the $Th_{2/3}$ cytokine IL-10 in splenocytes from KLH immunised mice re-stimulated *ex vivo* with KLH. These data are in contrast to a previous report in rats demonstrating that MDMA selectively suppressed the Th_1 arm of the antigen-specific immune response, indicated by a selective suppression of IFN-γ and IgG_{2a} antibody production (Connor *et al.*, 2001). In this experiment treatment groups challenged with LPS and vehicle or MDMA were also included; both of these groups showed a greatly inhibited recall response to antigen compared to non-LPS treated counterparts, and this suppressive effect of LPS was not further augmented by MDMA. The suppressive effect of LPS on antigen specific immunity was an unexpected finding; in fact it was expected that LPS would act as an adjuvant and thereby enhance antigen specific immunity. Although the biological basis of this immunosuppressive effect of LPS is not clear at present, it may be that the sickness response induced by LPS may have acted as a stressor on the animals, thereby reducing T-cell activation in response to KLH. In addition, there is a report in the literature indicating that LPS can induce apoptosis in macrophages following *in vivo* administration (Ayala *et al.,* 1996) - this phenomonen requires further study.

To examine the role of β-adrenoceptor activation on the suppressive effect of MDMA on antigen-specific cytokine production, mice were pre-treated with the β-adrenoceptor antagonist nadolol prior to administering MDMA and KLH. LPS injected groups were not included due to the fact that LPS suppressed antigen-specific cytokine production in the previous study, and failed to act as an adjuvant as expected. In this study MDMA decreased antigen-specific IFN-γ, IL-5 and IL-10 production, and the suppressive effect of MDMA on IFN-γ production, but not IL-5 or IL-10 was abrogated by prior treatment with nadolol. These results are in keeping with literature indicating that increased levels of noradrenaline control $CD4^+$ T-cell function by suppressing IFN-γ and IL-2 production from Th_1-cells, however studies have indicated that there is no direct effect on noradrenaline on

Th_2 cells due to the absence of β-adrenoceptor expression on these cells (Elenkov *et al.*, 2000, Kohm *et al.*, 2000; Sanders *et al.*, 1997).

Conclusion

MDMA suppresses the innate IFN-γ response via a β-adrenoceptor mediated increase in the anti-inflammatory cytokine IL-10. Considering the critical role played by IFN-γ in host defence to infection (Biron and Brossay, 2001; Rosenzweig and Holland, 2005), and in anti-tumour immunity (Kim *et al.*, 2000; Tannenbaum and Hamilton, 2000; Ikeda *et al.*, 2002), the ability of MDMA to impair the innate IFN-γ response is a potentially serious concern for MDMA users. Our data also suggest that treatment with β-adrenoceptor antagonists could be useful strategy in combating MDMA induced immunosuppression. We also suggest that the mechanisms underlying the immunosuppressive effects of MDMA observed in this study may be of relevance to other sympathomimetic drugs of abuse such as amphetamine, methamphetamine and cocaine, all of which are known to elicit immunosuppressive effects (Nunez-Iglesias *et al.*, 1996; Pellegrino and Bayer, 1998, Yu *et al.*, 2002). Overall these findings further support the notion that MDMA has immunosuppressive properties (see Connor, 2004), and that a major target of its immunosuppressive actions is the cytokine network and antigen presentation.

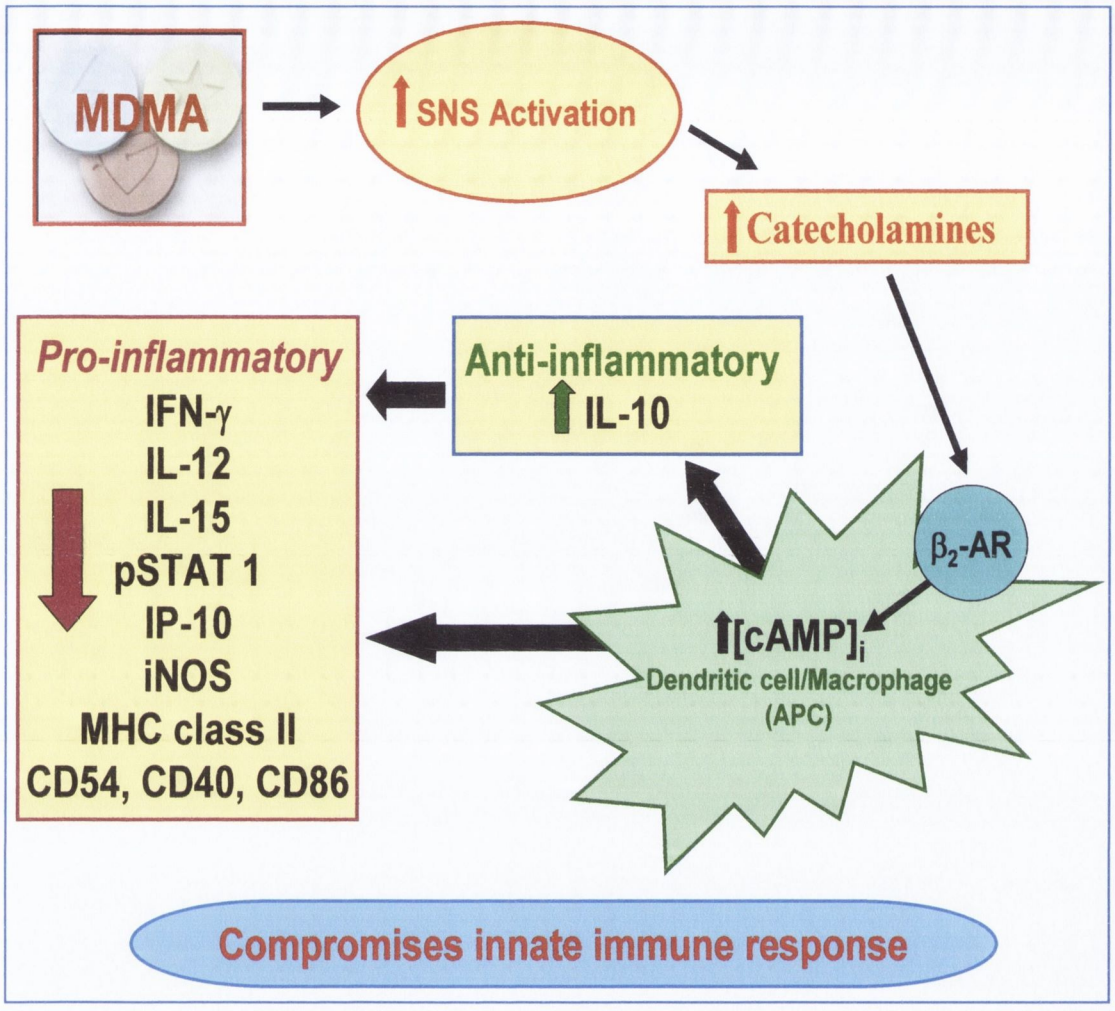


Figure 4.1: Summary of the mechanism of action of MDMA on innate immunity.

Future Directions

The research presented in this thesis has yielded a number of important leads for future research as outlined below.

1. In this thesis MDMA was always administered at the same time as the *in vivo* immunological challenge with LPS. In future studies it would be important to determine the duration of the immunosuppressive window following both a single administration of MDMA, and also following a binge dose of MDMA. Such studies would give an indication of how long the immunosuppressive actions of acute and repeated MDMA last for.

2. The results presented in this thesis demonstrate a role for β-adrenoceptor activation in mediating the immunosuppressive effects of the MDMA via increased IL-10 production. Since nadolol is a non-specific β-adrenoceptor antagonist, future studies should elucidate the contribution of β_1 vs. β_2 adrenoceptors in the immunosuppressive actions of MDMA. This could be achieved by pre-treating animals with selective β_1 or β_2 adrenoceptor antagonists prior to MDMA administration.

3. Since previous studies failed to identify the source of catecholamines that mediates the β-adrenoceptor-induced increase in IL-10 (Connor et al., 2005). The possibility that metabolism of MDMA could yield agents that have activity at β-adrenoceptors was raised. For instance, a major metabolite of MDMA is α-methyldopamine (see Monks et al., 2004), and evidence suggests that this compound can be transformed into a β-adrenergic effector following metabolism by monoamine oxidase (Langeneckert & Palm, 1968). Consequently it would be of great interest to determine if α-methyldopamine could mimic the immunosuppressive actions of MDMA via an action on β-adrenoceptors.

4. A pressing future aim of this research is to determine if the immunosuppressive effects of MDMA observed in this thesis and in previous studies alters disease susceptibility in a controlled setting. In this regard it will be important to evaluate

the impact of acute and repeated administration of MDMA on host resistance to bacterial and/or viral infections in animal models. Similarly, the impact of MDMA administration on tumor progression in a mouse tumor model should also be evaluated.

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IX Publications and presentations

N.T. Boyle, T.J. Connor (2006). MDMA ("Ecstasy") suppresses the innate IFN-γ response, and inhibits MHC class II and co-stimulatory molecule expression on antigen presenting cells: A role for IL-10?
Psychoneuroimmunology Research Society meeting, Miami, USA, May 2006.
Brain Behav. Immun. (In press).

N.T. Boyle, N.M. Curtin, T.J. Connor (2006). Pro-inflammatory actions of β-adrenoceptor antagonism *in vivo:* Enhancement of innate IFN-γ production and MHC class II and co-stimulatory molecule expression.
Psychoneuroimmunology Research Society meeting, Miami, USA, May 2006.
Brain Behav. Immun. (In press).

N.T. Boyle, T.J. Connor (2005) MDMA ("Ecstasy") suppresses innate IFN-γ production and signaling, and inhibits MHC class II expression on antigen presenting cells.
Irish Society of Immunology meeting, Dublin, October 2005.

N.T. Boyle, T.J. Connor (2005) MDMA ("Ecstasy") promotes a shift from a Th_1 to a $Th_{2/3}$ cytokine phenotype *in vivo.*
Royal Academy of Medicine in Ireland meeting, Galway, June 2005.
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N.T. Boyle, S.M. Todryk, T.J. Connor (2005) Methylenedioxymethamphetamine (MDMA; "Ecstasy") suppresses interleukin (IL)-12 and increases IL-10 production *in vivo*, and results in reduced stimulator capacity in the mixed lymphocyte reaction
Royal Academy of Medicine in Ireland meeting, Dublin, January 2005.
Ir. J. Med. Sci. (2005) 174 (1, e2): 23.

N.T. Boyle, S.M. Todryk, T.J. Connor (2004) Methylenedioxymethamphetamine (MDMA; "Ecstasy") suppresses interleukin (IL)-12 and increases IL-10 production *in vivo*, and results in reduced stimulator capacity in the mixed lymphocyte reaction
Irish Society of Immunology meeting, NUI, Maynooth, September 2004.