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Mechanisms Underlying Stress-Induced Changes in the Cytokine Network: Implications for Cancer Progression

by

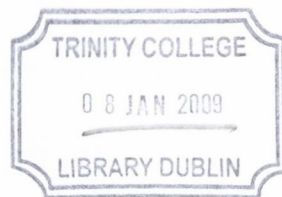
Niamh Curtin



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

Thesis submitted November 2008

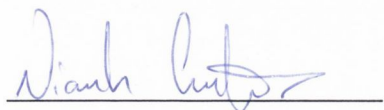
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II Abstract

Numerous studies have reported that exposure to psychological stress suppresses various aspects of the innate and adaptive immune responses, including impairment of the cytokine network. In agreement with these reports, in this thesis it was observed that exposure to acute and chronic restraint stress altered cytokine production following an *in vivo* LPS challenge in mice. The innate IFN- γ response was impaired, with reduced IFN- γ production, signaling, expression of IFN- γ inducible genes and the expression of IFN- γ inducing proteins IL-12 and IL-15. In addition, the anti-inflammatory cytokines IL-10 and the IL-10 family member, IL-19, were augmented following exposure to acute and chronic stress. It has been suggested that the stress-induced increase in IL-10 may mediate the stress-induced suppression of the innate IFN- γ response, due to its ability to inhibit the production of pro-inflammatory cytokines. However, the findings from this thesis indicate that stress-induced inhibition of the innate IFN- γ response occurs independent of IL-10 production. To further understand the mechanisms behind stress-induced alteration of the cytokine network, the contribution of the stress hormones were investigated. Glucocorticoids and catecholamines are released following stress-induced activation of the HPA and SAM axis and they can directly alter immune function via specific receptors found on immune cells. The findings from this thesis suggest that glucocorticoids and catecholamines play distinct, but parallel roles in the stress-induced alteration of the cytokine network. Glucocorticoids, acting via GR, contribute to stress-induced suppression of the innate IFN- γ response, however, catecholamines, acting via β_2 -AR, mediate stress-induced augmentation of IL-10 and IL-19. The meaningful consequences of stress-induced alteration of the cytokine network must be established to understand the implications for disease onset and progression. In this regard, the findings from this thesis indicate that exposure to chronic stress accelerates progression of a sub-cutaneous tumor in mice and alters the cytokine network which could suppress immunity in the tumor microenvironment. In contrast to the established immunosuppressive actions of chronic stress, some studies have indicated that an immuno-enhanced state can be induced under certain circumstances. In this thesis it has been demonstrated that an altered immune state becomes evident in the spleens of mice 24 hr following termination of chronic stress with increased IFN- γ and IL-12 production and decreased glucocorticoid sensitivity. Further investigation of this altered immune state needs to be carried out to understand the factors that contribute to this contradictory, immuno-enhanced state.

III Acknowledgements

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VII Abbreviations

Adr	Adrenaline
AIDS	Acquired immunodeficiency syndrome
ANS	Autonomic nervous system
AP-1	Activating protein
AR	Adrenoceptor
APC	Antigen presenting cell
AVP	Arginine-vasopressin
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BMDC	Bone marrow derived dendritic cell
CA	Catecholamine
CAMP	3'5'-cyclic adenosine monophosphate
CD	Cluster designation
CDZ	Chlordiazepoxide
CRH	Corticotrophin releasing hormone
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
FCS	Fetal calf serum
FoxP3	Forkhead box P3
GAS	Gamma-activated sequence elements
GC	Glucocorticoid
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
H ₂ O ₂	Hydrogen peroxide
Hr	Hour
HIV	Human immunodeficiency virus
HPA	Hypothalamic pituitary adrenal axis
HSV	Herpes simplex virus
HZO	Herpes zoster ophthalmicus
I κ B	Inhibitor of κ B
ICAM	Intracellular cell adhesion molecule

IFN- γ	Interferon gamma
IFNGR	Interferon gamma receptor
IgG	Immunoglobulin G
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
LC	Locus coeruleus
LFA	Lymphocyte function antigen-1
LPS	Lipopolysaccharide
Mal	MyD88 adapter like protein
MDA-7	Melanoma differentiation-associated gene-7
MDMA	3,4-Methyldioxymethamphetamine
MHC class II	Major histocompatibility complex class II
Min	Minute
MyD88	Myeloid differentiation factor 88
NA	Noradrenaline
NF κ B	Nuclear factor- κ B
NK	Natural Killer
NKT	Natural Killer T cells
NO	Nitric oxide
PKA	Protein kinase A
PNI	Psychoneuroimmunology
PVN	Paraventricular nuclei
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SAM	Sympathetic adrenal medullary
SERT	Serotonin transporter
SH2	Src-homology 2
SNS	Sympathetic nervous system

STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
TGF- β	Transforming growth factor-beta
T _H	Helper T-cell
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
T _{Reg}	Regulatory T-cell
TyK	Tyrosine Kinase

Chapter 1: Introduction

1.1 Brief Introduction to the Immune System

Our immune system is an extremely complex network of organs and cells that defend our bodies from foreign substances. It protects by immuno-surveillance, detection of foreign or non-host cells, and induction of appropriate responses to eradicate hazardous infectious agents. It also plays an important anti-neoplastic role where it detects and eradicates transformed or cancerous cells from the body. The immune system has been described as a 'sensory organ' due to its 'molecular machinery' that effectively and efficiently detect infectious agents or transformed cells as soon as they enter the body (Blalock, 1984). The immune response to an infectious or damaging stimulus is very fast due to the diffuse nature of the immune network which is spread throughout the body in immune organs and incorporates the vast numbers and types of immune cells or lymphocytes in the circulation.

1.2 Organs of the Immune System

The organs of the immune system are collectively referred to as the lymphoid system and they are the areas in which lymphocyte maturation, differentiation and proliferation take place. These organs can be divided into two further categories: primary and secondary lymphoid organs.

1.2.1 Primary Lymphoid Organs: Thymus and Bone Marrow

Within the primary lymphoid organs, immature lymphocytes mature and become committed to a particular antigenic specificity. There are two primary lymphoid organs responsible for the maturation of lymphocytes: the bone marrow and the thymus. Bone marrow is found in all major bones of the body and it contains pluripotent hematopoietic stem cells which are the common ancestral cells for all mature blood cells. In the bone marrow almost all of the cells of the innate immune system mature; monocytes, neutrophils, Eosinophils and basophils. Lymphoid progenitor cells give rise to T or B progenitor cells, however the differentiation and maturation of T-cells and B-cells takes place in different locations. B-cells mature in the bone marrow, and when they leave they express a unique antigen-binding receptor on their membranes. These mature B-cells circulate in the blood and reside in the lymphoid organs. In mammals, T-cells mature in the thymus. The thymus gland is a flat, bilobed organ

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situated above the heart and it facilitates the differentiation of progenitor cells into T lymphocytes (T-cells).

1.2.3 Secondary Lymph Organs: Spleen and lymph nodes

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-cells. In the secondary lymph organs, immune cells capture antigens and present the antigen when it is combined with major histocompatibility complex (MHC) class II molecules to T_H-cells during antigen presentation. Once activated, these T_H-cells can activate B-cells which rapidly divide and drive a specific immune response. The lymph nodes and the spleen are the most highly organised of the secondary lymphoid organs.

The lymph nodes are encapsulated, bean-shaped structures containing a network of lymphocytes, macrophages and DCs. Clusters of nodes are found at junctions of the lymphatic vessels around the body: neck, axillae, groin, mediastinum and abdominal cavity. They are the first organised lymphoid structure to encounter antigens that enter tissue spaces and they provide an ideal environment for lymphocytes to efficiently encounter and respond to trapped antigens. The spleen is a large, ovoid secondary lymphoid organ situated in the left abdominal cavity. It is specialised in filtering blood and trapping blood-borne antigens and therefore it is able to respond to systemic infections. It is highly efficient in trapping and concentrating foreign substances carried in the blood, and is the primary site where antibodies are synthesized and released into circulation. More circulating lymphocytes pass daily through the spleen than through all the lymph nodes combined. The structure of the spleen allows the immune system to effectively assess the blood as it is filtered and passed through the spleen and allows the efficient removal of older erythrocytes from the circulation and leads to the efficient removal of blood-borne microorganisms and cellular debris (Mebius and Kraal, 2005). The two arms of the immune system, the innate and the adaptive responses, can be simultaneously activated in the spleen, thus it is an essential organ for maintaining immune homeostasis.

1.3 Innate (Non-specific) Immunity

The activities of the immune system can be broadly categorised into two phases; the innate immune response and the adaptive immune response. The innate immune response provides immediate defense against infection and the cells of the innate system recognise and respond to pathogens in a generic way. However unlike the adaptive immune response it does not confer long-lasting or protective immunity to the host. Initially, when a host comes in to 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 defence (Dempsey *et al.*, 2003). It comprises of three types of defensive barriers: anatomic, physiologic and inflammatory. The anatomic barriers are physical barriers that prevent the entry of pathogens into an organism and are the first line of defence against infection. The skin and mucosal membrane surfaces are two types of physical barriers and they are effective at preventing the entry of most micro-organisms. The physiological barriers that contribute to innate immunity include temperature, pH and various soluble factors, such as histamine, bradykinin and prostaglandins. These prevent infections by different mechanisms, for instance normal body temperature and fever inhibit the growth of some pathogens.

An important part of the innate response to infection is the inflammatory response. This complex sequence of events is initiated following tissue damage caused by a wound or invasion of a foreign substance. Inflammation is stimulated by the chemical factors released by injured cells which sensitise pain receptors, cause vasodilation of the blood vessels at the scene of injury and attract innate immune cells. The innate immune cells that are initially attracted to the site of infection are the neutrophils and phagocytes. However the innate immune response encompasses many other cells of the immune system which provide immediate host defence, such as Eosinophils, monocytes, macrophages, complement, cytokines, and acute phase proteins. An important part of the innate immune response is the phagocytosis or ingestion of foreign particulate material or whole pathogenic microorganisms. The majority of phagocytosis is conducted by specialised immune cells, such as blood monocytes, neutrophils and tissue macrophages.

1.3.1 Innate Immune Cells:

1.3.1.1 Neutrophils

A central feature of the innate immune reaction is recruitment and activation of neutrophils to the site of infection to eradicate pathogens, including bacteria, fungi, protozoa, viruses, virally infected cells and tumor cells (Witko-Sarsat, 2000). These cells are usually the first to arrive at the site of inflammation as they represent 50-60% of the total circulating leukocytes. Neutrophils develop in the bone marrow, which takes about two weeks, and neutrophil concentrations increase in the bone marrow during acute inflammation. Upon release from the bone marrow to the circulation the cells are in a nonactivated state and circulate freely in the blood, or migrate along the vascular endothelium. Therefore, to migrate to a site of infection, neutrophils use a multistep process involving proinflammatory mediators, adhesion molecules, chemoattractants, and chemokines. Like macrophages, neutrophils are active phagocytic cells and upon arrival at the site of infection they engulf and destroy antigens. However, neutrophils also have a huge arsenal of antibiotic proteins stored in granules within their membrane. The primary and secondary granules are lysosomes containing lytic enzymes and bactericidal substances. Respiratory burst activity occurs within the granules and is an oxygen dependent mechanism involving nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This results in the formation of reactive oxygen species (ROS) such as hydrogen peroxide and hydroxyl radicals that are extremely toxic to infectious agents (Ward and Lentsch, 1999).

1.3.1.2 Monocytes / Macrophages / Dendritic cells

Monocytes are produced by the bone marrow from haematopoietic stem cells and they have two main functions in the innate immune response: (1) to replenish resident macrophages and dendritic cells (DCs) under normal states, and (2) in response to inflammation signals, monocytes can move quickly (8 – 12 hr) to sites of infection in tissues and divide / differentiate into macrophages and DCs to elicit an immune response. The function of macrophages is to engulf, internalise and destroy foreign particles. They remove particulate antigens and process and present antigenic peptides to T-cells. The monocytes circulate in an inactive state and when they encounter an antigen, they become activated and can internalise the antigen by phagocytosis or receptor-mediated endocytosis and degrade it through lysosomal proteolysis.

Phagocytosis of an antigen serves as an initial activating stimulus. However, macrophage activity can be further enhanced by cytokines secreted by other immune cells, by mediators of the inflammatory response and by components of the bacterial cell wall. Macrophages have also been shown to secrete pro- and anti-inflammatory mediators such as hydrolytic enzymes, complement components, oxygen metabolites, nitric oxide, nucleosides and cytokines that act as the 'red alert' of infection (Adrem, 2001). Activated macrophages express higher levels of MHC class II molecules which allow them to function more effectively as antigen presenting cells (APCs).

DCs are the most efficient professional antigen presenting cells (APCs) involved in the immune response to an infectious stimulus (Steinman *et al.*, 1978). They are particularly important for the activation of naïve T-cells and therefore are responsible for the type of adaptive immune response that is mediated from the innate immune response (Ohteki, 2007). DCs in different locations have different forms and functions. Immature DCs are located in the periphery where they capture antigens. They express high levels of MHC class II molecules and for this reason, are more potent APCs than macrophages and B-cells. Stimulation of DCs by pathogens triggers DC maturity, after which they migrate to lymphoid organs and prime naïve T-cells and initiate the immune response (Mellmann *et al.*, 1998). During this process, DCs lose antibody capturing/processing ability as they differentiate into mature, fully stimulatory APCs.

1.3.1.3 Natural Killer Cells

Natural Killer (NK) cells are large, granular lymphocytes that mediate certain functions overlapping with those delivered by T-cells. However, they do not bear a specific antigen receptor or CD3 complex (Trinchieri, 1989). Therefore, they respond as innate immune cells. NK cells have been shown to play important protective roles early in host defence against tumor cells and virally infected cells (Leung and Ada, 1981). They respond within 2-3 days of infection to kill virus-infected cells and to produce cytokines that will begin to initiate and enhance subsequent, specific anti-viral immune responses (Biron *et al.*, 1999).

NK cells recognise abnormal cells in two different ways. Firstly, they can use NK cell receptors to distinguish abnormalities, such as a reduction of MHC class I molecules or

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the unusual profile of surface antigens displayed by tumor cells. NK cells can also recognise potential targets by detecting antiviral or antitumor antibodies bound to the surface of tumor cells or cells infected with viruses (Moretta *et al.*, 1996). In addition, NK cell lytic activity is induced in response to early endogenously expressed Interferon (IFN) α / β which mediate elevated lysis of sensitive cells (Grilund *et al.*, 1978). When NK cells recognise a target, they display intrinsic cytotoxic activity and can lyse the target (Tosi, 2001). This is achieved by secretion of perforins onto the surface of the cell to which the NK cell has adhered. Perforins make holes in the cell membrane and granzymes are injected through the pores and cause apoptosis in the target (Trapani and Smyth, 2002).

Activated NK cells produce various cytokines, such as IFN- γ , tumor necrosis factor (TNF)- α and granulocyte macrophage colony stimulation factor (GM-CSF) which further enhance their cytotoxic effects and can modulate both innate and acquired immune responses (Lauwerys *et al.*, 2000). NK cells are the primary source of IFN- γ which plays critical roles in the host's response to an infectious stimulus. In turn, the proliferation and activation of NK cells are controlled by other cytokines, such as interleukin (IL) -12 and IL-18. The vital NK-activating activity of these cytokines was observed in mice deficient in IL-12 and IL-18 as they displayed impaired NK activity (Takeda *et al.*, 1998).

Investigations into activating factors of NK cells revealed that exposure to certain cytokines can derive a distinct population of NK cells, called NKT cells (Biron, 1999). This population of cells are derived in the presence of inducing cytokines such as IL-2 and IL-15 and they express both NK and T-cell (V α -24 chain paired with V β -11) surface markers. NKT cell activity depends on the nonclassical MHC class I (CD1) molecules which can present lipid structures, such as bacterial cell wall components to the NKT cell (Maher and Kronenberg, 1997). NKT cells have the potential to produce key cytokines of both T_H1 and T_H2 cells and are involved in the control of several types of immune responses (Senio *et al.*, 2006). In addition, NKT cells have been shown to have strong anti-tumor immune responses through direct cytotoxicity and indirect activation of a cascade of antitumor effector cells such as NK cells and CD8⁺ cytotoxic T-cells (Hong and Park, 2007).

1.4 Adaptive (Specific) Immune Response

Adaptive or specific immunity is capable of recognising and selectively eliminating specific foreign micro-organisms. Unlike innate immune responses, adaptive immune responses are initiated by specific antigenic challenges and improve on each successful encounter with the same pathogen. The specificity and memory capabilities of the adaptive immune response make it advantageous over the innate immune response, as it has the ability to recognise and remember specific pathogens and mounts stronger attacks each time the pathogen is encountered. However, it is important to note that the adaptive response does not act independent of the innate response. The two phases 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. The primary mediators of the adaptive immune system are the lymphocytes; T lymphocytes (T-cells) and B-lymphocytes (B-cells), and the antigen presenting molecules.

1.4.1 Antigen Presentation

During an effective adaptive immune response T-cells must be able to recognise a foreign antigen to mount an immune challenge against it. However, T-cells only recognise antigens when they are presented on the surface of APCs, such as macrophages, DCs and B-cells. APCs present antigen associated with MHC class II molecules. To achieve association, they engulf pathogens by endocytosis and degrade them into small peptides upon contact with lysosomal proteases. The endosome then fuses with MHC class II molecule-containing vesicles, which 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). (Figure 1.1). Following T-cell recognition of presented antigen, T-cells become activated and clonal expansion occurs. It is essential that T-cells have T-cell receptor (TCR) signalling to enable the cells to survive and proliferate as there is an interaction between the TCR and MHC class II molecules, and this is stabilised by cluster designation (CD) 4. In addition, interaction between the co-stimulatory molecules, CD80 and CD86, CD40 and ICAM-

1 (CD54) and their counterparts on T-cells is crucial for immunological communication (Larsen *et al.*, 1992).

1.4.2 Adaptive Immune Cells: Lymphocytes

Lymphocytes are the central cells of the immune system responsible for acquired immunity and the immunological attributes of diversity, specificity, memory and self / non-self recognition. As previously mentioned, there are two major classes of lymphocytes responsible for the recognition of antigens: B-cells and T-cells. Following maturation in the bone marrow and thymus, lymphocytes migrate via the circulation and afferent lymphatic vessels and populate in secondary lymphoid tissue (Parkin and Cohen, 2001). Lymphocytes express surface molecules 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.

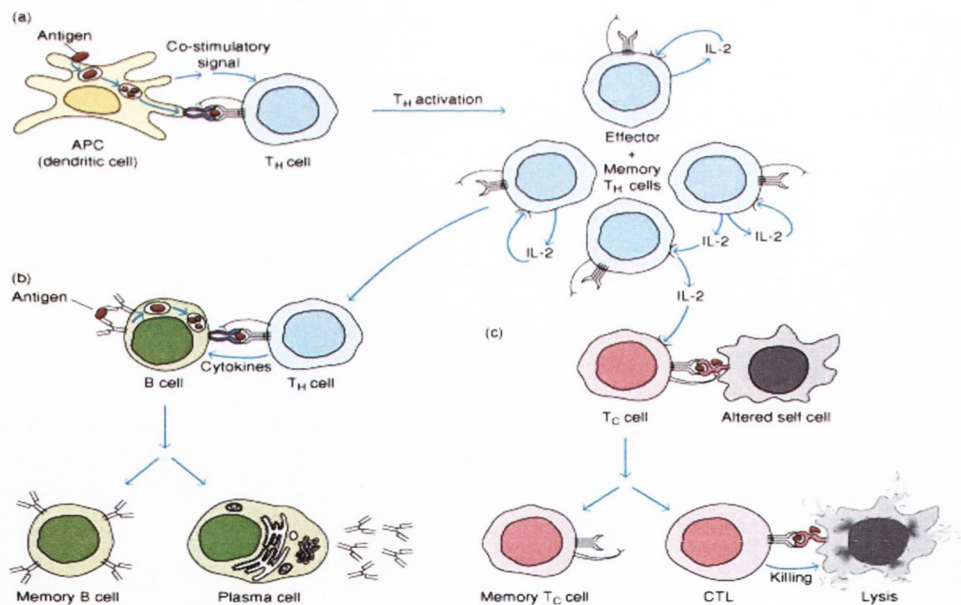


Figure 1.1: Activation of immune responses; innate detection of antigen, presentation to T-cell (a) and adaptive immune activation by T_H1 and T_H2 cells (b) and clonal expansion of T-cells (Duane, 1997)

1.4.2.1 T-lymphocytes

During maturation in the thymus, T-cells develop a unique antigen binding molecule called the TCR on its membrane. TCRs cannot recognise free antigens, only antigen that is bound to particular classes of self-molecules, such as MHC molecules. Upon engagement with self-MHC molecule, T-cells become activated slowly over 2-3 days and perform several effector mechanisms to protect against infection and instruct the adaptive immune response.

There are many well defined subpopulations of T-cells that respond differently to the detection of an antigen. Helper T-cells (T_H) and cytotoxic T lymphocytes (CTLs) are two well defined subpopulations and they are distinguished by the expression of $CD4^+$ or $CD8^+$ cell surface markers respectively. $CD8^+$ CTLs recognise endogenous antigens presented by MHC class I molecules. All nucleated cells express MHC class I, therefore any cell that is infected with a virus or other intracellular pathogen, or is producing abnormal tumour antigens, can present these antigens with class I, and be removed by cytotoxic attack. CTLs can eliminate virally infected cells through engagement of death-inducing ligands on the surface of the infected cell and CTLs can also secrete soluble factors such as β -chemokines and the $CD8^+$ antiviral factor (CAF) that suppress viral binding and transcription (Gulzar and Copeland, 2004). With their important virus detection abilities CTLs have been demonstrated to play a substantial role in the immunological control of immunodeficiency virus infections (see review Brown *et al.*, 2005).

$CD4^+$ T_H cells can bind to antigen-MHC class II complex and activate T_H cells to secrete various immune stimulating factors, or cytokines which help other T-and B-cells multiply into large clones and activate the immune response. There are two classical subdivisions of the $CD4^+$ T_H cells that are characterised by the cytokines that they secrete (Mosmann and Sad, 1999). T_H1 cells secrete IFN- γ and IL-12, and are responsible for many cell-mediated functions. T_H2 cells secrete IL-4, IL-5, IL-6 and IL-10, and are involved in the humoral immune response where they stimulate eosinophil activation and differentiation, provides help to B-cells, and promotes the production of antibodies (Avni and Rao, 2000). In addition, recent evidence has demonstrated that T_H cells can be further differentiated when other stimulating factors

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are introduced. The pro-inflammatory cytokine IL-23 was reported to stimulate the proliferation of a subset of $CD4^+$ T_H cells that display distinct immune stimulating properties from T_H1 or T_H2 cells. These T-cells are characterised by the production of IL-17 and are called T_H17 cells (Sutton *et al.*, 2006). T_H17 cells appear to promote an adaptive immune response to pathogens that is characterized by a heavy reliance on cells thought to function primarily as mediators of the innate immune response (Aggarwal *et al.*, 2002).

Recent evidence indicates that there is a further sub-division of T-cells that can control adaptive immune responses – regulatory T-cells (Von Herrath and Harrison, 2003). In addition, there are sub-divisions of this population that are both natural and inducible. Natural regulatory T-cells (T_{Reg}) express the cell-surface marker CD25 and the transcriptional repressor FoxP3 (Sakaguchi *et al.*, 1995; Hori *et al.*, 2003). Unique cytokine-production profiles have been used to define two populations of inducible regulatory T-cells. Inducible T_H3 cells were identified as a distinct population from T_H2 cells due to the large amounts of transforming growth factor (TGF) $-\beta$ they produced and the varying amounts of IL-4 and IL-10 (Chen *et al.*, 1996). Inducible T_R1 cells were identified as a distinct population from T_H1 cells due to the large amounts of IL-10 produced (Groux *et al.*, 1997). There is also evidence for an immunosuppressive function of $CD8^+$ regulatory T-cells that secrete either IL-10 or TGF- β (Garba *et al.*, 2002). The function of all the regulatory T-cells is to suppress T-cell effector mechanisms (see review Mills, 2004). For example, T_R1 cells and T_{Reg} cells have been shown to suppress the proliferation and cytokine production by naïve $CD4^+CD25^-$ T-cells, or antigen specific T_H1 and T_H2 cells. In addition, the immunosuppressive cytokines that are secreted by the regulatory T-cells can inhibit proliferation of, and cytokine production by effector T-cells.

1.4.2.2 B-lymphocytes

Mature B-lymphocytes are the primary producers of membrane-bound immunoglobulin (antibody) molecules which serve as receptors for antigens. Each B-cell expresses antibodies for unique antigen specificity. Interaction between an antigen and the membrane-bound antibody on the surface of a B-cell activates the B-cell, and the cell differentiates into a plasma cell, which secretes large amounts of a soluble form

of the antibody. 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).

1.5 Soluble Mediators of the Immune System: Cytokines

Cytokines are soluble proteins used in cell-cell communication in the immune system. They are secreted by many different cell types and can mediate short and long range messages that include growth, differentiation, effector function, and cell survival. Cytokines exert their effects on a target cell by binding to specific receptors on the membrane of the target cell and triggering signal-transduction pathways that ultimately alter gene expression. Their effects can be modulated in both a synergistic or inhibitory fashion by other cytokines. Cytokines comprise a large family of immunologically similar molecules including ILs, IFNs, TNFs, chemokines and growth factors (Balkwill, 2000). They are stimulated by a variety of immune cells and they communicate a wide range of immune functions, both pro-inflammatory and anti-inflammatory in nature. Pro-inflammatory cytokine signals are secreted by immune cells in response to an inflammatory stimulus and promote an immune response to eliminate or slow the spread of a pathogen. However, anti-inflammatory cytokine signals are also released during an immune response and these cytokines play an important regulatory role. Anti-inflammatory cytokines can prevent the production of pro-inflammatory cytokines, thus limiting the size of the immune response. The immune response must be tightly controlled because over activation of the inflammatory response can result in severe inflammation and damage to healthy host tissue.

1.5.1 Cytokines

1.5.1.1 IFN- γ

IFN- γ is a member of the IFN family, of which there are three members –IFN- α , IFN- β and IFN- γ . The nomenclature was derived from the ability of the molecules to “interfere” with viral activity. IFN- γ plays many important roles in orchestrating cell-mediated immunity. It stimulates macrophage functions including phagocytosis, respiratory burst activity, antigen presentation and cytokine secretion facilitating an

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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 T_H1 cell differentiation (Boehm, 1997). IFN- γ is predominantly produced by NK cells and NKT cells as part of the innate immune response and by CD4⁺ T_H1 cells and CTL effector T-cells once antigen-specific immunity develops (Schoenborn and Wilson, 2007).

NK cells are the most potent inducers of IFN- γ during the early stages of viral, parasitic or bacterial infection (Farrar *et al.*, 1993). NK cells release IFN- γ following stimulation with inflammatory signals, such as the pro-inflammatory cytokine IL-12. In addition, IFN- γ is part of a positive feedback loop that augments its own release by acting on NK cells to stimulate further release. It has been reported that other cytokines can act synergistically with IL-12 and IFN- γ to enhance IFN- γ release from NK cells, such as the pro-inflammatory cytokines IL-15 and IL-18 (Micallef *et al.*, 1996). Another major source of IFN- γ are NKT cells which have been demonstrated to stimulate IFN- γ release and can also activate APCs, such as DCs and macrophage cells, to secrete IL-12 (Kitamura *et al.*, 1999). The importance of these cell populations for IFN- γ production was shown in a report by Varma *et al.*, 2002 where it was reported that almost 60 % of IFN- γ producing cells are NK cells and 25 % are NKT cells following challenge with lipopolysaccharide (LPS) in mice.

Most of the remaining IFN- γ producing cells are T-cells, macrophages and DCs (Munder *et al.*, 1998). As previously discussed, naïve T-cells activated in the presence of IFN- γ differentiate into T_H1 effector cells (Bradley *et al.* 1996). In turn, T_H1 cells produce high amounts of IFN- γ to promote expression of MHC class I and II, activation of macrophages and production of IgG_{2a} by B-cells (see review by Adams and Hamilton, 1984). Macrophages and DCs are regarded as minor sources of IFN- γ in comparison to NK-cells and NKT-cells (Munder *et al.*, 1998). It has been demonstrated that IFN- γ is secreted from DCs and macrophages in an IL-12 dependent manner, since the IL-12 receptor is expressed on mouse DCs and macrophages (Grohmann *et al.*, 1998). Therefore, similar to NK cells, IL-12 derived from DCs is associated with autocrine activation between IL-12 and IFN- γ . In addition, it has been shown that DCs

have also been shown to release IFN γ in response to a synergistic action of both IL-12 and IL-18 (Stober *et al.*, 2001).

IFN- γ induces a cellular response by binding to the specific IFN- γ receptors that are found on the surface of most immune cells. Binding of IFN- γ to its receptors results in the recruitment and activation of the Janus Kinase (JAK) - signal transducer and activator of transcription (STAT) pathway and it is this pathway that is responsible for the biological effects of IFN- γ (Schlindler *et al.*, 1992). The JAK family consist of four members, JAK 1, JAK2, JAK 3 and tyrosine kinase 2 (TYK2) (Shuai and Liu, 2003). The JAK1 and JAK2 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 (Darnell *et al.*, 1994). There are seven members of this family; STAT-1, 2, 3, 4, 5A, 5B and 6 (Plantias, 2005). Of these, STAT-1 is the most crucial transcription factor during IFN- γ signalling.

The IFN- γ receptor is composed of 2 subunits, the IFN- γ R1 and IFN- γ R2, which are unassociated in the absence of a ligand. IFN- γ R1 is associated with the inactive JAK1 and IFN- γ R2 is associated with the inactive JAK2. Binding of extracellular IFN- γ to the receptor induces the receptor to oligomerize, leading to the activation 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 STAT-1 via its SH2 domain. After JAK-mediated tyrosine phosphorylation, STAT-1 dissociates from the receptor and forms homodimers. Dimeric STAT-1 translocates to the nucleus where it binds to γ -activated sequence (GAS) elements and it regulates gene expression (Plantias, 2005). (Figure 1.2)

In vivo studies have indicated that mice deficient in IFN- γ , IFN- γ R1, IFN- γ R2 or STAT-1 have dysfunctional immune responses characterised 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). These mice have very low rates of survival because of their high susceptibility to infection. Moreover, mutations in the human IFN- γ receptor-signalling pathway results in severely impaired immune responses to bacterial infections. This can be potentially fatal early in childhood due to

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the host not being able to control mycobacterium infections (Newport *et al.*, 1996; Jouanguy *et al.*, 1996; Pierre-Audigier *et al.*, 1997).

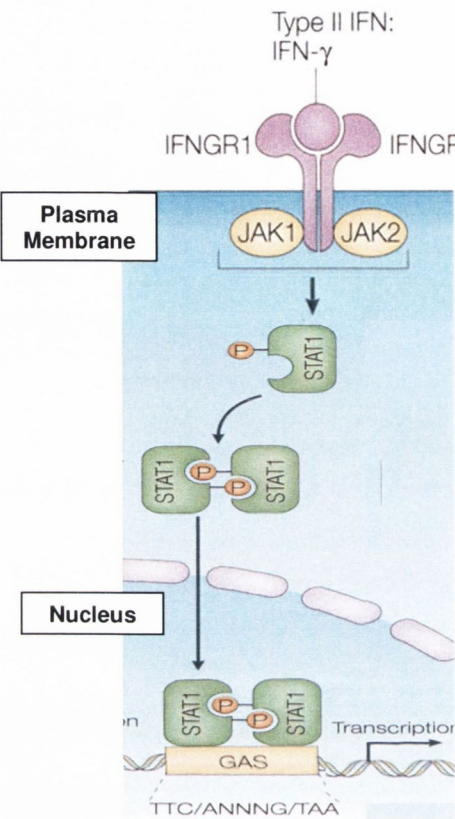


Figure 1.2: Activation of the JAK-STAT signalling pathways by IFN- γ (Platanias, 2005).

1.5.1.2 IFN- γ inducible genes

Activation of the JAK-STAT pathway results in the transcription of IFN- γ -inducible genes which can elicit antiviral, antiproliferative and immunomodulatory responses. For example, IFN- γ signalling can activate the transcription of chemokines, cytotoxic molecules and cytokines. One such IFN- γ -inducible gene is CD40 which is a costimulatory molecule for antigen presentation that is induced by inflammatory stimuli, such as LPS and its expression is also upregulated by cytokines, such as IFN- γ (Nguyen and Benveniste, 2000). CD40 is constitutively expressed by a wide variety of cells, such as B-cells, macrophages, microglia, DCs, fibroblasts and tumor cells and its expression is up-regulated following stimulation. It has been implicated to participate in many human diseases, particularly autoimmune diseases (Grewal and Flavell, 1998). Blocking the interaction between CD40 and its ligand, CD40L (CD154), with anti-

CD40 antibody has been found to be beneficial in several animal models of autoimmune diseases (Benveniste *et al.*, 2004). These studies illustrate the importance of CD40-CD154 interactions for homeostasis of immune responses.

One chemokine that is induced by the JAK-STAT pathway is IFN- γ -inducible protein 10 (IP-10). IP-10 is constitutively expressed at low levels in thymic, splenic and lymph node stroma and its expression can be strongly induced by type I and II IFNs and by LPS (Gattass *et al.*, 1994). IP-10 is a chemoattractant for activated T-cells and it has been demonstrated to be a critical factor for the generation and delivery of effector T-cell responses. Neutralisation of IP-10 inhibited T-cell infiltration into infected tissues and impaired Ag-specific T-cell effector functions, resulting in massive increase in tissue parasite burden, leading to increased mortality (Khan *et al.*, 2000).

Inducible nitric oxide synthase (iNOS) expression is induced by a combination of factors, including LPS and IFN- γ (Langermans *et al.*, 1992). iNOS produces the free radical nitric oxide (NO) from APCs. NO is an important messenger molecule involved in many physiological and pathological processes within the mammalian body both beneficial and detrimental (Hou *et al.*, 1999). For example, NO has been demonstrated to inhibit T-cell proliferation but does not alter T-cell cytokine production (Van Der Veen, 2001). However, it is becoming apparent that the role of NO in tissue inflammation and immunity is highly controversial. If NO does not form peroxynitrite it can have a role as an immunoregulatory molecule. For example, iNOS-KO mice had increased severity of experimental autoimmune encephalomyelitis (EAE) and therefore there were some protective role for NO (Fenyk-Melody *et al.*, 1998).

1.5.1.3 IL-12

IL-12 plays a vital role in a host's response to an inflammatory signal because it bridges the gap between the innate and adaptive immunity (Trinchieri *et al.*, 1995). As discussed in the previous section, one important function of IL-12 is the stimulation and regulation of IFN- γ production from immune cells (Jacobson *et al.*, 1995). It is also a major regulator of T_H1 differentiation and enhances T_H1 cell proliferation (Itoh *et al.*, 2004). IL-12 is primarily produced from monocytes / macrophages but also to a lesser

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extent from DCs, B-cells, Langerhans cells, polymorphonuclear neutrophils and mast cells (Cella *et al.*, 1996).

IL-12 is a heterodimeric cytokine of 70kDa (p70) consisting of two subunits, a 40kDa heavy chain (p40) and 35kDa light chain (p35) (Kobayashi *et al.*, 1989). Both the p40 and p35 chains must be expressed in the same cell to ensure that heterodimeric IL-12 is produced and its biological activity is intact (Wolf *et al.*, 1991). The IL-12p35 gene is thought to be expressed in all cells, however, IL-12p40 transcription is tightly controlled and is detected only in cell types with biological activity of IL-12 (Aste-Amezaga *et al.*, 1998). IL-12p40 can be secreted as a monomer or a homodimer in the absence of p35, whereas p35 can only be secreted 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 (Picotti *et al.*, 1998), and similarly for resistance to infection with mycobacteria (Cooper *et al.*, 2002).

The IL-12 receptor (IL-12R) consists of β 1 and β 2 subunits (Presky *et al.*, 1996) and is mainly expressed on NK cells, T-cells and NKT cells (Grohmann *et al.*, 1998). IL-12p40 interacts with β 1 subunit, while p35 binds to β 2 subunit to activate the receptor and induce the JAK-STAT pathway. The IL-12R is associated with the intracellular protein tyrosine kinases, Tyk2 and JAK2 which are linked with the β 1 and β 2 subunits of IL-12R respectively (Zou *et al.*, 1997). Activation of these JAKs results in the phosphorylation of STAT-3 or STAT-4, and they translocate to the nucleus where they can regulate gene transcription. Most studies have documented that STAT-4 molecule is primarily associated with IL-12 signalling (Bacon *et al.*, 1995). Moreover, STAT-4 knockout mice present deficits in IFN- γ production and a reduced capacity to induce a T_H1 immune response (Kaplan *et al.*, 1996).

The functions of IL-12 are activation, proliferation and cytotoxicity of pre-activated NK cells, and antigen-specific T_H cells (Bertagnoli *et al.*, 1992). It is a potent inducer of IFN- γ production from these cells (Trinchieri, 2003). In fact, the original term for IL-12 was the NK 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). Regulatory mechanisms of IL-12 production have been studied in detail. For instance, IL-12 secretion is enhanced by several cytokines, including IFN- γ , IL-1 β , IL-4 and IL-13 (Schnurr, *et al.*, 2005). Furthermore, IL-12 secretion is down-regulated by IL-10, TGF- β and ligand binding to G_{as}-linked G-protein coupled receptor (Aste-Amezaga *et al.*, 1998). IL-12 is an important cytokine involved in the development of cell-mediated immunity against intracellular pathogens such as *Mycobacterium tuberculosis*, *Listeria monocytogenes* and human immunodeficiency virus (HIV) (Trinchieri, 2001; 1997; Biron and Gazzinelli, 1995; Cooper *et al.*, 2002).

1.5.1.4 IL-15 and IL-18

IL-15 and IL-18 are pro-inflammatory cytokines produced primarily by macrophages during an innate immune response and subsequently profoundly influence adaptive immunity. They have been shown to play an influential part in the inflammatory response and have also been implicated in chronic inflammation, for example during rheumatoid arthritis (RA) (Liew and McInnes, 2002).

IL-15 is a pro-inflammatory cytokine which plays a crucial role for the development and proliferation of NK cells, NKT cells, memory CD8⁺ T-cells and also for the maturation of DCs and macrophages (Ohteki, 2001). It is produced in a concerted fashion with IL-12 and it has been shown to induce IFN- γ secretion (Fehniger *et al.*, 1999). Studies by Ohteki *et al.*, (2001) have shown that DCs 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 fact that the IL-12R β 1 is not properly expressed on the cell surface of cells from these mice. Therefore, it is thought that IL-15 induces IL-12R β expression on immature NK cells and results in the production of maximal concentrations of IFN- γ following stimulation with IL-12 (Ohteki *et al.*, 2001). IL-15 and IL-2 share a common cytokine receptor γ subunit; however the IL-15 receptor (IL-15R) has a unique α -chain (IL-15 α) which binds with high affinity to IL-15 (Liew and McInnes, 2001). Upon activation of the receptor, it induces the phosphorylation of Jak1 and Jak3, thereby enhancing the formation of STAT-5 homodimers which translocates to the nucleus and alters gene expression (Waldmann, 2006).

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IL-18 was originally coined as IFN- γ inducing factor (IGIF) due to its ability to produce large amounts of IFN- γ from NK and T-cells (Dinarello, 1999). Because of these IFN- γ inducing properties, IL-18 is considered a member of the T_H1 cell inducing family of cytokines along with IL-12, IFN- γ and IL-15. The majority of IL-18 is produced from monocytes or macrophages following stimulation with an immune stimulus or immune challenge such as LPS. IL-18 production alone induces low levels of IFN- γ from NK cells. However, in the presence of co-stimulants such as IL-12 and IL-15, IFN- γ production is greatly enhanced. For instance, IL-18 and IL-12 have been shown to act synergistically to induce IFN- γ production from bone marrow derived macrophages (Munder *et al.*, 1998). This synergistic activation is achieved by IL-18 and IL-12 activating the IFN- γ promoter at different sites. IL-18 activates the IFN- γ promoter by binding of c-jun to AP-1 (activating protein-1) and IL-12 activates by binding to the STAT4 site (Dinarello, 1999). The important role for IL-18 in the stimulation of IFN- γ was highlighted by studies conducted in IL-18-deficient mice. These mice produce little or no IFN- γ in response to an injection with LPS, with normal levels of IL-12 detected (Takeda *et al.*, 1998).

1.5.1.5 IL-10

IL-10 is an important immunoregulatory cytokine that limits and terminates inflammatory responses. This regulation of immune responses is vital to ensure that healthy host cells or tissue is not damaged while a robust immune response is mounted against pathogens. IL-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. Many immune cell populations secrete IL-10, including T-cells, B-cells, monocytes, kupffer cells, keratinocytes, mast cells, thymocytes, tumour cell lines, certain melanomas, ovarian and colon carcinomas (Moore *et al.*, 2001). Its production is induced by pro-inflammatory stimuli such as mitogens, LPS and certain viruses. The actions of IL-10 are mediated by its specific cell surface receptor complex, of which there are 2 subunits; IL-10R1 and IL-10R2 (Kotenko *et al.*, 1997). It has been demonstrated that the IL-10R2 chain is essential for IL-10 mediated effect in IL-10R2-deficient mice display the same phenotype as IL-10 deficient mice (Spencer *et al.*, 1998). However, both subunits need to be present for downstream signalling to occur (Kotenko *et al.*, 1997). The IL-10 / IL-10R interaction activates the tyrosine kinases Jak1 and Tyk2, which in turn activates the transcription factors STAT 1, 3 and 5, and

results in translocation and gene activation (Asadullah *et al.*, 2003). In addition, IL-10 / IL-10R interaction has also been shown to suppress the production of pro-inflammatory cytokines by other molecular mechanisms, including down-regulating nuclear translocation of nuclear factor (NF)- κ B which stimulates mRNA breakdown of pro-inflammatory mediators. Several investigators have reported that IL-10-induced inhibition of NF- κ B, is the primary molecular target by which IL-10 induces its immunoregulatory actions (Opal *et al.*, 1998).

IL-10 has been shown to promote the development of a T_H2 or humoral based immune profile and suppress the T_H1 or cellular based immune response via the inhibition of pro-inflammatory cytokines which shifts the immune profile from a T_H1 to a T_H2 (Abbas *et al.*, 1996). This alteration of immune response can result in activation and growth of mast cells and eosinophils, and increased antibody production by inducing differentiation of antibody secreting B-cells, and switching B-cell derived Ig to IgE antibody. In addition, IL-10 suppresses cell based immunity by inhibiting macrophage activation and T-cell proliferation, and suppressing the antigen-presenting capacity of monocytes and DCs. In addition, recent evidence has shown that IL-10 can induce T_{Reg} and T_R1 subpopulation of T-cells. These cells secrete IL-10 and TGF- β which are known to inhibit proliferation and cytokine production from T_H1 or T_H2 cells (Mills, 2004). A primary function of IL-10 is to deactivate macrophage pro-inflammatory cytokine production. Studies have indicated that IL-10 inhibits the production of pro-inflammatory cytokines, such as TNF- α , IL-1 and IL-12 from macrophages, and IFN- γ from NK and T_H1 cells by acting on antigen presenting cells (Fiorentino *et al.*, 1991; de Waal Malefyt *et al.*, 1991). IL-10 plays an important role during infection as it is a dominant factor in suppressing IFN- γ and enhancing antibody production. IL-10 inhibits activation of T-cell mediated responses and consequently enables the longevity of several pathogens including HIV, *Bordetella pertussis*, *Mycobacterium tuberculosis* and *Leishmania donovani* (McGuirk *et al.*, 2002). In contrast, more recent studies implicate a role for IL-10 in hepatitis C virus (HCV) development (Rigopoulou *et al.*, 2005). 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 the potential inflammatory responses (Redpath *et al.*, 2001). Similarly, tumor cells are proficient in secreting IL-10, hence escaping cell-mediated immunity (Williams *et al.*, 2004). Studies in several animal models of inflammation have been

conducted demonstrating the anti-inflammatory effects of IL-10 in sepsis, EAE, inflammatory bowel disease and collagen induced arthritis (Gerard *et al.*, 1993, Cua *et al.*, 1999). In contrast, studies have found a strong correlation between IL-10 and progression of human infectious diseases including malaria, *visceral leishmaniasis*, leprosy and tuberculosis (Moore *et al.*, 2001).

1.5.1.6 IL-10 family members; IL-19, IL-20 and IL-24

The family of IL-10-related cytokines include five novel human molecules structurally related to IL-10; IL-19, IL-20, IL-22, IL-24 (MDA-7) and IL-26 (AK155). They have conserved structural homology, however they have been demonstrated to have distinct biological functions. For example, the biological activities of IL-19 seem to be in regulating immune activity, IL-20 with skin biology and IL-24 in tumor apoptosis.

IL-19 was originally identified in 2000 while searching for IL-10 homologues and it has subsequently been found to be secreted by monocytes and to a lesser extent, by B-cells but not T-cells (Wolk *et al.*, 2002). It is produced following stimulation with an immune challenge, such as LPS or GM-CSF (Jordan *et al.*, 2005). IL-19 signalling occurs through a receptor complex composed of IL-20R1 and IL-20R2 chains which can also be utilised by IL-20 and IL-24 (Commins, *et al.*, 2008). The receptor is found on a number of tissues and on monocytes (Parrish-Novak *et al.*, 2002). The induction of IL-19 by LPS is up-regulated by the T_H2 -type cytokines, IL-4 and IL-13, whilst being down-regulated by the T_H1 -type cytokine, IFN- γ (Gallagher *et al.*, 2004). This indicates that IL-19 plays a role in the T_H1/T_H2 system and influences the maturation of T-cells in favour of T_H2 type. The induction of IL-19 activates monocytes to release IL-6, IL-1, TNF- α and numerous reactive oxygen species (Liao *et al.*, 2002). IL-19 has also been demonstrated to regulate the immune response by inducing IL-10 production and through auto-induction of its own expression (Jordan *et al.*, 2005). The IL-19-induced T_H2 profile could be of great interest in a range of autoimmune and infectious diseases. For example, IL-19 has been shown to have a role in joint inflammation, such as in rheumatoid arthritis (RA), by inducing IL-6 production from synovial cells and decreasing synovial cell apoptosis (Sakurai *et al.*, 2008). In addition it has been demonstrated that there is an upregulation of IL-19 in patients with allergic asthma (Liao *et al.*, 2004).

The primary function of IL-20 is the stimulation of keratinocyte proliferation and differentiation (Kunz *et al.*, 2006). Similarly to IL-19, the actions of IL-20 are mediated via the receptor complex of IL-20R1 and IL-20R2. The receptor for IL-20 results in activation of STAT-3 downstream signal-transduction pathway (Rich *et al.*, 2001). Recent investigations have suggested pathogenic roles of IL-20 in psoriasis. For example, over-expression of IL-20 in transgenic mice showed neonatal lethality, as well as severe skin abnormalities similar to those of psoriasis (Blumberg *et al.*, 2001). Moreover, IL-20 receptor mRNA is markedly upregulated in human psoriatic skin compared with normal skin (Wei *et al.*, 2005). Therefore, IL-20 has been suggested as a potential target for psoriasis treatment as blocking IL-20 signalling in psoriasis improves the condition (Stenderup *et al.*, 2007).

IL-24 was initially designated as melanoma differentiation-associated gene-7 (MDA-7) as it is expressed by melanocytes. When it was originally discovered it was considered as a potential therapeutic target for cancer because it was shown it could selectively target and reduce colony numbers of melanoma cells (Jiang *et al.*, 1995). A number of more recent studies reported that the majority of human cancer-derived cell lines, including melanoma, prostate, breast, cervical, lung, fibrosarcoma, pancreatic, colorectal and glioblastoma lines, undergo apoptosis upon exposure to IL-24 (Elmekcioglu *et al.*, 2001). It was also shown to induce the production of IL-6 and TNF- α from monocytes, which is antagonistic to that the action of IL-10 (Oral *et al.*, 2006).

1.5.1.7 TGF- β

The TGF- β superfamily consists of a large group of extracellular growth factors controlling multiple aspects of development. There are different isoforms which are expressed in different tissues and among these isoforms, TGF- β 1 is predominantly expressed in the immune system (Govinden and Bhoola, 2003). TGF- β is an important immunoregulatory cytokine with diverse effects on immune cells. Its pivotal function is to maintain tolerance to self or innocuous antigens via the regulation of inflammatory responses. It succeeds in this via the regulation of chemotaxis and macrophages, mast cells and granulocytes. However, overactivation of TGF- β pathway can lead to several immunopathologies under physiologic conditions including cancer progression, making it an attractive target for antitumor therapies (Wrzesinski *et al.*, 2007).

TGF- β exerts the greatest impact on T-cells as is evident in mice with a T-cell specific blockade of TGF- β signalling (Gorelik and Flavell, 2000). TGF- β impairs T-cell proliferation, differentiation and survival. With regard to differentiation, TGF- β inhibits differentiation of T_H1 and T_H2 cells and it also induces FoxP3 expression and the generation of T_{Reg} cells (see review Li *et al.*, 2005). It has been known for some time that TGF- β acts on NK cells to inhibit IFN- γ expression (Bellone *et al.*, 1995). Furthermore, a major phenotype resulting from the development of TGF- β -deficient mice was massive overexpression of IFN- γ (Shull *et al.*, 1992).

1.6 Challenging the Immune System with LPS

When our immune system is challenged by an invading substance, there is activation of the appropriate immune cells and immune mediators for the detection and destruction of the specific antigen. LPS is the major virulent factor of gram-negative bacteria and is a component of the bacterial cell wall. LPS is a well characterised immune challenge which is often used in research to mimic the initial immune response to a bacterial infection. LPS toxicity induces many well documented, physiological changes including hypotension, fever, tissue necrosis and death (Heumann and Roger, 2002). In the event of infection, the host cells orchestrate immediate immune signals to activate the systemic responses and control the traumatic insult. The initial response to LPS detection includes behavioural effects, such as anorexia, somnolence, lethargy and the induction of fever in response to neuroendocrine activity and production of neuropeptides. This consolidation of non-specific symptoms is collectively referred to as 'sickness behaviour' (Hart, 1988) and it is induced by cytokines, such as IL-1 β and TNF- α . Mast cells are one of the primary cells to respond at 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 produced 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).

An important initial stage for the immune response to LPS is detection of the antigen. The primary host proteins involved in LPS recognition are lipid binding proteins (LBP). LBP is an acute-phase protein, produced in the liver, which circulates in the bloodstream where it recognizes and forms a high-affinity complex with the lipid A component of LPS, as free molecules, fragments, or still bound to the outer membrane of intact bacteria (Heumann *et al.*, 2002). LBP aids the binding of LPS to its receptor by forming a tertiary complex with CD14 co-receptor found on the membrane of immune cells or in a soluble form. The LBP/LPS/CD14 complex are able to present the LPS to a Toll-like receptor (TLR) situated on the immune cell membranes. TLRs recognise conserved pathogen-associated molecular patterns (PAMPs) shared by large groups of microorganisms and play an instructive role in induction of innate and adaptive immune responses (Aderem and Ulevitch, 2000). TLRs are made up of extracellular leucine rich repeat (LRR) domain and intracellular Toll/interleukin IL-1 receptor (TIR) domain (Medzhitov, 2001). TLR4 is essential for the recognition of LPS and TLR4 is expressed on many immune cells, including DCs, monocytes and T-cells (Ahmad-Nejad *et al.*, 2002; Zanin-Zhorov *et al.*, 2007).

LPS-TLR4 intracellular signalling requires the presence of molecules known as adapter proteins, such as lymphocyte antigen 96 (MD2), which are essential components of the receptor complex. The LPS-induced TLR4 signalling can occur via two methods. Firstly, there is an early signalling response which is dependent on myeloid differentiation factor 88 (MyD88). Secondly there is a delayed signalling response which is independent of MyD88 but uses other adaptor proteins (Figure 1.3). 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 (Fitzgerald *et al.*, 2001). Following activation, the complex induces the activity of the signaling molecules IL-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor (TRAF6) resulting in activation of inhibitor of κ B (I κ B). As a result, the protein degrades and releases cytoplasmic NF- κ B. Once activated, NF- κ B translocates to the nucleus where it binds to promoter regions of pro-inflammatory genes and regulates the expression of such mediators (see review Palsson-McDermott and O'Neill, 2004). 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)

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enhance the activity of TRAF6 and TBK1 which in turn induce the activity of NF- κ B. This event is not as potent as that of the early phase in the presence of MyD88.

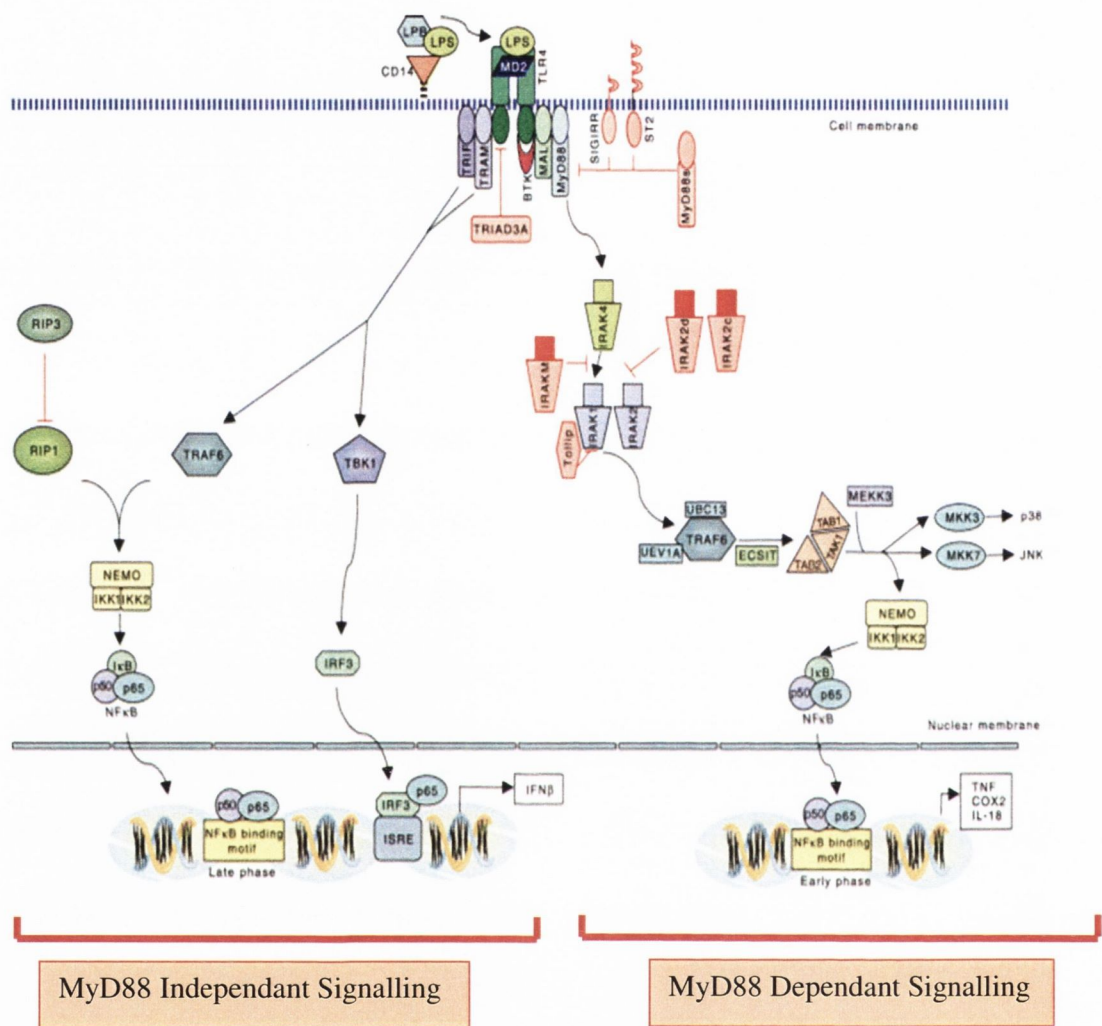


Figure1.3: LPS signalling via TLR4 with LPS/LBP/CD14 receptor complex presenting LPS to TLR4 on cell surface membrane (adapted from Palsson-McDermott and O'Neill, 2004)

The activated LPS-TLR4 intracellular signalling can have many downstream effects and it has been demonstrated that it can ultimately result in the production of pro-inflammatory cytokines such as IL-1, TNF- α , IL-12 from innate immune cells such as monocytes, macrophages and dendritic cells (Latz *et al.*, 2003 and Akira *et al.*, 2003). Moreover, stimulation of TLR4 promotes up-regulation of MHC proteins and co-stimulatory molecules, such as CD40, 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, chemotaxis and apoptosis. Furthermore, it plays a major role in up-regulating cell trafficking and recruitment by stimulating the expression of pleiotropic cytokines and chemokines.

1.7 Psychological Stress

Psychological stress is very prevalent in modern day life. It is accepted as part of our everyday, busy lives where we deal with the pressures of work, financial worries and family difficulties. Everyone understands what is implied by the term psychological stress, however it is difficult to define because it is a subjective experience. Stress was originally defined 70 years ago by Hans Selye in his short article in *Nature* in 1936 as the “non-specific response of the body to any demand made upon it”. He coined the term ‘stress’ to more accurately describe the ‘strain’ that an organism is under in response to a non-specific insult. The wording of the definition for stress has changed over the years since his original description, but the meaning has remained the same. The working definition of stress used in this thesis is ‘the physiological response to events or environmental demands that exceed an individual’s perceived ability to cope’. This definition is useful because it states that the same physiological stress response is activated with various types of stressful inputs – whether physical or psychological. It also implies that stress is subjective and an event is only perceived to be stressful when it is too much for a person to deal with. Therefore, an event which is stressful for one individual, may not be sufficient to activate the stress response in another individual.

Selye’s pioneering investigations of psychological stress uncovered the underlying areas of the brain implicated in the stress response. He also highlighted the nonspecificity of this response and named any stress causing agent, a ‘stressor’. His concept of a stressor exceeded the thinking at the time that the primary cause of a stress response was physical stress, such as excessive heat or cold, forced exercise and chemical agents. He concluded that a perceived psychological stress produced the same physiological response as a physical stressor. He also recognised that both negative and positive stressors (i.e. distress and eustress) could elicit virtually identical stress responses as physical stressors (Selye, 1936).

1.8 The Stress Response

When a stressor is perceived by the brain, our bodies prepare for action and the 'fight or flight' response is activated. This is a primitive survival response that gives a body the ability to fight to the death or make a desperate flight from certain defeat. During stress there are many beneficial physical and behavioural responses that prepare our body for action and improve functioning. Attention is enhanced, senses are heightened and our brains focus on the immediate perceived threat. Cardiac output and respiratory rates are accelerated and glucose and fat metabolism are increased to give fuel and energy to the aroused brain, heart and muscles. Endorphins are released to reduce pain, and blood vessels to the skin are restricted to reduce potential blood loss (Chrousos and Gold, 1992). Finally, functioning of systems, such as the digestive system and kidneys, which are non-essential responses to an acute threat, are shut down to allow vital organs and muscles to perform preferentially. These automatic peripheral changes that occur in response to stressor detection adjust the homeostasis of the body in order to improve chances for survival (Tsigos and Chrousos, 2002). When we face very real dangers to our physical survival, the fight or flight response is invaluable. However, in modern life, most of the stressors we perceive are not a threat to our physical survival. Today's stressors consist of psychological pressures and worries, such as financial worries, exam stress, caring for the unwell or death of a loved one. Nonetheless, these modern day stressors result in the activation of our stress response as if our physical survival was threatened.

1.8.1 The Stress System

The stress response is initiated and performed by the stress system. The major components of the stress systems include the CNS regions that perceive the stressful input and communicate the signal to the periphery and the organs in the periphery that produce stress signals (Figure 1.4). The system functions to maintain homeostasis, both at rest and during stress, primarily through the production of corticotropin-releasing hormone (CRH), glucocorticoids (GCs) and catecholamines. These substances are the major regulators of behaviour, fuel metabolism, cardiovascular function and thermogenesis. The system can respond to a great diversity of distinct circadian,

neurosensory, blood borne and limbic signals and adjust the functions according to need (Elenkov and Chrousos, 2006).

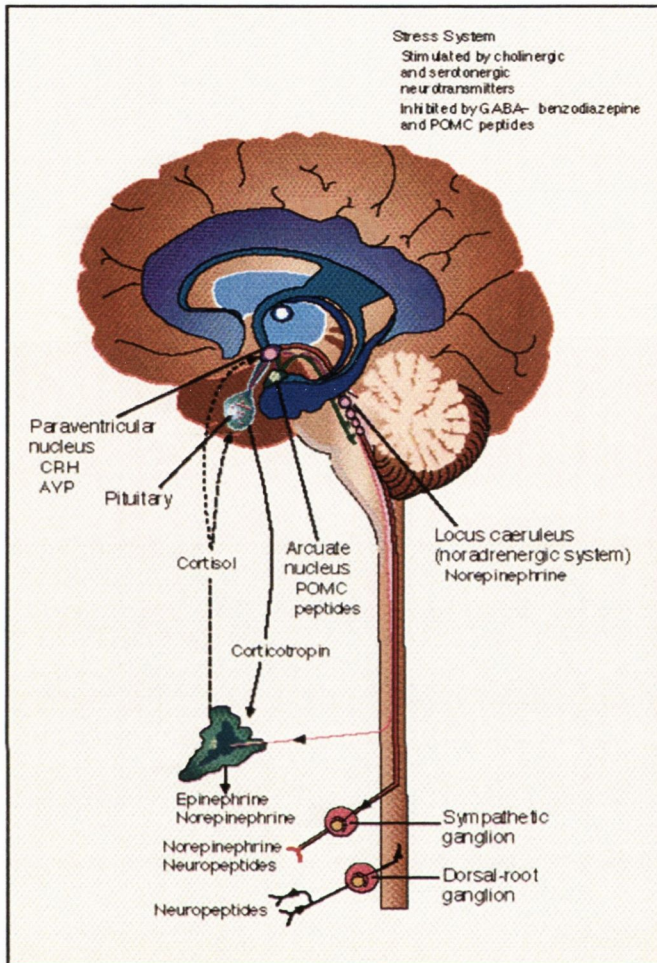


Figure 1.4 Major components of the central and peripheral stress system (Chrousos, 1995)

The central control stations of the stress system are located in the hypothalamus and the brain stem. The main brain circuits that initiate and maintain the stress response are the parvocellular neurons of the paraventricular nuclei (PVN) of the hypothalamus, that release CRH and arginine-vasopressin (AVP), and locus coeruleus-noradrenaline (LC-NA) autonomic systems. The hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) axis are the two major effector limbs of the stress system (Herman and Cullinan, 1997). However, the brain also activates a subset of vagal nerves that mediate the gut response to stress (Habib *et al.*, 2001). The HPA

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and SAM axis are the two primary pathways via which the brain influences all body organs in response to a stressor and their main function is to maintain homeostasis.

1.8.1.1 The HPA Axis

The HPA axis is the main efferent arm of the stress response. The pathway originates in the hypothalamus which is an important area in the brain for maintaining homeostasis. It is responsible for the regulation of factors such as blood pressure, body temperature, fluid and electrolyte balance, and body weight. These factors are held to a precise value and it is remarkably fixed from day to day. To achieve homeostasis, the hypothalamus must receive inputs about the state of the body, and must be able to initiate compensatory changes (Chrousos, 1992). When the hypothalamus detects a stressor, it can alter the homeostasis of the body and prepare the body for the threat. The principle hypothalamic output to the pituitary–adrenal axis is CRH. CRH is released locally to the pituitary gland where it stimulates the production of adrenocorticotrophic hormone (ACTH) from the anterior pituitary and into the blood stream. ACTH in turn, stimulates the adrenal cortex to release GC hormones, mainly cortisol in humans and corticosterone in rodents. AVP is a potent synergistic factor with CRH in stimulating ACTH secretion; however, AVP alone can stimulate little ACTH production (Lamberts *et al.*, 1984). In non-stressful situations, both CRH and AVP are secreted in the portal system in a circadian, pulsating fashion, with a frequency of about two to three secretory episodes per hour (Horrocks *et al.*, 1990). Under resting conditions, the amplitude of the CRH and AVP pulses increase in the early morning hours, resulting finally in ACTH and cortisol secretory bursts in the general circulation. These diurnal variations are altered by changes in lighting, feeding schedules and activity and are disrupted by stress. During acute stress, the amplitude and synchronization of the CRH and AVP pulsations in the hypophyseal portal system markedly increases, resulting in increases of ACTH and cortisol secretory episodes (Tsgosis and Chrousos, 1994).

1.8.1.2 The SAM Axis

The SAM axis is the sympathetic division of the autonomic nervous system. The pathway originates in the brain stem where the LC-NA system is located. Stimulation of the LC-NA activates the axis and the cells of the adrenal medulla synthesise and

secrete the principle end products of the SAM, the catecholamines, such as noradrenaline (NA) (Padget and Glaser, 2003). At rest catecholamines maintain homeostasis by regulating fuel metabolism, heart rate, blood vessel tone and thermogenesis. However, when homeostasis is disrupted by an internal or external challenge, the SAM axis is activated, resulting in increased peripheral levels of catecholamines (Elenkov *et al.*, 2000). The catecholamines act as a neural alarm system to activate the stress response. NA decreases neurovegetative functions, such as eating and sleeping, and increases autonomic and neuroendocrine responses to stress, including HPA axis activation. NA also activates the amygdala, the principal brain locus for fear-related behaviours (Tsiogios and Chrousos, 2002). As well as the hormonal component of the SAM axis, there is also a neuronal component that regulates peripheral activity by direct sympathetic innervations (Sternberg, 2006). Immune organs, such as the lymph nodes and spleen, have been shown to be hard wired with SAM innervations that result in the local release of NA into the organs. Regional release of these catecholamines results in local functional alteration of the immune organs and other tissues.

1.9 Neuro – Immune Communication

Exposure to psychological stress has long been associated by clinicians with health problems for humans, including many mental, emotional and physical problems. Long term exposure to stress increases risk for heart diseases and hypertension. Stress can cause or aggravate diseases of the digestive tract including gastritis, stomach and duodenal ulcers and irritable bowel syndrome. Stress has also been shown to trigger mental and emotional problems such as insomnia, headaches, depression and anxiety and been linked to problems with asthma, hair loss, reproductive organs and skin (Rabin, 1999). More recently it has been reported that stress can impact on our immune system and result in suppression of various aspects of immunity. This can have serious implications for the onset and progression of various illnesses and diseases (Glaser and Kiecolt-Glaser, 2004).

However, the complex interactions between a stress detected in the central nervous system and the immune system are only beginning to be understood. This area of research is called psychoneuroimmunology (PNI), and PNI research is uncovering the

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mechanistic evidence about how stressors can result in physiological changes in the immune response to an infection. A number of reports have established that cross-talk exists between the central nervous system and the immune system (Elenkov *et al.*, 2000; Kohm and Sanders, 2000; Freidman and Irwin, 1997). The communication that occurs between the two major adaptive systems of the body is bi-directional (Figure 1.5). As previously stated, when a stressor is perceived by the CNS, the HPA and SAM axes are stimulated to release NA and GC hormones into the periphery. These end products of the stress response have been shown to be able to directly alter the functioning of immune cells via specific receptors that are located on immune cells (Elenkov *et al.*, 2000). The two stress hormones are released in concert following exposure to stress and they act together to enhance the immunomodulating effects that would be produced by either one alone. For example, it has been demonstrated that neither splenic nerve cut nor adrenalectomy alone could block the effects of stress on immune function. However, the combination of these two manipulations significantly abrogated the immunosuppressive effect of stress on cytokine production (Meltzer *et al.*, 2003). In addition, histofluorescence studies reported that immune organs are innervated by sympathetic / noradrenergic transmissions from the CNS and there is local release of stress hormones to the immune organs (Felten *et al.*, 1985). For example, the spleen is richly innervated by the sympathetic nervous system and has been the focus of neuro-immune research over the last number of years (Felten, 1987). In turn, the immune system can communicate to the CNS via soluble factors, such as cytokines, that are released from immune cells and can enter the CNS and stimulate the hypothalamus and brainstem (Besedovsky *et al.*, 1986). It has been demonstrated that both the HPA and SAM axis can be triggered by peripheral signals through a complex CRH-dependant pathway (Chrousos, 1995). Most of the HPA axis-stimulating activity in the periphery comes from cytokines that are produced in response to an inflammatory stimulus; TNF- α , IL-1 and IL-6.

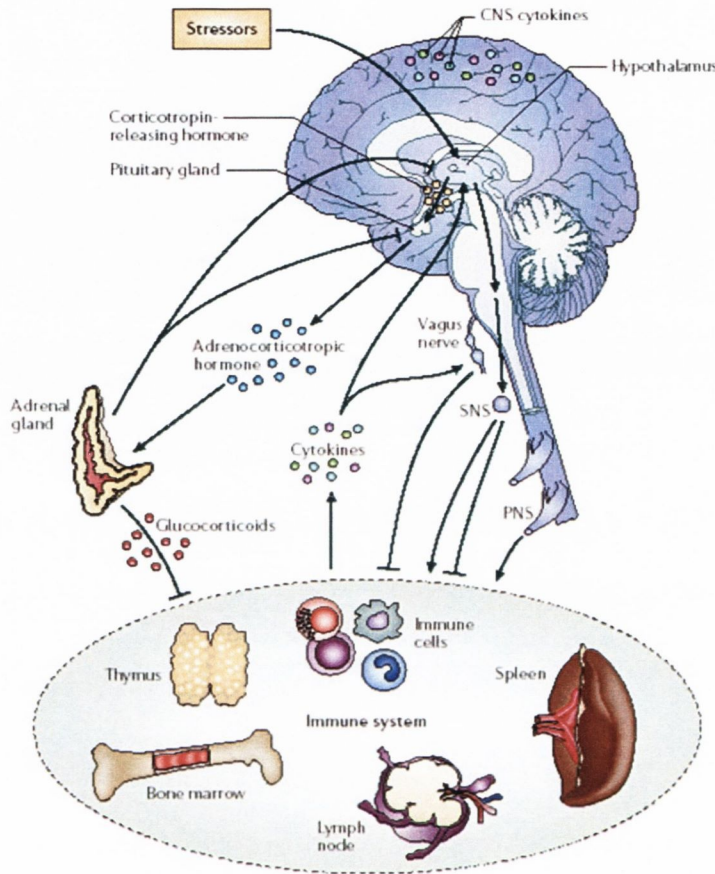


Figure 1.5: Neuro-immune communication indicating the signalling pathways involved (Sternberg, 2006)

1.9.1 GCs and GC receptors

Stress-induced activation of the HPA axis results in a large increase in peripheral GCs (Rivier, 1993). GCs are potent immunosuppressive agents capable of directly affecting the function of many immune responses. They belong to the most commonly and effectively used drugs in the clinic to relieve inflammation and various immune disorders, such as rheumatoid arthritis and inflammatory bowel diseases (Neeck, 2002). GCs have vast immunosuppressive effects because the GC receptor (GR) is expressed by nearly all cells in the body, including most immune cells (Ballard *et al.*, 1995). However, there is evidence that there is a dual role of GCs on immune function depending on the amount of GC produced. High doses of GC are immunosuppressive, however low doses of GCs are immunostimulating and it has been demonstrated that exposure to minor stress can increase course of an inflammatory disease, such as RA.

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The GR belongs to the family of steroid hormone receptors, and in its unliganded resting state it is present in the cytoplasm in an inactive complex with chaperones and co-chaperone molecules (Smith *et al.*, 1998). There are two types of GR (Type I and Type II) and they vary in their affinity to corticosterone. Type I has a much greater affinity for corticosterone with a 6 to 10 fold greater affinity than Type II GR (Spencer *et al.*, 1991). However, Type II receptors are more widespread than Type I receptors. Type I receptors are not detectable in the thymus, but Type II receptors are highly expressed in the thymus, spleen and lymph node (Spencer *et al.*, 1991). Ligand binding results in receptor hyperphosphorylation and activation. The activated GR can bind to specific DNA sequences – GC-response elements (GRE) and activate transcription of genes (Kunicka *et al.*, 1993).

GCs are of major importance for protection of the body against stress by regulating metabolic actions, behaviour, brain function, tissue maturation, wound healing and inflammatory and immune responses (De Bosscher *et al.*, 2003). The GC-induced modulation of the immune cells involves cross-talk with transcription factors, effects on cytokine receptor expression, regulation of thymocyte and lymphocyte survival, selection and function and interference with eosinopoiesis (Almawi *et al.*, 1996). If optimally balanced, GC-dependant functions will contribute to resolution of infection or trauma. However, disruption or malfunction of these dynamic interactions may result in a fatal outcome of acute inflammation or may predispose for autoimmunity reactions (Ballard *et al.*, 1995).

1.9.2 Noradrenaline and adrenergic receptors

NA is released in large quantities from the adrenal medulla into the blood following stress-induced activation of the SNS. The actions of NA are mediated via two types of adrenergic receptors (AR) of – α -AR and β -AR. There are two subtypes of α -ARs, the α_1 and α_2 and three subtypes of β -ARs, β_1 , β_2 and β_3 . β -AR expression has been detected on most lymphoid cell types, except T_H2 cells and evidence indicates that β_2 subtype mediates many of the suppressive effects of stress on the immune cells (Kohm and Sanders, 2000). However, NA can have an immunoenhancing effect when low doses of NA are present. The α -AR has a high affinity for NA, therefore when low doses are present, NA binds to α -AR and increases pro-inflammatory responses, such

as increasing TNF and IL-18 production (Nance and Sanders, 2007). In contrast, when high doses of NA are present, such as found following exposure to stress, the β -AR are activated which induce immunosuppressive effects. Although all lymphocytes have AR, differential density and sensitivity of AR expression may affect responsiveness to stress among cell subsets. For example, NK cells have both high density and high affinity β_2 -AR, B-cells have high density, but lower affinity and T-cells have the lowest density (Anstead *et al.*, 1998; Landman, 1992; Maisel *et al.*, 1989).

Once NA has bound to its receptors on immune cells they activate their signalling pathway which can alter functioning of the immune cell. The signalling pathway following β -AR stimulation is conducted via guanine nucleotide-binding proteins (G-proteins) that activate the intracellular enzyme, adenylyate cyclase (Elenkov *et al.*, 2000). Adenylyate cyclase stimulates the production of the second messenger cyclic adenosine 5'-monophosphate (cAMP), 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_2 AR on immune cells, cAMP levels are increased (Fedyk *et al.*, 1996 and Yang *et al.*, 2000). Production of cAMP is associated with an anti-inflammatory immune phenotype as it promotes the production of cytokines that can suppress cell mediated immune responses (Suberville *et al.*, 1996 and Platzner *et al.*, 2000). Therefore, high doses of NA which act on the β_2 -AR and β_2 -AR agonists are also anti-inflammatory agents in nature. For instance, it has been demonstrated that activated monocytes and DCs exposed to NA have reduced IL-12 concentrations, in parallel with increased production of the IL-10 (Elenkov *et al.*, 1996). This alteration of the cytokine network could potentially diminish the cell-based immune response, thus augmenting the chances for susceptibility to viruses, fungi and bacteria (Romani *et al.*, 1994).

1.10 Experimental models of stress

Investigations of the immunomodulating effects of stress are commonly carried out in animal models of stress as it is difficult to experimentally induce stress in a quantifiable way in humans. However, some studies have been carried out in humans to investigate the effect of exposure to life stress on health. In these experiments, researchers often investigate the psychological and immunological responses of individuals who are experiencing a distress-generating event, for example students

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taking an examination or more chronic stressor such as caring for a partner who has Alzheimer's disease (Segerstrom and Miller, 2004).

There are many different animal models of stress that vary in their duration, type and severity. However, all of the animal models of stress rely on the activation of the HPA and SAM axis and the subsequent release of glucocorticoids and catecholamines. There are two main categories of animal models of stress – physical and psychological stress. Animal models of physical stress include tail suspension, tail shock, forced swim stress, hot plate, immobilisation and restraint stress. However, restraint stress also has some psychological aspects associated with it. These stressors produce reliable activation of the HPA and SAM axis and increase the concentrations of corticosterone and catecholamines in the plasma and spleen of the mice exposed to the stress. For example, exposure to acute restraint stress has been shown to increase plasma corticosterone concentrations (from ~200 ng/ml to ~1000 ng/ml) and catecholamine concentrations in the spleen (from ~15 ng/mg to ~30 ng/mg) (Silberman *et al.*, 2003).

Exposure to chronic physical stress has been shown to result in adaptive changes and it is known to alter basic mechanisms of the stress response (Tannenbaum *et al.*, 2002). A substantial literature has focused on anatomical, behavioural and neuroendocrine changes associated to exposure with chronic stress. For example, it has been demonstrated that chronic stress exposure decreases body weight and food intake, induces adrenal hypertrophy, involution of the thymus, spleen and lymph nodes and affects neuronal plasticity (Bhatnagar and Dallman, 1998). However, despite these adaptive changes, it has been demonstrated by many groups that the concentrations of corticosterone and catecholamines remain elevated in the plasma and in the organs of mice exposed to chronic stress (Thaker, 2006; Marin *et al.*, 2006).

Animal models of psychological stress include social disruption stress, social confrontation and paired fighting. These stress models more closely mimic psychological stress in humans and therefore they are argued to be more relevant. However, long term exposure to psychological stress can only be investigated because it has been demonstrated that there is a much smaller response than exposure to an

acute physical stress (Blanchard *et al.*, 1998). However, with repeated exposures the intensity of psychological stress grows larger (Sato *et al.*, 1996).

1.11 The Effect of Stress on the Innate Immune Response

As previously discussed, exposure to stress activates the HPA and SAM axis to release stress signals to the periphery which can directly affect the function of immune cells. There are many reported alterations in the innate immune response following exposure to stress due to the susceptibility of the innate immune cells to the suppressive effects of stress hormones. Macrophage and neutrophil activity have been shown to be altered in response to stressful stimuli. For instance, there is a decrease in macrophage number and activity and the production of superoxides from macrophages is decreased following exposure to restraint stress (De Castro *et al.*, 2000). It has also been demonstrated that exposure to stress suppresses the expression of MHC class II molecules on macrophages (Zwilling *et al.*, 1993). Stressful stimuli also alter the chemotaxis of neutrophils and disrupt the recruitment of neutrophils to the site of infection (Shijo *et al.*, 1998). The activity of NK cells is influenced by a variety of physical and psychological stressors. For example, exposure to acute electric foot shock leads to a 25-50% suppression of splenic NK activity in rats (Dishman *et al.*, 2000; White-Welkley *et al.*, 1995). Psychological stressors such as social confrontation and social isolation have also been demonstrated to suppress cytotoxic activity of NK cells in rats (Oishi *et al.*, 2003). In addition, exposure of humans to psychological stress, such as severe academic stress, can down-regulate NK cell lysis. This reduction in NK cell activity is related to impaired cell trafficking in peripheral blood of stressed individuals (Kieckolt-Glaser *et al.*, 1986). It has been reported that exposure to stress reduces the number of lung NK cells via activation of β -AR, and the β -AR antagonist propranolol reversed this stress-induced reduction (Kanemi *et al.*, 2005).

In addition to the effect of exposure to stress on immune cell function, it has also been demonstrated that stress can alter cytokine production from immune cells. An alteration of the cytokine network can have significant effects on immune function as they control the response to an infection stimulus. The production and expression of many cytokines is altered in response to stressful stimuli, but in general there is a shift in cytokine production from a T_H1 to a T_H2 profile. In a study by Maestroni (2002) it was shown that NA can affect the production of cytokines from DCs in response to LPS *in vitro*.

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Bone-marrow DCs exposed to NA had a shift in their cytokine production from T_H1 profile to a T_H2 profile, with decreased IL-12 production and increased IL-10 production (Maestroni, 2002). In agreement with this finding, a number of other studies have also demonstrated that *in vivo* there is a stress-induced alteration in cytokine production towards a T_H2 profile. Following an *in vivo* challenge with LPS in rats, stress has been demonstrated to suppress the production of pro-inflammatory cytokines, such as IL-1 β and TNF- α (Goujon *et al*, 1995; Johnson *et al*, 2002) and increase the production of the anti-inflammatory cytokine IL-10 (Connor *et al*, 2005).

Stress-induced alteration of the cytokine network can have significant consequences on the innate immune response. IL-1 β and TNF- α are important signalling molecules in initiating and co-ordinating a range of immune responses against infectious stimuli (Hamblin and Henderson, 1994). Many studies have reported that the stress-induced inhibition of IL-1 β and TNF- α can have significant effects on response to an infectious stimulus and can result in defective host resistance to infection (Denis and Ghadirian, 1994). A stress-induced increase in IL-10 can suppresses cytokine synthesis from activated NK cells, monocytes/macrophages and DCs (Sato *et al.*, 1999). IL-10 can also block the ability of monocytes and DCs to act as APCs via down-regulation of MHC products and costimulatory molecules. In addition, the presence of IL-10 inhibits monocytes from differentiating into DCs and consequently they do not express CD1a cell marker and express lower levels of MHC class II (Allavena *et al.*, 1998).

1.12 Effect of Stress on the Adaptive Immune Response

Adaptive immune cells are also susceptible to the suppressive effects of stress hormones. It has been reported by many groups that B-cell and T-cell function are altered in response to stress. Following exposure to stress, B-cell proliferation is suppressed and there is reduced MHC class II expression on the surface of B-cells (Zhang *et al.*, 1998). In addition exposure to stress has been shown to have an impact on B-cell activation to a viral infection, leading to altered kinetics of the anti-viral antibody responses and diminished antigen specific plasma cell responses (Sheridan *et al.*, 1991).

T-cells have been shown to be particularly vulnerable to stress and their proliferation and differentiation can be altered in response to a stressful event. Initial studies on CD4⁺ T-cells show that these cells express β -AR which binds NA and increases intracellular

concentrations of cAMP resulting in an inhibition of proliferation and production of IL-2 (Bartik *et al.*, 1993). In addition, recent investigations have demonstrated that different subset of CD4⁺ T-cells respond differently to stimulation with NA due to differential expression of the β -AR. It was demonstrated that following exposure to a β_2 AR agonist, T_H1 cell clones accumulate cAMP while T_H2-cell clones did not (Sanders *et al.*, 1997). To expand on this finding, it was further demonstrated that resting and activated T_H1 cells express the β_2 AR binding site while T_H2 cells do not (Ramer-Quinn *et al.*, 1997). These results define a mechanism by which NA can decrease the level of cytokine production from T_H1 cells which inhibits IL-2 and IFN- γ production and cell-mediated immune responses. However, NA does not alter T_H2 cell function and increases the production of IL-4 and IL-5 and directs humoral immune responses (Ramer-Quinn *et al.*, 2000).

In addition to these studies, T-cell function was also demonstrated to be affected by stress *in vivo*. In a study by Sheridan *et al* (1991) it was demonstrated that exposure to restraint stress reduced anti-viral cellular immune responses, but not humoral immune responses to influenza virus. T-cell cytokine gene expression, cytokine secretion and the development of CTL cells were all impaired, however there was no change in the humoral response to the virus (Sheridan *et al.*, 1991). Exposure to stress has also been shown to alter human T-cell activity. *In vitro* treatment of human peripheral blood leukocytes with catecholamines results in the suppression of IL-12 synthesis and increase in IL-10 production (Elenkov *et al.*, 1996). These cytokines are activators of T_H2 cells and the stress-induced increase in production can cause a shift in the phenotype of CD4⁺ T_H cells from a T_H1 to a T_H2 profile. These results were supported by Glaser *et al.*, (2001) where they showed that medical students taking exams produced a shift in the cytokine balance toward a T_H2 profile. This stress-induced decrease in T_H1 cytokines results in a diminished cell-mediated immune response.

Evidence over the last 10 years indicates that GCs may cause selective suppression of the T_H1 cellular immunity axis and cause a shift toward a T_H2 mediated humoral immunity, rather than a generalised immunosuppression (Elenkov, 2004). This is a result of GC-induced inhibition of IL-12, IFN- γ , IFN- α and TNF- α by APCs and T_H1 cells, and GC-induced upregulation of IL-4, IL-10 and IL-13 by T_H2 cells. This means

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that during an immune response, the production of GCs may protect the organism from overshooting with T_H1 proinflammatory cytokines by causing a shift to T_H2 profile. However, exposure to chronic stress, which is associated with significant increases in GC levels, can modulate the T_H1/T_H2 balance and may affect the susceptibility to or the course of infections as well as autoimmune and atopic diseases (Calagni and Elenkov, 2006).

1.13 Stress-Induced Effects on Health

Animal and human studies have provided convincing evidence that stress-induced immune alterations can influence health and increase the susceptibility to infections agents, the severity of disease and the progression of the disease. For example, it has been demonstrated that stressors can diminish the strength of immune responses to vaccines, reactivate latent herpes virus and slow wound healing (Glaser and Kiecolt-Glaser, 2005). Stress-induced immunological alterations have also been considered as major risk factors for immune-related diseases, such as cancer, autoimmune disorders and infection (Mason, 1991).

Moreover, it was demonstrated in an animal models of stress that exposure to restraint stress can enhance the risk of developing infectious disease and they can prolong infectious episodes (Konstantinos and Sheridan, 2001). Restraint stress altered the immune response to a mouse model of influenza-virus, including the kinetics of the antibody response and suppression of both pro-inflammatory and anti-inflammatory cytokine responses. In addition to the mouse data on stress and influenza virus, it has also been demonstrated in human participants that the influenza-virus vaccine is less effective in stressed participants. In a study by Kiecolt-Glaser *et al.* (1996), it was shown that chronically stressed adults that have been caring for a partner with dementia have diminished cellular and humoral immune responses to an influenza-virus vaccine compared with controls. It has also been shown that stress can modify the antibody and T-cell responses to other antiviral vaccines, such as hepatitis B virus and rubella virus (Glaser *et al.*, 1992; Morag *et al.*, 1999). In agreement with these vaccine studies, it has also been shown that stress can alter an individuals susceptibility to infection with respiratory viruses (Cohen *et al.*, 1991; Stone *et al.*, 1992). Individuals who developed

cold symptoms following inoculation with rhinovirus had higher numbers of recent stressful life events than those who did not.

Herpes simplex virus (HSV) differs from other viruses in that it remains in a latent state in the body after primary infection. Stress is known to exacerbate HSV infection and studies in animal models and in humans have been developed to investigate the pathophysiology of the virus. Stress increases the development and severity of HSV infection, in both the peripheral nervous system and the CNS (Kusnecov *et al.*, 1992). It also suppresses the components of the primary and memory cytotoxic T-cells responses to HSV infection (Bonneau *et al.*, 2001). In addition, it has been demonstrated that stress can reactivate latent HSV in mice, whereas in non-stressed controls there is no reactivation of the virus (Padgett *et al.*, 1998).

Exposure to stress has been demonstrated to delay wound repair by disrupting the production of pro-inflammatory cytokines, such as IL-1, that are important for wound healing. In mouse studies investigating wound healing of a punch hole wound, it was found that exposure to restraint stress delayed the healing by 27% compared with non-stressed controls (Padgett *et al.*, 1998). Consistent with these findings in rodent models, it was also demonstrated that in Alzheimer's care givers it took 24% longer to heal a small wound than in matched controls (Kiecolt-Glaser *et al.*, 1995). These studies demonstrate that stress has substantial adverse effects on wound repair and in agreement with these laboratory findings, several studies have shown that greater fear or distress before surgery is associated with poorer outcomes, including longer hospitalization, more post-operative complications and higher rates of rehospitalisation (Kiecolt-Glaser, 2005).

1.13.1 The Effect of Stress on Immune-Based Diseases

Stress events or an inadequate response to them and anxiety have been associated to worsening of a variety of immune-based diseases including allergic asthma, psoriasis, atopic dermatitis and autoimmune diseases. Atopy is a genetic and environmental predisposition to a number of clinically expressed disorders including rhinitis, atopic dermatitis or eczema and allergic asthma. There is provocative evidence that psychological stress constitutes an increased risk for atopy (Wright *et al.*, 2004). This

Introduction

risk is thought to be mediated by the effects of stress on neuroimmunoregulation which in turn modulates the hypersensitivity response which is fundamental to atopy (Rosaland and Wright, 2005). Allergic diseases and asthma are caused by dysregulated T_H2 -biased immune responses to environmental allergens (Umetsu and DeKruyff, 2006). Therefore, stress-induced production of T_H2 cytokines would favor the inflammatory milieu characteristic of asthma and allergic diseases. In one study it was shown that in response to exam stress, asthmatic subjects demonstrated immunological changes such as decreased NK cell cytotoxicity and cytokine alterations (Marshall *et al.*, 2000).

Psoriasis is a mainly a T_H1 cell mediated chronic inflammatory skin disease characterized by epidermal hyper proliferation and psoriatic plaques (Krueger and Bowcock, 2005). Activated T-cells generated from psoriatic lesions secrete high concentrations of IL-2, TNF- α and IFN- γ indicating a significant role of T_H1 cell-mediated inflammatory processes in the psoriatic skin (Lowes *et al.*, 2004). It is widely accepted that psychosocial stress has an impact on psoriasis and may trigger exacerbation of the disease. In this regard, it has been demonstrated that psychological stress increases monocyte number and number of $CD4^+$ T-cells in PSO sufferers than in controls and this was accompanied by a significant decrease of $CD3^+/CD25^+$ cells (Buske-Kirschbaum *et al.*, 2006). In addition this study reported that exposure to stress resulted in elevated PBLM production of IFN- γ and IL-2, and moreover, a decreased production of IL-10 and IL-4 following exposure to phytohemagglutinin (PHA).

1.13.2 Effect of stress on cancer progression

For many years, doctors have been reporting an association between exposure to psychological stress and cancer onset and progression (see review Antoni, 2006). In addition, epidemiological data collected in the last 10-15 years indicates that exposure to psychological stress is linked with differential cancer onset and progression and mortality. For example, a 2 fold increase in breast cancer risk is evident after disruption of marriage owing to divorce, separation or death of a spouse (Lillberg *et al.*, 2003) and a 9 fold increase in risk with extreme stress and low social support (Price *et al.*, 2001). More recently, animal models have provided evidence for the involvement of chronic stress on tumorigenesis. For example, immobilisation stress in rats that were

given a carcinogen, diethylnitrosamine, increased both the incidence and rate of tumor growth (Laconi *et al.*, 2000). Many animal models of cancer have been investigated with exposure to different types of stressors and have found that exposure to stress increases the progression of many tumors in animal models. Some of the reported effects of stress are summarised the Table 1.1.

Experimental manipulation	Tumor type	Effect on tumor growth	Reference
Confrontation	Breast	↑ metastasis of tumor cells to lung	Stefanski and Ben-Eliyahu, 1996
Social isolation	Ehrlich	↑ growth	Palermo-Neto <i>et al.</i> , 2003
Restraint	Mammary	↑ tumor growth	Steplewski <i>et al.</i> , 1985
	Ovarian	↑ tumor growth	Thaker <i>et al.</i> , 2006
	Skin	↑ incidence, number, size and density	Saul <i>et al.</i> , 2005
Forced swim	Leukaemia	↑ mortality	Ben-Eliyahu <i>et al.</i> , 1991
Abdominal surgery	Mammary	↑ metastasis of tumor cells to the lung	Ben-Eliyahu <i>et al.</i> , 1991

Table 1.1 The reported effects of exposure to stress on cancer in animal models

The stress-induced increase in cancer progression is attributed to multiple components in the tumor microenvironment. It has been reported that exposure to stress alters the function of immune cells, cancer cells, oncogenic viruses and blood vessels. As previously described, immune cell function is altered in response to stress with impaired antigen presentation, alteration in T-cell proliferation to a T_H2 or humoral mediated immune response and compromised NK-cell function (Elenkov, 2002). NK-cell cytotoxicity is essential for effective anti-tumor immunity and NK-cell activity has been demonstrated to be compromised following exposure to stress in both human and animal studies (Ben-Eliyahu *et al.*, 1999). It has also been demonstrated that exposure to stress can increase cancer cell migration and invasion with stress-induced increases in proteases, such as MMPs (Sood *et al.*, 2006). In addition, stress hormones can activate oncogenic viruses, with increased transcription, replication and host-cell

cycling of viruses (Romero *et al.*, 1992). Finally, exposure to stress has been associated with stimulation of blood vessel angiogenesis by inducing production of pro-angiogenic cytokines, such as VEGF and IL-6 (Lutgendorf *et al.*, 2002).

The molecular mechanisms responsible for mediating the effects of stress on tumor tissues are not fully understood. However, it has been demonstrated that β -ARs which mediate most of the effects of catecholamines, are present on breast and ovarian cancer cells (Badino *et al.* 1996). In addition, β -AR agonists, such as metaproterenol show dose-dependant increases in lung tumor metastases (Ben-Eliyahu *et al.*, 1991). Pre-treatment of animals with β -AR antagonists, which blocks SNS activity, also blocked the effect of exposure to stress on lung tumor metastasis (Melamed *et al.*, 2005).

1.14 Pro-inflammatory effects of acute and chronic stress

As previously discussed, stress has been long associated with an immunosuppressive state. However, several studies claim that under certain circumstances, stress can be immunoenhancing. The findings from these studies indicate that the impact of stressor exposure on the immune response depends on a variety of factors.

The duration of stressor exposure seems to be important in determining the immune response and it has been reported that acute stress enhances, whereas chronic stress inhibits immune responses (Dhabhar and McEwen, 1999). For example, acute stress increased mitogen-induced T-cell proliferation, T-cell dependant delayed-type hypersensitivity response and antibody production (Millan *et al.*, 1996; Bauer *et al.*, 2001). The immunoenhancing effects of exposure to acute stress are hypothesised to prepare the immune system for immediate challenges (e.g. wounding) that may be imposed by a stressor (e.g. an aggressor) (Dhabhar, 2002). Consistent with this hypothesis, it has been reported that exposure to acute stress can result in redistribution of blood leukocytes and enhancement of skin cell mediated immunity (Dhabhar and McEwen, 2000). However, other authors found acute stress exposure did not lead to immunoenhancement. For example, acute exposure to footshock could decrease T-cell dependant antigen depending on the severity of the stressor (Zalcman and Anisman, 1993). It has also been shown that acute swim stress suppresses aspects of both innate and adaptive immunity in rodents (Ben-Eliyahu *et al.*, 2000; Connor *et al.*, 1997;

Salman *et al.*, 2000; Shu *et al.*, 1993). In addition, in human studies it has been shown that short-term acute stress exposure can have significant immunosuppressive effects on immune cell function and proliferation (Kennedy *et al.*, 1988). Therefore it appears, that while acute stress can enhance some aspects of the innate immune response, it impairs other aspects of the innate immune response and the adaptive immune response. This conclusion is in agreement with a meta analysis study conducted by Segerstrom and Miller (2006) where they showed that in human studies of stress, an altered immune profile could be induced depending on the time frame of stressor exposure. Acute, time-limited stressors triggered upregulation of some innate immune responses and suppression of specific immunity, brief naturalistic stressors or real-life short term challenges could cause a cytokine shift and chronic stress caused global immunosuppression (Segerstrom and Miller, 2006).

Another factor that appears to determine the stress-induced immune profile is the type of experimental stressor that is used. Some reports have shown that physical stressors such as electroshock, swimming or immobilisation can provoke different responses than psychological stressors such as social isolation and social confrontation (Oishi, *et al.*, 2003). For example, studies have shown that exposure to chronic social disruption stress and paired fighting stress results in altered splenocyte and function (Avitsur *et al.*, 2002a). It is reported that in response to exposure to social stress increased splenic monocytes and neutrophils and decreased lymphocytes (Avitsur *et al.*, 2002b). These findings may have implications for the healing of bite wounds that often are associated with social stress in rodents. However in the studies using social stress, and in other studies that show an immunoenhancing effect of exposure to chronic stress, the temporal proximity between the stressor termination and the immune challenge is delayed. It has been reported that an immunoenhanced state becomes evident within 24 hr of stressor termination (Zalcman *et al.*, 1998). Therefore, an immune challenge administered immediately following stress and 24 hr after stressor termination could give alternative immune responses.

1.15 The Objective of this Thesis

Exposure to acute and chronic stress suppresses various aspects of the immune response, including cytokine production (Goujon *et al.*, 1995). The overall objective of this thesis is to further investigate the effects of acute and chronic psychological stress (restraint stress) on cytokine production following LPS challenge. In addition, we aim to investigate the mechanisms underlying this stress-induced immunosuppression and to assess the contribution of the stress hormones. Exposure to chronic stress is associated with increased risk for the onset of infection and accelerated progression of diseases and cancer (Glaser and Kiecolt-Glaser, 2005). Therefore, we also aimed to assess the effect of exposure to chronic stress on the progression of a tumor in mice. Finally, in this thesis, we investigated if there was any alteration in the immune response to an LPS challenge given 24 hr following termination of chronic stress. There is some evidence that a pro-inflammatory profile becomes evident in the hours following termination of stressor exposure (Zalcman *et al.*, 1989).

The specific aims of the thesis are:

1. To determine the effect of exposure to acute and chronic stress on the innate IFN- γ response and on the production of IFN- γ inducing cytokines, IL-12 and IL-15, following an *in vivo* challenge with LPS.
2. To investigate the effect of stress on the production of the anti-inflammatory cytokine IL-10 and to explore if IL-10 mediates the immunosuppressive effects of stress on the innate IFN- γ response.
3. To assess the roles of GCs and catecholamines as mediators of stress-induced suppression of the innate IFN- γ response.
4. To determine if exposure to chronic restraint stress can influence progression of cancer and alter cytokine production in the tumor microenvironment.
5. To investigate if alteration of the temporal proximity between an immune challenge and termination of a stressor can produce a pro-inflammatory effect by increasing IL-12 and IFN- γ production.

Chapter 2:

Materials and Methods

2.1 Materials

Animals

Balb/c mice	Bioresources, TCD
IL-10 KO mice	Bioresources, TCD
Balb/c mice / C57 Bl/6 mice	Harlan Labs, UK
Lab mouse diet	Red Mills, UK

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

Chlordiazepoxide	Sigma, Ireland
<i>Escherichia coli</i> Lipopolysaccharide serotype 0111:B4	Sigma, Ireland
Nadolol	Sigma, Ireland
Norepinephrine + bitartrate salt hydrate (99%)	Sigma, Ireland
Recombinant mouse IL-10	R & D Systems, UK
Recombinant mouse IL-12	R & D Systems, UK
Salbutamol	Sigma, Ireland

Cell Culture

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

ELISA

Mouse IL-10 ELISA Kit	Biosource, UK
Mouse IL-12 ELISA Kit	Biosource, UK
Mouse IFN-γ ELISA Kit	Biosource, UK
96 well maxisorp immunoplates	Nunc, UK

Western Blotting

Anti-mouse - β-actin antibody	Sigma, Ireland
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Materials

Anti-mouse IgG Horseradish Peroxidase antibody	Sigma, Ireland
Anti-rabbit IgG Horseradish Peroxidase antibody	Amersham, UK
β_2 -AR antibody	Santa Cruz, UK
Broad-range molecular weight standard	Biorad, USA
Filter paper	Whatman, UK
Glucocorticoid receptor antibody	Santa Cruz, UK
Hyper-film	Amersham, UK
Nitrocellulose membrane	Amersham, UK
Phospho-Stat1 (Tyr701) antibody	Cell Signaling, USA
Restore™ Western Blot stripping buffer	Pierce, USA
STAT1 antibody	Cell Signaling, USA
Supersignal® West Dura extended substrate solution	Pierce, USA
Standard grade No.3 filter paper	Whatman, UK

Chemicals

Acrylamide	Sigma, Ireland
N'N' Bis Acrylamide	Sigma, Ireland
Ammonium chloride	Sigma, Ireland
Ammonium Persulphate	Sigma, Ireland
Bicinchonic acid (BCA) protein assay	Pierce, UK
Bovine serum albumin (BSA)	Sigma, Ireland
Bromophenol blue	Sigma, Ireland
β -Mercaptoethanol	Sigma, Ireland
Diethyl Pyrocarbonate (DEPC)	Sigma, Ireland
DL-Dithiothreitol (DTT)	Sigma, Ireland
Dulbeccos PBS	Sigma, Ireland
Glycerol	Sigma, Ireland
Glycine	Sigma, Ireland
Hydrochloric acid (HCl)	BDH, UK
Methanol (MeOH)	BDH, UK
Protease inhibitor cocktail	Sigma, Ireland
Potassium Chloride (KCl)	Merck, UK
Potassium dihydrogen orthophosphate (KH ₂ PO ₄)	Sigma, Ireland
2-propanol	Sigma, Ireland

Sodium azide	Sigma, Ireland
Sodium carbonate (Na_2HCO_3)	BDH, UK
Sodium bicarbonate (NaHCO_3)	BDH, UK
Sodium phosphate monobasic monohydrate (NaH_2PO_4)	Sigma, Ireland
Sodium chloride (NaCl)	Sigma, Ireland
Sodium dodecyl sulfate (SDS) 99%	Sigma, Ireland
Sodium phosphate (biphasic)	BDH, UK
Sulphuric acid (H_2SO_4) 98%	Sigma, Ireland
N,N,N',N'-Tetramethylethylene-diamine (TEMED)	Sigma, Ireland
Tetramethylbenzidine	Dakocytmatation, UK
Tween-20	Sigma, Ireland
Tris-base	Sigma, Ireland
Trypan Blue	Sigma, Ireland
Thymidine (^3H)	New Dupont ISIS

Molecular Reagents

Absolute ethanol	Sigma, Ireland
Agarose	Condra
Biosphere filter tips (1000, 200 and 100 μl)	Sarstedt Inc, Ireland
Diethyl pyrocarbonate	Sigma, Ireland
Ethidium bromide	Sigma, Ireland
High capacity cDNA archive kit	Applied Biosystems
Loading dye (6X)	Promega, UK
Molecular grade water	Sigma, Ireland
Optical adhesive covers	Applied Biosystems
PCR tubes	Sarstedt Inc, Ireland
RNA <i>later</i> TM	Ambion, UK
RNase away	Invitrogen, UK
RNase-free 1.5ml and 2ml microfuge tubes	Ambion, UK
RNase Zap wipes	Ambion, UK
Total RNA isolation kit	Macherney-Nagel
TaqMan gene expression assays	Applied Biosystems

Materials

TaqMan universal PCR master mix

10X TBE buffer

96-well optical reaction plates

Applied Biosystems

Invitrogen, UK

Applied Biosystems

Laboratory Plastics

Sterile falcon tubes (15 & 50ml)

Sterile petri dishes

Sterile 48 well plates

Sterile 96 well plates

Serological Pipettes (10ml & 25ml)

Pipettes tips (1ml & 500 μ l)

Flat bottom 96 well plates

Sterile Round bottom 96 well plates

Plastic syringes (20ml & 1ml)

Polystyrene round bottomed tubes (5ml)

Microtubes (1.5ml & 0.5ml)

Sarstedt Inc, Ireland

Sarstedt Inc, Ireland

Sarstedt Inc, Ireland

Sarstedt Inc, Ireland

Sarstedt Inc, Ireland

Sarstedt Inc, Ireland

Sarstedt Inc, Ireland

Sarstedt Inc, Ireland

Becton Dickenson

Becton Dickenson

Sarstedt Inc, Ireland

2.2 Methods: *In vivo* studies

2.2.1 Animal husbandry

Adult male Balb/c mice (6-8 weeks) were obtained from the Bioresources Unit in Trinity College Dublin or Harlan Limited (Oxford, UK). C57BL/6 (C57) mice were obtained from Harlan (Oxford, UK) and IL-10 KO mice were bred and obtained from Bioresources Unit (Trinity College Dublin, Ireland). All mice were housed in hard-bottomed polypropylene cages with wood shavings as bedding. Animals were housed 6 to a cage under standard laboratory conditions, with an ambient temperature of 20-24°C Celcius (C) and a 12 hour (hr) light: 12 hr dark cycle (lights on 08.00, lights off: 20.00). Animals had free access to food and water and were fed a standard laboratory diet (Red Mills, Ireland).

2.2.2 Physiological assessments

A record of body-weight was maintained as a general indication of the health and well-being of each animal throughout the studies.

2.2.3 Acute restraint stress procedure

Physical restraint restricts an animal's movement and access to food and water. The restraint stress procedure that was utilized in the studies presented here was based on previous studies using physical restraint (Sheridan *et al.*, 1991). However, a 2 hr restraint time was chosen as this has been shown to be sufficient to result in immunomodulation (Thaker *et al.*, 2006).

For the restraint stress procedure, mice were placed in well ventilated 50 milliliter (ml) falcon tubes (Sarstedt Inc, Ireland) for 2 hr. The tubes were secured parallel to the table top in racks and placed in a bright room with an ambient temperature of 20-24°C. Individual mice were placed in tubes at 9:00 am and removed at 11:00 am. Control mice were food and water deprived during the same time period, however these mice were free to roam in their cages.

2.2.4 Chronic restraint stress procedure

For the chronic restraint stress procedure, adult male balb/c mice were exposed to a 2 hr restraint stress per day for 7 consecutive days. Each restraint stress involved the mouse being put into a well ventilated 50 ml falcon tube and the lid was closed tightly. The

Methods

restraint tubes were secured parallel to the table top in tube racks and placed in a bright room with an ambient temperature of 20-24°C for 2 hr.

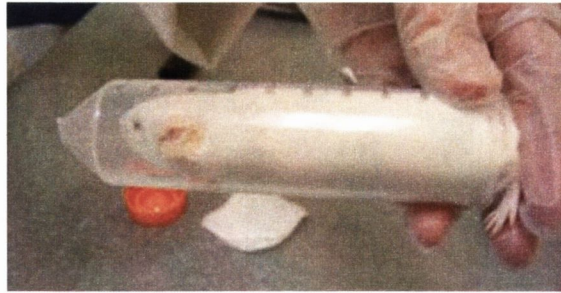


Figure 2.1 Photograph of Balb/c adult mouse undergoing restraint stress in a ventilated 50 ml falcon tube

2.2.5 Tumor injections with chronic restraint stress

Adult Balb/c mice were injected with human carcinoma cell line CT26 cells (100 μ l / mouse; sub cutaneous [s.c.]). The mice were immediately exposed to 2 hr restraint stress and stressed chronically for 2 hr per day for 24 days. The mice were sacrificed following the final session of stress and tumor mass and lymph nodes were dissected out. The freshly isolated tumor masses and lymph nodes were immediately prepared for FACs analysis as described in section 2.7.

2.2.6 Drug administration

2.2.6.1 LPS challenge

Mice were challenged with an injection of either the bacterial endotoxin lipopolysaccharide (LPS; Sigma, Ireland) (250 μ g/kg) or vehicle (0.89% NaCl in dH₂O) immediately following the 2 hr acute stress procedure described above. Injections were given via the intraperitoneal (i.p.) route using an injection volume of 10 ml/kg. Mice were sacrificed 1.5 hr or 8 hr post-LPS or vehicle injection.

2.2.6.2 Anti-IL-10 receptor antibody administration

Adult male balb/c mice were pre-treated with the anti-IL-10 receptor antibody (0.3 mg per mouse; i.p) or rat IgG1 isotype control (0.3 mg per mouse; i.p.) 2 hr prior to exposure to 2 hr acute restraint stress. LPS (250 μ g/kg) was administered immediately following stressor termination and the mice were sacrificed 8 hr post LPS administration.

2.2.6.3 Nadolol administration

Adult male balb/c mice were administered with nadolol (0.5 mg/kg; i.p.) or vehicle (0.89% NaCl; i.p.) 30 min prior to exposure to 2 hr acute restraint stress. LPS (250 µg/kg; i.p.) was administered immediately following stressor termination and the mice were sacrificed 1.5 hr post LPS administration.

2.2.6.4 Mifepristone administration

Adult male balb/c mice were administered with mifepristone (20 mg/kg; i.p.) or vehicle (0.89% NaCl; i.p.) 1 hr prior to exposure to 2 hr acute restraint stress. LPS (250 µg/ml; i.p.) was administered immediately following stressor termination and the mice were sacrificed 8 hr following LPS administration.

2.2.6.5 Chlordiazepoxide administration

Adult male balb/c mice were administered with 3 injections of chlordiazepoxide (CDP) (10 mg/kg; i.p.) or vehicle (0.89% NaCl; i.p.) at 48 hr, 24 hr and 2 hr prior to exposure to 2 hr acute restraint stress as described above in section 3.2.1. LPS (250 µg/ml; i.p.) was administered immediately following stressor termination and mice were sacrificed 1.5 hr following LPS administration.

2.2.7 Collection of trunk blood

Trunk blood was obtained by decapitating the mouse and collecting the blood into a 1.5 ml microtube. The trunk blood was centrifuged at 2000 rpm for 15 min at 4°C. The resultant serum was removed and stored at -80°C until cytokine analysis was carried out. ELISA's for IFN-γ and IL-10 and IL-12 were carried out as described in section 2.4.2.

2.2.8 Collection of spleen for protein analysis

Following sacrifice, mice were immediately dissected and the spleen was removed. The spleens were quickly transferred to 1.5 ml microtubes and snap-frozen on dry ice and stored at -80°C until protein analysis was conducted. The frozen spleens were removed from the freezer and prepared for western immunoblotting analysis as described in section 2.4.4.

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2.2.9 Collection of spleen portion for mRNA extraction

A small portion (~25mg) of the spleen from each mouse was placed in RNase-free tubes (Sarstedt, Germany) containing RNA later™ (Ambion, Japan) and stored for ~7 days at 4°C. All samples were then removed from the RNA later solution, transferred to fresh RNase-free tubes and frozen at -80°C until RNA extraction was performed as described in section 2.5. Real time PCR was carried out on the transcribed cDNA samples made from the extracted splenic mRNA samples to analysis the mRNA expression of the targets in table 2.2.1.

2.3 Methods: *In vitro* studies

2.3.1 Aseptic Techniques

Aseptic techniques were utilised during all cell culture work and also in the preparation of cell culture reagents. This is necessary to maintain a sterile environment free from fungal, bacterial and viral infections that can alter normal cellular functions. Aseptic techniques utilised, include the use of sterile disposable plastics, and sterilisation of glassware, plastics and H₂O by autoclaving at 121°C for 30-60 min. Dissection equipment was baked for a minimum of 2 hr at 200°C to ensure sterility. All cell culture work was carried out in a laminar flow hood (Hera Safe, category 2). This allows only filtered air to come into contact with cells, thus preventing contamination with airborne pathogens. The interior of the hood was wiped down with 70% ethanol (EtOH) before and after use. The hood surface was also exposed to ultraviolet (UV) light for 15-30 minutes after use. Any items taken into the flow hood were lightly sprayed with 70% EtOH to prevent introduction of any pathogens to the hood work area. Disposable latex gloves were worn and sprayed with EtOH before use. Gloves were changed regularly during cell culture work. Cells were maintained in a sterile Nuaire incubator (95% air, 5% CO₂ at 37°C) and any items put in the incubator were lightly sprayed with EtOH to prevent contamination with any pathogens. Both the incubator and laminar flow hood were regularly cleaned with Biocidal ZF (Invitrogen, Ireland) to maintain a sterile environment.

2.3.2 Preparation of bone marrow dendritic cells

Adult male balb/c mice were sacrificed by decapitation and their hind legs were removed and immediately stored in RPMI at 37°C. In a laminar flow hood, the flesh was removed from the legs to reveal the bone. Bone marrow was flushed from the bone

by inserting a 27 gauge (G) needle into one end and forcing media through it. The media was collected in Petri dish and the cell aggregates were broken up using a 19 G needle. The cell suspension was put into a 50 ml falcon tube and centrifuged at 1200 rpm for 5 min at room temperature (RT). The supernatant was removed and the pellet was disrupted before the addition of 2 ml of ammonium chloride (0.89%). After 2 min incubation with ammonium chloride, 10 ml of media was added and the suspension was centrifuged at 1200 rpm for 5 min at RT to wash the cells. This wash step was repeated and the final pellet was resuspended in 10 ml of RPMI. The white blood cells were counted using trypan blue (0.4% in Hanks balanced salt solution) and the final concentration of the cell suspension was adjusted to 10^6 cells / ml in 10 ml of complete RPMI 1640 medium (RPMI 1640 supplemented with 1% Pen/Strep and 10% Fetal Bovine Serum) + GM-CSF (40ng/ml). Cells were cultured in T75 tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO₂, and the medium was changed every 3 days. On day 6 cells were harvested, counted using the trypan blue dye exclusion method, and the cell concentration adjusted to 10^6 /ml. Cells were reseeded in new T75 tissue culture flasks and cultured in the same conditions for another three days. On day 10 cells were transferred to 24 well plates in order to facilitate drug treatments

2.3.3 Preparation of a single cell suspension of mouse splenocytes

Adult male Balb/c mice were sacrificed by decapitation and their spleens were removed and immediately stored in RPMI at 37°C. In a laminar flow hood, the spleens were mashed through a nylon sieve (40 mM cell strainer BD falcon) into a sterile Petri dish with 5 ml RPMI. The solution was titrated with a 10 ml pipette until all the clumps of cells were removed and a single cell suspension was obtained. The splenocytes solution was put in a 15 ml falcon tube and centrifuged at 1300 rpm for 5 min at RT. The supernatant was aspirated off and the red blood cells were lysed by the addition of 2 ml ammonium chloride (0.89%) for 2 min. The reaction was stopped by the addition of 5 ml of RPMI. The solution was centrifuged at 1300 rpm for 5 min at RT and the supernatant was removed. The cells were washed twice by resuspending them in 10 ml RPMI and centrifuging again. The final pellet was resuspended in 10 ml RPMI and the white blood cells were counted using a blood cell counter (Panvet lab). The final concentration of the cell suspension was adjusted to 2×10^6 cells / ml.

Methods

2.3.4 Preparation of culture media and PBS

RPMI 1640 (Invitrogen, Ireland) containing 10% fetal calf serum (FCS), 1% L-Glutamate and 1% penicillin-streptomycin (complete RPMI) was used for all cell cultures experiments. Briefly, 50ml of FCS, 5ml of L-Glutamate and 5ml of penicillin-streptomycin were sterile filtered using a 0.2 millimeter (mm) syringe filter and added to a 500 ml bottle of RPMI.

A working solution of PBS was prepared by adding 1 ml of Dulbecco's sterile concentrated (10x) PBS (100 mM NaCl, 80 mM Na₂HPO₄, 20mM NaH₂PO₄) to 9 ml of autoclaved distilled (d) H₂O.

2.3.5 Preparation of treatment compounds

2.3.5.1 LPS

25mg of Escherichia coli lipopolysaccharide (LPS) (Sigma, Ireland; serotype 0111:B4) was reconstituted in 1 ml of dH₂O to give a solution of 25 mg/ml. The solution was vortexed and when fully dissolved 500 µl was added to 62 ml of complete RPMI (1:125 dilution) to give a 200 mg/ml stock solution which was then sterile filtered using a 0.2 mm syringe filter. Stock solution was frozen at -20°C in 1 ml aliquots for future use.

2.3.5.2 Noradrenaline

A stock solution of noradrenaline (NA) [Formula weight (F.W.) is 337 g/ mole (M)] was prepared by adding 5 ml of dH₂O to 0.337 g of NA to give a stock solution of 200 mM that was then filter-sterilised using a 0.2 mm syringe filter. This stock solution was frozen at -20°C in 200 ml aliquots for future use.

2.3.5.3 Propranolol

A stock solution of propranolol [F.W. 259.34 g/M] was prepared by adding 5 ml dH₂O to 0.259g of propranolol to give a stock solution of 200 mM that was then filter-sterilised using a 0.2 mm syringe filter. This stock solution was frozen at -20°C in 200 ml aliquots for future use.

2.3.5.4 Salbutamol

A stock solution of salbutamol [F.W. 239.31g/M] was prepared by adding 5 ml d H₂O to 0.239g of Sal to give a stock solution of 200 mM that was then filter-sterilised using

a 0.2 mm syringe filter. This stock solution was frozen at -20°C in 200 ml aliquots for future use.

2.3.5.5 Corticosterone

A stock solution of corticosterone [F.W. 346.46 g/M] was prepared by adding 5 ml d H_2O to 0.346g of corticosterone to give a stock solution of 200 mM that was then filter-sterilised using a 0.2 mm syringe filter. This stock solution was frozen at -20°C in 200 ml aliquots for future use.

2.3.6 Cell treatments

2.3.6.1 Treatment of splenocytes with IL-10

Splenocytes were prepared from freshly isolated spleens of adult male balb/c mice as previously described in section 2.3.3. 475 μl of the single cell suspension of splenocytes were pre-treated with 25 μl of recombinant mouse IL-10 (1-10 ng/ml at 20 x concentration) prior to stimulation with 50 μl of LPS (1 $\mu\text{g}/\text{ml}$ at 11 x concentration). Cells were cultured at 37°C in 5% CO_2 atmosphere for 24 hr and 96 hr after which time the cell supernatant was removed from the culture plate and stored in 1.5 ml tubes at -80°C . ELISA's were carried out on the defrosted cell supernatant for IL-12 and IFN- γ as described in section 2.4.1.

2.3.6.2 Treatment of splenocytes with IL-12

Splenocytes were prepared from freshly isolated spleens of adult male balb/c mice as previously described in section 2.3.3. 475 μl of the single cell suspension of splenocytes were pre-treated with 25 μl NA (10 μM at 20 x concentration) for 30 min prior to stimulation with 50 μl LPS (1 $\mu\text{g}/\text{ml}$ at 11 x concentration). Following LPS administration the cells were incubated at 37°C in 5% CO_2 atmosphere and treated with 25 μl of recombinant mouse IL-12 (0.1-1 ng/ml at 25 x concentration) at 2 hr, 24 hr and 48 hr after LPS administration. The cell supernatant was harvested at 72 hr after LPS administration and stored in 1.5 ml eppendorfs at -80°C . ELISA's were carried out on the defrosted cell supernatant for IL-12 and IFN- γ as described in section 2.4.2.

2.3.7 Harvesting cell-free supernatants for cytokine ELISAs

After the required incubation time, the plates were removed from the incubator and supernatant removed and stored in 1.5 ml microtubes according to the treatment plan.

Methods

All samples were kept on ice before being centrifuged at 13,000 rpm for 3 min at 4°C to remove any cell debris. Samples were then put into aliquots (2-3 aliquots per sample) on 96 well plates for storage and frozen at -80°C until cytokine analysis.

2.3.8 Harvesting DC cells for mRNA analysis

The DC cells were harvested for mRNA immediately following the removal of the supernatant. Isolation of total RNA from Dendritic Cells on cell culture plates was carried out using Nucleospin RNA II kits (Macherey-Nagel) as per the protocol outlined below in section 2.5.2.

2.4 Analytical methods: Protein

2.4.1 Analysis of corticosterone concentrations by immunoassay kit

Corticosterone measurements were measured in serum samples and spleen homogenate using a corticosterone enzymeimmunoassay (EIA) kit (IDS, UK). Briefly, the samples were diluted 1:10 with sample diluent and vortexed. 100 µl of the diluted samples and standards were added to the antibody coated immunoassay plate (rabbit anti-corticosterone antibody coated plate) in duplicate. 100 µl of enzyme conjugate solution was added to all wells using a multichannel pipette. The plate was covered with an adhesive plate sealer and incubated at 2-8°C for 16-24 hr. The plate was washed 3 times with 250 µl wash solution. The plate was inverted and tapped firmly on absorbent tissue to remove excess wash solution. 200 µL of TMB substrate solution was added to all wells using a multichannel pipette and incubated at 18-25°C for 30 min 100 µL of stop solution (HCL) was added to all wells using a multichannel pipette and the absorbance of each well was measured at 450 nm using a microplate reader within 30 min of adding the stop solution. The percent binding (B/Bo%) of each calibrator, control and unknown sample was calculated as follows:

$$B/Bo\% = \frac{(\text{mean absorbance}) \times 100}{(\text{mean absorbance for '0' calibrator})}$$

A calibration curve was prepared by plotting B/Bo% on the ordinate against concentration of corticosterone on the abscissa. B/Bo% for each unknown sample was calculated and values were read off the curve in ng/mL and multiplied by dilution factor.

2.4.2 Analysis of cytokine concentrations by ELISA

The sandwich enzyme-linked immunosorbent assay (ELISA) method was used to determine cytokine concentrations in serum, spleen supernatants or cell culture supernatants.

2.4.2.1 Mouse IL-10 ELISA

The concentrations of IL-10 in serum, spleen supernatants or culture supernatants were determined using a commercially available mouse IL-10 ELISA kit (Biosource, Belgium). The capture antibody (rat anti-mouse IL-10 serum) was diluted to a working concentration of 1.25 µg/ml with bicarbonate coating buffer (51mM NaHCO₃, 50mM Na₂CO₃ pH 9.4). 100 µl of the capture antibody solution added to flat-bottomed 96 well plate (NUNC, F96 MAXISORP-immuno plate) and incubated overnight at 4°C. The wells were washed 4 times with 300 µl of wash buffer (PBS with 0.05% Tween-20) and excess wash buffer removed by blotting plate on a paper towel. Plates were blocked for a minimum of 2 hr at RT with 300 µl of blocking buffer (PBS with 5% BSA). The standards were prepared for loading on the plate by preparing the stock IL-10 (recombinant mouse IL-10). The stock was reconstituted in 1.6 ml of reagent diluent to give a concentrated reconstituted standard of 10,000 picograms (pg) / ml. The concentrated stock solution was diluted to a working concentration of 2000 pg/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. 100 µl of each standard and sample was loaded onto the plate in duplicate and incubated for 1 hr at RT. After incubation period the plates were washed as before and 100 µl per well of biotinylated detection antibody (0.125 mg/ml, in PBS containing 0.5% BSA, 0.1% Tween-20 and 5% fetal bovine serum) was added to the plates, which were then incubated for 1 hr at RT. Plates were washed as before and 100 µl of the working solution streptavidin-horseradish peroxidase (HRP) conjugate (1:2000 dilution in PBS with 0.5% BSA and 0.1% Tween-20) was added to each well and incubated for 45 min at RT, avoiding direct light. Following 4 washes, 100 µl of tetramethylbenzidine (TMB) substrate solution was added to each well. This was incubated for 20 min or prior to the colorimetric reaction reaching saturation. 100 µl of stop solution (1M H₂SO₄) was then added to each well and the absorbance measured at 450 nanometers (nm) using a microtitre plate reader (ELx800; Biotek, Germany). A standard curve was constructed by plotting the standards

Methods

against the absorbance and results obtained expressed as pg/ml of supernatant / serum or % control.

2.4.2.2 Mouse IL-12 ELISA

IL-12 concentrations were measured in serum and cell supernatant samples using a commercially available mouse ELISA kit (Biosource, Belgium). The capture antibody (rat anti-mouse IL-12 serum) was diluted to a working concentration of 1.25 µg/ml with bicarbonate coating buffer (51mM NaHCO₃, 50mM Na₂CO₃ pH 9.4). 100µl of the capture antibody solution was added to flat-bottomed 96 well plate (NUNC, F96 MAXISORP-immuno plate) and incubated overnight at 4°C. The next morning, the wells were washed 4 times with 300 µl of wash buffer (PBS with 0.05% Tween-20) and excess wash buffer removed by blotting plate on a paper towel. The plates were blocked with blocking buffer (5%BSA-PBS-T) for 2 hr at RT. Subsequently, plates were washed as mentioned previously. The standards were prepared for loading on the plate by preparing the stock IL-12 (recombinant mouse IL-12). The stock was reconstituted in 2.5 ml of reagent diluent to give a concentrated reconstituted standard of 500 pg/ml and used as the top standard, after which, 1:2 serial dilutions were carried out using the standard diluent assay buffer. 100 µl of each standard and sample was loaded onto the plate in duplicate and incubated for 1 hr at RT. After the incubation period, the plate was washed 4 times. The detection antibody (rat anti-mouse IL-12 biotin) was diluted in SDAB to a working concentration of 0.125 µg/ml and 100 µl of solution added to each well and incubated for 1 hr at RT. Another wash step was carried out on the plates. HRP was diluted in SDAB (1:2000 dilution in PBS with 0.5% BSA and 0.1% Tween-20) and 100 µl was added to each well and incubated for 45 min. Plates were washed again as before and 100 µl of TMB was added to each well for 30 min for the colorimetric reaction to occur. 100 µl of stop solution (1 M H₂SO₄) was added to stop the enzyme-substrate reaction. Following this, absorbance was measured using a microtitre plate reader at 450 nm wavelength. A standard curve was constructed by plotting the standards against the absorbance and results obtained were expressed as pg/ml of supernatant / serum using the software analysis package Graph Pad Prism.

2.4.2.3 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,

Belgium). The coating antibody (rabbit anti-mouse/rat serum) was diluted to a working concentration of 1.25 µg/ml with bicarbonate coating buffer (51mM NaHCO₃, 50mM Na₂CO₃ pH 9.4). Flat-bottomed 96 well plates (NUNC, F96 MAXISORP-immuno plate) were coated with 100 µl of the coating antibody and incubated overnight at 4°C. The wells were washed 4 times with 300 µl of wash buffer (0.5% NaCl in dH₂O with 0.05% Tween-20) and excess wash buffer removed by blotting plate on a paper towel. Following this, plates were blocked with 300 µl of blocking buffer per well for 2 hr at RT. The standards were prepared for loading on the plate by preparing the stock IL-12 (recombinant mouse IFN-γ). The stock was reconstituted in 0.79 ml of reagent diluent to give a concentrated reconstituted standard of 10,000 pg/ml. A further dilution of stock concentration was made to a working concentration of 1000 pg/ml and this was used as the top standard. Serial dilutions (1:2) were carried out using the standard diluent assay. 100 µl of each standard and sample was loaded onto the plate in duplicate. Immediately after the samples and standards were loaded on the plate, 50 µl of the detection antibody was added to each well and incubated for 2 hr. The detection antibody (rat anti-mouse IFNγ biotin) was diluted in SDAB to a working concentration of 0.125 µg/ml. The plates were washed 4 times to remove samples and detection antibody from the plate. HRP was diluted in SDAB (1:2000 dilution in PBS with 0.5% BSA and 0.1% Tween-20) and 100 µl was added to each well and incubated for 45 min. Plates were washed 4 times as before and 100 µl of TMB was added to each well for 30 min to allow the colorimetric reaction to occur. 100 µl of stop solution (1 M H₂SO₄) was added to stop the enzyme-substrate reaction and absorbance was measured using a microtitre plate reader at 450 nm 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.

2.4.3 Analysis of cytokine concentrations with Meso Scale (MSD)

MSD cytokine assays measure 1-10 cytokines on a 96-well multi-spot plate. The assays employ a sandwich immunoassay format as described above, where capture antibodies are coated in a patterned array on the bottom of a multi-spot plate. In the studies reported here, a 4-spot plate was used for assessment of IL-10 and IL-12p40 serum concentrations (MSD, USA).

Methods

The 96 well MSD plate was blocked by addition of 150 µl of 0.1 % (w/v) blocker b solution into each well of the MSD plate. The plate was sealed and incubated with vigorous shaking on a gyro-rocker (ST-R9 Stuart) for 1 hr at RT. The plate was then washed 3 x with 100 µl PBS + 0.05% Tween-20. 25 µl of each calibrator and sample were dispensed into a separated well of the MSD plate. The plate was sealed again and incubated for 2 hr with vigorous shaking at RT. Following incubation, 25 µl of 1 x detection antibody solution was dispensed into each well of MSD plate. The plate was sealed again and incubated for 2 hr with vigorous shaking at RT. The plate was washed 3 x with 100 µl PBS + 0.05% Tween-20. Finally 150 µl of read buffer T was added to each well of the MSD plate and the plate was read and analysed on the MSD sector imager 2400 (MSD, USA). The percent phospho protein in each the sample was calculated using the following formula: % Phospho protein = (Phospho signal / Total)*100.

2.4.4 Protein quantification - BCA protein assay

The BCA protein assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. Protein content was quantified using this assay. A working solution of 2000 µg/ml of bovine serum albumin (BSA) was prepared using lysis buffer stock as diluent. A set of dilutions were prepared to give final protein concentrations of: 2000, 1500, 1000, 750, 500, 250, 125, 25, 0 µg/ml BSA protein. Next 25 µl of the dilutions and samples were pipetted in duplicate into the wells of a new 96 well plate. A BCA working solution was prepared by adding 1 part of the BCA working reagent B to 50 parts of reagent A. 200 µl of this working solution was added to each sample/standard. The 96 well plate was then covered and incubated at 37°C for 30 min. The plate was subsequently cooled to RT and the absorbance read at 560 nm using a microtitre plate reader. A standard curve was constructed by plotting the standards against the absorbance and results obtained expressed as µg/ml of protein.

2.4.5 Analysis of protein expression with 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.

2.4.5.1 Tissue preparation for Western Blotting

Spleen samples were homogenized using a potter homogeniser at 800 rpm for 10-15 strokes in PBS buffer (PBS containing 1% protease inhibitors; 1% phosphatase inhibitors) and left to incubate on ice for 10 min before centrifuging at 13000 g for 15 min. 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 (Tris-HCl; glycerol; 10% SDS; β -mercaptoethanol; bromophenol blue) added to each and boiled at 90°C for 10 min to denature proteins. Samples were aliquoted and stored at -80°C until further analysis

2.4.5.2 SDS-PAGE 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 casting frame (Biorad, Ireland) and were held firmly on a casting stand. The gels were allowed to set for 30 min 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 electrode assembly and inserted into a clamping frame (Biorad, Ireland). 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 15 μ l of the equalised samples were loaded per well, with a different treatment group per lane. 5 μ l of molecular weight marker (Biorad, Ireland) 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 power pack (Biometra, Germany) at 55 millivolts (mV) for 40-45 min or until the blue dye of the sample buffer had run to the bottom of the gel.

Methods

2.4.5.3 Semi-Dry Transfer

Following electrophoresis, gels were removed and placed on a layer of immobilon PVDF transfer membrane (Millipore, UK) which had been activated in methanol for 15 sec and soaked in Milli-Q water (Millipore, UK) for 2 min. 2 pieces of filter paper were soaked in anode buffer I (0.3 M Tris, 10% methanol), 1 piece was soaked in anode buffer II (25 mM Tris, 10% methanol) and 3 pieces were soaked in cathode buffer (25 mM Tris, 40 mM glycine, 10% methanol) for at least 30 sec. The 2 pieces of filter paper soaked in anode buffer I was placed on the semi-transfer plate, with the 1 piece of filter paper soaked in anode buffer II on top. The nitrocellulose membrane was placed on top of these layers and the gel on top of this. Finally, the filter paper soaked in cathode buffer was placed on top of the gel as shown in Fig 2.2. 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 1.5 hr at 225 milliamps (mA).

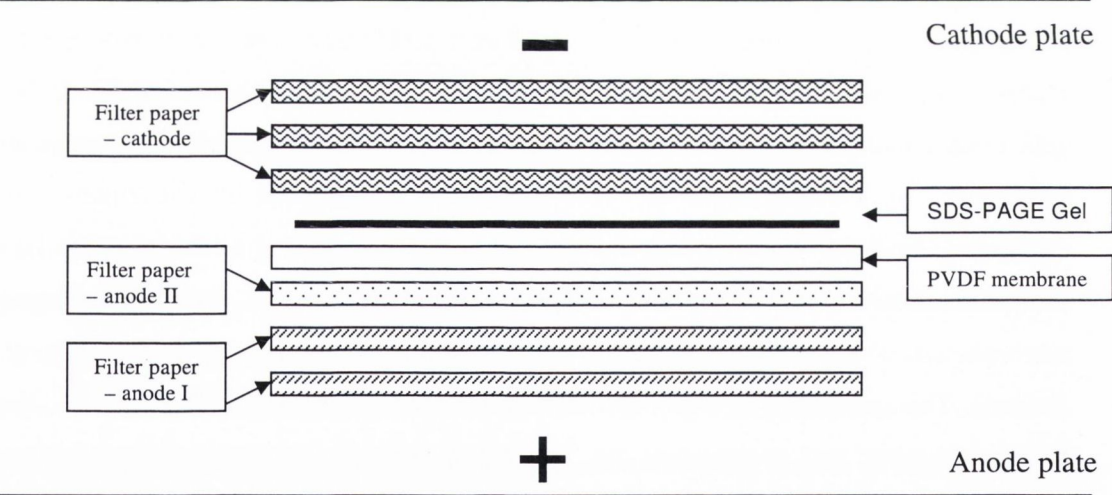


Figure 2.2: Diagram of sandwich preparation for semi-dry transfer of protein from SDS gel to nitrocellulose membrane.

2.4.5.4 Immunoblotting

Following the transfer of proteins, nitrocellulose membranes were removed and blocked for non-specific binding 2 hr in 5% BSA-TBS-Tween blocking buffer. The membranes were washed for 3 x 5 min washes. The membrane was then incubated in 5% BSA-

TBS-Tween (0.1%) with the appropriate primary antibody overnight at 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, the membranes were washed 4 times to ensure the primary antibody was completely washed off. The secondary antibody, which is a horseradish peroxidase-linked antibody that binds to the specific primary antibody was prepared and incubated on the membrane for 2 hr at RT. The membranes were washed a further 4 times to ensure sufficient removal of any secondary antibody. Super signal west dura chemiluminescence solution was added to membrane in order to detect bands (1 ml per blot) for up to 2 min, 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 the Gel Doc It Imaging System (UVP) and analysed using LabWorks software package.

Phosphorylated STAT-1 expression

To examine the phosphorylation of STAT1 molecules, the nitrocellulose membranes were blocked in 20 ml of 5% TBS-Tween with 5% BSA and allowed to incubate for 2 hr at RT. The membranes were washed with TBS-T wash buffer for 3 x 5 min incubation periods. The primary antibody, in this case phospho-STAT1 (Cell Signalling Technologies, USA), which detects endogenous levels of STAT1 only when phosphorylated 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 (10 ml) 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 5 min 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 (10 ml) and allowed to incubate for 1 hr at RT. The secondary antibody was removed from membranes and washes applied (3 x 5 min). 1 ml of supersignal west dura chemiluminescence was added to the membranes for 2 min, drained and placed in a cassette prior to exposure to hyperfilm in a dark room and developed using an automated developer.

Methods

STAT-1 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 ml of stripping solution and incubated for 15 min. 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 (10 ml) 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 5 min 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 (10 ml) and allowed to incubate for 1 hr at RT. The secondary antibody was removed from membranes and washes applied (3 x 5 min). 1 ml of supersignal west dura chemilluminescence was added to the membranes for 2 min, drained and placed in a cassette prior to exposure to hyper-film in a dark room and developed using an automated developer.

2.5 Analytical Methods: mRNA

2.5.1 Real-time polymerase chain reaction (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.

2.5.2 Total RNA extraction spleen cells

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 30 mg of tissue was homogenized in lysis buffer (350 µl RA1 buffer and 3.5 µl β-mercaptoethanol) using a Polytron for ~30 sec 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 min at 11,000

g. 350 μ l 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 sec at 11,000 g. This column now contains the RNA and it is desalted by applying 350 μ l membrane desalting buffer and centrifuging at 11,000 g for 1 min. Following this, any potential DNA contamination present was digested by adding 95 μ l Dnase reaction mixture directly onto the center of the silica membrane of the column and incubated for 15 min at RT. The column was washed with 200 μ l of RA2 buffer to inactivate the Dnase was added and centrifuged for 30 sec at 11,000 g and column was placed into a new collecting tube. A second wash was carried out and involved adding 600 μ l buffer RA3 to the column and centrifuging for 30 sec at 11,000 g and the final wash was with 250 μ l RA3 and centrifuged for 2 min at 11,000 g to allow the membrane to dry completely. The column was placed in a nuclease-free micro-centrifuge tube and RNA was eluted with 60 μ l Rnase-free H₂O and finally centrifuged for 1 min at 11,000 g. RNA was stored at -80°C .

2.5.3 Total RNA extraction from Dendritic Cells

Isolation of total RNA from Dendritic Cells on cell culture plates was carried out using Nucleospin RNA II kits (Macherey-Nagel) as per the protocol outlined above. RNA was harvested immediately following removal of the cell supernatant by addition of the lysis buffer directly to the wells of the plates.

2.5.4 Assessment of RNA quality and quantity

2.5.4.1 RNA Quality

The next stage following RNA extraction was to assess the integrity of the RNA and this was determined by electrophoresing a sample of RNA on agarose gels. 3 μ l of RNA was diluted with 2 μ l Rnase free H₂O and 1 μ l of loading dye and 4 μ l of this diluted RNA was loaded onto a 1% agarose gel and electrophoresed at 90 mV. When the gel is observed under UV light, 2 ribosomal RNA (rRNA) bands (Figure 2.3) can be seen, a dense 28S band on top and a lighter 18S band below as shown in figure.

2.5.4.2 RNA quantification

Total RNA was quantified using a nanodrop (Mason Technology, Ireland). 1 μ l of freshly isolated mRNA was loaded on to the reading platform and spectrometry was performed to determine the concentration in ng/ μ l. The purity was demonstrated by the

Methods

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 in the set of samples.

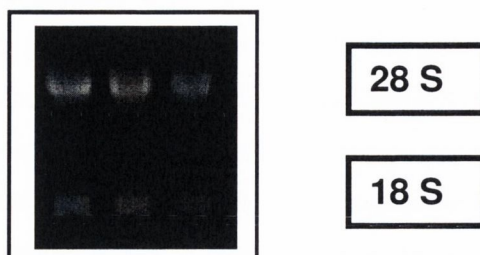


Figure 2.3: Sample blot demonstrating the integrity of extracted RNA from spleen (ribosomal 28S and 18S bands)

2.5.5 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_2O was prepared and stored on ice. RNA was diluted to a range between 0.02-0.2 $\mu\text{g}/\mu\text{l}$. Equal volumes of the master mix were added to an equivalent volume of diluted RNA, eg 25 μl of master mix was added to 25 μl 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 min at 25°C and step 2 set for 2 hr 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.

2.5.6 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 1:5 dilution of cDNA was prepared with sigma water. All real-time PCR was conducted using an ABI Prism 7300 instrument (Applied Biosystems). A 20 μl volume was added to each well (9 μl of diluted cDNA, 1 μl of primer and 10 μl of Taqman® Universal PCR Master Mix. Electronic pipettes (EDP3 20-200 μl , 2-20 μl and 10-100 μl) 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 min, 95°C for 15 sec for each cycle (denaturation) and finally the transcription step at 60°C for 1 min. β -actin

was used as an endogenous control to normalize gene expression data, and 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 ($2^{-\Delta\Delta C_t}$, where C_t is the threshold cycle).

There are various stages carried out during the PCR reaction, initially samples are heated to 95°C for 15 sec, which allows the double stranded cDNA to denature. In the next stage, 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).

2.5.7 Real-time PCR analysis

The $\Delta\Delta C_t$ 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 (C_t) difference between samples. Briefly, a threshold for fluorescence is set, against which C_t 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 C_t readings demonstrate high fluorescence, indicating greater amplification and hence, greater gene expression. When a PCR is 100% efficient a one-cycle

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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) 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 (CT). The CT difference (Δ CT) of the control is subtracted from itself to give 0, and subtracted from all other samples, this is the $\Delta\Delta$ CT value. The $\Delta\Delta$ CT (cycle difference corrected for β -actin) is then converted into a fold-difference. As a one-cycle difference corresponds to a 2-fold increase or decrease relative to control, 2 to the power of the $\Delta\Delta$ CT (difference in control and sample CT corrected for β -actin) gives the fold-difference in gene expression between the control and treated samples. The control sample always has a $\Delta\Delta$ CT value of 0, thus 0^2 gives a $2^{\Delta\Delta$ CT of 1, against which all other samples are referenced

Gene Symbol	Gene Name	Taqman Gene Expression ID
IFN- γ	Interferon gamma	Mm00801778_m1
IL-10	Interleukin 10	Mm00439616_m1
IL-12a	Interleukin 12p35	Mm00434165_m1
IL-12b	Interleukin 12p40	Mm00434170_m1
IL-12R β 1	Interleukin 12 receptor beta 1	Mm01351787_m1
IL-12R β 2	Interleukin 12 receptor beta 2	Mm01183807_m1
IL-15	Interleukin 15	Mm00434210_m1
IL-18	Interleukin 18	Mm00434225_m1
IL-19	Interleukin 19	Mm01288326_m1
IL-20	Interleukin 20	Mm00445341_m1
IL-24	Interleukin 24	Mm00474102_m1
CXC110	IFN Inducible protein 10 (IP-10)	Mm00445235_m1
CD40	Cluster of differentiation 40	Mm00441891_m1
iNOS	Inducible nitric oxide synthase (iNOS)	Mm00440485_m1

Table 2.1: List of genes used in single RT-PCR studies with the gene expression assay and genbank ref sequence numbers.

2.6 Immunophenotyping by fluorescent activated cell sorting (FACs)

FACs is a useful optical technique that allows live, intact cells to be monitored and provides sensitive and specific information about each single cell. Flow cytometers use lasers as their source to excite cells and when the light source hits a cell, the amount of light scattered to the side is detected in the side scatter (SS) (Fig 2.4). The information provided from side scatter describes the size and shape of the cell. 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 fluorescein, which is a common fluorochrome. 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 acquisition of samples. In addition, antibodies have fluorescent labels attached enabling the surface expression of specific cell markers.

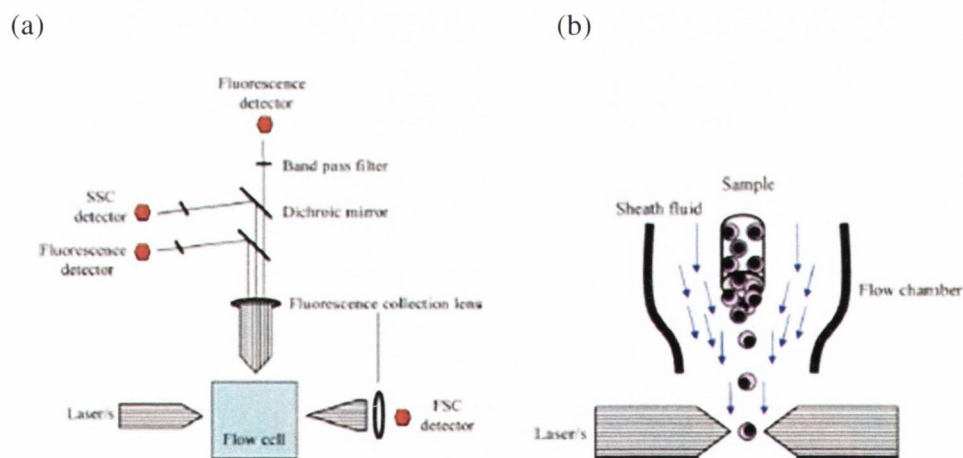


Figure 2.4: Diagram of the optics of flow cytometry (a) and how single cells suspended in sheath fluid are focused between the light-source (b) (Tarrant, 2005).

2.6.1 Preparation and staining of cells from tumor mass and lymph nodes

Tumor masses and lymph nodes were dissected from the CT26 injected mouse as previously described and single cell suspensions were prepared at a concentration of $2 \times 10^6/\text{ml}$. Cell suspensions were cultured for 2 hr with PMA (10 ng/ml) and ionomycin (1 $\mu\text{g}/\text{ml}$) and Brefeldin A (5 $\mu\text{g}/\text{ml}$) at 37°C and 5% CO_2 . Samples were centrifuged at $250 \times g$ for 5 min, supernatant decanted and pellets re-suspended in 10 ml FACs buffer (2% FCS, 0.1% NaN₃ in PBS) supplemented with 50% fetal calf serum to block non-

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specific binding. Samples were centrifuged again and washed 2 x with FACs buffer. Cells were finally re-suspended in 10 ml of FACs buffer and 500 µl of prepared cells were added to FACs tubes. The cell suspension was spun down and cell pellet was incubated with antibody solutions for CD4⁺ or CD8⁺ in order to identify T cell lineages. 1µl of the relevant antibodies was added to 100µl of FACs buffer and incubated for 30 min at 4°C. The cells were centrifuged as before and washed 2 x with FACs buffer. The cells were then stained for the intracellular targets IFN-γ and IL-10. (Table 2.2). Unstained cells from each sample were processed through all of the same steps as the stained cells. Tubes were stored on ice and covered with tinfoil at all times to prevent decay of flouorochromes and cell clumping, respectively.

2.6.2 Acquisition of cells on flow cytometer

Samples were acquired by FACs using a Dako Cytomation Cyan ADP, and FloJo software was used for analysis (Fig 2.3). The fluorescence was measured on a logarithmic scale with band pass filter 760 for phycoerythrin-cytomention 7 (PE-Cy7), 779 for Alexaflor 750 (Alexa 750), 575 for phycoerythrin (PE) and 660 for allophycocyanin (APC) (Table 2.2).

Cell marker	Primary monoclonal Antibodies	Conjugated to fluorescent tags
CD4+ T-cells	Rat anti-mouse CD4	PE-Cy7
CD8+ T-cells	Rat anti-mouse CD8a	Alexa 750
Interferon-γ	Rat anti-mouse IFN-γ	APC
Interleukin-10	Rat anti-mouse IL-10	PE

Table 2.2: List of primary antibodies and their relevant fluorescent tags used for staining tumor and lymph node cells.

Fluorochrome	Excitation Peak	Emission Peak	Laser Wavelength
PE-Cy7	488	760	707
Alexa 750	633	779	840
APC	633	660	780
PE	488	575	600

Table 2.3: List of fluorochromes used for immunophenotyping

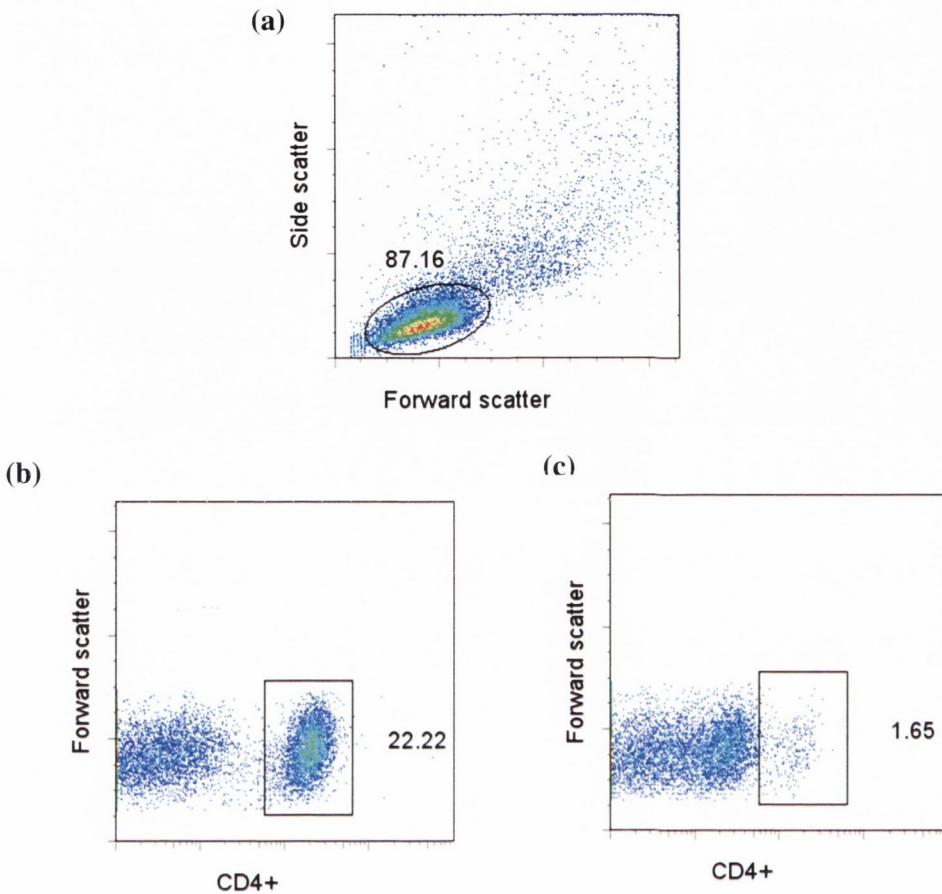


Figure 2.3: Sample dot plots indicating forward scatter (FS) versus side scatter (SS) of acquired lymph node cells (a), gated region showing CD4+ T-cells (PE-Cy7) (b), and fluorescence minus one (fmo) control for CD4+ (PE-Cy7) (c).

2.7 Statistical Analysis of Results

All data was analysed using a GB-STAT routine. Statistical comparisons were initially performed using a one, two, or three-way analysis of variance (ANOVA), with or without repeated measures, as indicated in the experimental sections. If significant changes were observed, the data was further analysed using Student Newman-Keuls or student t test post hoc test as appropriate. Data was deemed significant when $P < 0.05$. Results are expressed as means $\pm$ standard error of the mean (SEM) or mean percentage control and SEM.

Chapter 3: Acute Stress Results

3.1 Introduction

A vast literature indicates that exposure to acute stress has immunomodulating properties in humans and in animal models (Padgett and Glaser, 2003). Psychological stress has been shown to have many suppressive effects on the immune response to an infectious stimulus, including an alteration in the cytokine network. For instance, stress has been shown to impair the production of pro-inflammatory cytokines, such as IL-1 β and TNF- α (Johnson et al, 2002). However further characterisation of the stress-induced suppression of pro-inflammatory cytokines is needed to clarify the effects of exposure to stress on the immune system. Therefore, in this chapter, the innate IFN- γ response is investigated to see if this vital immune response to an infectious stimulus is impaired in response to acute stress exposure.

The mechanism behind the stress-induced suppression of pro-inflammatory cytokines is not fully understood. A possible mechanism for stress-induced suppression of pro-inflammatory cytokines is the increased production of anti-inflammatory cytokines. It has been previously demonstrated in rats that swim stress increases the production of the anti-inflammatory cytokine IL-10 (Connor et al, 2005). IL-10 has been shown *in vitro* and *in vivo* to have immunosuppressive effects (Fiorentino *et al.*, 1991; McGuirk *et al.*, 2002). Consequently, in this chapter we also consider IL-10 as a possible mediator of the stress-induced immunosuppression of the innate IFN- γ response. We also investigated the production of other anti-inflammatory cytokines, such as TGF- β and the IL-10 family members, to elucidate a possible role for these molecules as mediators of stress-induced immunosuppression.

Following perception of stress, two main physiological axis become activated, the HPA and SAM axis, which release glucocorticoids and catecholamine stress hormones respectively (Elenkov *et al.*, 2000). These end products of the stress response are released to prepare our body for stress but they can also directly impact on immune function because immune cells express receptors for these hormones (Ballard *et al.*, 1995; Kohm and Sanders, 2001). Glucocorticoids and catecholamines have known immunosuppressive effects. However in this chapter we investigate the contribution that each of these hormones plays in stress-induced immunosuppression.

3.2 Can exposure to restraint stress alter corticosterone concentrations?

In this thesis, a 2 hr restraint stress protocol was analysed because the physical immobilisation that the mice encounter has been established as a moderate psychological stressor (Sheridan *et al*, 2005). To ensure that this stress protocol resulted in activation of the stress response, the first aim of the studies presented here was to assess corticosterone concentrations in serum and spleen tissue from mice exposed to stress relative to home cage controls.

3.2.1 Exposure to acute restraint stress increased corticosterone concentrations in serum and in mouse spleen tissue

Exposure to 2 hr of restraint increased the concentration of corticosterone in the serum and in the spleen of mice. The concentrations of corticosterone in mice that were not exposed to stress were very low in the serum and spleen. However, exposure to restraint acute stress increased serum corticosterone (~ 8 fold) and splenic corticosterone (~ 5 fold). (Figure 3.2.1 a, b)

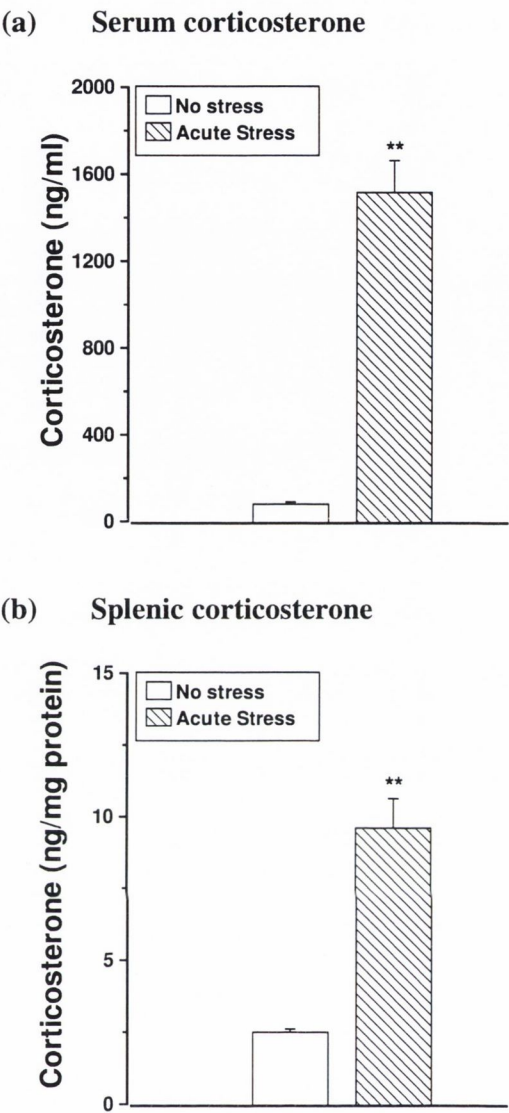


Figure 3.2.1 Exposure to acute restraint stress increased concentrations of corticosterone in the serum (a) and in spleen tissue (b)

Mice were either exposed to a 2 hr restraint stress or left in home cages and sacrificed immediately after stress. Data are expressed as mean $\pm$ SEM (n=6). **P<0.01 vs. non-stressed counterparts (Student-t test).

3.3 Can exposure to acute restraint stress alter the innate IFN- γ response?

It has been reported in many publications that exposure to stress can elicit an immunosuppressive state and can result in the onset or progression of many diseases (Glaser and Kiecolt-Galser, 2001). Therefore, we investigated the effect of exposure to restraint stress on the innate IFN- γ response. IFN- γ production is vital for an effective immune response to an LPS infection, as it bridges the innate and adaptive immune responses. To initially investigate the stress-induced effects on the LPS-induced IFN- γ response, IFN- γ mRNA expression in the spleen and IFN- γ serum concentrations of mice exposed to stress were assessed. IFN- γ mRNA transcription results in the production of IFN- γ protein and release of the protein into the serum. Therefore, serum concentrations of IFN- γ were also measured to demonstrate that the alterations in mRNA had translational effects on IFN- γ protein. IFN- γ signals via the phosphorylation of the transcription factor STAT-1 and the phosphorylation of this transcription factor was also assessed in the spleens of mice after exposure to stress. pSTAT-1 is a critical transcription factor required for the activation of many IFN- γ inducible genes. The down-regulation of STAT-1 phosphorylation indicates that the activation of inducible genes would also be impaired. Therefore, the mRNA expression of the IFN- γ inducible target genes iNOS, CD40, IP-10 and SOCS-1 were examined in the spleens of mice after exposure to acute stress.

3.3.1 Exposure to restraint stress impaired LPS-induced IFN- γ serum concentrations and IFN- γ mRNA expression in mouse spleen

Administration of LPS increased IFN- γ serum concentrations (~ 3 fold) and IFN- γ mRNA expression (~ 3 fold) relative to saline-treated counterparts. However, these LPS-induced increases were not evident in the serum or spleens from mice that were exposed to an acute restraint stress prior to LPS administration. In contrast, in mice that were exposed to restraint stress without an LPS challenge, IFN- γ serum concentrations and IFN- γ mRNA expression was not altered relative to saline-treated counterparts. (Figure 3.3.1 a, b)

3.3.2 Exposure to restraint stress impaired LPS-induced IFN- γ signalling

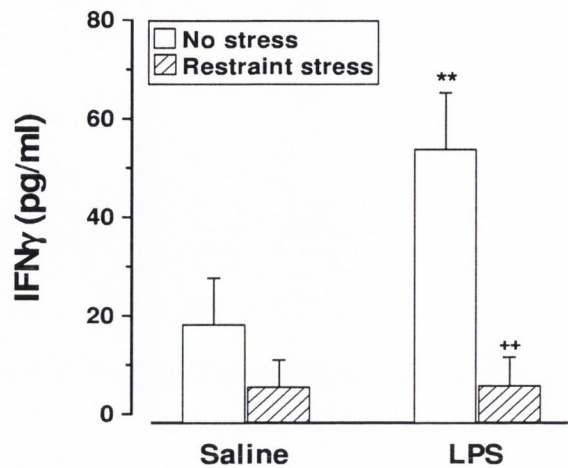
Administration of LPS increased STAT-1 phosphorylation in the spleen (~ 3 fold) relative to saline-treated counterparts. However, this LPS-induced increase in pSTAT-1

expression was not evident in the spleens from mice that were exposed to acute restraint stress prior to injection of LPS. In contrast, in mice that were exposed to restraint stress without an LPS challenge, pSTAT-1 expression was not altered relative to saline-treated counterparts. (Figure 3.3.2 b)

3.3.3 Exposure to restraint stress impaired LPS-induced mRNA expression of IFN- γ induced genes

Administration of LPS increased mRNA expression of iNOS, CD40 and IP-10 (~ 2 fold) in the spleen relative to saline-treated counterparts. However, the LPS-induced increases in expression of iNOS, CD40 and IP-10 were not evident in spleens from mice that were exposed to restraint stress prior to LPS administration. In contrast, in mice that were exposed to restraint stress without LPS challenge iNOS, CD40 and IP-10 mRNA expression was not altered relative to saline-treated counterparts. (Figure 3.3.3 a, b, c, d)

(a) Serum IFN- γ concentration



(b) Splenic IFN- γ mRNA expression

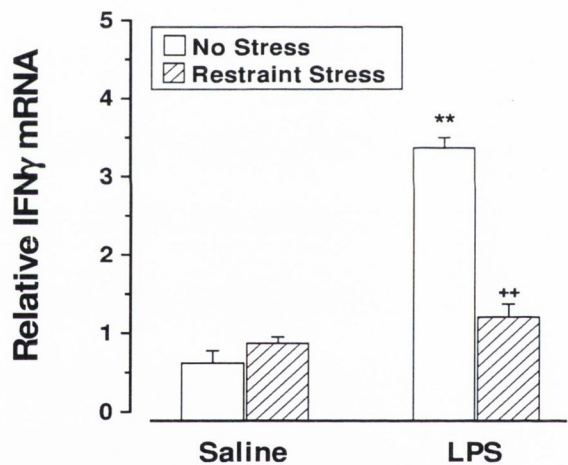
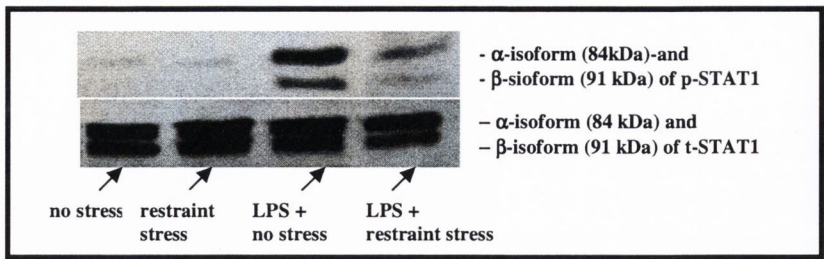


Figure 3.3.1 Exposure to restraint stress inhibited LPS-induced increases in serum concentrations of IFN- γ (a) and IFN- γ mRNA expression in mouse spleen (b) LPS (250 μ g/kg) was administered to mice immediately following a 2 hr restraint stress and the mice were sacrificed 8 hr after injection. A two-way ANOVA revealed a significant interaction effect between LPS treatment and restraint stress on serum concentrations of IFN- γ [$F_{(1,16)}=3.99$, $P<0.05$] and IFN- γ mRNA expression [$F_{(1,16)}=78.08$, $P<0.0001$]. Data are expressed as mean $\pm$ SEM (n=6). ** $P<0.01$ vs. saline counterparts, ++ $P<0.01$ vs. non-stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

(a)



(b)

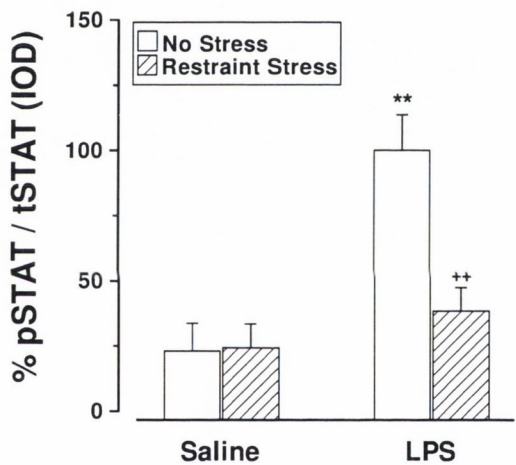


Figure 3.3.2 Exposure to restraint stress inhibited the LPS-induced increase in pSTAT-1 expression in mouse spleen

LPS (250µg/kg) was administered to mice immediately following a 2 hr restraint stress and the mice were sacrificed 8 hr after injection. (a) The sample immunoblot of pSTAT-1 and tSTAT-1 show the induction of pSTAT-1 expression following LPS and the decrease in expression with restraint stress administered prior to injection. The expression of tSTAT-1 is unaffected by treatment with LPS or stress. (b) Values are expressed the percentage of α + β pSTAT-1 divided by α + β tSTAT-1. A two-way ANOVA uncovered a significant interaction effect between LPS treatment and restraint stress [$F_{(1,16)}=4.34$, $P<0.05$]. Data are expressed as mean ±SEM (n=6). ** $P<0.01$ vs. saline counterparts, ++ $P<0.01$ vs. non-stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

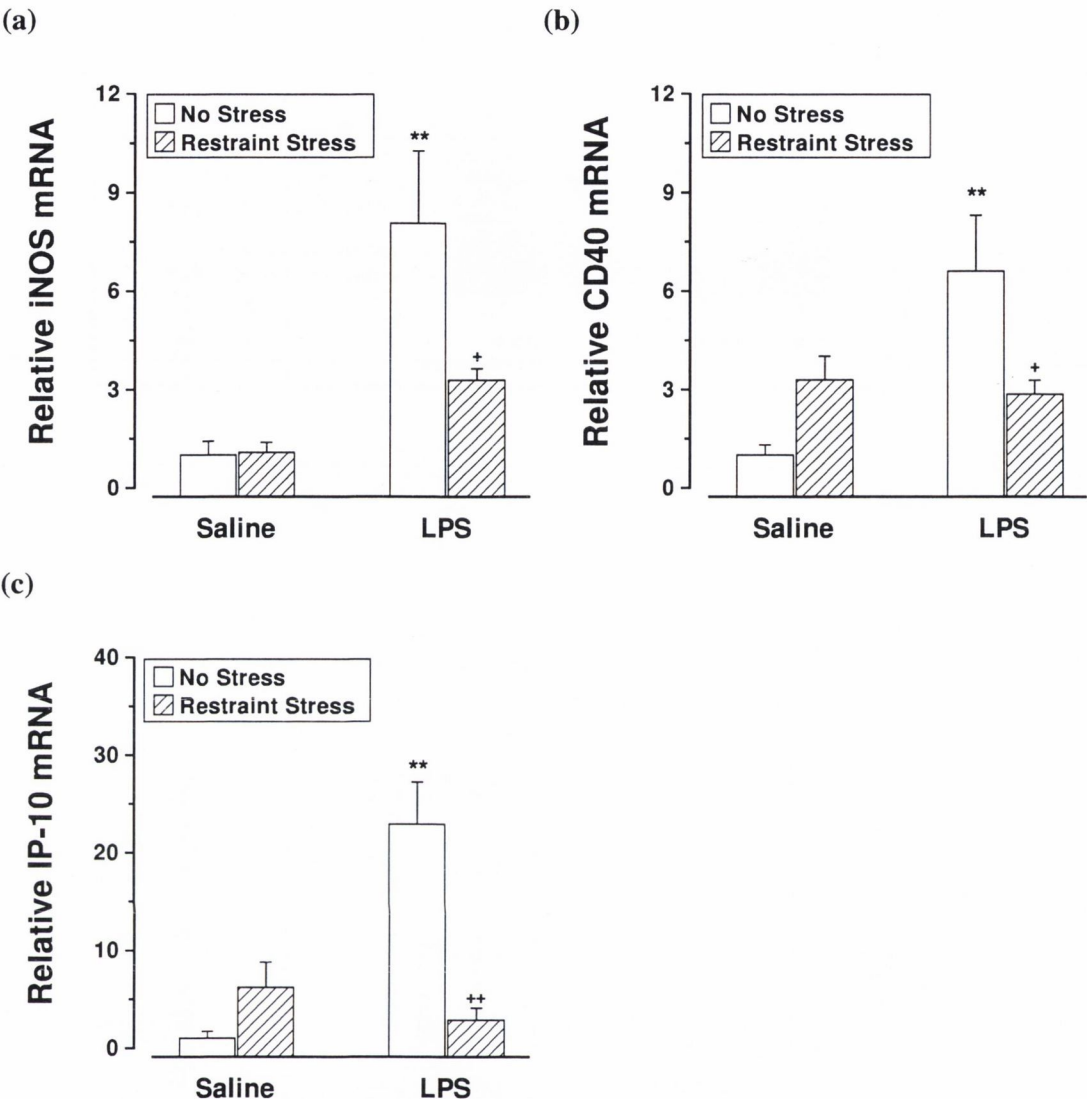


Figure 3.3.3 Exposure to restraint stress prevented LPS-induced increases in mRNA expression of IFN- γ inducible genes iNOS (a), CD40 (b) and IP-10 (c) in mouse spleen

LPS (250 μ g/kg) was administered to mice immediately following a 2 hr restraint stress and the mice were sacrificed 8 hr after injection. A two-way ANOVA revealed a significant interaction effect between LPS treatment and restraint stress on iNOS expression [$F_{(1,16)}=3.41$, $P<0.05$], CD40 expression [$F_{(1,16)}=9.74$, $P<0.005$] and on IP-10 expression [$F_{(1,16)}=23.68$, $P<0.001$]. Data are expressed as mean $\pm$ SEM (n=6). ** $P<0.01$ vs. saline treated counterparts, ++ $P<0.01$ vs. non-stressed LPS-treated counterparts, + $P<0.05$ vs. non-stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

3.4 Can exposure to restraint stress alter expression of IFN- γ inducing cytokines?

As shown in previous results, IFN- γ is released in response to LPS challenge. However it has been shown in other reports that its production is stimulated by LPS indirectly via IFN- γ inducing cytokines which act on immune cells, such as NK cells. IL-12 is the primary inducer of IFN- γ production from NK cells. IL-12 is composed of two sub-units IL-12p35 and IL-12p40. IL-12p40 appears to be the sub-unit which governs the biological activity of IL-12 whereas IL-12p35 is constitutively expressed on most cells (Aste-Amezaga *et al.* 1998). Due to the vital role that IL-12 plays in inducing IFN- γ from NK cells, the effect of restraint stress on the mRNA expression of IL-12p40 and IL-12p35 was examined. IL-12 exerts its biological effects by binding to specific IL-12 receptors of which there are two subtypes, IL-12R β 1 and IL-12R β 2. Therefore, the expressions of these two receptor subunits were examined in response to exposure to restraint stress. Although IL-12 is the primary inducer of IFN- γ from NK cells, IFN- γ has also been shown to be induced from two other cytokines, IL-15 and IL-18. Therefore, we also investigated the effect of restraint stress on the mRNA expression of these two cytokines.

In these investigations, the splenic mRNA expression of the cytokines was assessed to determine the stress-induced effects. The limitations in the amount of serum from a mouse determined this analytical method as there was not enough serum to analyse protein production for all targets. However, it has been demonstrated in the previous study shown here and by others that significant changes in mRNA expression directly determine protein production (Boyle and Connor, 2007).

3.4.1 Exposure to restraint stress inhibited LPS-induced increase in IL-12p40 mRNA expression

LPS administration induced IL-12p40 mRNA expression in mouse spleen after 1.5 hr (~40 fold) and 8 hr (~2 fold) relative to saline-treated counterparts. However, the LPS-induced increases in IL-12p40 mRNA expression were not evident at either time point in spleens from mice that were exposed to stress prior to LPS administration. Mice exposed to stress without LPS administration had no alteration in IL-12p40 mRNA relative to saline-treated mice. (Figure 3.4.1 a, b)

3.4.2 Exposure to restraint stress did not alter LPS-induced IL-12p35 mRNA expression

In contrast to the previous results, the mRNA expression of IL-12p35 subunit in mouse spleen was not altered by treatment with LPS or restraint stress at either 1.5 hr or 8 hr after LPS administration. (Figure 3.4.1 c, d)

3.4.3 Exposure to restraint stress inhibited the LPS-induced increase in IL-12 receptor β 1 and β 2 mRNA expression

LPS administration induced the expression of IL-12R β 1 mRNA (~ 10 fold) in mouse spleen and IL-12R β 2 mRNA (~ 4 fold) relative to saline-treated counterparts. However, the LPS-induced increase in expression of IL-12 receptor subunits was not evident in the spleens of mice exposed to restraint stress prior to the LPS administration. In contrast, in the spleens of mice exposed to stress without LPS administration, neither IL-12R β 1 nor IL-12R β 2 mRNA expression was altered relative to saline-treated counterparts. (Figure 3.4.2 a, b)

3.4.4 Exposure to restraint stress inhibited the LPS-induced increase in IL-15 mRNA expression

LPS administration induced IL-15 mRNA expression (~ 4 fold) in mouse spleen relative to saline-treated counterparts. However, this LPS-induced increase in IL-15 mRNA expression was not evident in the spleens of mice that were exposed restraint stress prior to LPS administration. In contrast, in the spleens of mice exposed to stress without LPS administration, IL-15 mRNA was not altered from saline-treated mice. (Figure 3.4.3 a)

3.4.5 Exposure to restraint stress did not alter IL-18 mRNA expression

LPS administration induced IL-18 mRNA expression (~ 2 fold) in mouse spleen relative to saline-treated counterparts. In contrast to IL-15 mRNA, the LPS-induced increase in IL-18 mRNA was not significantly altered by exposure to restraint stress. (Figure 3.4.3 b)

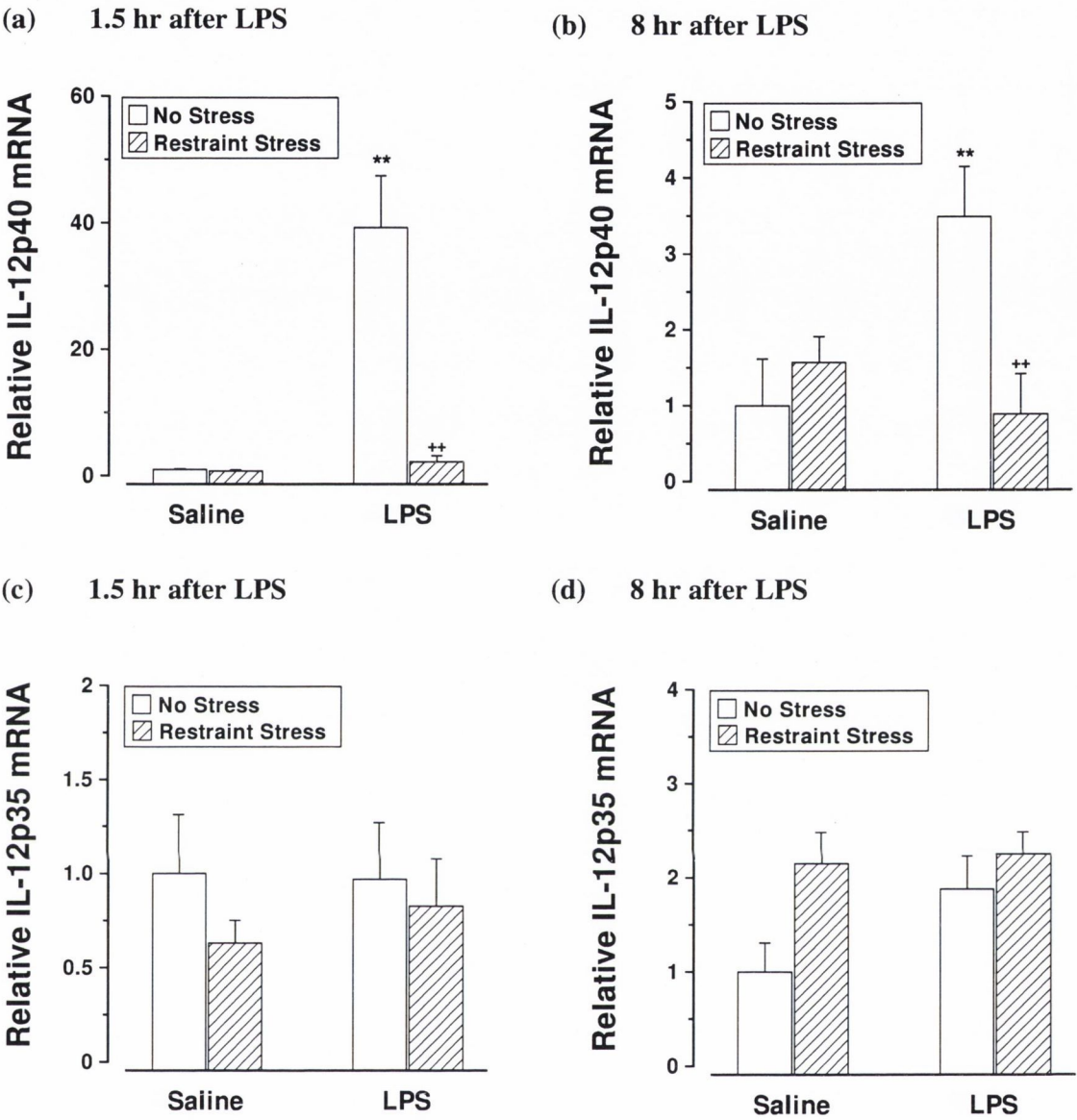


Figure 3.4.1 Exposure to restraint stress inhibited LPS-induced IL-12p40 expression, but did not alter IL-12p35 expression in mouse spleen

LPS (250 μ g/kg) was administered to mice immediately following a 2 hr restraint stress and the mice were sacrificed either 1.5 hr or 8 hr after injection. A two-way ANOVA revealed a significant interaction effect between LPS treatment and restraint stress on IL-12p40 expression at 1.5 hr [$F_{(1,15)}=28.50$, $P<0.0001$] and at 8 hr [$F_{(1,16)}=8.19$, $P<0.01$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P<0.01$ vs. saline counterparts, ++ $P<0.01$ vs. non-stressed LPS counterparts, + $P<0.05$ (Neuman-Keuls post hoc test).

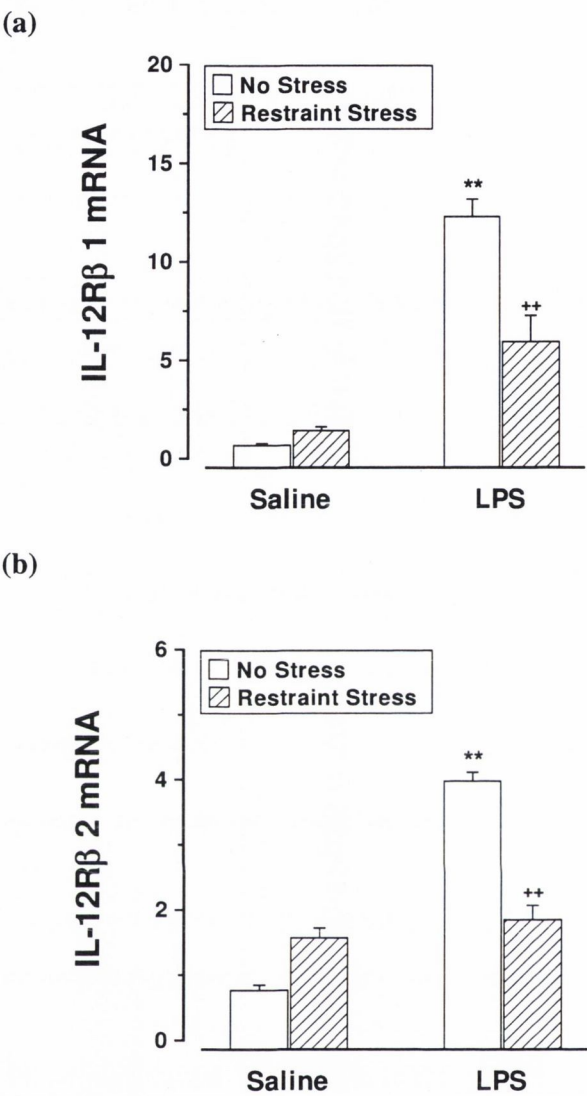


Figure 3.4.2 Exposure to restraint stress inhibited the LPS-induced expression of IL-12Rβ1 (a) and IL-12Rβ2 (b) in mouse spleen

LPS (250μg/kg) was administered to mice immediately following a 2 hr restraint stress and the mice were sacrificed 8 hr after injection. A two-way ANOVA revealed a significant interaction effect between LPS treatment and restraint stress on the mRNA expression of IL-12Rβ1 [$F_{(1,14)}=18.29$, $P<0.005$] and IL-12Rβ2 [$F_{(1,14)}=73.34$, $P<0.0001$]. Data are expressed mean $\pm$ SEM (n=6). ** $P<0.01$ vs. saline counterparts, ++ $P<0.01$ vs. non-stressed LPS counterparts (Neuman-Keuls post hoc test).

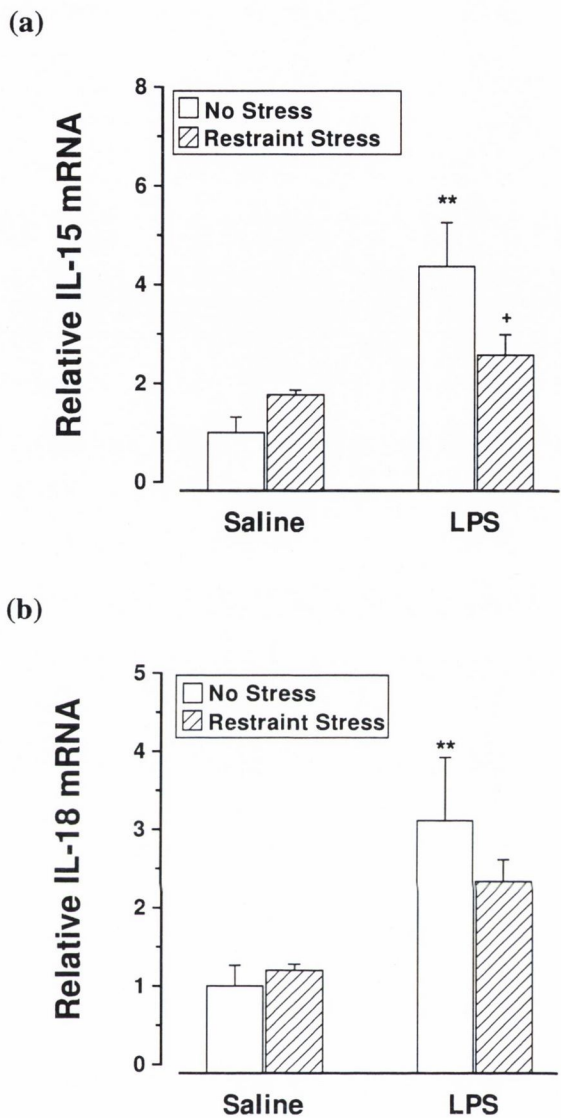


Figure 3.4.3 Exposure to restraint stress inhibited the LPS-induced increase in IL-15 expression (a), but did not significantly alter IL-18 expression (b) in mouse spleen

LPS (250µg/kg) was administered to mice immediately following a 2 hr restraint stress and the mice were sacrificed 8 hr after injection. A two-way ANOVA exposed a significant interaction effect of restraint stress with LPS challenge on IL-15 mRNA expression [$F_{(1,16)}=6.25$, $P<0.05$]. Data are expressed as mean $\pm$ SEM (n=6). ** $P<0.01$ vs. saline counterparts, + $P<0.05$ vs. non-stressed LPS counterparts (Neuman-Keuls post hoc test).

3.5 Does the stress-induced inhibition of IL-12 mediate the stress-induced inhibition of IFN- γ ?

Due to the vital role that IL-12 plays in stimulating IFN- γ production from immune cells, we proposed that the stress-induced inhibition of LPS-induced IFN- γ could be mediated by the stress-induced inhibition of LPS-induced IL-12. Therefore in the present study we exogenously administered mouse recombinant IL-12 protein to splenocytes cultured from mice exposed to stress. We administered a physiologically relevant dose of IL-12 to assess if it could rescue the stress-induced inhibition of IFN- γ .

3.5.1 IL-12 restored the stress-induced inhibition of LPS-induced IFN- γ

In agreement with the previous results, exposure to stress inhibited LPS-induced IFN- γ concentrations in splenocytes. Administration of IL-12 to splenocytes cultured from mice that had not been exposed to stress had ~ 3 fold increase in IFN- γ concentration. However, there was no stress-induced inhibition of IFN- γ in the splenocytes from mice exposed to stress compared with the IL-12-induced increase in IFN- γ . (Figure 3.5.1 a)

3.5.2 IL-12 restored the stress-induced inhibition of LPS-induced IL-12

In agreement with the previous results, exposure to stress inhibited LPS-induced IL-12 concentrations in splenocytes. Administration of IL-12 to splenocytes cultured from mice that had not been exposed to stress resulted in no significant increase in IL-12 concentrations. However, similar to the previous result, there was no stress-induced inhibition of IL-12 in the splenocytes from mice exposed to stress. (Figure 3.5.1 b)

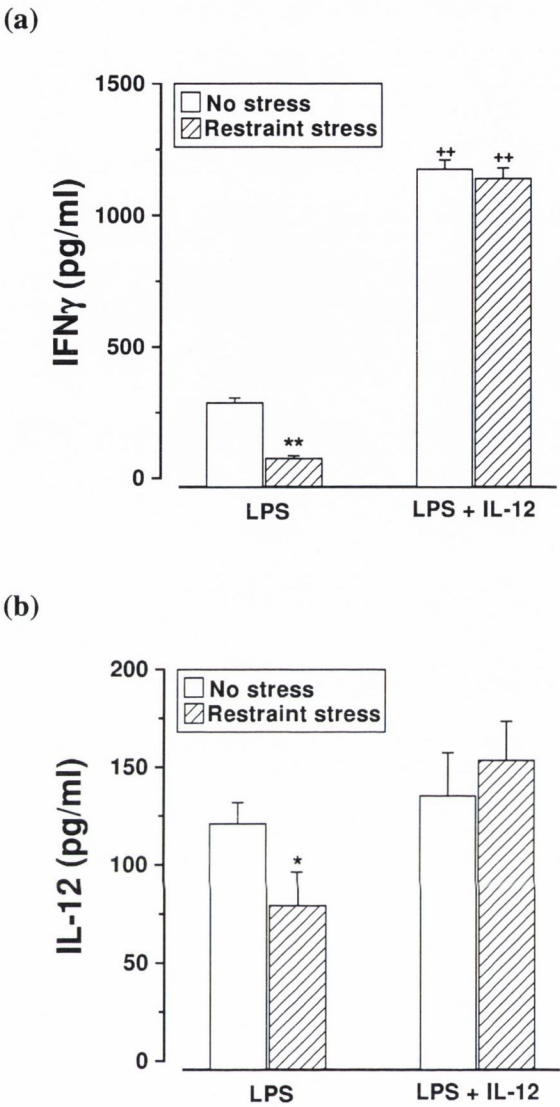


Figure 3.5.1 IL-12 administered to splenocytes restored stress-induced inhibition of LPS-stimulated IFN- γ and IL-12 production

Adult male balb/c mice were exposed to acute restraint stress and sacrificed immediately upon removal from restraint tubes. Spleens were dissected and splenocytes were prepared at a concentration of 2×10^6 cells / ml before stimulation with LPS ($1 \mu\text{g/ml}$) *ex vivo*. Mouse recombinant IL-12 (100 pg/ml) was administered 2 hr, 24 hr and 48 hr after LPS stimulation. The cell supernatant was harvested 72 hr after LPS stimulation. A two-way ANOVA exposed a significant effect of IL-12 on IFN- γ production [$F_{(1,22)}=420.25$, $P<0.0001$] and on IL-12 production [$F_{(1,22)}=4.04$, $P<0.05$]. Data are expressed as mean $\pm$ SEM ($n=8$). ** $P<0.01$ vs. LPS-treated counterparts, * $P<0.05$ vs. LPS-treated counterparts ++ $P<0.01$ vs. non-IL-12 treated counterparts (Neuman-Keuls post hoc test).

3.6 Does exposure to stress alter anti-inflammatory cytokine expression?

The anti-inflammatory cytokine IL-10 is an immunoregulatory cytokine that is known to have suppressive effects on the production of pro-inflammatory cytokines such as IFN- γ and IL-12. It was previously established that production of IL-10 is potently induced following an acute stressor, such as a swim stress in rats (Connor *et al.*, 2005). Therefore, it was investigated if exposure to restraint stress would have an effect on IL-10 serum concentrations or splenic IL-10 mRNA expression.

3.6.1 Exposure to restraint stress augmented serum IL-10 concentrations

LPS administration induced a significant increase in serum IL-10 concentrations after 45 min (~ 3 fold) and after 1.5 hr (~5 fold). Exposure to restraint stress prior to LPS administration augmented IL-10 serum concentrations after 45 min (~ 4 fold) and after 1.5 hr (~3 fold). In contrast, in mice that were exposed to stress without LPS challenge, there was no alteration in IL-10 compared to the saline-treated counterparts. (Figure 3.6.1 a, b)

3.6.2 Exposure to restraint stress augmented splenic IL-10 mRNA expression

There were similar changes in the IL-10 mRNA expression as IL-10 serum concentrations. LPS administration induced a significant increase in IL-10 mRNA expression (~ 3 fold) relative to saline-treated counterparts. In addition, exposure to restraint stress prior to LPS administration augmented the LPS-induced increase in IL-10 mRNA (~ 2 fold). In contrast, when mice were exposed to restraint stress without LPS administration, there was no alteration in IL-10 mRNA relative to saline-treated counterparts. (Figure 3.6.2)

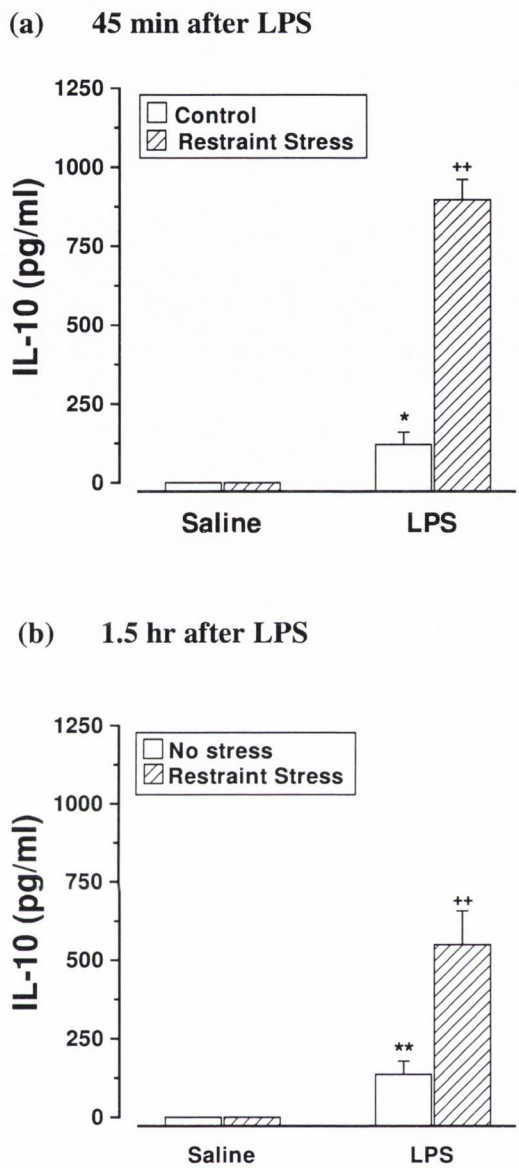


Figure 3.6.1 Exposure to restraint stress augmented the LPS-induced increase in serum IL-10 production after 45 min (a) and 1.5 hr (b)

LPS (250µg/kg) was administered to mice immediately following a 2 hr restraint stress and the mice were sacrificed 45 min or 1.5 hr after injection. A two-way ANOVA exposed a significant interaction effect of restraint stress with LPS challenge on IL-10 at 45 min [$F_{(1,16)}=84.14$, $P<0.0001$] and 1.5 hr [$F_{(1,16)}=12.93$, $P<0.001$]. Data are expressed as mean $\pm$ SEM (n=5). * $P<0.05$ vs. saline counterparts, ** $P<0.01$ vs. saline counterparts, ++ $P<0.01$ vs. non-stressed LPS counterparts (Neuman-Keuls post hoc test).

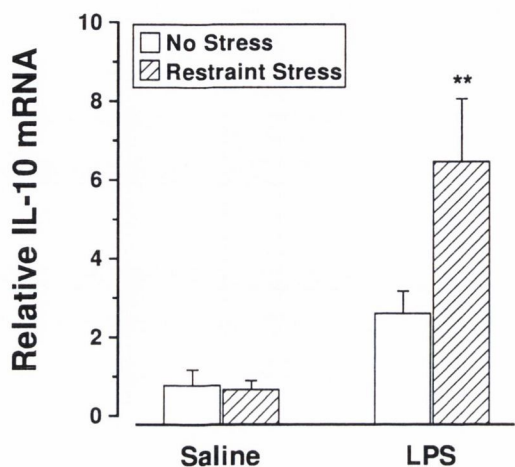


Figure 3.6.2 Exposure to restraint stress augmented the LPS-induced increase in expression of splenic IL-10 mRNA

LPS (250 μ g/kg) was administered to mice immediately following a 2 hr restraint stress and the mice were sacrificed 1.5 hr after injection. A two-way ANOVA exposed a significant interaction effect of restraint stress with LPS challenge on IL-10 at 45 min [$F_{(1,16)}=84.14$, $P<0.0001$]. Data are expressed as mean $\pm$ SEM (n=6). ** $P<0.01$ vs. LPS-treated counterparts

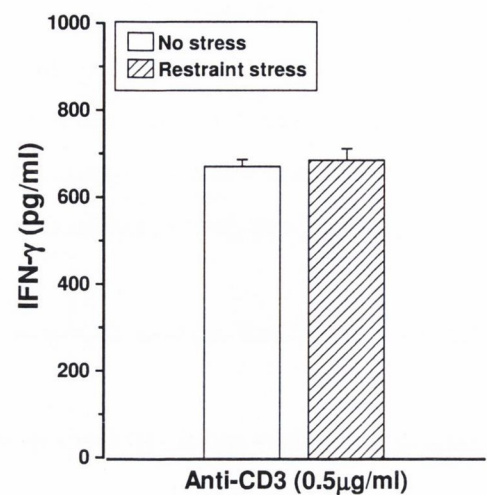
3.7 Does stress alter T-cell derived IFN- γ or IL-10?

The findings reported so far in this thesis have demonstrated that exposure to stress altered innate production of cytokines, however, T-cell derived production has not been investigated. Therefore, in the present section it was investigated if anti-CD3 stimulated splenocytes from stressed mice had altered T-cell derived production of IFN- γ or IL-10.

3.7.1 Exposure to stress does not alter T-cell derived IFN- γ or IL-10 production from splenocytes

Anti-CD3 stimulates T-cell production of IFN- γ and IL-10 from splenocytes. However, exposure to stress prior to culturing of splenocytes does not alter the T-cell derived increases in IFN- γ or IL-10 production. (Figure 3.7.1 a, b)

(a)



(b)

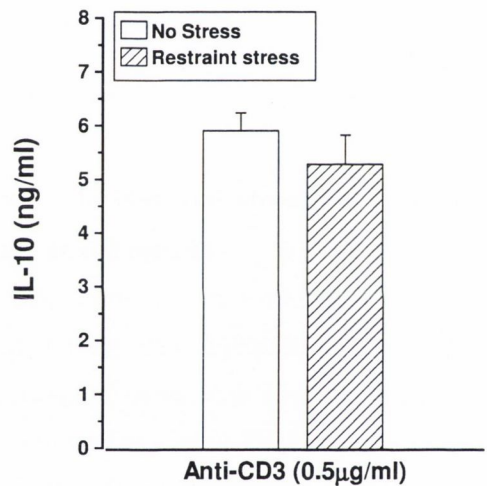


Figure 3.7.1 Exposure to restraint stress did not alter T-cell derived IFN-γ (a) or IL-10 (b) production from splenocytes

Adult male balb/c mice were exposed to acute restraint stress and sacrificed immediately upon removal from restraint tubes. Spleens were dissected and splenocytes were prepared at a concentration of 2×10^6 cells / ml, before stimulation with anti-CD3 (0.5 μg/ml) *ex vivo*. Data are expressed as mean $\pm$ SEM (n=8).

3.8 Can IL-10 suppress LPS-induced IFN- γ and IL-12?

IL-10 is known to have suppressive effects on the production of some pro-inflammatory cytokines *in vitro*. It has been shown in the results presented previously that IL-10 production is induced following acute restraint stress and it is produced early in the cascade of production of cytokines – as early as 45 min following LPS injection. Therefore, in the present study, the immunosuppressive effects of IL-10 were investigated to determine if the stress-induced increase in IL-10 could be the mediator of the stress-induced suppression of IFN- γ and IL-12. Therefore, mouse splenocytes were exposed to mouse recombinant IL-10 (mIL-10) *in vitro* to investigate the effect of exogenous IL-10 on IFN- γ and IL-12 production. Physiologically relevant doses of mIL-10 were chosen that mimic the *in vivo* concentrations following exposure to stress.

3.8.1 IL-10 suppressed LPS-induced IFN- γ and IL-12 concentrations

There were undetectable levels of IFN- γ and IL-12 in the supernatant from unstimulated splenocytes. However, stimulation with LPS induced concentrations of IFN- γ (~ 60 fold) and IL-12 (~ 4 fold) relative to saline-treated counterparts. The LPS-induced IFN- γ and IL-12 were not evident in the splenocytes that were pre-incubated with mouse recombinant IL-10 at all doses investigated (1, 3, 10 ng/ml). (Figure 3.8.1 a, b).

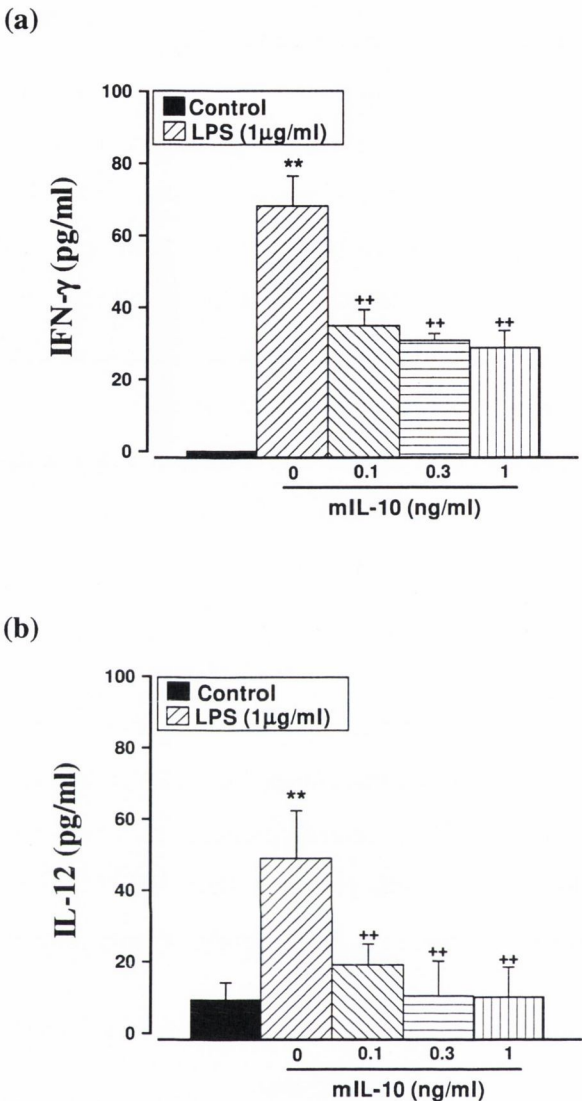


Figure 3.8.1 IL-10 suppressed LPS-induced IFN- γ (a) and IL-12 (b) concentrations from splenocytes

Splenocytes were cultured from freshly isolated spleens and the cell suspension was prepared to 2×10^6 cells / ml. The splenocytes were stimulated with LPS (1 μ g/ml) incubated for 96 hr in the presence or absence of recombinant mouse IL-10 (0.1-1 ng/ml). A one-way ANOVA demonstrated a significant effect of IL-10 treatment on IFN- γ [$F_{(4,27)} = 8.83$, $P < 0.001$] and IL-12 [$F_{(4,26)} = 13.9$, $P < 0.001$] production from splenocytes *in vitro*. Data are expressed as mean $\pm$ SEM ($n = 8$). ** $P < 0.01$ vs. non-LPS treated counterparts; ++ $P < 0.01$ vs. LPS treated counterparts (Newman-Keuls test).

3.9 Could IL-10 be the mediator of the stress-induced inhibition of IFN- γ and IL-12?

In the previous experiment, IL-10 was shown to inhibit LPS-induced IFN- γ and IL-12 concentrations *in vitro*. Therefore, in the present study, we investigated the effect of *in vivo* blockade of IL-10 receptor by administering the anti-IL-10 receptor antibody to mice. The anti-IL-10 receptor antibody binds to the IL-10 receptor, thereby preventing IL-10 from binding to its receptor and activating downstream signalling. In addition, IL-10 KO mice were exposed to stress to investigate the resultant LPS-induced IFN- γ and IL-12 cytokine production.

3.9.1 Anti-IL-10 receptor antibody failed to alter stress-induced inhibition of LPS-induced IFN- γ and IL-12 mRNA expression

Administration of the control anti-body (anti-IgG₁) prior to exposure to stress inhibited LPS-induced IFN- γ (~ 5 fold) and IL-12 (~ 2 fold) mRNA expression in mouse spleen relative to anti-IgG₁ alone treated counterparts. Similarly, administration of anti-IL-10R antibody prior to exposure to stress inhibited LPS-induced IFN- γ (~ 5 fold) and IL-12 (~ 10 fold) mRNA expression in mouse spleen relative to anti-IL-10R antibody treated counterparts. (Figure 3.9.1 a, b)

3.9.2 The stress-induced inhibition of LPS-induced IFN- γ serum concentrations and mRNA expression was not altered in IL-10 KO mice

As shown in previous results, control mice (C57 / Blk 6) exposed to stress have inhibited LPS-induced IFN- γ serum concentrations (~ 2 fold) and IFN- γ mRNA expression (~ 6 fold) relative to saline-treated counterparts. IL-10 KO mice have much greater LPS-induced IFN- γ serum concentrations (~ 25 fold) and IFN- γ mRNA expression (~ 750 fold) relative to control mice. However, exposure to stress has a similar stress-induced inhibition of IFN- γ serum concentrations and IFN- γ mRNA expression relative to LPS-treated counterparts. (Figure 3.9.2 a, b)

3.9.3 The stress-induced inhibition of LPS-induced IL-12p40 mRNA expression was not altered in IL-10 KO mice

As shown in previous results, control mice (C57 / Blk6) exposed to stress have inhibited LPS-induced IL-12p40 mRNA expression (~ 10 fold) relative to LPS-treated counterparts. IL-10 KO mice have much greater LPS-induced IL-12p40 mRNA

Chapter 3 Results

expression (~ 600 fold) relative to control mice. However, exposure to stress has a similar stress-induced inhibition of IFN- γ mRNA expression relative to LPS-treated counterparts. (Figure 3.9.3)

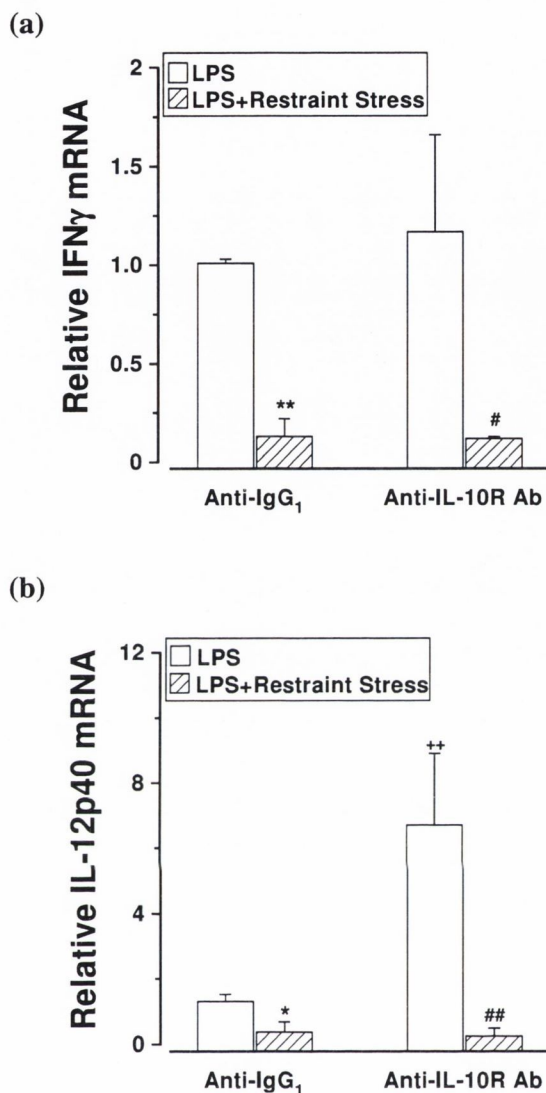


Figure 3.9.1 Anti-IL10 receptor antibody failed to alter the stress-induced inhibition of IFN- γ (a) or IL-12p40 (b) expression in mouse spleen

Anti-IL-10 receptor antibody (0.3 mg / mouse) was administered 30 min prior to receiving a 2 hr restraint stress and LPS (250 μ g/kg) was administered immediately following the stress. A two-way ANOVA exposed a significant effect of restraint stress with LPS challenge on the mRNA expression of IFN- γ [$F_{(1,16)}=5.27$, $P<0.01$] and IL-12p40 [$F_{(1,16)}=5.55$, $P<0.01$]. Data are expressed as mean $\pm$ SEM (n=5). ** $P<0.01$ vs. anti-IgG treated counterparts, * $P<0.05$ vs. anti-IgG treated counterparts, ++ $P<0.01$ vs. anti-IgG treated counterparts, ## $P<0.01$ vs. anti-IL-10R Ab treated counterparts, # $P<0.05$ vs. anti-IL-10R Ab treated counterparts (Neuman-Keuls post hoc test).

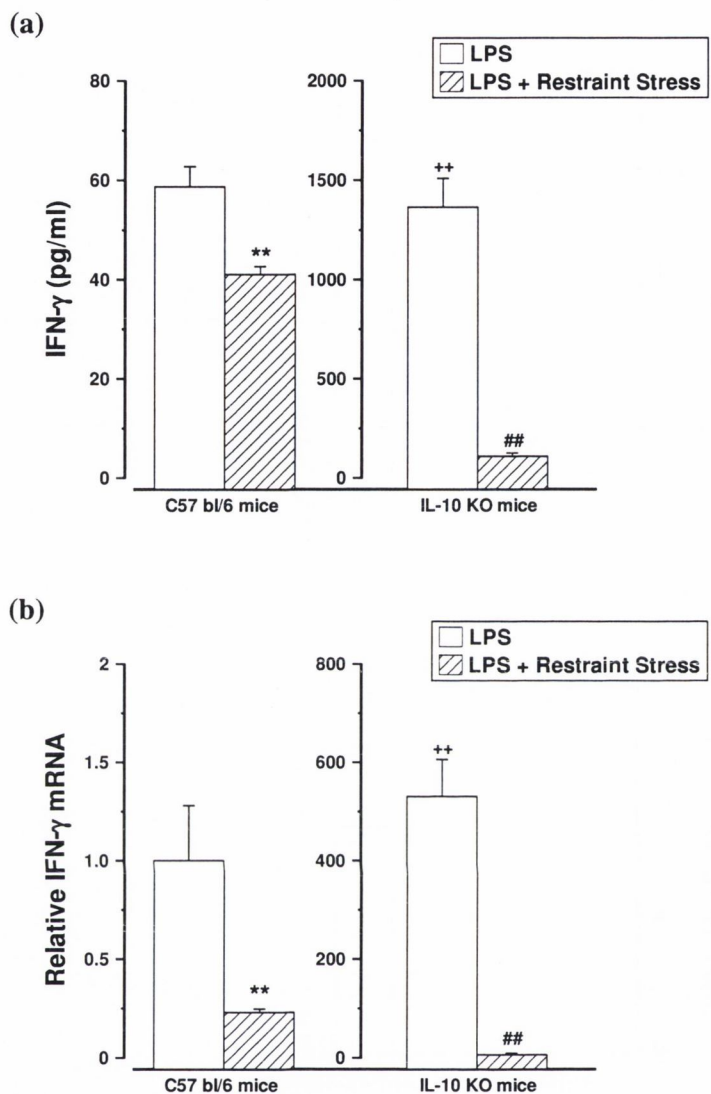


Figure 3.9.2 The stress-induced inhibition of IFN- γ serum concentrations (a) and IFN- γ mRNA expression (b) are not altered in spleen of IL-10 KO mice

IL-10 KO mice or C57/ blk6 control mice were exposed to 2 hr restraint stress and LPS (250 μ g/kg) was administered immediately following the stress. A two-way ANOVA exposed a significant effect of restraint stress in IL-10 KO LPS challenge on IFN- γ serum concentrations [$F_{(1,17)}=128.8$, $P<0.0001$] and IFN- γ mRNA expression [$F_{(1,16)}=78.5$, $P<0.0001$]. Data are expressed as mean $\pm$ SEM ($n=5/6$). ** $P<0.01$ vs. C57 LPS-treated counterparts, ++ $P<0.01$ vs. C57-counterparts, ## $P<0.01$ vs. IL-10 KO LPS-treated counterparts (Neuman-Keuls post hoc test).

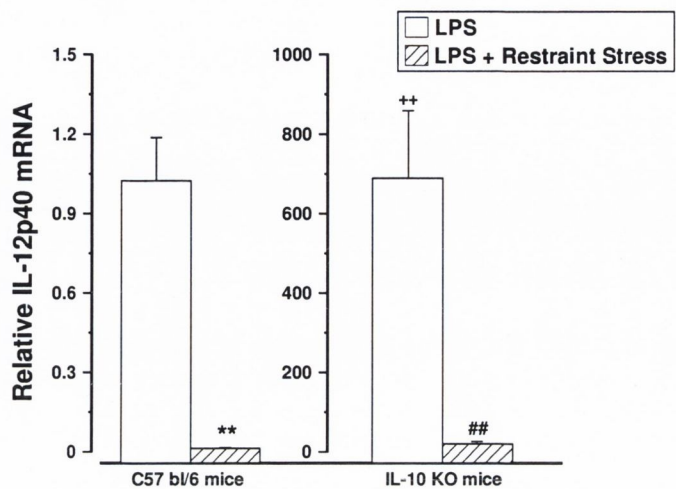


Figure 3.9.3 The stress-induced inhibition of IL-12p40 mRNA expression is not altered in spleens of IL-10 KO mice

Mice were exposed to 2 hr restraint stress and LPS (250µg/kg) was administered immediately following the stress. A two-way ANOVA exposed a significant effect of restraint stress in IL-10 KO LPS challenge on IL-12p40 mRNA expression [$F_{(1,17)}=28.3$, $P<0.0001$]. Data are expressed as mean $\pm$ SEM (n=5/6). ** $P<0.01$ vs. C57 LPS-treated counterparts, ++ $P<0.01$ vs. C57-counterparts, ## $P<0.01$ vs. IL-10 KO LPS-treated counterparts (Neuman-Keuls post hoc test).

3.10 Can exposure to stress alter production of other anti-inflammatory cytokines?

In the previous results we have shown that stress can increase the anti-inflammatory cytokine IL-10. However, the effect of stress on the production of other anti-inflammatory cytokines is not well elucidated. Therefore, in the present study, we investigated the effect of exposure to stress on TGF- β production. TGF- β is one of the most diverse acting pro-inflammatory cytokines and plays many important immunosuppressive roles (Govinden and Bhoola, 2003).

In addition, we examined the IL-10 family of cytokines as they might display a similar response to stress as IL-10. IL-10 shares structural homology to three other cytokines that have been identified as part of the IL-10 family – IL-19, IL-20 and IL-24. Although these members share structural homology, they have been shown to display differential biological functions. Therefore, we examined the effect of restraint stress on expression of these novel IL-10 family members.

3.10.1 Exposure to restraint stress failed to alter TGF- β expression

LPS administration had no significant alteration in splenic TGF- β mRNA expression relative to saline-treated mice. In addition, exposure to restraint stress with or without LPS failed to alter levels of TGF- β mRNA expression (Figure 3.10.1)

3.10.2 Exposure to restraint stress increased LPS-induced IL-19 mRNA expression

Exposure to restraint stress increased LPS-induced IL-19 mRNA expression after 45 min (~ 2 fold) and after 1.5 hr (~ 3 fold) relative to saline-treated counterparts. In contrast, when mice were exposed to restraint stress without LPS administration, there was no alteration in IL-19 mRNA expression relative to saline-treated counterparts. (Figure 3.10.2 a, b)

3.10.3 Exposure to restraint stress failed to alter IL-24 or IL-20 mRNA expression

There was very little IL-24 mRNA expression detected in the spleens of mice treated with saline, LPS or stress, and almost no IL-20 mRNA expression detected, as indicated by the low relative quantification (RQ) values. The amplification of cDNA in the spleen tissue could not take place because the targets were not present. This indicates that IL-24 and IL-20 are not involved in stress-induced immunosuppression. (Table 3.10.3)

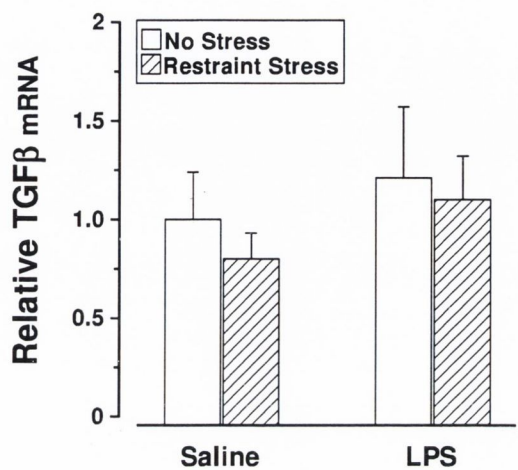


Figure 3.10.1 Treatment with LPS or restraint stress failed to alter TGF-β expression in mouse spleen

LPS (250μg/kg) was administered to mice immediately following a 2 hr restraint stress and the mice were sacrificed 1.5 hr after injection. Data are expressed as mean ± SEM (n=5).

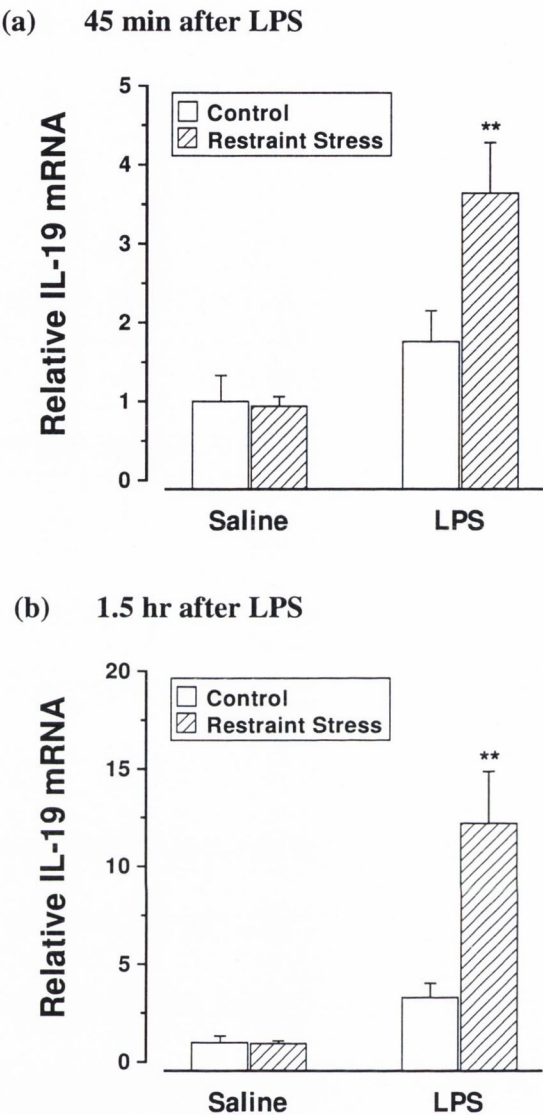


Figure 3.10.2 Exposure to restraint stress significantly augmented LPS-induced increase in IL-19 expression after 45 min (a) and 1.5 hr (b) in mouse spleen

LPS (250 μ g/kg) was administered to mice immediately following a 2 hr restraint stress and the mice were sacrificed 45 min or 1.5 hr after injection. A two-way ANOVA exposed a significant interaction effect of restraint stress with LPS challenge on IL-19 at 1.5 hr [$F_{(1,14)}=9.05$, $P<0.005$] and at 45 min [$F_{(1,16)}=$, $P<0.0001$]. Data are expressed as mean $\pm$ SEM (n=5). ** $P<0.01$ vs. saline-treated counterparts, * $P<0.05$ vs. non-stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

Treatment (RQ)	IL-19 (n=5)	IL-24 (n=5)	IL-20 (n=5)
Saline	1± 0.33	1±.4	1 ± 0.74
LPS	4.13 ± 0.72	2.4± 1.4	0.63 ± 0.63
LPS + restraint	12.3 ± 2.65 **	2.6 ± 1.6	0.81 ± 0.81

Table 3.10.3 Neither LPS nor restraint stress altered IL-24 or IL-20 mRNA expression

LPS (250µg/kg) was administered to mice immediately following a 2 hr restraint stress and the mice were sacrificed 1.5 hr after injection. Data are expressed as relative quantification (RQ) figures which are calculated from the cycle time (CT) of the target – CT of the endogenous control relative to that of saline-treated control mice. (n=5). A two-way ANOVA exposed a significant interaction effect of restraint stress with LPS challenge on IL-19 at 1.5 hr [$F_{(1,14)}=9.05$, $P<0.005$]. ** $P<0.001$ vs. LPS alone (Neuman-Keuls post hoc test).

3.11 Are the immunomodulating effects of stress mediated via stress-induced release of NA?

The physiological response to stressor exposure is the release of glucocorticoids and catecholamines into the periphery. The catecholamine NA has been implicated as a possible mediator of the stress-induced immune changes. Therefore, in the present study, the effect of NA on bone marrow dendritic cells (DCs) was investigated to see if it could mimic the effect of stress on the production of cytokines. DCs are professional APCs capable of stimulating resting T-cells. The hypothesis was that pre-treatment of DCs with NA could mimic the effect of stress on the cells and allow characterisation of the direct effect of NA on the production of cytokines. In the present study, the effect of NA on the mRNA expression of IL-10 and IL-12 were investigated.

3.11.1 NA increased LPS-induced IL-10 mRNA expression in DCs

Pre-treatment of DCs with NA did not alter IL-10 mRNA expression relative to saline-treated counterparts. In addition, administration of LPS did not significantly alter IL-10 mRNA expression relative to saline-treated counterparts. However, pre-treatment with NA significantly increased IL-10 mRNA (~ 5 fold) relative to LPS-treated counterparts. (Figure 3.11.1 a)

3.11.2 NA increased LPS-induced IL-19 mRNA expression in DCs

Pre-treatment of DCs with NA did not alter IL-19 mRNA expression relative to saline-treated counterparts. In addition, administration of LPS did not significantly alter IL-19 mRNA expression relative to saline-treated counterparts. However, pre-treatment with NA significantly increased IL-19 mRNA (~ 5 fold) relative to LPS-treated counterparts. (Figure 3.11.1 b)

3.11.3 NA inhibited LPS-induced IL-12p40 mRNA expression

Pre-treatment of DCs with NA did not alter IL-12p35 or IL-12p40 mRNA expression relative to saline-treated counterparts. However, treatment with LPS increased expression of IL-12p35 mRNA (~20 fold) and IL-12p40 mRNA (~100 fold) relative to saline-treated counterparts. The LPS-induced increases IL-12p35 and IL-12p40 were significantly reduced by pre-treatment with NA. This NA-induced inhibition of IL-12 mimics the inhibiting action of stress on IL-12p40 mRNA expression that was previously demonstrated *in vivo*. (Figure 3.11.2 a, b)

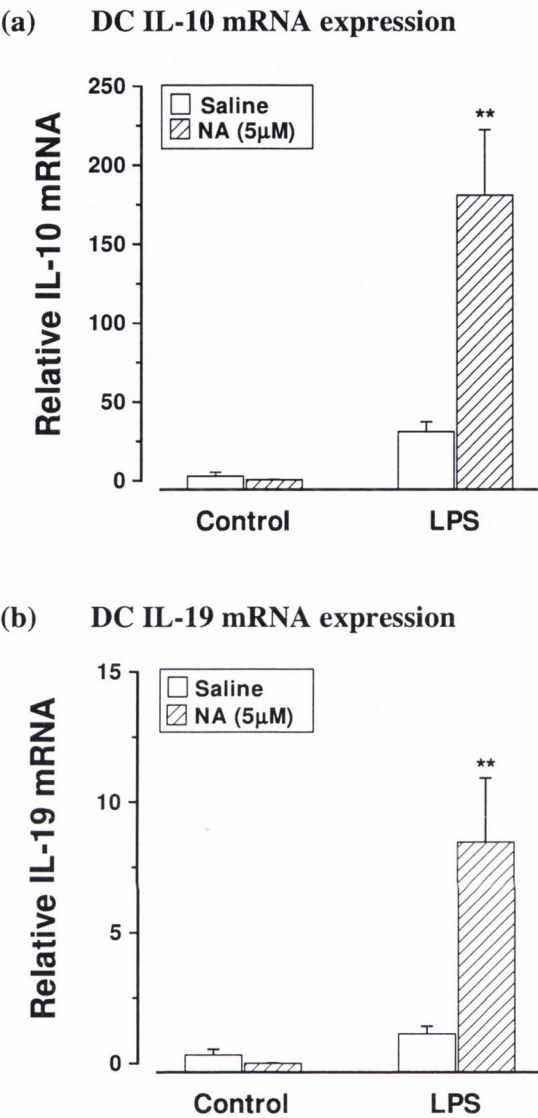
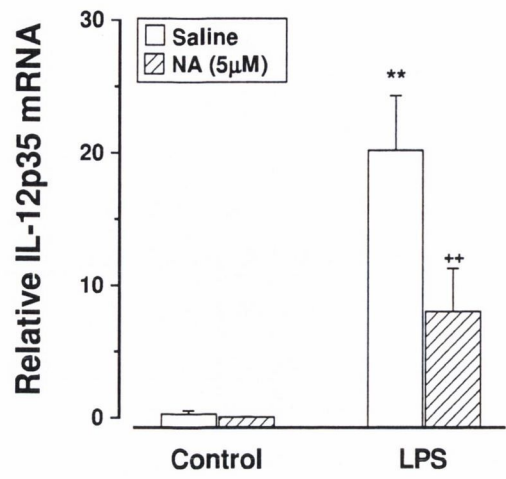


Figure 3.11.1 NA mimicked the augmenting effect of stress on LPS-induced IL-10 and IL-19 expression

DCs were pre-treated with NA (5µM) for 30 min prior to treatment with LPS (1µg/ml) for 6 hr. A two-way ANOVA uncovered a significant interaction effect between NA and LPS treatment on the expression of IL-10 mRNA [$F_{(1,14)}=7.55$, $P<0.01$] and IL-19 mRNA [$F_{(1,14)}=4.88$, $P<0.05$]. Data are expressed as mean $\pm$ SEM (n=4). ** $P<0.01$ vs. all groups (Neuman-Keuls post hoc test).

(a) DC IL-12p35 mRNA expression



(b) DC IL-12p40 mRNA expression

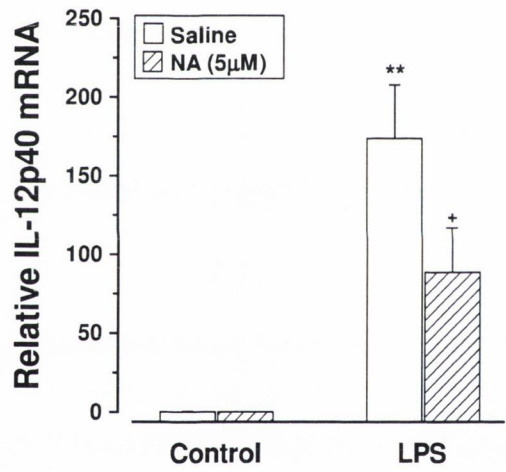


Figure 3.11.2 NA mimicked the suppressive action of stress on LPS-induced IL-12p35 and IL-12p40 expression

DCs were pre-treated with NA (5µM) for 30 min prior to treatment with LPS (1µg/ml) for 6 hr. A two-way ANOVA uncovered a significant interaction effect between pre-treatment with NA and LPS treatment on the expression of IL-12p35 mRNA [$F_{(1,14)}=5.41$, $P<0.05$]. A significant effect of LPS treatment was uncovered on IL-12p40 mRNA expression [$F_{(1,11)}=30.58$, $P<0.0001$]. Data are expressed as mean $\pm$ SEM ($n=4$). ** $P<0.01$ vs. non-LPS controls, ++ $P<0.01$ vs. LPS-treated counterparts, + $P<0.05$ vs. LPS-treated counterparts (Neuman-Keuls post hoc test).

3.12 Can the β -AR antagonist propranolol block NA-induced effects?

Following the demonstration that NA can modulate the production of cytokines from LPS stimulated DCs, we investigated the cellular target for NA on DC's. NA exerts its effects by binding to the β -AR and increasing intracellular cAMP. The immunomodulating functions of NA are mainly mediated via the β_2 -AR which is expressed on immune cells. Therefore, the aim of the present study was to investigate if we could block the NA-induced alterations in cytokine production using a non-selective eta-adrenergic receptor blocking agent, propranolol.

3.12.1 Propranolol impaired the augmenting effect of NA on LPS-induced IL-10 mRNA expression

In agreement with previous results, NA augments LPS-induced IL-10 mRNA expression (~5 fold) relative to LPS-treated counterparts. This NA-induced increase in IL-10 mRNA expression was not evident in DCs that were pre-treated with propranolol prior to NA and LPS administration. (Figure 3.12.1 a)

3.12.2 Propranolol impaired the augmenting effect of NA on LPS-induced IL-19 mRNA expression

In agreement with previous results, NA augments LPS-induced IL-19 mRNA expression (~6 fold) relative to LPS-treated counterparts. This NA-induced increase in IL-19 mRNA expression was not evident in DCs that were pre-treated with propranolol prior to NA and LPS administration. (Figure 3.12.1 b)

3.12.2 Propranolol impaired the suppressive effect of NA on LPS-induced IL-12p40 mRNA expression

In agreement with previous results, NA inhibits LPS-induced IL-12p40 mRNA expression (~2 fold) relative to LPS-treated counterparts. Pre-treatment with propranolol increased IL-12p40 mRNA expression ~2 fold relative to LPS-treated counterparts. However, the NA-induced inhibition of LPS-induced IL-12p40 was not evident in the DCs pre-treated with propranolol relative to the propranolol alone-treated counterparts (Figure 3.12.2)

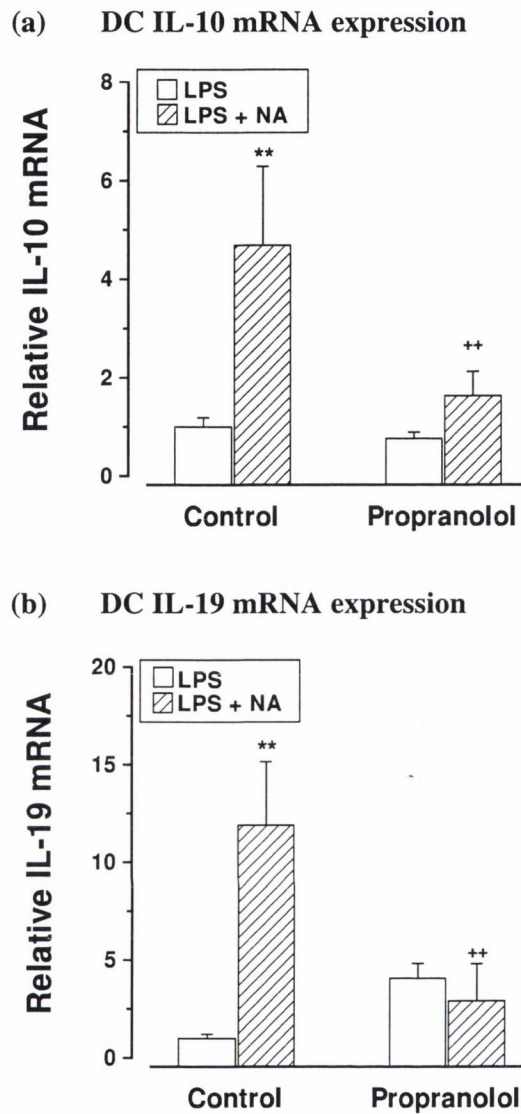


Figure 3.12.1 Propranolol inhibits the augmenting effect of NA on LPS-induced IL-10 (a) and IL-19 (b) mRNA expression

DCs were pre-treated with propranolol (5 μ M) for 15 min and NA (5 μ M) for 30 min prior to treatment with LPS (1 μ g/ml) for 6 hr. A two-way ANOVA uncovered a significant interaction effect between groups on the expression of IL-10 mRNA [$F_{(1,16)}=5.63$, $P<0.01$] and IL-19 mRNA [$F_{(1,16)}=4.22$, $P<0.01$]. Data are expressed as mean $\pm$ SEM ($n=5$). ** $P<0.001$ vs. saline-treated counterparts controls, ++ $P<0.001$ vs. non-propranolol – treated counterparts (Neuman-Keuls post hoc test).

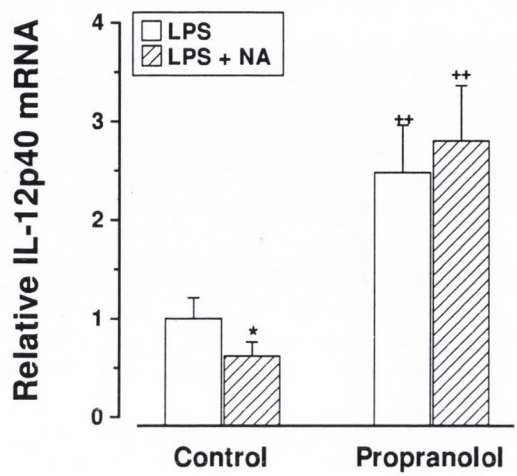


Figure 3.12.2 Propranolol inhibits the suppressive effect of NA on the LPS-induced inhibition of IL-12p40 mRNA expression

DCs were pre-treated with propranolol (5μM) for 15 min and NA (5μM) for 30 min prior to treatment with LPS (1μg/ml) for 6 hr. A two-way ANOVA uncovered a significant interaction effect between treatments on the expression of IL-12p40 mRNA [$F_{(1,17)}=47.15$, $P<0.0001$]. Data are expressed as mean $\pm$ SEM (n=5). * $P<0.05$ vs. saline controls, ++ $P<0.01$ vs. non-propranolol controls (Neuman-Keuls post hoc test).

3.13 Are the immunomodulating effects of stress mediated via the β -AR?

The previous results indicate that NA can mimic the immunosuppressant effects of stress *in vitro*. Therefore, NA could be the main mediator of the stress induced effects on the innate IFN- γ response that have been shown previously. NA actions on immune cells are mediated via the β -AR which is expressed by immune cells. The aim of the present study was to investigate if *in vivo* blockade of the β -AR could impair the stress-induced effect on LPS-induced production of cytokines. Therefore, it was investigated if the peripherally acting β -AR antagonist nadolol could block the stress-induced increase in IL-10 and IL-19 production and the stress-induced IL-12 and IFN- γ .

3.13.1 Nadolol inhibited the augmenting effect of stress on LPS-induced IL-10 serum concentrations and splenic IL-10 mRNA expression

As previously described, exposure to stress augmented LPS-induced IL-10 serum concentrations (~ 4 fold) and IL-10 mRNA expression (~ 2 fold) relative to LPS-treated counterparts. However, when nadolol was administered prior to exposure to stress and LPS administration, the stress-induced increases in IL-10 serum concentrations and IL-10 mRNA expression were not evident. In mice that were administered with nadolol and LPS but with no exposure to stress, IL-10 serum concentrations and IL-10 mRNA expression were comparable to LPS-treated counterparts. (Figure 3.13.1 a, b)

3.13.2 Nadolol inhibited the augmenting effect of stress on LPS-induced IL-19 mRNA expression

As previously described, exposure to stress augmented the LPS-induced IL-19 mRNA expression in the spleen (~4 fold) relative to saline-treated counterparts. However, when nadolol was administered prior to stressor exposure and LPS administration, the stress-induced increase in IL-10 was not evident. Mice administered with nadolol and LPS but with no exposure to stress had similar IL-10 serum concentrations to LPS-treated counterparts. (Figure 3.13.2)

3.13.3 Nadolol failed to alter the suppressive effects of stress on LPS-induced IFN- γ serum concentrations and IFN- γ mRNA expression

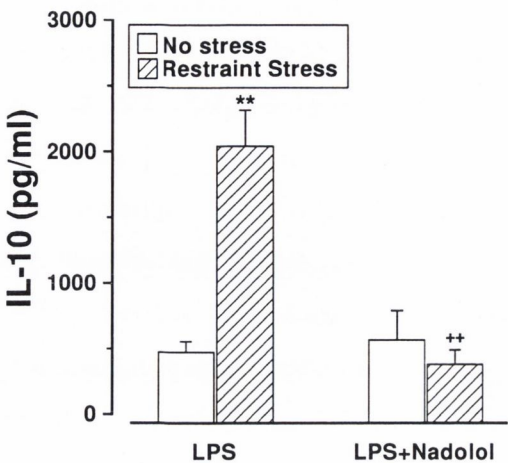
As shown in previous results, exposure to stress inhibited LPS-induced IFN- γ serum concentrations (~ 2 fold) and IFN- γ mRNA expression (~ 2 fold) relative to saline-treated counterparts. Exposure to stress with nadolol and LPS administration had a

similar stress-induced inhibiting effect on IFN- γ serum concentrations and IFN- γ mRNA expression relative to the mice without nadolol administration. Exposure to stress inhibited the nadolol + LPS-induced IL-12 serum concentrations (~ 2 fold) and IL-12p40 mRNA expression (~2 fold) relative to nadolol + LPS-treated counterparts. (Figure 3.13.3 a, b)

3.13.4 Nadolol failed to alter the suppressive effects of stress on IL-12 serum concentrations and IL-12p40 mRNA expression

As shown in previous results, exposure to stress reduced LPS-induced IL-12 serum concentrations (~3 fold) and IL-12p40 mRNA expression (~ 2 fold) relative to saline-treated counterparts. When nadolol was administered to mice prior to LPS there was an additive effect, with increased IL-12 serum concentrations (< 2 fold) and IL-12p40 mRNA expression (~ 3 fold) relative to LPS-treated counterparts. Exposure to stress following nadolol and LPS administration had a similar stress-induced inhibiting effect on IL-12 serum concentrations and IL-12p40 mRNA expression as the mice without nadolol administration. Exposure to stress inhibited the nadolol + LPS-induced IL-12 serum concentrations (~ 2 fold) and IL-12p40 mRNA expression (~2 fold) relative to nadolol + LPS – treated counterparts. (Figure 3.13.4 a, b)

(a) Serum IL-10 concentration



(b) Splenic IL-10 mRNA expression

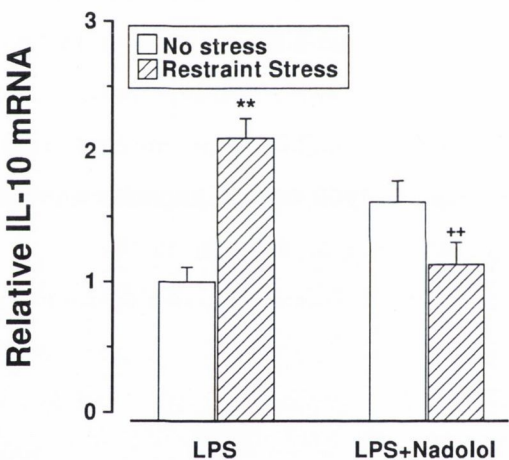


Figure 3.13.1 Nadolol impaired the stress-induced augmentation of LPS-induced serum IL-10 concentrations (a) and mRNA expression in mouse spleen (b)

Nadolol (0.5mg/kg) was administered to mice 30 min prior to exposure to 2 hr restraint stress. LPS (250µg/kg) was administered immediately after the stress and the mice were sacrificed 1.5 hr after injection of LPS. A two-way ANOVA exposed a significant interaction effect of restraint stress with nadolol + LPS on IL-10 serum concentration [$F_{(1,16)}=14.81$, $P<0.001$] and IL-10 mRNA expression [$F_{(1,16)}=27.05.11$, $P<0.0005$]. Data are expressed as mean $\pm$ SEM (n=5). ** $P<0.01$ vs. non-stressed LPS-treated counterparts, ** $P<0.01$ vs. stressed LPS-treated counterparts, + $P<0.05$ vs. stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

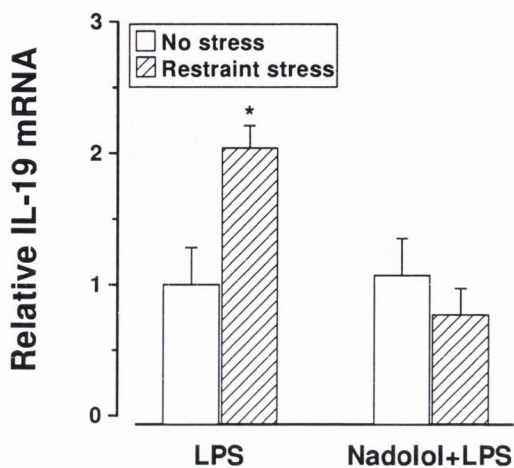


Figure 3.13.2 Nadolol impaired the stress-induced augmentation of the LPS-induced IL-19 mRNA expression in mouse spleen

Nadolol (0.5mg/kg) was administered to mice 30 min prior to exposure to 2 hr restraint stress. LPS (250µg/kg) was administered immediately after the stress and the mice were sacrificed 1.5 hr after injection of LPS. A two-way ANOVA exposed a significant interaction effect of restraint stress with nadolol + LPS on IL-19 mRNA expression [$F_{(1,20)}=7.11$, $P<0.01$]. Data are expressed as mean $\pm$ SEM (n=5). * $P<0.05$ vs. non-stressed LPS-treated counterparts, (Neuman-Keuls post hoc test).

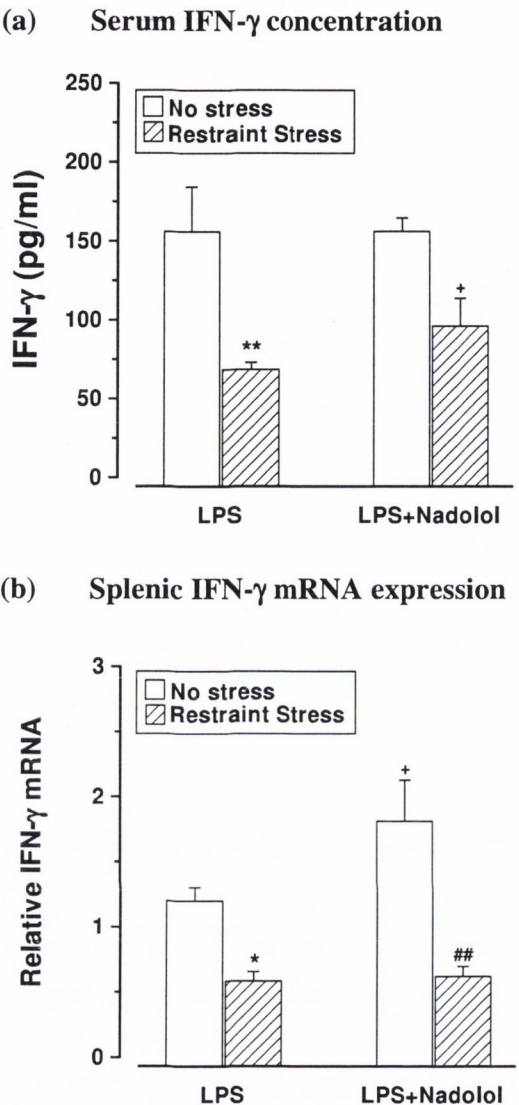


Figure 3.13.3 Nadolol failed to alter the stress-induced inhibition of LPS-induced IFN- γ serum concentration (a) or mRNA expression in mouse spleen (b)

Nadolol (0.5mg/kg) was administered to mice 30 min prior to a 2 hr restraint stress. LPS (250 μ g/kg) was given immediately after the stress and the mice were sacrificed 1.5 hr after injection of LPS. A two-way ANOVA revealed a significant interaction effect of nadolol and LPS challenge on IFN- γ serum concentrations [$F_{(1,17)}=17.88$, $P<0.0005$] and IFN- γ mRNA expression [$F_{(1,18)}=20.12$, $P<0.0005$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P<0.01$ vs. non-stressed LPS treated counterparts, * $P<0.05$ vs. non-stressed LPS treated counterparts, + $P<0.05$ vs. non-stressed LPS treated counterparts, ## $P<0.01$ vs. non stressed LPS + nadolol – treated counterparts (Neuman-Keuls post hoc test).

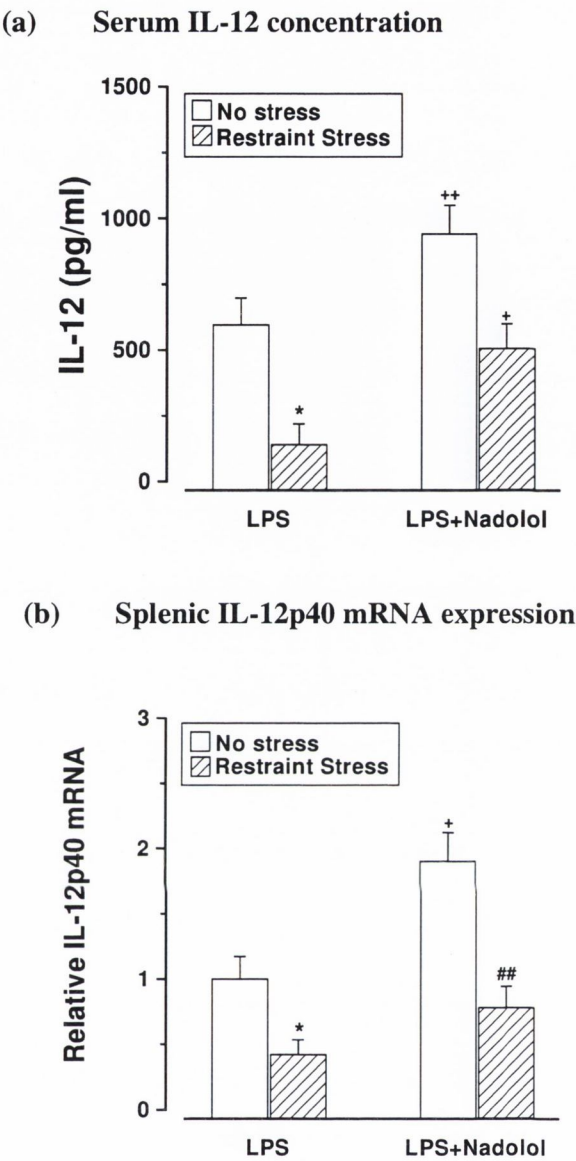


Figure 3.13.4 Nadolol failed to alter the stress-induced inhibition of LPS-induced IL-12p40 mRNA expression in mouse spleen

Nadolol (0.5mg/kg) was administered to mice 30 min prior to a 2 hr restraint stress. LPS (250µg/kg) was given immediately after the stress and the mice were sacrificed 1.5 hr after injection of LPS. A two-way ANOVA revealed a significant effect of nadolol and LPS challenge on IL-12 serum concentrations [$F_{(1,17)}=8.64$, $P<0.005$] and IL-12p40 mRNA expression [$F_{(1,17)}=3.58$, $P<0.01$]. Data are expressed as mean $\pm$ SEM (n=6). ** $P<0.01$ vs. LPS-treated counterparts, * $P<0.05$ vs. non-stressed LPS treated counterparts, † $P<0.05$ vs. non-stressed LPS + nadolol treated counterparts (Neuman-Keuls post hoc test).

3.14 Can corticosterone inhibit production of LPS-induced cytokines?

The previous results indicate that although NA does have a role to play in stress-induced immunomodulation, however it is not the main mediator of the stress-induced suppression of the cytokine network. Glucocorticoids, such as corticosterone in mice, are released into the periphery in response to a stressful input. The previous results have shown that exposure to acute restraint stress increased concentrations of corticosterone in serum (~150 fold) and in spleen (~ 5 fold) relative to non-stress counterparts. Corticosterone has been implicated as the main mediator of the stress-induced immunosuppressive effects. Therefore, in the present study, we investigated the ability of corticosterone to inhibit the LPS-induced IFN- γ and IL-12 concentrations.

3.14.1 Corticosterone inhibited LPS-induced IFN- γ and IL-12 concentrations from splenocytes

Splenocytes stimulated with LPS significantly induced IFN- γ (~ 70 fold) and IL-12 (~ 5 fold) concentrations relative to saline-treated counterparts. However, pre-treatment with corticosterone (0.01 – 1 μ M) prior to LPS administration inhibited the LPS-induced production of IFN- γ and IL-12 in a dose-dependant manner with complete inhibition of LPS-induced IFN- γ and IL-12 at 0.1 and 0.5 μ M respectively. (Figure 3.14.1 a, b)

3.14.2 IL-12 restored the corticosterone-induced inhibition of LPS-induced IFN- γ concentrations from splenocytes

As shown in the previous results, splenocyte LPS-induced IFN- γ concentrations are inhibited by pre-treatment with corticosterone. However, when IL-12 is administered following LPS stimulation, there is a dose-dependant increase in IFN- γ concentrations with increasing doses of IL-12.(Figure 3.14.2)

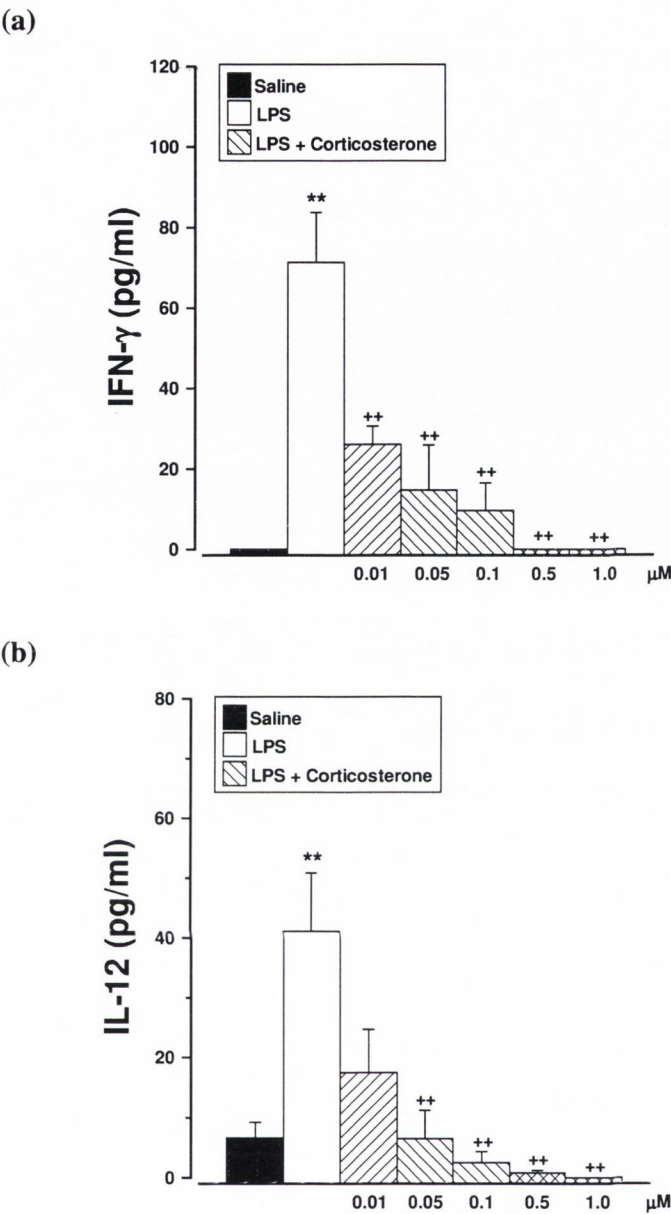


Figure 3.14.1 Corticosterone inhibited LPS-induced IFN- γ (a) and IL-12 (b) production from splenocytes

Splenocytes were cultured from freshly isolated spleens and the cell suspension was adjusted to 2×10^6 cells / ml. The splenocytes were pre-treated with corticosterone (0.01 – 1 μ M) for 30 min prior to simulation with LPS (1 μ g/ml). A one-way ANOVA revealed a significant effect of corticosterone with LPS administration on IFN- γ [$F_{(6,34)}=13.38$, $P<0.0001$] and IL-12 [$F_{(6,35)}=2.53$, $P<0.05$]. Data are expressed as mean $\pm$ SEM (n=5-6). ** $P<0.01$ vs. saline-treated counterparts, ** $P<0.01$ vs. LPS-treated counterparts (Neuman-Keuls post hoc test).

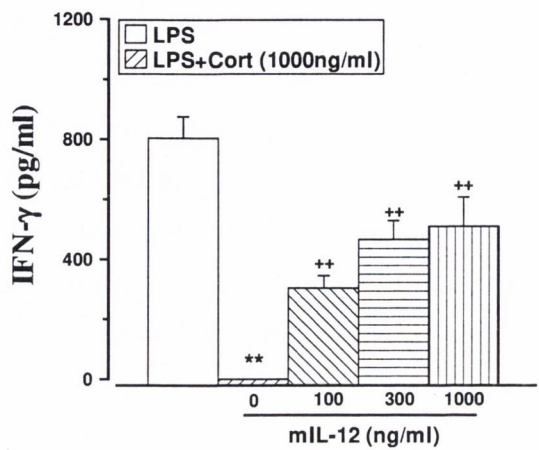


Figure 3.14.2 IL-12 restored corticosterone-induced inhibition of LPS-induced IFN- γ production from splenocytes

Splenocytes were cultured from freshly isolated spleens and the cell suspension was adjusted to 2×10^6 cells / ml. The splenocytes were pre-treated with corticosterone (0.01 – 1 μ M) for 30 min prior to stimulation with LPS (1 μ g/ml). IL-12 (100-1000 ng/ml) was administered to splenocytes 2, 24 and 48 hr following LPS stimulation and supernatant was harvested 72 hr following stimulation. A repeated one-way ANOVA revealed a significant effect of IL-12 with LPS and corticosterone administration on IFN- γ [$F_{(7,32)}=6.89$, $P<0.0005$]. Data are expressed as mean $\pm$ SEM (n=6). ** $P<0.01$ vs. LPS-treated counterparts, ++ $P<0.01$ vs. non IL-12-treated counterparts (Neuman-Keuls post hoc test).

3.15 Are the immunomodulating effects of stress mediated via GR?

The previous results have indicated that an increase in corticosterone can inhibit the production of IFN- γ and IL-12. This leads us to consider if corticosterone could be the main mediator of the stress-induced inhibition of IFN- γ and IL-12. Corticosterone actions are mediated by the GR receptor which is expressed by many immune cells. Therefore, in the present study we investigated the ability of a GR antagonist mifepristone to block stress-induced suppression of the innate IFN- γ response.

3.15.1 Mifepristone prevented the suppressive effect of stress on LPS-induced IFN- γ serum concentrations and splenic IFN- γ mRNA expression

Similarly to results shown previously, exposure to stress inhibited LPS-induced IFN- γ serum concentrations (~4 fold) and IFN- γ mRNA expression (~ 3 fold) relative to LPS-treated counterparts. Administration of mifepristone prior to LPS did not alter IFN- γ serum concentration or mRNA expression relative to LPS-treated counterparts. In addition, exposure to stress did not alter the LPS + mifepristone induced serum concentrations or mRNA expression. Therefore, mifepristone prevented the stress-induced inhibition of LPS-induced IFN- γ serum concentrations or mRNA expression. (Figure 3.15.1 a, b)

3.15.2 Mifepristone prevented the suppressive effect of stress on LPS-induced iNOS and IP-10 mRNA expression

As shown in previous results, exposure to stress inhibited LPS-induced expression of iNOS mRNA and IP-10 mRNA (~ 2 fold) relative to LPS-treated counterparts. Administration of mifepristone prior to LPS did not significantly alter iNOS or IP-10 mRNA expression relative to LPS-treated counterparts. In addition, exposure to stress did not alter this LPS + mifepristone level of expression. Therefore, mifepristone blocked the stress-induced inhibition of LPS-induced iNOS and IP-10 mRNA expression. (Figure 3.15.2 a, b)

3.15.3 Mifepristone prevented the suppressive effect of stress on LPS-induced IL-12p40 mRNA expression

As shown in previous results, exposure to stress inhibited LPS-induced IL-12p40 mRNA expression (~ 3 fold) relative to LPS-treated counterparts. Administration of mifepristone prior to LPS did not significantly alter IL-12p40 mRNA expression

relative to LPS-treated counterparts. In addition, exposure to stress did not alter this LPS + mifepristone level of expression. Therefore, mifepristone blocked the stress-induced inhibition of LPS-induced IL-12p40 mRNA expression. (Figure 3.15.2)

3.15.4 Mifepristone prevented the suppressive effect of stress on LPS-induced IL-12R β 1 mRNA expression

As shown in previous results, exposure to stress inhibits LPS-induced IL-12R β 1 mRNA expression (~ 3 fold) relative to LPS-treated counterparts. Administration of mifepristone prior to LPS did not significantly alter IL-12R β 1 mRNA expression relative to LPS-treated counterparts. However, exposure to stress increased this LPS + mifepristone (~2 fold) relative to non-stressed counterparts. Therefore, mifepristone blocked the stress-induced inhibition of LPS-induced IL-12R β 1 mRNA expression. (Figure 3.15.4 a)

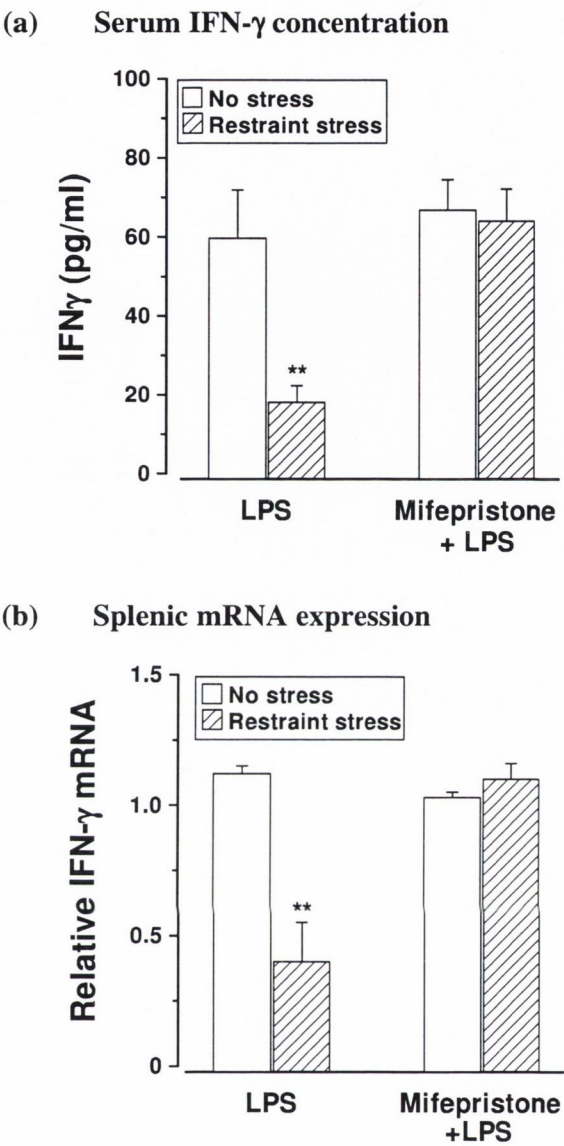


Figure 3.15.1 Mifepristone prevented the suppressive effect of stress on LPS-induced serum IFN- γ concentrations (a) and mRNA expression in mouse spleen (b)

Mifepristone (20 mg/kg; i.p.) was administered to mice 1 hr prior to exposure to restraint stress for 2 hr. LPS (250 μ g/ml; i.p.) was administered immediately following the restraint stress termination and mice were sacrificed 8 hr after LPS administration. A two-way ANOVA uncovered a significant interaction effect between groups on the expression of serum IFN- γ concentrations [$F_{(1,20)}=8.98$, $P<0.01$] and IFN- γ mRNA [$F_{(1,20)}=12.97$, $P<0.001$]. Data are expressed as mean $\pm$ SEM (n=6). ** $P<0.01$ vs. non-stressed LPS-treated counterparts (Fishers LSD post hoc test).

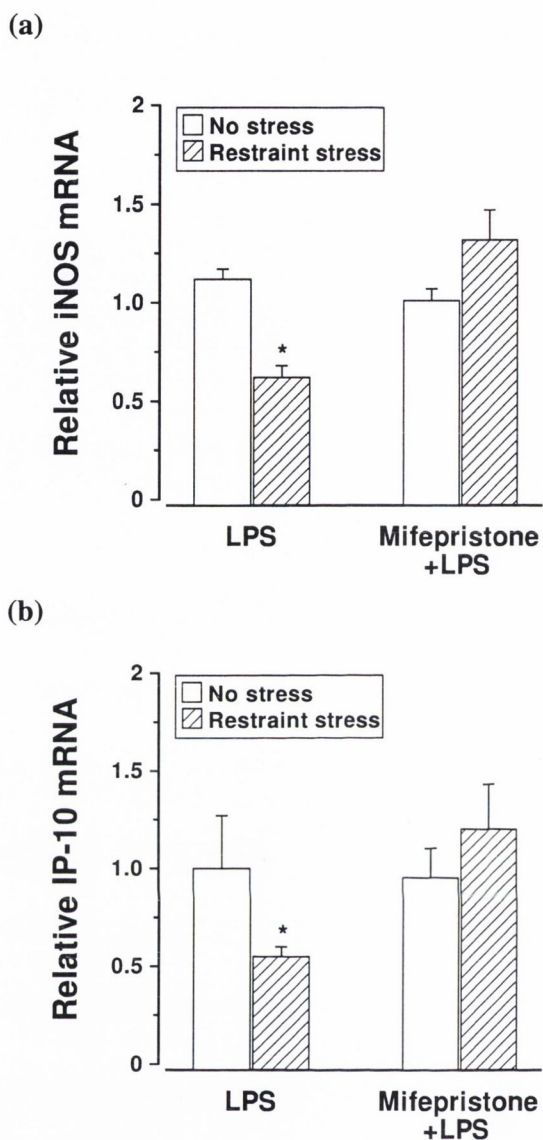


Figure 3.15.2 Mifepristone prevented the suppressive effect of stress on LPS-induced iNOS (a) and IP-10 (b) mRNA expression in mouse spleen

Mifepristone (20 mg/kg; i.p.) was administered to mice 1 hr prior to exposure to restraint stress for 2 hr. LPS (250 μ g/ml; i.p.) was administered immediately following the restraint stress termination and mice were sacrificed 8 hr after LPS administration. A two-way ANOVA uncovered a significant effect of mifepristone on the expression of IP-10 mRNA [$F_{(1,18)}=5.93$, $P<0.01$] and iNOS [$F_{(1,18)}=5.85$, $P<0.01$]. Data is expressed as mean $\pm$ SEM ($n=6$). * $P<0.05$ vs. non-stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

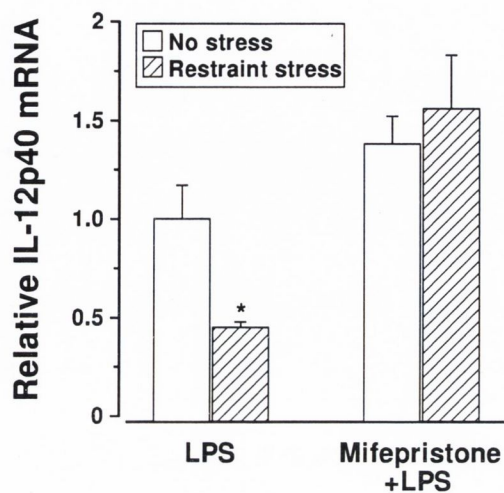


Figure 3.15.3 Mifepristone prevented the suppressive effect of stress on LPS-induced IL-12p40 mRNA expression in spleen

Mifepristone (20 mg/kg; i.p.) was administered to mice 1 hr prior to exposure to restraint stress for 2 hr. LPS (250 µg/ml; i.p.) was administered immediately following the restraint stress termination and mice were sacrificed 8 hr after LPS administration. A two-way ANOVA revealed a significant effect of mifepristone on the expression of IL-12p40 mRNA [$F_{(1,18)}=11.78$, $P<0.005$]. Data are expressed as mean $\pm$ SEM (n=6). * $P<0.05$ vs. non-stressed LPS-treated counterparts (Fishers LSD post hoc test).

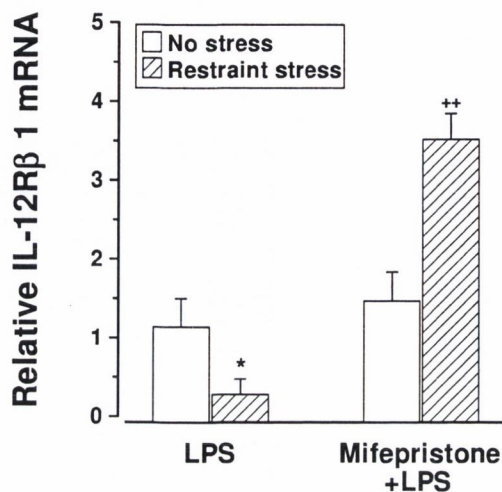


Figure 3.15.4 Mifepristone prevented the suppressive effect of stress on LPS-induced IL-12Rβ-1 mRNA expression in spleen

Mifepristone (20 mg/kg; i.p.) was administered to mice 1 hr prior to exposure to restraint stress for 2 hr. LPS (250 µg/ml; i.p.) was administered immediately following the restraint stress termination and mice were sacrificed 8 hr after LPS administration. A two-way ANOVA revealed a significant effect of mifepristone on the expression of IL-12Rβ1 mRNA [$F_{(1,16)}=21.51$, $P<0.0005$]. Data are expressed as mean $\pm$ SEM ($n=6$). * $P<0.05$ vs. non-stressed LPS-treated counterparts ++ $P<0.01$ vs. stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

3.16 Can an anxiolytic prevent the stress-induced alterations in the cytokine network?

The results in the present chapter have demonstrated that psychological stress can have immunosuppressive actions by enhancing the production of IL-10 and impairing the production of IFN- γ and IL-12. These actions can impact on a hosts' immune response to an invading organism. Therefore, in the present study a pharmacological intervention was employed to assess if blocking the perception of stress could prevent the immunosuppressive effect of stress. In these studies, the anti-anxiety drug, chlordiazepoxide was administered to prevent the perception of stress and to assess the consequences for the stress-induced immunosuppression.

3.16.1 Chlordiazepoxide prevented the stress-induced augmentation of LPS-induced IFN- γ serum concentrations and splenic mRNA expression

As demonstrated in previous results, stress suppressed LPS-induced IFN- γ serum concentrations (~ 2 fold) and splenic mRNA expression (~ 2 fold) relative to non-stressed counterparts. However, administration of chlordiazepoxide prior to LPS and exposure to stress, prevented the stress-induced effects and IFN- γ serum concentrations and mRNA expression were similar to non-stressed counterparts. In addition, when chlordiazepoxide and LPS were administered without exposure to stress, there was no significant alteration in IFN- γ serum concentrations or mRNA expression relative to LPS-treated counterparts. (Figure 3.16.1 a, b)

3.16.2 Chlordiazepoxide prevented the stress-induced inhibition of LPS-induced IL-12 serum concentrations and splenic IL-12p40 mRNA expression

As demonstrated in previous results, stress suppressed LPS-induced IL-12 serum concentrations (~ 4 fold) and IL-12p40 mRNA expression (~ 3 fold) relative to non-stressed counterparts. However, administration of chlordiazepoxide prior to LPS and stressor exposure prevented stress-induced inhibition of IL-12p40 mRNA expression which were similar to non-stressed counterparts. Administration of chlordiazepoxide prior to LPS and stressor exposure also prevented stress-induced effects on IL-12 serum concentrations, however they were significantly increased (~ 4 fold) relative to non-stressed counterparts. (Figure 3.16.2 a, b)

3.16.3 Chlordiazepoxide prevented the stress-induced augmentation of LPS-induced IL-10 serum concentrations and splenic mRNA expression

As demonstrated in previous results, stress augmented LPS-induced IL-10 serum concentrations (~ 2 fold) and splenic mRNA expression (~ 3 fold) relative to non-stressed counterparts. However, administration of chlordiazepoxide prior to LPS and stressor exposure prevented the stress-induced effects and IL-10 serum concentrations and mRNA expression were similar to non-stressed counterparts. In addition, when chlordiazepoxide and LPS were administered without exposure to stress, there was no alteration in IL-10 serum concentrations or mRNA expression relative to LPS-treated counterparts. (Figure 3.16.3 a, b)

3.16.4 Chlordiazepoxide prevented the stress-induced augmentation of LPS-induced IL-19 splenic mRNA expression

As demonstrated in previous results, stress augmented LPS-induced IL-19 mRNA expression (~ 3 fold) in mouse spleen relative to non-stressed counterparts. However, administration of chlordiazepoxide prior to LPS and stressor exposure prevented the stress-induced effects and IL-19 mRNA expression were similar to non-stressed counterparts. In addition, when chlordiazepoxide and LPS were administered without exposure to stress, there was no alteration in IL-19 mRNA expression relative to LPS-treated counterparts. (Figure 3.16.4)

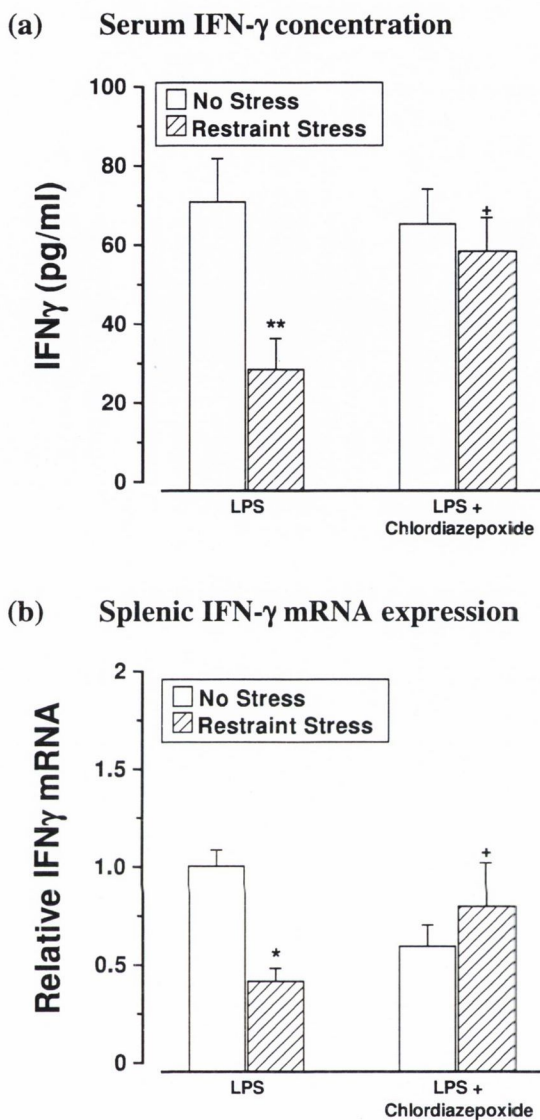


Figure 3.16.1 Chlordiazepoxide prevented the stress-induced suppression of serum IFN- γ concentrations (a) and mRNA expression in mouse spleen (b)

Chlordiazepoxide (10 mg/kg; i.p.) was administered 3 times to mice at 48 hr, 24 hr and 2 hr prior to exposure to acute restraint stress for 2 hr. LPS (250 μ g/ml; i.p.) was administered immediately following the restraint stress termination and mice were sacrificed 8 hr after LPS administration. ANOVA uncovered a significant interaction effect between groups on the expression of IFN- γ mRNA [$F_{(1,20)}=8.67$, $P<0.01$] and serum IFN- γ concentration [$F_{(1,25)}=3.99$, $P<0.05$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P<0.01$ vs. non-stressed LPS-treated counterparts, * $P<0.05$ vs. non-stressed LPS-treated counterparts, + $P<0.05$ vs. non-stressed LPS + chlordiazepoxide treated counterparts (Neuman-Keuls post hoc test).

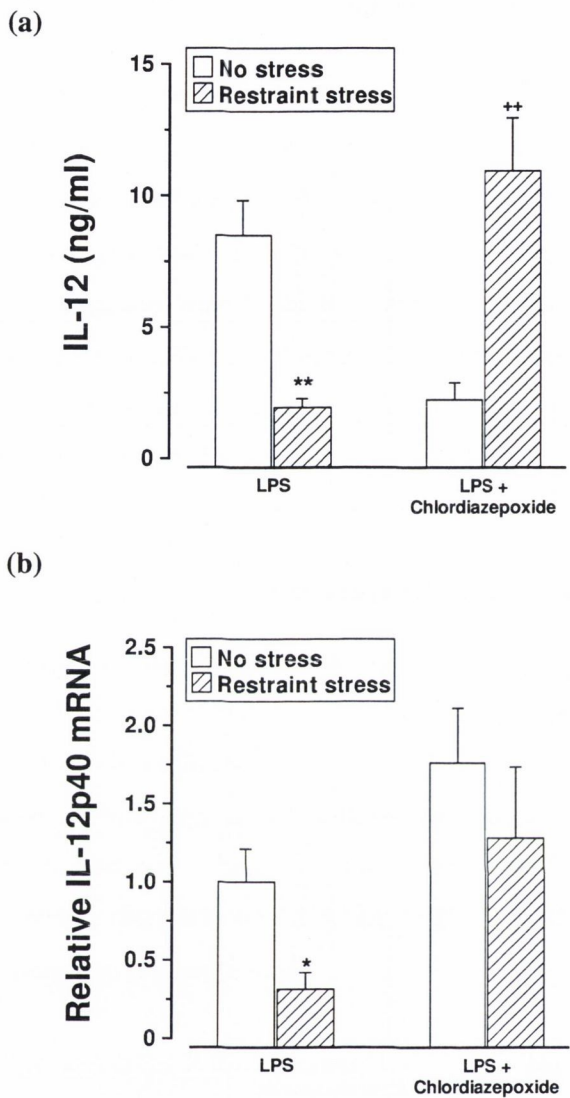
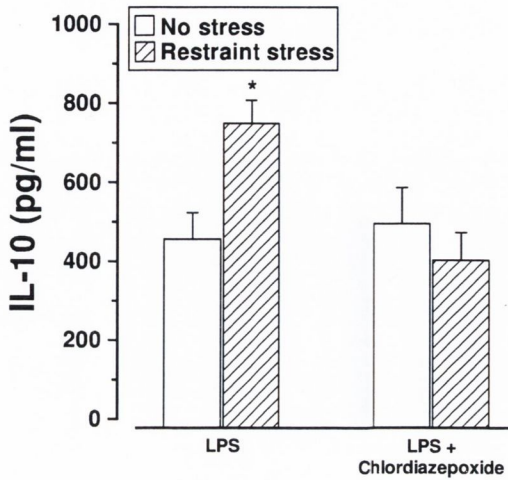


Figure 3.16.2 Chlordiazepoxide inhibited the stress-induced inhibition of serum IL-12 concentrations (a) and IL-12p40 mRNA expression in mouse spleen (b)

Chlordiazepoxide (10 mg/kg; i.p.) was administered 3 times to mice at 48 hr, 24 hr and 2 hr prior to exposure to acute restraint stress for 2 hr. LPS (250 μ g/ml; i.p.) was administered immediately following the restraint stress termination and mice were sacrificed 1.5 hr after LPS administration. ANOVA uncovered a significant interaction effect between groups on the expression of IL-12p40 mRNA [$F_{(3,14)}=3.56$, $P<0.05$] and serum IL-12 concentration [$F_{(3,16)}=13.5$, $P<0.001$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P<0.01$ vs. non-stressed LPS-treated counterparts, * $P<0.05$ vs. non-stressed LPS-treated counterparts, ++ $P<0.01$ vs. non-stressed LPS + chlordiazepoxide treated counterparts (Neuman-Keuls post hoc test).

(a) Serum IL-10 concentration



(b) Splenic IL-10 mRNA expression

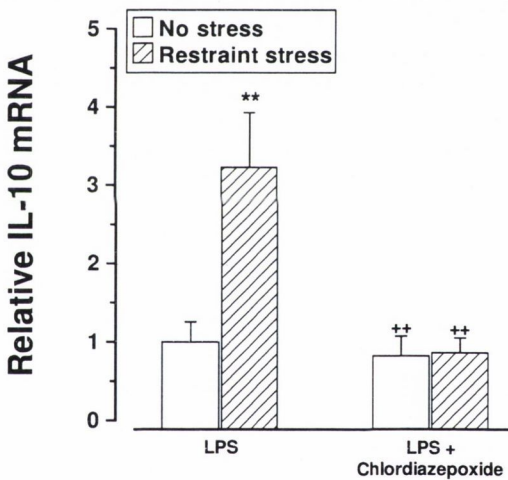


Figure 3.16.3 Chlordiazepoxide inhibited the stress-induced increase in serum IL-10 concentrations (a) and mRNA expression in mouse spleen (b)

Chlordiazepoxide (10 mg/kg; i.p.) was administered 3 times to mice at 48 hr, 24 hr and 2 hr prior to exposure to acute restraint stress for 2 hr. LPS (250 µg/ml; i.p.) was administered immediately following the restraint stress termination and mice were sacrificed 1.5 hr after LPS administration. A two-way ANOVA uncovered a significant interaction effect between treatments on the expression of IL-10 mRNA [$F_{(3,17)}=10.11$, $P<0.001$] and serum IL-10 production [$F_{(3,19)}=3.6$, $P<0.05$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P<0.01$ vs. non-stressed LPS-treated counterparts, * $P<0.05$ vs. non-stressed LPS-treated counterparts, ++ $P<0.01$ vs. stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

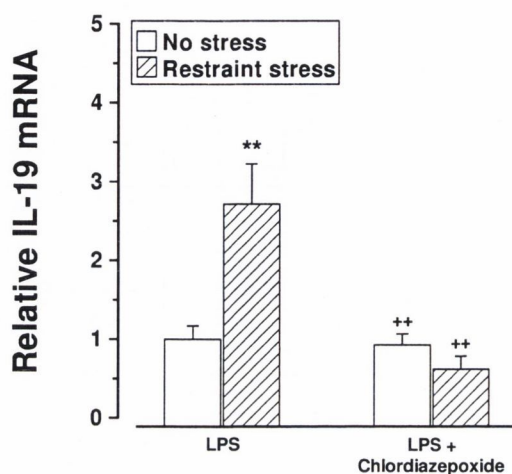


Figure 3.16.4 Chlordiazepoxide inhibited the stress-induced increase in IL-19 mRNA expression in mouse spleen

Chlordiazepoxide (10 mg/kg; i.p.) was administered 3 times to mice at 48 hr, 24 hr and 2 hr prior to exposure to acute restraint stress for 2 hr. LPS (250 µg/ml; i.p.) was administered immediately following the restraint stress termination and mice were sacrificed 1.5 hr after LPS administration. A two-way ANOVA uncovered a significant interaction effect between treatments on the expression of IL-19 mRNA [$F_{(3,15)}=11.21$, $P<0.0005$]. Data are expressed as mean $\pm$ SEM (n=6). ** $P<0.01$ vs. non-stressed LPS-treated counterparts, ++ $P<0.01$ vs. stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

Chapter 4:

Chronic Stress Results

4.1 Introduction

The results in the previous chapter have shown that exposure to acute psychological stress had significant immunosuppressive effects on the innate IFN- γ response and it also increased anti-inflammatory cytokine production. The immune altering effects of an acute stress are short lived and adaptive. However the consequences of long-term or chronic stress can be damaging (Dhabher and McEwen, 1997; 1999). Therefore, in the present study we investigated the effect of exposure to chronic stress on the innate immune response.

It has been widely reported in humans and in animal studies that exposure to chronic stress results in a sustained immunosuppressive state and this can result in the onset or progression of many diseases (Glaser and Kiecolt-Glaser, 2003). In addition, there is growing evidence that chronic stress may be associated with cancer pathogenesis and progression (Andersen *et al.*, 1994). For example a recent report has also shown that exposure to chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. (Thanker *et al.*, 2006). Therefore, in this chapter we also investigated the outcome of exposure to chronic stress on the progressive growth of a sub-cutaneous tumor mass in mice. We also examined the tumor mass and lymph nodes of affected mice for altered immune profile and changes in cytokine production.

4.2 Can exposure to chronic stress suppress the innate IFN- γ response?

In the previous chapters, acute stress was shown to significantly increase corticosterone concentrations and suppress the innate IFN- γ response. The consequences of exposure to acute stress are adaptive in the short-run, however the outcome of chronic stress could be longer lasting. Therefore in the present study the effect of exposure to chronic stress on IFN- γ and IL-12p40 mRNA expression was investigated.

4.2.1 Exposure to chronic stress inhibited LPS-induced serum IFN- γ concentrations and IFN- γ mRNA expression in mouse spleen

As previously demonstrated, LPS administration increased IFN- γ serum concentrations (~ 2 fold) and splenic mRNA expression (~ 5 fold) relative to saline-treated counterparts. This LPS-induced increase in serum IFN- γ and splenic IFN- γ mRNA is not evident in mice that were exposed to chronic restraint stress prior to LPS administration. When mice were exposed to chronic stress with no LPS administration, neither the serum concentrations nor mRNA expression of IFN- γ were not significantly altered from that of non-stressed counterparts. (Figure 4.2.1)

4.2.2 Exposure to chronic stress inhibited LPS-induced IFN- γ induced gene expression in mouse spleen

As previously demonstrated, LPS administration increased splenic mRNA expression of iNOS (~ 5 fold), CD40 (~ 2 fold) and IP-10 (~ 15 fold) relative to saline-treated counterparts. This LPS-induced increase in the IFN- γ induced gene mRNA expression was not evident in mice that were exposed to chronic restraint stress prior to LPS administration. However, exposure to chronic stress with no LPS administration had no significant alteration in mRNA expression of any of the targets relative to that of mice not exposed to stress. (Figure 4.2.2 a, b, c, d)

4.2.3 Exposure to chronic stress inhibited LPS-induced IL-12p40 mRNA expression in mouse spleen

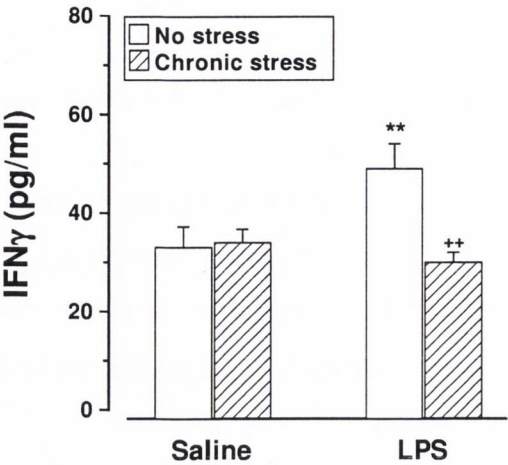
As previously demonstrated, LPS administration increased splenic IL-12p40 mRNA after 1.5 hr (~ 3 fold) and 8 hr (~ 3 fold) relative to saline-treated counterparts. This LPS-induced increase in IL-12p40 mRNA expression was not evident in mice that were exposed to chronic restraint stress prior to LPS administration. However, exposure to

chronic stress with no LPS administration had no significant alteration in IL-12p40 mRNA expression relative to mice not exposed to stress. (Figure 4.2.3 a, b)

4.2.4 Exposure to chronic stress inhibited LPS-induced IL-12 receptor expression in mouse spleen

As previously demonstrated, LPS administration increased splenic mRNA expression of IL-12R β 1 (~ 6 fold) and IL-12R β 2 (~ 2 fold) relative to saline-treated counterparts. This LPS-induced increase in IL-12 receptor mRNA expression was not evident in mice that were exposed to chronic restraint stress prior to LPS administration. However, exposure to chronic stress with no LPS administration had no significant alteration in IL-12R β 1 or IL-12R β 2 mRNA expression relative to mice not exposed to stress. (Figure 4.2.4 a, b)

(a) Serum IFN- γ concentrations



(b) Splenic IFN- γ mRNA expression

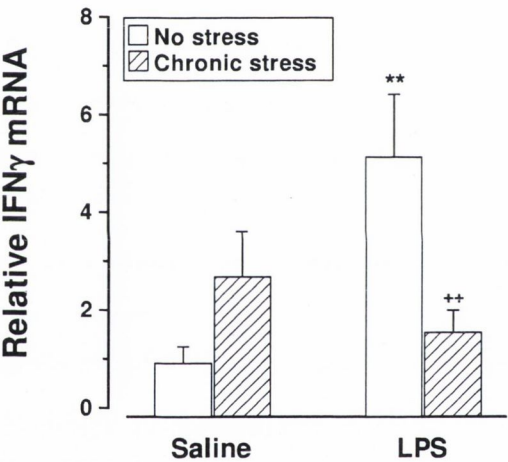


Figure 4.2.1 Exposure to chronic stress inhibited LPS-induced serum IFN- γ concentrations (a) and mRNA expression in mouse spleen (b)

Mice were chronically stressed by exposure to 2 hr restraint stress per day for 7 days. LPS (250 μ g/ml; i.p.) was administered immediately following termination of the final stress and the mice were sacrificed 8 hr after LPS administration. A two-way ANOVA exposed a significant interaction effect of chronic stress with LPS challenge on IFN- γ mRNA expression [$F_{(1,18)}=10.44$, $P<0.005$] and a significant effect of LPS treatment on IFN- γ serum concentrations [$F_{(1,20)}=9.97$, $P<0.005$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P<0.001$ vs. non-stress saline-treated counterparts, ++ $P<0.001$ vs. non-stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

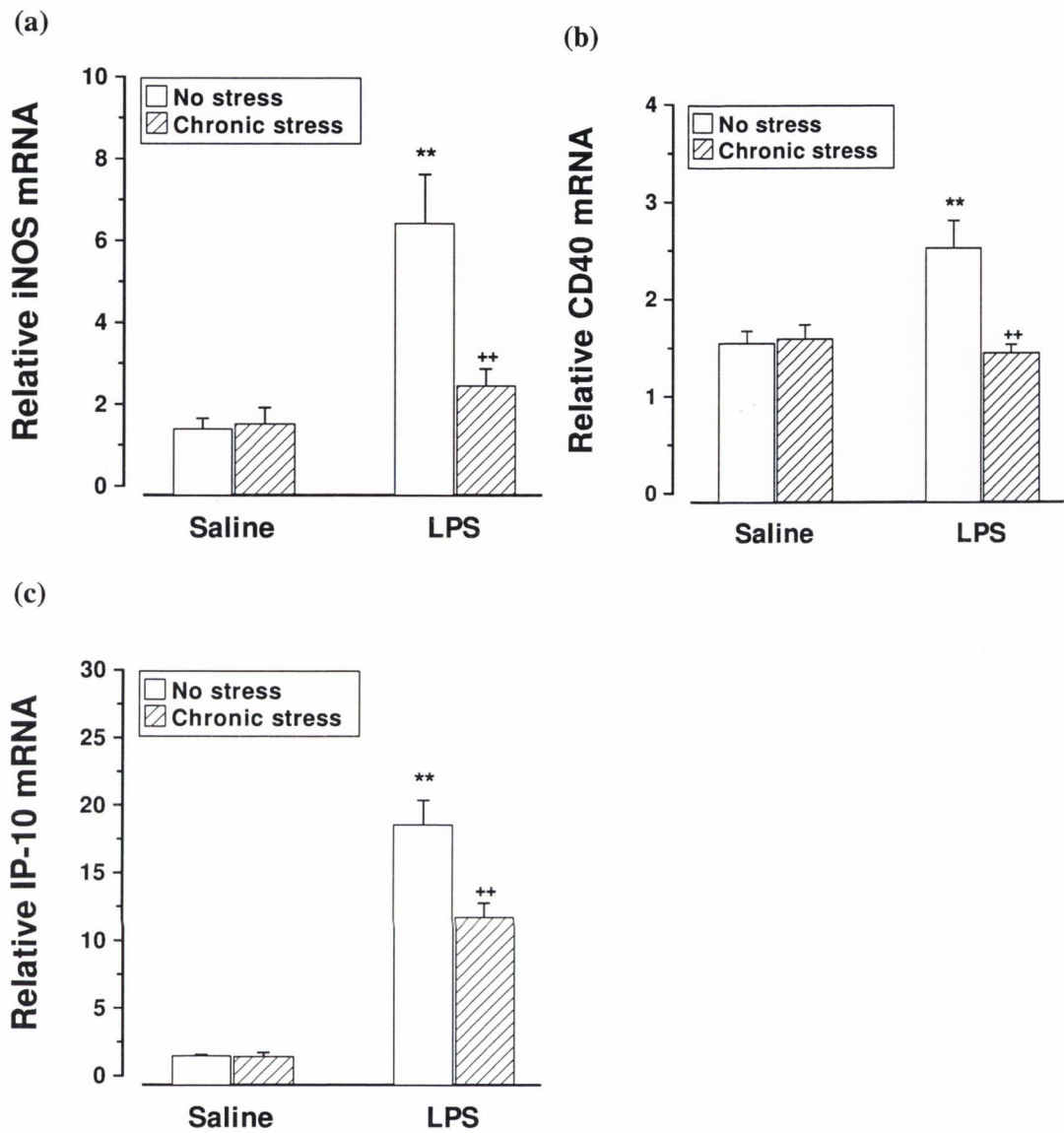
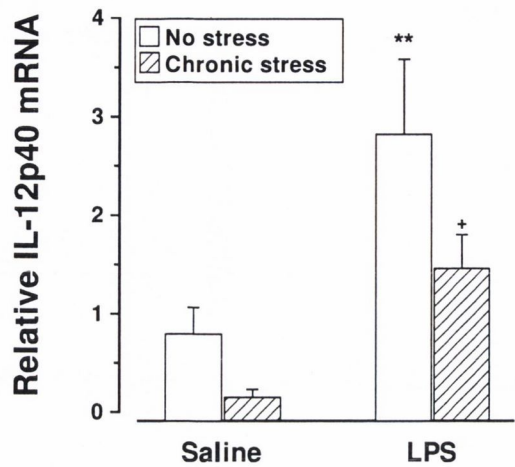


Figure 4.2.2 Exposure chronic stress inhibited LPS-induced mRNA expression of iNOS (a), CD40 (b), and IP-10 (c) in mouse spleen

Mice were chronically stressed by exposure to 2 hr restraint stress per day for 7 consecutive days. LPS (250 µg/ml; i.p.) was administered immediately following termination of the final stress and the mice were sacrificed 8 hr following LPS administration. A two-way ANOVA exposed a significant interaction effect of chronic stress with LPS challenge on iNOS mRNA expression [$F_{(1,17)}=9.75$, $P<0.005$], CD40 mRNA expression [$F_{(1,20)}=10.29$, $P<0.005$] and IP-10 mRNA expression [$F_{(1,19)}=8.91$, $P<0.01$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P<0.01$ vs. non-stress saline-treated counterparts, ++ $P<0.01$ vs. non-stressed LPS-treated counterparts + $P<0.05$ vs. non-stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

(a) Splenic expression 1.5 hr after stress



(b) Splenic expression 8 hr following stress

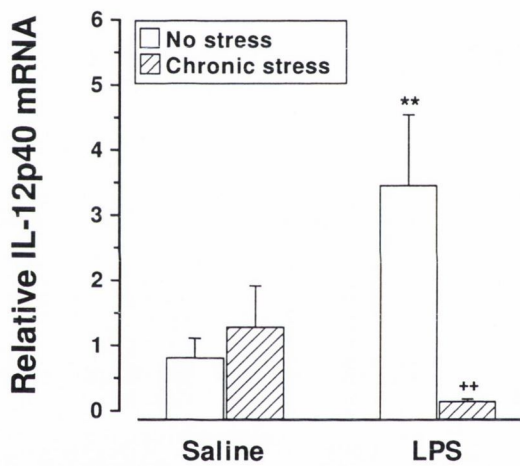
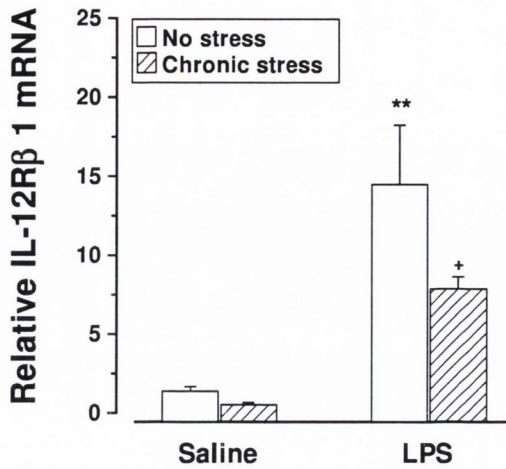


Figure 4.2.3 Exposure chronic stress inhibited LPS-induced IL-12p40 mRNA expression in mouse spleen

Mice were chronically stressed by exposure to 2 hr restraint stress per day for 7 consecutive days. LPS (250 $\mu\text{g/ml}$; i.p.) was administered immediately following termination of the final stress and the mice were sacrificed 1.5 hr or 8 hr following LPS administration. A two-way ANOVA exposed a significant interaction effect of chronic stress with LPS challenge on IL-12p40 mRNA expression 8 hr [$F_{(1,18)}=9.36$, $P<0.005$] and an LPS effect at 1.5 hr [$F_{(1,19)}=19.42$, $P<0.005$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P<0.01$ vs. non-stress saline-treated counterparts, ++ $P<0.01$ vs. non-stressed LPS-treated counterparts + $P<0.05$ vs. non-stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

(a)



(b)

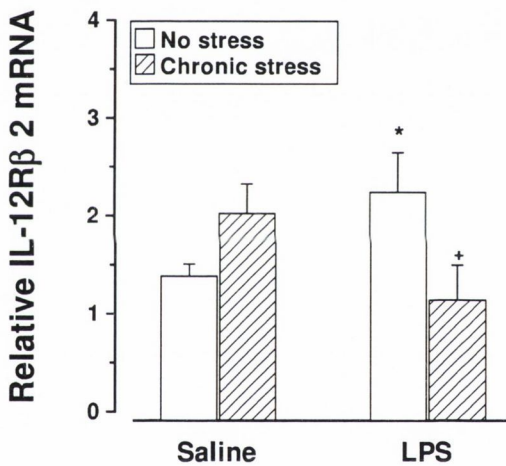


Figure 4.2.4 Exposure chronic stress inhibited LPS-induced mRNA expression of IL-12R β 1 (a) and IL-12R β 2 (b) in mouse spleen

Mice were chronically stressed by exposure to 2 hr restraint stress per day for 7 consecutive days. LPS (250 μ g/ml; i.p.) was administered immediately following termination of the final stress and the mice were sacrificed 8 hr following LPS administration. A two-way ANOVA exposed a significant interaction effect of chronic stress with LPS challenge on IL-12R β 1 mRNA expression [$F_{(1,20)}=21.37$, $P<0.0005$] and a significant effect of LPS on IL-12R β 2 mRNA expression [$F_{(1,19)}=7.11$, $P<0.01$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P<0.01$ vs. non-stress saline-treated counterparts, * $P<0.05$ vs. non-stressed saline-treated counterparts + $P<0.05$ vs. non-stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

4.3 Can exposure to chronic stress alter anti-inflammatory cytokine production?

In the previous results, chronic stress was shown to have similar immunosuppressive effects as acute stress on the innate IFN- γ response. Therefore the effect of exposure to chronic stress on the production of anti-inflammatory cytokines was investigated.

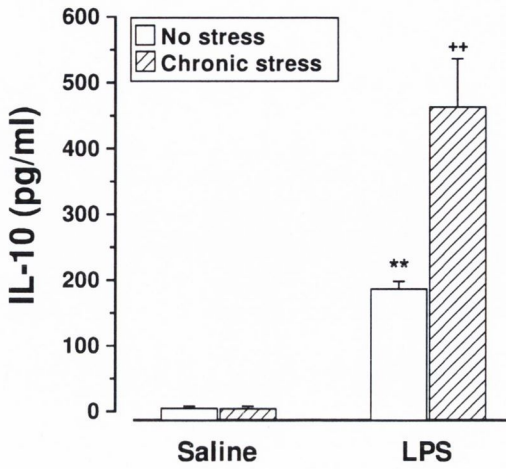
4.3.1 Exposure to chronic stress augmented LPS-induced serum IL-10 concentrations and IL-10 mRNA expression in mouse spleen

Administration of LPS increased IL-10 serum concentrations (~ 10 fold) and IL-10 mRNA expression (~ 3 fold) relative to saline-treated counterparts. Exposure to chronic stress prior to LPS administration augmented the LPS-induced increase in IL-10 serum concentrations (~ 2 fold) and IL-10 mRNA (~ 2 fold). In contrast, exposure to chronic stress with no LPS administration did not alter IL-10 serum or mRNA expression relative to non-stressed counterparts. (Figure 5.3.1 a, b)

4.3.2 Exposure to chronic stress augmented LPS-induced IL-19 mRNA expression in mouse spleen

Administration of LPS increased IL-19 mRNA expression (~ 4 fold) relative to saline-treated counterparts. Exposure to chronic stress prior to LPS administration augmented the LPS-induced increase in IL-19 mRNA (~ 2 fold). In contrast, exposure to chronic stress with no LPS administration did not alter IL-19 mRNA expression relative to non-stressed counterparts. (Figure 5.3.1 a, b)

(a) Serum IL-10 concentration



(b) Splenic IL-10 mRNA expression

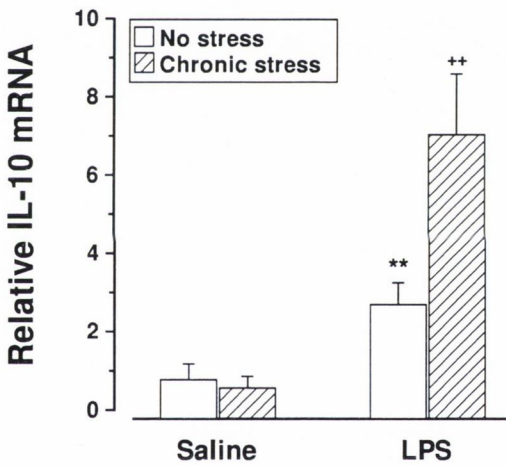


Figure 4.3.1 Exposure to chronic stress augmented LPS-induced serum IL-10 concentrations (a) and IL-10 mRNA expression in mouse spleen (b)

Mice were chronically stressed by exposure to 2 hr restraint stress per day for 7 consecutive days. LPS (250 µg/ml; i.p.) was administered immediately following termination of the final stress and the mice were sacrificed 1.5 hr after LPS administration. A two-way ANOVA exposed a significant interaction effect of chronic stress with LPS challenge on serum IL-10 concentrations [$F_{(1,19)}=26.7$, $P<0.001$] and IL-10 mRNA expression [$F_{(1,19)}=5.12$, $P<0.05$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P<0.01$ vs. non-stress LPS-treated counterparts, * $P<0.05$ vs. non-stressed LPS counterparts (Neuman-Keuls post hoc test).

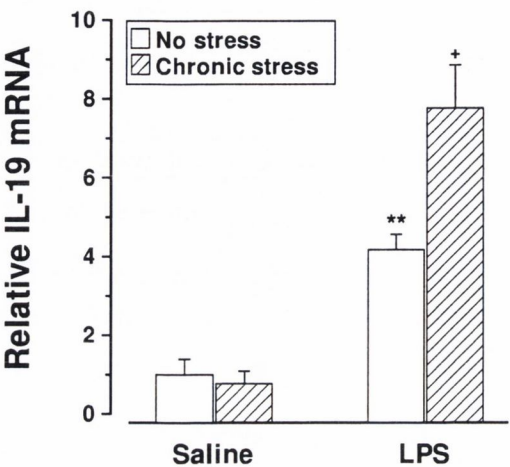


Figure 4.3.2 Exposure to chronic stress augmented LPS-induced IL-19 mRNA expression in mouse spleen

Mice were chronically stressed by exposure to 2 hr restraint stress per day for 7 consecutive days. LPS (250 $\mu\text{g/ml}$; i.p.) was administered immediately following termination of the final stress and the mice were sacrificed 1.5 hr after LPS administration. A two-way ANOVA exposed a significant effect of LPS on IL-19 mRNA expression [$F_{(1,19)}=3.41$, $P<0.05$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P < 0.01$ vs. non-stressed saline-treated counterparts, * $P < 0.05$ vs. non-stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

4.4 Can exposure to chronic stress alter progression of a tumor?

Many reports have indicated that exposure to chronic stress can lead to the onset or exacerbation of various diseases. More recently, the effect of exposure to chronic stress on cancer progression has been investigated. Exposure to chronic stress has been shown to accelerate the progression of cancer. Therefore, in the present study we investigated the effect of exposure to chronic stress on the progression of a sub-cutaneous tumor mass a mouse model of cancer.

4.4.1 Exposure to chronic stress increased tumor volume

The volume of a sub-cutaneous tumor mass was monitored in mice every second day after first palpable detection of tumor mass. Mice exposed to chronic stress had significantly increased tumor volume on days 19, 22 and 24 relative to non-stressed counterparts. (Figure 4.4.1)

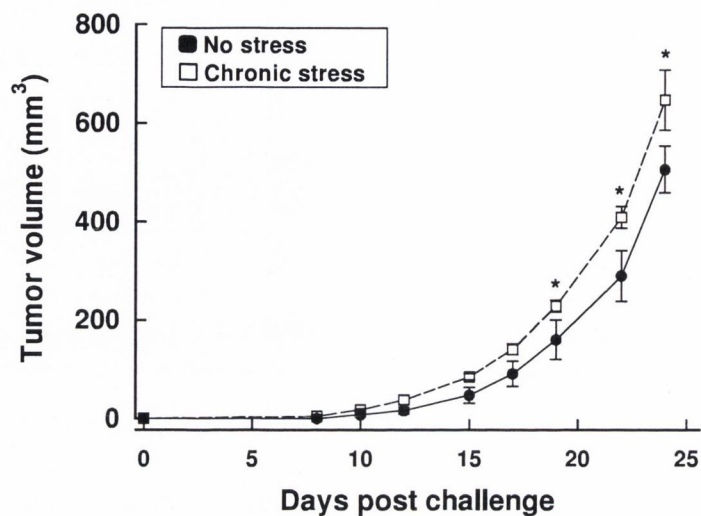


Figure 4.4.1 Exposure to chronic stress increased volume of a sub-cutaneous tumor in mouse model

Mice were injected sub-cutaneously (s.c.) with human carcinoma cell line CT26 cells (100 μ l / mouse) and immediately exposed to a 2 hr restraint stress. The mice were chronically stressed by exposure to 2 hr restraint stress per day for 24 days and the mice were sacrificed on final day of stress. A repeated two-way ANOVA revealed a significant interaction effect of stress exposure on each day [$F_{(7,24)}=2.71$, $P<0.05$]. Data are expressed as mean $\pm$ SEM ($n=4$). * $P<0.05$ vs. non-stressed CT26-treated counterparts (Tukey post hoc test).

4.5 Can exposure to chronic stress alter cytokine production in tumour mass?

The stress-induced increase in volume of the tumor indicates that there are some stress-induced alterations in the tumor microenvironment. T-cells are known to be important for anti-tumor immunity and constitute the main immune cell population attracted to the tumor site. The protective effector cells that are recruited to the tumor site include IFN- γ -secreting CD4⁺ and CD8⁺ T-cells. Therefore, in the present study, we investigated the effect of exposure to stress on the presence of IFN- γ -secreting and IL-10-secreting CD4⁺ and CD8⁺ T-cells.

4.5.1 Exposure to chronic stress failed to significantly alter IL-10-secreting CD4⁺ T-cells in tumor mass

High frequencies (16-31 %) of IL-10-secreting CD4⁺ T-cells were detected in the tumor mass of mice injected s.c. with CT26 cells. Exposure to chronic stress did not significantly alter IL-10⁺CD4⁺ cells (22-33 %), however there was a trend towards a stress-induced increase. (Figure 4.5.1 d)

4.5.2 Exposure to chronic stress failed to significantly alter IFN- γ -secreting CD4⁺ T-cells in tumor mass

In contrast to the previous results, a much lower frequency (2.1-3.9 %) of IFN- γ -secreting CD4⁺ T-cells were detected in the tumor mass of mice injected s.c. with CT26 cells. Exposure to chronic stress did not significantly alter the amount of cells (1.3–3.6 %), however there was a trend towards a stress-induced decrease. (Figure 4.5.1 c)

4.5.3 Exposure to chronic stress increased IL-10-secreting CD8⁺ T-cells in tumor mass

High frequencies (12-20 %) of IL-10-secreting CD8⁺ T-cells were detected in the tumor mass of mice injected with CT26 cells. In addition, exposure to chronic stress significantly increased the percentage of CD8⁺ T-cells that were IL-10-secreting (19-29 %). (Figure 4.5.2 d)

4.5.4 Exposure to chronic stress decreased IFN- γ -secreting CD8⁺ T-cells in tumor mass

In contrast to the previous results, a much lower frequency (2.6-4.1 %) of IFN- γ -secreting CD8⁺ T-cells were detected in the tumor mass of mice injected s.c. with CT26

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cells. Exposure to chronic stress significantly decreased the percentage of CD8⁺ T-cells that were IFN- γ -secreting (1.6–2.5 %). (Figure 4.5.2.c)

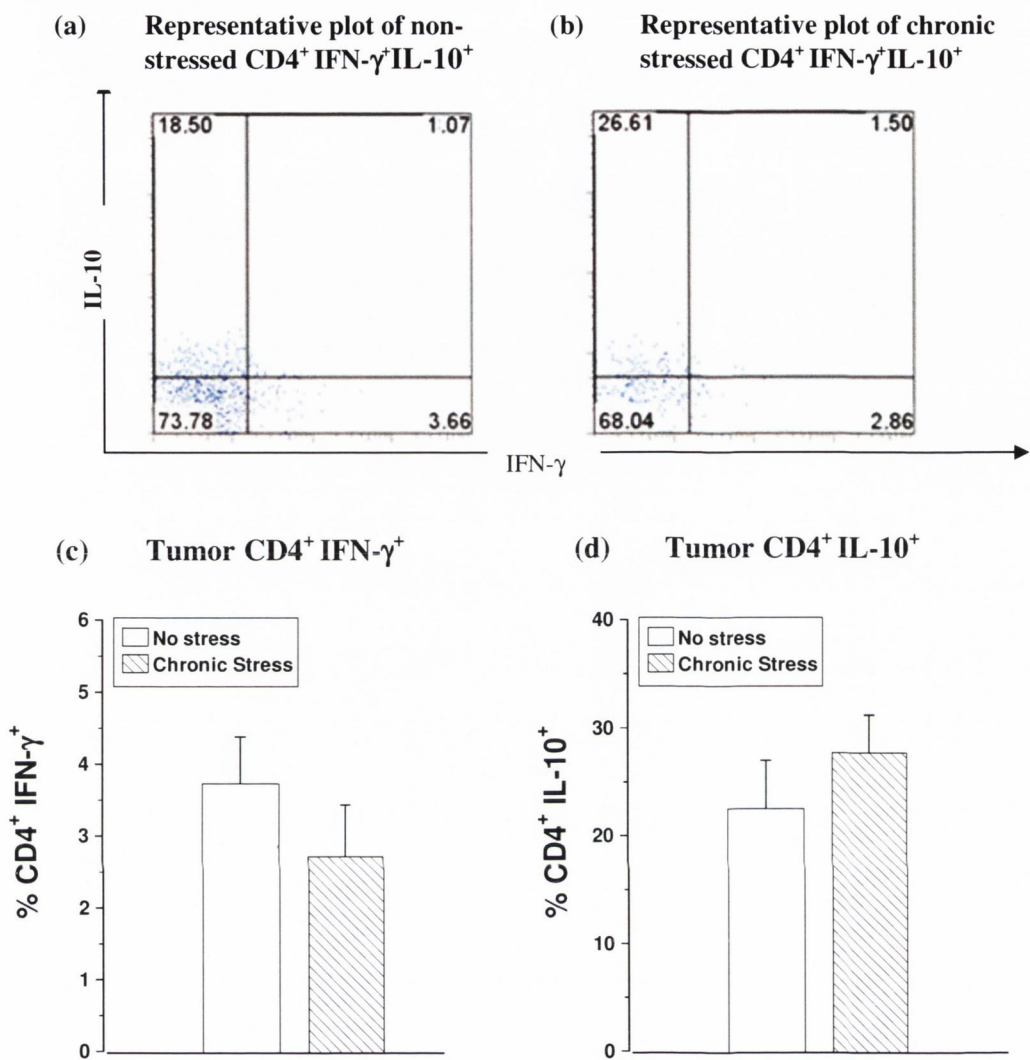


Figure 5.3.1.3 Exposure to chronic stress did not significantly reduce IFN- γ -secreting and or increased IL-10-secreting CD4⁺ T-cells in tumor mass

Mice were injected (s.c) with human carcinoma cell line CT26 (100 μ l / mouse) and immediately exposed to a 2 hr restraint stress. The mice were chronically stressed by exposure to 2 hr restraint stress per day for 24 days and the mice were sacrificed on the final day of stressing. (a) representative dot plot of tumor mass from non-stressed mouse with staining for IL-10-secreting and IFN- γ secreting CD4⁺ T-cells (b) representative dot plot of tumor mass from stressed mouse with staining for IL-10-secreting and IFN γ -secreting CD4⁺ T-cells (c) bar graph (n=4) of IFN- γ -secreting CD4⁺ T-cells from tumor mass (d) bar graph (n=4) of IL-10-secreting CD4⁺ T-cells from tumor mass. Data are expressed as mean $\pm$ SEM (n=4)

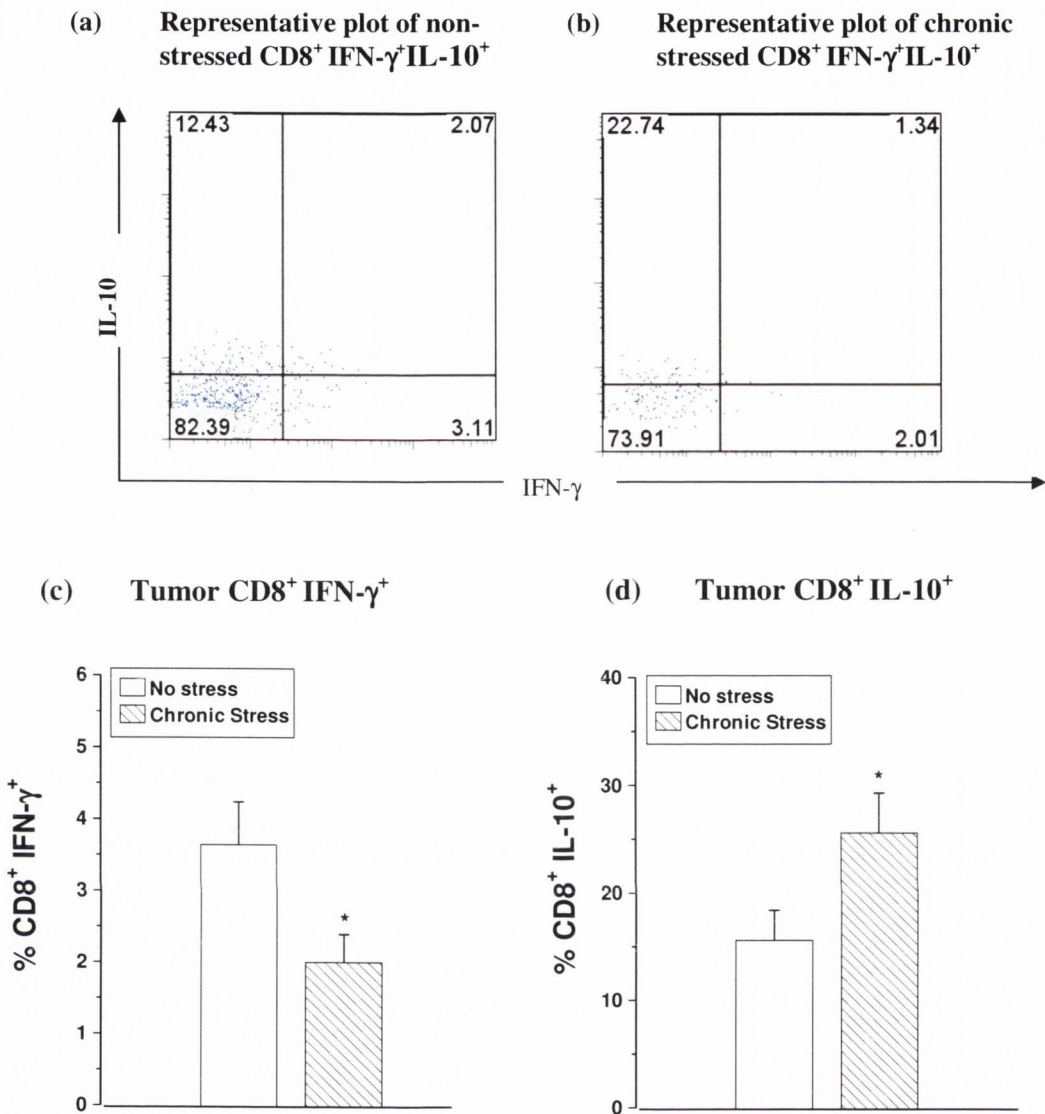


Figure 5.3.1.3 Exposure to chronic stress reduced IFN- γ -secreting and increased IL-10-secreting CD8⁺ T-cells in tumor mass

Mice were injected (s.c.) with human carcinoma cell line CT26 (100 μ l / mouse) and immediately exposed to 2 hr restraint stress. The mice were chronically stressed by exposure to 2 hr restraint stress per day for 24 days and the mice were sacrificed on final day of stressing. (a) representative dot plot of tumor mass from non-stressed mouse with staining for IL-10-secreting and IFN- γ secreting CD8⁺ T-cells (b) representative dot plot of tumor mass from stressed mouse with staining for IL-10-secreting and IFN γ -secreting CD8⁺ T-cells (c) bar graph (n=4) of IFN- γ -secreting CD8⁺ T-cells from tumor mass (d) bar graph (n=4) of IL-10-secreting CD8⁺ T-cells from tumor mass. Data are expressed as mean $\pm$ SEM (n=4). *P < 0.05 vs. non-stressed counterparts (Student-t test).

4.6 Can exposure to chronic stress alter cytokine production in the inguinal lymph node?

The inguinal lymph node filters blood coming from the site of an s.c. tumour mass. It traps cancer cells and other substances travelling from the tumour mass and therefore can give an insight into the tumour microenvironment. Therefore, in the present study we investigated the effect of exposure to stress on the presence of IFN- γ^+ and IL-10 $^+$ CD4 $^+$ and CD8 $^+$ T-cells in the lymph node.

4.6.1 Exposure to chronic stress significantly increased IL-10-secreting CD4 $^+$ T-cells in tumour mass

The frequency of IL-10-secreting CD4 $^+$ T-cells detected in the lymph node (5-15 %) of mice injected s.c. with CT26 cells was not as high as in the tumour mass. However, exposure to chronic stress significantly increased the percentage of CD4 $^+$ cells that were IL-10-secreting (10-22 %). (Figure 4.6.1 c)

4.6.2 Exposure to chronic stress failed to significantly alter IFN- γ -secreting CD4 $^+$ T-cells in the lymph node

Similar frequencies (2.3-3.4 %) of IFN- γ -secreting CD4 $^+$ T-cells were detected in the lymph nodes of mice injected s.c. with CT26 cells in comparison to those seen in the tumour mass. Exposure to chronic stress did not significantly alter the amount of cells (1.2–2.6 %), however there was a trend towards a stress-induced decrease. (Figure 4.6.1 d)

4.6.3 Exposure to chronic stress increased IL-10-secreting CD8 $^+$ T-cells in the lymph node

Similar frequencies (11-22 %) of IL-10-secreting CD8 $^+$ T-cells were detected in the lymph node of mice injected with CT26 cells in comparison to those seen in the tumour mass. In addition, exposure to chronic stress significantly increased the percentage of CD8 $^+$ T-cells that were IL-10-secreting (22-33 %). (Figure 4.6.2 c)

4.6.4 Exposure to chronic stress decreased IFN- γ -secreting CD8 $^+$ T-cells in the lymph node

Similar frequencies (1.9-3.4 %) of IFN- γ -secreting CD8 $^+$ T-cells were detected in the lymph node of mice injected s.c. with CT26 cells in comparison to those seen in the

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tumour mass. Exposure to chronic stress significantly decreased the percentage of CD8⁺ T-cells that were IFN- γ -secreting (1.6–2.5 %). (Figure 4.6.2 d)

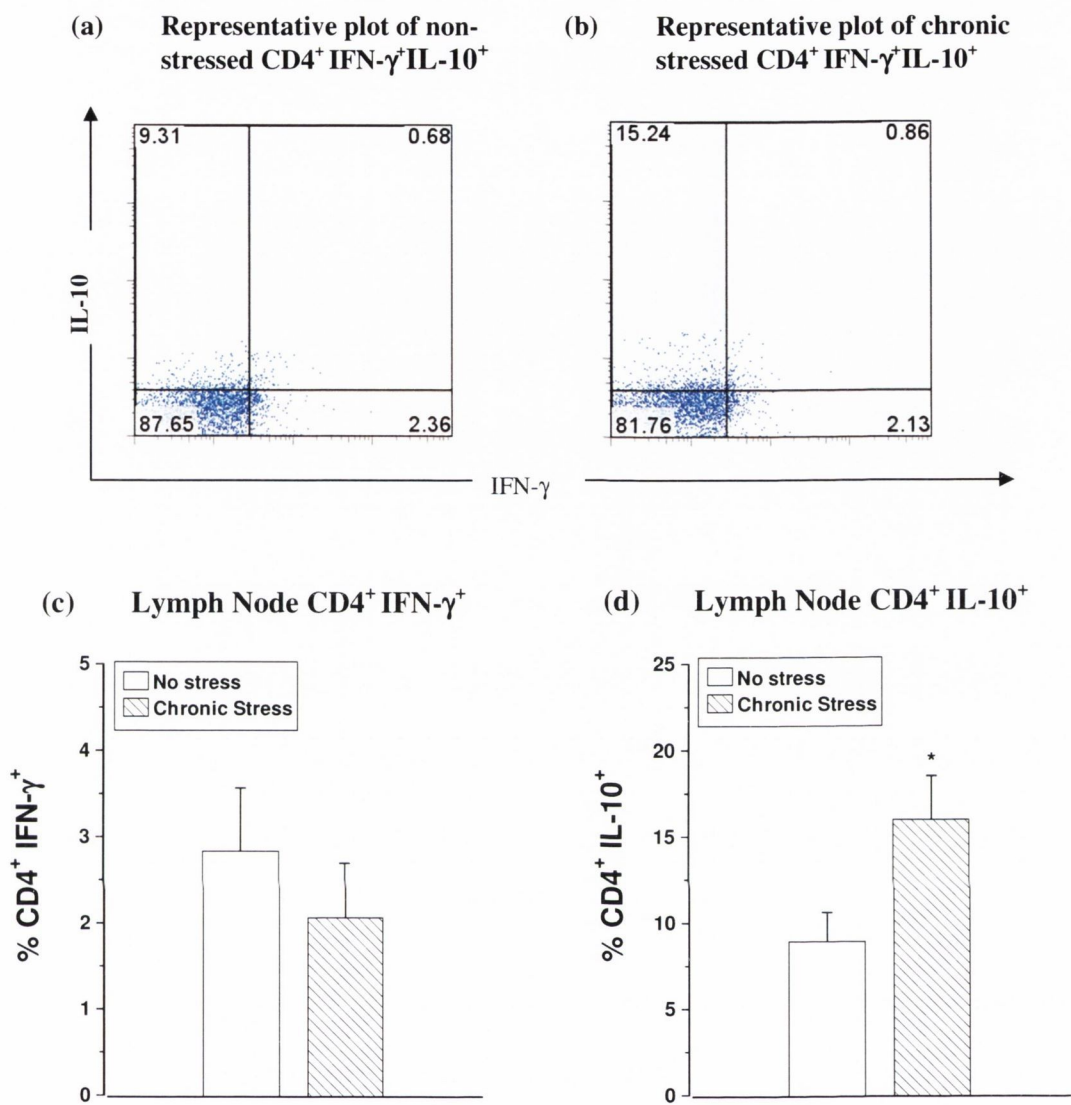


Figure 4.6.1 Exposure to chronic stress did increased IL-10-secreting CD4⁺ T-cells in lymph node

Mice were injected (s.c.) with human carcinoma cell line CT26 (100 μ l / mouse) and immediately exposed to a 2 hr restraint stress. The mice were chronically stressed by exposure to 2 hr restraint stress per day for 24 days and the mice were sacrificed on the final day of stressing. (a) representative dot plot of lymph node from non-stressed mouse with staining for IL-10-secreting and IFN- γ secreting CD4⁺ T-cells (b) representative dot plot of lymph node from stressed mouse with staining for IL-10-secreting and IFN γ -secreting CD4⁺ T-cells (c) bar graph (n=4) of IFN- γ -secreting CD4⁺ T-cells from lymph node (d) bar graph (n=4) of IL-10-secreting CD4⁺ T-cells from lymph node. Data are expressed as mean $\pm$ SEM (n=4). *P <0.01 vs. non-stressed counterparts (Student-t test).

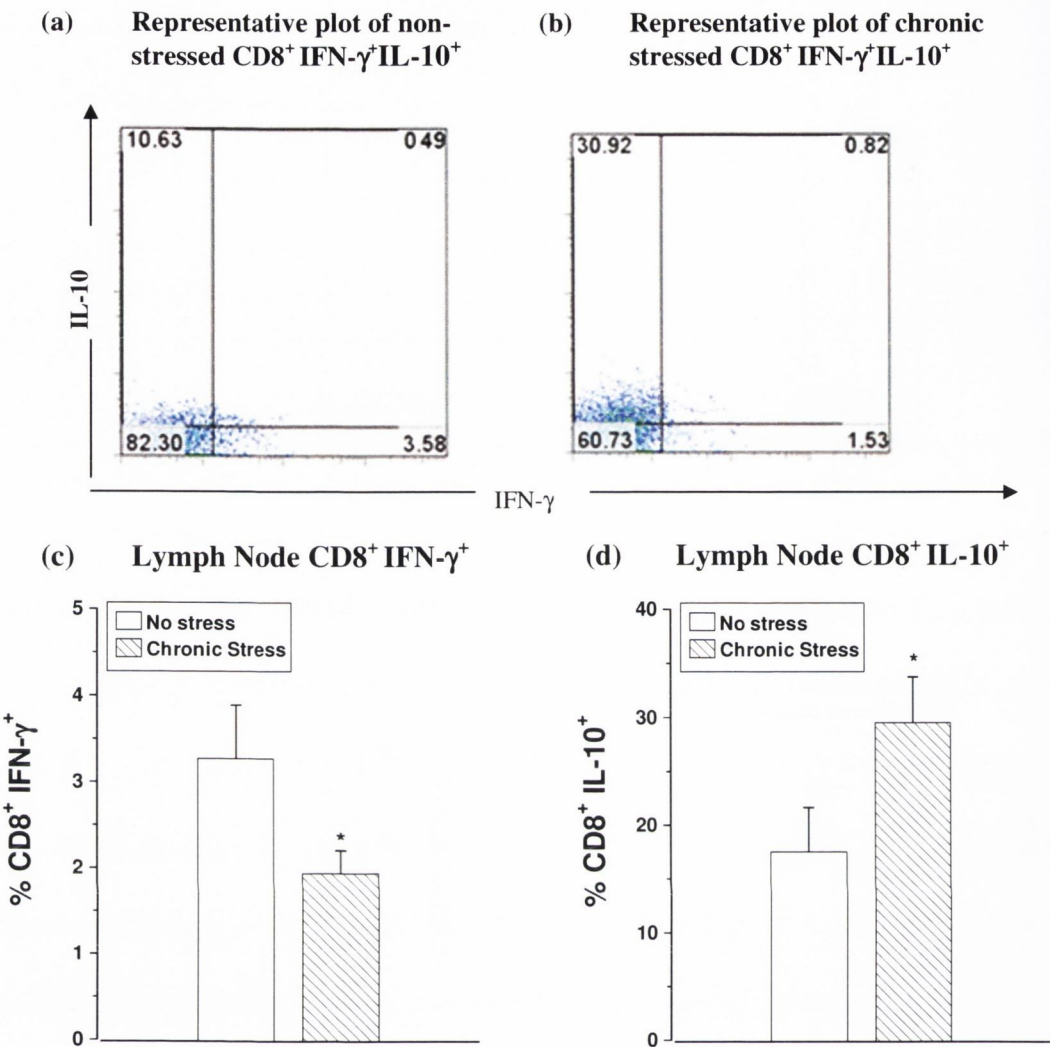


Figure 4.6.2 Exposure to chronic stress reduced IFN-γ-secreting and increased IL-10-secreting CD8⁺ T-cells in lymph node

Mice were injected (s.c.) with human carcinoma cell line CT26 (100 μl / mouse) and immediately exposed to a 2 hr restraint stress. The mice were chronically stressed by exposure to 2 hr restraint stress per day for 24 days and the mice were sacrificed on the final day of stressing. (a) representative dot plot of lymph from non-stressed mouse with staining for IL-10-secreting and IFN-γ secreting CD8⁺ T-cells (b) representative dot plot of lymph node from stressed mouse with staining for IL-10-secreting and IFN-γ-secreting CD8⁺ T-cells (c) bar graph (n=4) of IFN-γ-secreting CD8⁺ T-cells from lymph node (d) bar graph (n=4) of IL-10-secreting CD8⁺ T-cells from lymph node. Data are expressed as mean ± SEM (n=4). *P < 0.05 vs. non-stressed counterparts (Student-t test).

Chapter 5: Pro-inflammatory Results

5.1 Introduction

It has been reported in this thesis and by other groups that chronic stress can induce an immunosuppressive state which can lead to the onset of many diseases and increase the progression of cancer. However, other reports claim that stress can have immuno-enhancing actions under some circumstances. For example, it has been shown that exposure to chronic social disruption stress alters splenocyte phenotype and function with enhanced splenic monocytes and neutrophils and increased resistance to the suppressive effects of stress (Avitsur *et al.*, 2002).

The temporal proximity between stressor termination and the immune challenge has been suggested as one possible explanation for the immuno-enhancing effects of stress. Some reports have suggested that exposure to stress can induce a pro-inflammatory state that becomes evident within 24 hr of stressor termination (Avitsur *et al.*, 2005). Therefore in the present chapter we investigated the effect of an LPS challenge administered 24 hr after chronic stress termination.

The temporal delay between the termination of stress and immune challenge can alter the immune profile due to changes in receptor expression and response. This is the basis of the 'receptor desensitisation' hypothesis. It has been demonstrated that stress induces adaptations in receptor expression during exposure to chronic stress (Quan *et al.*, 2003). The 'receptor desensitisation' hypothesis states that these alterations in receptor expression remain for a period following stressor termination and consequently the normal physiologically levels of glucocorticoids and catecholamines are not sufficient to regulate immune responses. Therefore, in this chapter we further investigate this hypothesis by examining the splenic expression of receptors for stress hormones, IL-12 and LPS 24 hr following exposure to stress.

In addition we investigated the consequences of altered receptor expression and altered immune profile by examining the response of splenocytes. It has previously been demonstrated that splenocyte response is altered 18 hr following exposure to stress and glucocorticoid resistance is present (Avitsur *et al.*, 2001).

5.2 Is there an alteration in function of LPS stimulated splenocyte 24 hr following exposure to chronic restraint stress?

The previous results have shown that exposure to acute and chronic psychological stress suppressed LPS-induced IL-12 and IFN- γ concentrations in the hours immediately following stressor termination. In the present study we aimed to investigate if there was an altered immune response to LPS administration 24 hr following exposure to chronic stress. Therefore, the LPS-induced IL-12 and IFN- γ concentrations from splenocytes were investigated 1.5 hr and 24 hr following stressor termination.

5.2.1 Splenocytes harvested 1.5 hr following chronic stress exposure have reduced LPS-induced IL-12 and IFN- γ concentrations

As previously demonstrated, there is an immunosuppressive effect of exposure to chronic stress in the hours following stressor exposure. LPS administered to splenocytes significantly increased IL-12 concentrations ($P < 0.01$) and IFN- γ concentrations ($P < 0.01$) relative to saline-treated counterparts. However, this LPS-induced increase in IL-12 and IFN- γ was not evident in splenocytes cultured from mice immediately following stressor exposure. In contrast, when LPS was not administered to splenocytes cultured from mice exposed to stress, there was no alteration in IL-12 or IFN- γ concentrations relative to saline-treated counterparts. (Figure 5.2.1 a, b)

5.2.2 Splenocytes harvested 24 hr following chronic stress exposure have increased LPS-induced IL-12 and IFN- γ concentrations

As seen in the previous results, LPS administered to splenocytes significantly increased IL-12 ($P < 0.01$) and IFN- γ ($P < 0.01$) concentrations relative to saline-treated counterparts. In contrast to the previous results, there was a significant augmentation of the LPS-induced increase in IL-12 ($P < 0.05$) and IFN- γ ($P < 0.01$) in splenocytes cultured from mice 24 hr after exposure to stress. However, when LPS was not administered to splenocytes cultured from mice 24 hr after exposure to stress, there was no alteration in IL-12 or IFN- γ concentrations relative to saline-treated counterparts. (Figure 5.2.2 a, b)

5.2.3 Splenocytes harvested immediately following exposure to chronic stress have augmented LPS-induced IL-10 concentrations, however this is not present 24 hr following chronic stress

As seen in the previous results, LPS administered to splenocytes immediately and 24 hr following exposure to stress had significantly increased IL-10 concentrations ($P < 0.01$) relative to saline-treated counterparts. In addition, immediately following exposure to stress, LPS-induced IL-10 concentrations were significantly augmented ($P < 0.05$). However, splenocytes cultured from mice 24 hr after exposure to stress had no significant alteration in IL-10 concentrations. When LPS was not administered to splenocytes cultured from mice 24 hr after exposure to stress, there was no alteration in IL-10 concentrations at either time relative to saline-treated counterparts. (Figure 5.2.1 c, 5.2.2 c)

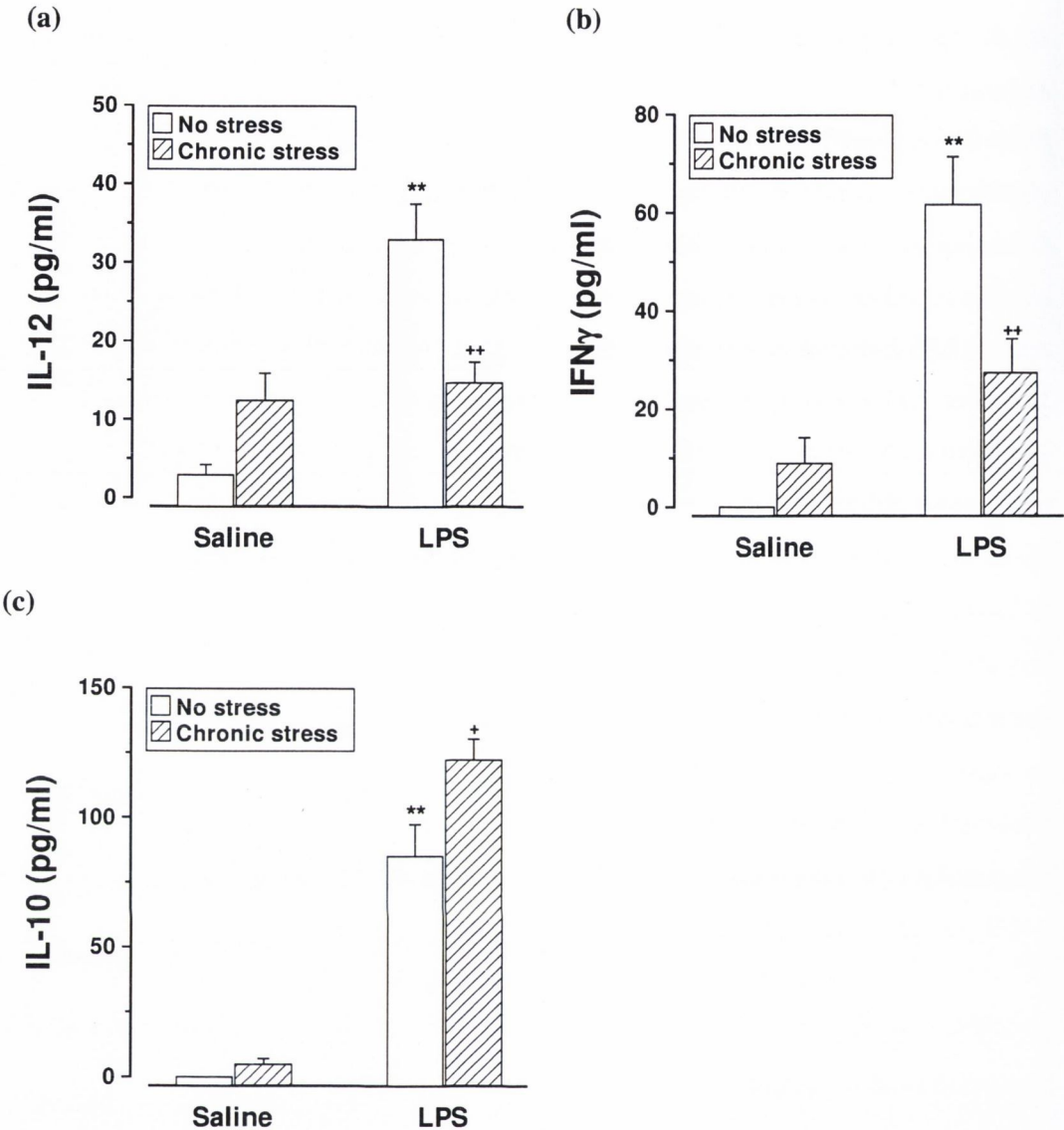


Figure 5.2.1 LPS-induced IL-12 (a) and IFN- γ (b) concentrations were inhibited and IL-10 (c) concentrations were increased in splenocytes cultured from mice 1.5 hr following exposure to chronic stress

Mice were exposed to chronic stress for 2 hr per day for 7 days and the mice were sacrificed 1.5 hr after termination of last stress. Splenocytes were cultured from freshly isolated spleens and treated with LPS (1 μ g/ml). Cell supernatant was harvested after 24 hr and 72 hr of incubation. A two-way ANOVA exposed a significant effect of chronic stress on IL-12 production [$F_{(1,20)}=22.5$, $P<0.001$], IFN- γ production [$F_{(1,21)}=14.2$, $P<0.005$] and IL-10 production [$F_{(1,20)}=126.7$, $P<0.0001$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P<0.01$ vs. non-stress saline-treated counterparts, ++ $P<0.01$ vs. non-stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

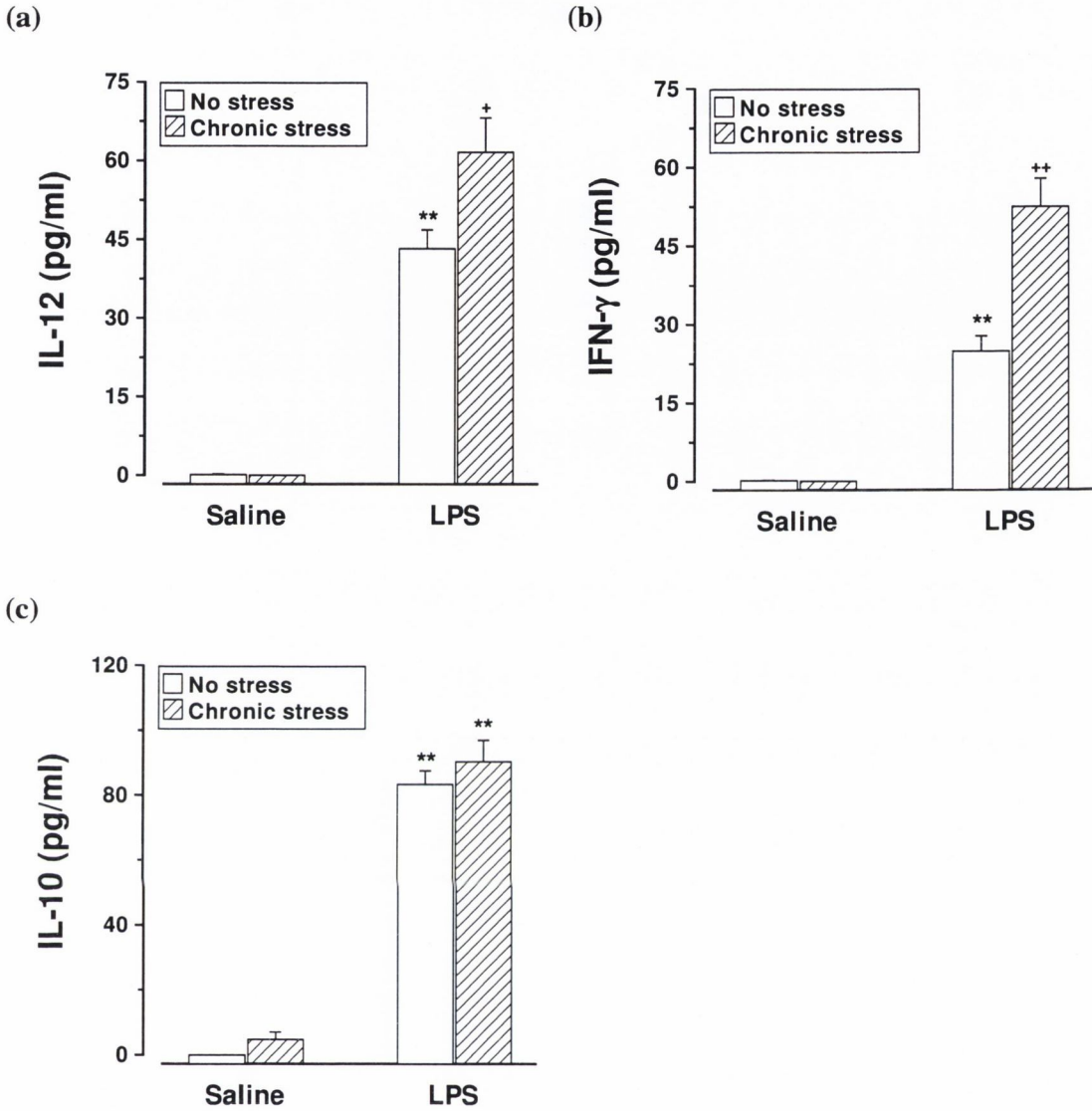


Figure 5.2.2 LPS-induced IL-12 (a) and IFN- γ (b) concentrations were augmented, however IL-10 concentrations were not altered in splenocytes cultured from mice 24 hr following exposure to chronic stress

Mice were exposed to chronic stress for 2 hr per day for 7 days and the mice were sacrificed 24 hr after termination of last stress. Splenocytes were cultured from freshly isolated spleens and treated with LPS (1 $\mu\text{g/ml}$). Cell supernatant was harvested after 24 hr and 72 hr of incubation. A two-way ANOVA exposed a significant effect of chronic stress on IL-12 production [$F_{(1,20)}=123.5$, $P<0.0001$], IFN- γ production [$F_{(1,19)}=42.2$, $P<0.0001$] and IL-10 production [$F_{(1,20)}=179.8$, $P<0.0001$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P<0.01$ vs. saline-treated counterparts, ** $P<0.01$ vs. non-stressed LPS-treated counterparts, * $P<0.05$ vs. non-stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

5.3 Are GR and β_2 -AR expression altered 24 hr following exposure to chronic restraint stress?

The previous results have shown that there is some immuno-enhancing effect in splenocytes cultured from mice 24 hr following exposure to stress. One possible explanation for the immuno-enhancing effect is the 'Receptor Desensitisation Hypothesis'. This hypothesis links the down regulation of GR and β_2 -AR expression that can occur 24 hr after stress exposure to the desensitisation of immune cells to immunosuppressive stress hormones. Therefore in the present study, we assessed the splenic expression of GR and β_2 -AR from spleens 24 hr following exposure to chronic stress.

5.3.1 β_2 -AR and GR protein expression were down-regulated 24 hr following exposure to chronic stress

There was a high frequency of GR (~ 60 %) and β_2 -AR (~ 70 %) protein expression in the mouse spleen relative to β -actin expression. However, in the spleens from mice 24 hr following exposure to stress there is a significant inhibition of GR ($P<0.01$) and β_2 -AR ($P<0.01$) expression. (Figure 5.3.1 a, b)

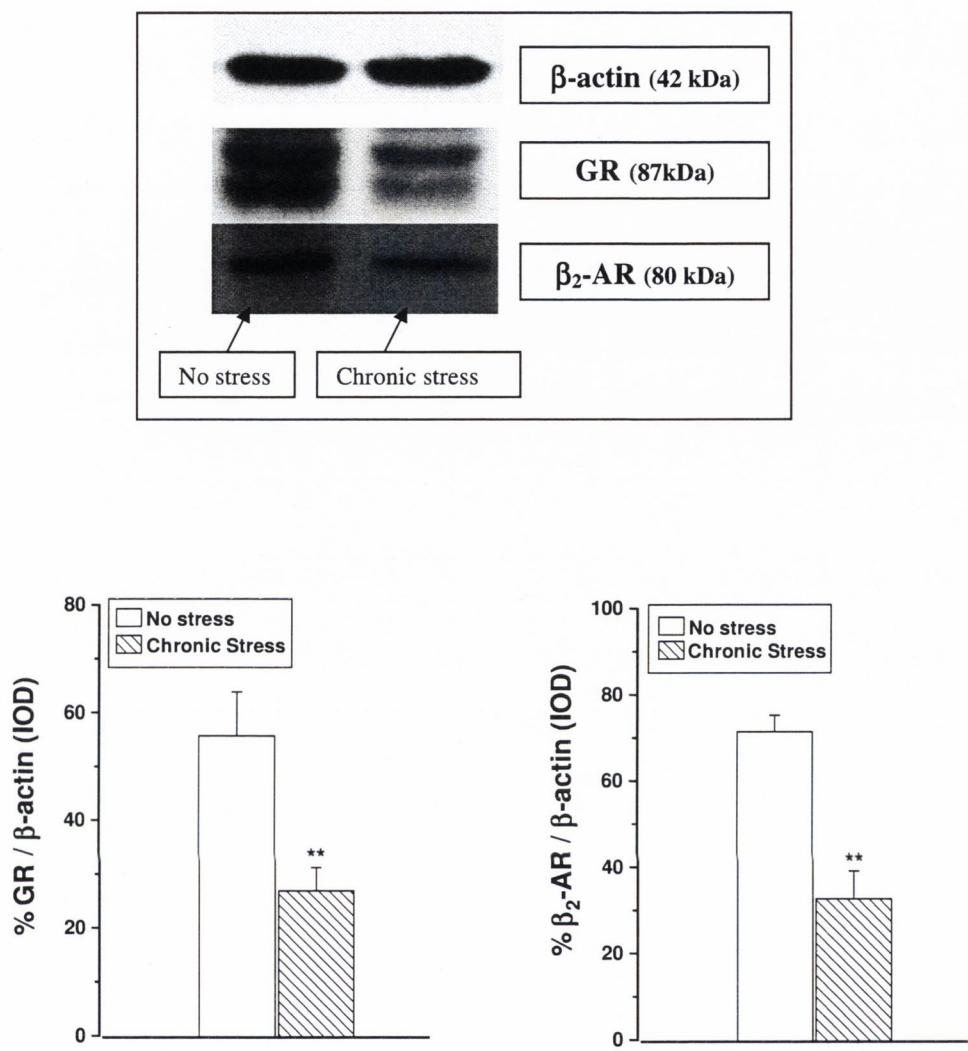


Figure 5.3.1 GR and β_2 -AR expressions were inhibited in the spleens of mice 24 hr following exposure to chronic stress

Mice were exposed to chronic stress for 2 hr per day for 7 days and the mice were sacrificed 24 hr after termination of last stress. Spleens were dissected and snap frozen before preparation for western blotting. Data are expressed as mean $\pm$ SEM (n=6). **P<0.001 vs. non-stressed counterparts (Student t-test).

5.4 Are the IL-12 receptor or LPS receptor expression altered 24 hr following exposure to chronic stress?

We have shown in chapter 3 that immediately following exposure to stress there was inhibition of LPS-induced splenic IL-12R expression. In addition, the previous results have shown that the stress hormone receptors are down-regulated following a 24 hr temporal delay before assessment of splenic expression. Therefore, in the present study we investigated if IL-12R expression was altered 24 hr following exposure to stress. In addition, it has been reported that exposure to stress alters immune response to LPS by altering receptor expression for LPS – TLR4 (Zhang *et al.*, 2008). Therefore in the present study we also investigated if splenic TLR4 expression was altered 24 hr following exposure to stress. As discussed in the introduction, CD14 receptor expression is essential for the presentation of a LPS molecule to the TLR4. Therefore, in the present study we also assessed the expression of splenic CD14 receptor expression.

5.4.1 IL-12R β 1 mRNA expression is increased 24 hr following exposure to stress

24 hr following exposure to stress there is an increase in IL-12R β 1 mRNA expression (~ 5 fold) relative to splenic mRNA expression of non-stressed mice. (Figure 5.3.1)

5.4.2 CD14 mRNA expression is not altered 24 hr following exposure to stress

24 hr following exposure to stress there no alteration in CD14 mRNA expression relative to splenic mRNA expression of non-stressed mice. (Figure 5.3.2 b)

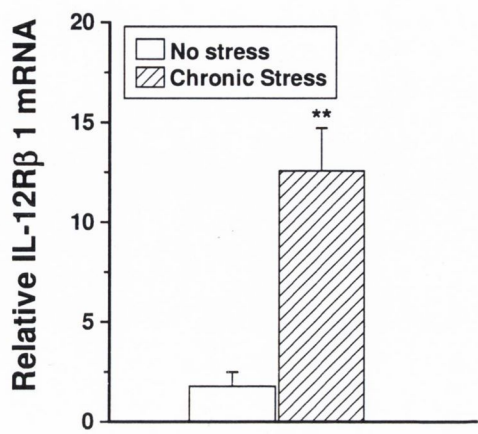


Figure 5.4.1 IL-12Rβ 1 mRNA expression was augmented in spleens of mice 24 hr following exposure to chronic stress

Mice were exposed to chronic stress for 2 hr per day for 7 days and the mice were sacrificed 24 hr after termination of last stress. Spleens were dissected and a portion was stored in RNA later for RNA extraction and real-time PCR. Data are expressed as mean ± SEM (n=6). **P<0.01 vs. non-stressed counterparts (Student t-test).

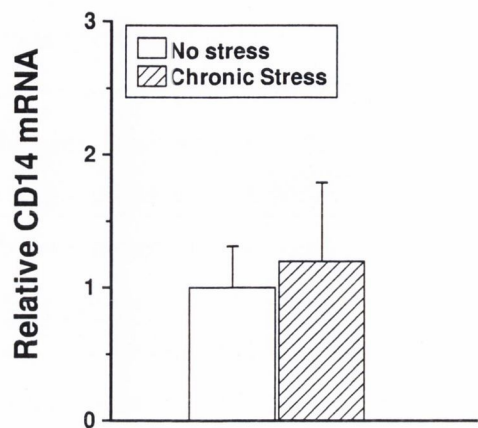


Figure 5.4.2 CD14 mRNA expression is not altered in spleens of mice 24 hr following exposure to chronic stress

Mice were exposed to chronic stress for 2 hr per day for 7 days and the mice were sacrificed 24 hr after termination of last stress. Spleens were dissected and a portion was stored in RNA later for RNA extraction and real-time PCR. Data are expressed as mean $\pm$ SEM (n=6). **P<0.01 vs. non-stressed counterparts (Student t-test).

5.5 Is splenocytes function altered 24 hr following exposure to chronic restraint stress?

The previous results have shown that 24 hr after stress there is some immuno-enhanced effects in the splenocytes and there is down-regulation of splenic GR and β_2 -AR. These findings indicate that there could be an altered response of splenocytes harvested 24 hr following exposure to stress. Therefore, in the present study we investigated the response of splenocytes cultured 24 hr following exposure to chronic stress to the suppressive effects of corticosterone and salbutamol. As previously shown, splenocytes stimulated with LPS have increased IFN- γ concentrations ($0 - 20.4 \pm 2.9$) and IL-12 concentrations ($0 - 43.2 \pm 3.6$) relative to saline-treated counterparts. However, splenocytes cultured from mice 24 hr following exposure to stress had increased LPS-induced concentrations of IFN- γ ($0 - 52.4 \pm 6.3$) and IL-12 ($0 - 61.9 \pm 6.7$) relative to non-stressed counterparts. Therefore we used % control LPS to compare the response of splenocytes to corticosterone and salbutamol.

5.5.1 *The suppressive effect of corticosterone on LPS-induced IFN- γ and IL-12 concentrations were impaired in splenocytes cultured 24hr following exposure to stress*

Corticosterone suppressed LPS-induced IFN- γ and IL-12 concentrations in non-stressed splenocytes in a dose-dependant manner. However, the LPS-induced IFN- γ and IL-12 concentrations from splenocytes cultured from mice 24 hr following exposure to stress were significantly increased with 0.1 and 0.5 μ M corticosterone. Therefore, there was reduced sensitivity of these splenocytes to the suppressive effect of corticosterone. However, LPS-induced IL-10 concentrations were not altered by corticosterone at any dose. (Figure 5.4.1 a, b, c)

5.5.2 *The suppressive effect of salbutamol on LPS-induced IFN- γ and IL-12 concentrations were impaired in splenocytes cultured 24 hr following exposure to stress*

Salbutamol suppressed LPS-induced IFN- γ and IL-12 concentrations in non-stressed splenocytes in a dose-dependant manner. However, the LPS-induced IFN- γ and IL-12 concentrations from splenocytes cultured from mice 24 hr following exposure to stress were significantly increased with 0.1-0.5 μ M and 0.05-1 μ M salbutamol respectively. Therefore, there was reduced sensitivity of these splenocytes to the suppressive effect of

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salbutamol. However, LPS-induced IL-10 concentrations were not altered by salbutamol at any dose. (Figure 5.4.2 a, b, c)

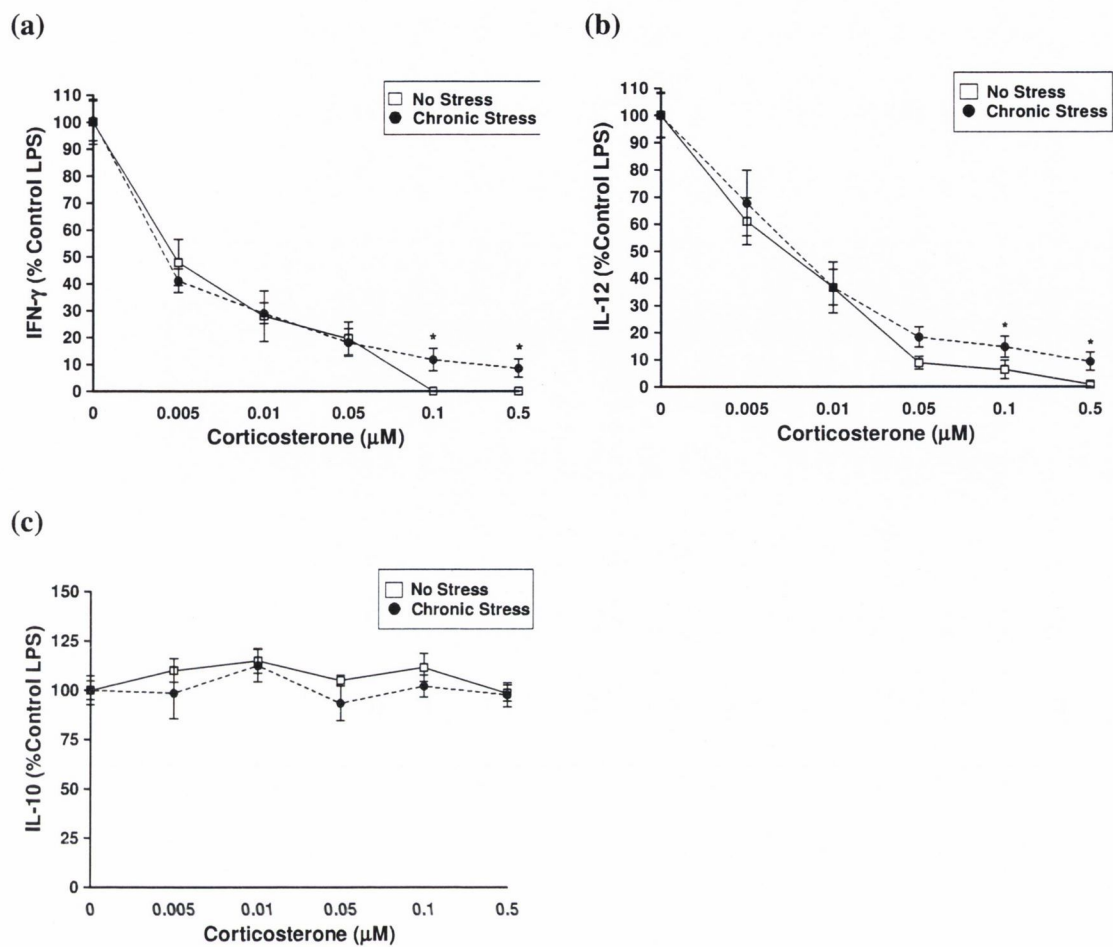


Figure 5.5.1 The suppressive effects of corticosterone on IFN- γ (a) and IL-12 (b) concentrations were impaired, however IL-10 concentrations (c) were not altered in splenocytes cultured from mice 24 hr following exposure to chronic stress

Mice were exposed to chronic stress for 2 hr per day for 7 days and the mice were sacrificed 24 hr after termination of last stress. Splenocytes were harvested from freshly isolated spleens and pre-treated for 30 min with corticosterone (0.005 – 0.5 μ M) or salbutamol (0.01 – 1 μ M) prior to treatment with LPS (1 μ g/ml). Cell supernatant was harvested after 24 hr and 72 hr of incubation. A two-way ANOVA revealed a significant effect of corticosterone [$F_{(7,91)}=32.6$, $P<0.0001$] and salbutamol [$F_{(7,93)}=58.5$, $P<0.0001$] on IFN- γ production. Data are expressed as mean $\pm$ SEM (n=6). * $P<0.05$ vs. non-stressed LPS-treated counterparts (Neuuman-Keuls post hoc test).

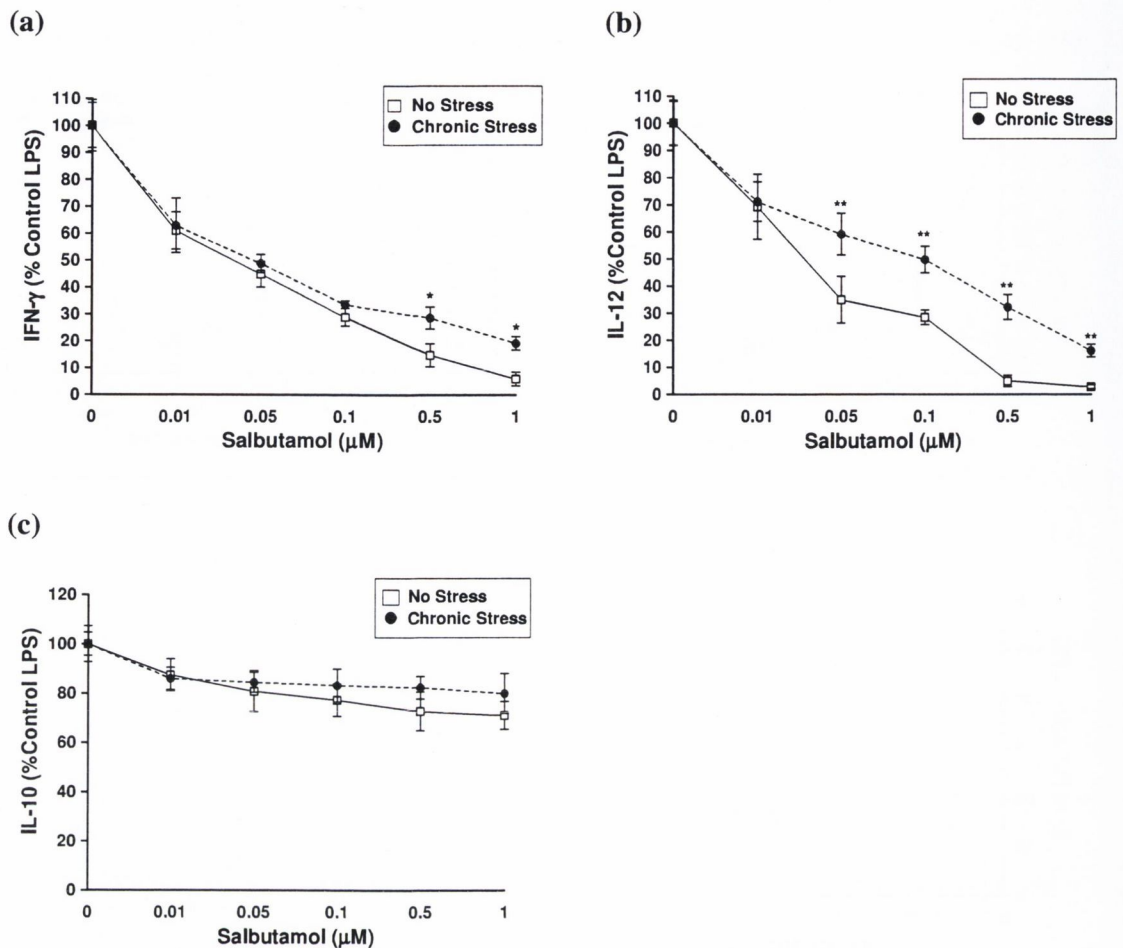


Figure 5.5.2 The suppressive effects of salbutamol on IFN- γ (a) IL-12 (b) concentrations were impaired, however IL-10 concentrations were not altered in splenocytes cultured from mice 24 hr following exposed to chronic stress

Mice were exposed to chronic stress for 2 hr per day for 7 days and the mice were sacrificed 24 hr after termination of last stress. Splenocytes were harvested from freshly isolated spleens and pre-treated for 30 min with corticosterone (0.005 – 0.5 μ M) or salbutamol (0.01 – 1 μ M) prior to treatment with LPS (1 μ g/ml). Cell supernatant was harvested after 24 hr and 72 hr of incubation. A two-way ANOVA revealed a significant effect of corticosterone on IL-12 production [$F_{(7,103)}=47.1$, $P<0.0001$] and a significant effect of salbutamol on IL-12 production [$F_{(7,96)}=64.3$, $P<0.0001$]. Data are expressed as mean $\pm$ SEM ($n=6$). ** $P<0.01$ vs. non-stressed LPS-treated counterparts, * $P<0.05$ vs. non-stressed LPS-treated counterparts (Neuman-Keuls post hoc test).

Chapter 6: Discussion

6.1 Exposure to acute restraint stress increased corticosterone concentrations

The studies presented in this thesis examined the immunosuppressive effects following exposure to restraint stress in mice. During the acute stressing protocol, mice were restrained or immobilized for 2 hr and the psychological stress that they encountered has been established as a moderate stressor (Thaker *et al.*, 2006). Perception of stress leads to activation of the HPA and SAM axes and the subsequent release of corticosterone, NA and Adr in mice (Chrousos, 1995). To confirm that the acute restraint stress paradigm used in this thesis produced a stress response, and activated the HPA axis, corticosterone concentrations in the serum and spleens of mice were measured immediately following exposure to 2 hr of restraint stress. It was demonstrated that exposure to acute restraint stress increased the concentrations of corticosterone in the serum (~ 8 fold) and in the spleen (~ 5 fold) relative to non-stressed mice. In agreement with these results, it was previously demonstrated that following 2 hr of restraint stress, corticosterone levels increased (~ 5 fold) in mouse spleen (Thaker *et al.*, 2006). In addition, this report also demonstrated that NA levels increased (~ 2 fold) in mouse spleen. However, the stress-induced increases in tissue and plasma concentrations of NA are highly unstable (Antoni *et al.*, 2006). The spleen is a model organ for testing brain-immune interactions as the spleen is innervated by sympathetic nerve fibres that are distributed in close proximity to immune cells (Nance and Burns, 1989; Meltzer *et al.*, 1997). In addition the observed time course for splenic cytokines in rodents follows a similar pattern for serum cytokines (Meltzer *et al.*, 2003). Therefore, in this thesis, the effect of stress on cytokine production in mouse spleen was investigated.

6.2 Exposure to restraint stress suppressed the innate IFN- γ response

Innate IFN- γ produced following LPS, is a vital response at the onset of infection, and serves as an essential link between innate and adaptive immunity. Innate IFN- γ is part of a positive feed back loop which amplifies antimicrobial immune responses by inducing phagocytosis and respiratory burst activity, antigen presentation and cytokine secretion by APCs (Boehm *et al.*, 1997). In addition, IFN- γ promotes secretion of IgG_{2a} antibodies by B-cells, induction of T_H1 cell differentiation, maturation of CTLs and activation of neutrophils (Boehm, *et al.*, 1997; Hasbold *et al.*, 1999). Therefore, any disruption or deficit of innate IFN- γ production can be perilous for the host defense (Rossow *et al.*, 2003; Jouanguy *et al.*, 1996; Kamijo *et al.*, 1993).

Discussion

The first objective of the studies presented in this thesis was to investigate the ability of acute restraint stress to impact upon LPS-induced production of IFN- γ . LPS is a potent inducer of IFN- γ (Farrar *et al.*, 1993) and in agreement with this report, the findings in this thesis demonstrate that *in vivo* challenge of LPS increased serum IFN- γ concentrations in mice. However, exposure to acute restraint stress impaired LPS-induced IFN- γ serum concentrations relative to non-stressed counterparts. This stress-induced reduction of innate IFN- γ serum concentrations was paralleled at mRNA level in mouse spleen, with suppressed LPS-induced IFN- γ mRNA expression in mouse spleen relative to LPS-treated counterparts. The findings from these studies are the first to report suppression of innate IFN- γ production following exposure to acute restraint stress. However, it has been reported that concanavalin A (con A) stimulated IFN- γ production from mouse splenocytes is greatly reduced following exposure to acute restraint stress (Iwakabe, *et al.*, 1998). This report also observed that the stress-induced suppression of IFN- γ was paralleled with a decrease in NK cell activity, but no alteration in NK cell numbers in the mouse spleen were found (Iwakabe *et al.*, 1998). In contrast to this report, in this thesis, it was demonstrated that anti-CD3 stimulated T-cell derived IFN- γ production from splenocytes was not altered following exposure to acute restraint stress. These contrasting reports are most likely due to the different durations of restraint stress used. Iwakabe *et al.* (1998) restrained the mice for 24 hr, which is more intense than the 2 hr stressing paradigm used in this thesis. Although we found that acute restraint stress does not directly alter T-cell production, it is likely that there is an indirect effect on T-cell differentiation and proliferation via alterations in the innate cytokine network. The stress-induced reduction of innate IFN- γ production could have implications on the type of adaptive immune response that is initiated in response to an infectious stimulus. In the presence of IFN- γ , CD4⁺ T-cells differentiate into T_H1 immune cells which mediate cell based immune responses with increased production of IL-2 and IFN- γ (Bradley *et al.*, 1996). However, the stress-induced inhibition of IFN- γ could lead to an altered immune profile and induce a shift in the immune response from a T_H1 to a T_H2 or humoral mediated immune response (Sheridan *et al.*, 1991; Rook *et al.*, 1991). This alteration in immune profile could delay clearing of a pathogen and leave a host vulnerable to opportunistic infection or accelerate disease progression.

LPS-induced production of IFN- γ activates the transcription of many IFN- γ inducible genes via the JAK-STAT signalling cascade, which converts IFN- γ 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 STAT-1. Upon phosphorylation, two STAT-1 molecules dimerise, and this dimer translocates to the nucleus where it binds to DNA and alters gene expression. In order to determine if the stress-induced reduction in IFN- γ production could impair IFN- γ signalling, phosphorylation of STAT-1 was assessed following exposure to stress. In agreement with previous reports, LPS administration increased the phosphorylation of STAT-1 in mouse spleen. Exposure to restraint stress suppressed LPS-induced STAT-1 phosphorylation. This is consistent with previous reports where it was demonstrated that administration of a chemical stressor (MDMA) suppressed LPS-induced STAT-1 phosphorylation in mouse spleen (Boyle and Connor, 2007). As predicted, exposure to stress did not alter total STAT-1 (tSTAT-1) expression and therefore there was a significant effect of exposure to stress on the pSTAT-1 as a percentage of tSTAT-1.

The JAK-STAT signalling pathway is associated with transcription of IFN- γ inducible genes such as iNOS, CD40 and IP-10 (Narumi *et al.*, 1992; Zhang *et al.*, 2005; Boss *et al.*, 1997). In this study, it was demonstrated that, in tandem with suppressing LPS-induced STAT-1 phosphorylation, restraint stress also significantly suppressed LPS-induced mRNA expression of iNOS, CD40 and IP-10 in mouse spleen. The stress-induced inhibition of these genes can have significant consequences on response to infection as they mediate various aspects of the innate immune response and play roles in initiating the adaptive immune responses. The stress-induced inhibition of LPS-induced iNOS that is observed in this thesis, is in agreement with a previous report where it was demonstrated that T-cell derived iNOS expression is inhibited following exposure to restraint stress (Zhang *et al.*, 1998). Stress-induced inhibition of iNOS is of particular interest because its induction plays a vital role in innate immunity (Hibbs *et al.*, 1987). iNOS can synthesise large amounts of nitric oxide in macrophages and prevents the growth of ectromelia, vaccinia and HSV-1 viruses (Palmer *et al.*, 1988; Bogdan, 2001). 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) and iNOS deficient mice do not survive following infection

due to proliferation of *Salmonella* (Mastroeni *et al.*, 2000). In addition, it has been shown that iNOS^{-/-} mice and IFN- γ R^{-/-} 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 stress to suppress iNOS expression is likely to have a significant negative impact on host defense to infectious diseases.

IP-10 is a chemokine that functions as a primary mediator in attracting effector T-cells to the site of the infection and therefore has an important role in linking the innate and adaptive immune responses (Taub *et al.*, 1993, Loetscher *et al.*, 1996). The importance of IP-10 was demonstrated in mice infected with *T.gondii* where it was shown that *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, studies have reported that macrophages stimulated with IP-10 have the ability to decrease parasitic infection and injection of IP-10 deferred the progression of disease in susceptible mice (Vasquez *et al.*, 2006). Moreover, studies have indicated that IP-10 is involved with the production of tumour-protective CD8⁺ T-cell mediated responses and therefore may have important functions during tumour surveillance (Pertl *et al.*, 2001; Guo *et al.*, 2004). Consequently, the ability of restraint stress 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. Consistent with our finding, administration of a chemical stressor (MDMA) also suppressed IP-10 expression in mouse spleen (Boyle and Connor, 2007). In addition to the stress-induced inhibition of IP-10, stress-induced inhibition of other chemokines has been demonstrated to suppress NK cell trafficking during viral infection (Hunzeker *et al.*, 2004) and delay local wound repair (Glaser *et al.*, 1999).

The stress-induced suppression of CD40 expression that was demonstrated in this thesis *in vivo* is in agreement with *in vitro* experiments that showed NA and GCs induced

suppression of co-stimulatory molecule expression, including CD40. For example, it was demonstrated that exposure of DCs to NA reduced LPS-induced expression of CD40 and the capacity to stimulate T_H cells (Maestroni, 2002). In addition, it has been demonstrated that GCs impair the expression of the co-stimulator CD40 from microglia and impaired T-cell proliferation and T_H1 polarisation (Maoquan *et al.*, 2007). 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 (McLellan *et al.*, 1996). In addition, CD40L *-/-* mice are susceptible to infection possibly due to the suppression of IL-12 production by APCs and therefore an impaired T_H1 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, the up-regulation of CD86 / CD80 co-stimulatory molecules and IL-12 production (Cella *et al.*, 1996). In this study it was demonstrated that restraint stress suppressed mRNA expression for CD40 in spleen, thus inhibition of gene transcription represents a mechanism by which stress can suppress CD40 cell surface expression on APC's. The ability of stress to suppress co-stimulatory molecule expression is a significant finding, as these molecules are essential for effective T-cell activation.

6.3 Exposure to restraint stress suppressed the production of the IFN- γ inducing cytokines - IL-12 and IL-15

Recent reports have indicated that LPS-induced IFN- γ production is predominantly derived from NK cells (60%) and NKT-cells (20-25%), with T-cells and DCs representing minor contributors (5%) (Varma *et al.*, 2002; Fukuda *et al.*, 2005). However, NK cells cannot respond directly to LPS challenge as they do not express functionally responsive TLR4 receptors (O'Connor *et al.*, 2006). Therefore, the LPS-induced IFN- γ response occurs secondary to inducing factors, such as IL-12, IL-15 and IL-18 when they are produced by accessory cells, such as macrophages and DCs in response to LPS detection. This secondary induction of IFN- γ by its inducers explains why it takes some time (8 hr) before the peak IFN- γ response to LPS is observed *in vivo* (Fukuda *et al.*, 2005; Kim *et al.*, 2000).

Discussion

In this thesis, the ability of stress to impact upon production of the pro-inflammatory cytokines IL-12, IL-15 and IL-18 in response to an *in vivo* LPS challenge was investigated as these three cytokines are established IFN- γ inducers (Kubin *et al.*, 1994; Chan *et al.*, 1991; Dinarello, 2000; Fehinger *et al.*, 1999). IL-12 is the most potent inducer of IFN- γ production from NK cells and is also an important immunoregulatory cytokine that promotes cell-mediated immunity. In this thesis it was found that restraint stress suppressed LPS-induced IL-12p40 mRNA expression in spleen at 1.5 hr and 8 hr following LPS administration. In contrast, stress failed to alter LPS-induced expression of IL-12p35 mRNA. However, this is in line with previous reports where it was shown that IL-12p35 is constitutively expressed in mouse spleen (Varma *et al.*, 2002). The p40 subunit is the inducible subunit of IL-12 and its gene expression is tightly controlled, and largely governs the biological activity of IL-12 (Trinchieri *et al.*, 2003; Brombacher *et al.*, 2003). Consistent with the findings presented in this thesis, exposure to restraint stress has been previously reported to impair IL-12 production following viral infection (Hunzeker *et al.*, 2004) and *Listeria monocytogenes* infection (Zhang *et al.*, 1998). In addition, it has been demonstrated that β -AR agonists impair IL-12 production *in vivo* and *in vitro* (Hasko *et al.*, 1998; Panina-Bordignon *et al.*, 1997) and GCs suppress production of IL-12 (Maoquan *et al.*, 2007; DeKruyff, *et al.*, 1998). The ability of stress to suppress IL-12p40 mRNA represents a key mechanism by which it can interfere with the development of an effective immune response. This is due to the fact that IL-12 is a pivotal cytokine in inducing IFN- γ production and in driving a T_H1 cell-mediated immune response and in host resistance to infectious disease (Trinchieri *et al.*, 2003; Magram *et al.*, 1996). Therefore, any alteration in IL-12 production can suppress cell mediated immunity. For example, it has been demonstrated that *in vitro* treatment with catecholamines suppressed IL-12 production and induced a shift in the immune response from a T_H1 to a T_H2 response (Elenkov *et al.*, 1996).

The proposed mechanism for the induction of IFN- γ in response to a bacterial challenge is that the early production of IL-12 at 1.5 hr following LPS administration stimulates IFN- γ production from NK cells at a later time point, such as 8 hr post LPS. To provide evidence for the proposed induction of IFN- γ by IL-12, mouse recombinant IL-12 was administered to splenocytes cultured from the spleens of mice exposed to stress. The concentration of IL-12 (100 pg/ml) that was administered was approximate to the observed concentrations before stress. The findings demonstrated that IL-12 could

restore stress-induced inhibition of IFN- γ production. Suggesting that stress-induced inhibition of IFN- γ production is mediated via IL-12.

The activities of IL-12 are mediated by a high-affinity receptor composed of two subunits - $\beta 1$ and $\beta 2$. The two receptor subunits are expressed on NK and T-cells and both are required for IL-12 bioactivity (Gately *et al.*, 1998). Studies of IL-12R $\beta 1^{-/-}$ and IL-12R $\beta 2^{-/-}$ mice confirmed the essential role for the two subunits in mediating the biological functions of IL-12 on NK and T-cells (Wu *et al.*, 2000). However, IL-12R $\beta 1$ is required for effective binding of IL-12p40 which is the inducible subunit of the cytokine (Zou *et al.*, 1997). In the studies presented in this thesis, it is demonstrated that exposure to restraint stress significantly inhibited LPS-induced splenic mRNA expression of both IL-12 receptor $\beta 1$ and $\beta 2$ in mouse spleen. This finding is consistent with a previous report where it was demonstrated that treatment of human T-cells with the synthetic GC, dexamethasone, down-regulated IL-12R $\beta 1$ expression and suppressed STAT-4 phosphorylation (Fahey *et al.*, 2006). It could be suggested that the stress-induced down-regulation of IL-12R expression could alter the immune response to an infectious stimulus because the immunostimulatory function of IL-12 would be impaired. In addition, there could be reduced responsiveness to IL-12 even when normal physiological levels of IL-12 are restored due to the down-regulation of the IL-12R. However, the findings from this thesis suggest that the normal concentration of IL-12 produced following LPS, could restore the stress-induced inhibition of IFN- γ concentrations, therefore the down-regulation of IL-12R did not impair the action of IL-12.

The pro-inflammatory cytokines IL-15 and IL-18 have been shown to enhance IFN- γ production from NK cells and T-cells, however, they are more often shown to induce IFN- γ in synergy with IL-12 (Fehniger *et al.*, 1999; Ohteki, 2001). Therefore, in this thesis the effect of stress on LPS-induced IL-15 and IL-18 mRNA expression was examined in mouse spleen. The results demonstrate that exposure to restraint stress suppressed LPS-induced expression of IL-15 mRNA in mouse spleen. The stress-induced suppression of IL-15 could have implications on NK cell function as IL-15 is known to be a potent NK growth factor. In line with this conclusion, it has been demonstrated that treatment of peripheral blood mononuclear cells (PBMCs) with GCs suppressed IL-15 production from NK cells (Liu *et al.*, 2008). In addition, the NK-

stimulating activity of IL-15 on human mixed lymphocytes was demonstrated to be inhibited by treatment with dexamethasone (Lewis *et al.*, 2001). The stress-induced suppression of IL-15 could have long term implications on adaptive immune responses because IL-15 is dedicated to the prolonged maintenance of memory T-cell responses to invading pathogens (Waldmann, 2006). Conversely, the results presented in the thesis demonstrate that splenic IL-18 mRNA was not altered in response to stress, suggesting that IL-18 is not a key mediator of IFN- γ production in response to LPS and that IL-18 is less sensitive to modulation by psychological stressors than inducible genes such as IL-12p40 and IL-15. In accordance with this view, it has recently been demonstrated that IL-18 does not directly induce IFN- γ production, but serves to amplify IL-12 and IL-15 induced IFN- γ production (Strengell *et al.*, 2003). These results are in contrast to previous studies which have shown that plasma concentrations of IL-18 and IL-18 mRNA expression are altered following exposure to stress (Sugama *et al.*, 2000; 2006). However, in these studies a more severe stress regime was employed that combined immobilisation stress with water immersion stress to achieve differences in the levels of IL-18. Comparison of both results suggests that IL-18 is a target of severe psychological stress and not moderate stress, as used in the studies presented in this thesis. While IL-18 production was unaltered by our moderate psychological stress, it is clear that IL-18 could not compensate or substitute for the reduction in IL-12 and IL-15 induced by stress.

6.4 Exposure to restraint stress increased production of the anti-inflammatory cytokine IL-10

IL-10 is an immunosuppressive cytokine that inhibits several macrophage functions, such as pro-inflammatory cytokine production (Bogdan *et al.*, 1992; Moore *et al.*, 2001). In response to an LPS challenge, the inhibition of pro-inflammatory cytokines is accompanied by increased production of anti-inflammatory cytokines, such as IL-10 (Connor *et al.*, 2005; D'Andrea *et al.*, 1993). The production of IL-10 limits the immune response to an inflammatory stimulus and ensures that there is no overshoot in pro-inflammatory cytokine production (Moore *et al.*, 2001). Therefore, any manipulation that alters IL-10 production is also likely to impact upon normal functioning of the immune system. For instance, IL-10 deficient mice develop severe colitis as a result of uncontrolled inflammation (Kuhn *et al.*, 1993). The results presented in this thesis demonstrate that exposure to restraint stress augmented LPS-

induced IL-10 serum concentrations and IL-10 mRNA expression in mouse spleen relative to LPS-treated counterparts. These findings provide further evidence that an alteration in mRNA expression parallels protein concentration changes. The stress-induced increase in IL-10 serum concentrations and mRNA expression were induced very rapidly, as early as 45 min following LPS administration. This finding is consistent with previous research where exposure to a brief swim stress or a chemical stress (MDMA) profoundly increased LPS-induced IL-10 production in rats (Connor *et al.*, 2005). In addition, it has been demonstrated that β -AR agonists increase the release of IL-10 in mice (Szabo *et al.*, 1997).

Taking this finding along with the previous results, there is strong evidence that exposure to acute restraint stress promotes an immunosuppressive cytokine profile, with increased IL-10 and impaired IFN- γ and IL-12. This is in line with previous reports that exposure to acute stress, such as swim stress, footshock and restraint stress suppressed LPS-induced pro-inflammatory cytokine expression in rodents (Connor *et al.*, 2004; Connor *et al.*, 2005; Goujon *et al.*, 1995 and Meltzer *et al.*, 2004). However, other studies have demonstrated that there is an enhancement of LPS-induced pro-inflammatory cytokine production following exposure to an acute stress, such as inescapable tail-shock paradigm (Johnson *et al.*, 2002; Johnson *et al.*, 2003). The findings from this thesis suggest that the interval post-stress at which LPS was administered may be a key factor underlying the divergent results obtained between studies. For instance in the studies presented in this thesis and in the studies conducted by Connor *et al.* (2005), Goujon *et al.* (1995) and Meltzer *et al.* (2004), LPS was administered immediately prior to stressor exposure. In contrast, in the studies that observed a stress-induced increase in LPS-induced pro-inflammatory cytokine production, LPS was administered a number of hours following exposure to stress. In addition, Johnson *et al.*, (2002) and Johnson *et al.*, (2003) both reported GC resistance following exposure to their stress paradigm. Therefore, it is likely that GC resistance underlies the altered response to LPS when there is a temporal delay before LPS administration.

6.5 IL-10 does not mediate stress-induced inhibition of the innate IFN- γ response

The stress-induced augmentation of IL-10 serum concentrations and mRNA expression could have profound effects on the immune response. For example, IL-10 has been

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shown to promote the development of a T_H2 or humoral based immune profile via suppression of T_H1 cytokines such as IL-12, IFN- γ and TNF- α (Abbas *et al.*, 1996). More recently IL-10 was demonstrated to induce the production of T_{Reg} cells which inhibit the proliferation and cytokine production from T_H1 and T_H2 cells (Mills, 2004). The stress-induced increase in IL-10 would enhance the immunosuppressive actions of IL-10 and could have consequences for the course of an immune response to an infectious stimulus. For instance, IL-10 has been demonstrated to suppress the production of pro-inflammatory cytokines *in vitro* and *in vivo* (D'Andrea *et al.*, 1993). In agreement with these reports, in this thesis it was demonstrated that exogenous administration of mouse recombinant IL-10 to splenocytes could inhibit LPS-induced production of IFN- γ and IL-12 with all doses of IL-10 investigated (0.1 – 1 ng/ml). The doses of IL-10 chosen were relevant to the concentrations observed following stress and therefore are physiologically relevant.

These findings suggest that IL-10 can mimic the suppressive effect of stress on the production of pro-inflammatory cytokines and therefore IL-10 could be the key mediator in the stress-induced suppression of the innate IFN- γ response. In addition, it has been demonstrated that stress-induced augmentation of IL-10 concentrations is induced as early as 45 min in the cascade of cytokine production and it precedes the stress-induced suppression of IL-12 and IFN- γ . Therefore, it is hypothesised that IL-10 could mediate the immunosuppressive effects of stress. It is suggested that the increase in IL-10 production induced by stress reduces IFN- γ synthesis indirectly by inhibiting production of IFN- γ -inducing factors, such as IL-12 and IL-15 from macrophages / DCs. In this regard, it was previously demonstrated that IL-10 suppressed LPS-induced IFN- γ production in tandem with reducing IL-12 production (Shibata *et al.*, 1998).

However, the results from this thesis demonstrate that IL-10 is not the mediator of stress-induced inhibition of IFN- γ or IL-12. Firstly, the anti-IL-10R antibody was used to investigate if IL-10R blockade could prevent the stress-induced inhibition of IL-12 and IFN- γ . However, the results from these studies demonstrate that the anti-IL-10R antibody did not alter stress-induced inhibition of LPS-induced IFN- γ and IL-12 mRNA expression in mouse spleen. The anti-IL-10R antibody increased IFN- γ and IL-12p40 mRNA expression relative to control antibody treated mice which is most likely due to its ability to inhibit the suppressive actions of basal IL-10. This is consistent with

previous reports indicating that IL-10 deficiency increased IFN- γ and IL-12 production (Varma *et al.*, 2002; Berg *et al.*, 1995) and demonstrates that the concentrations of anti-IL-10R antibody employed was efficacious in blocking IL-10R. However, blockade of the IL-10R alone was not sufficient to impair the stress-induced inhibition of IFN- γ or IL-12. To confirm that IL-10 does not mediate stress-induced inhibition of IFN- γ and IL-12, the effect of stress on the innate IFN- γ response was investigated in IL-10 knock out (KO) mice. The results demonstrate that LPS-induced IFN- γ serum concentrations and mRNA expression in mouse spleen are inhibited following exposure to stress in IL-10 KO mice and in C57BL/6 controls. Moreover, LPS-induced IL-12p40 mRNA expression in mouse spleen is inhibited following exposure to stress in IL-10 KO mice and in C57BL/6 mice. These results confirm the findings from the anti-IL-10R antibody study, and indicate that IL-10 does not mediate the stress-induced inhibition of the innate IFN- γ response. They also re-confirm that IL-10 deficiency results in increased IFN- γ and IL-12 production, as IL-10 KO mice have much greater concentrations of IFN- γ and mRNA expression of IFN- γ and IL-12p40.

Taken together, these studies suggest that IL-10 does not govern stress-induced immunosuppression and that other factors are involved in the stress-induced suppression of the innate IFN- γ response. This finding is consistent with previous reports where it was shown that there is mechanistic dissociation between the ability of exposure to swim stress to increase production of IL-10 and suppress production of the pro-inflammatory cytokines IL-1 β and TNF- α in rats (Connor *et al.*, 2005a). A number of other studies have also demonstrated that various pharmacological stimuli suppress LPS-induced pro-inflammatory cytokine production independent of increased IL-10 production (Connor *et al.*, 2005b; Shames *et al.*, 2001). Taken together, these results demonstrate that the increase in IL-10 induced by pharmacological means or psychological stressors does not mediate the suppression of pro-inflammatory cytokines, such as IFN- γ or IL-12.

6.6 IL-19 mimics the stress-induced augmentation of IL-10

IL-10 shares structural homology to five other molecules that have recently been identified as part of the IL-10 family. They are IL-19, IL-20, IL-22, IL-24 (MDA-7) and IL-26 (AK155) and so far little is known about their biological activities. Even though these members share structural homology with IL-10, they have been demonstrated to

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have some distinct biological functions. Therefore, in the studies presented here, the effect of stress on the IL-10 family members was investigated to identify if they could play a role in the immunosuppressive effects of stress. It is demonstrated that exposure to restraint stress augmented LPS-induced IL-19 mRNA expression in mouse spleen, which was similar to the effect of stress on IL-10 production. As no antibodies are available that react against IL-19 for the development of a specific ELISA, IL-19 mRNA levels were examined using quantitative real-time PCR. To the best of my knowledge, this is the first report of a stress-induced alteration in IL-19. IL-19 is an anti-inflammatory cytokine whose expression is induced from monocytes in response to LPS (Gallagher *et al.*, 2004). It has been shown to alter the balance of T_H1/T_H2 cells in favour of T_H2 or humoral mediated immune response (Gallagher *et al.*, 2004). In agreement with this theory, IL-19 was demonstrated to dose-dependently promote T_H2 responses by up-regulating IL-4 and down-regulating IFN- γ in T-cells (Liao *et al.*, 2004). IL-19 has also been implicated in the regulation of immunity through auto-induction of its own expression, as well as inducing the production of IL-10 (Jordan *et al.*, 2005). In turn, IL-10 functions to negatively regulate IL-19 production. Thus, whilst IL-19 up-regulates itself, implying a positive feedback mechanism, it also induces IL-10, which acts to down regulate IL-19 production. In relation to the results presented in this thesis, it can be seen that there is a larger induction of splenic IL-19 at 90 versus 45 minutes. This could be a result of the auto-reactive positive feedback mechanism of IL-19.

The stress-induced augmentation of IL-10 and IL-19 are not associated with a general increase in anti-inflammatory cytokines. For example, in this thesis, it has been demonstrated that TGF- β mRNA expression is not altered in mouse spleen following exposure to stress. In addition, there was no stress effect observed on the production of the two other IL-10 related cytokines that were investigated - IL-20 and IL-24. There was undetectable or very little amplification of IL-20 or IL-24 mRNA in response to LPS or stress. This indicates that these cytokines do not play an important role in the immune response to a bacterial challenge and are not altered in relation to stress hormones. These findings are in agreement with known functions of the IL-10 family members so far. The biological activities of IL-19 seem to be in regulating cellular immune activity, however IL-20 is associated with skin biology and IL-24 in tumour apoptosis (Wolk *et al.*, 2002).

6.7 NA mediates the stress-induced increase in IL-10 and IL-19, but not the stress-induced suppression of innate IFN- γ

When a stressful event is detected by the CNS there is activation of the SAM and the HPA axis. The end products of these pathways, such as the catecholamines and GCs, are often implicated as mediators of stress-induced immunosuppression. For instance, the SAM axis and its principle neurotransmitter NA have been shown to modulate immune function both *in vivo* and *in vitro* (see review Madden *et al.*, 1995). NA has been demonstrated to be increased in mouse spleen following exposure to stress, for example, following exposure to 2 hr of restraint stress the concentration of NA in the spleen increased ~2 fold (Thaker *et al.*, 2006). Immune cells are vulnerable to alteration by NA because they express specific functional receptors for NA. The effect of NA on immune cells is primarily mediated by β -AR activation and increased intracellular cAMP (Siegmund *et al.*, 1998). NA can alter many phases of the immune response, from initiation to proliferation and effector response, by alteration of cytokine production, lymphocyte proliferation, cell trafficking and antibody secretion (Madden, 2003). For instance, *in vitro* studies have demonstrated that NA can increase IL-10 production from macrophages in response to LPS challenge and suppress IL-12 production (Maestroni, 2002; Elenkov *et al.*, 1996). In addition, NA has been demonstrated to suppress the production of IFN- γ serum concentrations and IFN- γ mRNA expression in mouse spleen (Gan *et al.*, 2002; Dokur *et al.*, 2004). To further investigate the possible role of NA as a mediator of stress-induced immunosuppression, *in vitro* experiments were carried out to assess the production of cytokines from NA-treated DCs. The results in this thesis demonstrate that NA mimics the effect of stress on the production of pro- and anti-inflammatory cytokines from bone marrow derived DCs (BMDCs). Specifically, pre-treatment of BMDCs with NA augmented the LPS-induced increase in IL-10 and IL-19 mRNA, and significantly inhibited the LPS-induced increase in IL-12p40 mRNA expression relative to non-NA treated cells. In addition, the non-selective β -AR antagonist propranolol could prevent NA-induced changes in cytokine production. Propranolol inhibited NA-induced production of IL-10 and IL-19 mRNA expression from DCs and also successfully prevented NA-induced inhibition of IL-12p40 mRNA expression. The findings from these studies demonstrated that NA modulates immune cell function via β -AR and *in vitro* blockade of β -AR can prevent NA-induced alterations in IL-10, IL-19 and IL-12. As discussed previously, IL-

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12 is the primary inducer of IFN- γ and this thesis has demonstrated that the stress-induced alterations in IL-12 were paralleled with changes in IFN- γ production. This indicates that the NA could play a role in stress-induced immunosuppression of the innate IFN γ response.

To further elucidate if the stress-induced increase in NA could be responsible for the immunosuppressive effects of stress, the ability of the β -AR antagonist nadolol to prevent the immunosuppressive actions of stress *in vivo* were evaluated. The results from these experiments demonstrate that blockade of β -AR prevented the stress-induced augmentation of LPS-induced serum IL-10 concentrations and IL-10 mRNA expression in mouse spleen. In addition, pretreatment with nadolol prevented the stress-induced augmentation of LPS-induced IL-19 mRNA expression. However, nadolol did not prevent the stress-induced inhibition of LPS-induced IFN- γ serum concentrations or IFN- γ mRNA expression in mouse spleen. In addition, nadolol did not prevent stress-induced inhibition of LPS-induced IL-12 serum concentrations or IL-12p40 mRNA expression in mouse spleen. These data demonstrate that β -AR activation mediates the stress induced increase in IL-10, but not the suppression of pro-inflammatory cytokine production. Of note in these studies was the fact that nadolol administration alone increased LPS-induced IL-12p40 expression which 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 -AR blockade augmented production of the pro-inflammatory cytokine TNF- α following LPS administration in mice (Bencsics et al., 1997). The findings from these studies are in agreement with those of the immunoneutralization study and illustrate that there is dissociation between the ability of a psychological stress to increase IL-10 and suppress the innate IFN- γ response. Moreover, these results are in agreement with previous reports where it was shown that acute swim stress could increase IL-10 independent of a suppression of the pro-inflammatory cytokines TNF- α and IL-1 β in rats (Connor *et al.*, 2005a). Considering all of these findings, it seems unlikely that NA is the primary mediator of the stress-induced suppression of the innate IFN- γ response. However, catecholamines clearly mediate the stress-induced increase in peripheral IL-10 and IL-19 production via β -AR.

6.8 Corticosterone mediates the stress-induced suppression of the innate IFN- γ response

As previously discussed, following detection of a stressor and activation of the HPA and SAM axis, there is release of NA and GCs, such as corticosterone in mice. The findings discussed previously have suggested that NA does not mediate the stress-induced suppression of the innate IFN- γ response. Therefore, the role of corticosterone in stress-induced immunosuppression was investigated. In this thesis and in other reports, it has been demonstrated that following exposure to acute restraint stress there is increased corticosterone concentrations in mouse serum and in mouse spleen (Thaker *et al.*, 2006). Corticosterone is a potent immunosuppressive agent that is capable of altering immune cell function via GR activation (Ballard *et al.*, 1995). In this thesis, the immunosuppressive effects of corticosterone were characterized to investigate its ability to alter LPS-induced production of IL-12 and IFN- γ from splenocytes. The findings from these studies demonstrated that corticosterone inhibited LPS-induced IL-12 and IFN- γ in a dose-dependant manner. This finding is consistent with other groups that have shown that corticosterone can inhibit the production IL-12 from DCs and functionally compromise DC maturation (Elenkov *et al.*, 1996; Elftman *et al.*, 2007). The corticosterone-induced reduction of IL-12 can also inhibit IFN- γ production because IL-12 is extremely potent in enhancing IFN- γ (Elenkov, 2004). In addition, in this thesis it was demonstrated that IL-12 can restore the inhibition of LPS-induced IFN- γ elicited by corticosterone. This suggests that corticosterone-induced reduction of IL-12 is primarily responsible for the reduction of IFN- γ . The suppression of IL-12 and IFN- γ may interfere with the ability of DCs to prime naïve T-cells which is critical in the initiation of an adaptive immune response against viral infection (Trevejo *et al.*, 2001).

To investigate the role that GCs play in stress-induced immunosuppression *in vivo*, a GR antagonist, mifepristone, was assessed for its ability to alter the stress-induced production of IFN- γ and IL-12. Mifepristone prevented the stress-induced suppression of LPS-induced IFN- γ serum concentrations, IFN- γ mRNA expression and the expression of the IFN- γ inducible genes, iNOS and IP-10 in mouse spleen. In addition, mifepristone prevented the stress-induced suppression of LPS-induced IL-12p40 and IL-12R β 1 mRNA expression in mouse spleen. These findings suggest that the stress-induced suppression of the innate IFN- γ response is primarily mediated via the action of

GCs on the GR. In agreement with our findings, it has been suggested that GCs are primary inhibitors of pro-inflammatory cytokines in response to stress (Molina and Abumrad, 2000). However, adrenalectomy does not prevent all of the changes in the immune system induced by stress (Zwilling *et al.*, 1993; Meltzer *et al.*, 2003). In addition, it has been demonstrated that administration of mifepristone to rats failed to block the increase in IL-10 induced by swim stress (Connor *et al.*, 2005a). However, this was not surprising considering the observation that β -AR activation mediates this effect. These findings are consistent with a previous study by Elenkov *et al.* (1996), where it was shown that the GC dexamethasone (a synthetic GC) inhibited LPS-induced IL-12 production in a dose-dependant fashion in whole human blood. In addition, the GC-induced reduction of IL-12 production was antagonized by RU 486, a GC-receptor antagonist, suggesting it was mediated by GR. However, there was no effect of dexamethasone or RU-486 treatment on IL-10 production (Elenkov *et al.*, 1996). These authors also reported that treatment with NA and adrenaline could dose-dependently increase the production of IL-10 and this effect was completely blocked by propranolol (Elenkov *et al.* 1996). Therefore, taking these findings in addition to the results presented in this thesis, it is suggested that GCs and catecholamines regulate the immune response during stress. This is in agreement with studies by Meltzer *et al.* (2004) where they demonstrated that neither splenic nerve cuts nor adrenalectomies alone blocked the effects of stress on splenic immune function. However, the combination of these two manipulations significantly abrogated the immunosuppressive effects of stress on cytokine production (Meltzer *et al.*, 2004).

IL-12 and IFN- γ induce T_H1 or cellular immune responses, however IL-10 inhibits T_H1 responses and stimulates T_H2 or humoral immune responses. Therefore, the GC-induced suppression of IFN- γ and IL-12 and the NA-induced increase in IL-10 can cause a shift in the immune response from a T_H1 to a T_H2 or humoral immune responses. The substantial T_H2 driving force of endogenous stress mediators can be amplified during certain conditions and may play a role in increased susceptibility of the organism to various infections that are normally cleared by T_H1 responses. In addition, some conditions that contribute to a substantial increase or decrease in concentrations of these mediators may play a role in induction, expression and progression of certain autoimmune diseases, allergic / atopic reactions and tumour growth (Elenkov *et al.*, 2000). These conditions include acute or chronic stress, cessation of chronic stress,

chronic hypoactivity of the stress system, severe exercise, serious surgical procedures, traumatic injuries, major burns, severe ischemia or hypoxia, pregnancy and the postpartum period. Thus, stress hormone-induced inhibition or upregulation of T_H cytokine production may represent an important mechanism by which stress affects disease susceptibility, activity and outcome of various immune-related diseases (Calcagni *et al.*, 2006). However, the findings from this thesis suggest that any stress-induced alteration of T_H immune responses would occur indirectly as T-cell derived cytokine production is not altered from splenocytes following exposure to stress. Therefore, the alteration of innate cytokine production would mediate the alterations in immune profile.

6.9 Anxiolytic treatment prevented stress-induced immunosuppression

As previously discussed, exposure to stress can suppress the immune response to an infectious stimulus and this can have implications for the onset and progression of disease. Therefore, prevention of stress perception, by pharmacological or other means, could prevent the activation of the stress response and thus prevent the immunosuppressive effects induced by stress hormones (Connor *et al.*, 2008). Benzodiazepines have been extensively used as anti-anxiety and anti-stress agents for many years and their action is mediated via high affinity receptor sites linked to GABA_A-chloride channels in the CNS (Tripathi, 1999). Chlordiazepoxide (CDP) is a benzodiazepine anxiolytic commonly used to relieve anxiety and to control agitation caused by alcohol withdrawal. Chronic treatment with CDP is known to prevent the psychological and behavioral aspects of stress and to prevent the production of stress hormones following exposure to stress. For example, in rodents treatment with chlordiazepoxide prevented the increase in plasma corticosterone levels following exposure to elevated platform stress (Degroot *et al.*, 2004). In addition, CDP has been demonstrated to attenuate the novel stress-induced elevation of NA and adrenaline (de Boer *et al.*, 1990). In these studies, and in others (Rodgers *et al.*, 2002), a dose of 10 mg/kg of CDP was used to attenuate the release of stress hormones, therefore a dose of 10 mg/kg was also chosen for use in this thesis.

The findings from the results presented in this thesis demonstrate that treatment with CDP prevented the stress-induced augmentation of LPS-induced IL-10 serum concentrations, and IL-10 mRNA expression in mouse spleen. Similarly, CDP treatment

prevented the stress-induced augmentation of LPS-induced IL-19 mRNA expression. In addition, CDP prevented the stress-induced inhibition of LPS-induced IL-12 serum concentrations and IL-12p40 mRNA expression in mouse spleen. A number of other studies suggest that treatment with benzodiazepines reverse or attenuate stress-induced immunosuppression. For example, chronic treatment with alprazolam, diazepam or midazolam protects mice against the stress-induced suppression of NK cell activity (Freire-Garabal *et al.*, 1993). This occurs in stress conditions because the centrally acting benzodiazepine agonists interact with their neuronal receptors and prevent release of GCs and catecholamines into the periphery. Taken together, these findings suggest that CDP treatment prevents the stress-induced immunosuppressive state by preventing the perception of stress and the subsequent release of GC and catecholamines.

6.10 Exposure to chronic stress suppressed the innate IFN- γ response

The data discussed previously has demonstrated that exposure to acute restraint stress suppressed the immune response to an LPS challenge, with impaired IFN- γ and IL-12 production, and increased IL-10 production. However, the immune altering effects of exposure to acute stress are short lived and may persist from minutes to days. For example, exposure to stress can result in elevated serum corticosterone, altered serum proteins, and altered activity of T-cells, B-cells and APCs that persists for several days after stressor cessation (Deak *et al.*, 1997; Fleshner *et al.*, 1995; Fleshner *et al.*, 1998). In contrast, the consequences of exposure to chronic stress can have long term implications on health (Dhabhar and McEwen, 1997). Exposure to chronic stress can have long term adaptive changes in the immune response, and it is reliably demonstrated to result in global immunosuppression (see review Segerstrom, 2004). For many years, clinicians have documented an association between exposure to chronic stress and disease onset or progression. However, there has been very little research on the implications of stress-induced immunosuppression for disease susceptibility in healthy individuals. Some studies have convincingly established that exposure to chronic stress alters the immune response and leaves individuals vulnerable to adverse medical outcomes. For example, exposure to chronic psychological stress predicts a greater expression of illness and an increased production of IL-6 in response to an upper respiratory infection (Cohen *et al.*, 1999). In addition, many human and animal studies have established that exposure to chronic stress can increase susceptibility to infectious agents, influence the severity of infectious diseases, diminish the strength of immune

responses to vaccines, reactivate latent herpes virus and slow wound healing (see review Glaser and Kiecolt-Glaser, 2005).

The specific mechanisms that mediate stress-induced suppression of the immune system are not fully elucidated and insight into these mechanisms could have implications for amelioration of stress related immunosuppression and in stress-related disorders. Therefore, the data presented in this thesis investigated the alterations in LPS-induced cytokine production following exposure to chronic restraint stress for 7 days. It has previously been established that exposure to repeated restraint stress for 2 hr / day for 7 days activates the HPA and SNS axis which is characteristic of chronic stress (Thaker *et al.*, 2006). HPA and SNS activity were measured following 7 days of restraint stress and it was demonstrated that the concentrations of corticosterone increased ~8 fold and NA concentrations increased ~ 4 fold in mouse spleen (Thaker *et al.*, 2007). In addition, it has been demonstrated that plasma concentrations of corticosterone to a novelty stressor increased ~ 6 fold following exposure to chronic restraint stress for 10 days (Marin *et al.*, 2007; Tournier *et al.*, 2001).

The findings from this thesis demonstrated that exposure to chronic stress suppressed the LPS-induced IFN- γ response, with impaired IFN- γ serum concentrations, IFN- γ mRNA expression and the expression of IFN- γ inducible genes, iNOS, CD40 and IP-10 in mouse spleen. In addition, exposure to chronic stress impaired LPS-induced expression of IL-12p40 and IL-12R β 1. These results parallel the findings from the acute stress studies and are in agreement with previous reports where it has been demonstrated that exposure to chronic stress impairs production of pro-inflammatory cytokines following various stimuli (Elenkov and Chrousos, 2002). For example, it has been demonstrated that exposure to chronic stress impairs IFN- γ production following challenge with Con A stimulus (Zhang *et al.*, 2008), tetanus toxin vaccine (Tournier *et al.*, 2001) and *Listeria monocytogenes* (Zhang *et al.*, 1998). In addition, it was observed by Zhang *et al.* (1998) that exposure to chronic stress reduced the expression of iNOS from peritoneal exudate cells following bacterial infection with *Listeria monocytogenes*. Exposure to chronic stress has also been demonstrated to impair IL-12 production following *Listeria monocytogenes* infection (Zhang *et al.*, 1998). Taking these findings along with the results from this thesis, it is clearly demonstrated that exposure to chronic stress impairs both the innate IFN- γ response and IL-12 mRNA expression in

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mouse spleen. The stress-induced alterations in mRNA expression in the spleen have been demonstrated to occur independent of alterations in the composition of lymphocytes in the spleen and the percentages of different subsets of lymphocytes in the spleen were shown not to be effected by exposure to chronic stress (Fukui *et al.*, 1997; Tournier *et al.*, 2001). However, in blood there was a general decrease in B-cells and T-cells and there was decreased leukocyte trafficking (Tournier *et al.*, 2001; Dhabhar *et al.*, 1996).

The stress-induced inhibition of IL-12 and IFN- γ can have consequences for the immune response to an infectious stimulus. For example, it has been demonstrated that mice exposed to chronic restraint stress have impaired IFN- γ concentrations and compromised survival of CD8⁺ T-cells and thereby the control of herpes simplex virus type 1 (HSV-1) infection is compromised (Freeman *et al.*, 2007). In another study, it was demonstrated that mice receiving corticosterone treated DCs generated fewer cytokine producing CD8⁺ T-cells and fewer cytotoxic CD8⁺ T-cells. This suggests that elevated corticosterone concentrations, found *in vivo* following chronic stress, impair DC maturation and DC priming of naïve T-cells, which contributes to the inefficient CTL-mediated immunity found following stress (Elftman *et al.*, 2007). A delay or failure to efficiently prime CTL responses can compromise the successful control of an infection (Anglen *et al.*, 2003). In addition, it has been demonstrated that mice exposed to chronic restraint stress have a shift in the T_H1/T_H2 balance towards T_H2 dominance which is mediated by impairing NK cell activity and reduced IL-12 and IFN- γ production (Iwakabe *et al.*, 1998; Calcagni and Elenkov, 2006). In agreement with this animal model of stress, observations by Marshall *et al.* (1998) suggest that psychological stress in medical students undergoing exams can produce a shift in the T_H1/T_H2 cytokine balance toward a T_H2 response. This report demonstrated that PBLs, isolated from healthy individuals exposed to exam stress, had decreased IFN- γ concentrations accompanied with increased IL-10 production. This decreased T_H1 cytokine response could result in weakened cell-mediated immunity, resulting in increased susceptibility to virus, fungus and mycobacteria infection (Barnes *et al.*, 1993; Elenkov *et al.*, 1996; Romani *et al.*, 1994). For example, it has been demonstrated that exposure of mice to chronic stress increased bacterial translocation from the intestine and skin into local lymph nodes and spleen (Bailey *et al.*, 2006). This can contribute to the development of diseases such as pneumonia, and in this regard, it has been shown

that chronic stress-induced bacterial translocation was associated with the development of pneumonia and this is associated with a significant reduction in the ability of splenocytes to produce IFN- γ (Kiank *et al.*, 2008).

6.11 Exposure to chronic stress augmented LPS-induced IL-10 and IL-19 production

The chronic stress-induced suppression of IFN- γ and IL-12 pro-inflammatory cytokine production is often paralleled with increased anti-inflammatory cytokine production (Calcagni and Elenkov, 2006). Therefore, in this thesis, the consequence of exposure to chronic stress on LPS-induced IL-10 and IL-19 production was investigated. The results from these experiments demonstrate that exposure to chronic stress augmented LPS-induced IL-10 serum concentrations and IL-10 and IL-19 mRNA expression in mouse spleen. These results are consistent with the findings following exposure to acute restraint stress, and correlate with other studies where exposure to chronic stress increased IL-10 production following LPS (Elenkov *et al.*, 1996) and influenza viral infection (Dobbs *et al.*, 1996). In addition, increased IL-10 production has been demonstrated following GC and catecholamine administration (Salicru *et al.*, 2007). However, to the best of my knowledge, this is the first paper to report that chronic stress stimulated LPS-induced augmentation of IL-19. IL-19 has been demonstrated to mimic some of the effects of IL-10 *in vitro* and IL-19 can impair the production of T_H1 cytokines, such as IFN- γ , and increase the production of T_H2 cytokines, such as IL-10, IL-4 and IL-13 (Gallagher *et al.*, 2004; Liao *et al.*, 2004). This IL-19 influenced shift in T_H1 / T_H2 immune responses could have implications in the pathogenesis of diseases, such as asthma (Liao *et al.*, 2004) and psoriasis (Wei *et al.*, 2005). Specifically, IL-19 levels in asthmatic patients were twice those of healthy controls and were positively correlated with levels of IL-4 and IL-13 which are two major T_H2 cytokines (Liao *et al.*, 2004). In addition, IL-19 levels in the skin of patients with psoriasis are increased and the increased production is thought to contribute to the inflammatory process by upregulating keratinocyte growth factor from CD8⁺ T-cells (Ghoreschi *et al.*, 2007). Therefore, IL-19 is likely to play a role in the pathogenesis of these diseases and a stress-induced increase in its production could amplify the course of the disease.

The stress-induced increase in IL-10 could have implications for health as elevated IL-10 levels would further inhibit T_H1 differentiation via inhibition of pro-inflammatory

cytokine production and enhance T_H2 mediated immune responses (Mosmann and Sad, 1996). This would inhibit cell mediated immunity, as described previously, and leave a chronically stressed host vulnerable to infection. For example, it has been demonstrated that chronically stressed mice are unable to mount an effective anti-bacterial response due to multiple immune defects including apoptotic loss of T-cells, impaired T-cell proliferation, failure to produce sufficient amounts of IFN- γ , IL-6 or TNF, diminished phagocytic capacity, reduced oxidative burst response and increased anti-inflammatory cytokine production (Kiank *et al.*, 2006). This altered immune profile increases the risk of septic complications in chronically stressed mice following *Escherichia coli* infection (Kiank *et al.*, 2006). Moreover, this mimics the clinical situation where postoperative peritonitis patients have increased risk of septic complications which is characterized by increased IL-10 and reduced TNF production (Docke *et al.*, 1997; Hotchkiss and Karl, 2003). In addition, it has been demonstrated that exposure to chronic restraint stress increased periodontal disease in rats via increased IL-6 and IL-10 and impaired IFN- γ production (Peruzzo *et al.*, 2008).

6.12 Exposure to chronic stress increased progression of a tumour in mice

There is growing evidence that, in addition to the role that chronic stress plays in the pathogenesis of infectious diseases, it may also be associated with cancer pathogenesis and progression (Thaker *et al.*, 2006; Anderson *et al.*, 1994). Data from animal models suggest that stress can modulate the growth of certain tumours via neuroendocrine regulation of the immune response to tumour cells (Antoni *et al.*, 2006). In this thesis, the effect of chronic restraint stress on the progression of a sub-cutaneous tumour in mice was investigated. The results show that chronically stressed mice had increased tumour volumes from day 20 until day 24 when the experiment was terminated. This suggests that exposure to chronic stress increased the progression of the tumour, but not the onset of tumour proliferation. This finding is in agreement with reports from other animal studies where it was demonstrated that exposure of mice to restraint stress increased the rate of tumour growth following administration of a carcinogen, diethylnitrosamine (Laconi *et al.*, 2000). In addition, other experimental stressors, such as swim stress, social confrontation, surgical stress and hypothermia have been demonstrated to increase tumour metastasis and impair survival following cancer onset (Ben-Eliyahu *et al.*, 1991; Ben-Eliyahu *et al.*, 1999; Page *et al.*, 1999; Stefanski *et al.*, 1996). Data from patients with existing tumours also indicates that exposure to stress

can increase cancer progression. Cancer diagnosis and treatment can cause substantial distress and patients who tended toward depressive coping mechanisms and self reported inescapable stress had increased risk for accelerated disease progression (Reiche *et al.*, 2004). By contrast, positive factors such as social support and optimism predict longer survival (Allison *et al.*, 2003).

Exposure to chronic stress has been demonstrated to induce alterations in cellular immunity during cancer, including decreased T-cell response to mitogen stimulation, decreased NK cell cytotoxicity and altered production of cytokines (Herbert and Cohen, 1993). However, the mechanisms through which stress may promote malignant progression are poorly understood. It has been demonstrated that cytokines play a pivotal role in the development of tumour by regulating the expression of T_H1 and T_H2 cells (Loskog *et al.*, 2004; Reome *et al.*, 2004). Polarization of an immune response from a T_H1 to a T_H2 phenotype contributes to decreased cellular immunity against cancerous cells and impairs immunosurveillance of tumours (Gor *et al.*, 2003; Liew *et al.*, 2002). In the tumour microenvironment, there is increased production of IL-10, IL-4, IL-5 and IL-13 and decreased production of IFN- γ , TNF- β and IL-2 (Reome *et al.*, 2004; Lathers *et al.*, 2003; Ren *et al.*, 2001). In addition, cancer patients have been demonstrated to have a higher frequency of cytokine producing T_H2 cells and a lower frequency of cytokine producing T_H1 cells (Ito *et al.*, 1999; Sheu *et al.*, 2001). This finding is paralleled with cytokine production measured in the serum and in the local tumour regions of patients where it was demonstrated that there were increased IL-10, IL-4 and IL-13 production and reduced IFN- γ , TNF- β and IL-12 (Shibata *et al.*, 2002; Filella *et al.*, 2000). These findings suggest that there is an immunosuppressive state present in the tumour microenvironment, with impaired CD4⁺ T-cell mediated immune responses. As discussed previously, exposure to chronic stress also impairs CD4⁺ T-cell mediated immune responses. Therefore, stress could amplify the immunosuppressed tumour microenvironment and impair anti-tumour cell mediated responses. In addition, the stress-induced alteration in CD4⁺ T-cell mediated responses can influence other components of anti-tumour immunity. For instance, CD4⁺ T-cells control the effector function, memory and maintenance of CD8⁺ T-cells (Antony *et al.*, 2005). Effective CD8⁺ T-cell responses are vital for anti-tumour immunity. However, it has been demonstrated that chronic stress reduces CD8⁺ cytotoxic responses (Nunez *et al.*, 2006).

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In the present study, the effect of exposure to chronic stress was examined on the frequency of cytokine secreting CD4⁺ and CD8⁺ effector T-cells in the tumour mass and in the tumour draining lymph node. The results from these experiments suggested that exposure to chronic stress is associated with reduced cellular mediated immunity, with impaired infiltrating CD4⁺ and CD8⁺ T-cells in tumour mass and lymph nodes. Specifically, chronic stress significantly decreased the frequency of IFN- γ producing CD8⁺ T-cells in the tumour mass and in the draining lymph node. This was accompanied with an increased frequency of IL-10 producing CD8⁺ and CD4⁺ T-cells in the lymph node. The reduction of IFN- γ -secreting CD4⁺ and CD8⁺ T-cells has also been demonstrated in the plasma of socially stressed rats (Stenanski and Engler, 1999). However, these findings are in contrast to studies where it was demonstrated that exposure to chronic stress resulted in a reduction of CD4⁺, but not CD8⁺ T-cells (Saul *et al.*, 2005; Silberman *et al.*, 2002). The differences observed may be explained due to the different experimental stressors utilised. Chronic mild stress was used in the two reports where CD4⁺ T-cell reduction was shown without alteration in CD8⁺ T-cells. However, in the findings presented in this thesis, a moderately stressful restraint stress protocol was applied which is suggested to have more severe immune alternating effects.

IFN- γ has many protective roles in antitumour immunity, exerting anti-proliferative, anti-angiogenic, and pro-apoptotic effects on a wide variety of tumour cells (Beatty and Paterson, 2001; Ikeda *et al.*, 2002). In this study, a low frequency of IFN- γ secreting CD4⁺ and CD8⁺ cells infiltrated the tumour mass and were recruited to the draining lymph nodes in stressed and non-stressed mice. In addition to this report, it has previously been demonstrated that in CT26-induced tumours, T_H1 responses were suppressed to an unrelated antigen (Jarnicki *et al.*, 2006). Taking these findings together, it indicates that the growing CT26 tumour induced an immunosuppressive environment that suppressed T-cell responses to both tumour cells, and other antigens. Therefore, the stress-induced reduction of IFN- γ ⁺ CD8⁺ T-cells in the tumour and the lymph node would amplify this tumour-induced immunosuppressive state. The amplified immunosuppressive state in the tumour mass following exposure to stress could be explained by increased incidence of IL-10 secreting T-cells. During tumour progression, IL-10 production from T-cells and NK cells is significantly increased (Wei *et al.*, 2005). In agreement with this, the findings in this thesis show that there was a high frequency of IL-10 secreting CD4⁺ and CD8⁺ T-cells in tumour mass and lymph

nodes from non-stressed mice. Exposure to chronic stress amplified the frequency of IL-10 secreting CD4⁺ and CD8⁺ T-cells, and this could account for the impaired frequency of IFN- γ secreting T-cells. In agreement with this theory, it has been reported that the production of IL-10 from CD8⁺ and CD4⁺ T-cells can contribute to suppression of anti-tumour immunity (Jarnicki *et al.*, 2006). Therefore, the stress-induced up regulation of IL-10 producing CD8⁺ and CD4⁺ T-cells reported in this thesis could have significant implications for the progression of a tumour.

6.13 There is an altered immune profile 24 hr following exposure to chronic stress

As previously discussed, stress has long been associated with an altered homeostatic state of an individual, including behavioral, endocrine and immunological changes. However, the impact of stressor exposure on the development of an immune response depends on a variety of factors, such as the duration of stressor (Dhabhar and McEwen, 1999), the animal model used (Lu *et al.*, 1998) and the type of immunological challenge (McEwen, 1998). Thus, several studies have investigated factors that contribute to stress-induced alterations in cellular and humoral immunity in laboratory models. It has been suggested that the temporal proximity to immune challenge following stressor termination is important for the type of immune response. For example, it has been demonstrated that a pro-inflammatory state becomes evident within 24 hr of stressor termination (Zalcman and Anisman, 1993; Zalcman *et al.*, 1989; Zalcman *et al.*, 1988). In addition, studies have reported a pro-inflammatory state following exposure to social stress when the immune challenge was administered 18 hr following stressor termination (Avitsur *et al.*, 2004). Consistent with these findings, in this thesis it was demonstrated that 24 hr following termination of chronic restraint stress, there was evidence of a pro-inflammatory response to stress in mouse spleen. Specifically, LPS-induced IL-12 and IFN- γ concentrations were increased from splenocytes harvested 24 hr following termination of chronic stress. However, in agreement with the previous results, LPS-induced IL-12 and IFN- γ concentrations are reduced from splenocytes harvested immediately following termination of chronic stress. In addition, the stress-induced augmentation of LPS-induced IL-10 production observed in splenocytes harvested immediately following stressor termination is not evident in splenocytes harvested 24 hr following termination of stress. Therefore, it appears that some cellular alteration is occurring during the recovery period following stress, which can alter the immune response to an infectious stimulus such as LPS.

One explanation for this altered immune profile is the 'receptor desensitisation hypothesis'. This hypothesis explains the altered immune state as the cellular adaptation that occurs during exposure to chronic stress and during the subsequent recovery from stress. Exposure to chronic stress which chronically activates the HPA axis, has been shown to result in substantial increases in systematic GC levels by both enhancing the release of GC hormones and by decreasing the plasma binding capacity of corticosterone binding globulin (Stefanski, 2000; Spencer *et al.*, 1996). This has been demonstrated to lead to adaptive changes in immune cells with reduced nuclear translocation of the GR and impaired immunosuppressive action of the GR in monocytes. This stress-induced reduction of GR expression and function is an adaptive response to chronic stress to alleviate immunosuppressive effects. When exposure to stress is terminated, the concentrations of GCs return to pre-stress, basal levels. However, the cellular adaptations may persist for some time and during this interim there may be an immunoenhanced state as a result of the GR receptor desensitisation to normal physiological levels of GCs. Similarly, chronic activation of the SAM axis results in substantial increases in catecholamine concentrations, and this can lead to adaptive changes in the expression of β_2 -AR. For example, it has been demonstrated that continuous exposure to catecholamines leads to a reduced receptor response by a process of receptor phosphorylation, internalization and down-regulation (Bawa-Khalfe *et al.*, 2007).

In agreement with this theory, in this thesis it has been demonstrated that 24 hr following exposure to chronic stress, there is down-regulation of GR and β_2 -AR protein expression in mouse spleen. This finding is consistent with reports from Quan *et al.* (2001) where they demonstrated that GR mRNA expression is down-regulated in mouse spleen 18 hr following exposure to chronic social stress (Quan *et al.*, 2001). They also reported that GR mRNA expression was reduced in hippocampus which is one of the most important neural structures for the negative feedback action of GCs (De Kloet *et al.*, 1998). Chronic stress-induced down-regulation of GR protein and mRNA in mouse hippocampus has also been demonstrated by other groups and therefore it is believed to be involved in the negative feedback response to exogenous GCs (Sapolsky *et al.*, 1984, 1986; Herman *et al.*, 1995; Kitraki *et al.*, 1999). It is established that GC induces down-regulation of GR and it has been demonstrated that

patients treated with GCs in the long term have reduced hormone responsiveness (Bellingham *et al.*, 1992; Silva *et al.*, 1994). Studies have shown that GC treatment decreases the GR mRNA expression in different tissues and leads to a reduction of GR protein concentrations via the ubiquitin-proteasome-dependent pathway (Dong *et al.*, 1998; Vedeckis *et al.*, 1989; Burnstein *et al.*, 1994). In addition to these findings on GR down-regulation, it has been demonstrated that 24 hr following termination of a forced exercise regime, which can be seen as a stressor for laboratory animals, there decreased β_2 -AR expression (Itoh *et al.*, 2004). Therefore, the stress-induced reduction of β_2 -AR could be involved in the negative feedback response to catecholamines.

In addition to the down-regulation of the receptors for stress hormones, other receptors could be altered in response to stress, and therefore could play a role in the 'receptor desensitisation' hypothesis. For example, it has been suggested that the LPS receptor TLR4 mediates restraint stress-induced immunosuppression and TLR4-deficient mice have been demonstrated to be resistant to stress-induced lymphocyte reduction of T_H1 and T_H2 cytokines compared with restraint stressed wild type mice (Zhang *et al.*, 2008). Moreover, the expression and release of the LPS receptor antigen CD14 are suppressed by GCs *in vivo* and *in vitro* (Nockher *et al.*, 1997). Therefore, it is possible that exposure to chronic stress can induce alterations in LPS cellular targets, and this may be involved in the immunoenhanced state reported in this thesis. However, the findings in this thesis demonstrate that CD14 mRNA expression is not altered in mouse spleen 24 hr following exposure to stress. CD14 receptor expression is essential for the presentation of an LPS molecule to TLR4. Therefore, any alteration in CD14 can impact on the LPS-induced immune activation. However, the findings in this thesis have demonstrated that LPS signalling was not altered following exposure to chronic stress. It was observed that immediately following exposure to chronic stress LPS-induced IL-12R expression is reduced in mouse spleen and this could play a role in the stress-induced immunosuppression of IL-12. Therefore, IL-12R could be another cellular target that could contribute to the altered immune state that is seen 24 hr following exposure to stress. In this regard, the findings in this thesis have demonstrated that IL-12R β 1 mRNA expression is increased in mouse spleen 24 hr following exposure to stress. The increase in IL-12R β 1 could contribute to the enhanced T_H1 immune response 24 hr following stress as IL-12 is a major T_H1 inducing cytokine (Itoh *et al.*, 2004).

One of the functional consequences of down-regulation of GR and β_2 -AR is that there is reduced sensitivity to the inhibitory actions of GCs and catecholamines. In the present thesis, it has been demonstrated that the suppressive effect of corticosterone and the β_2 -AR agonist, salbutamol on LPS-induced IFN- γ and IL-12 concentrations were impaired from splenocytes cultured 24 hr following exposure to stress. However, there was no alteration in LPS-induced IL-10 concentrations from splenocytes. In agreement with these findings, it has been established that GC resistance develops in the spleens of mice exposed to chronic stress (Avitsur, 2003). Specifically, 18 hr following exposure to stress, LPS-stimulated splenocytes were less sensitive to the inhibitory effects of corticosterone on cell proliferation and viability (Avitsur *et al*, 2001). The findings from these studies show that there are functional consequences to the down-regulation of GR. In addition, a number of stress-related diseases, including autoimmune, infectious and inflammatory disorders as well as certain neuropsychiatric disorders, such as major depression have been associated with decreased responsiveness to GCs (Pace *et al.*, 2006). This is believed to be related in part to impaired function of GR. GC hypersecretion is a common and reliable finding in patients with major depression (Pariante and Miller, 2001). These patients are found to have reduced GC responsiveness (dexamethasone nonsuppression) approximately 60 % of the time (Maier and Watkins, 1998).

6.14 Concluding Remarks

Exposure to acute and chronic stress suppresses various aspects of immunity including alteration of cellular trafficking, reduced antibody production, suppression of T-cell proliferation, suppression of cellular immunity and impaired cytokine production (see review Padgett and Glaser, 2003). The findings from this thesis are consistent with these reports and it was demonstrated that exposure of mice to acute and chronic restraint stress suppressed the innate IFN- γ response, with impaired IFN- γ production, IFN- γ signaling, the induction of IFN- γ inducible genes and the production of IFN- γ inducers IL-12 and IL-15. In addition, it was demonstrated that exposure to acute and chronic stress increased production of the anti-inflammatory cytokine IL-10 and the IL-10 related cytokine IL-19. IL-10 has been suggested as the mediator of stress-induced inhibition of the innate IFN- γ response due to its known immunosuppressive action on pro-inflammatory cytokines (Connor *et al.*, 2006). However, in this thesis it was demonstrated that the stress-induced suppression of the innate IFN- γ response was not mediated via the stress-induced increase in IL-10. To further understand the mechanisms behind the stress-induced immunosuppression, the contribution of the mediators of the stress response were investigated i.e. the GCs and the catecholamines. These stress hormones are released following activation of the HPA and SAM axis and directly induce suppressive effects on immune cells via specific receptors found on immune cells (Chrousos *et al.*, 1995). The findings from this thesis suggest that GCs acting via the GR mediate stress-induced suppression of the innate IFN- γ response demonstrated by the fact that the GR antagonist mifepristone prevented the stress-induced inhibition of IFN- γ and IL-12. In addition, it was demonstrated that catecholamines, acting via the β_2 -AR mediated the stress-induced augmentation of IL-10 and IL-19 expression (see figure 6.1). These findings give a great insight into the mechanisms underlying the ability of stress to impact on the cytokine network and highlight potential targets for therapeutic intervention to prevent stress-induced immunosuppression.

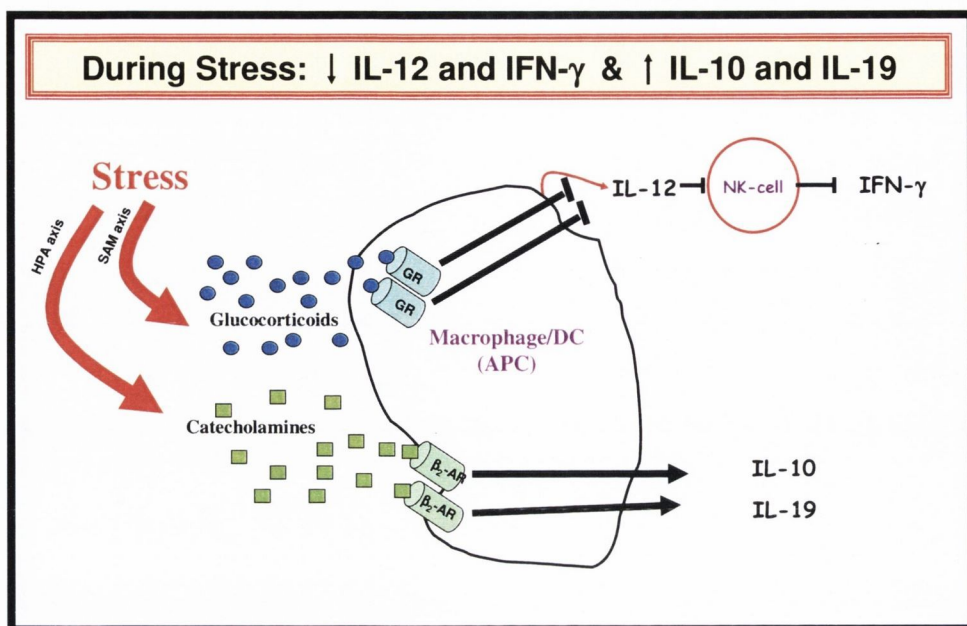


Figure 6.1 – During stress, the HPA and SAM axes are activated to release GCs and catecholamines which act on GR and β₂-AR respectively. GR activation reduces IL-12 and IFN-γ production and down-regulates its own expression. β₂-AR activation increases IL-10 production and down-regulates its own expression..

Exposure to chronic stress leads to a sustained immunosuppressive state that can leave an individual vulnerable to disease onset and exacerbation and the progression of cancer (Glaser and Kiecolt-Glaser, 2006). However, it is important to establish the extent to which stress-induced changes in the immune system have meaningful implications for disease susceptibility. Therefore, in the present thesis it was demonstrated that exposure to chronic stress increased the progression of a tumour in a mouse model, and also altered the tumour microenvironment by impairing the cellular immune responses. These findings could be of relevance to cancer sufferers and it is suggested that interventions that overcome the deleterious effects of stress on immunity could help prevent tumour progression. Such interventions could include psychological / behavioral-cognitive approaches or pharmacotherapies (such as benzodiazepines) to modulate stress responses. In this regard, in this thesis it was demonstrated that administration of chlordiazepoxide could prevent the suppression of IL-12 and increase in IL-10 by acute stress.

The main focus of this thesis has been on the immunosuppressive effects of chronic stress, however, some reports have indicated that a pro-inflammatory state becomes evident within 24 hr of chronic stress termination (Zalcman *et al.*, 1988). Consistent with this, in this thesis it was found that immunoenhancement is evident in the splenocytes harvested from mice 24 hr following termination of stress, with increased LPS-induced IL-12 and IFN- γ production. This altered immune state could be a result of altered receptor expression. For instance, in this thesis, it was demonstrated that 24 hr following termination of chronic stress there is down-regulation of GR and β_2 -AR expression in mouse spleen. According to the ‘receptor desensitization’ hypothesis, the down-regulation of receptor expression could enhance the immune response because there is reduced sensitivity of immune cells to the suppressive effects of normal physiological concentrations of GCs and catecholamines. Therefore, this temporary altered immune profile could produce a different outcome to an immune challenge (see figure 6.2).

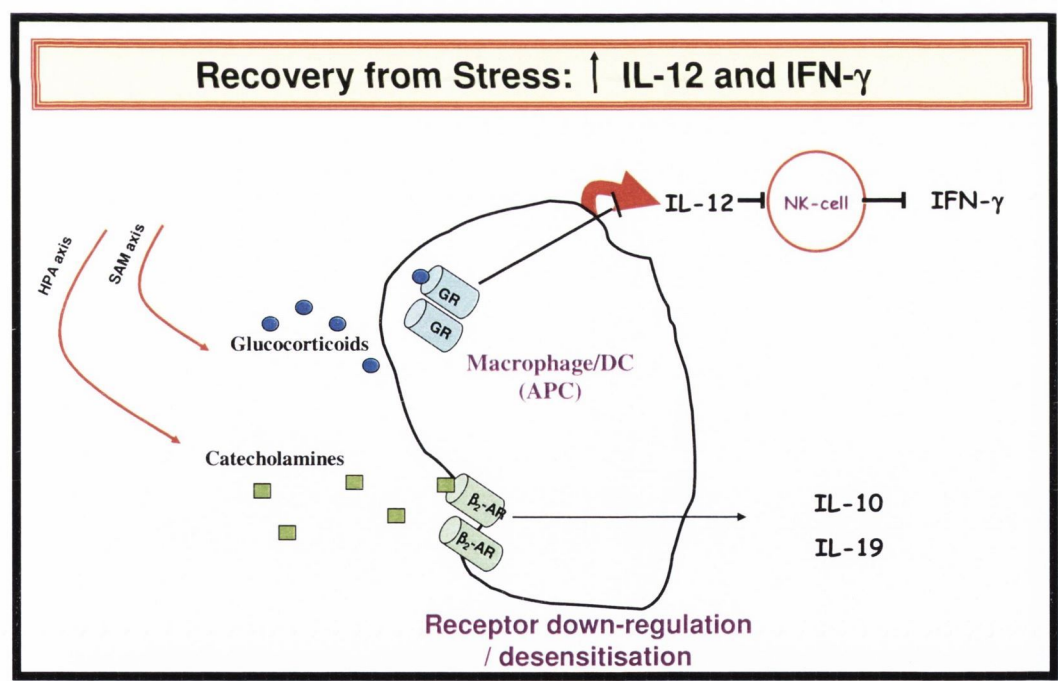


Figure 6.2 – During recovery from stress, GC and catecholamine concentrations return to basal levels. However, GR and β_2 -AR remain down-regulated for a period following stress. Therefore, IL-12 and IFN- γ production is increased and IL-10 production is decreased.

Chapter 7: Future Directions

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

1. The findings from this thesis suggest that stress-induced immunosuppression is mediated by a combination of the GCs and catecholamines. Therefore, in future research, it would be interesting to co-administer the GR antagonist and the β_2 -AR antagonist to see if combined blockade of the stress hormone receptors could alleviate the stress-induced effects. Alternatively, a combined adrenalectomy and splenic nerve cut would establish the roles played by the HPA and SAM axis and their respective hormones – corticosterone and NA.
2. The results from this thesis suggest that chlordiazepoxide can prevent the suppression of IL-12 and increase in IL-10 observed following exposure to stress. However, future research should be conducted to establish if the stress-induced suppression of the innate IFN- γ production and signalling is also prevented by chlordiazepoxide treatment. In addition, it should be determined if administration of chlordiazepoxide prevents the activation of the HPA and SAM axes and the production of the stress hormones.
3. Future research should determine if exposure to chronic stress could alter leukocyte distribution in mouse spleen. For instance, it is possible that an influx of IFN- γ producing NK cells into the spleen could account for the increase in IFN- γ production from mouse splenocytes stimulated with LPS 24hr following chronic stress.
4. The biological significance of increased tumor progression following exposure to chronic stress should be assessed by evaluating the survival rates of mice in a terminal mouse model of cancer following exposure to chronic stress. In addition, the therapeutic relevance of this finding should be assessed by developing a pharmacotherapy (e.g. benzodiazepines) to overcome the deleterious effects of stress on the immune system.
5. The pro-inflammatory effects demonstrated 24 hr following termination of chronic stress should be further investigated to establish the time course of how long the altered immune state persists for. In parallel, it is necessary to determine how long the down-regulation of GR and β_2 -AR expression persists for following termination of the chronic stress regimen.

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IX Presentations and publications

N.M. Curtin, K.H.G. Mills, T.J. Connor (2008) Chronic stress reduces glucocorticoid and β_2 -adrenergic receptor expression, and induces an inflammatory state characterised by increased IL-12 and IFN- γ production. *Psychoneuroimmunology Research Society abstract*

Oral presentation at the annual PNIRS international meeting, Madison, WI, USA

N.M. Curtin, K.H.G. Mills, T.J. Connor (2008) Differential effects of acute and chronic stress on immune function and the timing of administration of the immune challenge. *Journal of Neuroimmunology* 197;2 159-176

Poster presentation at the International Neuroscience Symposium, Dublin, Ireland

N.M. Curtin, K.H.G. Mills, T.J. Connor (2007) Interleukin-10 and Interleukin-19: Novel mediators of stress-induced immunosuppression. *The Psychoneuroimmunology Research Society abstracts*

Poster presentation at the annual PNIRS international meeting, Arcachon, France

N.M. Curtin, K.H.G. Mills, T.J. Connor (2007) Psychological stress suppresses the innate IFN- γ response: A role for the anti-inflammatory cytokine IL-10? *Royal Academy of Medicine in Ireland abstracts*

Awarded 1st prize for oral presentation at the RAMI Winter meeting, Dublin, Ireland

N.M. Curtin, K.H.G. Mills, T.J. Connor (2007) Noradrenaline and the β_2 -adrenoceptor agonist salbutamol suppress innate IFN- γ production by mouse spleen cells. *Royal Academy of Medicine in Ireland abstracts*

Poster presentation at the RAMI winter meeting, Dublin, Ireland

N.M. Curtin, K.H.G. Mills, T.J. Connor (2007) The effect of stress on the immune system. *Science speak public competition*

Runner up for oral presentations in Institute of Neuroscience, Trinity college, Dublin, Ireland

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N.M. Curtin, K.H.G. Mills, T.J. Connor (2006) Psychological stress increase production of IL-10 and IL-19: A role for the sympathetic neurotransmitter noradrenaline. *Irish Society for Immunology abstracts*

Poster presentation at the annual ISI meeting, Belfast, Ireland

N.B. 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 abstracts*

Poster presentation at the annual PNIRS international meeting, Miami, FL, USA

J.B. O'Sullivan, E.N. McNamee, N.M. Curtin, T.J. Connor (2005) Noradrenaline suppresses IL-1 β and TNF- α in primary glial cells: A role for cAMP signalling or anti-inflammatory cytokines? *Irish Society for Immunology abstracts*

Poster presentation at the annual ISI meeting, Dublin, Ireland