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The role of Interleukin 15 in three murine models of Inflammation

**Richard Walker B.Sc (Hons)
University Department of Medicine
Glasgow Royal Infirmary
Glasgow**

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Glasgow for the degree of M.Sc research
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Abstract

Research into the causes and roots of inflammation has provided a vast literature from which to draw. In spite of this data, major human diseases such as rheumatoid arthritis or the effects of sepsis following injury continue to cause suffering and sometimes death.

Research has led to several main contenders for the title of “protein at the top of the inflammatory cascade.” but again, reading the vast knowledge that others have uncovered, it is plain to see that there are redundancies, and a legion of control mechanisms operating to join all the major cytokines and cell types that constitute the mammalian inflammatory response. Amongst one of the recently pro-inflammatory proteins is Interleukin-15. (IL-15)

IL-15 was discovered in 1994 and has since been associated with up-regulation of inflammation and postulated as a controller of tumour necrosis factor alpha. The IL-15 protein is similar in structure to the IL-2 protein in several mammalian species. However in spite of its structural homology, there is little or no sequence homology with IL-2.

The experiments here are performed in the murine models of human diseases. The murine IL-15 shares approx 73% sequence homology with the human IL-15.

Thus this project set out to clone what at the time, was commercially unavailable, namely murine recombinant IL-15 and to raise antibodies against this protein. It was the intention to use these antibodies as therapeutic agents in three murine models of inflammation; the Collagen Induced model, the LPS shock model of sepsis and finally the septic arthritis model.

Recombinant murine IL-15 was cloned, purified and used to raise rabbit anti-muIL-15 antibodies. These anti sera were used in the CIA model. Furthermore, the cloning process uncovered a mutant form of murine IL-15 which had the native binding characteristics but had no ability to activate T cells. This protein was also used therapeutically in the CIA model as a putative therapeutic agonist to IL-15. Neither experiment produced clear indications that the anti -IL-15 anti bodies or the mutant agonist were successful in abrogating the symptoms of CIA however there was a trend for early and subtle amelioration of symptoms.

The difficulties with purification of recombinant protein and particularly solubility indicated a change to the original protocol. Instead of using the recombinant IL-15 & its antibody to continue the investigation in the remaining two models, the more readily purified soluble IL-15 Receptor alpha (IL-15R α) was used as the therapeutic agent.

In both the LPS shock model and the septic arthritis model, the therapeutic inoculation of the mice with IL-15R α , seemed to modify the mortality associated with high dose LPS shock in the LPS model; severity and incidence of sepsis in both models. There was also a trend for reduced incidence and severity of arthritis in the septic arthritis model.

Furthermore, IL-15R α therapy appeared to alter the distribution of *S. aureus* in the soft tissues of the septic arthritis mice. In this instance, *S. aureus* was found in the IL-15R α treated group tissues but not in the control group, suggesting IL-15 removal had some effect on the ability of the tissues examined to clear the bacteria. This finding was also either time dependant or related to the cessation of therapy as the differential

distribution generally disappeared following the cessation of therapy and then resembled the controls. IL-15R α administration also appeared to affect the ability of the spleen cells from mice in the septic arthritis mice also to react to the mitogens, SEA and CON-A, with the treatment mice showing less activity than the control mice during the treatment period.

In the many determinations of the effects of IL-15 throughout this study, there have not been any constantly obvious and statistically significant differences, however, an overall conclusion can be drawn that there is a persistent trend for the amelioration of sepsis and arthritis in the CIA, LPS and Septic arthritis models when IL-15 depleting agents such as anti-IL-15 antibodies, IL-15 antagonist and soluble receptor are administered therapeutically. This study therefore, may provide the basis for better defined experiments to be carried out, perhaps with better power and sampling and reagents, strengthening this trend into a fact.

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List of Abbreviations

5-Bromo-4-Chloro-3-indolyl- β -galactopyranoside.....	X-GAL
Adenosine triphosphate.....	ATP
Adenosine.....	A
Alanine-amino transferase.....	ALT
Alkaline phosphotase buffer.....	CIP
Antigen presenting cell.....	APC
Aquired immune deficiency syndrome.....	AIDs
Aspartate-amino transferase.....	AST
Base pairs (bp).....	bp
Bicinchoninic Acid assay.....	BCA
Bovine serum albumin.....	BSA
Carbon dioxide.....	CO ₂
Collagen induced arthritis.....	CIA.
Colony forming units.....	CFUs
Complementary DNA.....	cDNA
Concanavalin-A.....	CON-A
Copper sulphate.....	CuSO ₄
Creatine Kinase.....	CK
Cytosine.....	C
Cytotoxic T lymphocyte line.....	CTL
Dalton.....	Da
Deoxynucleictriphosphate.....	dNTP
Deoxyribonucleic acid.....	DNA
Diethylpyrocarbonate.....	DEPC
Dithiothreitol.....	DTT
Ethylenediaminetetracetic acid.....	EDTA
Effective dose.....	ED
Enzyme linked immunosorbent assay.....	ELISA

Escherichia coli.....	E. coli
Fetal calf serum.....	FCS
Granulocyte/monocyte colony stimulating factor.....	GMC-SF
Guanosine.....	G
High salt-Phosphate buffered saline.....	HS-PBS
Horse radish peroxidase.....	HRP
Human lymphotropic virus HTLV-1	HTLV-1
Human serum albumin.....	HSA
Immunoglobulin.....	Ig
Inducible nitric oxide synthase.....	iNOS
Interferon gamma.....	IFN γ
Interleukin.....	IL
Interleukin-15 receptor alpha.....	IL-15R α
Interleukin-2 receptor alpha.....	IL-2R α
Intracellular adhesion molecule 1.....	ICAM-1
Isopropyl-B-D-thiogalactopyranosamide.....	IPTG
Janus kinase 3.....	JAK-3
Leucocyte Antigen-1	LFA-1
Lipopolysaccharide.....	LPS
Litre.....	L
Long terminal repeat	LTR
LPS Binding Protein.....	LBP
Lymphotoxin.....	LT
Magnesium chloride.....	MgCl $_2$
Magnesium sulphate.....	MgSO $_4$
Major Histo-compatibility molecule.....	MHC
Messenger ribonucleic acid.....	mRNA
Microlitre.....	μ l
Micromolar.....	μ M
Milliliter.....	ml
Millimolar.....	mM

Murine.....	mu
Nanometers.....	nM
Nitric oxide.....	NO
Optical density.....	OD
Phosphate buffered saline.....	PBS
Pico gram	pg
Potassium Chloride.....	KCl
Potassium.....	K
Reverse transcriptase polymerase chain reaction.....	RT-PCR
Sodium carbonate.....	NaCO ₃
Sodium Chloride.....	NaCl
Sodium dodecyl sulphate polyacrylamide gel electrophoresis	SDS-PAGE
Sodium hydroxide.....	NaOH
Staphylococcal enterotoxin-A.....	SEA
Staphylococcus aureus.....	S. aureus
T helper cell 2.....	Th2
T-cell receptor.....	TCR
T-helper cell 1.....	Th1
Thymidine.....	T
TOLL like Receptor.....	TLR
Toxic shock syndrome toxin.....	TSST
Transforming Growth Factor beta	TGF- β
Transgenic.....	TG
Tumour necrosis factor alpha.....	TNF α
Untranslated region	UTR
Uracil.....	U

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Chapter 1

The role of IL-15 in murine models of inflammation.

1.1

Inflammation

The Latin words, *rubor*, *calor*, *tumor* and *dolor* describe the results of the biological process of inflammation. Redness, heat, swelling and pain are the physical symptoms accompanying the stimulation of immune system components by either foreign bodies such as microbes, or aberrant reactions to normally innocuous antigens derived from self or the environment. The processes involved in the generation of the inflammatory response can loosely be divided into three areas (1):

- ◆ Recognition of the antigen followed by activation of components of the immune system. (Can be non-specific or Antigen specific).
- ◆ Amplification of the immune response through development of immune-effector cells and immune-active molecules and their recruitment to the site(s) of inflammation.
- ◆ Outcome of the response. E.g. Pathology, fatality or eradication/control of the pathogen etc.

The inflammatory response is a normal and necessary reaction enabling defence of the body against pathogens and foreign bodies. The recognition of antigen, initiation of the inflammation response and the removal of the pathogen are all processes mediated by specific cells and soluble factors of the immune system. Before discussing inflammation in terms of disease situations, it is important to present an overview of the mechanism of the

inflammatory response in the context of a healthy immune system. For instance: A macrophage in tissue near the site of a skin wound encounters an invading bacterium. The bacterium is ingested by the macrophage by phagocytosis. The macrophage may then enter the lymph capillary system that drains the tissue of interstitial fluid and cellular debris; Alternatively, a skin wound occurs and becomes infected by bacteria, for example *S. aureus*. As the infection proceeds, some of the bacteria migrate into the interstitial spaces of the lower dermis. Fragments of dying bacteria enter the draining lymph capillaries and travel with the lymph to a draining lymph node, usually found locally with respect to the injury site. Within the lymph node, the bacterial antigen might be engulfed by one of the resident macrophages.

Either of the macrophages described above would enzymatically digest the protein components of the bacterium and display some of the products on their surface membranes, in conjunction with specialised Major Histo-Compatibility class II molecule (MHC-II). This macrophage is now functioning as an Antigen Presenting Cell (APC).

The lymph node is a site of interaction between antigen presenting cells and T-cells, where these and other cells of the immune system can monitor the presence of antigens entering the lymph. These antigens may be processed by antigen presenting cells prior to arriving in the lymph node or may arrive independently with the lymph. If no antigen is detected by the T cells, antigen presenting cells return to the circulation or tissues. However if antigen such as those derived from the ingested bacterium are detected, the following set of events may occur, recognising the antigen and initiating an immune response.

Naive CD4+ve T cells regularly exit the blood circulation and migrate through the lymphoid organs and nodes. However, a CD4+veT cell specifically able to recognise a bacterial peptide may encounter the APC displaying its specific antigen and a T cell driven inflammatory response may be initiated. (Generally, the T-cells cannot recognise antigen unless it is displayed by an APC e.g. macrophage.) (1)

The T cell Receptor (TCR) is specific for its antigen. The TCR binds the antigen/MHC complex on the APC. Adhesion molecules such as LFA/1 and ICAM/1 (2) (3) followed by “Co-stimulatory” molecules e.g. CD28 and B7 (T cell and APC respectively) draw the two cells together and allow cell-cell “communication”. This set of signals starts the antigen recognition process, which in turn activates the formerly quiescent naive T cell.

The activated T cell now begins to express the mitogenic cytokine IL-2 and IL-2 receptor alpha protein. The IL-2 α receptor protein migrates within the plane of the membrane to join with the beta and gamma IL-2 receptor subunit to form a high affinity IL-2 receptor. The newly synthesised IL-2 acts via this receptor allowing clonal expansion of the T cell. At this stage, the naive T cell moves into a proliferative state. The new clone of T cells formed by this proliferation is also reactive to the bacterial antigens presented by the APC, but no longer requires co-stimulatory signals such as the CD28/B7 interaction.

The activated or “primed” T-cell clones re-enter the circulation and can develop in several ways. Activated CD4 cells may differentiate as T helper (Th) cells of which there are generally held to be two types, Th1 and Th2 (4). The direction of differentiation depends upon the cytokine milieu existing in the tissues in which the T cell clones find themselves. (5)

Some pathogens induce macrophages and natural-killer cells to produce IFN γ and IL-12 and this causes T cells to mature into Th1 type cells. Th1 cells have pro-inflammatory activities, inducing more IFN γ and IL-12, resulting in a pro-inflammatory cycle. These cytokines activate macrophages and more T cells are activated by the macrophages' increased antigen presentation and enhanced non-specific phagocytic events. Furthermore, this activation of macrophages by the Th1 cells enables them to produce more TNF α and TNF receptor. TNF α acts in an autocrine fashion in synergy with IFN γ from the T cells to induce production/release of antimicrobial molecules such as Nitric Oxide (NO) and Oxygen radicals such as O \cdot .

In the presence of cytokines such as IL-4, Th2 type cells may develop from the new clone. These cells, although having some pro-inflammatory activity, tend to eventually express cytokines such as TGF- β and IL-10, which can damp down the inflammatory response.

B cells often require T cell help to generate antibodies against their specific antigens (6) (T cell independent activation is possible by antigen cross-linking of receptors) (7). Antigen is identified by its specificity to the B cell surface Immunoglobulin. The immunoglobulin and the antigen are internalised and antigenic peptides are displayed on MHC class one molecules. An activated Th2 cell, reactive to the peptides displayed on the B cell MHC, can bind the MHC/antigen via its TCR. CD40/CD40-ligand costimulatory contact may then occur between these cells and, when supported by Th2 cell derived cytokines, the B cell may develop into a plasma cell producing antibodies against the antigen.

A further subset of the activated T cell clones may become memory cells, an expanded population of cells within the body, able to recognise the antigen that initially invoked the clonal expansion. These cells are identified by protein markers on their cell surfaces (for example CD45RO) which are different from those of the naive T cell.

Thus, the immune response can be seen to have an antigen recognition stage and this is followed by an initiation of inflammation by cellular action (macrophage activation and soluble factors such as pro-inflammatory cytokines) (1).

The last stages of the healthy immune response include the production of antibody. This allows the mopping up of antigen by non-specific mechanisms, such as complement and increased phagocytosis. This is often accompanied by the production of inhibitory cytokines such as TGF- β and IL-10 that act against the pro-inflammatory cytokines leading to reduction of proliferation events and inflammatory cytokine production. The residual reactive memory

T cell clones can also be considered as an outcome of the healthy immune response, ensuring a rapid response against any further encounter with the original antigen. (1)

1.1a Cytokines defined and their diversity of action.

Cytokines are often defined as chemical messengers. They are diverse and numerous and not limited to production by cells of the immune system alone. Janeway and Travers (1) describe cytokines as “small soluble proteins produced by one cell that alter the behaviour or properties of another cell”

Cytokines is a term referring to all these chemical messengers however, those termed interleukins are those derived from T cells. E.g. IL-15. However, we know that IL-15 is found in many tissue types so it is probably best to stick to the general term of cytokine and accept the name interleukin for convenience! The table (i) below (adapted from (1)) highlights some of the diversity of cytokines and their origin and targets.

Cytokine	Produced by	Effects on			
		B Cells	T Cells	macrophages	Haemopoetic cells
Interleukin-4 (IL-4)	Th2	Activation, growth	Growth & survival	Inhibits activation	Growth of mast cells
IL-5	Th2	Differentiation IgA production	-	-	Eosinophil differentiation and growth
IL-6	Th2	Growth and differentiation	Co- stimulator	-	Induces colony stimulating factor
IL-10	Th2	Upregulates MHC class 2	Inhibits Th1	- Inhibits cytokine release	Costimulates mast cell growth
IL-2	Th1	growth	growth	-	Stimulates NK cell growth
Interferon γ	Th1	Differentiation and Ig2a synthesis	Kills	activation	Activates NK cells
Tumour necrosis factor	Th1, Th2	-	-	Activates and induces NO production	-
Granulocyte macrophage colony stimulating factor	Th1 & Th2	differentiation	Inhibits growth	activation	Stimulates production of granulocytes and macrophages

Table (i) adapted from Janeway and Travers (1) showing a selection of common cytokines, their originating cell types and their effects on other cells in the immune system.

The effects of cytokines on the effector cells of the immune system are very important in disease. It can be seen that there is a target for therapeutic intervention through either supplementing or blocking of cytokines. Monoclonal antibodies against tumour necrosis factor such as that marketed as Remicade by Schering Plough, have increasingly wide indications in human treatment for immune disease. The licensed indications for Remicade (generic name infliximab) currently include Rheumatoid arthritis, Ankylosing spondylitis, Crohn's disease and just recently psoriasis.

1.2

The discovery of IL-15 and its characterisation

This thesis concerns the cytokine IL-15 and the role it may play in the immune response, explored through three murine models of inflammatory disease.

The discovery of IL-15 is documented in the literature in May 1994. Burton et al, (8) demonstrated that the conditioned culture supernatant from HuT-102 cells (an adult leukaemia T cell line) could induce the proliferation of the normally IL-2 dependant cell line CTLL. This factor was designated IL-T. In the same journal edition, Bamford reported (9) that the IL-2receptor β chain was not only used by IL-2, in stimulating the Kit 225 cell line but also by the novel IL-T. In the same month, May 1994, Kenneth Grabstein et al, (10) cloned a cytokine, purified from the supernatants of the simian kidney cell line CV-1/EBNA. These supernatants had demonstrated proliferative activity on the IL-2 dependent, murine cell line CTLL. Purification by molecular weight and charge characteristics revealed this activity resided in a protein with mobility of 14-15kDa on SDS PAGE. N-terminal amino acid sequencing led to cloning and publication of the IL-15 cDNA sequence in several mammalian species. The murine IL-15 shares 73% homology with the human in both nucleic and amino acid sequence. (11)

The IL-15 and IL-2 molecules share a number of biological functions. The gene coding for IL-15 exists as a single copy gene (11) & 12); because of the single gene status and the absence of homology with the IL-2 gene sequence implies that there is unlikely to be any evolutionary link to IL-2 via gene duplication and subsequent mutation from or to the IL-2 gene. However, IL-15 undoubtedly has an important relationship to IL-2, as not only is it encoded on the same chromosome, but also shares to a remarkable degree the same biological

functions. These include T cell stimulation (8), B cell proliferation and differentiation (13) and induction of natural killer cell proliferation. (14)

When the IL-15 secondary structure (inclusive amino acid sequence of IL-15) was processed by 3D-analysis software, (10) a “4-alpha helix bundle” structure was predicted, similar to the structure of IL-2. In addition to molecular shape, IL-15 and IL-2 share some important biological activities, as noted earlier. These similarities are likely, in part, to be a function of the redundancy of the IL-2 and IL-15 receptor subunits. Within the trimeric IL-2 receptor, only the IL-2 receptor alpha (IL-2R α or CD-25) subunit is exclusive to IL-2 activity. The IL-2 gamma-subunit (also known as Gamma C or Common) is a component of the receptors of IL-4 and 7 (15) and IL-15. In addition to the gamma subunit, the IL-15 receptor also utilises the IL-2 beta subunit (16)

The distinction of the IL-15 receptor from the IL-2 receptor lies in the IL-15 receptor alpha (IL-15R α) subunit. The IL-15R α receptor subunit protein is related in tertiary structure to the IL-2R α , but has little sequence homology. However IL-15/IL-15R α exhibits 1000 times the binding coefficient of IL-2/IL-2R α . The tissue distribution of IL-15R α by mRNA analysis shows that, compared to the IL-2R α , the IL-15R α is ubiquitous, whereas the former is largely restricted to T cell containing tissues (17).

Although sometimes described as a macrophage-produced cytokine, the tissue distribution of IL-15 reflects that of its receptor alpha subunit. Grabstein (10) details IL-15 mRNA expression in diverse tissues, including kidney epithelia, placenta, heart, lung and liver cells. However, this ubiquity of IL-15 mRNA is not readily demonstrated by IL-15 protein. IL-2 is produced by T-cells, whereas IL-15 is not. This may be an example of an extremely tight biochemical control mechanism, perhaps functioning to prevent widespread induction of unwanted IL-15 action in major body organs and tissues. The IL-15 mRNA is present, but not readily translated to protein. A mechanism for this regulation has been

suggested by the work of Bamford et al (18) who discovered that the HTLV-1 virus transformed T-cell line HuT-102 produced IL-15 active protein at 51pg/ml (between 6-10 fold more protein than non-transformed cells) when assayed by Enzyme Linked Immunosorbent Assay (ELISA). However, further investigation revealed that between 214 and 216 base pairs (bp) of the 5' untranslated IL-15mRNA had been replaced with viral DNA derived from the Long Terminal Repeat (LTR) section of the HTLV viral gene sequence. Within the native IL-15 UTR lie 10 AUG motifs of which 8 had been deleted by the viral insertion. These AUG motifs were found to influence IL-15 production. This was shown when these elements were serially deleted and transfected as constructs into COS cells. Increases of up to 4.3 fold IL-15 production were demonstrated when the AUGs were deleted.

In addition to the AUG translation inhibition control mechanism, alternative splicing of the mRNA is also reported. An example of this is described by Meazza (19) who reported the inclusion of an additional exon of 119bp found in 9 of 11 small lung cell cancer cells. This exon conferred a new open reading frame to the elongated mRNA and translated into a shorter signal peptide sequence. Bio-active IL-15 was not reported in connection with this variant IL-15mRNA by either ELISA (sensitivity 50ng/ml) nor the highly sensitive IL-15 CTLL bioassay) leading to speculation that effect of the added exon may be a negative control mechanism

The literature mentions several other variant forms of IL-15mRNA, including three described by Nishimura (20) where an alternative exon 5 insertion leads to a slightly increased RNA/protein-translation efficiency. The discrepancy between observed levels of IL-15mRNA and detected protein and the variations in the efficiency of translation are indicative of complex transcriptional and translational and post-translational control mechanisms that are at present unclear. However, recently Musso et al (127) have shown that

differential forms of the leader sequence upstream of the coding region of the mRNA for IL-15 have can lead to expression of either a secreted form or a membrane-bound form of IL-15 in normal human monocytes. The membrane bound form could not be eluted by either Trypsin or acetate buffers, indicating that the IL-15 was an integral membrane protein. The membrane bound form of IL-15 was noted to be up regulated by IFN γ . Musso speculates that this location of the protein may prevent association with the IL-15R complex but furthermore that the membrane for was biologically active and was able to stimulate in-vitro proliferation of T lymphocytes. Differential production of membrane and cytosolic IL-15 isoforms was also reported by Kurowska M et al (128) in human foetal brain tissue.

These reports may indicate the reason for apparent ubiquitous distribution of mRNA for IL-15 yet often low detection by ELISA.

1.3

Rheumatoid arthritis and cytokines

The pro-inflammatory effects of IL-15 suggest it may have a role in inflammatory disease. This is exemplified by a series of experiments focusing on Rheumatoid Arthritis (rheumatoid arthritis) and models of this common disease. In order to examine any role of IL-15 in rheumatoid arthritis, it is necessary to describe the immunological events believed to lead to the establishment and progress of the disease. The diagram below Figure (i) shows a normal, joint on the left side and the right side shows the rheumatoid arthritis joint, including an enlarged section showing the site of immune cell infiltration and development of pannus as described in the text immediately below.

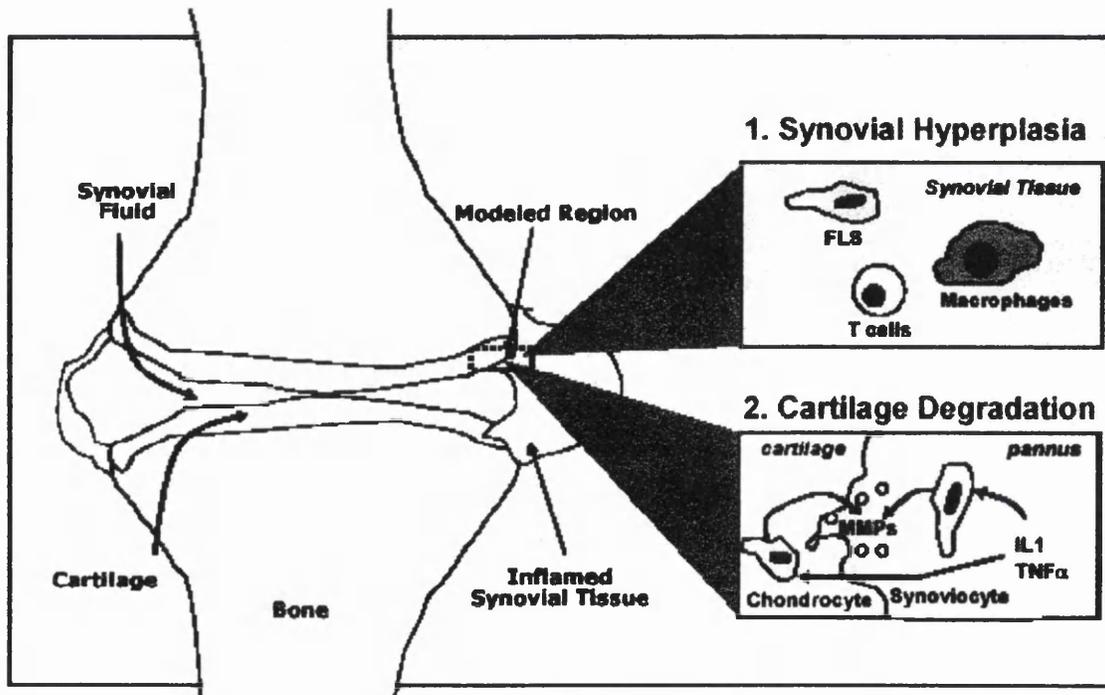


Figure 1: Diagram of a prototypical RA joint illustrating clinical outputs and major cell types modeled. The model extrapolates from activity in the area near the cartilage-synovial tissue junction, indicated by the dotted rectangle.

(figure i, adapted from Malaysian Arthritis foundation review, 7, May, 2005)

Rheumatoid arthritis is a disease where the initiating event is unclear. The major focus of the disease pathology is on the destruction of the joints (although there are a range of non-joint effects associated with rheumatoid arthritis). The destruction begins with the infiltration of the normally sparsely cellular synovium (some macrophages and fibroblasts) with activated cells of the immune system. These include CD4 and CD8 T-cells, dendritic cells and macrophages. In later stages of the joint inflammation, B-cells and mast cells are detected in the synovium. These activated cells produce destructive enzymes, such as collagenases, that destroy the joint cartilage and a cytokine milieu that promotes aggressive attacks on the cartilage and bone within the joint. The healthy synovial membrane is very thin, principally comprising of fibroblasts and fibrous tissue and a lining layer, often just one cell thick. In rheumatoid arthritis, the synovial membrane changes. The lining layer is often 10 cells thick and containing many macrophages. Many immune cells, including T cells, infiltrate and swell the stroma. During rheumatoid arthritis, this expansion of the synovium eventually impinges on the surface of the cartilage in the joint. This mass of cells develops a dense region, in juxtaposition to the cartilage known as "Pannus". Cartilage and joint destruction leads to impaired joint function (21)/(22).

The orchestration of the above events is conducted by cytokines, the messengers of the immune system which function through both auto and paracrine action. As with the immune system as a whole, they can have beneficial or derogatory effects. For example, Granulocyte /Macrophage-Colony Stimulating Factor (GM-CSF) can up-regulate MHC class II molecules in a pro-inflammatory action. However, it can also induce up-regulation of IL-1 receptor antagonist, leading to a down regulation of an IL-1 driven inflammatory response (21). Investigations into the cytokines found within the rheumatoid joint indicate a profile including IL-1 (23) GM-CSF, (24) IL-6 (25) IL-8 (26) and TNF- α (27)/ (28).

Chu et al (29) emphasise the importance of these cytokines by localising them to the site of joint destruction, i.e. the Cartilage/Pannus Junction (CPJ). The elucidation of cytokine types and their localisation to the joint stimulated discussion regarding the type of cells responsible for initiation and propagation of the inflammation in the joint. The relative absence of IL-2 and Interferon gamma (IFN γ), traditionally held as the primary T-cell activators, from the synovial cytokine profile has led to differing opinions regarding the main cellular players in the aetiology of rheumatoid arthritis. For instance, Firestein et al (30) argue for the ascendancy of the macrophage in the progression of the disease because of the absence of IL-2 and the presence of GM-CSF. Panayi et al (31) have proposed the T-cell as the prime initiator and propagator of the synovial inflammation citing direct T-cell Receptor (TCR) activation and Fibronectin binding events in the context of T-cell activation within the joint.

The discovery and characterisation of IL-15 has allowed a further arm of the discussion to be developed where a macrophage-produced cytokine similar to IL-2 could account for the recruitment, activation and propagation of an active population of T-cells within the rheumatoid joint. Several experiments in this laboratory have illuminated aspects of IL-15 action and presence in rheumatoid tissue samples and models of this disease.

The generation of synovitis would require recruitment of T-cells into the joint. IL-2, signalling through the IL-2 receptor along a chemical gradient, is a very potent chemo-attractant of T-cells (32). Wilkinson and Liew (33) demonstrated that IL-15 exhibited similar activity when T-cell locomotion and polarity were assessed on collagen gel matrices. Compared to MIP1 α and IL-8 (both T-cell chemo-attractants believed to be present in the synovium), IL-15 had greater weight for weight chemo-attractant activity.

McInnes et al (34) reported the presence of IL-15 in the joint tissues of rheumatoid arthritis patients when detected by ELISA. This cytokine was localised by immune-

histochemical staining to macrophages, i.e. cells counter-stained with CD68 and also amongst T-cell aggregates (counter-stained with CD3). It was proposed that this IL-15 was either macrophage generated in close proximity to the T-cells or that it was IL-15 receptor bound on the T-cell surface.

The *in-vitro* chemotactic evidence was reflected *in-vivo* when DBA/1 mice were sensitised with heat killed *C. parvum* prior to a 500ng, recombinant, simian-IL-15 injection, administered intradermally to the footpad. This led to a vigorous T-cell infiltration to the site of the IL-15 inoculation (34).

Later, McInnes et al (35) proposed a mechanism relating the presence of IL-15 within the joint to the macrophage production of TNF- α . In this hypothesis, T-cells became exposed to IL-15 and, following IL-15 exposure, cell-cell contact with macrophages induced TNF- α production. *In vitro* cell contact experiments using dual chambered slides indicated soluble factors were not likely to be responsible for this and that the cell contact was indeed necessary. Levels of up to 400pg/ml TNF- α production were induced in this manner in peripheral blood monocytes (PBMCs) when they were placed in contact with the same patients' synovial T-cells (35).

The cytokines involved in inflammation are believed to act in a cascade mechanism where the initiating events are controlled by one or two cytokines at the apex of the cascade. This is reviewed by Feldmann (36). Here, TNF- α was described as exerting dominant control over IL-1 and other pro-inflammatory cytokines. The work of McInnes et al (35) noted above, indicates that IL-15 may indeed be another dominant regulator in context of its ability to control the production of TNF- α via T-cell/macrophage cell/cell contact and induction, and therefore possibly involved in the initiation of inflammatory events relating to rheumatoid arthritis.

Animal models of inflammation

1.4 Collagen Induced Arthritis model

The collagen induced arthritis model (CIA) has been developed from experiments in the rat (37). The CIA model has frequently been used in mice. Use of DBA/1a genetic background mice for this model has become a standard technique. The DBA/1a type mice show no immunologically aberrant behaviour and, therefore are widely accepted as suitable tools for inflammation research. (The methodology is described in the materials and methods section below). As described by Sewell & Trentham (38), the CIA model demonstrates: sustained proliferative synovitis; pannus formation; and cartilage and bone destruction. These are all salient features of the human rheumatoid disease, although CIA is in most cases transient, resolving after a few weeks, compared to rheumatoid arthritis which tends to be chronic over very long periods of time.

This model has several features that imply that T-cells are involved in the pathogenesis of the attendant joint disease. In their review, Staines and Wooley (39) describe how the adoptive transfer of either T-cells or anti-collagen antibodies from a mouse with CIA to genetically similar mice with no disease will independently induce CIA in the recipient mouse. This implies that T-cells reactive against collagen are involved, possibly by direct recognition of collagen as antigen, (activated Th1 type reaction) or by Th2 cell induction of anti-collagen antibodies by collagen reactive B-cells.

Another line of evidence of T-cell involvement in pathogenesis of CIA is the dependence on the MHC for disease incidence within an experimental species. Staines and Wooley also note that the B10.R111 (H2r) strain of mouse is completely resistant to CIA induced by chicken collagen, but is reactive to deer, bovine and porcine collagen. The DBA/1

H2q mice will react to all tested collagens. This differential susceptibility is suggestive of collagen reactive T-cells' ability to recognise collagen antigenic epitopes being controlled by differential expression of MHC class II proteins i.e. the phenotypic expression of the genetic background of the mice H2q vs. H2r.

A third line of evidence suggesting a central role for T-cells is the ability to modify the CIA disease progression by anti-T cell antibodies such as anti-CD4 (40) and CD40-ligand which is the molecule synthesised by T-helper cells in order to induce B-cell proliferation (41).

Immune cells do not work in isolation but utilise soluble factors such as cytokines to influence their activities. The cytokines may form a more defined target for therapy possibilities and researchers have been attempting to reveal such a target as described in the discussion of the immunology of rheumatoid arthritis above. TNF- α has been a focus due to its position as a dominant regulator of the pro-inflammatory response. Williams et al (42) clearly demonstrated a role for TNF- α in the CIA when they administered neutralising anti-TNF- α monoclonal antibodies, before and after the induction of arthritis. The therapy was clinically effective in established arthritis two days after treatment commenced.

As noted above, McInnes (35) proposes a control mechanism whereby IL-15 exposed T. cells drive macrophages to produce TNF- α . Therapeutic anti-TNF therapy of the CIA model and the recent success of clinical trials using anti-TNF in human rheumatoid disease indicate the pre-eminence of TNF- α in the pathology of rheumatoid arthritis and its model CIA. Ruchatz et al (43) tested the hypothesis that IL-15 might control TNF- α production through modification of CIA. A soluble form of IL-15 receptor alpha subunit (rIL-15R α) was administered as a preventative agent that resulted in the reduction of arthritis development during the therapy period. Proliferative responses to the collagen type II antigen were also

reduced. This provided evidence that IL-15 may play a role in the collagen induced arthritis and by inference in rheumatoid arthritis.

1.5 Lipopolysaccharide shock model

In humans, septic shock (also known popularly as blood poisoning or LPS shock) is a major cause of death in hospitals after cardiogenic shock. Typically, septic shock is caused by infections such as peritonitis occurring as a result of instrumental abortion, intestinal wounds and ruptures or via the infection of the genital tract. Gangrene or urinary infections are another common cause. Patient symptoms include high fever, significant vaso-dilation throughout the whole body increased cardiac output (in response to the vaso-dilation), blood "sludging" and clotting. The clotting causes blockages in the small capillaries and venules of the major organs. The result of the vasodilation is blood pools in the veins. Perfusion of the essential organs fails, leading rapidly to death. Endotoxic shock is similar in pathogenesis to these and a frequent cause in humans is when a portion of the gut becomes strangulated and the coli-form bacteria of the gut multiply out of control. These mostly gram-negative bacteria, frequently *E. coli*, release endotoxin into the tissues and subsequently the blood, initiating the septic shock response described above. It is highly likely that cytokines are involved in the evolution of the septic shock. The dependence of the pathogenesis of endotoxic shock on these mediators is discussed below (44). The role of the lipopolysaccharide (LPS) itself is demonstrated by the fact that the symptoms of the endotoxic shock can be reproduced by LPS injection into mice. Furthermore, these symptoms can be abrogated by the prior administration of anti-LPS antibodies in primates as demonstrated by Tracey et al (45).

Septic shock is a self-propagating disease where the vasodilatory effects on the circulation and cytokine production lead to further shock i.e. more vasodilation and cytokine release, and rapidly lead to death. Standiford et al (46) studied the regulatory effect of IL-10. Neutralisation of this cytokine increased lethality in murine models of septic shock. This, along with investigations into TNF- α discussed below, point to the importance of the cytokines in the progression of septic shock

Therefore, septic shock occurs in humans when endotoxins are released from invading bacteria, usually gram negative (70%) and sometimes gram-positive species. Endotoxins are called so because they consist of a chain of oligosaccharides linked to a lipid moiety and reside within the membrane of many gram-negative species. The most commonly studied endotoxin is lipopolysaccharide (LPS) and this is released by bacteria into the surrounding tissues/serum. (47)

The symptoms seen in the model following LPS administration are dose dependent and often lethal. Fever, diarrhoea, lethargy, mucus encrustation around the eyes, cyanosis and weight loss are frequently seen. TNF- α and IL-1 are commonly attributed as principal mediators of endotoxin induced inflammation (48), (49) & (50).

Beutler et al (51) injected 200ul rabbit anti-TNF- α enriched immune serum by the intra-peritoneal (IP) route into BALB/c mice, both 3-6 hours prior and post IP injection of 400ug LPS. Significant protection from LPS induced lethality was attained when the anti-TNF- α enriched sera were injected prophylactically, but no protection was evident if the antisera were administered post or concurrent with the LPS injection. Interestingly, the antisera did not have any marked effect on the symptoms of shock. This led the author to propose that lethality and some of the other sub-lethal effects of LPS shock might be under differential regulation.

Freundenberg et al (52) suggested that the majority of the septic shock pathologies were mediated by macrophages. They transferred endotoxin sensitive macrophages into mice previously insensitive to LPS, which then succumbed to endotoxic shock. As macrophages are a major producer of $\text{TNF}\alpha$, this is not inconsistent with many papers over the last decade, which indicates $\text{TNF}\alpha$ as a major factor in Endotoxic shock. Several authors have used neutralising anti-bodies or gene deletions to elicit the roles of $\text{TNF-}\alpha$, $\text{IL-1}\alpha$ and β , IL-6 , and $\text{IFN}\gamma$ and some consensus views can be gained from a study of the results of these investigations. One such study, investigating sub-lethal Euthyroid syndrome, illustrates the cytokine pattern induced by LPS insult. Following IP injection of 75ug *E. coli* LPS into 25g (average weight) Swiss mice (a sub lethal dose), Boelen et al (53) reported that $\text{TNF-}\alpha$, $\text{IL-1}\alpha$ & β , IL-8 and IL-6 production is increased. Boelen's study also monitored the weight loss associated with LPS induced illness and found that although $\text{TNF-}\alpha$ production was inhibited by prophylactic anti- $\text{TNF-}\alpha$ neutralisation, neither IL-6 production nor weight loss parameters were affected. Zanetti (54) also studied the relationship between cytokines and shock induced by either LPS or gram negative bacteria. Following Intra-Venous (IV) injection of LPS (range 10 -500ug/mouse LD = 200-500ug), serum levels of $\text{TNF-}\alpha$ peaked at up to 45ng/ml at 1.5-2 hours post LPS injection. Levels then fell below detectable levels in survivors (at 0+6hrs) and maximum 100pg (at 0+6hr) in mice that eventually died. $\text{TNF-}\alpha$ was not detectable in serum at the time of death. IL-1 and IL-6 also increased in level, peaking after the $\text{TNF-}\alpha$ but remaining detectable [in survivors] until 0+15 and 9 hrs respectively. These cytokines were also detectable in serum at the time of death. When bolus injection of *E. coli* by IP route was used to induce shock, the level of $\text{TNF-}\alpha$ production was 50-100 fold lower than that seen during IV-lipopolysaccharide induced shock. In this case (IP), the $\text{TNF-}\alpha$ production did not peak and fade as with the intravenous LPS or *E. coli*

induction of shock, but persisted at a lower level until death. Treatment of these groups with anti-TNF- α neutralising antibodies showed a dichotomy as the IV challenged group were completely protected from death but no protection was exhibited in the IP-live group. The anti-TNF- α significantly reduced IL-1 and IL-6 in the IV-LPS challenged mice, but no such reduction was seen in the IP *E. coli* experiment. There are limitations here as one group (IP) are using a single dose of LPS and the other a challenge of live bacteria, constantly producing more pathogenic antigens. Furthermore, the IP 1000xLD50 LPS/anti-TNF- α group were sensitised by co-injection of 15mg D-galactosamine with the LPS (D-galactosamine reduces liver metabolism giving another variable). (55)

The live *E. coli* experiment is perhaps more akin to physiological disease than the bolus LPS shock experiment. If the same antigen (LPS) is injected by different routes or produced by a live organism rather than a single injection, the immune response may also operate in subtly different ways. Doherty (56) also demonstrated protection from LPS induced lethality by anti-TNF- α administration 6 hours prior to a 40mg/Kg LPS injection by the IP route, as did Smith et al (57), when 90% protection was conferred by pre-treatment with anti TNF α in LPS challenged mice. Tracey et al (45) reported that complete protection from lethal septic shock, induced by intravenously injected *E. coli* in baboons, was conferred by prophylactic injection of anti-TNF- α antibodies. This reflects the IV results of Zanetti noted above (54). Tracey et al (50) demonstrated that recombinant TNF- α could produce all the symptoms of Endotoxic shock. When this evidence is placed alongside the evidence for the modification of the LPS induced responses described briefly above, there can be no doubt as to the importance and pre-eminence of TNF- α in septic shock, irrespective of the route of disease induction.

The focus on TNF- α as a presumptive controller of lethality may now move towards some other cytokine(s) and more specifically that the control of IL-1 and 6 production may

not necessarily be directed by TNF- α in the live bacterial infection. For example, other variations are seen in the septic shock cytokine pattern, specifically IL-6 and IFN γ . IL-6 is often reported as increased during LPS sepsis. Zanetti (54) reports LPS induced IL-6 decreased in response to anti-TNF- α treatment (in the IV LPS model but not IP), but Boelen (50) reports no effect on IL-6 when anti TNF- α treatment is used in IP low dose LPS induced shock. Amiot et al, (58) when injecting mice with disrupted TNF- α and lymphotoxin genes with LPS, found no difference in the IL-6 production between wild type and TNF α -/-.

Another complication relevant to the Endotoxic shock story is the fact that mice can be pre-sensitised to LPS by priming them with heat killed bacteria, e.g. *C. parvum* (59), BCG (60), or Actinomycin D (61). These priming steps lower the lethal dose by many times and the LPS induced cytokine pattern in these experiments may be altered in comparison to LPS induction of shock without priming. Both Wysocka (60) (via IL-12 in response to LPS) and Smith (59) (*C. parvum*) report that priming induces IFN γ and claim roles for IFN γ in shock pathogenesis by synergy with other cytokines or as a means of potentiating TNF- α production (59). A role for IFN γ was also demonstrated by Doherty, (56) who found anti-IFN γ completely protected against recombinant TNF- α and endotoxin induced shock. Doherty also suggests the possibility that IFN γ may enhance the lethal effects of endotoxin or LPS. Furthermore, Doherty et al (62) demonstrated murine-IL-15 production by macrophages can be induced by IFN γ priming and incubation in LPS independently of T cells in a non-specific manner. Therefore, both IFN γ and TNF α , which from the evidence discussed above can be seen to have a major role to play in the pathogenesis and lethal effects of septic shock, could also be associated with IL-15 activity through its ability to up-regulate TNF α via activated T cells. This thesis attempts to explore such a role for IL-15 in septic shock through the septic shock model whose methodology is described in chapter 2.

1.6 The septic arthritis model

A logical continuation in the investigation of the role of IL-15 in inflammation was to investigate IL-15 action in the murine model of septic arthritis. The human disease has two components: development of a rapid and aggressive arthritis; and a risk of mortality from septic shock induced multiple organ failure. In a review, Goldenberg, (63) describes how bacteria enter the joint spaces from the blood having migrated from elsewhere in the body, e.g. an infected wound or skin infection site. Within the hours following entry of the bacteria into the joint, inflammatory synovitis occurs. The synovial membrane thickens by cell proliferation and inflammatory cells invade the synovium. These cells then secrete pro-inflammatory cytokines and proteases. Subsequent to these events, cartilage and then bone are destroyed in the afflicted joint. The bacteraemia causing such arthritis also may lead to shock like symptoms similar to those described in the section on LPS shock above. The gram positive nature of the most common cause of septic arthritis, *S. aureus*, means that the major toxins causing shock to occur are super-antigens such as Staphylococcal Enterotoxins A and B (SEA & SEB) and Toxic Shock Syndrome Toxin (TSST).

75% of septic arthritis in humans is caused by *Staphylococcus* species Goldenberg, (64). Patients receiving injected, intra-articular therapy such as Glucocorticosteroids may be at higher risk as bacteria can be accidentally introduced directly into the joint with the injection. Furthermore, the introduction of immune-suppressive drugs into the joint environment may add a risk factor for septic arthritis development simply because of the immunosuppressive nature of the drugs. Ostensson and Geborek (65) studied patients undergoing treatment for rheumatoid arthritis and concluded that there was one septic arthritis incident associated with every 2000 injections within their study group. One patient in their study (patient 1) received an injection of crystalline glucocorticosteroid into a

rheumatoid shoulder. Within two days, high fever had developed as had septic arthritis in the original shoulder and furthermore, acute and severe arthritis occurred in one knee and ankle within one day. Septic arthritis can be a serious threat to human health. For example, a study by Gupta et al (129) followed 75 patients over 2 years following diagnosis of Septic arthritis by positive identification of *S. aureus* within synovial fluid culture. Joint disease was reported in 46% of this group. Fever and leg ulcers were also represented in these patients. 11% of the patients died. The poor prognosis has led to a further prospective study in Septic arthritis patients Gupta et al (130) recommending the treatment of patients exhibiting clinical signs suggestive of Septic arthritis to be treated as such even in the absence of actual *S. aureus* culture isolate.

A murine model of the human septic arthritis has been developed by Bremell et al. (66) Bremell describes how bolus injections of live *S. aureus* are administered into the blood via the tail vein of Swiss mice. Symptoms of bacteraemia and arthritis appear after a few days. Bremell later characterises some aspects of the model with description of the histological evidence of arthritic joint destruction, accompanied by mononuclear cell infiltration of the joint. He also describes serological changes such as increased IL-6 production and massive induction of IgG, indicating B cell activation (67). Many of the early papers focus on histological changes in the model relating to the arthritis seen in human disease. More recently many investigators have studied the more life-threatening aspects of the disease and its model, for example sepsis, shock and the host response to the pathogenic bacteria.

The role of IL-15 demonstrated by Ruchatz (43) in the modification of Collagen Induced arthritis in mice, invites the question as to whether IL-15 plays a similar modulating role in septic arthritis. Furthermore, the symptoms of sepsis exhibited by the mice in the septic model are not dissimilar to those displayed by the mice in the LPS induced shock

model. (High fever, lethargy, eye secretions etc.). Serologically, TNF- α and IFN γ are up regulated (68). Bremmell (67) reports increased IL-6 and Zhao et al (69) reports increased TNF- α and IL-1.

There appears to be, as in the LPS shock model of sepsis, a picture of pro-inflammatory cytokines mounting a co-ordinated attack on the pathogen. This may be a host response to the spread of toxins from the bacteria, entering the bloodstream with resultant shock and multiple organ failure. However in this disease, there are also the consequences of joint destruction due to the localisation of the bacteria to the joint cavity and the local inflammation.

If IL-15 does have a place in the regulation of the host response via cytokine cascade control, then the negative effects of the immune response to the bacteria and their toxins may be ameliorated by the therapeutic intervention of IL-15Receptor- α (IL-15R α). However, even if there is a reduction of TNF- α driven immune responses, there may also be a concomitant reduction of the ability of the host to mount sufficient immunity against the invading pathogen. In this case bacteria might continue to colonise the host unchecked with eventual death or severe disease from shock and only a temporary respite from the initial effects of sepsis. Indeed, Docke et al (70) note that monocyte function may be down-regulated during septic shock citing evidence that sepsis patients were unable to produce high amounts of TNF- α and IL-1. They also correlate with this inability of patients to produce pro-inflammatory cytokines resulting in increased morbidity and mortality. Within a mouse model for septic arthritis, mice deficient in TNF α and Lymphotoxin (LT) were injected with toxic shock strain of *S. aureus*. Mice deficient in TNF α /LT were observed to have 67% mortality and this was associated with reduced phagocytosis. In comparison, control mice (wild type) were able to clear *S. aureus* from the bloodstream. Control mice (wild type) were also observed to have far more severe arthritis *symptoms*. (131) Thus the implication for

TNF α having a role in the pathogenesis of Septic arthritis is promoted by these data but the amelioration of the arthritis by therapeutic intervention may come at a risk of greater sepsis and possible mortality.

Interestingly, the evidence for a lead role for TNF α and IL-1, the main "usual" inflammatory protagonists is queried by the report by Kimura et al (132) Here, anti TNF α antibodies and IL-1R antagonist, when co injected with heat killed *S. aureus*, reduced leukocyte infiltration by 80% in rabbits compared to controls. This effect was temporary and progressive cartilage destruction was not ameliorated by a subsequent injection of antiTNF α /IL-1R antagonist at 20 hrs post *S. aureus* injection. Therefore, although TNF α was involved actively in the early stages of infiltration, TNF alpha was not the main inflammatory protagonist 20hours following the *S. aureus* inoculation.

Verdrengh et al (133) investigate the role of Macrophages (putative IL-15-secreting cells in inflammation) in the pathogenesis of septic arthritis and sepsis. Mice were either inoculated with *S. aureus* or a buffered saline control. Within these mice, monocytes and macrophages were depleted by treatment with etoposide. The monocyte /macrophage deficient mice were noted to exhibit less arthritis symptoms when compared to the non-depleted mice. However, the mice exhibiting milder arthritis symptoms were also associated with higher *S. aureus* infection linked mortality. Furthermore, the mice with increased sepsis linked mortality also had reduced serum levels of TNF α and IL-6. This indicates that Macrophages while playing a destructive role in the pathogenesis of *S. aureus* induced arthritis were likely to be providing a protective role in clearing *S. aureus* from the blood and kidneys. Thus removal of Macrophages was detrimental to survival, and the concomitant link to reduced TNF α and IL-6 indicate that these cytokines may also have a similar protective role. TNF alpha, seems to have an early destructive role in promoting inflammatory cell infiltration but only to a much lesser extent 20 hours later.

Therefore, the septic arthritis model presents the conundrum; how to reduce inflammation associated with Arthritis while not reducing the host inflammatory response against the pathogen?

With the experiments described above in mind, it is interesting to speculate how IL-15 receptor alpha based on its arthritis reducing activity shown by Ruchatz (43) in the collagen induced arthritis model might influence the septic arthritis model. If IL-15 neutralisation was to occur and TNF were reduced, might infection of the mice continue and mortality increase? These experiments in all these murine models start from the premise that IL-15 somehow influences TNF α , previously shown to have a controlling role in all these disease models.

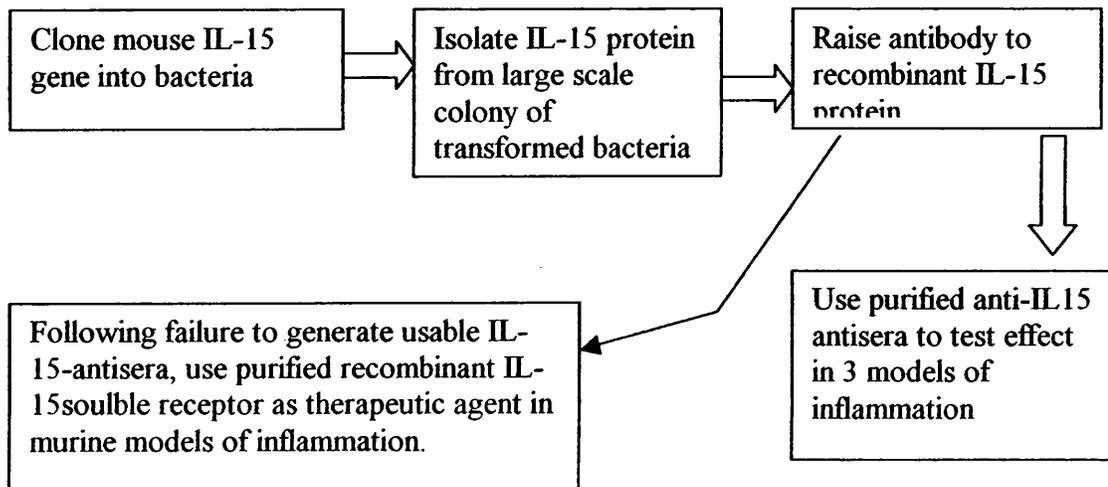
1.7 Aim

The pro-inflammatory effects of IL-15 are unlikely to be associated only with rheumatoid disease. This thesis attempts to further explore the role of IL-15 in inflammation through the Collagen Induced Arthritis (CIA) model of rheumatoid disease, the role of IL-15 in the Lipopolysaccharide (LPS) Shock model and the Septic Arthritis murine models of bacterially induced inflammation. The aim of these experiments is to detect any modification of the normal disease outcomes of the models of inflammation described above, caused by the removal of IL-15 by therapeutic intervention of either anti-IL-15 anti-body, IL-15 antagonist or IL-15-Receptor-alpha protein to mice.

Chapter 2

Materials and methods

Table ii Summary of steps for experiments in murine models of inflammation



2.1 Cloning of the murine IL-15 gene

In order to produce recombinant murIL-15, it was first necessary to clone the gene from murine cells.

2.2 Cell culture

A mouse macrophage cell line, J774 was cultured according to standard techniques. Cells were cultured to confluent density in Corning 75cm² (Corning Costar, Cambridge, MA, USA) plastic tissue culture flask in RPMI (GIBCO, Life Technologies, Paisley, Scotland.) medium containing Penicillin, Streptomycin, L-glutamine and 10% foetal calf serum (All additives from Gibco).

Induction of IL-15 mRNA was reported in murine macrophage cells in response to IFN γ and LPS stimulation. (62) Based on this method, J774 cells were washed in RPMI medium and seeded at 1×10^7 cells per 75cm^2 flask as described above then rested overnight. IFN γ @ 10ng ml^{-1} (final concentration) was added followed by LPS 4 hours later at $2 \mu\text{g/ml}$ (final concentration). The cells were harvested after six hours by scraping and then pelleted by gentle centrifugation (4°C , 1500rpm, five minutes) The supernatant was discarded and the cells frozen at -70°C .

2.3 RNA preparation

The cell pellet was thawed on ice and RNA extracted using Trizol reagent (Gibco Life Technologies, Paisley, Scotland) according to the manufacturer's recommendations.

1ml of Trizol reagent was added to the cell pellet and homogenised by repeated pipetting and then incubated for five minutes at 22°C. A 20% volume of chloroform (Sigma Poole, Dorset, UK) was added and after 15 seconds vigorous shaking, incubated at room temperature for three minutes. The two-phase solution was separated by centrifuging at 12000 rpm for fifteen minutes at 4°C. (MSE, Micro Centaur, Phillip Harris, Aberdeen, Glasgow UK). The upper, aqueous phase containing the RNA was then transferred to a fresh eppendorf and precipitated by adding 0.5ml isopropanol (BDH Chemicals LTD, Poole, England).

After incubation at room temperature for ten minutes, the RNA precipitate was pelleted by centrifuging (MSE, Micro Centaur, Phillip Harris, Aberdeen, Glasgow UK) at 12000 rpm for fifteen minutes at 4°C. The supernatant was discarded and the pellet washed in 70% ethanol (BDH Chemicals LTD, Poole, England).

Then, after mixing, the tube was spun at 7500rpm for five minutes at 4°C. The pellet was air dried for three to five minutes and dissolved in 30ul of Diethyl Pyrocarbonate (DEPC) (Sigma Poole, Dorset, UK) DEPC treated water and incubated at 60°C for ten minutes to ensure solubilisation of the RNA. The quality and quantity of the recovered RNA were tested by comparing the amount and ratio of UV light absorption by the sample in the 260 and 280nm wavelengths. (Spectrophotometer, LKB, Pharmacia, Upsala, Sweden) The samples were analysed at 1/500 dilution. After correction for the dilution factor, the amount of RNA recovered was calculated by reference to the classical formula; Absorbance of 1.0 @260nm indicates 40 µg/ml RNA. The RNA quality was estimated to be most free from protein contamination when the ratio of A260/280nm was equal or close to 1.8. (71)

2.4 Amplification of muIL-15 by RT-PCR

Isolated RNA was transcribed to cDNA by Reverse Transcriptase-PCR (RT-PCR) according to the standard protocol (Superscript RT-PCR kit Gibco, Paisley, Scotland). In summary; a random primer/RNA template reaction was prepared; 5µg total RNA from J774 cells cultured as described previously, was added to 3µl random primers (50ng/ul) and adjusted to 12µl final volume with DEPC treated water, mixed and incubated at 70°C for ten minutes. A first strand synthesis reaction was prepared; 2µl 10X PCR buffer, 2 µl MgCl₂(25mM), 1µl dNTP mixture (10 mM) and 2µl Dithiothreitol 0.1M(DTT) (All supplied with Superscript kit, Gibco). Seven µl of this reaction was added to the RNA/Primer reaction and incubated for five minutes at room temperature (25°C) 200Units of Reverse Transcriptase (Gibco, Life Technologies, Paisley, Scotland.) were added to the reaction, mixed and followed by a further incubation of ten minutes at room temperature. The reaction was transferred to a water-bath (Grant Instruments, Cambridge, England) and incubated at 42 degrees for fifty minutes followed by termination by heat inactivation at 70°C for fifteen minutes in a Heat Block (Grant Instruments, Cambridge, England). Oligonucleotide primers were designed to define the mature coding region of the IL-15 mRNA according to the IL-15 gene sequence published by Krause et al (72)

Two cloning strategies were used to attain recombinant IL-15. In the first instance, primers were designed to enable a PCR product to be cloned directly into the TA- cloning vector (Invitrogen, 9351 NV Leek, The Netherlands) followed by sub-cloning to the Qiagen Protein expression vector . It was therefore important that the first set of primers captured the entire coding portion of the IL-15 gene. It was also necessary that the ligated ends of the gene would match the sequence of the “sticky ends” required to correctly orientate the sequence

into the plasmid for transformation of the e coli. The second primers were also stringent in their requirement to match the expression vector ends. Furthermore, the start codon needed to be captured by the upstream primer, thus the sequences below were chosen.

TA cloning vector primers

Primer 1 5' A GGA TCC ATG TGG ATA GAT GTA AGA TAT 3'

Primer 2 3' GCAGTCAGGACGTGTTGATG 5'

Primers for direct insertion to pQE 60 expression vector

Primer 1 5' G GCC ATG GGC ATA GAT GTA AGA TAT GAC CTG G 3'

Primer 2 3' GTC AGG ATC CGT TGA TGA ACA TTT GGA CAA TG 5'

These primers were diluted to a working stock of 1uM. These were included in the following PCR reaction mixture. This is detailed in Table 1 below.

Reagent/sample	Amount (μl)
10 X PCR buffer	10
MgCl ₂ (25mM)	6
DNTP mix(10mM)	2
Primer 1	5
Primer 2	5
Taq polymerase	1

CDNA	2
Sterile water	69

Table 1 PCR reaction mixes in a total volume of 100ul were mixed to this formula.

PCR reactions were carried out on a Techne (Techne (Cambridge) Ltd, Cambridge, England) thermal cycler without a heated lid. Therefore all reactions were overlaid with mineral oil (Sigma, Poole, Dorset, UK.) The PCR reaction mixture omitted Taq polymerase prior to addition of mineral oil. Reaction samples were heated to 95°C for two minutes. Whilst cooling, Taq polymerase was added, followed by oil. The tubes were loaded into the thermal cycler and denatured for three minutes at 95°C then 35 cycles of;

- ◆ Denaturation.....one minute @ 95°C
- ◆ Annealing.....two minutes @ 56°C
- ◆ Extension.....three minutes @ 72°C
- ◆ One final cycle extension step of ten minutes at 72°C.

These conditions were also used for β -Actin control reactions using primers synthesised according to published murine- β -actin sequence data. The results were analysed on 1% agarose gels under UV light and Ethidium Bromide staining.

2.5 PCR Product Purification for direct cloning in to vectors

PCR products of the correct size were purified in the following manner. As reactions were usually carried out in duplicate, the two positive reactions were pooled and 200ul Phenol/Chloroform (Sigma, Poole, Dorset, UK.) added, shaken hard for 15 seconds. The

tubes were centrifuged for two minutes at 13000 rpm @ room temperature. The upper aqueous phase was transferred to a fresh tube and re-extracted with 200ul chloroform. The upper aqueous phase was transferred to a fresh tube and 20ul 3M Na- Acetate pH7 and 440ul 100% Ethanol. After incubation for ten minutes in liquid nitrogen, the tubes were centrifuged at 15000rpm for thirty minutes at 4°C. The supernatant was discarded and the PCR product pellet washed in 75% ethanol and re-centrifuged for ten minutes at 4°C and 12000rpm. The pellet was then dried under negative pressure for five minutes at 55°C and re-suspended in 30µl distilled water.

2.6 Ligation of the muLL-15 product into pCR 2.1 TA cloning Vector

The TA cloning vector (Invitrogen, 9351 NV Leek, The Netherlands) provides a convenient system for rapid incorporation of a gene of interest from PCR amplified form into a plasmid. This is achieved because the linearised plasmid has 3' Thymidine residues in multiple "A" cloning-site. A consequence of PCR amplification using Taq polymerase with a 10-minute final extension step is the addition of an Adenine (A) residue at the 5' end of each of the double strands of DNA. During ligation, these "A"s form "Watson and Crick" complimentary base pairs with the Thymidine (T) residues of the plasmid strands. It was decided to persue this strategy in the first instance prior to insertion of the gene into a protein expression system using the following method.

A 50µl PCR reaction using the Primers designed for TA cloning (primers 1&2 described above), produced a product of 356 base pairs, visualised by agarose gel analysis. In line with Invitrogen's recommendations, the PCR cycles were restricted to 30. Ligation into pCR2.1 (the code name for TA cloning vector) was carried out according to the manufacturer's protocol. This plasmid carries both Ampicillin and Kanamycin resistance gene open reading frames in the plasmid sequence, allowing negative selection of transformants when the plasmid is transformed into non-ampicillin or non-Kanamycin resistant strains of host bacteria in the presence of one, or a combination of these antibiotics. 3µl of fresh PCR product was added to 1µl 10X ligation buffer, 2µl of pCR2.1, 3ul sterile water and 1 µl of T4 ligase (4 units) with all reagents supplied by Invitrogen (Invitrogen, 9351 NV Leek, The Netherlands). (This reaction gives approximately a 1:1 ratio of PCR product to vector) The reaction was gently mixed and placed in a 14°C water bath overnight.

2.7 Transformation

A 50 μ l aliquot of Competent *E. coli* strain "One shot Top INV α F" (Invitrogen) were thawed on ice. 2 μ l of 0.5M B-mercaptoethanol was added to the competent cells with very gentle mixing by sterile pipette tip. 2 μ l of the overnight ligation reaction was then added with further gentle mixing over ice and then incubated on ice for thirty minutes. After the incubation the Transformation reaction was heat shocked for exactly thirty seconds in a 42 $^{\circ}$ C water bath with the reaction then being placed onto ice for two minutes. 450 μ l of SOC medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCL, 10mMMgCl₂, 10mMMgSO₄ and 20mM glucose at room temperature) was added to the reaction and then shaken horizontally (Gallencamp, Dorset, UK) for one hour at 37 $^{\circ}$ C @ 225rpm followed by storage on ice.

LB agar plates were prepared. 50 μ g/ml ampicillin (Sigma Poole, Dorset, UK.), 40 μ g/ml 5-Bromo-4chloro-3-indolyl- β -galactopyranoside 15 μ g (X-Gal), (Boeringher-Mannheim, Lewis, East Sussex, UK) and Isopropyl-B-D-thiogalactopyranosamide (IPTG) (Bioline, UK) were added as the plates cooled. The plates were pre-warmed to 37 $^{\circ}$ C and 50 μ l of the transformation reaction was pipetted and then spread onto the plate surface. After absorption of liquid into the agar, the plates were inverted and incubated overnight at 37 $^{\circ}$ C. The plasmid used here contained the Lac Z gene, (which allows bacteria incorporating this to metabolise the plate constituents), described above to form a blue product. If there has been successful insertion of the IL-15 sequence into the plasmid, the Lac Z gene is disrupted and therefore the host bacterium cannot metabolise the chromogenic substrate in the plate and as a consequence will remain white instead of blue.) Therefore, 12 white colonies were selected and inoculated into 2ml 2xYT medium with 100ng/ml ampicillin and grown in shaken

suspension overnight. The cells were harvested by centrifugation at 1500 rpm for two minutes at room temperature.

2.8 Plasmid Mini-prep

A standard mini-prep protocol was used to isolate plasmid DNA from the host *E. coli* for analysis. All medium was removed by pipette and cells were re-suspended in 200µl GTE buffer, (18g Glucose, 4ml 0.5M EDTA, 5ml 1M Tris, add sterile water to 500ml). (Chemicals by BDH Chemicals LTD, Poole, England.) 400µl of freshly prepared lysis buffer (8ml sterile water, 1ml 2M NaOH, 1ml 10%SDS (BDH Chemicals LTD, Poole, England.) was added, shaken and incubated on ice for five minutes. After exactly five minutes, 300µl of neutralising solution (K-acetate) was added followed by further 5-minute incubation on ice. The samples were centrifuged at 13000rpm for five minutes at 4°C. The supernatant was transferred to a fresh tube and the pellet discarded. 900µl of 100% ethanol was added to precipitate the plasmid DNA, mixed well then centrifuged as previously. The supernatant was discarded and the pellet washed in 75% ethanol and re-centrifuged. The supernatant was discarded again and the pellet dried under reduced pressure at 55°C for five minutes. The pellet was re-suspended in 40µl of distilled water with 1 µl DNase free RNase (Boehringer-Mannheim, Lewis, East Sussex, UK)

2.9 Restriction Digest analysis of cDNA

To identify any clones positive for IL-15cDNA sequence, analysis was initially performed by restriction enzyme digest of the plasmid DNA. This method was also used to check orientation of the insert within the plasmid. (Because the nature of T/A cloning insertion is not orientation specific)

Restriction-Digest reactions were set up to determine orientation and aid identification of clones positive for an IL-15 insert. For example, 3µl of plasmid DNA was added to 2 µl of

restriction enzyme buffer B (Boehringer-Mannheim, Lewis, East Sussex, UK), 14µl dH₂O and 1µl BamHI (Boehringer-Mannheim, East Sussex, UK) This was incubated at 37°C for 2 hours. Samples of the same plasmid DNA were also analysed in a similar reaction containing the enzymes, EcoRI and RsaI (Boehringer-Mannheim, Lewis, East Sussex, UK), 1µl of each in 2µl of buffer A, 13µl of water and 3 µl of DNA. Several samples of IL-15 PCR product were prepared for sequencing using the Qiagen mini-prep system according to the manufacturers instructions (Qiagen. Crawley, England). Of the 3 inserts sequenced, 1 was identical for the sequence of muIL-15 (No 6) and another was identical except for a 48 base pair, in-frame deletion. Further transformations and plasmid DNAs were produced and digested followed by sequencing using the manual Sanger Di-deoxy Nucleotide method, described below.

2.10 Di-deoxy DNA sequencing

The di-deoxy Sequencing method (also known as the Chain Termination Method) devised by Sanger et al (73). A standard mini-prep (See above) was carried out on 2ml overnight cultures of the transformant colonies, believed to carry the IL-15cDNA insert. 9µl of Plasmid/TE buffer was added to 1µl of 2M NaOH and incubated for fifteen minutes at 37°C to remove contaminating RNA and denature the DNA. Sequencing primers were constructed according to the sequence recommended by the plasmid suppliers allowing sequencing of the plasmid from 200 base pairs 5 prime to the insert, through the insert and then ending around 200bp downstream of the insert. See below for primer sequences.

- ◆ M13 Reverse Primer CAG GAA ACA GCT ATG AC
- ◆ M13 Forward primer(-20) GAC CGG CAG CAA AAT G

A priming reaction was set up. 1 μ l of each primer was added to the denatured samples and mixed by vortex. 3 μ l of 3M K-acetate (pH4.8) was then added to neutralise the alkali denaturant. The DNA was then precipitated with 75 μ l 100% ethanol and collected by centrifugation at 13000 rpm at room temperature for ten minutes. The supernatant was discarded and the pellet washed in 75% ethanol. After centrifugation, the supernatant was again discarded and the pellet re-suspended in 8 μ l sterile water. The sequencing then followed the protocol recommended by the suppliers of the sequencing reagent (Sequenase version 2.0 DNA sequencing kit, Amersham Life science), 10 μ l of the priming reaction was added to 1 μ l DTT, 0. 2 μ l diluted labelling mixture, 0.5 μ l 35 S dATP, 2 μ l diluted Sequenase-polymerase. A 96 well-U bottomed plate (Corning Costar, Cambridge, MA, US) was labelled with A, C, G and T for both the forward and reverse primed reaction. As suggested by Sambrooke, Fritsch & Maniatis (71), 2.5 μ l of each of these nucleotides were then dispensed to the bottom of each of the wells as per the labelling scheme. 3.5 μ l of the labelling/template reactions were added to the top of the wells, taking care not to allow the two reagents to meet. The plate was then briefly spun in a centrifuge (Mistral 3000 MSE, Micro Centaur, Phillip Harris, Aberdeen, UK) to start the reactions simultaneously. The reaction was incubated for five minutes at room temperature and meanwhile 4 μ l of the formamide stop solution was added to the opposite side of each well. A quick spin precipitated the stop formula into the reaction allowing all the reactions to be stopped simultaneously. The sequences were analysed on 6M-urea polyacrylamide denaturing gels using standard procedure (71).

2.11 Transformation into Qiagen Expression Vectors

Two expression vectors were used to transform M15 strain *E. coli* with murine IL-15 cDNA to enable expression of IL-15 recombinant protein. These vectors differ in the orientation of the 6-histidine residue affinity purification tag with respect to the cDNA insert. The 6-Histidine tag is added to the recombinant protein at the 5 prime end when the pQE-30 is used and 3 prime end when the pQE60 is used. The secondary structure of the recombinant protein may dictate that one orientation of the tag may affect bioactivity less than the other orientation and therefore both vectors were used here to explore any advantage conferred by this facility.

The pQE-series of plasmids carry a β -Lactamase sequence open reading frame that confers ampicillin resistance in successful transformants. In turn the host *E. coli* species used here; M15 is selected for pREP4, which expresses Kanamycin resistance activity. Thus these two antibiotics can be used for accurate selection and to reduce high background cloning events.

2.12 Sub Cloning TA Vector insert to pQE 30-expression vector

From the pCR2.1 transformed bacteria (confirmed by digest pattern and sequence analysis) one clone IL-15 coding for the correct IL-15 sequence and another clone, IL-15, -48 which coded for IL-15 with a 48 bp deletion were selected for sub cloning into the expression vector pQE-30. (Qiagen, Crawley, England) These clones were inoculated into 2 ml of LB broth with ampicillin and cultured overnight in shaken suspension at 37°C. A mini prep (described above) was used to isolate plasmid DNA. A digestion reaction was set up in total volume of 70 μ l including 7 μ l Restriction enzyme buffer B, 5 μ l BamH1 (Boeringher-Mannheim, Lewis, East Sussex, UK), 33 μ l sterile water and 25 μ l of plasmid DNA. Concurrently, 2 μ l of the vector pQE-30 was digested in a volume of 60 μ l of the same reaction mix. The plasmid reactions were incubated to completion at 1.5 hours @37°C in a water bath whilst the vector was digested for 3 hours in a 37°C water bath.

To prevent self-ligation of the vector, 55 μ l BamH1 digested vector was de-phosphorylated with 1 μ l CIP (Alkaline Phosphatase) (Boeringher-Mannheim, Lewis, East Sussex, UK), 10 μ l CIP buffer and 34 μ l Tris 10mM pH8.3. Incubated at 37 $^{\circ}$ C for 2 hours and the reaction stopped with 1 μ l 0.5M EDTA with heating to 75 $^{\circ}$ C for ten minutes. Meanwhile, the BamH1 digested IL-15-cDNA fragments were gel purified by running out on a 1% agarose gel. Following electrophoresis, bands of the predicted size were visualised under UV light and cut from the gel. DNA was extracted by the "Freeze Squeeze" method, briefly, the gel fragments containing the excised bands were placed into freeze squeeze buffer (0.3M Na-Acetate in 1mM EDTA pH 7.0) in separate eppendorfs and incubated in the dark for ten minutes at room temperature. The buffer was discarded and the tubes containing the gel fragments placed into a (dry ice CO₂/ethanol bath (-80 $^{\circ}$ C) until frozen. The frozen gel fragments were transferred to a Spinex (Corning Costar, Cambridge, MA, USA) 0.2 μ m filter tube and spun at 13000 rpm for ten minutes at room temperature. The gel fragments were discarded and the liquid residue containing the IL-15 fragments made up to 400 μ l per sample with TE buffer. The IL-15 and the de-phosphorylated vector were then Phenol-chloroform extracted and ethanol precipitated as described earlier for PCR purification. The vector pQE-30 was re-suspended into 20 μ l sterile water and the DNA inserts into 15 μ l each. 3 μ l of each of these were run out on an agarose gel to determine concentration ratios for ligation (ideally a 1:1 ratio).

A ligation reaction was set up as described for pCR2.1 above resulting in the production of the IL-15/pQE-30 and IL-15-48/pQE-30 constructs. A control self-ligation with no insert was also incubated and used for transformation control. Transformation of *E. coli* strain M15 (Qiagen) with these constructs was carried out to the manufacturer's protocol summarised here. A 100 μ l aliquot of thawed transformation competent M15 (Qiagen, Crawley, England) cells were added to 10 μ l of ligation reaction and incubated on ice for twenty minutes. The cells were heat shocked for 90 seconds at 42 $^{\circ}$ C in a water bath and following this incubated in 500 μ l of Psi broth. (LB bacterial growth medium (Gibco Life Technologies, Paisley, Scotland) with 4mMMgSO₄ and 10mM KCl. (BDH Chemicals LTD,

Poole, England). Samples were then placed into shaken suspension at 37°C for ninety minutes. 50µl of each of the transformation reactions were plated out on agar plates containing 100µg/ml ampicillin (Sigma Poole, Dorset, UK.) and 25µg/ml Kanamycin (Gibco, Life Technologies, Paisley, Scotland.) The plates were incubated overnight at 37°C and positive colonies selected (negative selection) for protein expression screening.

In the case of transformation with expression vector pQE-60, the procedure was exactly the same as above except the IL-15 PCR product was generated to include the restriction enzyme sites, Nco I and Bgl II (Boehringer-Mannheim, Lewis, East Sussex, UK). These were inserted into the 5' and 3' primer sequences (primer sequences noted on page 28). A construct was prepared by linearising the pQE-60 vector by digestion with these enzymes in a two-stage digestion reaction followed by ligation with the similarly digested PCR product. Sequencing of the insert to confirm IL-15 sequence integrity was performed directly on the pQE-60/IL-15 constructs (as compared to the pCR.2.1 sequencing that was carried out prior to sub cloning into the pQE-30 vector).

Small-scale expression cultures were set up to identify transformants (selected by sequencing), which were able to express recombinant IL-15. Single colonies were picked from agar plates of successful transformants (including vector controls) and cultured overnight in LB broth with antibiotics (Penicillin and Kanamycin as described above). A fresh flask with 10ml medium was inoculated with 500µl overnight culture and grown at 37°C for thirty minutes and then periodically measured by spectrophotometry until OD @600nm was approx. 0.6 indicating log growth phase had been attained and cultures were at similar densities. Cultures were prepared in duplicates enabling one to be induced with isopropyl-β-D-thiogalactoside (IPTG) (Bioline London, England) to a final concentration of 2mM and one to serve as non-induced control. Following induction of recombinant protein expression, cultures were grown for 3 - 6 hours and then harvested by centrifugation at 10000rpm for 1 minute at 4°C. (Centrifuge, Becton-Dickinson ultracentrifuge & JA 10 rotor, Oxford UK). The cells were then lysed in Lysis buffer B (8M urea @pH8.0) The lysate was centrifuged at 13000rpm /room temperature for ten minutes and the pellet of cell debris

discarded. The supernatant was transferred to a fresh tube. 40µl of a slurry of Ni NTA agarose resin (Qiagen) (Qiagen, Crawley, England) (pre-equilibrated in buffer B) was added and rotated for thirty minutes at room temperature. Centrifuging for 10 seconds at 13000 rpm pelleted the resin. The unbound fraction was removed and stored on ice for analysis. The resin was washed 3 times with buffer C (buffer B at pH6.4) and the washings kept for analysis. 20ul buffer C with 100mM EDTA was added and mixed with the resin to elute any protein and incubated at room temperature for two minutes. After a 10 second spin at 13000, the supernatant was transferred to a fresh tube and mixed with 5 µl of 5X-PAGE sample buffer (5ml Glycerol, 2.5ml Mercaptoethanol, 1.5g SDS 6.25ml 0.5M TRIS, made up to 25ml with H₂O and pH6.8). Following heat denaturation (100⁰C water bath) for seven minutes the samples were loaded onto a 15% polyacrylamide SDS gel and separated by gel electrophoresis. The proteins eluted were visualised by Coomassie blue staining.

2.13 Protein production

Transformed bacteria established by Coomassie gel analysis to be expressing recombinant IL-15 proteins were grown in large-scale cultures. IL-15 protein was purified and characterised to further establish its identity by anti-IL-15 binding, and ability to bind the IL-15 specific receptor subunit, recombinant IL-15 Receptor alpha. (IL-15R α) Bioactivity was also tested in the Cytokine dependent CTLL murine T cell line.

2.14 Large scale production and purification of recombinant IL-15

Glycerol treated stocks of IL-15 expressing bacteria were stored at -80⁰c. Large-scale culture was established by inoculating 100ml LB broth/antibiotics with a loop of bacterial stock, grown overnight with shaking at 37⁰C. 900ml (or multiples of these amounts) of pre-warmed broth were inoculated with 100 ml overnight culture and growth continued for 2 hours (OD@ A_{600nm}=approx 0.6). This log phase culture was then induced by addition of IPTG to final concentration of 2mM. Induction was continued for 4 hours. Cells were harvested by centrifugation at 4000rpm at 4⁰C for twenty minutes (Beckman JA 10 Rotor). The supernatant was discarded and the pellets re-suspended with 30ml Buffer B (8M urea,

0.1M NaH₂PO₄, 0.01M Tris.Cl, pH8.0) per cell culture in sterile 50 ml tubes. Lysis was achieved by rotation at room temperature overnight. Cell debris was pelleted by centrifuging at 14000rpm for thirty minutes at room temperature (Becton-Dickinson ultracentrifuge & JA 17 rotor, Oxford UK). The supernatant was transferred to a fresh 50ml tube and 500ul NiNTA resin was added per original litre of culture. Then followed by incubation at room temperature with gentle rotation for periods in excess of one hour. The resin was collected by brief centrifugation and the “unbound” portion discarded. The resin was washed in 40 ml buffer B and loaded into a Qiagen 10 ml affinity purification column. The filter collected the resin as the wash buffer/resin system was poured into the column. As the washing buffer voided from the column fresh buffer B @ pH8.0 was added to the column to about 10 column volumes. Periodically, (i.e. every 2-column volume), samples of the washings were measured for protein by Absorbance @260nm. When the OD was <0.01, buffer C (As buffer B but at pH6.4) was added to the column and further OD observations made. At OD<0.01 the protein was deemed to be purified to a greater degree and after a pre-wash of Buffer C with 50mM Imidazol, the remaining resin-bound protein was eluted in 4ml Buffer C/300mM Imidazol. Samples were taken and analysed for molecular weight and purity. Dialysis against PBS induced precipitation of the protein subsequently; PBS with 30% glycerol was used for dialysis.

Constant problems with precipitation and low yield resulted in the purification strategy being changed to more severe conditions. The revised protocol included Lysis and solubilisation in Buffer A (6M Guanidine hydrochloride) (Qiagen. Crawley, England), two extra washing buffer steps; Buffer D and E (As buffer B but pH 5.9 and 4.5 respectively) and elution in buffer F, (6M Guanidine Hydrochloride/acetic acid 0.2M = approx. pH 2.8). Analysis by electrophoresis required dialysis against PBS/30% glycerol. This was because guanidine was precipitated as crystal salts by SDS in reducing-polyacrylamide gels. (See Western Blotting, section 2.17 below)

2.15 Production of murine IL-15 receptor alpha

5 Litres of LB medium with antibiotics (25ng/ml Kan & 100ng/mlAmpicilin) were inoculated 10% overnight culture of E. coli (M15 strain, transformed with truncated murine IL-15 receptor alpha cDNA in pQE 30 expression plasmid; kindly donated by Holger Ruchatz). After 2 hours growth at 37°C protein production was induced by 2mM IPTG added to the log phase culture. Growth was continued for two more hours at 37°C with shaking at 225rpm. Cells were pelleted by centrifugation (4000 rpm @ 4°C for thirty minutes) in a J10 rotor in a Beckman ultracentrifuge) media discarded and the cell pellet frozen at -70°C overnight.

Pellets were directly lysed by 8M urea (buffer B) (Qiagen) @30ml per 1L culture of cells. Lysis achieved by rotation for 4 hours. A cleared lysate was prepared by centrifugation (14000rpm@25°C for forty minutes J17 rotor). Cell debris discarded. 0.30ml of Ni-NTA agarose was added to each 30ml-cleared lysate and incubated with rotation for 1hour. The lysate/Agarose slurry was centrifuged at 2500rpm and the glutinous, unbound fraction of lysate discarded. The remainder was diluted with 30ml buffer B and re-centrifuged to pellet the agarose. The unbound fraction was again discarded. The resin/slurry was then added to a Qiagen column. The column was washed with approx. 200ml buffer B followed by 200ml buffer C (Qiagen) (i.e. $A_{280} < 0.01$) The column was washed with 10ml buffer C/20mM Imidazol followed by a wash with 5ml of bufferC/50mM Imidazol. The column bound fraction was then eluted with 8ml Buffer C/ 300mM Imidazol. The eluate was then placed in dialysis tubing and dialysed against High salt PBS buffer at 25°C for 30 hours with 1 change of buffer. (PBS pH7.2/2% NaCl)

2.16 Protein assay

Protein concentration was determined by either of two methods.

The Coomassie protein assay

A standard curve of BSA diluted in dialysis buffer with concentration range 1.5mg - 0.1mg/ml was constructed. (74) 100µl of each standard and each dialysed sample was mixed with 5ml Coomassie reagent (Pierce, Rockford, Illinois, USA). Absorbance was read immediately at A595nm and protein content of the samples determined in relation to the standard curve absorbencies. As the curve was most linear at values of 0 to 500µg/ml, dilutions of the samples were made to enable readings in this section. This method is less susceptible to solvent interactions and could be used well for quick determinations e.g. success of purification.

BCA protein assay.

This assay may be affected by Guanidine in excess of 4M but is very reproducible and useful for accurate protein determinations such as Bioassay or western blotting preparation. 10 ml Bicinchoninic acid (Sigma Poole, Dorset, UK.) was mixed with 200µl 4% CuSO₄ (aq) according to the manufacturers recommendations. In volumes of 500µl, standards were prepared with double dilution of BSA in whatever sample buffer was being used, in concentrations from 200ul and down. The sample was diluted 1/10 and sample and dialysis blank prepared. 500µl of reagent was added to each sample/blank/standard tube, mixed and incubated for thirty minutes at 37°C. Absorbance was read at 562nm and sample concentration determined against that of the standards.

2.17 Western Blotting

It was necessary to confirm the identity of the recombinant protein by an affinity method as well as the mobility evidence of Coomassie stained gels. A 15% polyacrylamide/SDS gel was prepared. Samples of dialysed recombinant IL-15 were denatured in 2X sample buffer and amounts corresponding to 1 μ g protein loaded onto the gel. Rainbow, pre-stained molecular weight markers (Amersham, Little Chalfont, Bucks, England) were run and when the markers indicated the 14kDa proteins had travelled approximately 3/4 of the gel surface, the current was stopped. The stacking portion of the gel was discarded and the gel washed briefly in Tris/glycine/methanol transfer buffer. The separated proteins were transferred onto pre-wetted nitro-cellulose membrane (Biorad) by liquid-transfer at 200-300 milli-amps overnight. The nitro-cellulose membrane was incubated in blocking buffer (PBS 0.1% Tween 20 and 5% dried milk (casein) for one hour with orbital shaking at room temperature conditions for all incubation and washing steps).

The membrane was washed in PBS 0.1%Tween 20 (PBS-T) for fifteen minutes and then three times with fresh PB-T for five minutes. The primary detection monoclonal antibody; biotinylated-rat anti-murine IL-15 (PharMingen, San Diego, California, USA) was diluted 1/1000 v/v into blocking buffer and the membrane incubated for 2.5 hours. Washing was then as before. Extravidin HRP conjugate (Sigma, Poole, Dorset, UK) was diluted in PB-T and the membrane incubated for one hour. The membrane was washed as before with 1 extra 5-minute wash. The blot was transferred to a darkroom with detection by the ECL method (ECL, Amersham, Little Chalfont, Bucks, England). The membrane was dried of excess wash buffer. Equal volumes of the detection reagents were mixed together (0.125ml per cm² membrane) and poured over the membrane and incubated for 60 seconds in a clean petri dish. The membrane was dried of excess detection reagent and placed protein side down over Clingfilm. The Clingfilm was folded in an envelope around the membrane and the protein side placed face up into an auto radiograph cassette. Autoradiography film (Kodak, Ilford, Essex, England) was placed over the membrane/envelope and exposed for variable amounts of time.

2.18 ELISA

The bioactivity of the recombinant protein was tested initially by its ability to bind the IL-15 specific receptor subunit IL-15 Receptor alpha (IL-15R α) in an ELISA based assay. A 96 well plate (Immulon) was coated with 50 μ l per well with IL-15 Receptor alpha @ 1 μ g/ml in 0.1M NaHCO₃ pH8.2 and incubated at 4 $^{\circ}$ C overnight. The plate was washed 3 times in PBS-Tween20 (0.05%) and non-specific binding blocked by incubation at 37 $^{\circ}$ C for one hour with 200 μ l per well 10% Foetal Calf Serum in PBS. The plate was washed 4 times and samples of recombinant IL-15 and a Human commercial IL-15 standard curve, both diluted into 10% FCS/PBS added with serial dilution across the plate. After 3 hours incubation at 37 $^{\circ}$ C, the plate was washed four times and rat anti-mouse IL-15 biotinylated monoclonal anti-body (PharMingen, San Diego, California, USA) diluted to 1/2000v/v in 10% FCS/PBS was added at 50 μ l per well. This was incubated for one hour at 37 $^{\circ}$ C. the plate was washed four times and a 1/1000 dilution of Extravidin peroxidase (Sigma Poole, Dorset, UK.) in 10% FCS/PBS was then added at 50ul per well and incubated for forty five minutes at 37 $^{\circ}$ C. The plate was washed six times and the chromogenic substrate, TMB-Microwell added at 100 μ l per well. After ten minutes development time, the plate was read for absorbance at 405nm enabling calculation of IL-15 and an assessment of binding which in turn infers a degree of bioactivity.

2.19 CTLL assay

This T cell line, originally cloned from a C57bl/6 mouse (1977) is IL-2 dependent. However proliferation of this cell line is also supported by IL-15 (10). It was possible therefore, to assess bioactivity of the recombinant protein murine IL-15 by measuring 3H

thymidine uptake by CTLL cells in a proliferation assay. Briefly, cells were cultured in the presence of IL-2 (10ng/ml) for 3 days in RPMI/10% FCS and antibiotics. The cells were harvested, washed twice in RPMI (No IL-2) and re-suspended at 1×10^5 cells/ml. In a 96 well culture plate, a standard curve of Human IL-15 or IL-2 was constructed using serial dilutions of cytokines in RPMI. Samples of dialysed recombinant protein were diluted in the same manner. CTLL cells were added to the plate and incubated overnight at 37°C. A pulse of tritiated thymidine, (1uCi per well) was added with incubation at 37°C for 6 or more hours.

Cells were harvested on a Wallac cell harvester and thymidine uptake determined by scintillation counting on a Wallac Betaplate scintillation counter.

2.20 Generation of Polyclonal anti-murineIL-15 anti-body

A New Zealand White rabbit (Harlan, Olac, Huntingdon, Cambridge) was bled for pre-immune serum sampling. Then it was inoculated with 92µg of recombinant IL-15 in 250 µl of PBS/glycerol diluted into Freund's complete adjuvant (Difco) with a total volume of 500µl. Nineteen days later, a booster injection of 140µg recombinant IL-15 in Freund's incomplete adjuvant (Difco, USA) was given. Two subsequent booster injections were given at similar intervals. Serum-levels of anti-IL-15 antibody were measured by ELISA. A 96 well plate was coated 50µl/well, half with 1µg/ml recombinant IL-15 and the remainder with recombinant IL-18 (negative control) and incubated overnight. Following the washing regime described above, a 1/100 Rabbit serum sample was added and then serially diluted across the plate and incubated for 2 hours at 37°C. The plate was probed with 1/1000 Sheep/anti-rabbit HRP conjugate (SAPU, Scottish Antibody Production Unit, Law, Scotland) and anti-body titre analysed by spectroscopy at 405nm as previously described. Anti-body was purified from serum by protein A column chromatography (Sigma Poole, Dorset, UK), following the manufacturers' recommendations (except eluted anti-body fraction was not dialysed against

Azide). The Anti-body was dialysed against PBS and total IgG recovered measured by Coomassie assay (Pierce, Rockford, Illinois, USA) as described earlier.

Murine models of Inflammation

To investigate the role of IL-15 in inflammatory diseases, three models were used.

2.21 Collagen Induced Arthritis

Male DBA/1 mice (Harlan, Cambridge, England) at 6-8 weeks old (75) were inoculated intradermally with 200ug bovine collagen type II (Sigma Poole, Dorset, UK) in complete Freund's Adjuvant (Difco, USA). The mice were challenged 21 days later by Intra-peritoneal (IP) injection of 200ug collagen in Freund's incomplete adjuvant (Difco, USA). Therapeutic doses of anti-IL-15 polyclonal anti-body or -48 IL-15 mutant were administered daily starting one day after challenge.

Following the collagen challenge, the mice were monitored daily for weight change, signs of arthritis including paw thickness (calliper measurement of paw diameter) and incapacitation. Arthritis was assessed by the following severity score system, 0 = normal paw appearance, 1= erythema, 2= erythema and swelling 3 = severe swelling and /or loss of function. Incidence of arthritis was also recorded daily.

2.22 LPS induced Endotoxic shock model

LPS shock was induced in 10-week-old Balb/c mice (Harlan, Cambridge and In-house bred mice) by intraperitoneal injection of 18mg/Kg LPS in PBS (Sigma Poole, Dorset, UK) (48). Immediately following induction the mice were treated with either IL-15 receptor alpha or PBS. The therapeutic dose regime varied in time and amount according to the exact experiment, (See results). Frequent observation was used to assess the effect of the treatment using the indices of septicaemia shown in the table (Table 2) below. Initially mortality was

the only informative parameter. In later experiments, biochemical analysis of Serum samples were obtained by exsanguination of the mice following dislocation of the neck. Serum was diluted 1/10 in 7% BSA (Sigma Poole, Dorset, UK) and levels of serum enzyme indicators of liver damage determined (kind assistance of Glasgow Royal Infirmary, Routine Biochemistry). A general matrix of indicators of sepsis was also derived and used to determine severity. In some experiments in this model, BALB/c mice of the same age were primed with 1mg/mouse heat killed *C. parvum* suspended in PBS and injected by IV route. Seven days later doses between 0 and 5 mg/Kg LPS were injected by IP route and therapeutic IP injections of IL-15 receptor alpha (or control) were initiated. The observation and post-mortem analysis of the procedure was identical to that for that described above.

Healthy		Unhealthy	
Actively running around	0	Moving slowly	1
Inquisitive	0	Not Inquisitive	1
React to touch	0	No reaction to touch	1
Moving around at 60 hours	0	No spontaneous movement at 60 Hours	4
Normal stools	0	Diarrhoea	1
Clear eyes	0	Encrusted eyes	1
Skin warm to touch/relaxed	0	Skin cold/Hunched aspect	1
Weight loss <30%	0	Weight loss >30%	7
No shivering when picked up	0	Shivers uncontrollably on picking up	5
Mobile at 72 hours	0	Immobile at 72 Hours	7

Table 2 Table of scoring system used to monitor mice during this experiment based on previous results and Morton-Griffith index and the Rodent Protection Document. All animals scoring –7 were scored and humanely killed. The data was recorded on an “intention to treat” basis.

2.23 Septic arthritis model

S. aureus LS-1 strain the TSST producing strain of *Staphylococcus aureus* previously used in Bremell's murine model of septic arthritis (66) were cultured on blood agar plates at 37°C and re-suspended to 5×10^8 bacteria/ml determined by spectrophotometry. 4-6 week old MF1 mice were inoculated with 5×10^7 bacteria per animal. In one experiment, 40 µg of IL-15-receptor-alpha was injected immediately following and three days after the bacterial inoculation by the IP route. In a later experiment IL-15 receptor treatment was given daily for 7 days following the bacterial inoculation. The mice were carefully observed (where possible by a blinded observer) for symptoms of incidence and severity of Arthritis by the scoring system described above and septicaemia by definition of lack of spontaneous mobility, presence of ocular discharge, starey coat and hunched position. Weight loss/gain, mortality, and the incidence and severity of sepsis were also recorded. Observations were continued for periods up to two weeks.

Whole blood obtained by exanguination was analysed for *S. aureus* colony forming units. 1/10 serial dilutions of whole blood were plated out on blood agar and incubated overnight at 37°C. *S. aureus* colonies were kindly identified by Professor C Gemmell.

At post-mortem, spleens were removed from each carcass, pooled per treatment/control group and forced through nylon mesh with a syringe plunger into a single cell suspension. 1g of tissue suspension was weighed out and serial dilutions at 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} & 10^{-7} into sterile PBS were made. 100 µl of each of these dilutions were spread onto pre-warmed blood agar plates and incubated overnight at 37°C. *S. aureus* colonies were counted the following day. Kidneys and liver were obtained and treated the same way.

Hind limbs were removed above the ankle joint. The paw was cut across the foot midway between the heel and toes. The freshly cut face was then streaked across the surface of a pre-warmed blood agar plate. After over night incubation at 37°C, the number of *S. aureus* colony forming units were counted.

Spleen cells from the suspension described above were re-suspended in RPMI at 1×10^6 cells /ml and plated out in a round bottomed 96 well culture plate. Spleen cells were stimulated with dilutions of Staphylococcal Enterotoxin-A (SEA) (Sigma), Toxic Shock Syndrome Toxin (TSST) Sigma, Concanavalin-A (Sigma), Simian IL-15 (Immunex) or medium alone. Incubations were for 24 or 48 hours at 37°C . Detection of proliferation was by ^3H Thymidine pulse 6 hours prior to cell harvesting followed by scintillation counting on a Wallac Beta counter.

2.24 Statistical analysis

In order to assess significance of the experimental results, the following tests have been used.

1. Mann Whitney U
2. Chi square
3. Kruskal Wallace

These tests were performed on raw data using a PC supplied by Gateway Computers, UK and using the statistical analysis programme, “Minitab” provided by the University of Glasgow. To confirm significance, a p value of less than 0.05 was required.

Chapter 3

Results

3.1 Generation of murine IL-15 and murine -48 IL-15 mutant

A PCR primer pair (listed in chapter 2 page-26) was synthesised corresponding to parts of the published sequence of murine IL-15 and predicted a product of 355bp (gene-bank accession number U14332). Following PCR amplification using these primers, products with mobility of approximately 360bp were identified by agarose gel electrophoreses. Fig 1 shows a photograph of such a gel.

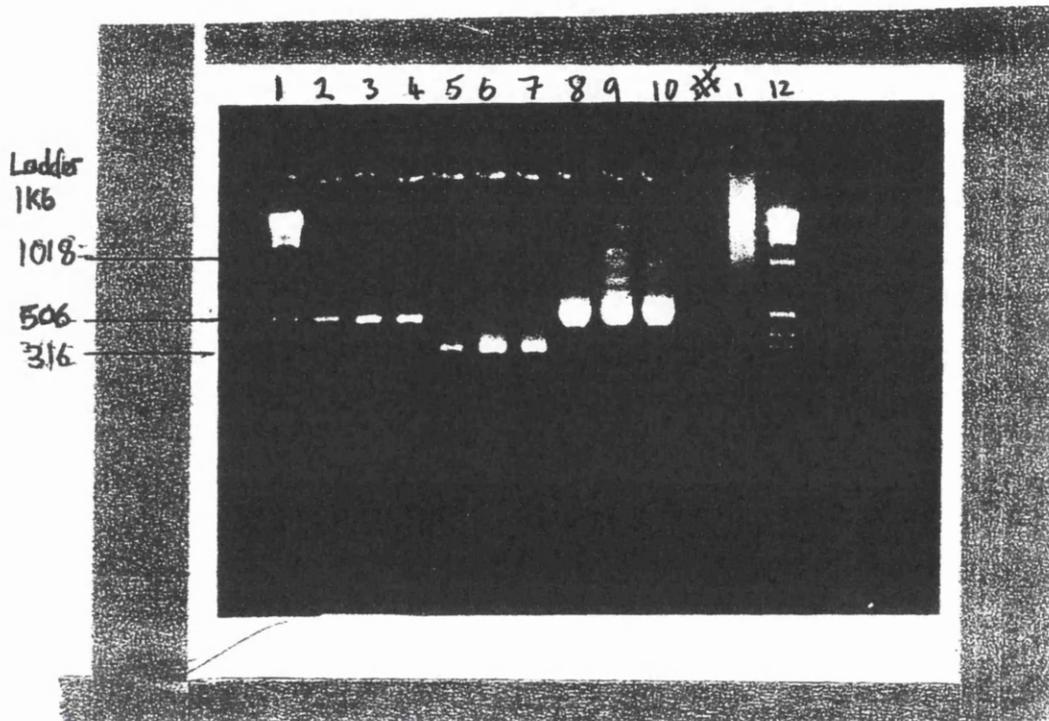


Fig 1 PCR products amplified from J774 macrophage total cDNA using IL-15 and β -actin specific primers. Lane 1; 1Kb ladder lane 2,3 and 4; IL-15 cDNA including signal sequence product 510bp, lanes 5,6 and 7 mature coding sequence. Lanes 7,8 and 9; β -actin. Lane 12 1Kb ladder (Ethidium bromide stained 1% agarose gel illuminated by UV light)

These PCR products were ligated into the TA cloning vector, pCR2.1 and transfected into E.coli according to the protocols listed in the previous chapter (page 33). Plasmid DNA was extracted from overnight cultures of successful transformants by the mini-prep method described earlier. Two restriction enzyme digest reactions were carried out to further confirm the identity of the PCR products as IL-15 derived. A single restriction enzyme BamH1 (Boehringer Mannheim, Germany) digest confirmed that the insert was reverse orientated within the plasmid and had a mobility of around 400bp (including some 3' plasmid sequence). The murine IL-15 cDNA sequence has a naturally occurring Rsa1 restriction enzyme cut site at 770bp. When the plasmid DNA was digested with both EcoR1 and Rsa1, a pair of products of 199 and 170 bp were predicted. Figure 2 below indicates the locations of these restriction sites and their digestion product sizes.

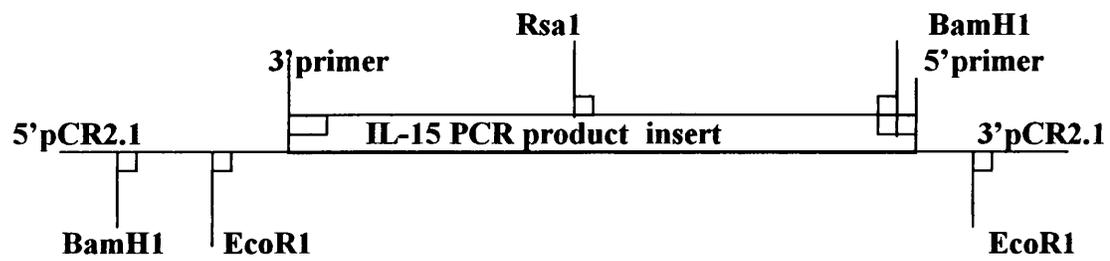


Figure 2 Schematic representation of the pCR.1/IL-15 plasmid and insert with locations of restriction enzyme sites listed.

Fig 3 below shows the products of a digestion with EcoR1/Rsa1. As predicted, lanes numbered 6 and 7 give products of 190 and 170bp. However the lane labelled 8 has one product at 190 and a smaller one at approximately 130 bp. This alternative digestion product size suggested that as well as IL-15, a mutant had possibly been cloned as a result of a PCR artefact or an alternative IL-15 mRNA transcription event. This alternative fragment was called IL-15-48 for the purposes of this thesis.

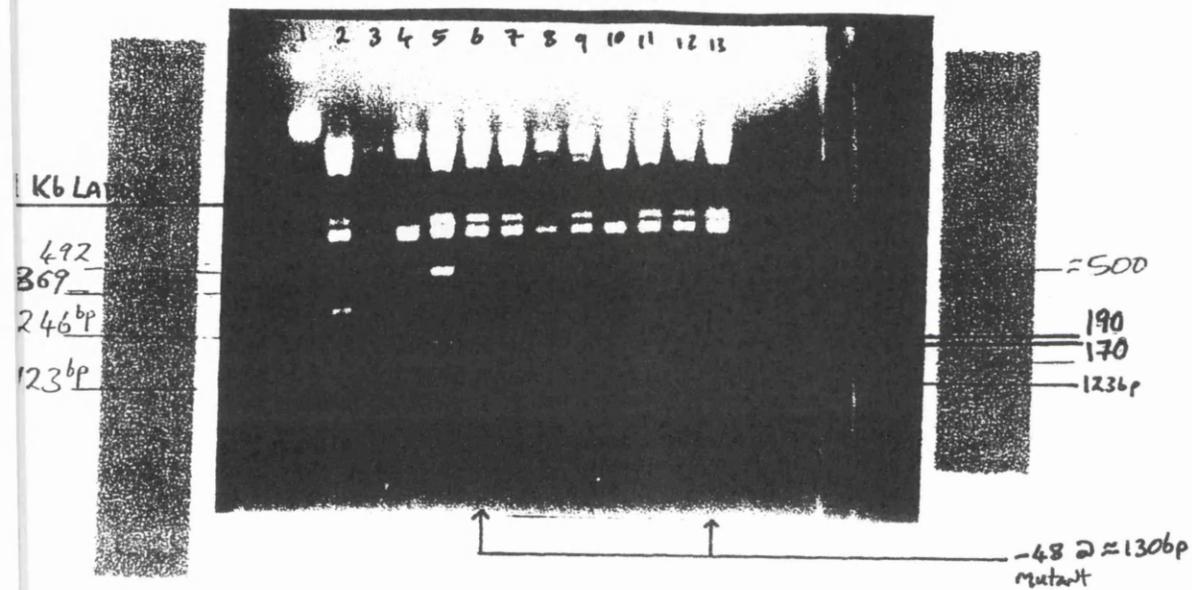


Fig 3 Agarose gel (2%) stained with Ethidium bromide and illuminated with UV light. Lanes 11 and 12 show predicted digestion products of 170 and 190bp when pCR2.1/IL-15 is digested with restriction enzymes EcoR1 and Rsa1. The characteristic "double banding" is visible. Lane 6 and 13 contain a product at 190bp and one below at approximately 130bp (IL-15-48).

3.2 Sequencing

The clones contained in the plasmids labelled 6, 7 and 8 in figure 3 above, were sequenced by automated sequencing (Strathclyde University, Glasgow) (Sequencing gel not shown).

Clone 6 and 7 were confirmed as 100% homologous with the published sequence for murine IL-15. Clone 8 was found to be identical to murine IL-15 sequence except for an in-frame deletion of 48 base pairs, corresponding to 16 amino acids from (bp)99 to 147 inclusive hence IL-15-48. Figure 4 below shows the sequence of clone 6 which is homologous to the murine IL-15 sequence published by Anderson et al (Accession number to gene bank= U14332). The recombinant clone excludes the 5' un-translated region (UTR) found in the natural gene product. The figure also shows in red and brackets, the in frame deletion of 48 bp corresponding to 16 amino acids from the normal mature protein.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
ATG tgg ata gat gta aga tat gac ctg gag aaa att gaa agc ctt att caa																	
Met	trp	ile	asp	val	arg	tyr	asp	leu	glu	lys	ile	glu	ser	ile	gln	ser	
18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
tct att cat att gac acc act tta tac act gac agt gac ttt cat {ccc agt tgc																	
ser	ile	his	ile	asp	thr	thr	leu	tyr	thr	asp	ser	asp	phe	his	pro	ser	cys
36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	
aaa gtt act gca atg aac tgc ttt ctc ctg gaa ttg cag} gtt att tta cat																	
lys	val	thr	ala	met	asn	cys	phe	leu	leu	glu	leu	gln	val	ile	leu	his	
53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	
gag tac agt aac atg act ctt aat gaa aca gta aga aac gtg ctc tac ctt																	
glu	tyr	ser	asn	met	thr	leu	asn	glu	thr	val	arg	asn	val	leu	tyr	leu	
70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	
gca aac agc act ctg tct tct aac aag aat gta gca gaa tct ggc tgc aag																	
ala	asn	ser	thr	leu	ser	ser	asn	lys	asn	val	ala	gln	ser	gly	cys	lys	
87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	
gaa tgt gag gag ctg gag gag aaa acc ttc aca gag ttt ttg caa agc ttt																	
glu	cys	glu	glu	leu	glu	glu	lys	thr	phe	thr	glu	phe	leu	gln	ser	phe	
104	105	106	107	108	109	110	111	112	113	114							
ata cgc att gtc caa atg ttc atc aac acg tcc tga----- plasmid-----}																	
ile	arg	ile	val	gln	met	phe	ile	asn	thr	ser	Stop						

Figure 4

cDNA sequence of clone 6 showing amino acid translation, homologous to gene bank sequence number U14332. The translation to amino acids is indicated by green, small standard abbreviations below cDNA sequence.

Bases and amino acids highlighted in red indicate the deletion found in the cDNA sequence of clone 8. This deletion, between amino acids 33 and 48 inclusive, is in-frame with both the recombinant start codon and the natural sequence.

3.3 Hydropathicity

In figure 5 below, a Kyle and Doolittle predictive plot of Hydropathicity of the primary structure of the cloned product sequence shows that the amino acids deleted from the standard IL-15 approximately correspond to a major hydrophobic domain.

The significance of this deletion is unknown. It may be an artefact of the cloning process which happened to be in frame and coincide with a hydrophobic domain of the primary protein structure. However, it is interesting to speculate, as later experiments described below show, that the translated deletion mutant retained the ability to competitively bind the IL-15receptor in an antagonistic mode when in competition with the native protein. The lower Hydropathicity of IL-15-48 mutant may lead to greater solubility compared to that of the native sequence.

Kyte and Doolittle Hydrophobicity Plot

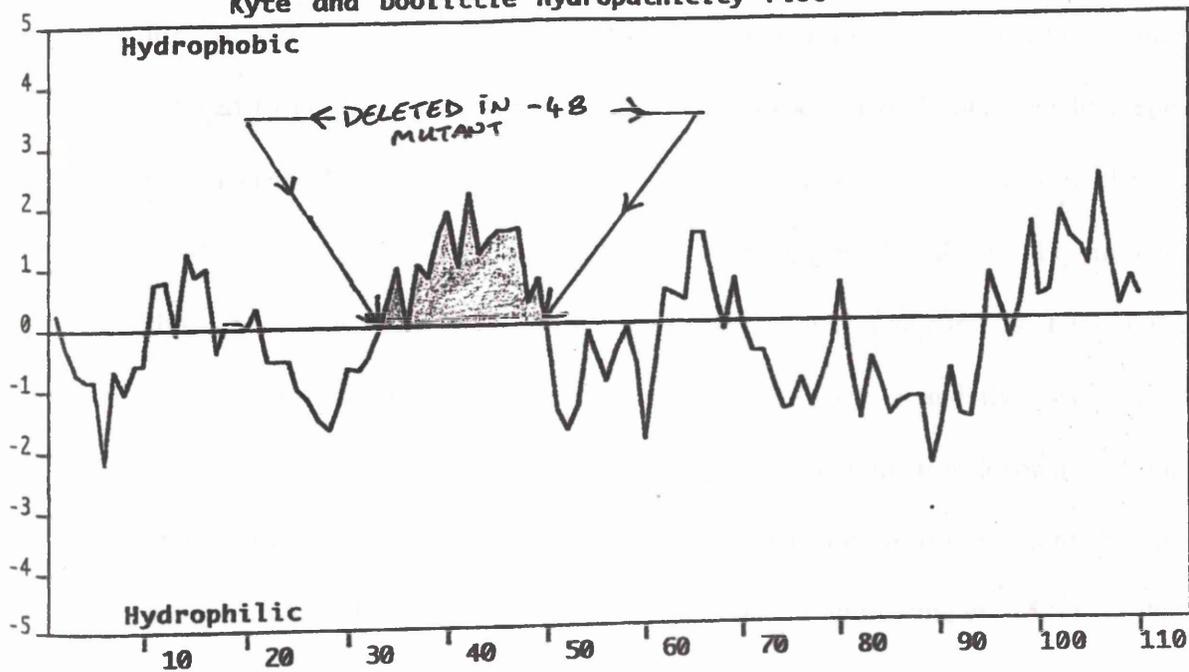


Figure 5

Doolittle plot of the relative hydrophobicity of the protein predicted by the sequence derived from clone 6

3.4 Protein expression

Sequenced clones containing IL-15 or the IL-15-48 were directionally sub-cloned into the Qiagen expression vector pQE30 and transformed into E.coli (M15 strain) by the methods described in chapter 2. Following the manufacturer's protocol for fast colony screening (see chapter 2), cultures were selected which could be induced to produce recombinant protein of the expected molecular weight range for recombinant IL-15, i.e. 14 -15 kDa. The small overnight cultures were pelleted and lysed in SDS sample buffer and run out on a 15% polyacrylamide gel. Figure 6 below shows a coomassie stained 15% polyacrylamide gel with five cultures. These cultures were sampled pre-protein production-induction with IPTG and then two and four hours after induction. The fifth culture is a control culture transformed with empty pQE30 vector. It can be seen that there is heavy induction of proteins after 2 hours at approximately 13kDa in the IL-15-48 cultures and at approximately 15kDa in the IL-15 cultures when compared to the non-induced samples of these cultures. The fifth culture, the vector control shows no such induction. Treatment of the samples is described above in the materials and methods. Briefly, pellets were dissolved into 0.5ml SDS sample- buffer and heated for 7 minutes at 100⁰c. Samples were loaded at 20ul per well into a 15% polyacrylamide gel. Gel was stained with coomassie blue and vacuum dried

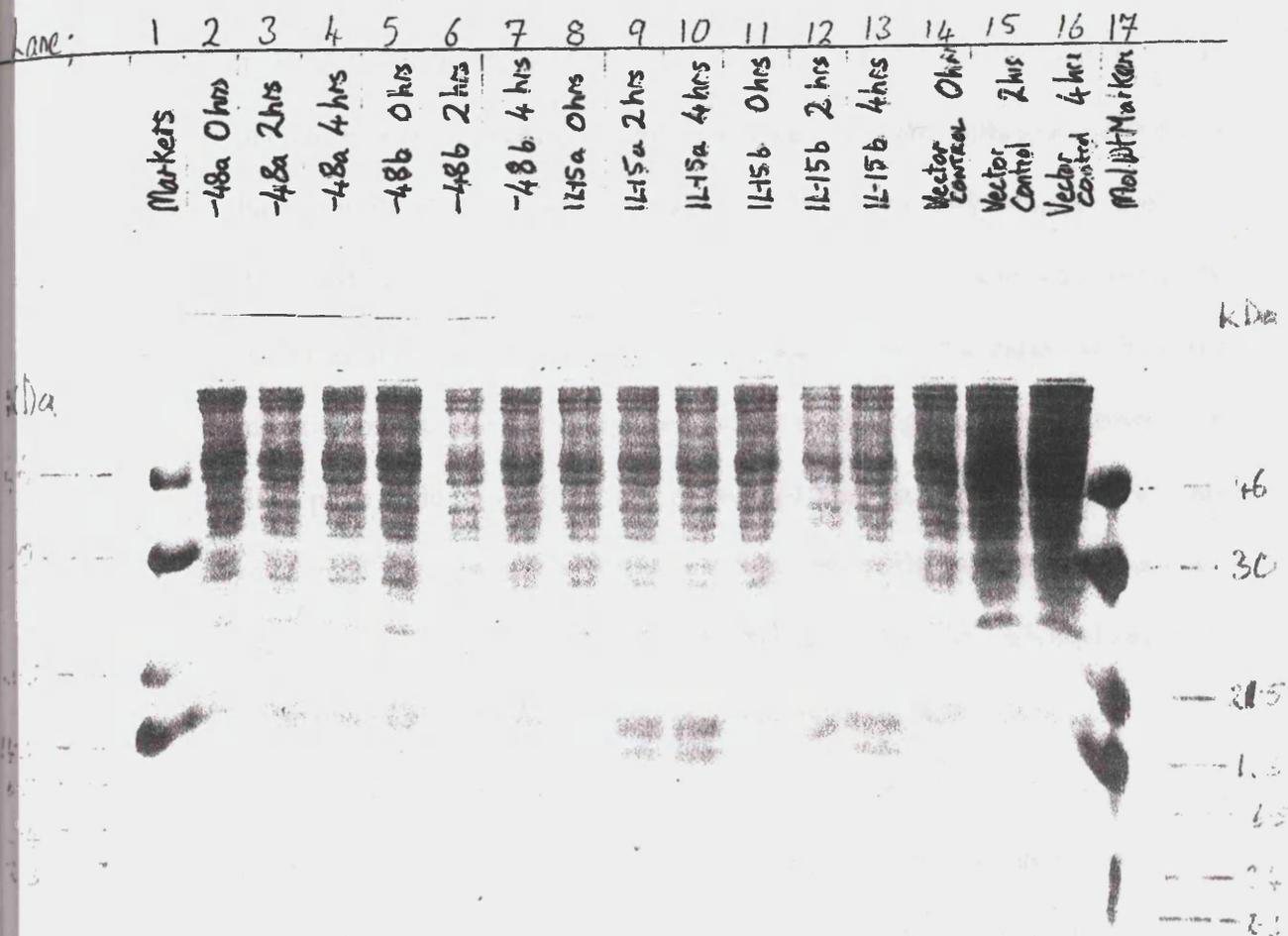


Fig 6 Polyacrylamide gel electrophoresis separation of total protein from five pQE30 transformed *E. coli* cultures including a vector control transformation. Lanes 1 & 17 Molecular weight markers. Lanes 2-4 & lanes 5-7 two cultures transformed with pQE30/IL-15-48. Lanes 8-10 & 11-13 cultures transformed with pQE30/IL-15 and lanes 14-16 control culture transformed with an empty pQE30 vector. The first lane in each group was sampled before protein induction by 2mM IPTG and the second and third lanes of each group are sampled at 2 and 4 hours post induction respectively. Protein was induced at 2 and 4 hours in all IL-15-48 and IL-15 cultures at approximately 13 and 15 kDa respectively. No protein induction is seen in the vector control transformant culture. 2ml culture samples were taken and pelleted by centrifugation and pelleted by brief centrifugation.

Having identified colonies producing inducible protein in the predicted molecular weight range, larger size colonies of both IL-15 and IL-15-48 mutant expressing cultures were established to gain sufficient protein for further analysis and characterisation. The protocols and volumes used are described in chapter two. It was found that the protein yield when using the Ni-NTA affinity purification system was much lower than expected from the intensity of the staining in induced cultures when protein was extracted by SDS page buffer. (Fig 5) It was considered that the recombinant IL-15 was only slightly soluble in the 8M urea "Buffer B" of the standard protocol and therefore a more stringent lysis and washing procedure was adopted using the Guanidium Chloride "Buffer A" system described in chapter 2.

This increased the protein yield from below 100µg/ml to levels exceeding 300µg/ml. Purity was also increased but solubility was only achieved in Phosphate Buffered Saline (PBS) at pH 7.4 when 30% glycerol was added to the dialysis buffer. The IL-15 -48 deletion mutant, however, was soluble in the urea buffer at 400µg/ml and remained soluble when dialysed into PBS/Glycerol 30%. This again, perhaps due to the observed missing hydrophobic domain seen in the kyle Doolittle plot on pg 60. Because glycerol and traces of urea are believed to interfere with some protein assays, such as the Bicinchoninic Acid (BCA) Assay, the wider spectrum Coomassie Plus assay, (Pierce Reagents USA) was used to determine protein concentrations of recombinant IL-15 and -48 mutant. An example of this assay is shown below in figure 7

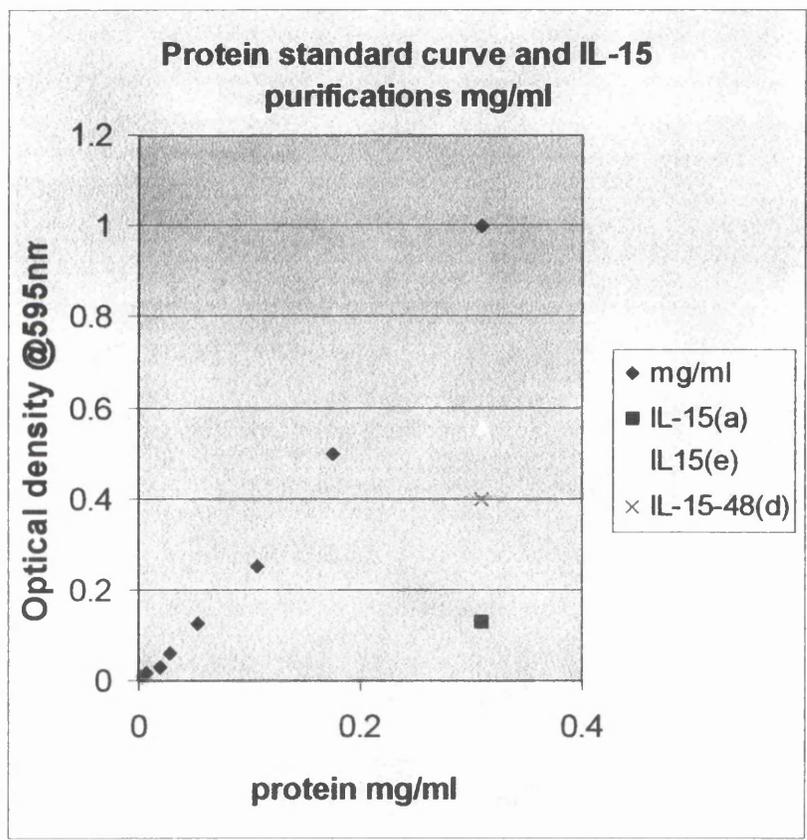


Fig. 7 A typical concentration determination of recombinant IL-15 and IL-15 -48 mutant. A standard curve was constructed by serial dilution of Bovine Serum Albumin in PBS/30% glycerol dialysis buffer. 100µl of dialysed sample was added to 5000µl Coomassie plus reagent, vortex mixed and Optical Density (OD) measured by spectrophotometry at 595nm against a reagent/buffer control.

3.5 Western Blotting

In order to confirm further the identity of the putative recombinant IL-15 or its variant IL-15-48, a western blot was made using IL-15 and IL-15-48 samples and appropriate controls. Below in figure 8A, the samples were run out on one of a pair of duplicate 15% polyacrylamide gel. The other of these gels, figure 8B, (Same batch of gel mixture and samples run simultaneously in a dual gel apparatus) was transferred and western blotted according to the protocols described in chapter two. When the gel was blotted with a commercial anti murine IL-15 monoclonal antibody, the characteristic double bands, as noted in all the IL-15 purification performed in relation to this thesis, stained intensely when visualised by ECL-auto-radiography. There was no staining of the control samples and it was concluded that the antibody was detecting recombinant murine IL-15 and a mutant IL-15-48, clearly seen to have a lower molecular weight in both the coomassie and western blotted gels. Due to time elapsed between write up and experiment, the gels no longer were readable and were discarded therefore no data can be shown other than the recorded observations above.

3.6 Recombinant murine IL-15 Binding assay

The western blot evidence in conjunction with the coomassie-blue stained gel molecular weight evidence (Fig 6) strongly suggested that the purified recombinant protein was murine IL-15.

To further confirm the identity of the presumptive recombinant IL-15 and mutant IL-15-48 molecules, an ELISA type assay was designed to establish any binding activity of the recombinant molecules to a soluble murine IL-15 receptor alpha protein cloned and expressed by this laboratory.

Half of the wells in an Immulon 4 (Corning Costar, Cambridge, MA , USA), 96 well plate were coated at 10µg/ml with recombinant IL-15Ra and the remainder with recombinant murineIL-18 at the same concentration. After overnight incubation and washing and blocking as described in the ELISA protocol, a duplicate set of wells in the receptor and IL-18 coated part of the plate, were incubated with serial dilutions of either recombinant IL-15 or IL-15-48 mutant. These were added at a starting concentration of 1mg/ml and serially diluted across the plate and incubated @37⁰c for 2.5 hours. The presence of IL-15 or mutant IL-15-48 bound to the bound IL-15-receptor alpha or IL-18 control was detected by a sheep anti-IL-15Rα antibody (SAPU, Scotland) and development of the assay with the chromogenic substrate TMB-microwell (KPL, Gettysburg, Maryland USA).

Figure 9 below reveals binding of IL-15 and IL-15-48 to the receptor but little binding was evident in the IL-18 control wells.

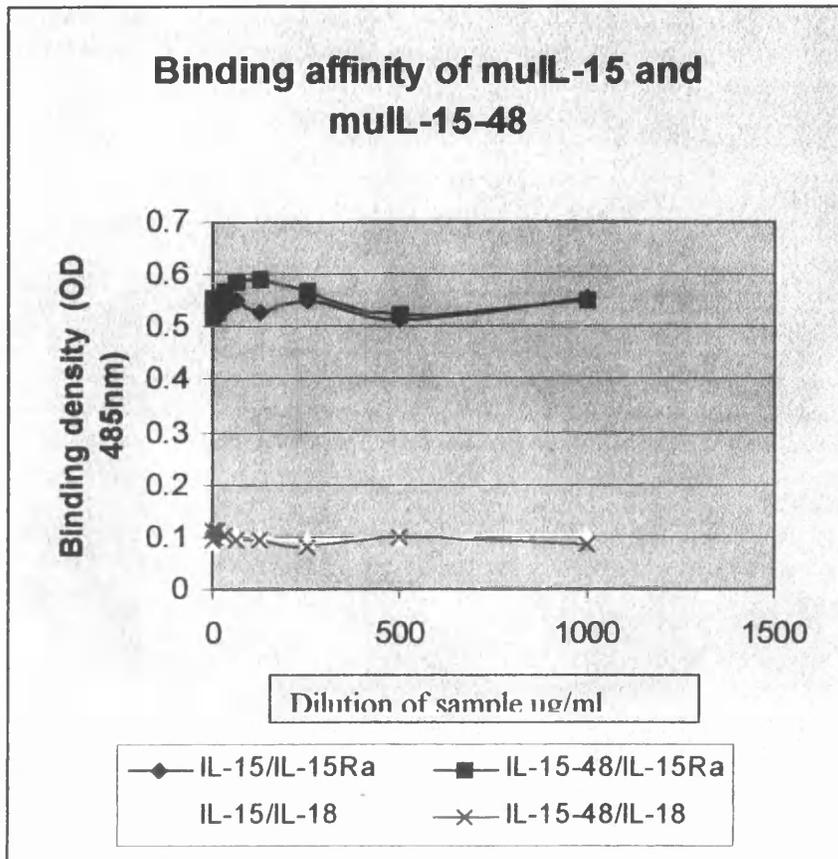


Figure 9 Shows results of ELISA to determine binding affinity of purified samples of rmuIL-15 and rmuIL-15-48 mutant to the soluble IL-15Receptor alpha. Rmu IL-18 was used as a control molecule. The recombinant IL-15 and IL-15 mutant exhibited strong binding to IL-15Ra at all concentrations tested. However a weak binding of these molecules was observed to the control. The significance of this was not realised at the time but with hindsight it may have indicated some LPS contamination of the samples following purification.

Having demonstrated that the recombinant IL-15 and mutant were both able to recognise the IL-15 receptor, it was necessary to ascertain if the recombinant protein had IL-15 bioactivity.

Characterisation of Bio-activity of recombinant murine IL-15

3.7 CTLL proliferation assay.

The CTLL assay is based upon a murine Cytotoxic T Lymphocyte Line cell line, which is commonly used as an IL-2 bioassay (82). This is because these cells have an absolute dependency in a cell culture medium for exogenous IL-2 for proliferation and survival. However, this cell line is also responsive in the same way to IL-15 as demonstrated by Grabstein et al (10), as IL-15 and IL-2 share many of the same cell proliferative activities.

CTLL cells were cultured for three days following recovery from frozen storage (-80°C), in RPMI with penicillin and streptomycin, 10% foetal calf serum and L-glutamine and in the presence of 10ng/ml recombinant IL-2 (Genzyme, UK). The cells were then cultured for a further three days in the absence of IL-2 and washed twice in fresh RPMI pipetted onto a 96 well plate at 5×10^4 cells per well. Prior to addition of the cells, recombinant murine IL-15 and IL-2 were serially diluted, starting from 10 and 250 ng/ml final concentration, across the triplicate, 6-well sections of the plate in volumes of 100µl/well. The recombinant-mutant molecule, murine IL-15-48, was added to three of the IL-15 groups of wells at 60, 125 and 250ng/ml in each of the selected wells. These were pre-incubated with the IL-15 dilutions. In one set of wells, IL-15-48 mutant was added alone. Cells were cultured in 5% CO₂ @37°C overnight and then pulsed with 1µCi tritiated-thymidine per well. Cells were harvested and proliferation activity was measured by beta scintillation counting in counts per minute (Wallac Beta Counters, USA). The induction of

proliferation of the CTLL cells by murine IL-15, murine IL-2 and murine IL-15-48 mutant is shown below in figure10.

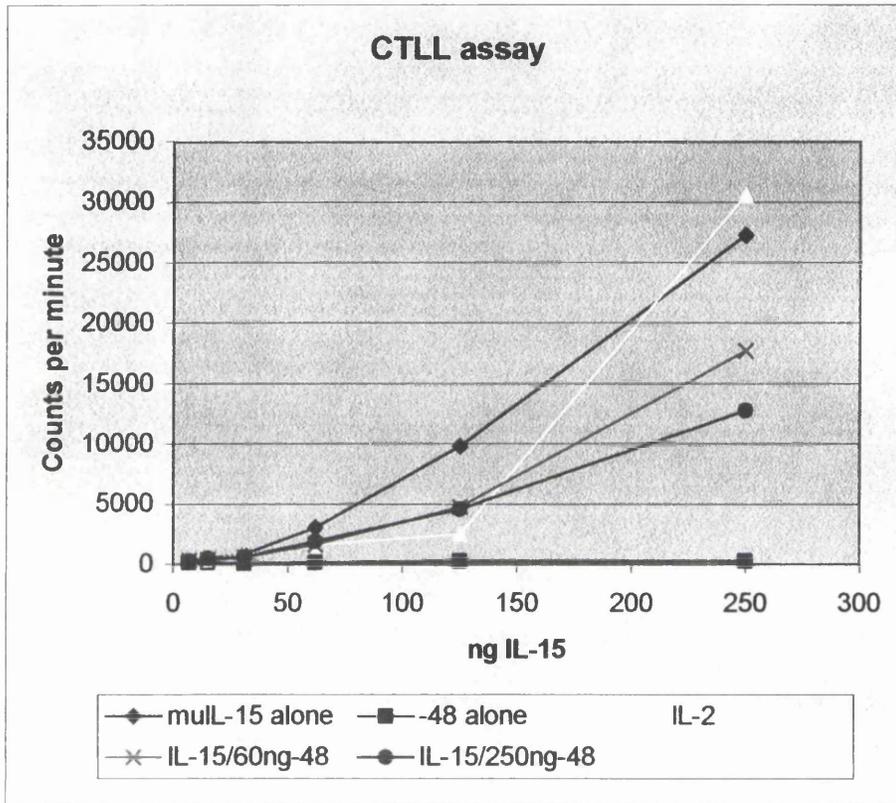


Figure 10

When CTLL cells were incubated with recombinant murine IL-15 or IL-2, a dose dependent proliferation curve is seen. If the murine IL-15 is pre-incubated with mutant murine IL-15-48 before adding to the cells, a reduction of proliferation activity is seen. The mutant molecule itself demonstrated negligible proliferative activity.

Figure 10 shows that the CTLL cells were induced to proliferate by the recombinant murine IL-15 in the absence of IL-2, indicating that it had IL-15 bioactivity and therefore, further confirming the recombinant molecule as murine IL-15. It has been suggested that the identity of this molecule as IL-15 could have been strengthened by the addition of further controls such as IL-2 receptor or neutralising antibody in the assay. The mutant molecule, IL-15-48 had a defined, in-frame deletion mutation of 16 amino acids. It was interesting to see if this deletion affected the ability of the mutant to bind its receptor or indeed had any IL-15 bioactivity. In the previous section, the ELISA assay (figure 9) demonstrated that the mutant could bind to the IL-15 soluble receptor with similar affinity to the standard recombinant murine IL-15. Here in figure 10 however, where the normal IL-15 could induce proliferation measured by cpm >30000, the mutant molecule induced little or no proliferation above the cell only background count of 150cpm.

This implied that the site of interaction between IL-15 and its receptor could be affected by the deletion.

In order to investigate the possibility that IL-15-48 could act as an antagonist to IL-15, a sample of IL-15-48 was added to murine IL-15. In this assay, 250ng/ml recombinant IL-15 had induced proliferation > 27000cpm. However in wells where the dilutions of IL-15 were pre-incubated with mutant IL-15-48 protein prior to cells being added, there was a suppression of proliferation. This suppression was present at 60ng/ml IL-15-48 and at 125ng/ml. At 125ng/ml IL-15-48 mixed with 250ng/ml IL-15, there was almost 50% suppression. At higher ratios of IL-15-48 to recombinant murine IL-15 (500 or 250ng/ml IL-15-48 with dilutions of muIL-15 250ng/ml), the

suppression effect was lost. (Not shown in figure 10) This may have been due to precipitation of the IL-15-48 protein at these concentrations in the glycerol free RPMI cell growth medium. However, suppression was demonstrated when the cells were incubated by IL-15-48 at concentrations of 60 and 250ng/ml.

The low stimulation activity of recombinant IL-15 relative to human and commercial products was investigated in the following experiment. Briefly, 200ng of recombinant murine-IL-15 was diluted into 1ml of either RPMI or PBS. Then 12ul of each of these suspensions was removed following mixing and settling and loaded into a PAGE gel, as described in the materials and methods. The relative amount of recombinant murine-IL-15 remaining in each of the samples was compared by transfer of the electrophoresed proteins to a western blot. The blot showed that there was relatively little recombinant IL-15 in the RPMI compared to the PBS/glycerol sample. If the murine IL-15 was indeed precipitating in physiological solution, this might explain the disparity noted above between the activities of the recombinant IL-15 produced here and the commercial murine-IL-15 product in the CTLL cell bioassay. Due to degradation of the gel autoradiograph, this data is not illustrated.

3.8 The generation of anti-IL-15 antibodies.

Polyclonal, anti-murine IL-15 antibody generation was carried out according to the protocol described in the methods and materials section 2.20. Briefly, a white New Zealand rabbit was immunised with recombinant murine IL-15. Subsequent booster injections of mu-IL-15 were made at 3-week intervals, as described in the methods and materials section 2.6. The anti-IL-15 antibody titre of the test-bleeds was determined two weeks after each IL-15 booster injection by coating a 96 well culture plate with wells containing 50µl of either murine IL-15 or murine IL-18 at a concentration of 1mg/ml. The plate was then incubated overnight at 4⁰C. A serial dilution of a 1/100 dilution of IL-15 injected rabbit serum was then added across the plate. Detection of antibody binding was by incubation with 1/1000 anti-rabbit HRP conjugate, washing and then with 1/2000 anti rabbit HRP conjugate, followed by washing and incubation with 1/2000 Extravidin peroxidase. The assay was developed with TMB-microwell (KPL, Gettysburg, Maryland USA). An example of an ELIZA demonstrating binding of the Rabbit anti-serum to mu-IL15 and not to IL-18 is shown below in figure 11B below where 1/100 dilution of the serum was made into PBS, as described in section 2.20 of materials and methods and briefly above.

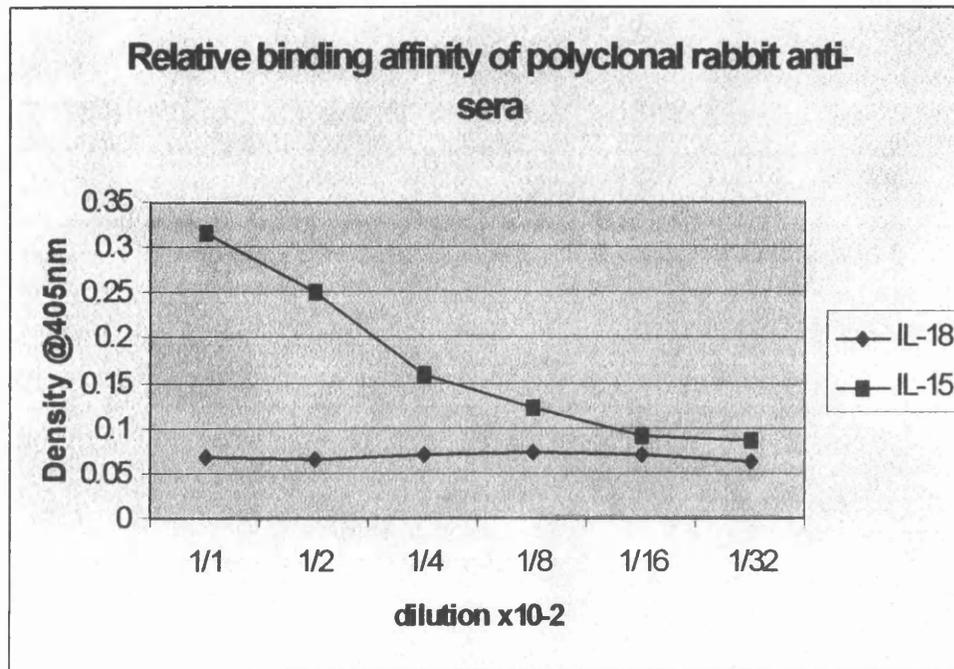


Figure 11B Rabbit polyclonal anti sera was found to have binding affinity for muIL-15 but only background readings for IL-18 when tested by ELISA. Duplicate results only thus no error bars can be shown. Initial dilution of the anti sera was 1/100 (dilution 1) and subsequent dilutions were doubling dilutions. Polyclonal anti-mu-IL-15 titre indicated at convergence with background at 1/1600 A pre bleed serum was taken prior to inoculation of the rabbit with muIL-15 there is no data available for this serum sample. However, the graph demonstrates dose dependant binding effect approaching a similar level of anti IL-15 versus IL-18 which indicates specificity for its ligand. A second rabbit was inoculated but did not develop a significant antibody titre.

After the fourth booster, the serum anti-IL-15 titre was indicated as 1/500000 (not shown) and this crude serum was IgG enriched by affinity purification on a Protein A column (Sigma, Poole, Dorset, UK) and recovered by dialysis against PBS. The manufacturer's recommendations were followed, as described in materials and methods section 2.20, only omitting the addition of azide as the anti sera was to be used in an in-vivo experiment.. The anti-IL-15 activity of the purified serum was measured by the method described above and the results are shown below in figure 12. From this figure the anti-IL15 titre of the serum was estimated as 1/32000.

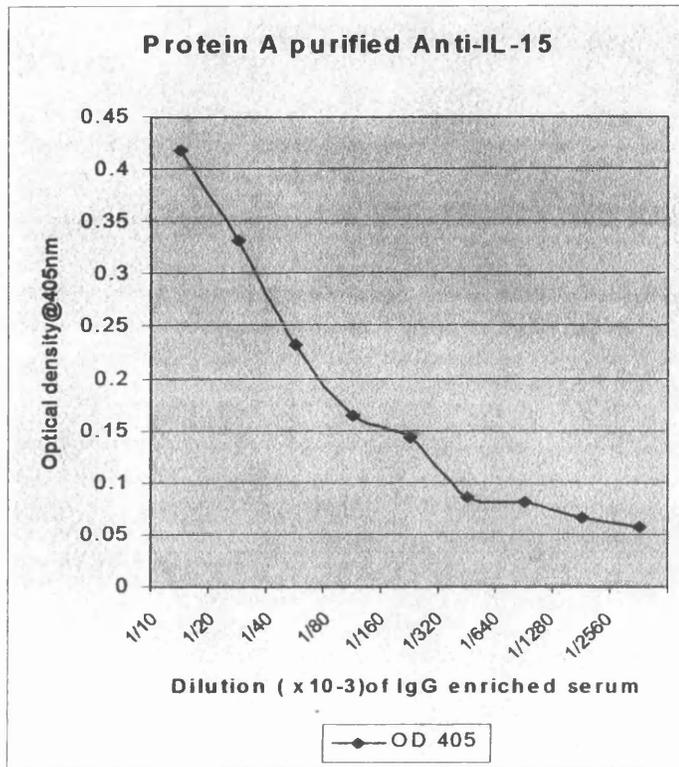
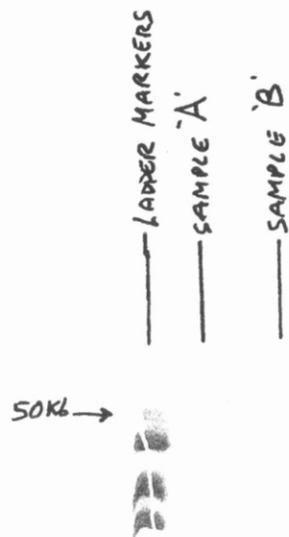


Fig 12 Anti IL-15 titre of the fifth bleed. Serum titre =1/32000 following purification against Protein A column. This is reduced from 1/50000 prior to IgG enrichment

The anti-IL-15 IgG rich sera were then tested for protein concentration using the Bicinchoninic Acid Assay (Pierce) described in materials and methods section 2.16. Briefly, a Bovine Serum Albumin (BSA) standard curve was constructed and duplicate samples of the purified anti IL-15 serum were measured against the standard curve. The concentration of the IL-15 serum IgG samples was 140 μ g/ml. protein A purified samples were loaded onto a PAGE gel apparatus to determine molecular weight of the antibody fraction. Figure 13 shows that a clean major band in the region of 50kDa could be observed. This is the molecular weight expected for IgG molecules, confirming a successful purification of IgG. It has been learnt subsequently that silver staining could enhance the definition and sensitivity of this gel.



α IL-15 Pst A Purified

Fig 13b Two (A & B) samples of Protein A purified rabbit anti-murine IL-15 serum separated on a 15% polyacrylamide gel. The majority of protein in the samples corresponds to the expected weight for IgG molecules on denaturing gels of around 50kDa.

3.9 Purification and assay summary

3.9 Purification and assay summary

The restriction enzyme digestion analysis, Sanger sequencing, Western blotting and molecular weight evidence noted above indicated that the products obtained from cloning were indeed recombinant murine interleukin 15 and a novel variant, IL-15-48.

Commercial muIL-15 (Immunex, Seattle, USA), which became available following the start of these experiments, was able to induce maximal stimulation of the CTLL cell proliferation indicated by 390000 counts per minute (cpm) as a result of adding just 0.01ng/ml IL-15. The recombinant muIL-15 produced for this thesis was also assayed and CTLL stimulation was attained, indicating bioactivity of this non-commercial recombinant muIL-15 molecule. However, it required concentrations between 125ng/ml and 2.7µg/ml to induce comparable stimulation (370000 counts per minute, a decrease in activity of at least 10000-fold). This indicated that there was much lower bioactivity in the muIL-15 produced here when compared to the Immunex product. One possibility is that this may be attributable to the reduced solubility of the laboratory recombinant muIL-15 in the physiological solution used in the assays.

The CTLL assay testing stimulation of recombinant muIL-15 also demonstrated that the IL-15-48 mutant had no proliferation induction activity and, moreover, IL-15-48 was able to competitively suppress proliferation of CTLL cells when it was co-incubated with the recombinant muIL-15 at the concentrations listed above in figure 10. This suppression occurred despite IL-15-48 being able to bind to the IL-15 receptor. It is interesting to note that there is an analogous protein, IL-1 receptor antagonist (IL-1Ra). This variant was found to have binding activity for the type 1 Interleukin 1 receptor. However it was also discovered that IL-1Ra could not

activate the receptor and, furthermore was shown to have a suppressive effect in some murine models of inflammatory disease. (83)

Finally, the testing of semi-purified anti sera obtained from a rabbit previously inoculated with our murine recombinant IL-15 resulted in purification of IgG enriched antibodies with anti-IL15 activity.

Results for Murine models of Inflammation

3.10 Results observed in collagen induced arthritis, treated with murine IL-15 – 48bp mutant and rabbit anti-mouse IL-15 antisera

Two experiments were set up to investigate the role of IL-15 in inflammation. It was noted above that the recombinant IL-15-48 mutant exhibited binding to the IL-15 receptor alpha sub-unit with similar affinity to the standard recombinant murine IL-15. It displayed no sign of proliferative activity in the CTLL assay but did appear to act as an antagonist to IL-15 induced CTLL cell proliferation. In the light of these characteristics, an experiment was set up to investigate the possibility that this IL-15 – 48 mutant might modify the progress of the Murine model for collagen induced arthritis. This model is described in chapter 1 section 1.4 and uses the protocol described in section 2.21 of chapter 2. Briefly, 10, 9 week old, male DBA/1 mice were sensitised with 200µg bovine collagen suspended in 100µl Freund's Complete Adjuvant (Difco, USA) injected intradermally on day 1. Subsequently, these mice were challenged with 200-µg collagen in Freund's incomplete adjuvant by intraperitoneal injection on day 21. Also on day 21, five of the mice received a therapeutic dose of IL-15-48 and five received a control dose of Human Serum Albumin (HSA). These injections were 300µg in 200µl PBS/Glycerol 40% and were given daily, starting on day 22 for 14 days. Measurement and observation of the mice was carried out daily using the assessment code described in chapter 2 section 2.21 to define severity and incidence of arthritis.

3.11 Body weight measurement

Body weight was measured to 1/10th gram daily for IL-15-48 and control groups following collagen challenge on day 22. In fig 14 below, it can be seen that there was no apparent difference in the weight loss pattern between the two groups. Both groups lost weight at the same rate, indicating that the IL-15-48 molecule conferred no advantage compared to the HSA-control during the duration of the experiment with respect to weight loss.

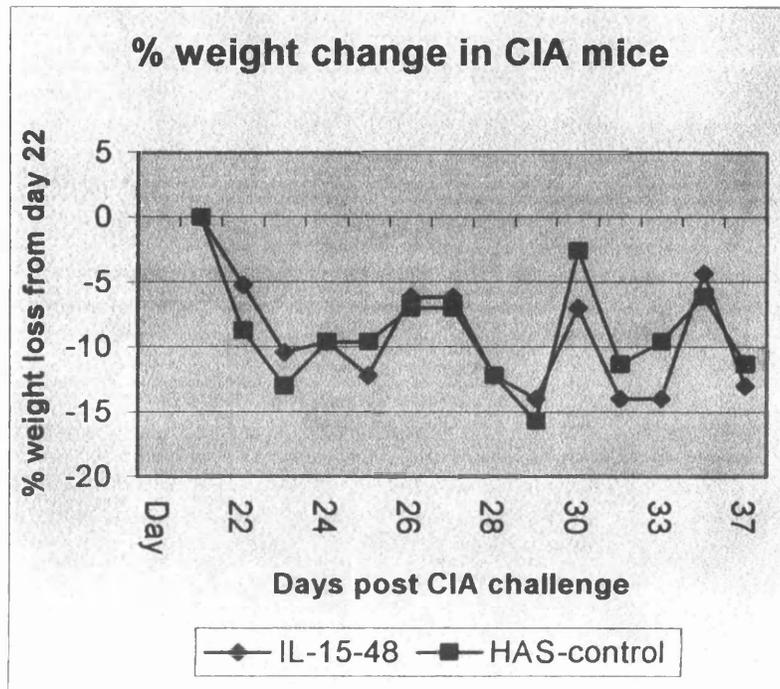


Figure 14
Change of body weight expressed as % weight change per day per mouse.

3.12 Severity of arthritis

The arthritis score of each mouse was determined by measuring footpad thickness by dial calliper (Kroeplin, Munich, Germany). As described previously in chapter 2, each limb was assessed for symptoms of arthritis according to the scheme 0= no arthritis, 1=Erythema (redness), 2=Erythema and swelling, 3=severe swelling and/or loss of limb function.

In figure 15 below, it can be seen that initially the IL-15-48 treated group showed a lower average score over the first 5 days of treatment, ranging 0.0-0.2 compared to the HSA control group with scores of 0.2-1.0 for the same period. The difference, however, was very small and the two groups were not distinguishable after day 27. Even though there was an apparent difference at day thirty-four. When these results were tested by the Mann Whitney U test, there were no significant data at any time point ($p = >0.05$).

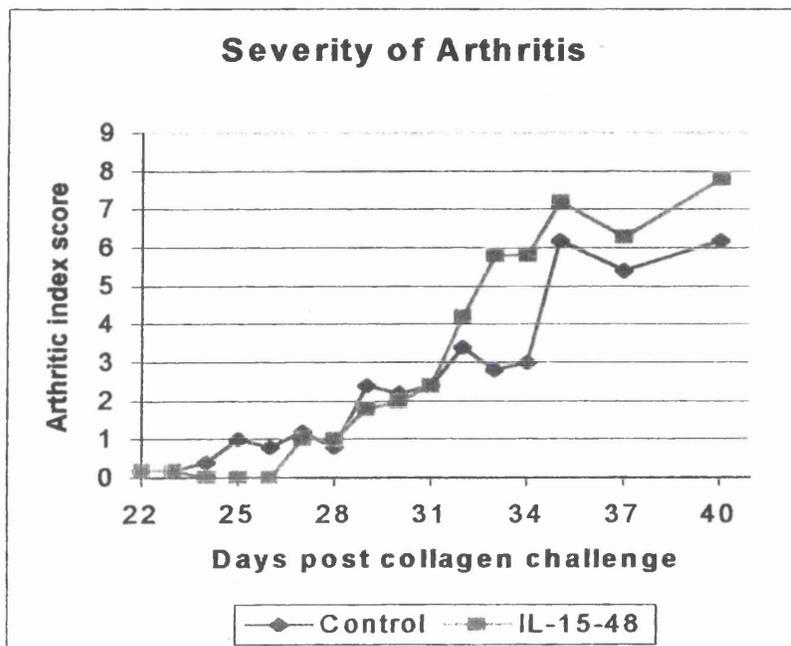


Figure 15

The mean arthritis severity score per group per day is presented here. No statistical difference was demonstrated between the two groups

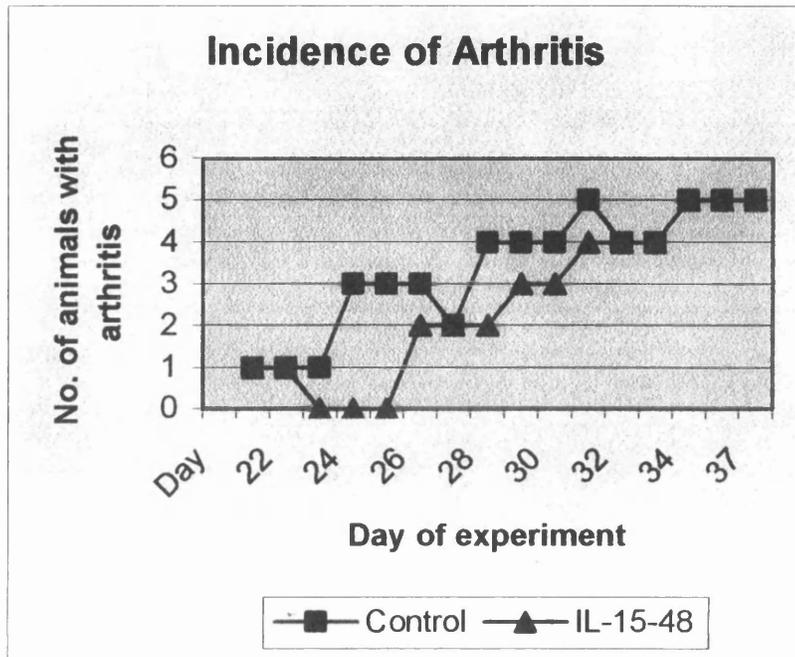


Fig 16

Graph of the number of animals with symptoms of arthritis in each group per day. A trend for lower incidence is consistent but no statistically significant data points demonstrated.

13.14 Treatment of Collagen arthritis mice with rabbit anti mouse IL-15 anti sera.

Targeting certain types of immune-cell and soluble factors such as TNF- α has been shown to modify the CIA model, as discussed in the introduction section, 1.4.1.

Ruchatz et al successfully modified the progress of the CIA by treatment with soluble, murine recombinant IL-15 receptor (Ruchatz 1998).

It follows that neutralisation of endogenous IL-15 by anti-IL-15 antibodies might also modify the progress of collagen induced arthritis. Therefore, in a similar experiment to that described above, fifteen, male, 12 week old, DBA1 mice were injected with collagen as in the previous experiment and as described in the materials and methods. These mice were challenged with a further collagen injection in the same manner as the experiment above (except the challenge was carried out on day 28 not 21.) Following the collagen challenge, eight of the mice were injected daily for 7 days with polyclonal Rabbit anti-murine IL-15 serum which had been IgG enriched on a protein-A column The remaining 7 mice were injected daily with an irrelevant control antibody, normal Rabbit IgG, (Sigma). Both the anti-IL-15 and IgG control were injected intraperitoneally @ 250 μ g antibody in sterile PBS. Observations were recorded daily as in the previous experiment and are presented below in figures 17 &

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13.15 Body weight measurement

Body weight was measured to 1/10th gram daily for both anti-sera and control groups following collagen challenge on day 28. The weight loss profile of this experiment was similar to the previous IL-15-48 collagen arthritis trial in that there was no apparent difference between the group treated with anti-IL-15 or the IgG control group. These data are shown below in figure 17.

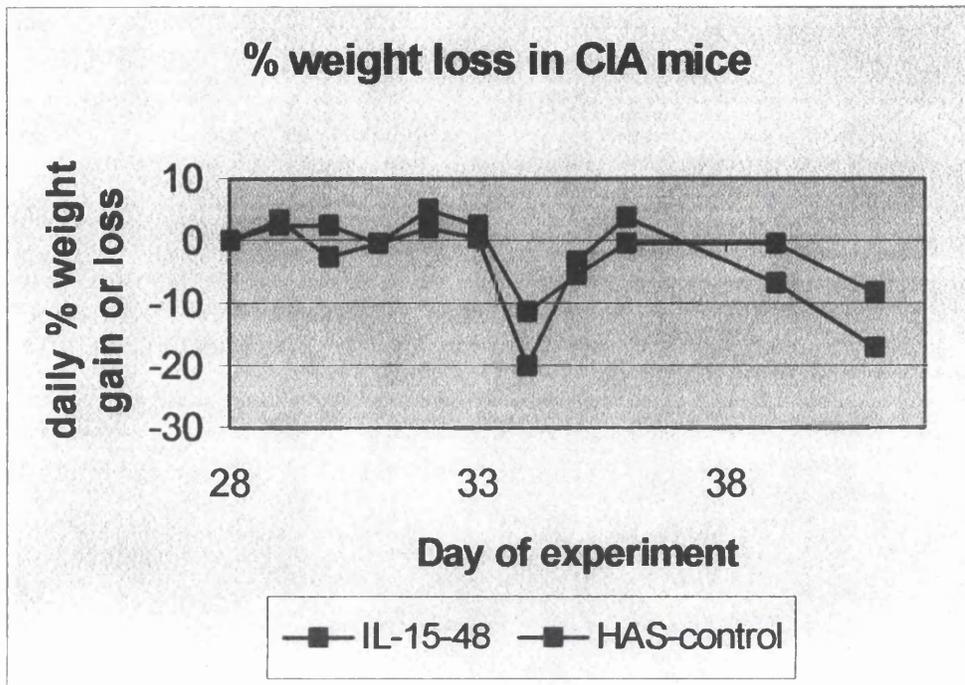


Fig 17 Body weight was measured to 1/10th gram daily. There was no apparent difference in percentage weight-loss during the experimental period between the two groups.

3.16 Severity of arthritis

The arthritis score for each mouse was determined by measuring footpad thickness by dial calliper (Kroeplin, Munich, Germany). As described previously, each limb was assessed for arthritis. At first a trend developed where the severity of the arthritis was lower in the treatment group than the control group. This occurred at most time-points until 11 days after the collagen challenge when the groups became indistinguishable. Although most data points demonstrate no significant difference, on day 32 and 36, the treated mice showed significantly less severe symptoms of arthritis. These data are shown below in figure 18.

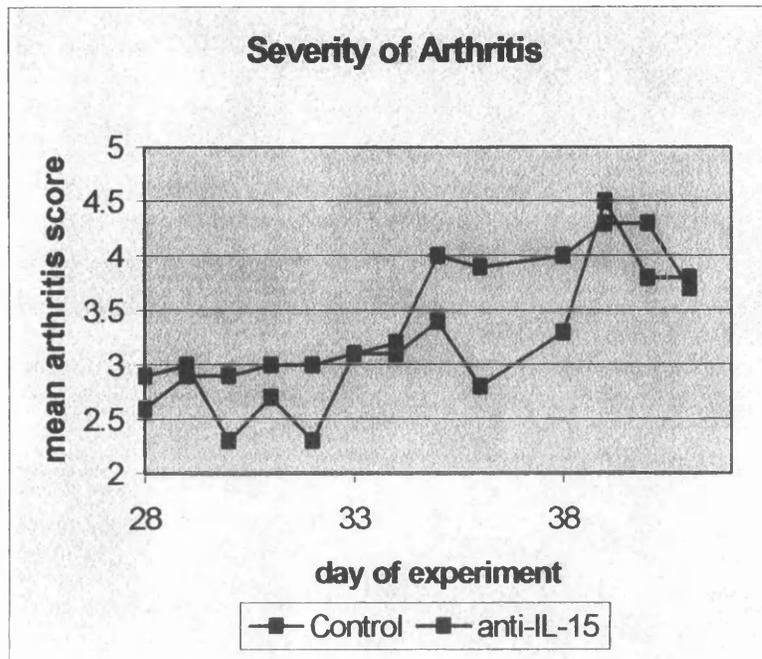


Figure 18

The arthritis score of each mouse was determined. There was a trend for lower severity of arthritis in the anti-serum treated group and this attained statistical significance on days 32 and 36 (*). $P < 0.05$ and $p < 0.05$ respectively, Mann Whitney U).

13.17 Incidence of Arthritis

Using the scoring system for arthritis mentioned above, the number of mice per group displaying any sign of arthritis was calculated daily. The graph of incidence of arthritis is shown below in figure 19, expressed as a percentage. Initially, there was no difference between the mice treated with anti-IL-15 anti-sera and the incidence of arthritis observed in the control group, although between the days 36 and 38, the anti-IL-15 treated group had a transiently lower incidence of mice with arthritis than the control group. However there were no significant data points when tested by the Chi square test ($p > 0.05$), shown below in figure 19.

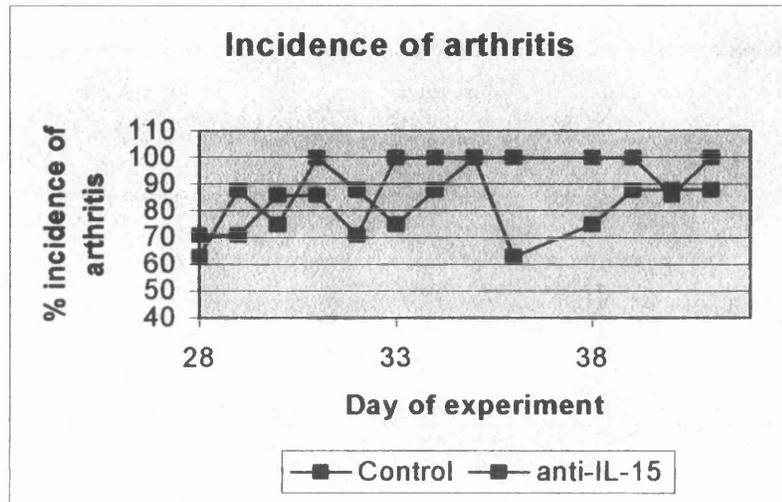


Fig 19

Graph showing the number of animals with symptoms of arthritis in each group per day. Figures are expressed as a percentage of the animals in the group. No significant data points are detectable. Measurements were not made prior to day 28.

13.18 Assessment of footpad swelling as an indicator of severity of arthritis

As described previously, each footpad was measuring by paw thickness with a footpad dial calliper (Kroeplin, Germany). The measurements in mm for each limb were totalled per group per day and are presented here as a percentage increase or decrease over the initial measurement on day 28. The percentage increase or decrease in footpad size as an indicator of severity and disease progress indicates that there was little or no difference between the placebo and the anti-IL-15 serum treated groups. There was no evidence of any advantage conferred by the anti-serum. When the two groups were compared by Mann Whitney U analysis, there were no significant data points ($P = >0.05$). The significance of the reduction in footpad thickness between day 28 and 33, shown in figure 20, is unknown and highly likely to be due to measurement inconsistencies.

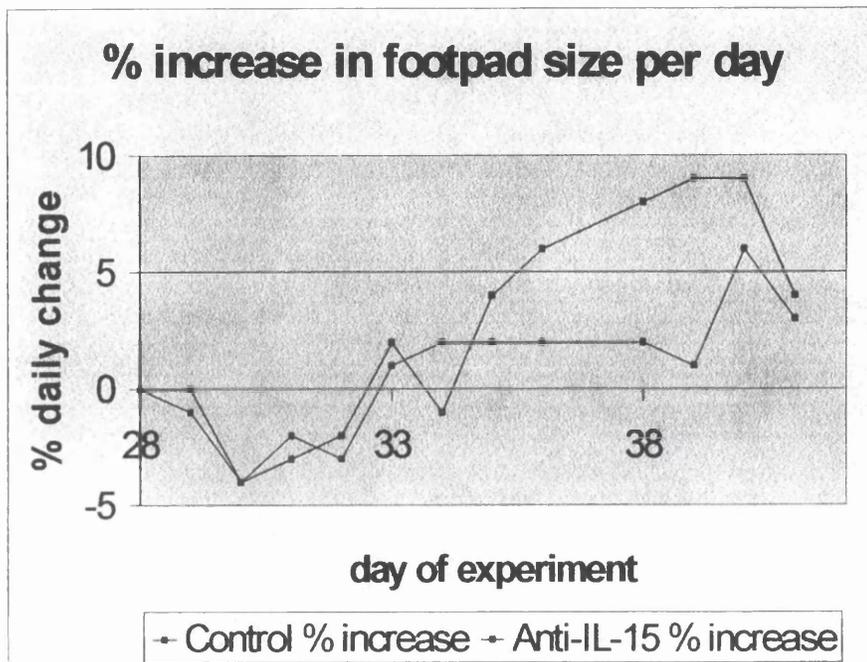


Figure20

The increase of footpad size as a percentage of that measured at day 28. The footpad size of the anti IL-15 and the control groups increased but the significance of this is unclear.

13.19 Summary of Collagen arthritis model experiments

The collagen induced arthritis experiments were carried out with the –48 IL-15 mutant or with rabbit anti-sera enriched in anti-murine IL-15 antibodies raised against the recombinant IL-15 produced as described above. At no point in the weight loss data was there any suggestion that the administration of either the IL-15 antagonist IL-15–48 mutant or the anti-IL-15 anti-sera modified the progress of weight loss associated with collagen induced arthritis in mice.

When severity of arthritis was determined by the scoring method referred to above, there was a transient trend for lower severity of arthritis in the antagonist treated group compared to that of the control group. The trend was reversed 5 days later and the antagonist treated group exhibited more severe arthritis than the control group until the end of the experiment. However, no significant data points were observed.

A trend for lower severity of arthritis was also demonstrated when mice were treated with the anti-IL15 anti-sera in the second experiment. This trend was sustained through the majority of time-points until the end of the experiment and obtained statistical significance on days 32 and 36.

The assessment of footpad swelling as an indicator of arthritis severity in the IL-15 anti-sera experiment did not indicate any trend for reduced severity in the treatment group compared to the controls, but again, no statistically significant data points were observed.

The measurement of incidence of arthritis in mice treated with the –48 antagonist reveals a similar trend for lower incidence of arthritis on 6 out of 16 days of the experiment compared to the control. The same determination for incidence of arthritis

in the experiment where mice were treated with IL-15 anti-sera also reveals a trend for lower arthritis incidence in the treated group compared to the controls. However, the trend is less defined here, becoming apparent only for three days at the end of the experiment with no evidence of statistically significant data points.

In summary, either the antagonist IL-15-48 mutant or the anti murine IL-15 anti-sera had theoretical potential to modify any inflammation characteristics associated with IL-15 in the CIA model. However, only non-statistically significant and very transient trends for lower arthritis symptoms were exhibited in mice treated with these compounds compared to their respective control animals.

The unknown nature of the antagonist –48 mutant makes it difficult to speculate about the possible reproducibility or value of the trend for lower arthritis incidence noted above.

The rationale for using the IL-15 anti sera as a treatment for CIA was based on its specific ability to bind IL-15 and not IL-18 (Figure 11). However, in a later analysis, a sample of the Protein A purified antiIL-15 anti sera used in the experiment above was further purified by passing it through an immobilised E.coli lysate (Pierce USA). Much of the IgG band seen after Protein A purification of the serum was removed (data mislaid), indicating that the majority of the IgG antibody recovered from the muIL-15 rabbit serum had anti-E.coli activity rather than the expected anti-IL-15 component. The concentration of specific anti-IL-15 antibody in the IgG fraction injected to the mice may therefore contribute to the inability of the anti-IL-15 serum to modify the CIA and explain the weak trends exhibited here compared to that reported by Ruchatz (1998) when treating the CIA with soluble IL-15Ralpha.

13.20 Conclusions

The problems associated with the purification of murine IL-15 including its low solubility in any kind of physiological solution and commensurate low proliferative activity in the CTLL bioassay rendered it unsuitable for use as a reliable agent in the investigation of the role of IL-15 in inflammation. The low activity per unit of the recombinant murine IL-15 compared to the commercially prepared murine IL-15 indicated that it was unsuitable for use in meaningful in-vivo or in-vitro experimentation.

Treatment of CIA mice with the antagonist IL-15-48 mutant established a transient, but non-statistically significant trend for lower incidence of arthritis compared to the controls. A similar trend was also transiently exhibited for less severe arthritis in the treatment group.

Treatment of CIA mice with anti-IL-15 enriched rabbit/anti-mouse anti-sera established a slightly stronger, but still non-statistically significant trend for lower incidence of arthritis compared to control mice. A weak but more sustained trend was observed for less severe arthritis in the treatment group compared to the controls, this again not reaching statistical significance.

The slightly more robust trends for lower incidence of and less severe arthritis in the anti-sera treated mice indicate that a statistically significant difference in arthritis severity and incidence might be observed if a more pure form of anti-IL-15 antibody could be administered to CIA mice in a similar experiment. Inclusion of an affinity purification step using an immobilised E.coli lysate should be an integral part of any future attempt to raise antibodies against a recombinant protein which itself had been cloned from an E coli expression system.

The indifferent result using mutant IL-15-48 and the anti -IL-15 antiserum raised against the recombinant IL-15 in the rabbit also made it unlikely that these reagents would provide informative results for the other murine models of inflammation planned for this thesis. The laboratory had recently cloned and expressed a soluble recombinant murine IL-15 receptor alpha molecule. This was readily purified from *E coli* and soluble in the near physiological solution 2mMNaCl/PBS. It was, therefore decided to use this receptor to further the investigation of the role of IL-15 in the LPS shock and the septic arthritis models of inflammation, as previously described in materials and methods, chapter 2.

Chapter 4

4.1 Treatment of LPS Shock with soluble murine IL-15 Receptor alpha protein

Chapter three described the treatment of collagen-induced arthritis (CIA) with the recombinant IL-15-48 mutant and subsequently with rabbit anti-murine, polyclonal anti-IL15 antibodies. The results of these two experiments implied a trend for less severe arthritis (figure 15) and a lower incidence of arthritis (figure 16) and to some degree, similar trends in the IL-15 anti-sera treated groups. In order to investigate further the role of IL-15 in murine inflammatory disease, further experiments in the septic shock (LPS) and septic arthritis models were set up (See Chapter 2, 2.22 & 2.23 for protocols). The active treatment was soluble, murine IL-15-Receptor alpha (IL-15R α), recently cloned and expressed by Holger Ruchatz of this laboratory. This molecule was shown to be readily purified from E.coli and soluble in the near physiological solution, 2mM NaCl PBS.

Ruchatz et al (43) reported that CIA was modified by both prophylactic and during-disease treatment with IL-15-receptor-alpha, providing strong evidence that IL-15 was an important cytokine in inflammatory disease and in particular, a disease model associated with tumour necrosis factor. is widely believed to be a pre-eminent cytokine in the pathogenesis of LPS shock. (51) (55) (76) (77) (53). The importance and central role proposed by Freundenberg (52) of macrophage action in LPS shock when considered alongside the role shown for IL-15 in inducing macrophage TNF- α production (35) suggest that IL-15 may have a role in LPS shock. The following experiments address that possibility.

Three experiments were undertaken to see if the course of LPS induced septic shock could be modified by neutralising endogenous IL-15 following injection of recombinant soluble IL-15 receptor alpha post LPS injection.

In a preliminary experiment, 48 young adult (10-12 week old) healthy, female Balb/C (Harlan, UK) mice were divided to 4 groups. Groups 1 and 2 were injected intraperitoneally (IP) with 18mg/Kg lipopolysaccharide (LPS). (Sigma Poole, Dorset.) Groups 3 and 4 were injected with 20mg/Kg LPS. Half an hour post LPS injection groups 1 and 3 (controls) were injected (IP) with 100µg Human Serum Albumin (HSA) suspended in 100µl HS- PBS. Groups 2 and 4 were injected intraperitoneally at a similar time interval with 100µg IL-15R α suspended in 100µl HS-PBS. The HSA and IL-15R α injections were repeated at 24-hour intervals for a total of three days. This protocol is summarised below in table 3.

Inject LPS (IP) Inject IL-15Rα or control as below (IP)		Inject IL-15Rα or control as below		Inject IL-15Rα or control as below		Terminate all groups
0Hours	12Hours	24Hours	36Hours	48Hours	60Hours	72 Hours
Group No.	LPS mg/Kg	IL-15Rα or PBS injections				
1	18	100ug HSA/PBS (Control)				
2	18	3x40ug IL-15R α (Treatment)				
3	20	100ug HSA/PBS (Control)				
4	20	3x40ug IL-15R α (Treatment)				

Table 3 Table detailing groups receiving LPS at 0 hours and treatment with IL-15R α or High salt-PBS control 24 and 48 hours. Observations of mice performed at 12-hour intervals. The experiment terminated at 72 hours.

4.2 Mortality in LPS shock mice

With the exception of 1 of the mice in group 3, all the mice that had been injected with the greater dose (20mg/Kg) of LPS (groups 3 & 4) succumbed to LPS induced shock within 72 hours. This indicated the maximal informative dose of LPS was exceeded at 20mg/Kg.

The mice treated with 18mg/Kg showed different survival characteristics between the treatment and control groups in mortality at 72 hours post LPS injection. As can be seen in figure 21 below, the HSA treated control group exhibited 75% LPS induced mortality but the IL-15R α treated group showed 25% mortality. $P=0.013$ (Kruskal-Wallace) indicating that IL-15R α was in this instance able to modify the ability of mice to survive LPS induced shock mortality. This protective effect was not apparent when the LPS dose was at 20mg/Kg. with hindsight, it would have been useful to establish a dose response curve to determine LD 50 to set this experiment against other author's work.

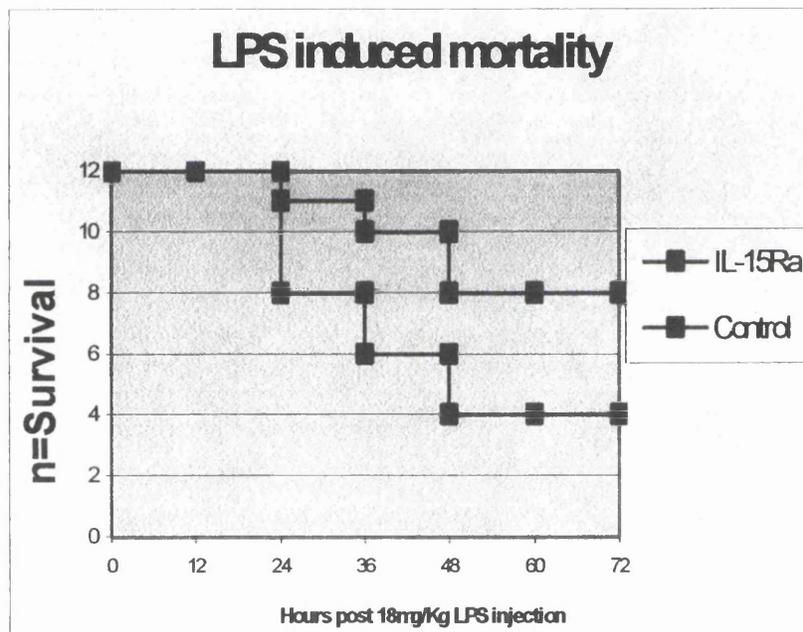


Figure 21
Number of mice surviving measured in hours following LPS injection. From 48 hours, 8 mice survived in the IL-15R α treated group compared to 4 of the control group. $P=0.013$ (Kruskal Wallance)

Observations in the above experiment indicated that the effect of LPS injection at 18mg/Kg when sustained over 5 days of the experiment was deleterious to the welfare of the mice leading to an extended period of severe illness. After discussion with Home Office veterinary staff, it was suggested that this was not consistent with best practice. Furthermore, the high doses of LPS used in the previous experiment and their associated morbidity prevented measurements other than mortality being made. One of the features of LPS shock is liver damage. In order to assess this useful biochemical indicator of disease severity, blood samples must be taken and this had proved refractive in the mice treated with high doses of LPS due to the hypotensive nature of LPS shock pathogenesis (78). Many authors, including Smith SR. (79) and Tsutsui H (80) have used a priming injection of heat killed *C. parvum* to sensitise mice to the effects of LPS. Whereas many of these experimenters were using lethal doses, the aim here was to reduce the dose of LPS used to induce shock symptoms to an approximately sub-clinical level and therefore reduce the severity of the LPS-shock symptoms and their associated high mortality. Therefore, the mice in the following experiment were injected intravenously with heat killed *Corynebacterium parvum* (*C parvum*) 1mg/mouse. This new method was expected to allow symptoms of LPS shock to develop at a lower severity induced by a 5 or 10 fold lower dose of LPS. One week later LPS was injected at 0.0, 2.5 or 5mg/Kg.

Briefly, in this experiment, *C parvum* was injected into the tail vein of 31 of 34 Balb/c mice. 7 days later, the mice were pre-treated with IL-15R α , PBS-control or nothing, as described in materials and methods (section2.22) and summarised below in table 4. Furthermore, mice were also segregated to receive two different doses of LPS in this dose ranging experiment and lastly, with or without *C parvum* priming.

Weight was monitored over the 24 hours post LPS challenge as an indicator of shock.

Liver damage, amongst other major organ failures is a common feature of the pathogenesis of LPS shock. This has been observed in most species and is reviewed (for humans) by Parrillo (80).

Therefore, as described in the introduction, three biochemical indicators of liver damage were assessed by measuring serum samples for evidence of increased levels of Aspartate-aminotransferase (AST), Alanine-aminotransferase (ALT) and Creatine Kinase. These enzymes were measured with the kind help of Dr Dennis O'Reilly, Glasgow University Hospital trusts. A Kodak slide forms a biochemical test for these markers of liver damage and whereas the role of the liver breakdown enzymes AST and ALT are common indicators of liver disease, the role of CK is not understood however it does seem to fluctuate with AST and ALT in all these determinations as seen below. This experiment also addressed the question of whether IL-15R α treatment could modify LPS shock when administered prophylactically compared to the therapeutic treatments of the experiments described earlier.

Prime Mice And inject IL-15R α or control as below	Inject IL-15R α or control as below	Inject IL-15R α or control as below	Inject LPS as below & terminate non-LPS groups	Terminate LPS groups			
Day1	Day2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Group No.	<i>C.parvum</i> mg/mouse	IL-15R α or PBS injections			LPS mg/Kg		
1 n=5	1	3x40mg IL15R α			5		
2 n=5	1	3x40mg IL-15R α			2.5		
3 n=5	1	3x40mg IL-15R α			0		
4 n=4	1	100ml HS-PBS			5		
5 n=4	1	100ml HS-PBS			2.5		
6 n=5	1	100ml HS-PBS			0		
7 n=3	0	0			5		
8 n=3	0	0			0		

Table 4.

Table detailing groups receiving *C. parvum* on day 1 and subsequent treatment with IL-15R α or High salt-PBS control on days 1,3 and 5. LPS was administered IP to the groups described above on day 6 and following 24 hours observation, the experiment terminated on day 8.

4.3 Mortality in C parvum treated LPS shock mice

As expected, and detailed in figure 22 below, mortality was not a major feature of this experiment and only 3 mice succumbed to LPS shock. This distribution exhibits no differences between the groups and does not attain statistical significance ($p > 0.05$ Kruskal Wallace). It could be noted that all the dead mice were from groups treated with LPS. (One each from the groups of mice treated with IL-15R α +CP+5mgLPS, those treated with PBS+C/P+5mgLPS and those treated with PBS+C/P+2.5mgLPS). Although it was expected that the mortality amongst the control mice especially and the treated mice generally would be reduced compared to the earlier experiment, it must be noted that there is a possibility that variation in the weight for weight toxicity of the batch of LPS used could introduce another variable.

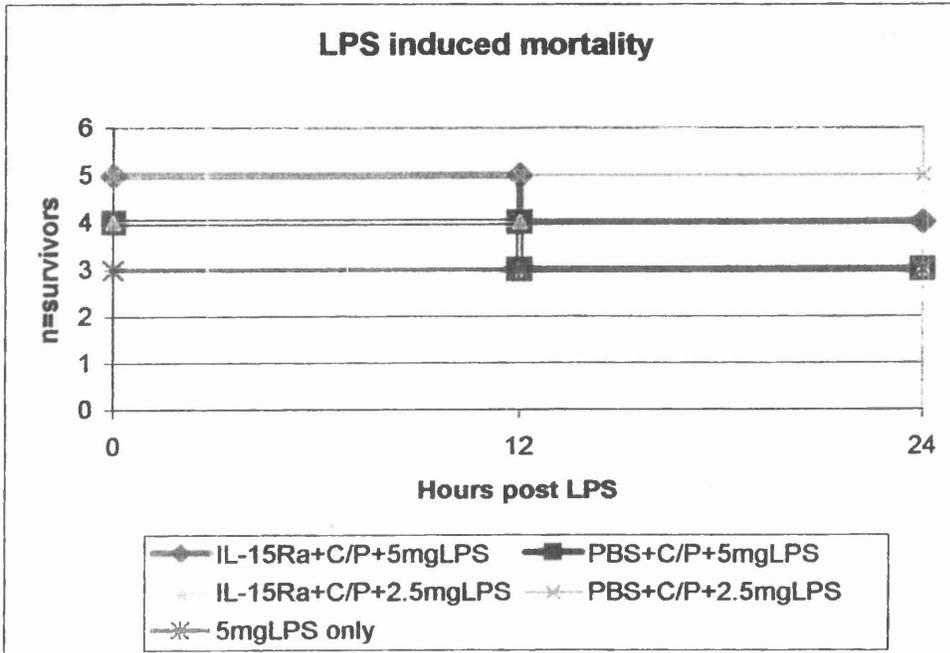


Figure 22

LPS induced mortality was exhibited in 3 mice, one from each group. There was no statistical difference indicating that mortality was not a reliable indicator of LPS shock in *C. parvum* primed mice.

4.4 Weight loss in C parvum primed LPS Mice

Weight loss or gain was recorded as change in weight from the time of LPS injection (base) and 18 hours later. Primed mice treated with IL-15R α and receiving 5mg/Kg LPS were compared to those receiving the same priming and LPS but PBS control instead of IL-15R α . There was no statistically significant difference between the two groups. (not shown; $p \geq 0.05$ Mann Whitney U). As indicated in figure 23 below, when the weight-loss characteristics of the control and treatment mice injected with the lower 2.5 mg of LPS/Kg were compared, the IL-15R α treated mice lost more weight in the 18 hours following LPS shock induction than the control mice of group 5 ($p = < 0.05$, Mann Whitney U).

The significance of difference in weight loss is not clear, although it infers that there is a greater weight loss in the presence of IL-15R α in the context of therapy against LPS induced shock. The presence of 5mg/Kg LPS may have overwhelmed any effect of IL-15R α operating in this model, resulting in no difference between the two groups.

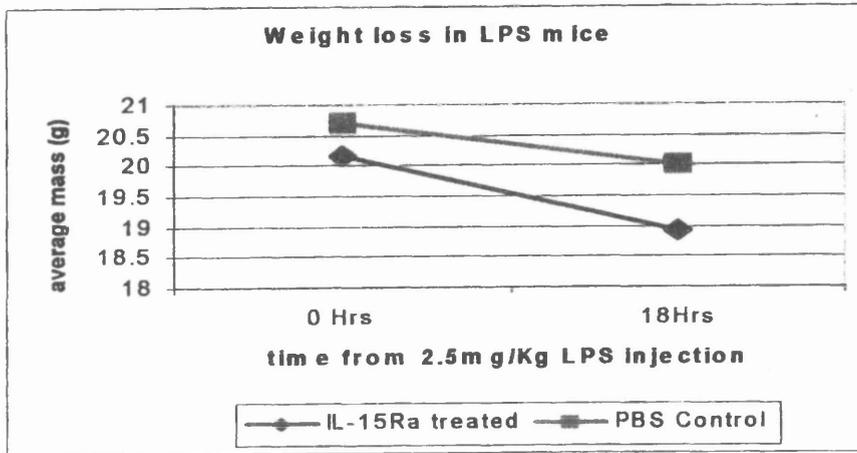


Figure 23

Differential weight loss in the LPS shock model. IL-15R α treated mice lost more weight than PBS treated controls. ($P < 0.05$, Mann Whitney U)

4.5 Biochemical Liver damage indicators in C parvum primed LPS shock mice

To test the independent effects of C Parvum, IL-15soluble receptor and two different doses of LPS, the following series of experiments were devised. Serum samples were taken following exanguination of the mice. As described in the materials and methods (section 2.22), serum samples were diluted 1/20 into 7% BSA/Saline (Sigma) and analysed on a photo assay device (Kodak, Ilford, Essex) and the numbers of international units of enzyme recorded per ml of serum sample. These results are displayed below in figure 24. Georg Meffert et al, (135) have very recently suggested that creatine kinase as a marker of liver injury is very unreliable due to isoforms interereng with assays. The creatine kinase measured in these experiments was done so because it was an assay included in the liver damage kit supplied by Kodak. This part of the results may now not be relevant however AST and ALT are still perceived to be markers of liver disease.

4.6 The effect of IL-15/C parvum on liver damage in the absence of LPS

As expected, in all three of the liver enzyme measurements, control mice receiving neither LPS, C parvum or active treatment/placebo exhibited the lowest levels of liver damage enzyme indicators. For the purposes of this experiment, these must be considered as the base line

Five mice were also primed with C. parvum and 7 days later received the active, muIL-15 soluble receptor treatment, but were not challenged with LPS. As seen below in figure 24, there was a 3-6 fold increase in the serum levels of the three measured liver damage enzymes detected, compared to the mice in the control group receiving no treatment or priming. A further group of 5 mice were also primed with

C. parvum and was not challenged with LPS but received PBS placebo injections in place of the IL-15R α . The serum AST, ALT and CK levels detected were similar to the active treatment group described above. Thus it can be seen that merely priming the mice with *C. parvum* has an effect and that there was no vast difference in the serum liver damage enzyme parameters between the active treatment and the PBS placebo groups. As these data are derived from pooled samples, no statistical analysis can be applied.

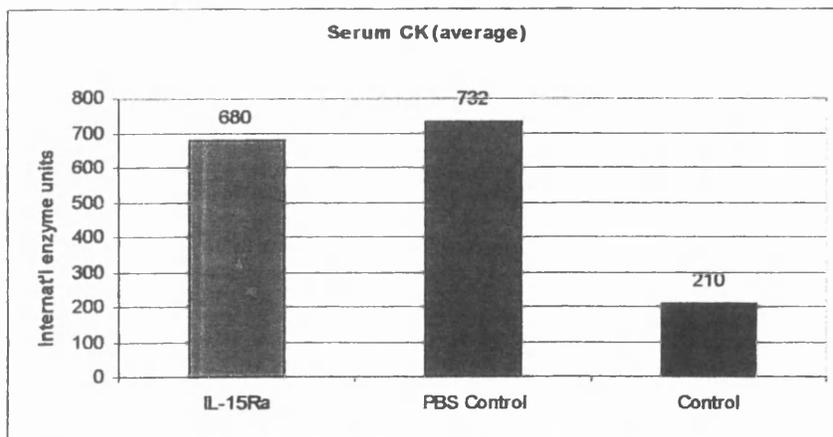
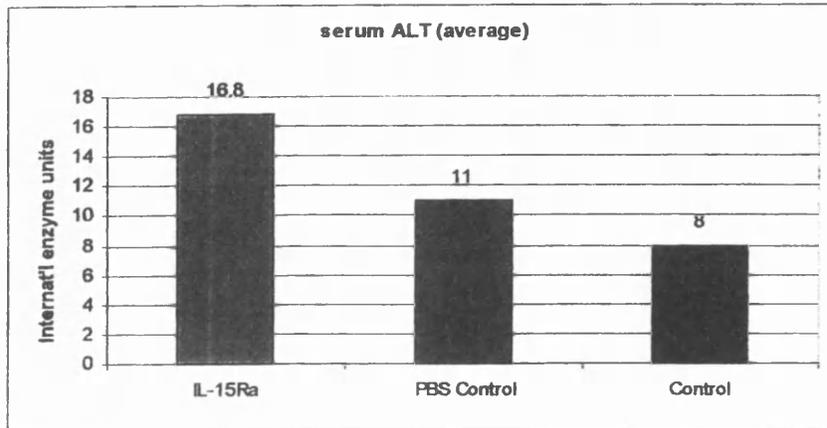
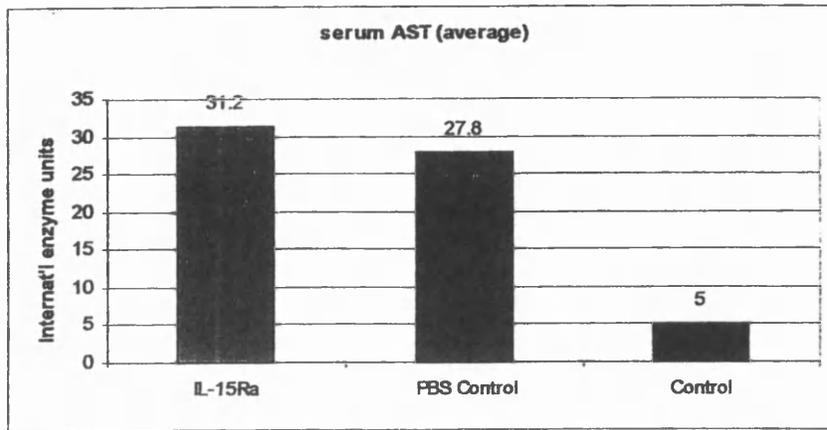


Figure 24 Serum ALT, AST and CK determinations

	IL-15Ra	PBS-Control	No C parvum control
C Parvum priming	+++	+++	---
LPS	---	---	---
PBS	---	+++	---
IL-15Ra	+++	---	---

Data derived from pooled samples.

The untreated C parvum controls showed less liver damage enzymes than the IL-15 or PBS treated mice which exhibited similar levels of serum liver enzymes.

4.7 The effect of IL-15R α on liver damage induced by 2.5mg/Kg LPS

Five mice primed with *C. parvum* and then challenged with 2.5mg/Kg LPS received soluble IL-15 receptor active treatment. Four control mice were treated with PBS control instead of IL-15R α . With reference to figure 25 below, comparison of the ALT, AST and CK levels indicate that the IL-15 treated group exhibited approximately 50% lower serum levels of damage enzymes than the placebo controls. These lower levels detected in the IL-15 treated group were also similar to the group of five primed+PBS-control mice that were not challenged with LPS. Thus, the presence of soluble IL-15R α appeared to reduce the level of liver damage induced by 2.5mg/Kg LPS when compared to placebo.

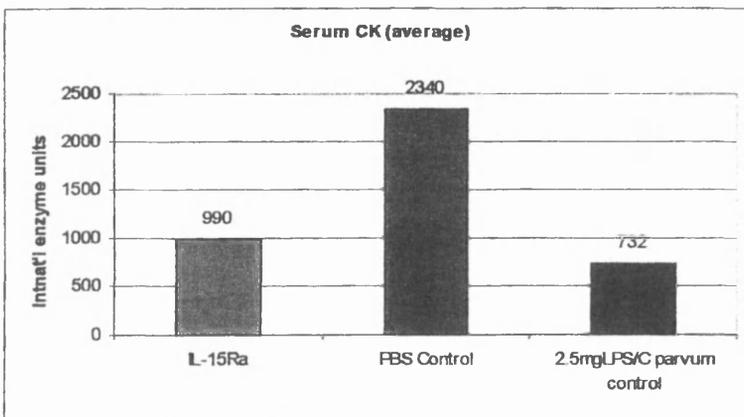
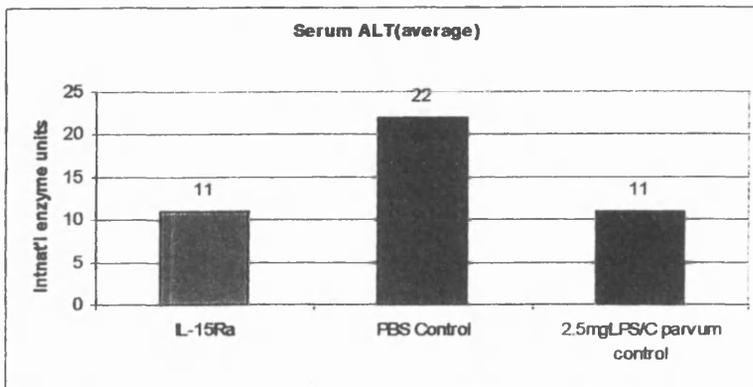
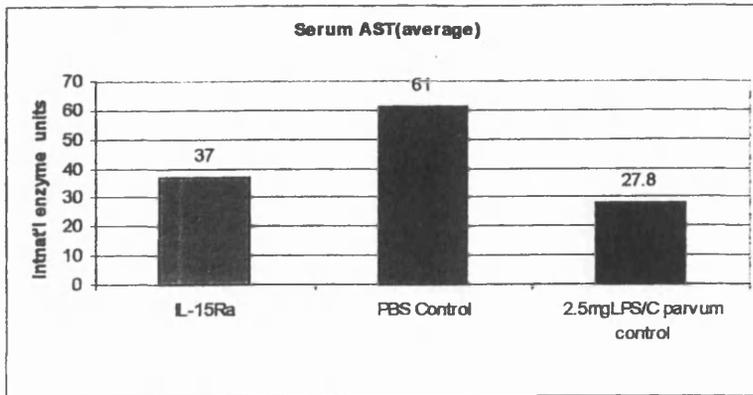


Figure 25 Serum ALT, AST and CK determinations

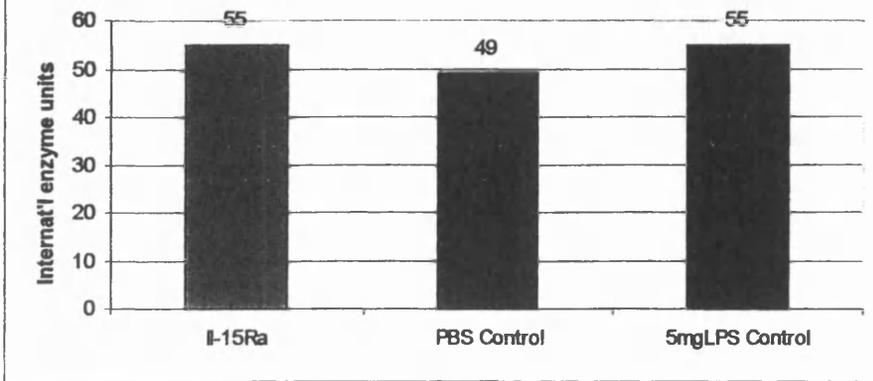
	IL-15Ra	PBS-Control	C parvum control
C Parvum priming	+++	+++	+++
LPS 2.5mg/Kg	+++	+++	---
PBS	---	+++	+++
IL-15Ra	+++	---	---

Data derived from pooled samples.

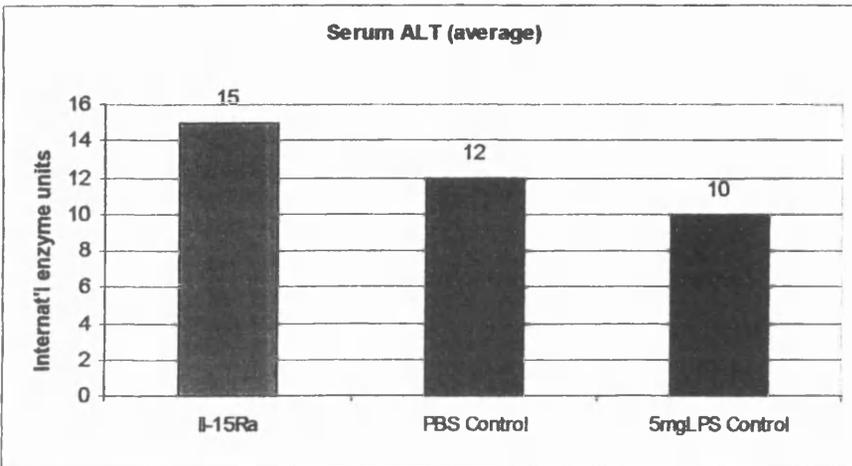
The IL-15Ra treated C parvum-primed mice showed approx. 50% lower liver damage enzymes than the PBS treated control mice. The IL-15Ra treated mice serum levels of liver damage enzymes were similar to those observed in the non-LPS challenged controls.

4.8 The effect of IL-15R α on liver damage induced by 5mg/Kg LPS

Five mice primed with *C. parvum* and then challenged with 5.0mg/Kg LPS received muIL-15 receptor active treatment. A control group of four mice also primed with *C. parvum* and injected with 5mg/Kg LPS were treated with PBS. A further control group of 3 mice were not primed nor treated with either IL-15 or PBS placebo but were challenged with 5mg/Kg LPS. With reference to figure 26 below, comparison of the ALT, AST and CK levels indicate that the IL-15 treated and the PBS-placebo treated group exhibited similar serum levels of damage enzymes to that of 5mg/Kg LPS only mice. These data suggest that in the presence of 5mg/Kg LPS, there was no trend for the IL-15 treated mice for less liver damage compared to the PBS controls when observed in *C. parvum* primed mice. However, the levels of damage were similar to that observed to be induced by 5mg/Kg LPS alone in the absence of *C. parvum*. This was in contrast to the difference seen above in figure 25, where in the presence of 2.5mg/Kg LPS, there was a trend for lower liver damage in the IL-15R α treated group compared to the PBS control mice. No statistical analysis was possible to define these trends as the data was derived from pooled samples.



Serum ALT (average)



Serum CK (average)

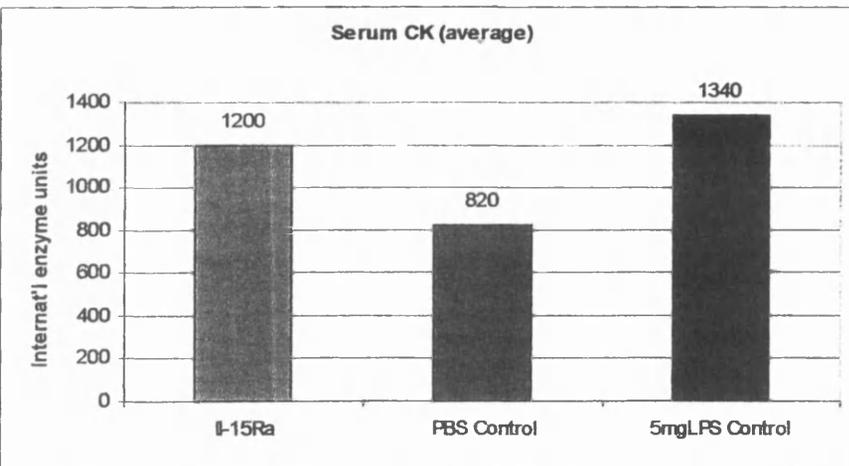


Figure 26 Serum ALT, AST and CK determinations

	IL-15Ra	PBS-Control	C parvum control
C Parvum priming	+++	+++	---
LPS 5mg/Kg	+++	+++	+++
PBS	---	+++	---
IL-15Ra	+++	---	---

Data derived from pooled samples.

The IL-15Ra treated C parvum-primed mice showed similar serum levels of liver damage enzymes to both the PBS treated control mice and the non-primed controls. IL-15Ra showed no protective effect in terms of a trend for lower serum liver damage enzymes in the presence of 5mg/LPS and priming.

4.9 LPS model measurement using septic shock index

A further experiment was developed to examine the modification of LPS shock by IL-15R α based on the findings of the two previous experiments. Further changes were sought to investigate ways of improving the welfare of the mice whilst gaining better quality data.

Some experimenters use a rectal probe to monitor core temperature in measurement of septic shock. This was felt to be invasive and likely to be stressful for the mice. Others, often in nutrition fields, use a blood pressure cuff around the tail vein. As LPS shock features hypotension with blood supply tending to be restricted to core regions, this was also felt to be impractical. The Morton-Griffith index (84) provides a framework for assessment of animal welfare during experiments such as LPS induced shock. Based on these papers, a scoring index was devised allowing a detailed assessment of LPS shock features and enabling prediction of lethal shock. The aim for this was to prevent dead or very sick mice being found in cages, as this prevented serum sampling and subsequent biochemical analysis.

The index functions by “0” being scored for each “healthy-parameter”. However if a mouse showed signs of an unhealthy parameter then a negative score was given, as shown in table 5 below. If any mouse scored “-7” it was humanely killed as experience from the experiments above indicated that this score predicted death.

Healthy		Unhealthy	
Actively running around	0	Moving slowly	-1
Inquisitive	0	Not Inquisitive	-1
React to touch	0	No reaction to touch	-1
Moving around at 60 hours	0	No spontaneous movement at 60 Hours	-4
Normal stools	0	Diarrhoea	-1
Clear eyes	0	Encrusted eyes	-1
Skin warm to touch/relaxed	0	Skin cold/Hunched aspect	-1
Weight loss <30%	0	Weight loss >30%	-7
No shivering when picked up	0	Shivers uncontrollably on picking up	-5
Mobile at 72 hours	0	Immobile at 72 Hours	-7

Table 5 Table of scoring system used to monitor mice during this experiment based on previous results and Morton-Griffith index and the Rodent Protection Document. All animals scoring -7 were humanely killed

Using the above table (as described in the methods and materials section 2.22), 27 Balb/C mice (Harlan Olac, Cambridge) were primed with 1mg/mouse C parvum IV route. The protocol is summarised in table 6 below. These mice were injected with 2.5mg/Kg LPS and segregated randomly to receive either IL-15R α or PBS control immediately post LPS injection and at 24 hours later.

Day1		Day 7 (0 hours)	Day 8 (24 Hours)	Day 9 (48 Hours)
Prime Mice		Inject LPS as below & Inject IL-15R α or control	Inject IL-15R α or control	Terminate Experiment
Group No.	<i>C.parvum</i> mg/mouse	IL-15R α or PBS injections	LPS mg/Kg	
1 n=14	1	2x40mg IL15Ra	2.5	
2 n=13	1	2x100ml HS-PBS	2.5	

Table 6 Table detailing groups receiving IV *C. parvum* on day 1 LPS was administered on day 7 and immediate treatment with IL-15 receptor or High salt-PBS control at t=0 and t=24. All animals were terminated at t=36 hours

4.10 Analysis of Weight data in mice assessed by septic shock index

As noted in the materials and methods, weight was recorded at 0, 12, 24 and 36 hours post LPS challenge. The body masses recorded are shown below in figure 27.

Both the treated and placebo groups lost weight over the course of the experiment. When individual weight changes of the mice were compared across the two groups, Mann Whitney U analysis shows that, although there was no difference between the groups at 0, 24 and 36 hours ($p > 0.05$), at 12 hours the IL-15receptor treated group lost less weight than the controls ($P < 0.011$). The significance of this differential weight loss data is unknown and was only exhibited transiently. Mortality was only by termination.

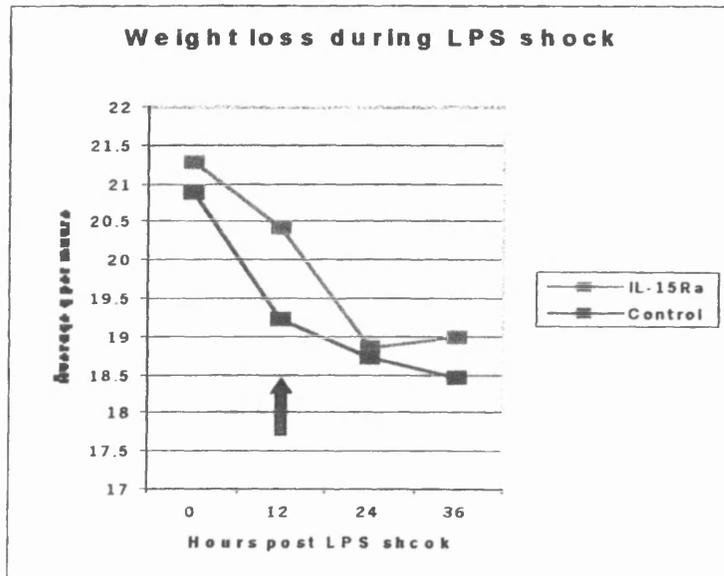


Figure 27

Weight loss measure in mice following LPS shock.

Mice treated with IL-15Ra exhibited less weight loss at 12 hours than controls ($p=0.011$, Mann Whitney U analysis), however at 24 and 36 hrs, there was no significant difference.

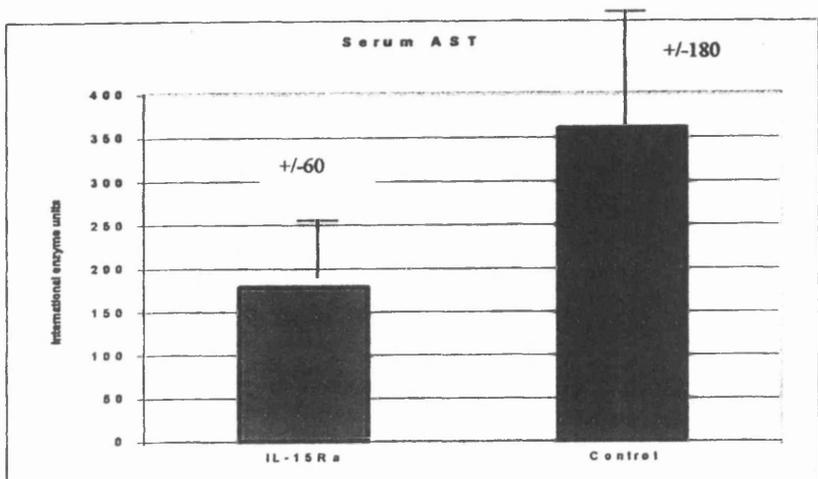
4.11 Biochemical Liver damage indicators in mice assessed by septic shock index

Serum samples were taken at termination of each mouse, diluted into 7% BSA and assayed for serum levels of ALT, AST and Creatine Kinase as indicators of severity of septic shock, induced by 2.5mg/Kg LPS challenge. These results are shown below in figure 28 .

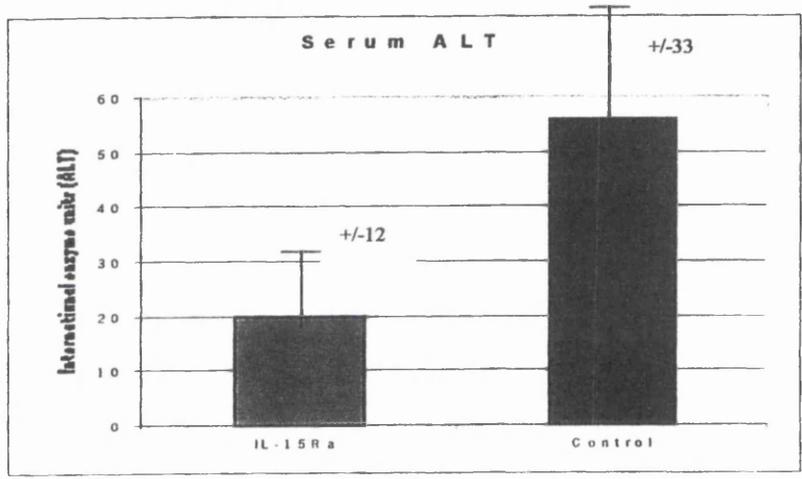
There was a significant difference found when the IL-15R α treated group was compared to the control group in the assay for both ALT and AST (P=0.0011 Mann Whitney U, respectively). However, there was no statistical difference between the two groups demonstrated by the assay for Creatine Kinase (P >0.05 Mann Whitney U), although the trend is for lower liver damage as indicated by the lower serum level of Creatine Kinase in the receptor treated group.

In the previous experiment, (figures 24, 25 &26), analogous assays were made for these enzymes and similar differences were shown for IL-15R α treated mice receiving 2.5mg/Kg LPS, but the former serum samples were grouped blood as opposed to the latter samples which allowed statistical analysis, as they were derived from individual mice. The other difference between these two experiments is that the former was sampled at 24 hours compared to 48 in the latter. The average AST enzyme unit levels were much higher in the second experiment (average range 178 to 360) compared to (average range 27to 61) in the previous determination. The significance of this difference is unknown and may be due to sampling error or the longer elapsed sampling time. In summary, there was a trend for lower liver damage enzymes in IL-15R α treated mice in the 2.5mg/Kg LPS group of the first experiment shown in the pooled samples. This trend is further confirmed by the lower serum

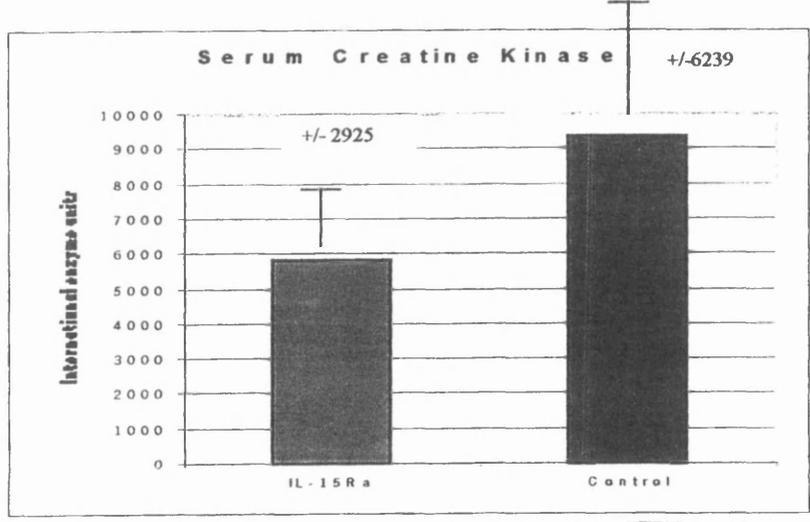
levels of AST, ALT and CK in the IL-15R α treated, 2.5mg/Kg LPS group of the second experiment when compared to the control mice. (ALT p= < 0.0011, AST p=< 0.0011 and CK p= > 0.05 respectively Mann Whitney U analysis.)



P= <0.0011
Mann Whitney-U
Error bar = Std Dev



P= <0.0011
Mann Whitney-U
Error bar = Std Dev



P = >0.05
Mann Whitney-U
Error bar = Std Dev

Figure 28
Serum levels of AST, ALT and Creatine Kinase following LPS shock. Sampling at 48 hours post LPS injection. The data indicate lower liver damage enzyme levels in the IL-15R α group compared to the controls with P=0.0011 in AST and ALT (Mann Whitney U) tests. The difference in CK is not significant P=>0.05) But the trend is maintained for lower serum-CK in the IL-15R α treated group .

4.12 Septic shock index

As mentioned in the text above, an index was devised to measure the severity of LPS-induced septic shock during this experiment. Assessments were made at 0, 12, 24, 36 and 48 hours following LPS injection. These data are presented below in figure 29. Comparison of IL-15R α treated groups and the control reveals that there were less severe features of septic shock in the receptor treated group at all time points. At 12 hours and at the end of the experiment at 48 hours, these differences are statistically significant ($P= 0.0011$ Mann Whitney U test). The dead mice scored -7 and continued to be included at each time point on the basis of intention to treat.

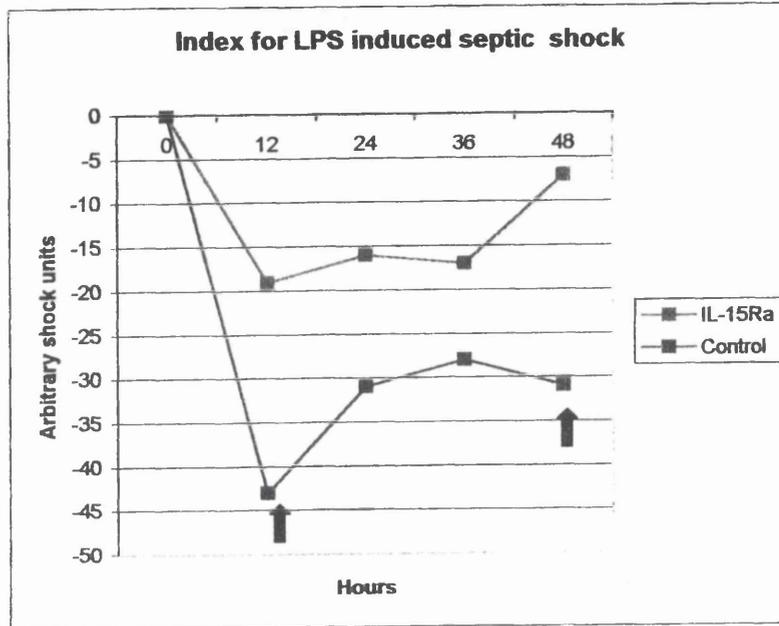


Figure 29

Graph of severity of septic shock measured by arbitrary score index. The symptoms of shock were less severe in the IL-15R α treated group at 12 and 48 hours ($P = <0.0011$ Mann Whitney U) The trend for less severity was maintained at all time points.

4.13 Summary of results from the experiments in LPS induced shock

In the preliminary LPS induced shock experiment, it was shown that injecting 3x40ug doses of IL-15R α reduced mortality of Balb/C mice by 40% when compared with controls. (P= <0.013 Kruskal-Wallace test) This modification of mortality was demonstrated when the initiating dose of LPS was 18mg/Kg but in the presence of 20mg/Kg LPS dose all the mice succumbed to LPS shock.

Although this was informative in that IL-15R α was seen to have a protective effect, the severity of the illness of the mice was unacceptable and furthermore, dead mice in cages prevented any further observations being made. Therefore, in the second experiment, *C. parvum* priming was used to reduce the amount of LPS needed to induce disease and to shorten the duration of the LPS induced septic-shock experiment. Mortality and morbidity were markedly reduced, with only three mice succumbing to shock, distributed amongst control and treatment groups. The lower overall severity of the septic shock in this experiment allowed serum sampling of the mice and therefore analysis of biochemical indicators of LPS shock: serum liver enzyme levels. Unfortunately, in the first of the *C. parvum* primed experiments, the samples from most informative groups (IL-15R α and controls injected with 2.5 or 5mg/Kg LPS) were insufficient for individual mouse levels to be assayed. Therefore group data was derived without statistical analysis being possible. However these results showed approximately 50% lower serum levels of liver damage enzymes in the receptor treated group compared to the control where LPS shock had been initiated by a 2.5mg/Kg mouse bodyweight dose. Interestingly, this was not observed in the IL-15R α treated group injected with 5mg/Kg LPS. This reflected the finding of the first

experiment where the protective effect of IL-15R α treatment did not enhance survival in mice with shock induced by 5 mg/Kg LPS, as noted above.

The final experiment was again carried out with *C parvum* primed Balb/C mice. These were injected with LPS. Shock symptoms were induced by a dose of 2.5mg/Kg LPS- mouse weight. During 48 hours observation following induction of LPS shock, there was a trend for greater weight loss in the control group compared to the controls, this transiently attaining statistical significance at 12 hours. In this experiment, it was possible to extract serum samples from each mouse at 48 hours and assess the serum concentrations of ALT and AST and Creatine Kinase. The mice treated with IL-15R α showed significantly lower levels of serum ALT and AST than the control group, ($P < 0.0011$ for both, (Mann Whitney-U)). There was also a trend for lower Creatine Kinase in the sera of the receptor treated group compared to the controls. This result reflects the trend for a protective effect of IL-15R α treatment demonstrated by the lower serum enzyme levels of ALT and AST (and CK) in the first *C parvum* primed LPS experiment. In addition to the above indication that IL-15R α was able to modify the response to LPS induced septic shock in the mice, the septic index of severity also demonstrated that there was reduced severity of shock features in the receptor treated mice. The graph of severity (figure 29) shows that this was maintained at all time points and statistically significant at two time points ($P < 0.0011$ at 12 and 48 hours Mann Whitney U).

In conclusion, soluble-recombinant IL-15R α was able to protect mice following induction of septic shock by administration of low doses of LPS both with or without priming with *C. parvum*. This protective effect was seen when IL-15R α was introduced prophylactically and as an active treatment post LPS induction of shock. The nature of this protection was exhibited by reduction in mortality, as

disclosed by the first experiment and features of liver damage, and demonstrated by the lower serum levels of enzyme indicators described in the latter two experiments. Weight loss, if considered as an indicator of disease severity, was also attenuated (transiently) in the IL-15R α treated mice of the second *C parvum* primed experiment. Furthermore, the general features of morbidity were less severe in the IL-15R α treated mice as demonstrated by the lower septic-shock severity scores in the latter experiment. These data show a robust indication of the trend for reduced disease severity in mice treated with IL-15 antagonists, demonstrated in the collagen induced arthritis model of inflammation in the previous chapter. In addition to demonstrating IL-15R α modification of LPS shock, using the *C parvum* priming of the mice, priming of mice in this way can increase the response of mice to subsequent injection of LPS shock (55). This increases TNF α production in response to LPS challenge as shown by Rossol (81). Here, they reported that in general, *C parvum* primed mice began to die within 6 hrs of LPS (20ug/mouse) challenge, in contrast to the majority of mice they had pre-treated with either anti-TNF α or IFN γ antibodies. In the context of this experiment, priming enabled a more humane, shorter time scale to be followed for the LPS model experiment. However, although shorter in time, this enhanced the usefulness of the model, because serum is more readily available from less severely ill mice therefore allowing a greater chance of success in obtaining serum for cytokine ELISAs and other biochemical tests. The establishment of a scoring system for pathological data derived from liver and kidney sections might also be a good indicator of disease severity. This too would drive the focus away from mortality as a de facto end-point in this animal model of inflammatory disease.

Chapter 5

5.1 Treatment of septic arthritis mice with IL-15R α recombinant protein

In chapter 3, the Collagen Induced Arthritis model (CIA) was used to see if the treatment with antagonists of murine IL-15 could modify this disease. Although there was no statistically significant difference between the disease progression between the two groups, a trend was recognised where the group treated with the recombinant IL-15-48 mutant molecule exhibited lower incidence and severity of arthritis in the early stages of the disease. This was shown to be transient and after a few days, there was no difference between the control and treated groups. A second experiment treating collagen arthritis with polyclonal anti-sera (Chapter 3, section 3.10) failed to support this trend. However, treatment of collagen induced arthritis with recombinant IL-15R α , reported by Ruchatz et al (1998), indicated that a strong, down-regulatory response occurred, induced by IL-15 receptor therapy. In chapter 4, the IL-15R α molecule was used to treat LPS induced shock. The symptoms of this acute inflammatory response were modified by IL-15R α therapy. These mice exhibited a lower incidence and severity of arthritis and furthermore, less liver damage than the control animals.

The two experimental diseases discussed above demonstrate the effect of IL-15R α in disease models that are initiated by a single dose of defined antigen, against which the host makes a vigorous inflammatory response. It was felt to be of interest to see if the same trends might exist in a disease model that was induced by a live pathogen. In order to investigate this, two experiments were devised using the septic arthritis Model. In one experiment, a bolus dose of therapeutic IL-15R α concurrent

with induction of septicaemia and a second therapeutic bolus on day three. The second experiment featured a prolonged treatment of IL-15R α , which was initiated following induction of septicaemia. The progression of clinical symptoms of the induced disease was measured in groups treated with either with recombinant IL-15R α or a control substance. The results are described below in section 5.5, an experiment extending over 7 days and section 5.6, a further experiment extending over 14 days.

5.2 Induction of septic arthritis

Murine IL-15R α was prepared and purified according to the protocol set out in chapter two, diluted to a working stock of 400 μ g/ml and tested by ELISA for IL-15 binding activity which was found to be equivalent to that obtained in previous preparations. The methods for this are described in chapter 2, but briefly here: *E. coli*, transformed with the gene for murine IL-15R α , was incubated in shaken suspension. Production of IL-15 was induced by IPTG and following expression, muIL-15 was affinity purified on a nickel-agarose column. The purified protein was resuspended into high salt PBS.

5.3 Animals

30, female Swiss mice, 4-6 weeks old were segregated into 4 groups. Mice were segregated into 5 per cage and fed on standard laboratory diet and water ad-libitum. Twenty of the mice (groups 1 and 2) were injected with 5×10^7 live *Staphylococcus aureus* (*S. aureus*) by intravenous injection via the tail-vein. The 10 remaining mice of groups 3 and 4 were injected in the same manner with 1×10^7 heat killed *S. aureus*.

Shortly after the *S. aureus* inoculation, the mice of group 1 and 3 were injected intraperitoneally with 40 μ g murine IL-15R α suspended in 100 μ l PBS. Groups 2 and 4 were injected with an equivalent volume of PBS control solution. The injection of IL-15R α or control PBS was repeated on day three after the *S. aureus* injection. This protocol is summarised in Table 7 below.

The mice were monitored for any symptoms of septicaemia from day one and arthritis from day three and onwards. Previous studies had shown symptoms of arthritis were most likely to appear, (67). At day seven the mice were euthanased and

examined at post-mortem. Septicaemia was assessed daily by observation of coat condition, body stance, eye discharge and spontaneous movement. Body mass was measured every second day.

The presence of joint disease was assessed by the scale of clinical scores used for collagen induced arthritis. Mice were monitored on days 1, & 3-7 for symptoms of septic arthritis and assigned severity scores according to the index described in materials and methods section 2.21, but briefly: 0 = no swelling or red colour; 1= erythema or slight swelling; 2= marked swelling or erythema; and 3 = severe swelling and or loss of limb function. Each limb was examined daily by a blinded observer. Each limb of each animal was assessed daily by this method.

Day 1 20 mice 5×10^7 live aureus (TSST⁺ve)(IVroute) &. 10 mice 5×10^7 heat killed S. aureus (TSST⁺ve)(IVroute)

10each mice 40mg IL-15R α or equivalent volume of High-Salt PBS (IP route)

Monitoring for sepsis and arthritis

Day 2 Monitoring for sepsis and arthritis

Day 3 25 mice 40mg IL-15R α or equivalent volume of High-Salt PBS. Monitoring for sepsis and arthritis.

Day 4 Monitoring for sepsis and arthritis

Day 5 Monitoring for sepsis and arthritis

Day 6 Monitoring for sepsis and arthritis

Day 7 Monitoring for sepsis and arthritis All mice from each group terminated.

Table 7 Summary of schedule for bolus injection septic arthritis model experiment.

Features of Septicaemia

5.4 Mortality

There was no evidence of mortality in either of the groups treated with heat killed bacteria (data not shown). In the groups treated with live *S. aureus*, there was 30% mortality in the PBS control group at one week, but 0% in the receptor treated group. The occurrence of this mortality is shown below in figure 30 and there was no significant difference in the two groups ($p > 1.0$, Kruskal-Wallis test).

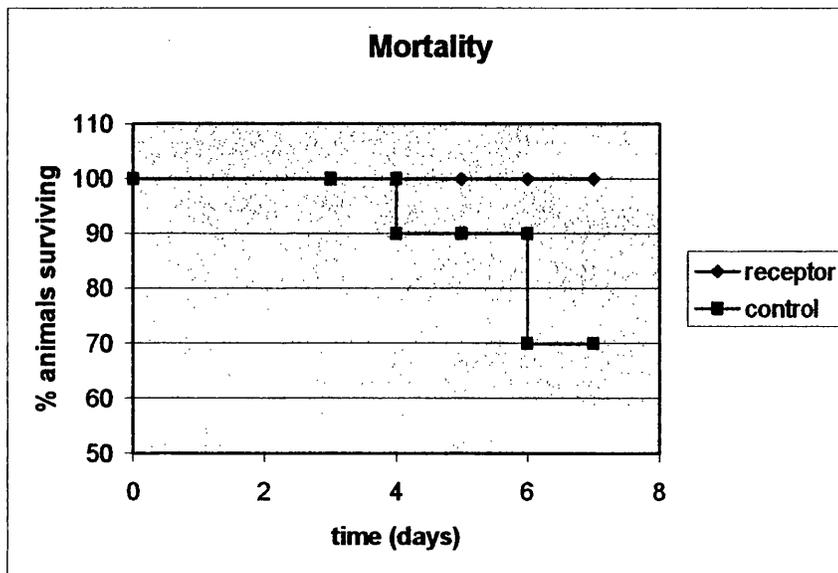


Figure 30
Mortality data for the receptor and PBS control treated groups that had previously been inoculated with live *S. aureus* bacteria LS-1 strain. There was no significant difference in mortality

5.5 Incidence of septicaemia

The incidence of septicaemia was calculated daily and expressed below in figure 31. A trend for lower sepsis incidence in the receptor treated group was seen, but the difference between the two groups was not statistically significant. ($p > 0.05$ chi squared)

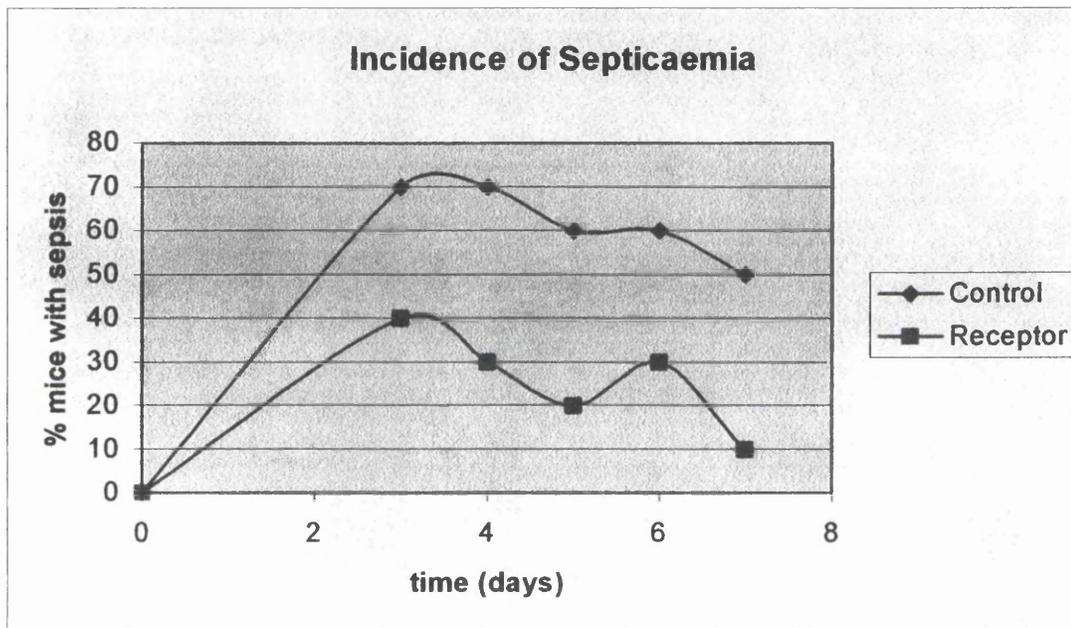


Figure 31

Incidence of septicaemia in receptor and PBS control treated group inoculated with 5×10^7 live *S. aureus*.

Chi square test indicates no significant data points i.e. $p > 0.05$.

5.6 Weight loss data

The body-mass of the mice was recorded on days 3, 5 and 7. Figure 32 below indicates the change as percentage difference to the previous day's weight. The greater weight loss in the control group data reflected the incidence of sepsis data, shown above in figure 31. However, no statistical significance was demonstrated when these data were compared by the Mann Whitney U test $p > 0.05$.

Two groups of mice (1&2) inoculated with heat-killed *S. aureus* showed no features of septicaemia. Both the receptor and the control treated groups (1&2) had gained 2% body weight by the end of the experiment. (Data not shown)

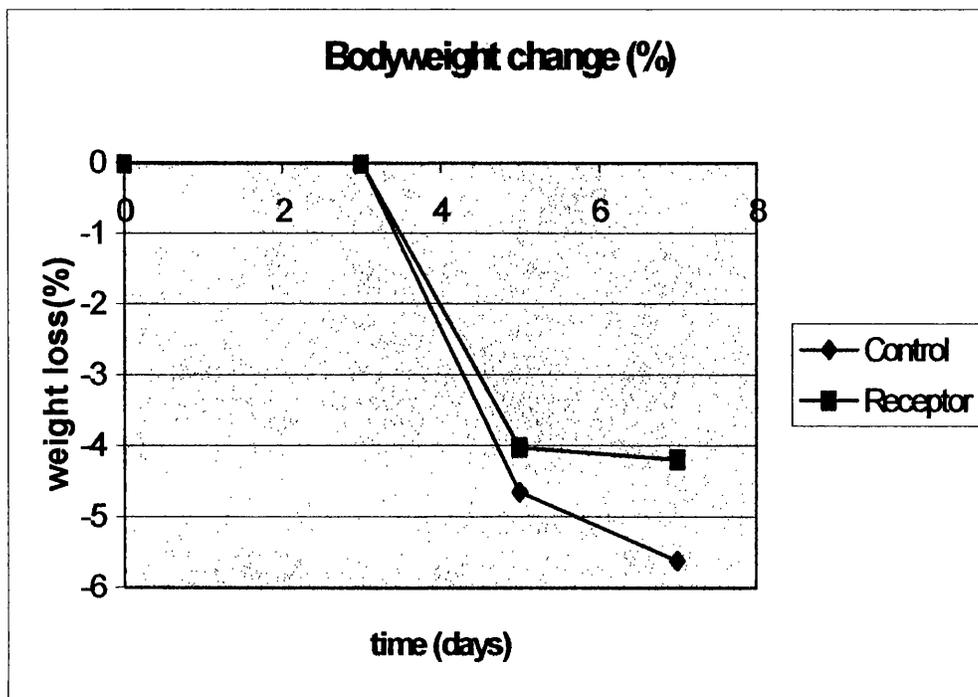


Figure 32
The body weight of each mouse was measured on days 3, 5 & 7. The actual change in grams from the previous days reading is presented here as a percentage.

5.7 Severity of septicemia

The severity of sepsis over time was also determined according to the scoring system described in the materials and methods (section 2.22, table 2), by a blinded observer on days 1, 3 and 7. The mice that succumbed were counted as scoring 4: from the day of death until the end of the experiment. (i.e. analysis was on an intention to treat basis.) Briefly, Starey-coat, hunched aspect, lack of spontaneous movement and mucotaneous eye secretion, each scored one index point. The average score was calculated daily for each mouse with a maximum score of four. These data are shown below in figure 33. This figure shows that as with the septicemia incidence and weight loss data above, there was a trend for lower severity of septicemia in the receptor treated group when compared to the control. This difference reached statistical significance on day five where $p = <0.02$. (Mann Whitney U analysis), emphasising the strength of the trend.

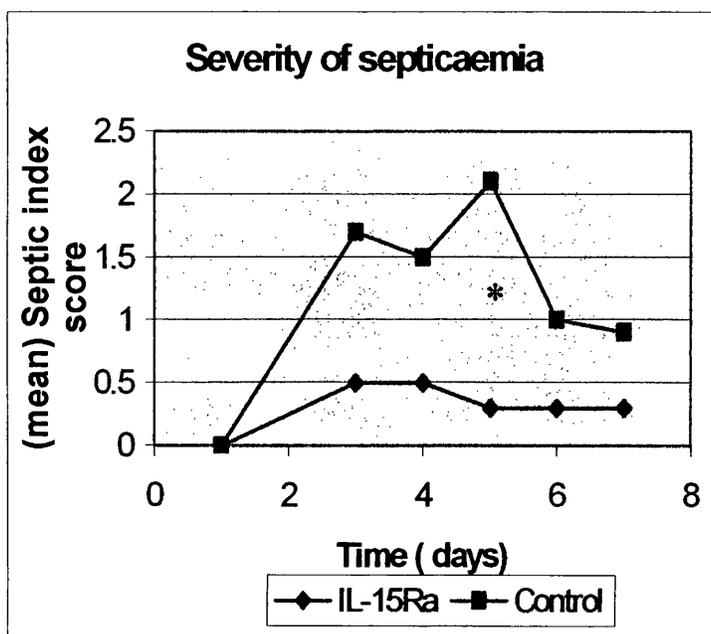


Figure 33

The severity of septicemia was assessed by the septic index described in the text above. The difference between the two groups reached statistical significance on day 5(*) ($p < 0.02$) (Mann Whitney U). A trend for lower sepsis severity was maintained on all other days of the experiment.

Arthritis

The animals were also monitored for evidence of arthritis. Mice were monitored by a blinded observer on days 1, & 3-7 inclusive for symptoms of septic arthritis and assigned severity scores according to the index in materials and methods section 2.21. Briefly: 0 = no swelling or red colour; 1= erythema or slight swelling; 2= marked swelling or erythema; and 3 = severe swelling and or loss of limb function. The severity of arthritis is shown later in figure 35.

5.8 Incidence of septic arthritis

The incidence of arthritis was calculated by expressing the percentage of mice in each group with arthritis at each time point. Analysis by Mann Whitney-U indicated that there was no significant difference in the incidence of arthritis between the two groups ($p > 0.05$) as shown immediately below in Figure 34. However, a trend for lower incidence of arthritis in the receptor treated group compared to the control group is apparent.

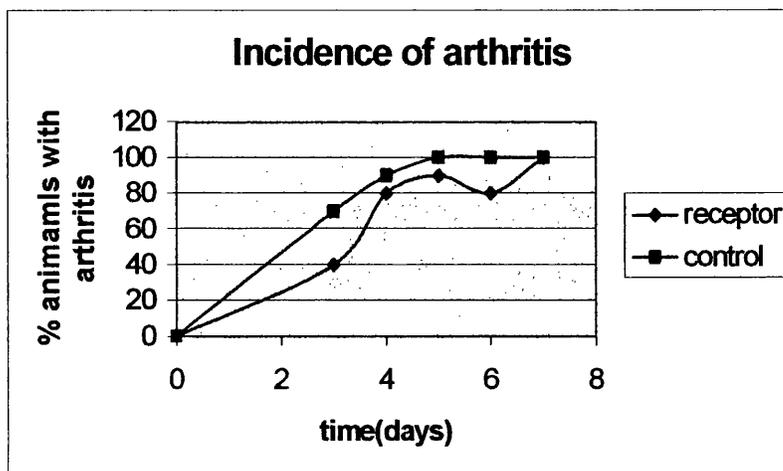


Figure 34

Incidence of arthritis No significant difference was demonstrated but the receptor treated group initially exhibited less incidence of arthritis.

5.9 Severity of septic arthritis

Severity of arthritis was also calculated using the arthritic index described. The mean score per mouse was calculated daily and is presented below in figure 35. As with the incidence of arthritis, there was a clear trend in which the receptor treated group incurred less severe arthritis than the control group. When these data were compared by the Mann Whitney U test with statistical significance was attained on day six ($P < 0.05$). However, by day seven, there was no difference between the two groups.

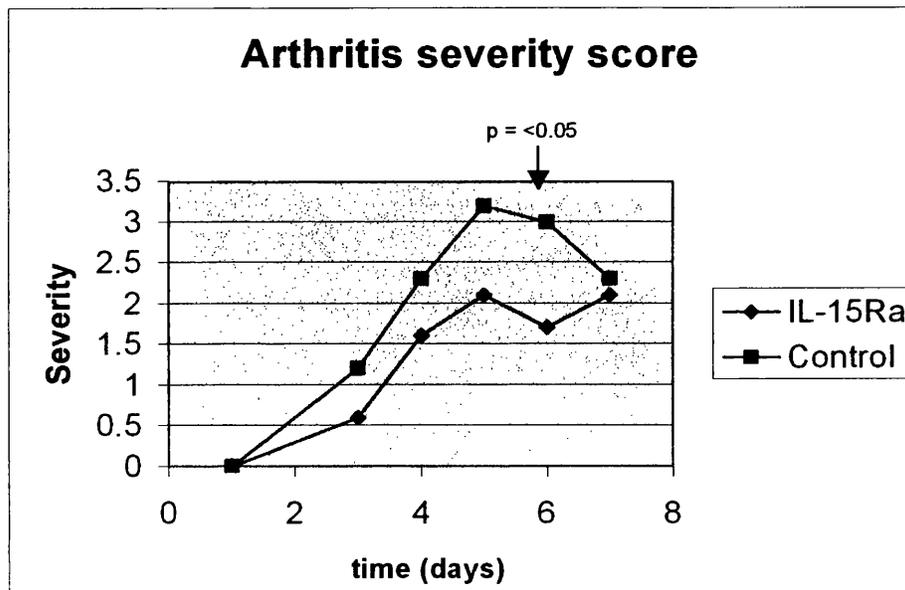


Figure 35

The severity of arthritis is assessed by the arthritic index described in the text above.

The difference between the two groups reached statistical significance on day 6 ($p < 0.05$)

(Mann Whitney U) The IL-15R α group trend for lower arthritis severity was maintained on all other days of the experiment until day 7.

Severity of arthritis was also calculated in terms of the average number of arthritic limbs per group of mice at each time point. This is illustrated below in figure 36. A trend existed with fewer limbs involved per mouse in the receptor treated group compared to the control group. The difference was statistically significant on days 5 and 6 ($p = <0.05$ and <0.03 respectively. (Mann Whitney U). This reflected the severity and incidence data presented above.

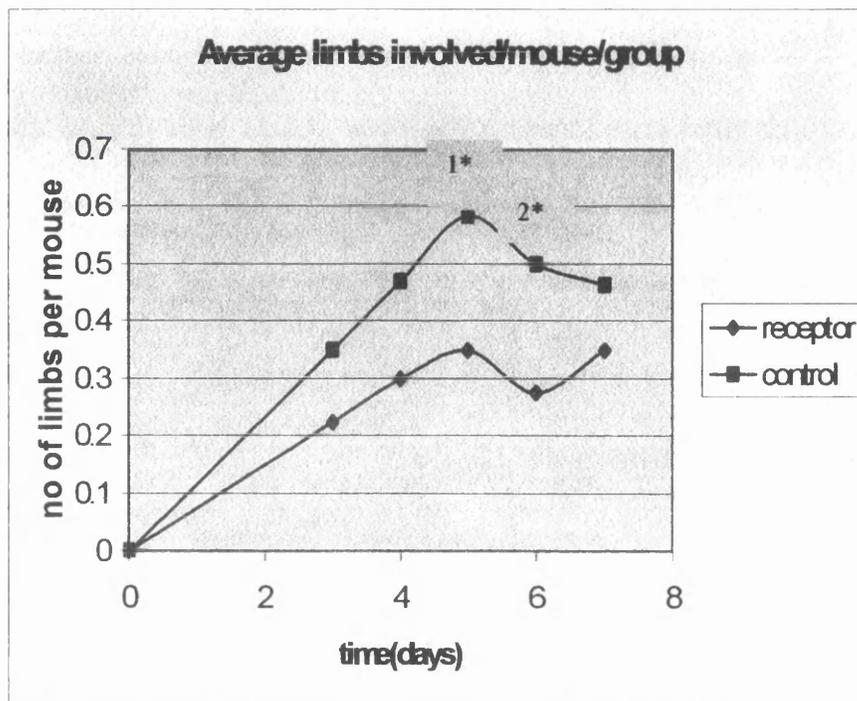


Figure 36

The number of limbs with arthritis was calculated and is expressed here as mean limbs involved per mouse.

1* $p < 0.05$

2* $p < 0.03$

(Mann Whitney U) all other data points not significant.

There was no evidence of septic arthritis in the groups inoculated with heat killed *S. aureus*. This is in contrast to the groups treated with IL-15 receptor or the PBS control that were inoculated with live *S. aureus*.

The trend for reduced septicaemia and lower arthritis severity and incidence suggests that the IL-15R α may act in mice to reduce the symptoms of septicaemia and arthritis associated with the septic arthritis model, caused by inoculation with live *S. aureus*. Furthermore, this trend was reinforced by statistical significance at several time points.

The experiment above can be described as bolus administration of the receptor. A further experiment was designed to see if prolonging the IL-15R α treatment would identify more clearly, any modification of septic arthritis progression by IL-15R α administration. The following experiment features 7 daily injections of IL-15R α (or control) and the observation period was extended to two weeks.

5.10 The effect of prolonged injection of IL-15R α in the septic arthritis model

In a second experiment, 50 female Swiss mice, 4-6 weeks old, were segregated into 4 groups, 5 mice per cage and fed on standard laboratory diet and water ad-libitum.

All of the mice were injected with 5×10^7 live *Staphylococcus aureus* (S. aureus) by intravenous injection via the tail-vein, as shown below in table 8.

Shortly after this inoculation, 25 of the mice were injected intraperitoneally, with 40mg murine IL-15-receptor alpha suspended in 100 μ lPBS and the remainder with an equivalent volume and concentration of murine serum albumin (MSA) (Sigma) suspended in PBS. The injection of IL-15R α or control PBS was repeated daily at 24-hour intervals for seven days after the S. aureus injection.

Day 1 All mice 5×10^7 live *S. aureus* (TSST⁺) (IV route) then 25 mice 40mg IL-15R α / High-Salt PBS (IP route). and 25 mice equivalent volume of High-Salt PBS.

Assessment of sepsis and arthritis.

Day 2 as day 1 for IL-15R α or control injection with assessment of sepsis and arthritis. (No further *S. aureus* inoculation) and continue until day 7

Day 3 |

Day 4 |

Day 5 |

Day 6 |

Day 7 ▼ 10 mice from each group terminated. Cease injection of receptor and control.

Day 8 Monitoring for sepsis and arthritis and continue until day 14

Day 9 |

Day 10 |

Day 11 |

Day 12 |

Day 13 |

Day 14 ▼ remaining 15 mice per group terminated

Table 8.

Summary of schedule and reagents used during an experiment in prolonged injection of therapeutic soluble IL-15R α /control in the septic arthritis model experiment.

The mice were monitored for symptoms of septic arthritis and septicaemia from day one, as described in the materials and methods section 2.23. Therapeutic injection of IL-15R α and control ceased on day 7 and 10 mice from each treatment group euthanased for analysis.

Subsequently, this group was referred to as the “1 week group”. The remaining 15 were monitored for a further 7 days before euthanasia and these mice were subsequently known as the “2 week group”.

The occurrence of septicaemia was assessed by a blinded observer using the septicaemia index, described in section 2.22, table 2. Body mass was measured every day and coat condition, body stance, eye discharge and the presence or absence of spontaneous movement were also assessed. At post-mortem, organs, blood and joints were examined for live colony forming units of *S. aureus*. Spleen cells were also taken at post-mortem to examine proliferative activity against a range of cellular mitogens.

The presence of joint disease was assessed by the scale of clinical scores used for collagen-induced arthritis described in materials and methods section 2.21, materials and methods

5.11 Mortality during IL-15R α prolonged therapy

There were four deaths among the 50 inoculated mice. One mouse in the control group died on day 5 and two further mice died in the control group on day 11, as did one mouse in the receptor group. This is shown in figures 37 & 38 below where the surviving animals are plotted as a function of time. When these data were compared, there was no statistical difference in the two treatment groups for mortality. (Kruskal-Wallis test)

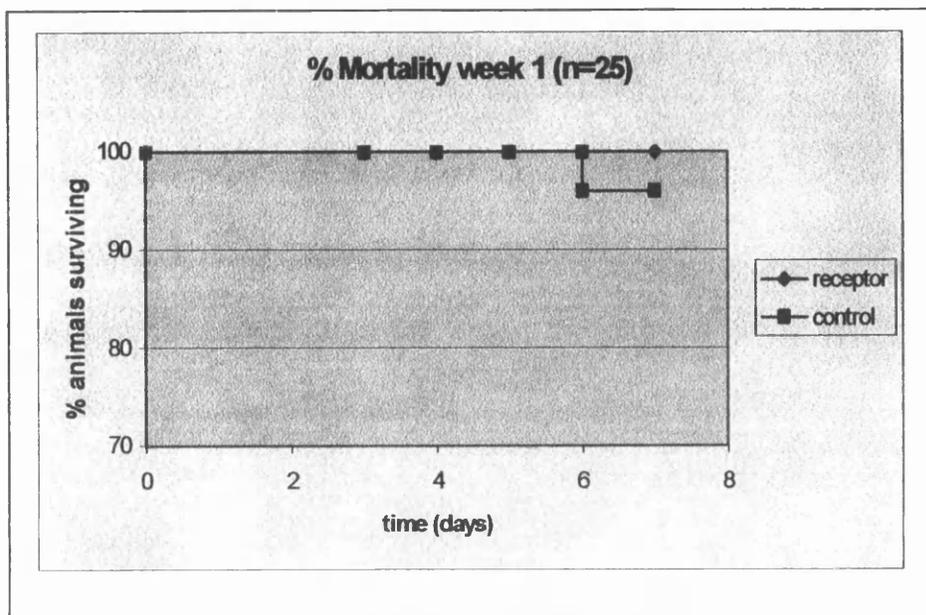


Figure 37 Mortality described as % mice surviving in week ONE. 25 mice per group. No statistical difference was demonstrated.

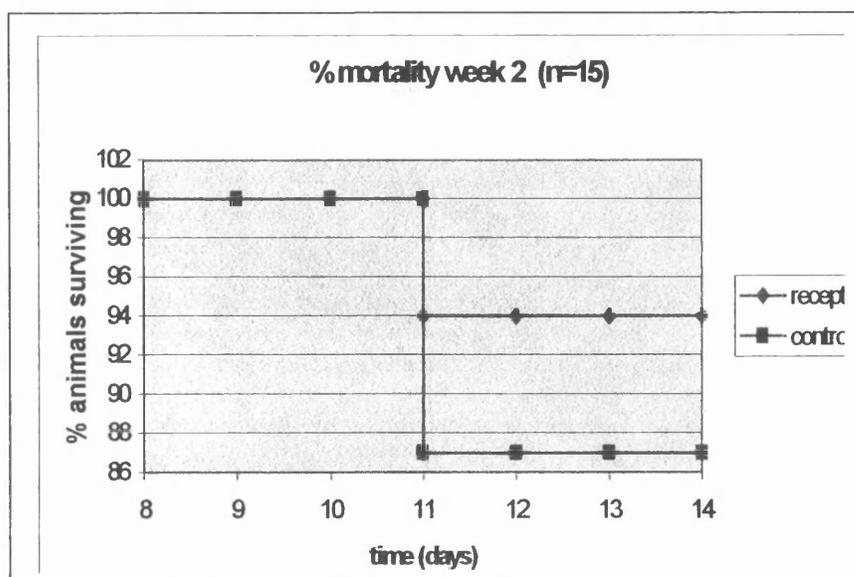


Figure 38 Mortality described as % mice surviving in week TWO. 15 mice per group. No statistical difference was demonstrated.

5.12 Incidence of septicaemia during IL-15R α prolonged therapy

The status of four symptoms of bacterially induced septicaemia was monitored daily and recorded for each mouse, as described for LPS induced septicaemia in section 2.22, materials and methods. The presence of any one or more of these symptoms was recorded daily and the percentage of mice in either treatment group exhibiting septicaemia at each time point are presented in figure 39 below. Dead mice continued to be included as scoring "4" because assessment was carried out on an intention to treat basis as before.

The incidence of sepsis was observed to be lower in the receptor treated group on days 1-4, but none of these data points reached statistical significance ($p > 0.05$ Mann Whitney U). After day four, there was no obvious difference between the two groups.

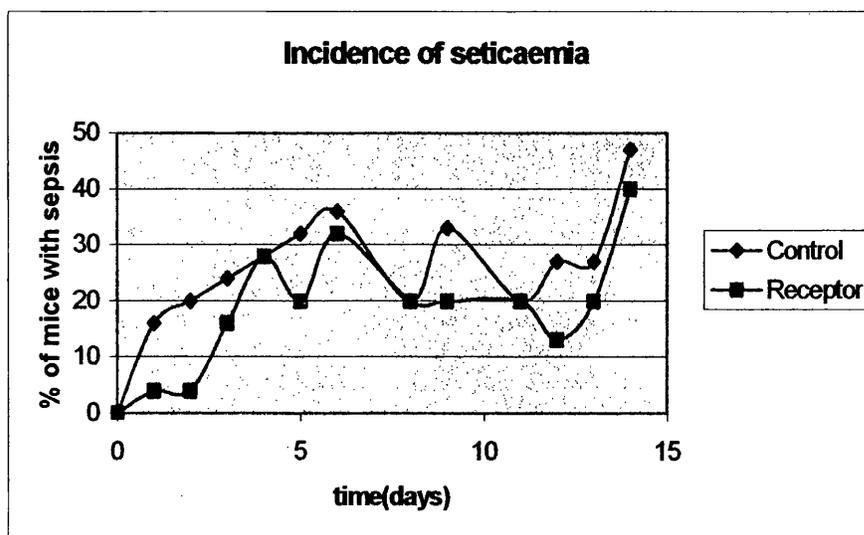


Figure 39

The actual number of mice expressed as a percentage of mice in each group with symptoms of sepsis over time. No significant data points were recorded

5.13 Changes in body mass during IL-15R α prolonged therapy

In figure 40 below the daily change in weight was plotted against time. The general pattern indicated that both groups lost body mass initially and from day seven (coinciding with cessation of injection therapy), both treatment and control arms of the two week group began to gain weight. At no point during the experiment did the data attain statistical significance ($p > 0.05$ Mann Whitney U).

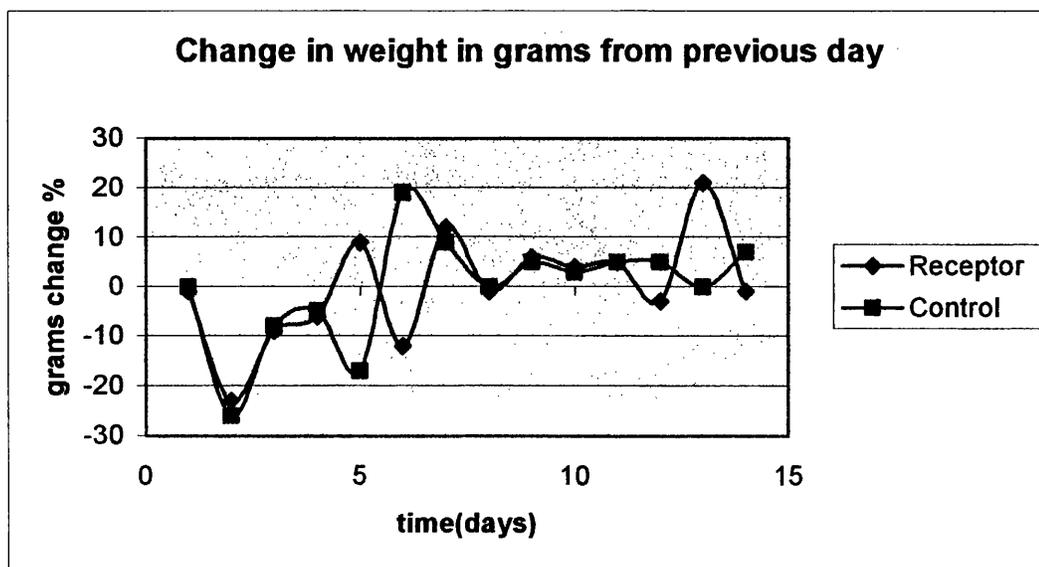


Figure 40

The actual body weight for all the mice in each group presented as change in grams from the previous day's total weight over time. No significant data points were demonstrated

5.14 Severity of septicaemia during IL-15R α prolonged therapy

The severity of septicemia was calculated at each time point for the two groups. This was achieved using the mean septic score per mouse derived from the index described in section 2.22, materials and methods. The data shown in Figure 41 below indicated that between day 1 and day 8, the receptor group consistently appeared to exhibit less severe sepsis than the control group. After day 8, (1 day after cessation of receptor treatment), the difference in severity of septicaemia was less apparent between the two groups. There were no significant data points when the two groups were compared using the non-parametric test; Mann-Whitney U ($p \geq 0.05$).

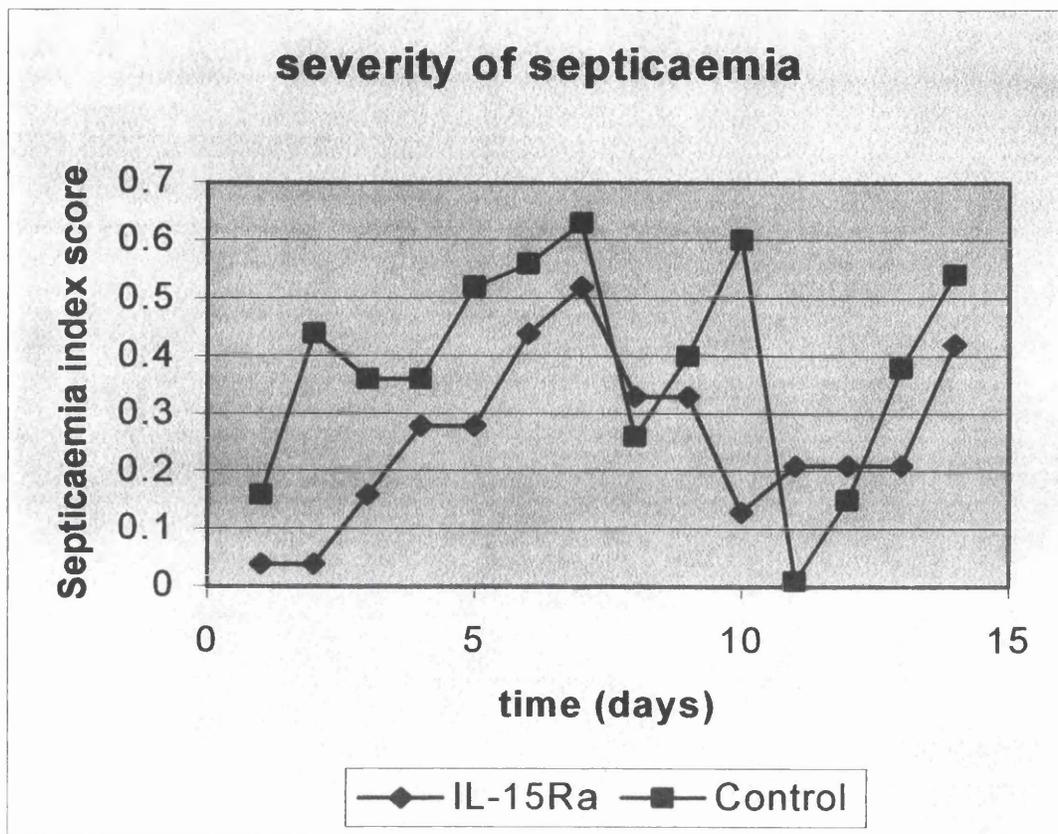


Figure 41. The mean septicaemia score shown in this table was calculated as the average score of the surviving mice in each group per day. In spite of the early trends for difference, No significant data points were recorded.

5.15 Incidence of Arthritis during IL-15R α prolonged therapy

Both the receptor and control groups of mice in this septic arthritis model were assessed for clinical signs of arthritis using the arthritic-index and by incidence of arthritic animals in each group over time and finally, the mean number of limbs with arthritis per mouse as a function of time.

In figure 42 below, the incidence of arthritis is described by the percentage of surviving animals in each group showing any signs of arthritis at each daily time point.

Statistical analysis of this data by Chi² test reveals that the lower incidence of arthritis in the receptor group illustrated in figure 42 below, was significant ($p < 0.0002$) on day two. This data indicated there was a delay in the incidence of arthritis in the earlier stage of the disease when the receptor group was compared to the controls. There is no difference between the groups after day five ($p > 0.05$).

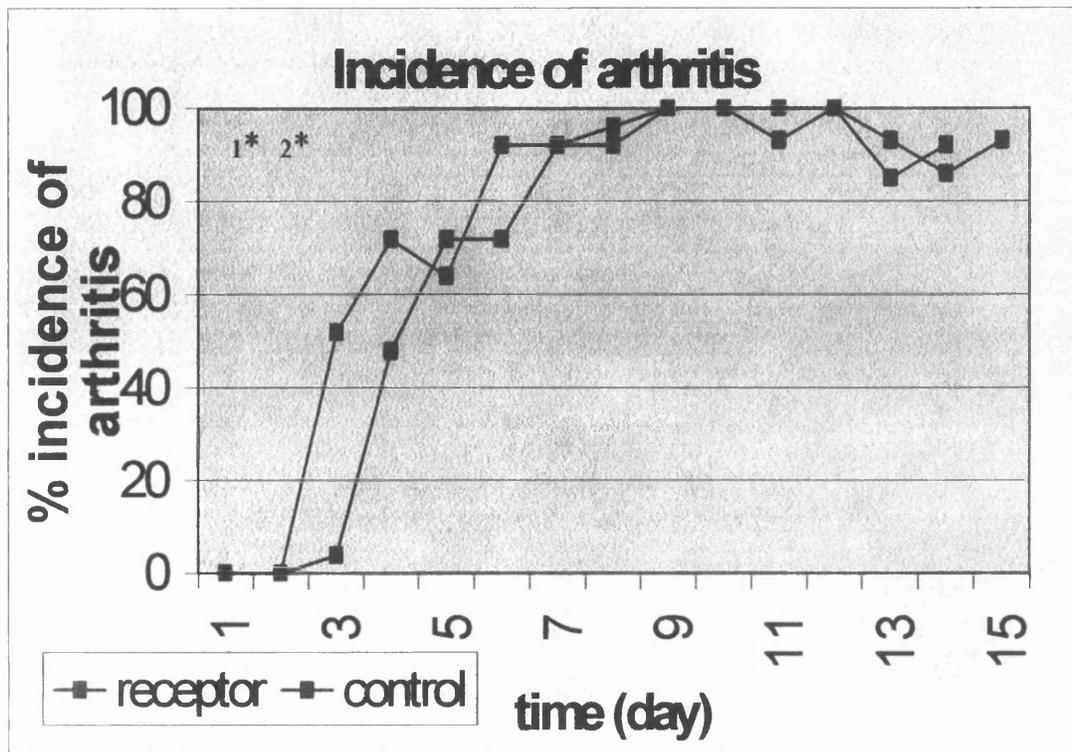


Figure 42

There are fewer incidences of arthritis in the receptor group on days 2,3 &5 compared to the control group. Day 2 (1*) $p < 0.0002$
 Day 3 (2*) $p > 0.05$. Following day five, there are no statistically significant data points

5.16 Severity of Arthritis during IL-15R α prolonged therapy

Figure 43 below shows the mean arthritic index score calculated from the sum of the individual daily index scores of each mouse divided by the number of surviving mice to give the mean on each day of the experiment. Although there was a trend whereby the severity of arthritis was lower in the receptor group compared to the control on days 2 and 3 (accurately reflecting the data in figure 42 above) , statistical analysis using the Mann Whitney U test fails to show statistical significance. ($P > 0.05$)

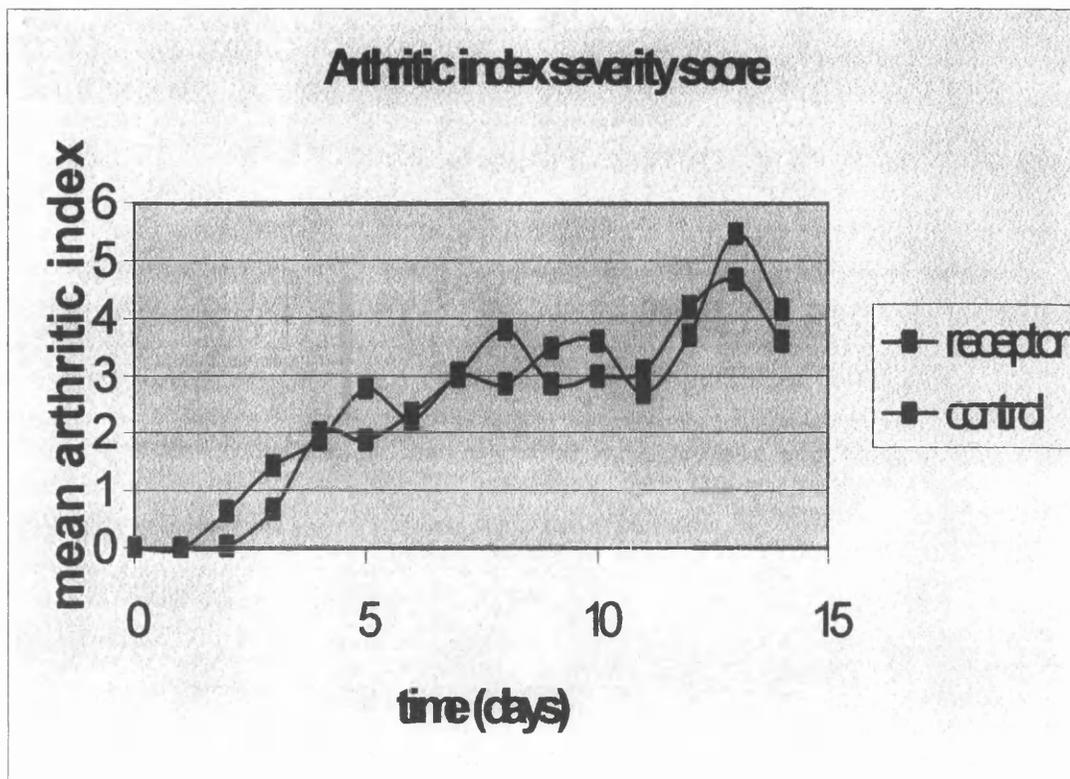


Figure 43

The severity of arthritis was measured by the index described on page 147. There was initially a transient trend for lower severity in the receptor group compared to the control. No statistically significant data points were observed.

5.17 Severity of Arthritis defined as limbs involved during IL-15R α prolonged therapy

Severity of arthritis was also assessed by calculating the mean number of limbs involved per mouse at each time point. This is illustrated below in figure 44. Concurring with the graphs of arthritic incidence (figure 42) and severity (figure 43), the data in figure 44 indicated that there were initially, less limbs involved per mouse in the receptor group compared to the number of limbs involved in the control. This difference was statistically significant on day 2 ($p < 0.0035$ Mann Whitney U). On day three there was less difference ($p = >0.05$) and from day 4 onwards there was no difference between the groups.

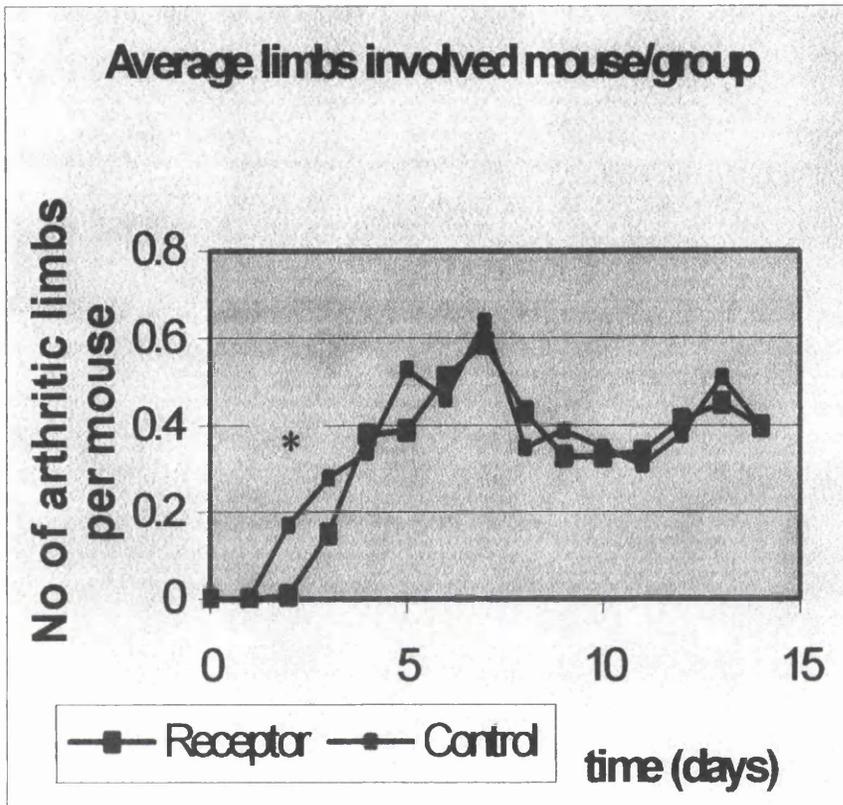


Figure 44

Average limbs per mouse per group

The difference on day two (*) was significant at $p < 0.0035$ but there were no significant differences after day 3.

5.18 Bacteriological observations in septic arthritis model mice during prolonged therapy with IL-15R α

In addition to clinical indicators of infection, the presence of *S. aureus* -colony forming units (CFUs) was also investigated by culture of various tissues at post mortem, as described in section 2.23 of materials and methods, chapter 2. Briefly, following euthanasia of the 1 and 2 week groups of mice on day 7 and 14 respectively, samples of tissue and blood were taken from 10 mice in the treatment and control groups at both time points. These tissues were then pooled. Homogeneous, single cell suspensions were made from kidney, spleen and liver. 1g samples of each tissue were diluted in 10ml sterile PBS and then serially diluted to 1×10^{-2} through to 1×10^{-7} . 100 μ l of whole blood was obtained by tail bleeding immediately prior to euthanasia. This was diluted as above into 900 μ l sterile PBS and then serially to 10^{-7} as above. 100 μ l of each dilution of tissue or blood was then spread over a pre-incubated blood-agar plate (Oxoid UK).

To estimate the presence of *S. aureus* CFUs in joints, the whole foot below the knee was removed at euthanasia and stored on ice. Using a sterile scalpel, a fresh cut was made through the forefoot of each hind limb. The exposed bone surface was then streaked across a pre-incubated blood agar plate (Oxoid UK) in a regular pattern.

The agar plates were incubated overnight at 37⁰C and colonies identified with the kind assistance of Dr Curtis Gemmell of the Department of Microbiology of Glasgow Royal Infirmary. The colonies positively identified as *S. aureus* were counted. These results are presented below in figure 45 – 49. In whole blood (figure 45), Kidney (Figure 46) Liver (figure 47) and spleen (figure 48), there is a trend for higher *S. aureus* CFUs in the receptor treated group compared to these tissues in the

control group. Furthermore, this trend continues into the second week for the blood and kidney compartments, albeit at lower CFU levels than the day seven analysis. The CFUs present in the joints assessed offer no support to any trend seen in the other tissues and the significance of this observation is unclear.

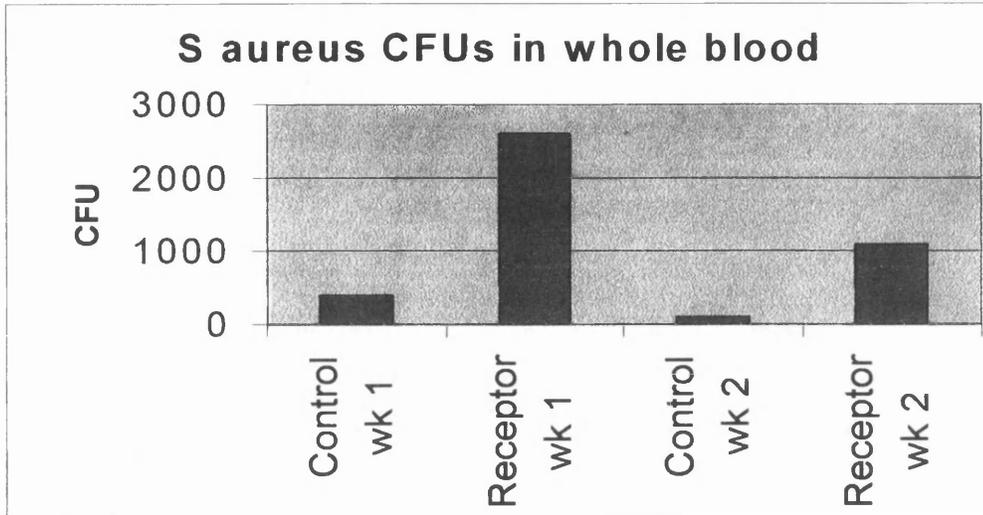


Figure 45 *S. aureus* colony-forming units in 100 μ l pooled, whole blood taken at 7 and 14 days after *S. aureus* inoculation from 10 mice per group. (I.e. not individual mice) In both week one and two groups, more bacteria were observed in the receptor treated mice than the control

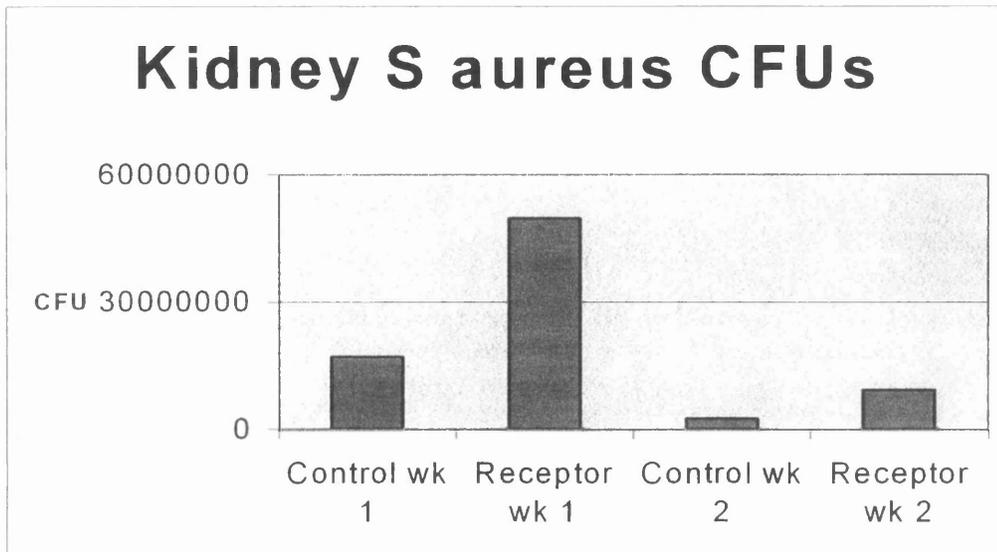


Figure 46 *S. aureus* colony-forming units in 1g combined kidney cells (I.e. not individual mice) from 10 mice per group. There were far greater numbers of CFUs in the receptor treated week 1 group compared to the control group. A similar observation of lower magnitude is seen in the results of the two week group

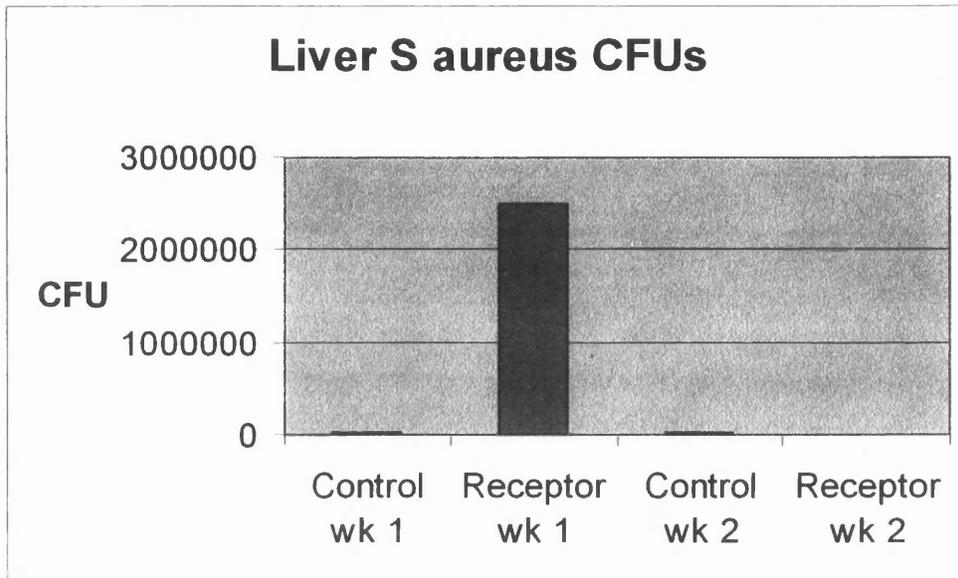


Figure 47 *S. aureus* colony-forming units in 1g combined liver cells (I.e. not individual mice) from 10 mice per group. The One week receptor treated group demonstrated increased CFUs compared to the control group. There were no evident CFUs in the two week group. Treated or control arms

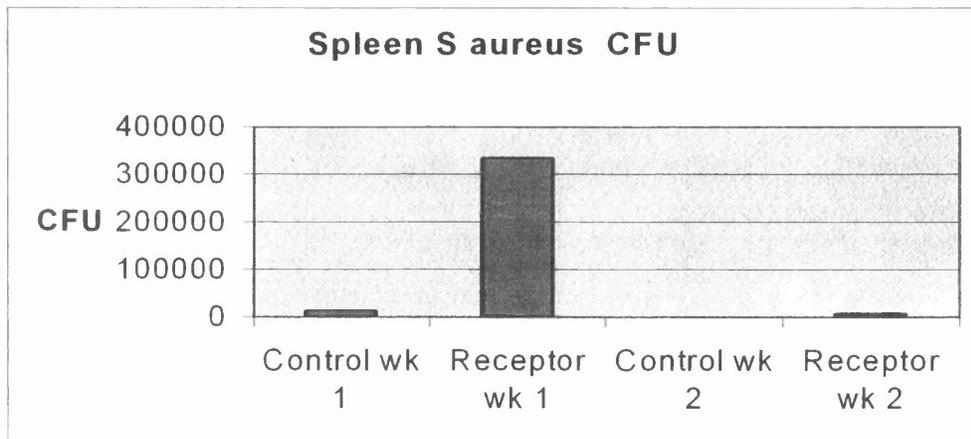


Figure 48 *S. aureus* colony-forming units in 1g combined spleen cells (I.e. not individual mice) from 10 mice per group. Increased CFUs were only observed in the One week Receptor treated mice.

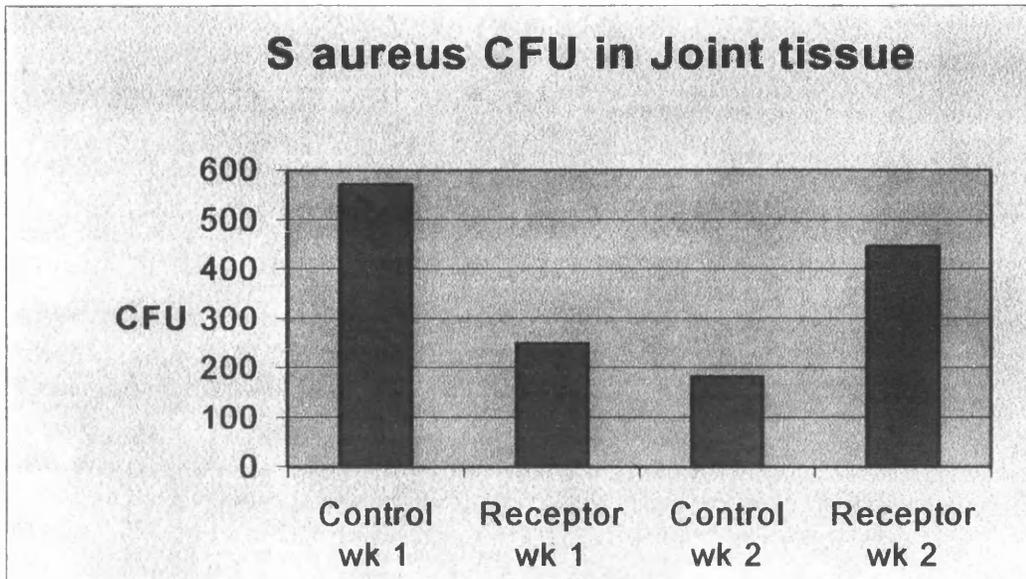


Figure 49 *S. aureus* colony-forming units in freshly cut joints at seven and 14 days post infection. In week one there were more CFUs isolated from the control mice than the receptor treated mice. In contrast, the opposite was observed in the two week group.

5.19 Stimulation of Spleen cells

The spleens of ten mice from each group were collected on days seven and fourteen. These were processed into a single cell suspension according to the method described in the methods and materials section (2.23). Briefly, spleen cells were re-suspended in RPMI at 1×10^6 cells /ml and plated out in a round bottomed 96 well culture plate. Spleen cells were stimulated with serial dilutions of Staphylococcal Enterotoxin-A (SEA) (Sigma), Concanavalin-A (Sigma) or medium alone. Incubations were for 24 or 48 hours at 37°C . Detection of proliferation was by ^3H Thymidine pulse 6 hours prior to cell harvesting followed by scintillation counting on a Wallac Beta counter. (Wallac, USA).

The counts per minute induced by spleen cell proliferation in response to exogenous SEA or the mitogen: CON-A were plotted as a function of concentration.

Figures 50 - 53 below indicate that there was a reduction in the ability of the spleen cells of the receptor-treated group to proliferate in response to both the Non-specific T cell mitogen CON-A and the *S. aureus* specific molecule, Staphylococcal Enterotoxin-A (SEA), when compared to the control group. The receptor group's lower spleen cell proliferation was marked in the day 7 spleens. This pattern was reversed in the day 14 spleen cells; Approximately 50% lower in SEA and 30% lower in CON-A week 1 and changing to approximately 10% higher in SEA and 12% higher in CON-A at the end of week two.

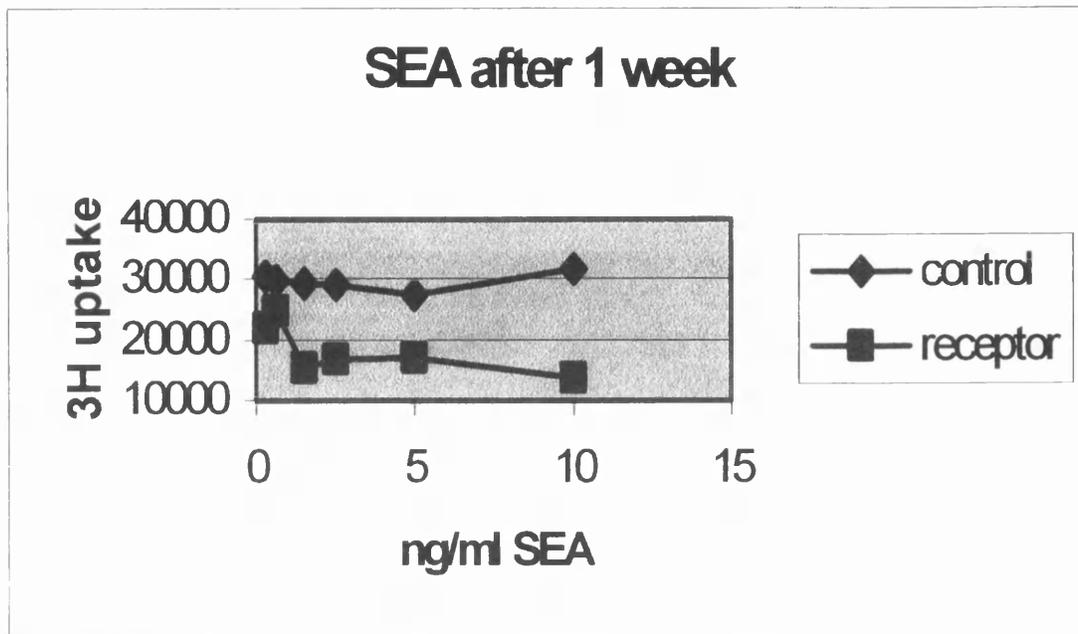


Figure 50 There was a trend for lower in-vitro Spleen cell activation in mice treated with IL-15R α when pooled spleen cells from the one week group of septic arthritis mice were challenged with SEA

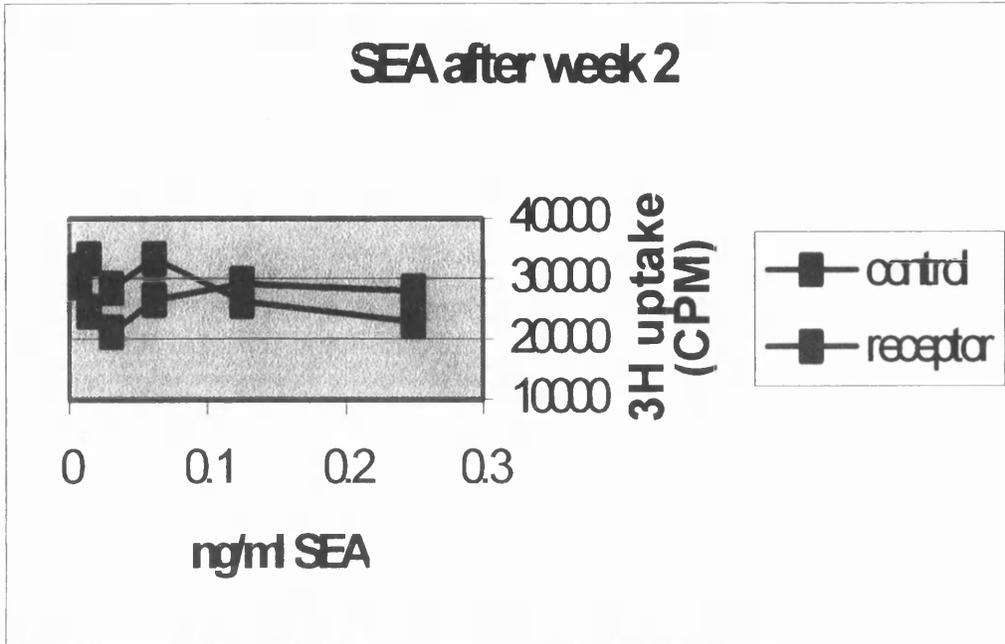


Figure 51 At low concentrations of SEA, there was a trend for lower in-vitro Spleen cell activation in mice treated with IL-15R α when pooled spleen cells from the two week group of septic arthritis mice were challenged with SEA. However, this was not confirmed at higher concentrations of SEA where the opposite was observed.

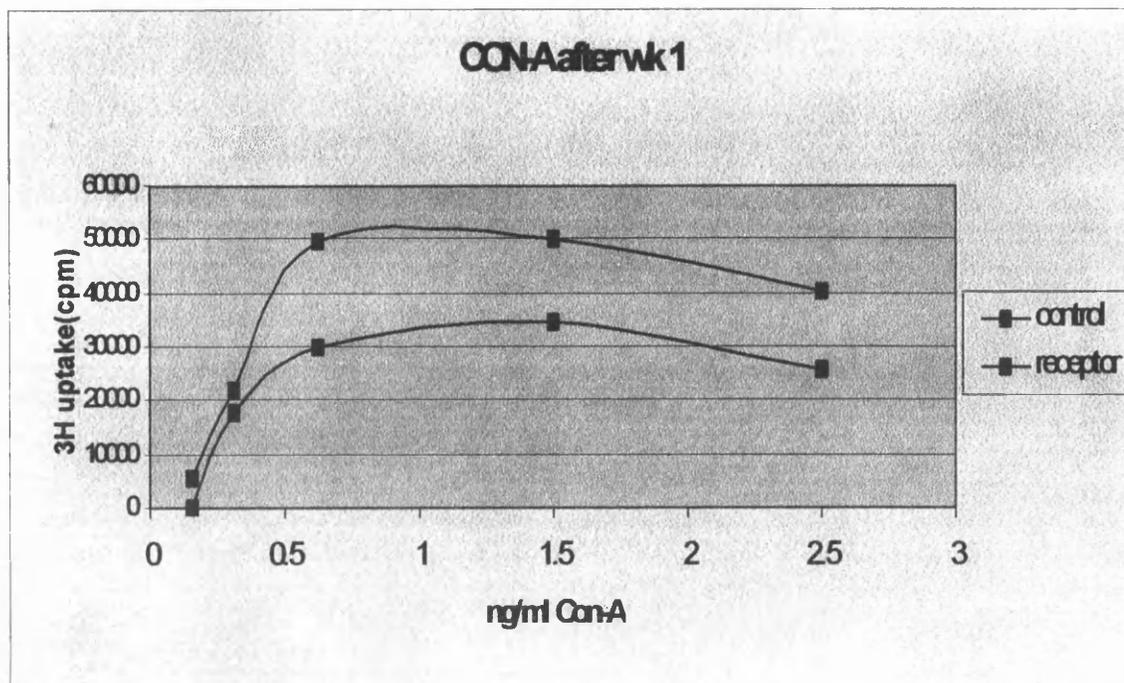


Figure 52 There was a trend for lower in-vitro Spleen cell activation in mice treated with IL-15R α when pooled spleen cells from the one week group of septic arthritis mice were challenged with CON-A. This trend was observed continuously across the range of CON-A used to stimulate the cells.

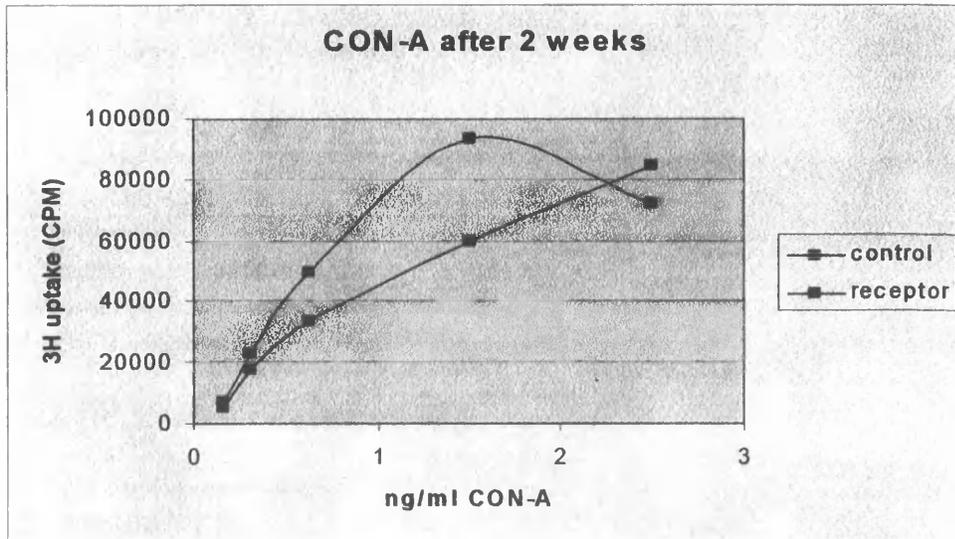


Figure 53 There was a trend for lower in-vitro Spleen cell activation in mice treated with IL-15R α when pooled spleen cells from the one week group of septic arthritis mice were challenged with CON-A. This trend was observed most strongly at a range of 1-2ng/ml CON-A but the difference was not so apparent at the extreme concentration range used to stimulate the cells.

5.20 Summary of bolus and prolonged IL-15R α septic arthritis model experiments

A trend for reduced symptoms of septicaemia and arthritis was demonstrated in mice treated with 2 bolus injections of IL-15R α when compared to those treated with the carrier control. The incidence of septicaemia was decreased at every time point in the receptor treated group when compared to that of the control group. Although this difference did not attain statistical significance, the observations of severity of sepsis also exhibited the same trend with the receptor group exhibiting fewer features of septicaemia at each time point throughout the experiment when compared to the controls. Furthermore, the difference between the two groups did attain statistical significance on day 5 ($p < 0.02$, Mann Whitney U analysis).

The arthritis associated with the model was also reduced in the receptor treated group. The incidence of arthritis was reduced on all days of the experiment in the receptor treated group when compared to the controls. Although the trend was consistent, there were no significant data points. The severity of arthritis measured both by the arthritic index and the number of limbs involved per mouse, again exhibited a clear trend: less severe arthritis and less limbs involved per mouse in the receptor treated group contrasting with those observed in the control group. Furthermore, the data for arthritic severity was significantly different on day 6 ($p < 0.05$, Mann Whitney U analysis) and also for the number of limbs involved on day 5 ($p < 0.05$) and day 6 ($p < 0.03$) when subject to Mann Whitney U analysis.

In the experiment featuring prolonged therapy with IL-15R α , similar trends were exhibited during the first few days of the experiment to those described in the bolus experiment summarised above. There was a trend for lower incidence of

septicaemia in the receptor treated group on days 1-4 compared to the controls. A similar trend was observed for reduced severity of septicaemia in the receptor treated group compared to the controls, until day 7 after which no difference was seen.

Furthermore, reduced incidence of arthritis, severity of arthritis and of mean number of limbs involved with arthritis was observed in the IL-15R α therapy group compared to the control group between days 3-6. Although not statistically significant, on the days 2, 3 and 5, there was less severe arthritis in the receptor treated group when compared to the controls. There was also lower *incidence* of arthritis in the receptor treated group on days 3, 4 and 6, with day 3 ($p = <0.0002$) and day 4 ($p = < 0.05$) attaining statistical significance. The mean number of limbs involved was also less in the receptor treated group on days 2, 3 and 5 and the difference on day 3 was statistically significant ($p = <0.0035$).

In summary, it can be seen that the administration of IL-15R α to mice with septic arthritis reduced the incidence and severity of septicaemia and arthritis associated with the septic arthritis model in the first 3-7 days of the disease. This implies that the IL-15R α may act to modify host response to the *S. aureus*, resulting in less inflammatory disease.

Two interesting features can be discerned from this data. In the experiment, where two doses of IL-15R α were administered, a sustained difference was seen between the two groups with a trend for lower sepsis and arthritis extending over the 7 days of the first experiment. In the experiment featuring prolonged injection of IL-15R α , a similar trend was seen but observations of lower severity/incidence of symptoms associated with this model did not generally extend beyond the first few days of the experiment. A second feature is that the lower incidence/severity of sepsis

and arthritis was not apparent following the cessation of treatment. This might indicate that the host response to *S. aureus* was being suppressed by the administration of IL-15R α and that this effect was lost on cessation of treatment.

The clinical observations described above for the two experiments represent a repetitive trend for reduced inflammatory response leading to manifestation of septicaemia and septic arthritis when mice were treated with IL-15R α . The results for the non-clinical parameters investigated, *S. aureus* colony forming unit numbers and spleen cell proliferation, may also be reflective of this trend. (These measurements were derived from pooled samples and as a consequence, statistical analysis is impossible.) The *S. aureus* bacterial load was found to be greater in blood, kidney, liver and spleen at the end of week one in the therapy group compared to the control group. In the septicaemia and arthritis measurements, the receptor treated mice generally exhibited less severe symptoms of septicaemia and arthritis during the time that blood bacterial load was higher. In the second week following cessation of IL-15R α therapy, bacterial loads between the two groups became essentially identical. This “normalisation” of bacterial load, however, is demonstrated at a time point where there was no longer any evidence of lower arthritis or septicaemia in the receptor treated group.

These results may suggest that IL-15R α treatment transiently reduced inflammation (manifest as symptoms of septicaemia and arthritis), but concomitantly may have suppressed the ability of the mice to clear the infecting *S. aureus* or perhaps modified the distribution pattern of the bacteria in some way.

If the host response to *S. aureus* was indeed impaired by the IL-15R α , then further evidence for this hypothesis might be expected in the form of reduced spleen cell proliferation in the receptor treated mice.

To investigate this, spleen cells were pooled from 10 mice per group and their proliferative response against SEA and Con-A was measured. Figures 50 and 51 showed that in the One Week groups, that the spleen cell proliferative response of the receptor treated mice was reduced by 40 - 50% compared to the controls at the end of the treatment period (day 7). In the Two Week group, (figure 52 & 53) the reduced proliferative responses of the receptor treated group were observed to have similar or equal levels of response to SEA and CON-A to the controls by the end of week two, 7 days after the last IL-15R α injection. This data does not contradict the trends exhibited for septicaemia or arthritis. The proliferative ability of the spleen cells was reduced in the receptor group at the 7 day time point when the CFUs of the therapeutic group were markedly higher than the CFUs found in the control group. This coincides with the increase of inflammation and of reduction of bacterial load in this group to levels seen in the control group. As with the bacteriology data, the spleen samples used in these preliminary experiments were pooled from 10 mice and no statistical analysis can be applied. This preliminary CFU and proliferation data appears consistent with the modification of the host response to *S. aureus* by therapeutic injection of IL-15R α .

In conclusion, the administration of IL-15R α to mice with septic arthritis seems to modify host response to *S. aureus*, manifest by reduced symptoms of sepsis and arthritis. The reduction in inflammatory response demonstrated by the severity and incidence data was co-incident with the highest bacterial load. This point also coincided with a reduced ability of the spleen cells to proliferate in response to the

pathogen's antigen *in-vitro*. Cessation of treatment restored the proliferative response of the spleen cells resulting in reduced bacterial load and a restoration of the inflammatory response indicated by levels of sepsis and arthritis indistinguishable from control mice.

McInnes (68) examined the blood/organ bacterial load and spleen-cell proliferation profile of mice infected with the same strain of *S. aureus* used here. The experimental mice were genetically deficient in inducible NO Synthase (iNOS), an enzyme that produces Nitric Oxide. In their experiment, they found that the deficiency of NO production led to increased colony forming units (*S. aureus*) in blood, spleen and kidney compared to control mice, reflecting the pattern seen above in figures 45-49. In contrast to these experiments described above, McInnes et al found *increased* proliferation of spleen cells from the iNOS deficient mice in response to SEA when compared to control mice.

There is no relationship implied here between NO action in inflammatory response to live pathogens such as *S. aureus* and that of IL-15. However, it is possible to see that the bacterial profiles demonstrated by the removal of the pro-inflammatory Nitric Oxide are similar to the bacterial seen above in response to the removal of the pro-inflammatory murine IL-15 by administration of the IL-15-receptor. Comparison of the graphs of Severity of Sepsis in the first (fig 41) experiment and that of McInnes (1998 fig 2b) reveals a startlingly similar pattern of sepsis over seven days in both the wild type mice and the experimental groups. These similarities with the NO deficient model of Septic arthritis strengthen the possibility that the results are likely to be reproducible and informative, if repeated in a more robustly designed experiment.

Some logical steps to investigate the trends and patterns presented here would be to increase the dose of IL-15R α and to extend the observation period to 21 days to study the long term effect of greater bacterial load. The use of single spleens for proliferation and bacteriological assays would allow statistical calculations to be carried out.

Chapter 6

Discussion and Conclusions

This project investigated the possibility that IL-15 might play a modifying role in three murine models of human inflammatory disease; collagen induced arthritis, septic shock and septic arthritis, models for the human diseases of rheumatoid arthritis, septic shock and septic arthritis respectively. The possibility that IL-15 is implicated was based on the premise that IL-15 influences the production of TNF α . TNF α in turn being documented as a major cytokine component in pathogenesis of the diseases and/or their murine models investigated here.

Initially, the experimental design was to generate murine IL-15 and then derive monoclonal antibodies against IL-15 and use these as a neutralising agent in the murine models. Generation of a murine IL-15cDNA was achieved from J774 cells resulting in the cloning of a cDNA with a sequence homologous to that published by Anderson et al (17). Furthermore, a mutant form of IL-15 was cloned from the same culture of J774 cells.

6.1 Recombinant murine IL-15 deletion mutant

When analysed, this alternative cDNA contained the same sequence as the wild type murine IL-15 cDNA but was deficient in 16 codons translating to an in-frame deletion mutant of 16 hydrophobic amino acids. The significance of the presence of this mutant in the total RNA of the J774 cells is unclear. It may be an artefact of either the cloning procedure or perhaps the nature of the J774 cells that their continued culture may have generated mutations over time. However it is interesting in that the deletion is in frame and furthermore, deletes codons for a series

of predominantly hydrophobic amino acids from the secondary structure of murine IL-15. The deletion mutant, here named *IL-15-48*, was also tested for characteristic IL-15 T cell stimulating activity. It was found that this mutant was able to bind IL-15 receptor alpha with similar coefficient to the purified recombinant murine IL-15. However it did not activate CTLL cells. This suggests that the deleted 16 amino acids were not in binding to the IL-15R α but are important in generating the necessary signals to activate the T cell. Taking advantage of this unexpected phenomenon, the mutant was also used in a CIA experiment as a therapeutic antagonist to IL-15.

Another cytokine deletion mutant is well documented in the literature (85) IL-1R α is a naturally occurring mutant form of IL-1, which amongst other cells, is produced by macrophages (86). This mutant shares amino acid homology to IL-1 α and IL-1 β however, IL-1R α lacks one receptor binding motif compared to IL-1 α & β which have two. The omission of the motif in IL-1R α seems to prevent signal transduction when IL-1R α is bound (87). Therefore as IL-1R α has relatively similar affinity for the receptor as that of the IL-1 α & β (88) its binding can be seen to have an antagonistic effect on IL-1 activity in a cell system.

There is also evidence of alternative IL-15 splicing mutants in the literature. Meazza et al found splice variant IL-15 cDNA in the majority of small lung cell cancer cells that they examined. Meazza (19) discovered that there was an alternative exon, which coded for a shorter signal peptide when the IL-15 RNA was translated. The effect of the shorter leader sequence was not determined. It did not up-regulate expression or secretion of bioactive IL-15 in these cells. It is interesting to note that IL-15 RNA can be transcribed by cells such as T cells which do not secrete IL-15, demonstrating that the IL-15 control mechanisms are likely to be at a translation/secretion level rather than transcriptional (89).

The Immunex Corporation (Seattle, USA) has extensively tested PEGylated and mutein forms of IL-15. They found that targeting lysine residues adjacent to amino acids crucial for interaction with the IL-15 β subunit might account for the antagonist activity exhibited by PEG-IL-15. They concluded that there was scope for therapeutic molecules against inflammatory disease to be generated in this manner (90)

If however, IL-15-48 were a natural mutant, then it too would have a similar theoretical antagonist potential to that of the muteins above. However, the addition of PEG is known to have the effect of reducing immunogenicity and enhancing physiological half-life. It is not known that IL-15-48 is a naturally occurring mutant, neither is it known what function if any the deleted hydrophobic residues would have had within the tertiary structure of the mature protein if it had been produced. However, it can be seen that the binding function was not affected by the deletion and therefore it might be speculated that the IL-15-48 mutant might exist naturally as some form of regulatory agent for IL-15. The deletion sequence itself may be of interest to those who have insight into protein structure as it would define an area involved in the function of IL-15 but not related in any meaningful way to its capacity to bind IL-15R α . Since completion of the experimental section of this thesis, no studies have been published too offer any evidence that the IL-15-48 mutant observed here was anything other than an artefact. Recent work using site directed mutagenesis by Harb et al, (110) has elucidated amino acid sequences in several portions of the native *human* IL-15 poly peptide which interact in some way with the human soluble IL-15R α . Notwithstanding the lack of sequence homology between human and murine forms of IL-15, there is no obvious homology between the amino acids identified by Harb as being involved in the interaction between IL-

15 and its receptor. The hydrophobic nature of the IL-15-48 deletion mutant is not reflected in Harbs' sequences nor is the numerical position within the polypeptide chain. More analysis could be done here but the lack of other reports of any such similar molecule spontaneously arising in the 5 years since the first observation of IL-15-48 in this experiment would suggest that it was indeed an artefact of the J774 cells used for culture. However, the possibility of artificially constructing a similar mutant as a research tool, i.e. a molecule that binds its receptor and acts as an antagonist remains open as an alternative should an experiment require a non-antibody based protocol.

Although functional/non-functional mature protein IL-15 isoforms have been conspicuous by their absence, pre translation Isoforms have been observed. Recent research into IL-15 mRNA isoforms centre around 5' upstream modifications allowing eventual direction of mature IL-15 protein to different cellular compartments, leading to soluble or membrane bound isoforms. This has informed the debate around the discrepancies between IL-15 mRNA and mature protein in various tissues (such as macrophages where constitutive expression of IL-15 mRNA but little protein) has often been seen in the IL-15 literature. Musso et al (111) investigated this and show that monocytes do indeed have a membrane bound form of IL-15 but did not observe this form in the T and B cell subsets. It can be seen that a culture medium of macrophages might not exhibit much or any evidence of IL-15 activity even where there was evidence for mRNA however the role of macrophages and monocytes in immune responses may well be driven by cell-cell contact or soluble forms of IL-15R alpha.

The activities of IL-15 as a promoter of TNF α immune systems may be controlled by a soluble receptor acting as an antagonist. I.e. a differential in

concentration of IL-15 soluble receptor may allow or disallow interaction between membrane bound receptors on target cells with which for example macrophages displaying IL-15 might wish to activate. In 2004, Mortier et al (112), demonstrated a proteolytic release mechanism for soluble IL-15 in human cell lines and COS7 cells. This demonstrates a possible control mechanism for IL-15 activity and perhaps suggests a reason as to why the soluble receptor was so successful in the work of Ruchatz et al (43) in ameliorating the proposed IL-15 driven pathology of the collagen arthritis model. This regulation of pro-inflammatory cytokines is reflected in the homologous IL-2/IL-2R α system where spontaneous IL-2R α is cleaved leading to soluble antagonism to IL-2 activity (113). The difference here is that the IL-2/IL-2R α interaction is of low affinity and non-physiological concentrations must be generated to induce antagonism. In contrast, Mortier (112) proposes that the IL-15/IL-15 sSoluble receptor alpha interaction is of very high affinity and as such may be an example of a physiological control mechanism.

6.2 Recombinant Murine IL-15

Murine IL-15 cDNA was cloned and protein expression was induced according to the expected mobility on SDS-PAGE analysis. However, the protein

produced was highly insoluble and had very low bioactivity. It was not soluble in PBS and could therefore only be re-suspended in PBS with high concentrations of salt and glycerol present. The presence of these additives engendered great difficulty in pursuing meaningful experiments for activity and cell culture bioassays. Low bioactivity of the protein might be due to the presence of these additives and/or the harsh purification/resuspension steps needed to elute the murine IL-15 protein from the expression system. A more fundamental reason may be that the regulation of IL-15 secretion, widely acknowledged to be complex, suggests that using a prokaryotic expression system was possibly disadvantageous. In any future attempts to derive bioactive recombinant IL-15 an eukaryotic cell expression system such as drosophila cells could be used. However, since the completion of this thesis, several companies now offer commercial recombinant murine IL-15, including Cedarlane Laboratories, (USA), who now market an E. coli derived, recombinant murine IL-15 with 98% purity and an ED₅₀ value of <20ng/ml (CTLL assay), giving a specific activity of >5x10⁴units/mg. They also state an endotoxin value of <0.1ng/μg. Although these units of measurement are not available for our recombinant muIL-15, it would be interesting in any future attempts to repeat this work to compare and refer to the activity data of the Cedarlane muIL-15 product. It would also seem from these data that purification with immobilised anti E. coli media might be essential in gaining a purified and active protein. It had been suggested above that using an Eukaryotic recombinant protein system (such as bacculo/drosophila) to produce active muIL-15 might overcome stability, activity and solubility problems: the fact that the Cedarlane recombinant muIL-15 product was soluble in water when reconstituted from 10mM Tris at pH8 would suggest that there might not be such an advantage. (Data sheet available in the appendix). In spite of the low bioactivity, some of the recombinant

murine IL-15 protein was used to induce polyclonal rabbit anti-murine IL-15 antibodies. These were purified to a relative binding affinity of 1/32000 following protein-A enrichment of the IgG fraction and then used in the CIA experiment described in chapter 3.

As the conditions and reagents used in the purification of recombinant murine IL-15 were virtually incompatible with the in-vivo and in-vitro experimental use, it was decided to use the soluble form of murine IL-15R α . This protein was eluted from the Qiagen expression system and had activity in PBS at a physiological concentration. Therefore, the experiments within the LPS and the septic arthritis models were undertaken using IL-15R α a natural antagonist.

6.3 Collagen Induced Arthritis model

Previously, Ruchatz et al (43) reported modification of the symptoms of CIA when treating DBA1 mice with soluble IL-15R α . In the experiments described in chapter 3, neither the IL-15 mutant antagonist nor the purified IL-15 anti-sera demonstrated such a clear improvement in the symptoms of mice suffering from CIA. These experiments were carried out using the same strain of mice DBA/1 and welfare conditions and diet etc.

In terms of arthritis severity, the mice treated by placebo were reflective of placebo treated mice of the Soluble IL-15R α experiment by Ruchatz et al. There was a common pattern for a more rapidly increasing severity in the beginning of the second week of treatment.

The mice treated with IL-15-48 initially exhibited a flat curve for arthritis severity, however this trend was not statistically different from the placebo treated mice. This is in contrast to the mice treated with soluble IL-15R α , which showed a marked and increasing difference from those treated with placebo. The initial trend for reduced arthritis severity in IL-15-48-mutant treated mice may be of interest in the light of other score/observations shown in the anti-body treated CIA (below) and the effect that IL-15R α had on LPS shock and Septic arthritis. However, there was no statistical difference demonstrated by the therapeutic intervention of IL-15-48 compared to placebo.

In addition to severity, incidence of arthritis was also measured there was a trend for lower incidence initially but not statistically significant. Although not directly comparable, this is in contrast with the data for number of arthritic paws

shown by the soluble IL-15R α mice of Ruchatz et al who reported almost complete abrogation of arthritis incidence.

In a subsequent CIA experiment, rabbit anti-IL-15 polyclonal antiserum was also used. Similar to the IL-15-48 experiment, there were no statistical differences in any of the parameters measured between anti-IL-15 and placebo treated mice except a transient reduction of severity of arthritis in the anti-IL-15 treated group suggesting a trend for mice treated with anti-IL-15 to show less severe signs of arthritis than the placebo group.

Considering the ability of IL-15R α to abrogate almost completely the symptoms and signs of collagen induced arthritis, it was disappointing that there was such a weak trend for improvement when the IL-15-48 and anti-IL-15 were used under such analogous conditions. Endogenous IL-15 was again the presumptive target and it can really only be concluded that the therapeutic agents trialed here were of insufficient concentration or quality to neutralise any IL-15 pro-inflammatory activity.

The slightly stronger trend for lower severity in the anti-IL-15 treatment experiment indicates that there may be a mild abrogation of arthritis. The generation of a substantially more potent preparation of anti-IL-15 antibody might be a useful experiment and would possibly demonstrate a level of abrogation of collagen induced arthritis symptoms similar to that shown by the soluble IL-15R α . A sample of the polyclonal IL-15 anti-serum was run through an *E. coli* affinity purification column. The filtered eluate was found to have a much lower IgG concentration than prior to filtration (data not shown). This implies that the anti-sera used could have partly been comprised by anti-*E. coli* antibodies rather than the anti-IL-15 activity first supposed. The inclusion of an immobilised *E. coli* lysate step should be an integral part of any

future preparation of recombinant protein and antibody from an E. coli based expression system.

Any future experiments with anti-IL-15 or the mutant antagonist might also benefit from more extensive dose ranging trials, as toxicity at the doses used here was not seen to be a problem.

It is interesting to speculate that a crossover trial with both anti-IL-15 and anti-TNF α would be interesting with respect to any synergy arising in symptom abrogation. Interestingly, Williams et al (42), describe a dose dependent effect of anti-TNF α on CIA, suggesting that a higher concentration of neutralising, anti-IL-15 antiserum might be more effective in any future work. It would have been helpful to analyse cytokines by ELISA at early time points to generate more and comparable data in any repetition of these experiments. It is not possible to demonstrate how intervention of anti-IL-15 reduced (transiently) the severity of arthritis symptoms.

If IL-15 is important here, it may be through its ability to modify T cell activation and attraction (33). Thus it could be proposed that neutralising IL-15 reduced chemoattraction of T cells into the joint hence less arthritis. Less arthritis because removal of IL-15 could reduce expression of TNF α both by failing to induce T cell secretion of TNF α and reduction in T cell activation of Macrophages to produce TNF. Again, Fluorescence Activated Cells Sorting (FACS) analysis might illuminate more of the precise role of IL-15 in the pathogenesis of the CIA model. IL-15 is also reported to induce B cells to mature (13). Therefore as anti CD40 treatment has shown to reduce arthritis, then in addition to the reduction of TNF α production and T cell recruitment, B cell activation may also be reduced when IL-15 is neutralised. In addition, IL-15 has been shown to upregulate Neutrophil activity (91) If this were reduced by neutralisation of IL-15, then the pro-inflammatory effect of

neutrophils in the CIA model, as demonstrated by Schrier et al (92) in rats, might also be less severe.

From the above, the effects of IL-15 can be seen to be pleiotropic and its neutralisation might reduce the severity of symptoms in the CIA model. In relation to this project, that IL-15 can modify the symptoms of arthritis in this model is hinted at by the transient reduction seen in the results section. "Transient action" may be suggested by the results but the end of protective effect when the therapeutic injections ceased in the prolonged septic arthritis experiment results in chapter 5, would suggest the contrary. Dividing the study of effects of IL-15 into time sections in the arthritis model may also be helpful and informative in future experiments.

The fact that there was such a marked reduction in the symptoms with the sIL-15R α is interesting and points to the sIL-15R α as a more stable and effective trial molecule. Human trials are about to commence in Rheumatoid Arthritis patients to evaluate the therapeutic benefit of neutralisation of human IL-15 (134). All of the above points to implicit activity of IL-15 in arthritic disease and the cascade of activity where IL-15 activated T cells cause migration of active immune cells to the site of synovitis has been discussed in the literature search earlier. The most effective confirmation of the role of IL-15 in arthritis will be seen in the results of the forthcoming human IL-15 modulation trials.

6.4 LPS Shock model

In the original experimental design, anti-IL-15 anti sera would have been used in therapeutic intervention in the LPS shock model to elucidate any effects mediated by the neutralisation of endogenous IL-15. Because of the poor definition of any effect seen in the CIA experiment described above and the difficulties involved in production of murine recombinant IL-15 for testing such anti-bodies, soluble IL-15R α was used as the therapeutic agent in the septic arthritis and LPS experiments.

In a preliminary experiment, a highly significant difference was seen in the BALB/c mice treated with sIL-15R α (33% mortality compared to placebo treated mice: 85%) following insult with 18mg/Kg LPS. This was a highly significant difference ($p < 0.013$ Kruskal Wallace).

It was decided to pursue LPS challenge experiments using lower doses of LPS, (5 - 2.5 mg/Kg) in combination with *C. parvum* priming. This was in order to gain information through biochemical analysis of the liver damage enzymes in experiments with shorter duration and lower severity.

As expected, these mice exhibited no significant difference in mortality whether treated with srIL-15R α or placebo. The most informative results were derived from measurements of the liver damage associated with *C. parvum* primed mice, challenged with LPS. Serum levels of aspartate-aminotransferase (AST), alanine-aminotransferase (ALT) and creatine kinase (CK) were assessed as indicators of liver damage. The samples were pooled due to small blood volumes eluted from the tail vein. Several observations can be made from the results. Firstly the priming of the *C. parvum* does affect the level of liver damage indicators in its own right. Secondly, the placebo was comparable to the *C. parvum* alone treatment in its effect

on liver damage enzymes, lending credibility to the actual liver damage parameters observed in the active srIL-15R α treatment group in spite of the impossibility of statistical analysis of pooled samples. The reduction of serum levels of liver damage indicators in the IL-15R α treated mice; suggest that IL-15 is associated with liver damage and hence its use may be indicated in preventing acute hepatic injury

In the active-treatment or placebo mice, the results segregated to the mice insulted with 5mg/Kg LPS and those with 2.5mg/Kg LPS. There was no apparent difference between the PBS control and the active sIL-15R α treatment in the 5mg/Kg but in the 2.5mg/Kg experimental set, the sIL-15R α treated mice showed approximately 50% lower serum levels of all three indicators of liver damage indicators. This suggests that future experiments might need greater concentration of sIL-15R α in order to show a protective effect at 5mg/Kg but more importantly that the experiment was informative at 2.5mg/Kg LPS.

A further experiment sought to describe the clinical signs of non-lethal levels of LPS shock and to generate serum samples suitable for statistical analysis of serum liver damage levels.

The results showed that there was a significant protective effect against symptoms of septicaemia when IL-15R α was administered compared to placebo PBS ($p < 0.0011$) because the mice in the IL-15R α group consistently exhibited less severe symptoms of sepsis at all points in the duration of the experiment. As with the previous experiment, serum liver enzymes were measured, this time at 48 hours post LPS challenge. As a consequence of later sampling, one would expect higher serum enzyme activity due to the prolongation of the model. The serum enzyme levels were increased according to this expectation.

The trend for lower sepsis score in sIL-15R α treated mice, measured by the septic index mirrored the enzyme determinations; AST, ALT were reduced by 50 – 60% compared to the control mice levels ($p < 0.011$). The CK level was also reduced in the treatment group by about 33% compared to the control ($p < 0.05$).

As there was no ELISA assay to determine any serum cytokine changes occurring during the experiment, it is hard to determine how IL-15 might actually be influencing this apparent reduction in mortality, liver damage and signs of sepsis. It could be proposed that IL-15 following LPS injection acted to cause mortality at high LPS doses and merely liver damage at low LPS doses, through induction of TNF α . TNF α is implicated in liver injury, as shown by Ming et al (93) who concluded that TNF α was essential in the pathology of liver damage.

Taguchi et al (94) used very similar methodology to that used here, when they primed BALB/c mice with 1 mg/mouse P acnes (*C. parvum*) and challenged with LPS (1 μ g/ml IV tail vein) 7 days later. At 24 hours blood sampling showed that control mice exhibited similar resting serum levels of AST and ALT to the placebo/non-LPS mice of the experiments described here.

Following LPS challenge Taguchi et al demonstrated a 15-50-fold increase in serum liver damage enzymes in their mice. When Taguchi administered Rolipram, (an antidepressant phosphodiesterase inhibitor, known to cause liver damage in mice) these serum damage indicators were reduced for ALT and AST. In our experiments treatment by IL-15R α reduced ALT and AST, indicating an abrogation of the liver injury induced by LPS by treatment with sIL-15R α .

Taguchi also measured the levels of macrophage derived, in-vitro TNF α . This was reduced by 40% in the Rolipram treated group. It would be of great interest to investigate the effect of IL-15R α on in-vitro TNF α levels in analogous experiments to

Taguchi to see if similar reductions in liver injury might be related to a cascade effect of IL-15. If so, then treatment with anti-IL-15 in acute hepatitis may be beneficial.

It is believed that macrophages infiltrate the liver tissue several days after *C. parvum* priming. (95) These macrophages express the mac-2 activation marker 7 days following priming. Taguchi (94) showed that in a time course, cultured macrophage-TNF α production began within 2 hours of the LPS challenge. This reflected earlier work by Nagakawa (96) which showed that TNF α was transiently elevated and also that the amino-transferase enzymes measured here too, were also elevated in tandem with the rise in TNF α . TNF α neutralisation was found to be protective. Tiegs et al (97) in another experiment, link TNF α production and raised serum liver damage enzymes. Here, TNF α production is followed by increased AST and ALT serum levels. Tiegs demonstrated a dose dependant relationship between the treatment of mice with Ebselen, (a selenium based immune-suppressive compound, synthetic antioxidant inhibitor of free-radical induced apoptosis) and TNF α production and the activity of the liver enzymes, AST and ALT. The AST and ALT again followed a decrease in concentration as TNF α decreased. This was in the presence of increasing Ebselen levels.

The experiments in the literature indicate that TNF α has a direct effect on liver injury as shown by serum levels of AST and ALT. Removal of TNF α or use of TNFR1 receptor knockout mice shows a protective effect, underlining the importance of TNF α in liver injury. Macrophages may well be the source of the increased TNF α concentrations in inflammatory situations. The events or mediators influencing the increase in concentration of TNF α are likely to occur within 2 hours following LPS challenge. The findings in the author's experiments suggest that IL-15 may contribute to mortality and liver injury after the mice are challenged with LPS. At a high dose of

LPS, the presence of sIL-15R α conferred a protective effect against mortality compared to placebo. At a much lower dose of LPS, liver injury was observed in the mice treated with placebo however the mice treated with sIL-15R α exhibited much reduced levels of liver damage enzymes. This indicates that endogenous IL-15 might be acting to induce liver damage but neutralisation of IL-15 by the presence of sIL-15R α led to a protective effect. As no other cytokine determinations were made during the above experiments, it is a matter of speculation as to how the protective effects of sIL-15R α were acting in what can be seen from the literature quoted above, is widely acknowledged to be a primarily TNF α driven process.

For the future, evidence for any pro-TNF α production up-regulation by endogenous IL-15 might be sought out in the time preceding the first 2 hours following LPS insult in *C. parvum* primed mice. A natural progression might be to examine real time levels of TNF α . The recently developed "Real Time PCR" (98) could be an informative technique here. Then it might be possible to establish any connection with the reduced serum AST and ALT levels, modified by the presence of sIL-15R α and the TNF α influence on LPS induced liver damage. To investigate this area further, experiments might be informative when measuring TNF α levels in a time course concomitantly with the treatment with IL-15R α . The results reported here indicate that future experiments might be most informative at doses of 2.5mg/Kg or less.

The trend for lower liver damage and reduced symptoms of sepsis reported above provide a strong indication that removal of IL-15 does indeed modify the LPS shock process.

It is interesting to speculate on the nature of a possible link between IL-15 and TNF α in the LPS shock pathogenesis. The liver tissues are highly enriched in an LPS

receptor called SR-A. (99) These receptors may be highly prevalent in this organ in order to remove LPS from the bloodstream as it flows along the portal vein from the gut. It is further believed that LPS Binding Protein (LBP) transfers the LPS to CD14, either soluble form or membrane bound to liver macrophages. At these macrophages, LPS then interacts with TOLL like receptors and this interaction in turn leads to eventual elimination of the LPS in combination with HDL molecules. In the mouse, TLR4 is believed to be the TOLL Like Receptor responsible for LPS metabolism (100). This is demonstrated by the observation that TLR4 deficient mice are more susceptible to gram -ve infection. TLRs may have a central role in promogulating the inflammatory reaction. Signalling through these receptors has been associated with release of $\text{TNF}\alpha$, IL-1, 6, 8 and 12. TLRs have been shown to be essential for LPS responsiveness in mice.

Does IL-15 have a role, synergistically or otherwise, in the pathway from LPS binding, TOLL up-regulation and $\text{TNF}\alpha$ production by liver macrophages, leading to LPS induced liver damage? IL-15 upregulates TOLL 2 in cell lines as does LPS (100). Therefore might IL-15 be acting synergistically with LPS by up-regulating $\text{TNF}\alpha$ via a TOLL related cascade. This may explain the reason why, in Ruchatz' work, sIL-15R α was able to almost totally abrogate the inflammation resulting in arthritis in response to collagen challenge and mediated by $\text{TNF}\alpha$, pointing to a direct and possibly exclusive influence of IL-15 in $\text{TNF}\alpha$ expression. However, in the case of LPS shock, although biochemical indicators of liver damage were reduced, the symptoms of sepsis were reduced but not abrogated. This may be because $\text{TNF}\alpha$ driving liver damage was under the direct influence of IL-15 but the $\text{TNF}\alpha$ inducing the wider signs of sepsis was in fact being partly driven by signalling via LPS induced TOLL receptors or some other unknown system, independent of IL-15. Thus,

neutralisation of IL-15 might for instance, have reduced only activation of the TOLL 2 but not TOLL 4 for example. The possibility that TOLL proteins were involved in the pathogenesis of sepsis and associated liver injury might be investigated further in similar experiments to those of chapter 4 using mice deficient in the different TOLL receptors. The consensus of the literature indicates that the symptoms of LPS shock are driven by TNF α . The role of TNF α and IL-15 in LPS induced liver damage and shock symptoms might also become clearer if mice deficient in TNF α were used, either by pre-treatment with neutralising antibodies or genetic knockout. In a recent paper, Ajuwon et al (114), discover that following LPS insult of primary pig adipocytes, IFN γ induces IL-15 production. Interestingly, TNF α was not induced until at least 8 hours by the LPS. Where as the significance of this is unclear, it implicates upregulation of IL-15 in a LPS affected cell type yet without a direct link to TNF α production. Another recent study by Yajima et al (115), looks at the effect of over expression of IL-15 in BCG primed mice when exposed to LPS. The mice became more susceptible to liver injury when IL-15 was over expressed an example of an opposite situation to that seen in the experiment here when liver injury was reduced by the reduction of endogenous IL-15 by sIL-15 receptor therapy, supporting the observation of IL-15 involvement in LPS shock pathogenesis.

In 2002, Biber et al (116) demonstrated a lethal systemic inflammatory response in mice co-inoculated with IL-15 and IL-12. NK cells were activated into apoptosis and severe shock symptoms were induced. Subsequent blockade of interferon gamma and TNF α or IL-16 did not alter this response. Only the depletion of NK cells modified the shock response. They proposed that IL-15 and IL-12 were synergistically inducing lethality via a TNF α and IFN γ independent mechanism, via

the NK cells. Cooper et al (117) demonstrated how IL-15 deficient mice do not develop NK cells. It is odd that when IL-15 was co-injected with IL-12 NK cell apoptosis was induced. Many studies suggest that IL-15 has a central role in NK cell development.

The mode of IL-15 driven activation and development of NK cells has been further explored by Wary et al (118) who showed evidence of NK cell activation through the ERK 1 / 2 signal transduction pathway. Immune cell developmental effects of IL-15 were also reported by Hiromatsu et al (119). Apoptosis in liver, kidney, lung and spleen cells was significantly reduced in mice transgenically over expressing IL-15. These mice were resistant to induced E. coli shock and interestingly their TNF α levels were the same as non-transgenic IL-15 mice. Hiromatsu suggested that the over expression of IL-15 somehow reduced TNF α induced apoptosis. The protective effect of IL-15 exhibited here seems somewhat at odds with the protective effect of neutralising IL-15 in the LPS experiments reported here. It may be that some feedback loop becomes established where IL-15 reduced either TNF α or some other disruption in receptor activity trafficking.

In another twist of the story, Yajima et al (115) demonstrate an increased susceptibility to LPS induced liver injury using similar IL-15 transgenic mice when the mice were primed with BCG and insulted with LPS. The number of CD8 T cells in the livers of the IL-15 TG mice was elevated following LPS inoculation. However depletion of the cells saw complete abrogation of the TG mice's susceptibility to LPS shock. This work suggests a central role for CD8 T cells in the mechanism of IL-15 activity in LPS shock.

In the experiments here, almost complete resistance to lethal shock was observed in the non-BCG primed mice following insult with LPS. In the latter

experiments when BCG priming was used, the IL-15R α therapy again conferred protection from LPS induced liver injury and shock symptoms. In reference to the work of Yajima et al (115), it may be suggested that the susceptibility of the mice to LPS induced liver damage is mediated by an IL-15 driven CD 8 T cells infiltration. In Yajima's experiment, the depletion of these cells abrogated the liver damage; likewise, it is possible to speculate that following the therapeutic administration of soluble IL-15R alpha, an endogenous IL-15 driven migration of CD8 T cells was averted leading to the observed reduction in liver damage and associated sepsis symptoms. With this in mind, future experiments exploring the role of IL-15 in Shock may be served by using IL-15 $-/-$ mice to see if there is a difference in the CD8 T cell infiltration as reported by Yajima. In addition, the apoptosis rate of NK cells, the levels of TNF α and IL-12 could be measured in IL-15 $-/-$ mice. If exogenous IL-15 were administered it may be informative to see if the reported reduction of NK cell apoptosis and LPS shock susceptibility were restored to wild type responses.

If it could be confirmed that IL-15 was in fact able to reduce the severity of LPS induced liver injury, it may be that endogenous IL-15 be a possible therapeutic target in human incidence of septic shock. However as a caution, LPS shock model is a bolus insult with non-repeating assault on the immune system. The results of the septic arthritis model where the live pathogen was able to colonise far more effectively the essential organs of the mouse in the situation of reduced endogenous IL-15 following therapeutic inoculation of soluble IL-15 receptor.

It is also worth noting that alongside the research into IL-15, IL-17 has been identified as a possible therapeutic target alongside IL-15. Ferretti et al (120) showed that following LPS induced lung inflammation in mice, separate infiltrations of

Neutrophils and lymphocytes were observed at days +1 and +2. These immune infiltrations were accompanied by increased IL-17. The IL-17 pulse was however preceded by increased IL-15 messenger RNA, immediately after the LPS insult. This observation lead Ferretti et al to propose that the IL-15 triggers IL-17 which in turn drives the neutrophilia leading to the observed airway inflammation. Thus another therapeutic target is presented in the inflammatory cascade and a suggestion that Il-15 may play just a brief transitory role in the pathway to the immune response.

6.5 Septic arthritis Model

The LPS shock model is based on a bolus insult of LPS. In the final experiments conducted in the preparation of this thesis, the septic arthritis model was investigated. Here the initial insult is generated by the injection of *S. aureus*, however, unlike the “single shot” LPS model, the insult continues and extends as the bacteraemia progresses.

Unlike the protective effect against septicaemia observed when therapeutic sIL-15R α was used in the LPS model, there was no evidence of any similar effect here. As the *S. aureus* infection was not calculated to be a lethal dose (range 1-5 $\times 10^7$), a separate experiment would be required to quantify any effect on mortality associated with *S. aureus* induced sepsis. There were no dramatic differences in either symptoms of sepsis or arthritis following sIL-15R α therapy compared to placebo. This was the case for both the bolus sIL-15R α therapy and the prolonged sIL-15R α therapy. The summary table (table 9) below compares each of the results for the two experiments. A swift glance indicates that in the absence of distinct differences, there is evidence of a trend running throughout, indicating a partial abrogation of arthritis and sepsis. However, like the results for the LPS experiments, there is no complete abrogation of sepsis, leading to the possibility that endogenous IL-15 has a brief but not exclusive role in the pathogenesis of sepsis caused by *S. aureus* infection. This may suggest that in this model that the presumptive TNF α driving symptoms of sepsis is not exclusively under the influence of endogenous IL-15. This is similar to the hypothesis for incomplete abrogation of sepsis in the LPS shock model. Biochemical

parameters were informative in the LPS experiments above; it may therefore be profitable to make similar investigations in a future repetition of the Septic arthritis model too.

Symptom	Bolus (LPS)	Prolonged (S. aureus)
Mortality	NS	NS
Incidence of Sep	NS	NS Weak trend
Weight change	NS	NS
Severity Sep	Trend one day sig	NS Initial trend
Incidence arth	NS Weak trend	NS Initial trend
Severity Art	Sig Weak trend	No trend
Ave limbs inv'd	Sig Trends	Sig Initial trend
CFUs	N/A	CFUs higher in IL-15R α except joint tissue.
SEA wk 1	N/A	Initially reduced in IL-15R α
SEA wk 2	N/A	Initially reduced in IL-15R α
Con a wk 1	N/A	Reduced in IL-15R α
Con a wk 2	N/A	No trend NS

Table 9

Table comparing overall results in the various parameters investigated in the Bolus and the prolonged IL-15R α therapy experiments in the septic arthritis model.

It is interesting to consider the almost complete abrogation of arthritis induced by endogenous collagen in the CIA experiment of Ruchatz et al (43) in relation to the severity of arthritis demonstrated in the septic arthritis model here. In spite of the use of the same therapeutic agent at a similar dose and dosing regimen, there was little or no effect on the severity of symptoms of arthritis compared to placebo. One can only speculate that there is a separate system driving this damage that is related to the bacteria and their antigens. Bremmell (66) reports that *S. aureus* could bind directly to a bone protein called bone sialoprotein in the septic arthritis model. Furthermore, *S. aureus* strains have been shown to bind directly to collagen (102) and fibronectin (103). Bremmell suggests that it may be the direct binding of the bacteria to the joint/bone interfaces which directs the arthritis.

In 1994, Abdelnour et al (104) characterised the cell types found at the site of synovitis seen in the septic arthritis model. They reported that initially, Macrophages and Granulocytes, staining positively for production of TNF α and IL-1, had colonised the synovium. Later a population of CD4⁺ T cells were distinct in the synovium, some of which stained positively for IL-2. There was also very little histochemical evidence of MHC class II expression. Abdelnour et al also observed that if they depleted the CD4⁺ T cell population, then the synovitis was reduced in severity although not abrogated. They concluded that the appearance of the cellular infiltration of the septic model caused by injection of *S. aureus* was not dissimilar to the infiltrate known to be present in non-infectious arthritis disease models.

It is therefore very interesting to ask why the abrogation of arthritis was possible in the CIA model following sIL-15R α treatment and yet, using the same therapeutic agent in the septic arthritis model, there was no abrogation of the arthritic symptoms? There is of course the fact that the CIA is a bolus challenge of collagen

and the septic model there is an active and regenerating live bacterial challenge. However, if the pathogenic mediators of arthritis are proposed to be the same in both the septic and CIA models, would it not follow that there would be some greater modulation of the arthritis in the septic model by sIL-15R α ?

The direct binding of the *S. aureus* to the bone and joint components may be significant in driving a different sort of response to that seen in CIA and LPS shock leading to sepsis and synovitis respectively. The differences in cytokine induction by *S. aureus* derived SEA and LPS were examined by Bjork et al (105). They discovered that in-vitro; LPS induced predominantly four cytokines; IL-1 α and β , TNF α and IL-8. Peak levels of TNF α occurred within 4 hours. When SEA was used to induce cytokines, TNF α was not amongst the dominant cytokines induced by SEA. T-cells eventually produced TNF α but this was between 12 and 48 hours following the SEA stimulation. This differential expression pattern of cytokines in response to gram positive and gram negative toxins is suggested by Bjork et al to be a possible explanation of the differences seen in the speed of onset in the diseases (and models) that arise in response to either *E. coli* / LPS and *S. aureus*/SEA infection. Might this also be a clue to why the sIL-15R α was able to abrogate the liver damage of the LPS model but not the sepsis of the septic arthritis model? Interestingly, IL-2 was not found to feature in the LPS induced cytokine pattern but did in the cytokines induced by SEA. Perhaps again, in reference to the redundancy of IL-2 and IL-15, the immune response to SEA/*S. aureus* induced disease is focussed more on the release of IL-2 than IL-15. Hence, the neutralisation of IL-15 would have little effect as demonstrated by the results here.

Although removal of IL-15 by administration of sIL-15R α seems to have had little effect on the outcome of arthritis and sepsis with the exception of a non-

significant trend for slightly less severe symptoms of both, there is one aspect of the results that stands out and that is the presence/absence of *S. aureus* CFU and the *in-vitro* reactivity of the splenocytes to exotoxin (SEA and synthetic CON-A).

The presence of raised CFU levels has been previously reported: McInnes et al (68) also reported increased CFUs in the septic arthritis model following IV injection of *S. aureus* in iNOS knockout mice. In the experiment here, as with the iNOS deficient mice, the numbers of *S. aureus* colony forming units were markedly increased in blood, spleen and kidney tissues compared to both the placebo treated mice.

Takano et al (106) neutralised IL-15 using anti-IL-15 monoclonal antibodies. They showed that in mice treated with anti-IL-15-mab, there was a significant increase in *E. coli* CFUs in liver spleen and peritoneal cavities. Also they showed that there was a reduced proliferation of $\gamma\delta$ T cells in response to IL-15 neutralisation. The higher numbers of CFUs indicated a reduced ability of IL-15 deficient mice to clear *S. aureus*. The presence of higher *S. aureus* CFUs indicate that endogenous IL-15 has a role in the immune response leading to the clearance of bacteria from the blood and organs. The question remaining regards the exact role of IL-15 in the clearance process.

The spleen cells removed from sIL-15R α treated mice were less reactive to SEA and CON-A than spleen cells from placebo treated mice. It is interesting that Gerwein et al (107) reported that the IL-15 driven T cell mitogenesis was down regulated by SEA. Not all T cells respond to SEA only previously activated T cells. However in the presence of an experimental dose of IV injected *S. aureus*, one would expect there to be a sizeable population of activated T cells. The suggestion of Gerwein's results is that the ability of T cells to react to SEA was reduced by the

neutralisation of IL-15. This may indicate that the presence of endogenous IL-15 would normally be synergistic in either the activation of T cells or by promoting the high affinity MHC co-expressed SEA receptors which allow APC independent activation of the T cells. Gerwien et al had also shown that pre-treatment of T cells reduced the activation of Janus Kinase 3 (JAK 3) which is integral to IL-15 signalling.

It is also known that the presence of IL-15 upregulates IL-2R α receptor and down regulates the IL-15R α (108). It may be that the role of IL-15 in septic arthritis is very distinct from that suggested by the CIA and LPS shock models. From the results in chapters 3, 4 & 5, there seems to be some inconclusive pro-inflammatory action in general sepsis and arthritis but nothing as inflammatory as that seen in the liver damage of the LPS model or the mortality protection also exhibited there. Furthermore, the septic arthritis results in chapter 5 do not suggest that IL-15 is so fundamentally involved as it would appear to be in the arthritis of the collagen induced arthritis model. However, what can be proposed is that there is a role for IL-15 in the clearance of bacteria in gram-positive infections. The nature of this role is unclear. With reference to the differential cytokine pattern reported by Bjork et al (105), it may be that IL-15 exerts its effect through up-regulation of high affinity IL-2 receptors (Kumaki et al 108) and therefore has only a transitory role before IL-2 itself takes over the driving of the immune response to the pathogen. Also Takano's demonstration (106) of reduced $\gamma\delta$ T-cells proliferation may indicate that the role of these T cells might not so much to do with the pathogenesis of the arthritis but in fact to be associated with the normal clearance of invading bacteria. Hence the neutralisation of IL-15 might have been significant in the increased CFUs found in the tissues.

Whereas the LPS model literature showed a possible CD8 T Cell/IL-15 link, suggesting a mechanism for the observed protection from the LPS induced symptoms of shock, there was no such clear result or outcome in the two septic arthritis experiments; only that there was a slightest trend for amelioration of synovitis and sepsis and possibly of greater interest, the observed lack of bacterial clearance of *S. aureus* from the tissues of the mice in the therapy groups and the subsequent lack of reactivity of the spleen cells to bacterial toxins. At the time of the original write up of this project, all that could be surmised was that the IL-15 somehow could influence bacterial clearance.

In recent papers several other aspects of the bacterial proteins and host immune modulators have been examined for a role in the pathogenesis of sepsis and arthritis associated with the septic arthritis model. For example Calander et al (121), examined a group of staphylococcal proteins called exoproteases. When a *S. aureus* strain deficient in these proteases and lysins was tested in the septic arthritis model, there was no difference to the wild type control. Similarly, Gjertysson, Nitschke & Tarkowski (122) (investigated the possibility that polyclonal B cell and Ig humoral responses was important to the resolution of sepsis and arthritis in the event of septic arthritis. To investigate this they depleted the entire B cell complement. The pathogenesis of sepsis and arthritis was unchanged in spite of this when compared to the controls. The sialic acid binding protein CD22 was also tested for a role in the pathogenesis of septic arthritis but it's presence or absence was found not to signify any difference to the controls.

Two studies have looked at other *S. aureus* characteristics that can affect virulence. In 2002 Jonsson et al (123) studied the influence of sortase A, a staphylococcal coat protein which catalyses cell/bacterial attachment. Using a sortase

deficient mutant of *S. aureus* (*S. aureus* newman SMK3) in the septic arthritis model, they demonstrated that a mild response in both sepsis and arthritis compared to the wild type *S. aureus* newman controls.

Interestingly, the authors describe a rapid blood clearance of the sortase mutant in comparison to that of the control wild type bacterial clearance. They furthermore suggest that neutrophils are essential to the clearance because following depletion of neutrophils a severe systemic infection (in spite of which, the sortase mutants were unable to induce any great degree of septic arthritis!) Jonsson et al conclude that the sortase mutant was less virulent. In a later paper Jonsson et al (124) describe two distinct sortases; Srt A and Srt B. using mutants, they assigned Srt B as having a role in pathogenesis of infection and Srt A as being a critical virulence factor for the induction of septic arthritis.

So in summary, although recent research has shown that some virulence factors endogenous to the *S. aureus* species, are involved in the pathogenesis of sepsis and arthritis in the septic arthritis model, it is difficult to see exactly what if any influence the administration of soluble receptor had on the observed accumulation of colony forming units in the mice's tissues following therapeutic intervention.

In an analogous experiment to those these, Takano et al (106) used anti IL-15 antibodies to deplete endogenous IL-15 in the septic arthritis model. As here, they reported increased *S. aureus* colony forming units and interestingly also reduced SEA/T- cell reactivity.

One possible mode of action may depend on the bacteria subduing T cell activity allowing direct attachment of *S. aureus* to sialic acid residues (via CD22) and therefore directly initiating arthritis, furthermore, less reactivity of T cells to bacterial

toxins to SEA/TSST etc may allow faster and more effective infection of tissues and organs leading to greater sepsis such as that observed here. As these possible host immune defence reactions are exacerbated by administration of anti IL-15 antibodies it may also be worth investigating the possibility that persons succumbing to *S. aureus* infection and septic shock exhibit any polymorphism in their ability to generate endogenous IL-15. With respect to IL-15/bacterial interactions, the differential activities of Srt A and Srt B in sepsis and arthritis in the septic arthritis model, might be profitably investigated alongside presence or absence of IL-15 effect of soluble IL-15R alpha on levels of activity.

Following the septic arthritis experiment, the role of IL-15 in septic arthritis remains unclear. In collagen arthritis a repetition of experiments with better quality reagents might be informative and possibly mirror the effects of the receptor seen in the experiment of Ruchatz et al (44). In the case of LPS shock, there were observations of IL-15 acting in a pro-inflammatory role and concomitant abrogation or amelioration of the effects of LPS was observed following S IL-15 receptor therapy. In addition, these observations were not discordant with the literature. In summary, the role of IL-15 will be further explored by the results of ongoing phase two pharmaceutical trials of anti IL-15 therapy in human rheumatoid arthritis. In recent reviews, several authors have described many disease states in which IL-15 is suspected of having a contributing, pathogenic role including rheumatoid Arthritis, transplant rejection, cancer, irritable bowel disease, dermatitis, pulmonary inflammation and AIDS, (125) (126).

However it can be clearly seen that IL-15 lies to a greater or lesser degree deep within the complex cascades of immune regulators fundamental to the normal mammalian response to infection. Depletion of IL-15 in situations such as LPS shock models where the insult is non-repeated is informative however care should be taken when knowing out a pro-inflammatory protein that the host doesn't become subject to greater risk of infection such as evidenced here in the septic arthritis model and elsewhere as shown in the literature above. A consideration of the success of the anti TNF α therapies now commercially being used in inflammatory disease suggests there may be a cost/benefit analysis to be considered. Whilst the success is striking there are reports of unusual opportunistic infections in patients receiving these therapies. This should not reduce interest in elucidating the role of IL-15 and other pro-inflammatory cytokines and their pursuit as therapeutic targets, merely a caution that they were there for some reason and although this project has not uncovered any exact role it has shown that there are positive and negative effects to depletion of IL-15 in the LPS and septic arthritis models respectively. The most promising tools that may elucidate further the role of IL-15 in all these disease areas are the emergent techniques of proteomics and genomics, allowing real time visualisation of IL-15 genetic and protein products in their sites of activity and production.

6.6 Conclusions

In conclusion, the neutralisation of IL-15 with the mutant IL-15-48 antagonist, anti murine IL-15 anti serum and sIL-15R α seems to have a varied potential for modulation of the pathogenesis of all three models. However in each case there seems to be different pathways by which this modulation could be occurring. In future experiments, repetition of the above experiments using IL-15, TNF α and T cell knock out mouse strain might be informative. However it is worth paying considerable attention to the redundancy of IL-2 and another recently discovered cytokine IL-21 which are now defined as members of the same family of 4 alpha helix cytokines (109) and possible induction of IL-17 as a mediator of IL-15 presumptive functions.

References

- 1 Janeway & Travers 1996, Reviewed by Janeway & Travers, *Immuno-Biology*, 2nd edition, Current Books
- 2 Shimizu Y, van Seventer GA, Horgan KJ, Shaw S. *Immunol Rev* 1990 Apr; 114:109-43. Roles of adhesion molecules in T-cell recognition: fundamental similarities between four integrins on resting human T cells (LFA-1, VLA-4, VLA-5, VLA-6) in expression, binding, and costimulation
- 3 Linsley PS, Brady W, Grosmaire L, Aruffo A, Damle NK, Ledbetter JA, 1991, *J Exp. Med.* Mar 1;173(3):721-30 Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin2 mRNA accumulation
- 4 Abbas AK, Murphy KM, Sher A. 1996 *Nature* Oct 31; 383 (6603): 787-93, Functional diversity of helper T lymphocytes.)
- 5 Salgame P, Abrams J.S., Clayberger C., Goldstein H, Modlin R L, Convit J and Bloom B.R, 1991, *Science*, 254: 279-282, Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones
- 6 Parker DC, 1993 *Annual Rev. Immunol*; 11:331-60, T Cell dependent B cell activation)
- 7 Abbas AK, 1988, *Immunol Today*, Mar; 9(3): 89-94, A reassessment of the mechanisms of antigen-specific T-cell-dependent B-cell activation
- 8 Burton JD, Bamford RN, Peters C, Grant AJ, Kurys G, Goldman CK, Brennan J, Roessler E and Waldmann TA, 1994, *Proc. Natl. Acad. Sci. USA*. Vol. 91, pp4935-4939, May. A lymphokine, provisionally designated interleukin T and produced by a human adult T-cell leukemia line, stimulates T-Cell proliferation and the induction of lymphokine-activated killer cells
- 9 Bamford RN, Grant AJ, Burton JD, Peters C, Kurys G, Goldman CK, Brennan J, Roessler E & Waldmann TA, 1994, *Proc. Natl. Acad. Sci. USA*, Vol. 91, pp4940-4944, The Interleukin IL(2) receptor α chain is shared by IL-2 and a cytokine provisionally designated IL-T that stimulates T-cell proliferation and the induction of lymphokine-activated killer

- 10 Grabstein KH, Eisenman J, Shanebeck K, Rauch C, Srinivasan S, Fung V, Beers C, Richardson J, Schoenborn MA, Ahdieh A, Johnson L, Alderson MR, Watson JD, Anderson DM, Giri JG, 1994, *Science*, Vol. 264, 13th May, p965-968; Cloning of a T-cell growth factor that interacts with the b chain of the interleukin-2 receptor.
- 11 Dirk M Anderson, Lisabeth Johnson, Moira B Glaccum, Neal G Copeland, Debra J Gilbert, Nancy A Jenkins, Virginia Valentine, Mark N Kirstein, David N Shapiro, Stephan W Morris, Kenneth Grabstein & David Cosman, 1995, *Genomics* 25, p701-706, Chromosomal assignment and genomic structure of IL-15) Restriction and linkage data analysis in human and murine DNA indicate that IL-15 is located in the central region of chromosomes 4 and 8 respectively.
- 12 Krause (12) Krause H, Jandrig B, Wernicke C, Bulfone-Paus S, Pohl T, Diamantstein T, 1996, *Cytokine*, Vol. 8, No. 9, pp667-674. Genomic structure and chromosomal localisation of the human IL-15 gene.
- 13 Armitage RJ, Macduff BM, Eisenman J, Paxton R, and Grabstein KH, 1995, *Journal of Immunology*, 154: pp483-490. IL-15 has stimulatory activity for the induction of B II proliferation and differentiation.
- 14 (14) (Carson WE, Giri JG, Linndemann J, Linett ML, Ahdieh M, Paxton R, Anderson D, Eisenmann J, Grabstein K, and Caligiuri MA. 1994, *J. Exp Med.* Vol. 180, October 1994, pp1395-1403, Interleukin15 is a novel cytokine that activates Human Natural Killer cells via components of the IL-2 receptor
- 15 Leonard WJ, Noguchi M, Russel, SM, McBride OW, 1994, *Immunol. Review*, Apr: 138:61-86, The molecular basis of X-linked severe combined immunodeficiency and the role of the interleukin-2 receptor gamma chain as a common gamma chain, gamma c.
- 16 Giri JG, Ahdieh M, Eisenman J, Shanebeck K, Grabstein K, Kumaki S, Namen A, Park LS, Cosman D and Anderson D, 1994, *EMBO*, Vol 13, No. 12, pp 2822-2830, Utilization of the α and β chains of the IL-2 receptor by the novel cytokine IL-15.
- 17 Dirk M Anderson, Satoru Kumaki, Minoo Adieh, Jeanette Bertles, Mark Tometsko, Aaron Loomis, Judith Giri, Neal G Copeland, Debra J Gilbert, Nancy A Jenkins, Virginia Valentine, David N Shapiro, Stephen W Morris, Linda S Park & David Cosman, 1995, *J Biol. Chem*, Vol 270, No 50, December 15, p29862-29869. Functional characterization of the human interleukin-15 receptor α chain and close linkage of IL-15RA and IL-2RA genes.

- 18 Richard N Bamford, Andrew P Battiata, Jack D Burton, Harmesh Sharma & Thomas A Waldmann, 1996, Proc. Natl. Acad. Sci. USA, Vol 93, pp2897-2902, Interleukin 15/(IL-T) production by the adult T-cell leukaemia cell line HuT-102 is associated with a human T-cell lymphotropic virus type IR region/IL-15 fusion message that lacks many upstream AUGs that normally attenuate IL-15mRNA translation.
- 19 Raffaella Meazza, Simonetta Verdiani, Roberto Biassoni, Massimiliano Coppoleccia, Alessia Gaggero, Anna Maria Orengo, Mario P Colombo, Bruno Azzarone, Silvano Ferrini, 1996, Oncogene 12, p2187-2192, Identification of a small novel interleukin 15 (IL-15) transcript isoform generated by alternative splicing in human small cell lung cancer cell lines.
- 20 Nishimura H, Washizu J, Nakamura N, Enomoto A and Yoshikai Y, 1998, J. Immunology, 160: 936-942, Translational Efficiency is upregulated by alternative exon in murine IL-15mRNA.
- 21 Klippel J H, & Crofford L, 1997, Primer on the Rheumatic Diseases, Arthritis Foundation, USA
- 22 Ishikawa H & Ziff M, 1976: Arthritis Rheum. 1976 Jan-Feb; 19(1): 1-14, Electron microscopic observations of immunoreactive cells in the rheumatoid synovial membrane.
- 23 Buchan G, Barrett K, Turner M, Chantry D, Maini RN, and Feldmann M, 1988; Clin. Exp. Immunol: Sep; 73(3): 449-55; Interleukin-1 and tumour necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1 alpha
- 24 Xu W.D. Firestein GS, Taetla R, Kaushansky K, and Zvaifler, 1989: J. Clin. Invest. Vol. 83, March ; 876-882; Cytokines in chronic inflammatory arthritis
- 25 Hirano T, Matsuda T, Turner M, Myasaka N, Buchan G, Tang B, Shimizu M, Maini R, Feldmann M et al; 1988 Eur J Immunol. Nov; 18(11): 1797-80; Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid arthritis.
- 26 Brennan FM, Zachariae CO, Chantry D, Larsen CG, Turner M, Matsushima K, Feldmann M 1990, Eur J Immunol. Sep; 20(9): 2141-4; Detection of interleukin 8 biological activity in synovial fluids from patients with rheumatoid arthritis and production of interleukin 8 mRNA by isolated synovial cells and TNF-a

- 27 Chu CQ, Field M, Feldmann M, and Maini RN, 1991 *Arthritis and Rheumatism*, Vol. 34: No. 9, 1125-1132; Localization of Tumour Necrosis Factor α in synovial tissues and at the Cartilage-Pannus junction in patients with rheumatoid arthritis
- 28 Elliot MJ, Maini RN, Feldmann M, Long-Fox A, Charles P, Katsikis P, Brennan FM, Walker J, Bilj H, Ghayeb J, Woody JN, 1993; *Arthritis Rheum.* Dec;36(12):1681-90; Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha
- 29 Chu CQ, Field M, Allard S, Abney E, Feldmann M, Maini RN, 1992: *Br. J Rheumatology*, 31, p653-661; Detection of cytokines at the cartilage/pannus junction in patients with rheumatoid arthritis; Implications for the role of cytokines in cartilage destruction and repair.
- 30 Firestein GS, Xu WD, Townsend K, Brodie D, Alvaro-Gracia J, Glasebrook A, Zvaifler NJ, 1988, *J. Exp. Med.* November 1988; 1573-1586; Cytokines in chronic Inflammatory arthritis
- 31 Panayi GS, Lanchbury JS, and Kingsley GH, 1992, *Arthritis and Rheumatism*, Vol. 35 No 7: 729-735: The importance of the T cell in initiating and maintaining the chronic synovitis of Rheumatoid Arthritis.
- 32 Kornfeld H, Berman JS, Beer DJ & Centre DM, 1985, *J. Immunol.* 134: 3887-3890. Induction of human T lymphocyte motility by IL-2.
- 33 Wilkinson PC & Liew FY, 1995, *J. Exp. Med.* Vol. 181: 1255-1259, Chemoattraction of human blood T lymphocytes by Interleukin-15.
- 34 McInnes IB, AlMughales J, Field M, Leung B, Huang FP, Dixon R, Sturrock RD, Wilkinson PC & Liew FY, 1996 *Nature Medicine*, 2:175; The role of IL-15 in T cell migration and activation in Rheumatoid arthritis.
- 35 McInnes IB, Leung B, Sturrock RD, Field M and Liew FY, 1997 *Nature Medicine*, 3:189; Interleukin-15 mediates T cell dependent regulation of TNF α production in RA.)
- 36 Feldmann M, Brennan FM & Maini RN, 1996, *Ann. Rev. Immunol.* 14: 397; Role of cytokines in Rheumatoid Arthritis.
- 37 Trentham DE, Townes AS and Kang AH, 1977, *J. Exp. Med.*, 146: 857; Autoimmunity to type II collagen: an experimental model of arthritis

- 38 Sewell KL, Trentham DE Pathogenesis of rheumatoid arthritis *Lancet*. 1993 Jan 30;341(8840):283-6
- 39 Staines NA, Wooley PH, Collagen arthritis--what can it teach us? *Br J Rheumatol*. 1994 Sep;33(9):798-807
- 40 Trentham DE.; Novel immunomodulating and immunotherapies, and novel therapies and strategies for inflammatory arthropathy. 1992, *Curr. Opin. Rheumatol*. Jun; 4(3): 322-4
- 41 Durie FH, Fava RA, Foy TM, Aruffo A, Ledbetter JA, Noelle RJ. Prevention of collagen-induced arthritis with an antibody to gp39, the ligand for CD40. 1993, *Science* Sep 3;261(5126):1328-30;
- 42 Williams RO, Feldmann M, Maini RN, 1992; *Proc. Natl. Acad. Sci. USA*, Vol. 89 pp 9784-9788; Anti-tumour necrosis factor ameliorates joint disease in murine collagen induced arthritis
- 43 Ruchatz H, Leung BP, Wei XQ, McInnes IB, Liew FY, 1998 *J Immunol*. 1998 Jun 1; 160(11): 5654-60. Soluble IL-15 receptor alpha-chain administration prevents murine collagen-induced arthritis: a role for IL-15 in development of antigen-induced immunopathology
- 44 Guyton Arthur C, 1986, *Textbook of medical physiology*, WB Saunders company, Philadelphia, USA.
- 45 Tracey KJ, Fong Y, Hesse D, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A, 1987, *Nature*, Vol. 330: 662; Anti-cachetin/TNF monoclonal antibodies prevent shock during lethal bacteraemia
- 46 Standiford, TJ, Stietter RM, Lukacs NW, Kunkel, 1995 *J Immunol*. 155: 2222-2229; neutralization of IL-10 increases lethality in endotoxemia
- 47 Glauser MP, Zanetti G, Baumgartner Cohen J, 1991; *The Lancet*, Vol. 338, p 732; Septic Shock: pathogenesis
- 48 Michie HR, Manogue KR, Spriggs DR, Revhaug A, O'Dwyer S, Dinarello C, Cerami A, Wolff SM, Wilmore DW; 1988 *N Engl J Med*. 1988 Jun 9; 318(23):

1481-6; Detection of circulating tumor necrosis factor after endotoxin administration

- 49 Cannon JG, Tompkins RG, Gelfand JA, Michie HR, Stanford GG, van der Meer JW, Endres S, Lonnemann G, Corsetti J, Chernow B, et al. *J Infect Dis* 1990 Jan; 161(1): 79-84 Circulating interleukin-1 and tumor necrosis factor in septic shock and experimental endotoxin fever
- 50 Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, Hariri RJ, Fahey III TJ, Zentella A, Albert JD, Shires T, Cerami A; 1986, *Science* Vol. 234, p470; Shock and Tissue injury induced by recombinant human cachectin
- 51 Beutler B, Milsark IW, Cerami AC 1985 *Science* Aug 30;229(4716):869-71 Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin
- 52 Freudenberg MA, Keppler D, Galanos C, 1986 *Infect Immun* Mar;51(3):891-5 Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin
- 53 Boelen A, Platvoet-ter Schiphorst MC, Wiersinga WM 1997 *J Endocrinol* Apr;153(1):115-22 (1997) Immunoneutralization of interleukin-1, tumor necrosis factor, interleukin-6 or interferon does not prevent the LPS-induced sick euthyroid syndrome in mice
- 54 Zanetti G, Heumann D, Gerain J, Kohler J, Abbet P, Barras C, Lucas R, Glauser MP, Baumgartner JD 1992, *J. Immunol* Mar 15; 148(6): 1890-7; Cytokine production after intravenous or peritoneal gram-negative bacterial challenge in mice. Comparative protective efficacy of antibodies to tumor necrosis factor-alpha and to lipopolysaccharide
- 55 Lehmann V, Freudenberg MA, Galanos CJ 1987 *Exp. Med.* Mar 1; 165(3): 657-63 Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice
- 56 Doherty GM, Lange JR, Langstein HN, Alexander HR, Buresh CM, Norton JA 1992 *J Immunol* Sep 1;149(5):1666-70 Evidence for IFN-gamma as a mediator of the lethality of endotoxin and tumor necrosis factor-alpha
- 57 Smith SR, Calzetta A, Bankowski J, Kenworthy-Bott L, Terminelli C, 1993; *J Leukoc Biol* Jul;54(1):23-9 Lipopolysaccharide-induced cytokine production and mortality in mice treated with *Corynebacterium parvum*

- 58 Amiot F, Fitting C, Tracey KJ, Cavaillon JM, Dautry F, 1997; *Mol Med* Dec;3(12):864-75 Lipopolysaccharide-induced cytokine cascade and lethality in LT alpha/TNF alpha-deficient mice
- 59 Smith SR, Terminelli C, Denhardt G, Narula S, Thorbecke GJ; 1996 *Cell Immunol* Nov 1;173(2):207-14; Administration of interleukin-10 at the time of priming protects *Corynebacterium parvum*-primed mice against LPS- and TNF-a-induced lethality
- 60 Wysocka M, Kubin M, Vieira LQ, Ozmen L, Garotta G, Scott P, Trinchieri G, 1995; *Eur J Immunol*. Mar; 25(3): 672-6; Interleukin-12 is required for interferon-gamma production and lethality in lipopolysaccharide-induced shock in mice
- 61 Shalaby MR, Halgunset J, Haugen OA, Aarset H, Aarden L, Waage A, Matsushima K, Kvithyll H, Boraschi D, Lamvik J, et al 1991, *Clin Immunol Immunopathol* Oct;61(1):69-82; Cytokine-associated tissue injury and lethality in mice: a comparative study
- 62 Doherty TM, Seder RA, Sher A, 1996; *J Immunol* Jan 15; 156(2): 735-41 Induction and regulation of IL-15 expression in murine macrophages
- 63 Goldenberg DL, 1998; *Lancet*, 351: 197-202; Septic Arthritis [review]
- 64 Goldenberg DL. *Arthritis Rheum* 1989 Apr;32(4):496-502; Infectious arthritis complicating rheumatoid arthritis and other chronic rheumatic disorders.
- 65 Ostensson A, Geborek P. 1991; *Br J Rheumatol* Feb;30(1):35-8; Septic arthritis as a non-surgical complication in rheumatoid arthritis: relation to disease severity and Therapy.
- 66 Bremell T, Lange S, Yacoub A, Ryden C, Tarkowski A. 1991, *Infect Immun* Aug;59(8):2615-23 Experimental *Staphylococcus aureus* arthritis in mice
- 67 Bremell T, Abdelnour A, Tarkowski A. 1992; *Infect Immun* Jul;60(7):2976-85 Histopathological and serological progression of experimental *staphylococcus aureus* arthritis

- 68 McInnes IB, Leung B, Wei XQ, Gemmell CC, Liew FY 1998; *J Immunol* Jan 1;160(1):308-15 Septic arthritis following *Staphylococcus aureus* infection in mice lacking inducible nitric oxide synthase
- 69 Zhao YX, Ljungdahl A, Olsson T, Tarkowski A. 1996, *Arthritis Rheum* Jun;39(6):959-67 In situ hybridization analysis of synovial and systemic cytokine messenger RNA expression in superantigen-mediated *Staphylococcus aureus* arthritis
- 70 Docke WD, Randow F, Syrbe U, Krausch D, Asadullah K, Reinke P, Volk HD, Kox W. 1997 *Nat Med*. Jun; 3(6): 678-81 Monocyte deactivation in septic patients: restoration by IFN-gamma treatment
- 71 Sambrooke, Fritsch & Maniatis, (1989) *Molecular Cloning*, Cold spring Harbour laboratory press.
- 72 Hans Krause, Burkhard Jandrig , Catrin Wernicke, Silvia Bulfone-Paus, Thomas Pohl, Tibor Diamantstein 1996, *Cytokine*, Issue 9 p667-674; Genomic structure and chromosomal localization of the human interleukin 15 gene (il-15).
- 73 Sanger F, Nicklen S, Coulson AR. *Proc Natl Acad Sci U S A* 1977 Dec;74(12):5463-7 DNA sequencing with chain-terminating inhibitors.
- 74 Sedmak JJ, Grossberg SE. 1977, *Anal. Biochem*. May 1;79(1-2):544-52. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue.
- 75 Wooley PH, Luthra HS, Stuart JM, David CS. 1981, *J Exp Med* Sep 1;154(3):688-700 Type II collagen-induced arthritis in mice. I. Major histocompatibility complex (I region) linkage and antibody correlates.
- 76 Cerami A Beutler B, 1988, *Immunology Today*, Vol. 9, No 1, The role of TNF in endotoxic shock and cachexia
- 77 Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF & Cerami A, 1987, *Nature*, 330 17 Dec p 662; Anti Cachetin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia
- 78 Colman RW, 1989, *N Eng J Med*. May 4; 329(18) 1207-9, The role of plasma proteases in septic shock

- 79 Smith SR, Manfra D, Davies L, Terminelli C, Denhart G, Donkin J, 1997 *J Leucocyte Biology*, Vol. 61, p 24-32; Elevated levels of NO in both unchallenged and LPS challenged *C parvum* primed mice are attributable to the activity of a cytokine induced isoform of inos.
- 80 Parrillo, JE, 1993, *N Eng J Med*, Vol.238 No 20, p 1471. Pathogenetic mechanisms of septic shock.
- 81 Rossol S, Voth R, Brunner S, Muller WE, Gallati H, Meyer zum Buschenfelde KH, Hess G, 1990, *Eur J Immunol*. Aug; 20 (8) 1761-5; *C Parvum*; An inducer of TNF α in humans
- 82 S. Gillis K.A. Smith, *Nature* 268. 154, 1977
- 83 Reviewed by C A Dinarello, 1991 *Blood* 77, 1627 and animal models Ohlsson K et al, 1990, *Nature*, 348, 550
- 84 Morton and Griffiths (1985), *Veterinary Record* 116:431-43 Morton Griffiths index
- 85 Carter DB, Deibel MR Jr, Dunn CJ, Tomich CS, Laborde AL, Slightom JL, Berger AE, Bienkowski MJ, Sun FF, McEwan RN, et al. *Nature*. 1990 Apr 12;344(6267):584 Purification, cloning, expression and biological characterization of an interleukin-1 receptor antagonist protein
- 86 (Janson RW, Hance KR, Arend WP *J Immunol* 1991 Dec 15;147(12):4218-23 Production of IL-1 receptor antagonist by human in vitro-derived macrophages. Effects of lipopolysaccharide and granulocyte-macrophage colony stimulating factor
- 87 Dripps DJ, Brandhuber BJ, Thompson RC, Eisenberg SP *J Biol Chem* 1991 Jun 5;266(16):10331-6 Interleukin-1 (IL-1) receptor antagonist binds to the 80-kDa IL-1 receptor but does not initiate IL-1 signal transduction.
- 88 Granowitz EV, Clark BD, Mancilla J, Dinarello CA *J Biol Chem* 1991 Aug 5;266(22):14147-50 Interleukin-1 receptor antagonist competitively inhibits the binding of interleukin-1 to the type II interleukin-1 receptor
- 89 Onu A, Pohl T, Kause H, Bulphone-Paus S, 1997, *J. Immunology*, 158, 255-262; Regulation of IL-15 secretion via the leader peptide of two IL-15 isoforms

- 90 Pettit DK, Bonnert TP, Eisenman J, Srinivasan S, Paxton R, Beers C, Lynch D, Miller B, Yost J, Grabstein KH, Gombotz WR 1997; *J Biol. Chem.* Jan 24; 272(4): 2312-8; Structure-function studies of interleukin 15 using site-specific mutagenesis, polyethylene glycol conjugation, and homology modelling.
- 91 Cassatella MA, McDonald PP *Curr Opin Hematol* 2000 May;7(3):174-7; Interleukin-15 and its impact on neutrophil function
- 92 Gilbertsen RB, Lesch M, Fantone J : *Am J Pathol* 1984 Oct;117(1):26-9 The role of Neutrophils in type II collagen-induced arthritis in rats
- 93 (Ming Yin, Micheal D Wheeler, Hiroshi Kono, Blair U Bradford, andle M Gallucci, Micheal D Luster and Ronald G Thurman 1999, *Gastroenterology*, 117: 942-952, Essential role of TNF α in Alcohol-induced liver injury in mice
- 94 Taguchi I, Oka K, Kitamura K, Sugiura M, Oku A, Matsumoto M. 1999, *Inflamm Res.* Jul;48(7):380-5. Protection by a cyclic AMP-specific phosphodiesterase inhibitor, rolipram, and dibutyryl cyclic AMP against *Propionibacterium acnes* and lipopolysaccharide-induced mouse hepatitis
- 95 Ferluga J, Allison AC. 1978 *Lancet* Sep 16;2(8090):610-1 Role of mononuclear infiltrating cells in pathogenesis of hepatitis.
- 96 Involvement of tumor necrosis factor-alpha in the pathogenesis of activated macrophage-mediated hepatitis in mice. *Gastroenterology*. 1991 Apr; 100(4): 1153-4 Nagakawa J, Hishinuma I, Hirota K, Miyamoto K, Yamanaka T, Tsukidate K, Katayama K, Yamatsu I.
- 97 Tiegs G, Kusters S, Kunstle G, Hentze H, Kiemer AK, Wendel A. 1998 : *J Pharmacol Exp Ther* Dec;287(3):1098-104; Ebselen protects mice against T cell-dependent, TNF-mediated apoptotic liver injury
- 98 Kim DW *Exp Mol Med* 2001 Apr 21;33(1 Suppl):101-9 Real time quantitative PCR
- 99 Review by Anthony L Defranco, Locksley and Robertson, 2001 *Immunity, The Immune response to Infection*, New Science Press Ltd, London WIT 4LB.
- 100 Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S 1999; *J Immunol* Apr 1;162(7):3749-52 : Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product

- 101 Musikacharoen T, Matsuguchi T, Kikuchi T, Yoshikai Y 2001; *J Immunol* Apr 1; 166(7): 4516-24: NF-kappa B and STAT5 play important roles in the regulation of mouse Toll-like receptor 2-gene expression
- 102 Holderbaum D, Spech RA, Ehrhart LA *Coll Relat Res* 1985 Jun;5(3):261-71 Specific binding of collagen to *Staphylococcus aureus*
- 103 Kuusela P 1978. *Nature* 1978 Dec 14;276(5689):718-20 Fibronectin binds to *Staphylococcus aureus*.
- 104 Abdelnour A, Bremell T, Holmdahl R, Tarkowski A *Scand J Immunol* 1994 Apr;39(4):403-8 Role of T lymphocytes in experimental *Staphylococcus aureus* arthritis
- 105 Bjork L, Andersson J, Ceska M, Andersson U *Cytokine* 1992 Nov;4(6):513-9 Endotoxin and *Staphylococcus aureus* enterotoxin A induce different patterns of cytokines.
- 106 Takano M, Nishimura H, Kimura Y, Mokuno Y, Washizu J, Itohara S, Nimura Y, Yoshikai Y: 1998; *Infect Immun* Jul;66(7):3270-8 Protective roles of gamma delta T cells and interleukin-15 in *Escherichia coli* infection in mice
- 107 Gerwein J, Kaltoft K, Neilson M, Neilson M, Svejgaard A, Geisler C, Ropke C and Odum N: 1998 *Tissue Antigens*: 51 164-173 *Staphylococcus enterotoxin A* modulates il-15 induced signaling and mitogenesis in humans
- 108 Kumaki S, Armitage R, Ahdieh M, Park L, Cosman D, 1996, *Eur J Immunol* 26: 1235-1239.
- 109 Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, Johnston J, Madden K, Xu W, West J, Schrader S, Burkhead S, Heipel M, Brandt C, Kuijper JL, Kramer J, Conklin D, Presnell SR, Berry J, Shiota F, Bort S, Hambly K, Mudri S, Clegg C, Moore M, Grant FJ, Lofton-Day C, Gilbert T, Rayond F, Ching A, Yao L, Smith D, Webster P, Whitmore T, Maurer M, Kaushansky K, Holly RD, Foster D *Nature* 2000 Nov 2;408(6808):57-63 Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function
- 110 Harb, 2004 *J Biol Chem*, 2004 Jun 4; 279(23): 24313-22 Identification of an Interleukin-15 α Receptor-binding Site on Human Interleukin-15
J. Biol. Chem., Jun 2004; 279: 24313 - 24322

- 111 Musso T, Calosso L, Zucca M, Millesimo M, Ravarino D, Giovarelli M, Malavasi F, Ponzi AN, Paus R, Bulfone-Paus S. Human monocytes constitutively express membrane-bound, biologically active, and interferon-gamma-upregulated interleukin-15. *Blood*. 1999 May 15;93(10):3531-9.
- 112 Erwan Mortier, Jérôme Bernard, Ariane Plet, and Yannick Jacques. Natural, Proteolytic Release of a Soluble Form of Human IL-15 Receptor α -Chain That Behaves as a Specific, High Affinity IL-15 Antagonist. *J. Immunol.*, Aug 2004; 173: 1681 - 1688
- 113 Nelson et al, 1998 *Adv Immunology* 70:1
- 114 Ajuwon KM, Jacobi SK, Kuske JL, Spurlock ME. Interleukin-6 and interleukin-15 are selectively regulated by lipopolysaccharide and interferon-gamma in primary pig adipocytes. *Am J Physiol Regul Integr Comp Physiol*. 2004 Mar;286(3):R547-53. Epub 2003 Dec 04
- 115 Toshiki Yajima,^{1,2*} Hitoshi Nishimura,¹ Kimika Saito,¹ Hiroyuki Kuwano,² and Yasunobu Yoshikai¹ Overexpression of Interleukin-15 Increases Susceptibility to Lipopolysaccharide-Induced Liver Injury in Mice Primed with *Mycobacterium bovis* Bacillus Calmette-Guérin Infection and Immunity, July 2004, p. 3855-3862, Vol. 72, No. 7
- 116 Biber JL, Jabbour S, Parihar R, Dierksheide J, Hu Y, Baumann H, Bouchard P, Caligiuri MA, Carson W. Administration of two macrophage-derived interferon-gamma-inducing factors (IL-12 and IL-15) induces a lethal systemic inflammatory response in mice that is dependent on natural killer cells but does not require interferon-gamma. *Cell Immunol*. 2002 Mar-Apr;216(1-2):31-42
- 117 Megan A. Cooper, Jennifer E. Bush, Todd A. Fehniger, Jeffrey B. VanDeusen, Ross E. Waite, Yang Liu, Hector L. Aguila, and Michael A. Caligiuri. In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. *Blood*, 15 November 2002, Vol. 100, No. 10, pp. 3633-3638
- 118 Wary et al, *haematological* 2004, 89:101-103
- 119 Takashi Hiromatsu,^{1,2} Toshiki Yajima,³ Tetsuya Matsuguchi,¹ Hitoshi Nishimura,¹ Worawidh Wajjwalku,⁴ Toshiyuki Arai,² Yuji Nimura,² and Yasunobu Yoshikai. Overexpression of Interleukin-15 Protects against *Escherichia coli*-Induced Shock Accompanied by Inhibition of Tumor Necrosis Factor- α -Induced Apoptosis. *The Journal of Infectious Diseases* 2003;187:1442-1451

- 120 Stephane Ferretti, Olivier Bonneau, Gerald R. Dubois, Carol E. Jones and Alexandre Trifilieff¹ IL-17, Produced by Lymphocytes and Neutrophils, Is Necessary for Lipopolysaccharide-Induced Airway Neutrophilia: IL-15 as a Possible Trigger. *The Journal of Immunology*, 2003, 170: 2106-2112
- 121 Calander AM, Jonsson IM, Kanth A, Arvidsson S, Shaw L, Foster SJ, Tarkowski A Impact of staphylococcal protease expression on the outcome of infectious arthritis *Microbes Infect.* 2004 Feb;6(2):202-6
- 122 Gjertsson I, Nitschke L, Tarkowski A The role of B cell CD22 expression in *Staphylococcus aureus* arthritis and sepsis *Microbes Infect.* 2004 Apr;6(4):377-82
- 123 Jonsson IM, Mazmanian SK, Schneewind O, Verdrengh M, Bremell T, Tarkowski A On the role of *Staphylococcus aureus* sortase and sortase-catalyzed surface protein anchoring in murine septic arthritis *J Infect Dis.* 2002 May 15;185(10):1417-24. Epub 2002 Apr 22
- 124 Jonsson IM, Mazmanian SK, Schneewind O, Bremell T, Tarkowski A The role of *Staphylococcus aureus* sortase A and sortase B in murine arthritis *Microbes Infect.* 2003 Jul;5(9):775-80
- 125 Waldmann T. IL-15 in the life and death of lymphocytes: immunotherapeutic implications *Trends Mol Med.* 2003 Dec;9(12):517-21
- 126 McInnes and Gracie, Current McInnes IB, Gracie JA Interleukin-15: a new cytokine target for the treatment of inflammatory diseases *Curr Opin Pharmacol.* 2004 Aug;4(4):392-7
- 127 Musso T, Calosso L, Zucca M, Millesimo M, Ravarino D, Giovarelli M, Malavasi F, Ponzi AN, Paus R, Bulfone-Paus S Human monocytes constitutively express membrane-bound, biologically active, and interferon-gamma-upregulated interleukin-15 *Blood.* 1999 May 15;93(10):3531-9
- 128 Kurowska M, Rudnicka W, Maslinska D, Maslinski W Expression of IL-15 and IL-15 receptor isoforms in select structures of human fetal brain *Ann N Y Acad Sci.* 2002 Jun;966:441-5
- 129 Gupta MN, Sturrock RD, Field MA prospective 2-year study of 75 patients with adult-onset septic arthritis. *Rheumatology (Oxford).* 2001 Jan;40(1):24-30
- 130 Gupta MN, Sturrock RD, Field M. Prospective comparative study of patients with culture proven and high suspicion of adult onset septic arthritis. *Ann Rheum Dis.* 2003 Apr;62(4):327-31.

- 131 Olof Hultgren^{2*}, Hans-Pietro Eugster[†], Jonathon D. Sedgwick[†], Heinrich Körner[§] and Andrzej Tarkowski TNF/Lymphotoxin- α Double-Mutant Mice Resist Septic Arthritis but Display Increased Mortality in Response to *Staphylococcus aureus* *The Journal of Immunology*, 1998, 161: 5937-5942
- 132 Kimura M.; Matsukawa A.; Ohkawara S.; Takagi K.; Yoshinaga M Blocking of TNF-alpha and IL-1 Inhibits Leukocyte Infiltration at Early, but Not at Late Stage of *S. aureus* -Induced Arthritis and the Concomitant Cartilage Destruction in Rabbits *Clinical Immunology and Immunopathology*, January 1997, vol. 82, no. 1, pp. 18-25(8)
- 133 Margareta Verdrengh, Andrej Tarkowski Role of macrophages in *Staphylococcus aureus*-induced arthritis and sepsis *Arthritis & Rheumatism* Volume 43, Issue 10, Pages 2276 - 2282
- 134 IB McInnes, Abstract , ACR, September 2004
- 135 Georg Meffert, Frank N Gellerich , Raimund Margreiter and Markus Wyss. Elevated creatine kinase activity in primary hepatocellular carcinoma. *BMC Gastroenterology* 2005, 5:9

List of Abbreviations

5-Bromo-4-Chloro-3-indolyl- β -galactopyranoside.....	X-GAL
Adenosine triphosphate.....	ATP
Adenosine.....	A
Alanine-amino transferase.....	ALT
Alkaline phosphotase buffer.....	CIP
Antigen presenting cell.....	APC
Aquired immume deficiency syndrome.....	AIDs
Aspartate-amino transferase.....	AST
Base pairs (bp).....	bp
Bicinchoninic Acid assay.....	BCA
Bovine serum albumin.....	BSA
Carbon dioxide.....	CO ₂
Collagen induced arthritis.....	CIA.
Colony forming units.....	CFUs
Complementary DNA.....	cDNA
Concanavalin-A.....	CON-A
Copper suphate.....	CuSO ₄
Creatine Kinase.....	CK
Cytosine.....	C
Cytotoxic T lymphocyte line.....	CTLL
Dalton.....	Da
Deoxynucleictriphosphate.....	dNTP
Deoxyribonucleic acid.....	DNA
Diethylpyrocarbonate.....	DEPC
Dithiothreitol.....	DTT
Ethylenediaminetetracetic acid.....	EDTA
Effective dose.....	ED
Enzyme linked immunosorbent assay.....	ELISA

Escherichia coli.....	E. coli
Fetal calf serum.....	FCS
Granulocyte/monocyte colony stimulating factor.....	GMC-SF
Guanosine.....	G
High salt-Phosphate buffered saline.....	HS-PBS
Horse radish peroxidase.....	HRP
Human lymphotropic virus HTLV-1	HTLV-1
Human serum albumin.....	HSA
Immunoglobulin.....	Ig
Inducible nitric oxide synthase.....	iNOS
Interferon gamma.....	IFN γ
Interleukin.....	IL
Interleukin-15 receptor alpha.....	IL-15R α
Interleukin-2 receptor alpha.....	IL-2R α
Intracellular adhesion molecule 1	ICAM-1
Isopropyl-B-D-thiogalactopyranosamide.....	IPTG
Janus kinase 3.....	JAK-3
Leucocyte Antigen-1	LFA-1
Lipopolysaccharide.....	LPS
Litre.....	L
Long terminal repeat	LTR
LPS Binding Protein.....	LBP
Lymphotoxin.....	LT
Magnesium chloride.....	MgCl $_2$
Magnesium sulphate.....	MgSO $_4$
Major Histo-compatibility molecule.....	MHC
Messenger ribonucleic acid.....	mRNA
Microlitre.....	μ l
Micromolar.....	μ M
Milliliter.....	ml
Millimolar.....	mM

Murine.....	mu
Nanometers.....	nM
Nitric oxide.....	NO
Optical density.....	OD
Phosphate buffered saline.....	PBS
Pico gram	pg
Potassium Chloride.....	KCl
Potassium.....	K
Reverse transcriptase polymerase chain reaction.....	RT-PCR
Sodium carbonate.....	NaCO ₃
Sodium Chloride.....	NaCl
Sodium dodecyl sulphate polyacrylamide gel electrophoresis	SDS-PAGE
Sodium hydroxide.....	NaOH
Staphylococcal enterotoxin-A.....	SEA
Staphylococcus aureus.....	S. aureus
T helper cell 2.....	Th2
T-cell receptor.....	TCR
T-helper cell 1.....	Th1
Thymidine.....	T
TOLL like Receptor.....	TLR
Toxic shock syndrome toxin.....	TSST
Transforming Growth Factor beta	TGF-β
Transgenic.....	TG
Tumour necrosis factor alpha.....	TNFα
Untranslated region	UTR
Uracil.....	U