The role of Interleukin-15 in inflammation

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Abstract

Cytokines are important mediators of immune functions in humans and animals. Interleukin (IL)-15 is a proinflammatory cytokine, which is mainly produced by monocytes. It shares many of its functions with IL-2, which is partly due to the shared use of receptor subunits on target cells, and serves as a growth and survival factor for T lymphocytes. The type I IL-15 receptor is composed of the IL-2Rβ and γ subunits, which form a trimeric complex with the high affinity IL-15Rα chain. The expression of IL-15 is tightly regulated both at the transcriptional and translational level. The production of IL-15 is associated with immune responses against bacterial and parasitic pathogens but has also been associated with the pathology of human autoimmune diseases, in particular Rheumatoid Arthritis (RA). RA is characterized by chronic inflammation within the synovial membrane accompanied by infiltration of lymphocytes leading to progressive, erosive destruction of cartilage and underlying bone. The severity of RA is associated with the overexpression of proinflammatory cytokines within the synovial tissue, in particular tumor necrosis factor alpha (TNFα) which is thought to play a central role in maintaining the inflammatory processes within the arthritic joint. So far, little is known about the processes that initiate and perpetuate RA. IL-15 was found in the synovial tissue of RA patients where it stimulated the production of TNFα, placing IL-15 in a central position orchestrating the cytokine cascade that causes inflammation and pathology in RA. Antagonists to IL-15 may therefore have an important therapeutic potential for the treatment of RA in humans.

A major aim of this project has been to clone and express a recombinant IL-15 antagonist to use as a therapeutic agent in a murine model of RA closely related to the human disease, collagen-induced arthritis (CIA). A soluble IL-15Rα was cloned from a murine macrophage cell line and expressed in a bacterial expression system. The resulting protein has a molecular weight of 26kD and bound to IL-15 specifically. It also had a

neutralizing effect on IL-15-induced proliferation of T cell lines. Administration of soluble IL-15Rα to mice prevented the onset of CIA and had a suppressive effect on disease severity and incidence. Mice treated with the recombinant IL-15Rα also showed reduced serum cytokine production and altered humoral responses against collagen. These results consolidate the therapeutic potential of IL-15 antagonists for the treatment of inflammatory diseases. To further enhance the therapeutic properties of recombinant IL-15Rα, a second expression construct has been cloned fusing the extracellular region of native IL-15Rα to the constant region of the murine immunoglobulin heavy chain. This construct was expressed in a mammalian expression system and results in a product of 66kD, which also bound to IL-15.

The generation of knockout mice by gene targeting is a powerful tool to study the function of gene products *in vivo*. The Cre/lox system provides a novel strategy to generate inducible and tissue specific genomic alterations that allow the detailed analysis of gene function. The second part of this project was concerned with the generation of a mouse model lacking IL-15Rα in a tissue specific way by conditional mutagenesis in embryonic stem (ES) cells. Using cDNA encoding the extracellular domain of IL-15Rα as a radiolabeled probe, a murine genomic library was screened. Two clones containing part of the gene encoding IL-15Rα were characterized. A DNA construct was cloned to target the IL-15Rα gene in murine ES cells. Homologous recombination of the construct with the target locus resulted in the flanking of the critical regions of the IL-15Rα-gene by loxP sites. Cre-mediated recombination *in vitro* caused the deletion of loxP site flanked sequences within the genome of the targeted clone. Using this technique, two ES cell clones have been generated that allow the generation of mice that either lack IL-15Rα in all tissues or are suitable for conditional mutagenesis mediated by Cre recombinase. The resulting model may provide a useful tool to study the effects of IL-15 in inflammatory processes *in vivo*.

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Abbreviations

Ag	antigen
Ab	antibody
APS	ammonium-persulfate
AS	antiserum
BSA	bovine serum albumin
bp	base pairs
CFA	complete Freund's adjuvant
CIA	collagen induced arthritis
DEPC	diethyl-pyrocarbonate
DMSO	dimethyl-sulfoxide
dNTP	mix of deoxy-nucleotides (dATP, dCTP, dGTP, dTTP) at 10mM
DNA	deoxy-ribonucleic acid
DIA/LIF	differentiation inhibiting factor / leukemia inhibitory factor
DTH	delayed type hypersensitivity
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)- N',N',N',N'-tetraacetic acid
ER	endoplasmatic reticulum
ES cells	murine embryonic stem cells
FCS	fetal calf serum
GM-CSF	granulocyte/macrophage-colony stimulating factor
hCMV	human cyto-megalo virus
HLA	human leukocyte antigen
Hprt	hypoxanthine phosphoribosyl transferase
HRP	horse radish peroxidase
HSA	human serum albumin
HSV-tk	herpes simpex virus-thymidine-kinase
ICAM	intracellular cell adhesion molecule
i.d.	intra dermal
IFA	Incomplete Freund's adjuvant
IL	interleukin
IPTG	isopropyl thio-β-D-galactoside

i.p.	intra peritoneal
IRF	interferon regulatory factor
Jak	Janus kinase
LFA	leukocyte function associated antigen
mAb	monoclonal antibody
MCP	monocyte chemotactic protein
MCS	multiple cloning site
mRNA	messenger ribonucleic acid
Neo	neomycin-phosphotransferase
Ni-NTA	nickel-nitrilotriacetic acid
NK	natural killer
OD	optical density
PBS	phosphate-buffered saline
PBMC	peripheral blood mononuclear cells
PEG	poly-ethylene glycol
p.f.u.	plaque forming units
PGK	phospho-glycerokinase
PMSF	phenyl methyl sulphonyl fluoride
RA	rheumatoid arthritis
s.c.	sub cutaneous
SCID	severe combined immuno-deficiency
SD	standard deviation
SEM	standard error of the mean
SDS	sodium dodecyl sulfate
STAT	signal transducers and activators of transcription
TEMED	N,N,N',N'-tetramethylenediamine
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
UTR	untranslated region

M	molar
mM	millimolar
mg	milligram
ml	milliliter
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar
nm	nanometer
nM	nanomolar
pg	picogram

Chapter 1 General Introduction

1.1 Interleukin-15 and its Receptor

1.1.1 Historical perspective

IL-15 was first characterized as a soluble factor contained in culture supernatants derived from a simian kidney epithelial cell line, CV-1/EBNA (Grabstein, *et al.*, 1994) and an adult human T-cell leukemia line (Burton, *et al.*, 1994). While testing cell culture supernatants for cytokine activity it was found that these cells produced a soluble factor capable of inducing the proliferation of the IL-2-dependent cell line CTLL-2 (Gillis and Smith, 1977). Purification of the soluble factor with the CTLL-2-stimulating activity via anion-exchange and high-pressure liquid chromatography (HPLC) revealed that the stimulatory activity could be attributed to a protein of approximately 14-15kD. N-terminal amino acid sequencing and subsequent PCR using degenerate primers enabled cloning of a 92bp cDNA fragment, which was used to screen a cDNA library made from CV-1/EBNA cells. A full-length cDNA clone was obtained encoding a 162 amino acid residue precursor polypeptide. Further analysis showed that this precursor polypeptide contained a 48 amino acid leader peptide, which is cleaved to form the mature protein. Initially designated IL-T by Burton *et al.* (1994), the newly described cytokine was subsequently named IL-15. The similar biological functions of IL-15 and IL-2 are partly due to the sharing of receptor subunits. The IL-2 receptor is composed of three subunits IL-2Ra, -β and –γ, whereas the IL-15R contains the IL-2β and –γ chains in addition to a unique IL-15Rα subunit.

1.1.2 Characteristics of Interleukin-15

Like IL-2, IL-15 is a member of the 4α-helix bundle family of cytokines, which also includes IL-3, IL-4, IL-5, IL-6, IL-7 and IL-9. Accordingly IL-15 folding results in a protein consisting of three loops connecting four α-helices, which are arranged in an up-up

down-down-configuration (Grabstein, *et al.*, 1994). Although no sequence homology exists between IL-15 and IL-2, or any other member of the same cytokine family, IL-15 shares the same structural features as IL-2. Mature IL-15 contains 114 amino acid residues resulting in a molecular weight of 14-15kD. The mature protein is N-glycosylated on two asparagine residues and also contains two putative cystine disulfide cross-linkages at positions Cys42-Cys88 and Cys35-Cys85. The first of these disulfide bonds is homologous to a similar cross linkage found in IL-2. Human and simian IL-15 share 97% sequence identity and there is 73% sequence identity between simian and murine IL-15. In contrast to IL-2, which is produced by activated T cells (reviewed by Swain, 1991), mRNA encoding IL-15 can be detected by Northern blot analysis in many tissues and was found to be most abundant in human placenta and skeletal muscle with detectable levels in heart, lung, liver and kidney. High levels of IL-15 mRNA can also be found in adherent peripheral blood mononuclear cells (PBMC) and epithelial and fibroblast cell lines (Grabstein, *et al.* 1994).

1.1.3 Regulation of IL-15 expression

In humans, the gene encoding IL-15 was mapped on chromosome 4 by *in situ* hybridization, whereas the murine IL-15 gene is found on chromosome 8. In both species the gene encoding IL-15 contains nine exons covering a total area of approximately 35kb (Krause, *et al.*, 1996). The regulatory mechanisms that control the expression of IL-15 are very complex and occur mainly at the translational level. IL-15-mRNA expression in monocytes is upregulated by interferon gamma (IFNγ) and lipopolysaccharide (LPS) (Grabstein, *et al.*, 1994) as well as in response to several pathogens like *Mycobacterium tuberculosis* and *Toxoplasma gondii* (Doherty, *et al.*, 1996). Transcription of IL-15-mRNA is regulated by a number of transcription factors, most importantly IRF-1 (Ogasawara, *et*

al., 1998) and NF-κB (Azimi, *et al.*, 1998). Corresponding conserved elements serving as putative transcription factor binding sites can be found in both the human and murine IL-15 promoter regions. Mice with a targeted mutation of the IRF-1 gene are NK cell deficient. However, this deficiency can be compensated *in vitro* by culturing IRF-$1^{-/-}$ bone marrow cells with IL-15. Also, an IRF responsive element (IRF-E) has been located in the 5'control region of the IL-15 gene (Otheki, *et al.*, 1998). IL-15-mRNA transcription is elevated in T cells infected with the human T cell lymphotropic virus type I (HTLV-I). This elevation is mediated by the HTLV-1 associated Tax protein and can be abrogated by the mutation of a NF-κB consensus sequence located in the 5' regulatory region of the IL-15 gene (Azimi, *et al.*, 1998). .

However, regulation of IL-15 expression predominantly occurs post-transcriptionally at the level of translation and translocation (Grabstein, *et al.*, 1994; Bamford, *et al,* 1996). The observation that although IL-15-mRNA is readily expressed in many tissues, IL-15 cannot easily be detected at the protein level le d to the investigation of mechanisms that control translation of the IL-15 protein. A first level of translational control is constituted by the 5'untranslated region (5'UTR) of the IL-15-mRNA transcript. Whereas the 5'UTR of most mRNAs is short and does not contain many regulatory elements, the 5'UTR of the IL-15 transcript is relatively long and contains multiple AUGs. Five AUGs can be found in murine and 12 in the human IL-15 UTR. These AUGs are located upstream of the initiation AUG and are known to significantly reduce the efficiency of protein translation (Kozak, 1986, 1987, 1989, and 1991). They can also be found in the 5'UTR of mRNAs encoding several protooncogenes, growth- and transcription factors. Experimental deletion of the 5' AUGs in an IL-15 expression construct transfected into COS cells increased protein production 4-5 fold when compared to a control construct containing the wild type AUGs (Bamford, *et al.*, 1996). Two different signal peptides have been described that regulate IL-15 secretion and intracellular

trafficking. Initial studies demonstrated that the originally described 48 amino acid signal peptide of IL-15 strongly inhibits IL-15 generation. Expression constructs where the 48 amino acid signal peptide of IL-15 had been exchanged for the leader sequence of IL-2 resulted in a 17-20 fold increase in IL-15 expression in COS cells, whereas the IL-15 signal peptide inhibited IL-2 production 40-50 fold in reciprocal constructs (Bamford, *et al.*, 1998). An IL-15 splice variant has been described bearing an alternative second leader sequence containing 21 amino acid residues. This alternative signal peptide originates from alternative splicing of exon 4a resulting in an insertion of 119 nucleotides encoding an alternative start codon and 21 amino acid signal peptide. The murine alternative gene segment has a length of 136 nucleotides resulting in a shortened leader sequence of 26 amino acid residues (Prinz, *et al.*, 1998). Messenger RNA encoding both splice variants can be readily detected in T cells. However, IL-15 containing the shorter leader sequence is translated more efficiently than IL-15 containing the 48 amino acid sequence. The 21 amino acid signal peptide isoform is not secreted and remains within the cell (Onu, *et al.*, 1997). The short signal peptide isoform is preferentially expressed in tissues such as testis and thymus and is not secreted, but rather stored intracellularly, appearing in the nucleus and cytoplasmic components of the cell. IL-15 may therefore play a role as an intracellular signaling molecule. The long signal peptide isoform appears in the endoplasmatic reticulum (ER), regulates the rate of protein translation and functions as a secretory signal peptide (Tagaya, *et al.*, 1997; Nishimura, *et al.*, 1998; Gaggero, *et al.*, 1999). A third regulatory control mechanism influencing IL-15 expression has been described, which is located at the 3' end of the IL-15 coding sequence. For the purpose of antibody detection an IL-15 expression construct containing the FLAG coding sequence at its 3'end was cloned. The observation that this construct resulted in a 5-10 fold increase in IL-15 expression suggests the presence of a regulatory element located within the 3' end of the coding sequence (or the carboxyl-terminus of the IL-15 protein, respectively). This region, unintentionally disrupted by the FLAG coding sequence, appears to contribute to the

negative regulation of IL-15 expression. The molecular mechanism by which the C-terminal negative element inhibits IL-15 expression has not yet been defined but preliminary studies demonstrate that the stability of both IL-15-mRNA transcript and the resulting peptide are not altered by the presence of the C-terminal negative element (Bamford, *et al.*, 1998). This observation may indicate the presence of a comparatively large pool of IL-15-mRNA transcripts within the cell with only a low-level translational activity and peptide secretion. This pool of transcripts may allow for the secretion of large amounts of IL-15 within a short period of time in response to immunological stimuli, thus providing a means to rapidly activate NK and T cells.

IL-15 also exists in a membrane-bound form constitutively expressed on normal human monocytes, as well as on monocytic cell lines but not on human T or B cells. Cell surface-bound IL-15 can be upregulated *in vitro* by IFNγ stimulation. Membrane-bound IL-15 does not elute with acetate buffer or trypsin treatment excluding the possibility that surface IL-15 is associated with its own receptor. These results suggest the presence of an integral membrane protein that has not yet been defined. The membrane bound form of IL-15 is biologically active and stimulates T lymphocytes to proliferate *in vitro*. So far, no correlation has been found between the differential usage of signal peptides and the expression of membrane bound IL-15. Membrane bound isoforms have been described for other cytokines including IL-1, TNFα, and IL-10 (Kurt-Jones, *et al.*, 1985; Perez, *et al.*, 1990; Fleming, *et al.*, 1996). IL-15 may therefore be capable of mediating part of its biological activities in a localized, cell contact dependent manner as a membrane bound cytokine (Musso, *et al.*, 1999).

1.1.4 The IL-15 receptor

Two distinct receptors and corresponding signaling pathways have been described for IL-15. The type-1 IL-15 receptor mediates the functions of IL-15 in T-, B- and NK cells. It shares important receptor components with the IL-2R system (Bamford, *et al.*; Grabstein, *et al.*; Giri *et al.*, 1994). The type-1 IL-15 receptor contains three components, two of which, IL-2/15Rβ (CD122) and IL-2Rγ (CD132) are also part of the IL-2R complex. The murine IL-2Rβ- and γ- chains are proteins with a molecular weight of 110kD and 75kD respectively (Kono, *et al.*, 1990). They both belong to the hematopoietin receptor superfamily (D'Andrea, *et al.* 1989). Both chains are required for ligand internalization and signal transduction (Robb and Greene, 1987; Hatakeyama, *et al.*, 1989). The lack of IL-2Rγ in humans is associated with X-linked severe combined immunodeficiency (XSCID) underlining the importance of the IL-2R signaling complex (Noguchi, *et al.*, 1993). In addition to the IL-2Rβ and γ chain, both IL-2 as well as IL-15 bind to their own unique receptor subunits, IL-2Rα (CD25) or IL-15Rα, respectively, to form high-affinity heterotrimeric receptor complexes.

The shared usage of IL-2R subunits between IL-2 and IL-15 and the observation that many non-lymphoid cell types bind IL-15 but not IL-2 (Giri, *et al.*, 1994) suggested the existence of a unique IL-15-binding protein as a structural homologue to IL-2Rα. The murine T helper cell line D10 manifested a 10-fold higher dose responsiveness to IL-15 than to IL-2 (Giri, *et al.*, 1995). IL-15 labeled with ^{125}Iodine specifically bound to a protein of 58-60kD derived from D10 lysates. The gene encoding the IL-15 binding protein was cloned from COS-7 transfected with a murine cDNA expression library derived from D10 cells. A single clone derived from this library encoded a type I membrane protein with a predicted signal peptide of 32 amino acids, 173 amino acid extracellular domain, a

transmembrane domain of 21 amino acids and a cytoplasmic tail of 37 amino acid residues. The protein contains a single site for N-linked glycosylation and is extensively O-glycosylated. Little significant sequence homology of IL-15Rα to known proteins was found, however the extracellular domain of IL-15Rα exhibits a 45% similarity (28% identity) to bovine IL-2Rα. This homology is particularly pronounced within a conserved protein binding motif, also named 'sushi domain', glycoprotein (GP-)-1 motif or 'short consensus repeat (SCR) present in both IL-2Rα and IL-15Rα (Giri, *et al.*, 1995, Davic, *et al.*, 1986; Perkins, *et al.*, 1988) and is also present in several complement receptors (e.g. CR2, complement factor H, β-2-glycoprotein I). The protein encoded by the COS-7 clone was subsequently named IL-15Rα. The figure below shows a schematic representation of IL-15Rα and its subdivision into distinct functional domains compared to the IL-2Rα chain.

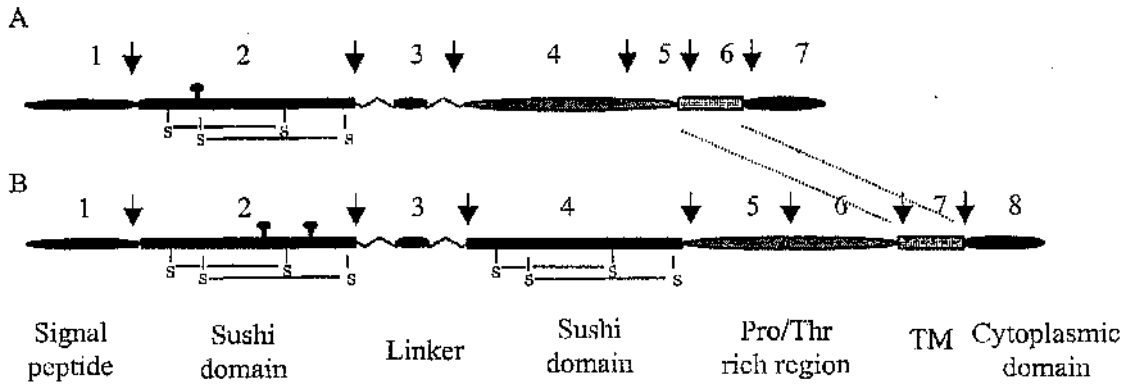


Figure 1.1 Schematic representation of IL-15Rα (**A**) and IL-2Rα (**B**) demonstrating the structural similarity between both cytokine receptors. Both cytokine receptors are composed of a signal peptide, extracellular domain, and a single membrane spanning segment (TM) and cytoplasmic tail. Furthermore, both proteins contain the conserved protein binding motif called 'Sushi domain', which characteristically contains disulfide bridges. One putative N-glycosylation site is located within the 'Sushi domain' of IL-15Rα, whereas IL-2Rα contains two sites of glycosylation. IL-15Rα contains a single 'Sushi domain', whereas two protein binding domains can be found in IL-2Rα. Arrows represent the intron-exon boundaries of the genes encoding IL-15Rα and IL-2Rα. The numbering between the arrows refers to the exon number containing coding sequence corresponding to each protein domain.

Transfection experiments and equilibrium binding experiments using [^{125}I]-simian IL-15 showed that IL-15 binds to IL-15Rα with high affinity (K_a~1x$10^{11}M^{-1}$). This affinity is comparable to the binding affinity of IL-2 to the IL-2R complex, however IL-2Rα binds IL-2 with low affinity (K_a~10^8M^{-1}) in the absence of IL-2Rβ and γ chains. The additional presence of IL-2Rβ and IL-2Rγ did not significantly enhance the affinity of IL-15 binding to its receptor or could not be demonstrated due to the intrinsically high affinity of IL-15 to IL-15Rα. IL-2Rβ and IL-2Rγ alone are unable to bind to IL-15 with any measurable affinity, although high concentrations of IL-15 may cause signaling even in the absence of the IL-15Rα chain (Anderson, *et al.*, 1995). IL-15Rα has a wide cellular distribution and unlike IL-2Rα is not only expressed on T cells, Macrophages and B cells, as well as thymic and bone marrow stromal cell lines express IL-15Rα-mRNA. In addition, mRNA encoding IL-15Rα can be detected in liver, spleen, heart, skeletal muscle, lung and activated vascular endothelium. In contrast to the wide tissue distribution of IL-15Rα, IL-2Rα is only found on T lymphocytes and NK cells. The induction of IL-15Rα on T and B-lymphocytes correlates with the activation of these cells. In contrast to resting cells, mitogen- activated macrophages, NK cells, $CD4^+$ and $CD8^+$ T cells express IL-15Rα chains (Chae, *et al.*, 1996). The expression of IL-15Rα-mRNA in T cells cultures is increased by the addition of anti-CD3 antibodies, phorbol myristyl acetate (PMA) or IL-2. In macrophages, treatment with IFNγ or IFNγ and LPS greatly stimulated the production of IL-15Rα-mRNA. IL-15 has been demonstrated to upregulate IL-2Rα but to downregulate its own high affinity receptor on human T and B cells (Kumaki, *et al.*, 1996).

The gene encoding murine IL-15Rα has been mapped on chromosome 2, whereas the human gene is located on chromosome 10p14-15. In both the murine and human genomes, the genes encoding IL-15Rα and IL-2Rα are located in close proximity (Anderson, *et al.*, 1995; Leonard, *et al.*, 1985). The gene encoding IL-15Rα is composed of 7 exons and 6 introns. The intron-exon boundaries relative to predicted structural domains of IL-15Rα and IL-2Rα show that the positions and coding sequence of several exons are in close agreement. The functional, structural and genetic similarities of IL-15Rα and IL-2Rα have led to speculation that the genes encoding the two proteins arose from a random gene duplication event that predated the phylogenetic separation of mouse and man. Human and murine IL-15Rα share an overall amino acid identity of 54%. This homology is particularly conserved within the region of the putative 'Sushi domain', where the amino acid identity between the two proteins derived from mouse and human reaches 82%.

Several differentially spliced mRNA transcripts for hIL-15Rα exist. In the original publication reporting the cloning and characterization of human IL-15Rα (Anderson, *et al.*, 1995), three splicing variants were described. Apart from the full-length mRNA-transcript encoding the complete IL-15Rα protein, a variant lacking exon 3 (linker/hinge region) has been found in a human cDNA library. A further cDNA fragment contains a 120bp-insertion encoding a shortened cytoplasmatic domain resulting from splicing of an alternate downstream-exon named E7'. E7' is 100bp shorter than E7, which encodes the cytoplasmic tail of IL-15Rα. RT-PCR using specific primers for all three splicing variants confirmed the existence of all three differentially spliced forms of human IL-15Rα in peripheral blood T cells, the NK-like YT cell line and the myelogenous leukemia cell line K562. All three splicing variants identified are capable of high affinity binding to IL-15.

Recently, Dubois *et al.* (1999) reported the existence of another five alternatively spliced IL-15Rα-transcripts. These splicing variants lack either E2, E3 or both exons and are found with either of the two exons (E7 or E7') alternatively encoding the cytoplasmic tail of IL-15Rα. All eight splicing variants are present in PBMCs and can be specifically amplified by RT-PCR. The most striking observation for these splicing events is the existence of a mRNA-transcript lacking exon 2, encoding the protein binding 'Sushi motif' of IL-15Rα. Subsequent cloning and expression of differentially spliced variants of IL-15Rα in COS-7 cells showed that proteins resulting from mRNA-transcripts lacking E2 where unable to bind IL-15. Analysis of the subcellular localization of the different IL-15Rα isoforms within COS-7 cells by confocal microscopy revealed that full-length IL-15Rα is mainly associated with the nuclear membrane compartment of COS-7 cells. Some of the IL-15Rα protein was also localized on the inner site of the nuclear membrane. In support of this observation, a putative nuclear localization sequence (NLS) lies within the sequence encoded by E2. These NLSs occur in the sequence of a number of ligands, including those that activate STAT transcription factors (Strehlow and Schindler; Johnson, *et al.*, 1998) and may be associated with the nuclear routing of proteins. In contrast, IL-15Rα lacking the E2-encoded 'Sushi' domain was mainly associated with ER, Golgi and cytoplasmatic vesicles, but not with nuclear membrane compartments. The deletion of E3 had no effect on the cellular localization of IL-15Rα, nor did it alter the binding capacity for IL-15. External labeling of COS-7 cells with [^{125}I]-iodine and subsequent immunoprecipitation showed that both the full-length IL-15Rα and the splicing variant lacking E2 are routed to the plasma membrane and are expressed at the cell surface of transfected cells at low concentrations. Although the biological functions of IL-15Rα without E2 remain uncertain, a regulatory role for both IL-15Rα-transcripts in the regulation of IL-15 mediated signaling events has been proposed. Truncated IL-15Rα may compete with the full-length transcript for the recruitment of IL-2Rβ and γ subunits.

Interestingly, a similarly truncated form of IL-2Rα has been described that results from differential splicing and lacks E4 of the IL-2Rα gene, which similarly encodes a protein binding 'Sushi' domain (Cullen, *et al.*, 1988). Since two isoforms of IL-15 have been described that differ in the length of their signaling peptides, secretion patterns and cellular location, IL-15Rα may bind to IL-15 intracellularly and, through its putative NLS, translocate the cytokine-receptor complex into the nuclear compartment of the cell. Since IL-15Rα is a large protein of approximately 60kD making diffusion into the nucleus unlikely, it has been speculated that an active transport mechanism mediating nuclear translocation of IL-15-IL-15Rα may exist.

IL-15 can also stimulate the proliferation of murine mast cell lines and normal bone marrow mast cells. However, the mast cell lines PT-18 and Mc/9 do not respond to IL-2 and mRNA encoding the IL-2 receptor (IL-2Rα, -β or γ) is undetectable in these cells. These results suggest that an alternative IL-15R complex exists on mast cells. To investigate the mechanism underlying IL-15 function in mast cell proliferation, IL-15 has been cross-linked to ^{125}Iodine and used with disuccinimidyl suberate in order to identify a putative mast cell-specific IL-15R. Using this technique, an IL-15-binding protein of 60-65kD was identified and subsequently named IL-15RX. This receptor, which mediates the functions of IL-15 on mast cells has also been referred to as type-2 IL-15 receptor (Tagaya, *et al*, 1996).

1.1.5 Signaling through the IL-15 receptor

Due to the shared usage of receptor subunits between the type 1 IL-15R and IL-2R, signaling through the type 1 IL-15R is characterized by intracellular events mediated by signal transduction through the IL-2Rγ chain. IL-2Rγ, also referred to as 'common gamma chain' is a component of other cytokine receptors and thus mediates signaling events triggered by IL-2, IL-4, IL-7, IL-9 and IL-15. Jak1 and Jak3 are known to be coupled functionally to the common gamma chain (Witthuhn, *et al.*, 1994). Addition of IL-15 or IL-2 to T cells expressing both cytokine receptor subunits triggered the phosphorylation and nuclear translocation of STAT3 and STAT5 (Johnston, *et al.*, 1995). Furthermore, common gamma chain-mediated signaling pathways in T cells involve the phosphorylation of cytoplasmic tyrosine kinases $p56^{lck}$ and $p72^{syk}$ as well as the expression of the anti-apoptotic protein bcl-2. Ras/Raf/MAP kinase pathways are also stimulated leading to the activation of fos/jun (Miyazaki, *et al.*, 1995).

The mast cell specific receptor for IL-15 does not signal through the common γ-chain cytokine receptor. Signaling events following the binding of IL-15 to IL-15RX therefore differ from those observed in T and B-lymphocytes. IL-15 stimulation of PT-18 and bone marrow mast cells caused the phosphorylation of Jak2 rather than Jak1/Jak3 as observed for events following IL-15 binding to IL-15Rα. In contrast to type 1 IL-15R signaling, no tyrosine phosphorylation and subsequent nuclear translocation of STAT3 was observed in mast cells limiting the activation event to STAT5. The distinct receptor usage and signaling cascade induced by IL-15 in mast cells further underline the pleiotropic functions of IL-15 and its action in various tissues (Tagaya, *et al.*, 1996).

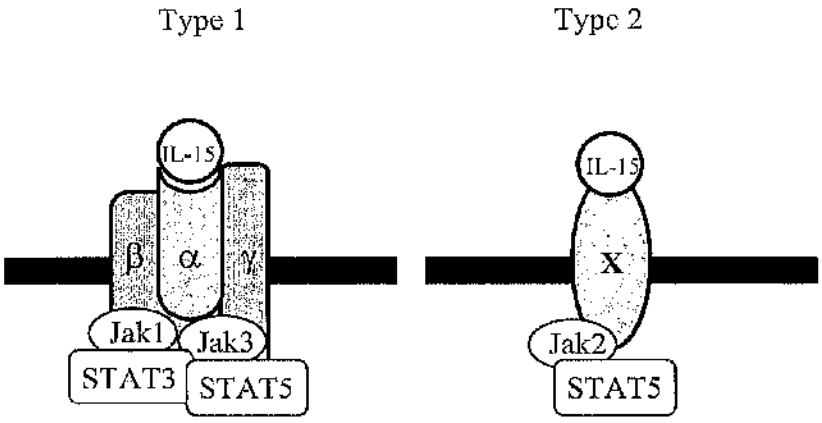


Figure 1.2 Schematic representation of the IL-15 receptors found in different tissues. The IL-15-type 1 receptor is found on lymphocytes and many other cell types. It is composed of the IL-2Rβ and γ chain and contains a unique IL-15Rα chain for high affinity binding to IL-15. Signaling through the type 1 IL-15R involves Jak1 and Jak3 as well as STAT 3 and STAT5. Mast cells express the IL-15-type 2 receptor, which does not contain any components of the IL-2R complex but is composed of a 60-65kD membrane spanning protein. Signal transduction in mast cells involves Jak2 and STAT5 (after Tagaya, *et al.*, 1996).

1.2 The functions of Interleukin-15

1.2.1 The biological role of IL-15

The initial cloning of IL-15 from CV-1/EBNA and adult human leukemia cells revealed a protein capable of replacing IL-2 as a cytokine inducing the proliferation of T cells and activation of lymphokine-activated killer cells. Due to the usage of common receptor subunits, most crucially the common γ chain required for signal transduction, the biological effects of IL-2 and IL-15 overlap. However there are major differences between the two cytokines in terms of site of synthesis, regulation of expression and receptor tissue distribution. IL-15 stimulated the proliferation of the IL-2 dependent cell line CTLL-2 and lymphokine-activated killer cells. It also induced the proliferation of activated $CD4^+$ and $CD8^+$ cells (Burton, *et al.*, 1994). B cell proliferation and antibody production were stimulated by IL-15 *in vitro* in combination with either CD40L or immobilized anti-IgM (Armitage, *et al.*, 1995). IL-15 upregulated CD69 expression on $CD45R0^+$ cells (Kanegane, *et al.*, 1996) and thus can recruit and expand memory T cells. Unlike IL-2, IL-15 causes the stimulation of memory-phenotype $CD44^{hi}$ $CD8^+$ but not $CD4^+$ cells *in vivo* and purified T cells *in vitro*, and this correlated with much higher expression of IL-2Rβ on $CD8^+$ cells than on $CD4^+$ cells (Zhang, *et al.*, 1998). Priming of naïve $CD4^+$ cells from TCR-transgenic mice by IL-15 led to enhanced IFNγ-, but not IL-4 production after subsequent stimulation with specific antigen (Seder, 1996). On the other hand activated human T lymphocytes were reported to produce both IFNγ and IL-4 after IL-15 stimulation (Borger, *et al.*, 1999).

The development and function of NK cells has been demonstrated largely to rely on IL-15. Deficiencies in the number of NK cells have been observed in IL-2Rβ- but not IL-2

knockout mice suggesting that IL-2Rβ-mediated signals that do not involve IL-2 are important for the differentiation and function of these cells (Suzuki, *et al.*, and Otheki, *et al.*, 1997). Mice lacking the transcription factor IRF-1 are deficient in NK cell development and also exhibit impaired IL-15 mRNA expression. However, the lack of IRF-1 affects the radiation resistant cells that constitute the microenvironment required for NK cell development rather than NK progenitors themselves. Culture of bone marrow cells derived from IRF-$1^{-/-}$ mice in the presence of IL-15 led to the generation of NK cells suggesting that IL-15 is crucial for the development of this cell type (Otheki, *et al.*; Ogasawara, *et al.*, 1998). Resting human NK cells have been shown to express the IL-15Rα chain required for high affinity binding of IL-15. Picomolar amounts of IL-15 but not other cytokines sustained NK cell survival for up to 8 days in the absence of serum. One mechanism by which IL-15 promotes NK cell survival may involve the maintenance of bcl-2 protein expression. IL-15 also stimulates IFNγ production by NK cells (Carson, *et al.*, 1997). The cytotoxicity of NK cells induced by herpesvirus 6 and 7 was dependent on IL-15, since the addition of anti-IL-15 Ab markedly reduced the herpesvirus-induced activation of NK cells (Flamand, *et al.*, 1996; Atedzoe, *et al.*, 1997).

IL-15 acted synergistically with IL-12 to promote the responses of human γδ T cells to non-peptide antigens and induced IFNγ production from these cells (Garcia, *et al.*, 1998). The importance of IL-15 in innate immunity is further underlined by its chemotactic properties. IL-15 is chemotactic for NK cells and promotes NK cell attachment to the endothelium (Allavena, *et al.*, 1997). IL-15 acted as a chemokine for human blood T cells (Wilkinson and Liew, 1995) and induced the production of IL-8 and MCP-1 from monocytes (Badolato, *et al.*, 1997). IL-15 also stimulated the expression of CC-, CXC-, and C-type chemokines and CC chemokine receptors, but not CXC chemokine receptors on T lymphocytes (Perera, *et al.*, 1999). In addition to its effect on T cells and NK cells,

IL-15 also had a stimulatory effect on neutrophils. These cells have been shown to express the high affinity IL-15Rα chain and to respond to IL-15 by undergoing morphological changes that are associated with neutrophil-activation. IL-15 but not IL-2 increased the phagocytosis of opsonized sheep red blood cells and stimulated *de novo* RNA synthesis and protein production (Girard, *et al.*, 1996).

A further important biological effect of IL-15 lies in its anti-apoptotic properties. IL-15 has been shown to protect activated T and B cells from apoptosis induced by anti-Fas, anti-CD3, dexamethasone or anti-IgM. In addition, administration of an IL-15-IgG2b fusion protein protected BALB/c mice from hepatic failure and death induced by anti-Fas injection and inhibited chemotherapy-induced apoptosis of keratinocytes (Bulfone-Paus, *et al.*, 1997; Lindner, *et al.*, 1998). The molecular mechanisms underlying the inhibitory effect of IL-15 on apoptosis are so far only partially understood. Apart from the stimulatory effect of IL-15 on bcl-2, a possible function of IL-15Rα may be to compete with the type 1 TNF receptor (TNFR1, p55) for the binding of TRAF2 (TNFR associated factor 2) thereby inhibiting TNF-mediated apoptosis. The cytoplasmic tail of IL-15Rα contains sequences homologous to the TRAF2 binding domains of CD30 and CD40. Binding of IL-15 to IL-15Rα leads to the binding of TRAF2 to the cytoplasmic domain of IL-15Rα (Bulfone-Paus, *et al.*, 1999).

Due to the wide tissue distribution of IL-15 mRNA expression, several biological functions have been described for IL-15 that do not directly affect cells of the immune system. The expression of IL-15Rα on vascular endothelial cells results in high affinity binding of IL-15 to these cells, which can not be observed for IL-2. In a murine system, IL-15 has been shown to promote angiogenesis *in vivo* and to induce tyrosine phosphorylation in endothelial cells (Angiolillo, *et al.*, 1997). As IL-15 is capable of binding to the type 2

IL-15R on mast cells and to induce mast cell proliferation *in vitro*, the stimulatory effect of IL-15 on the vascular system may be of some physiological importance. The expression of IL-15- and IL-15R-mRNA in murine brain microglia and human neural cell lines as well as the effect of IL-15 on NO production and growth *in vitro* may indicate a role for IL-15 in the central nervous system (Lee, *et al.*, 1996; Hanisch, *et al.*, 1997; Satoh, *et al.*, 1998). An anabolic effect of IL-15 on skeletal muscle has been described due to the finding that both IL-15 and its high affinity receptor are highly expressed by myocytes. IL-15 stimulates myogenic differentiation with low concentrations of IL-15 leading to fiber formation and MHC accumulation (Quinn, *et al.*, 1995 and 1997).

The biological activity of IL-15 has been further elucidated by the generation of mice lacking IL-15Rα using conventional gene targeting techniques (Lodolce, *et al.*, 1998). These mice appear healthy and breed normally. Despite the wide tissue distribution of IL-15Rα in normal mice, IL-15Rα$^{-/-}$ mice do not exhibit gross differences in the histology of brain, lung, liver, spleen, kidney or intestine. However, IL-15Rα$^{-/-}$ mice are markedly lymphopenic due to decreased proliferation and homing of lymphocytes to peripheral lymph nodes, thus leading to a reduction of peripheral lymph node size and cell content. This reduction in lymph node size, shown to vary from 30%-80% in IL-15Rα$^{-/-}$ mice, is not due to the total number of lymphocytes in IL-15Rα deficient mice, which is only reduced by 10%-15% when compared to wild type mice. Mutant mice lacking IL-15Rα have also been demonstrated to lack NK cells (CD3$^-$DX5$^+$ and CD3$^-$NK1.1$^+$) combined with a marked reduction in the number of intraepithelial lymphocytes, particularly γδT cells (5-10 fold) compared to wild type mice. Whereas the development of B cells in mice lacking IL-15Rα is not affected, mutant mice show a reduction of total lymphocyte numbers, particularly for the number of CD8$^+$ cells in thymus, spleen and periphery. A reduction in the number of CD8$^+$CD44^{hi} and CD8$^+$CD44^{int} population

indicates an important role for IL-15 in the development and function of normal $CD8^+$ and $CD8^-$ memory cells. Although IL-15 has been described as a cytokine with anti-apoptotic properties, mice lacking IL-15Rα show no signs of elevated apoptosis of lymphocytes.

Despite the functional similarities between IL-15 and IL-2 with respect to T cell regulation, the phenotype of IL-15Rα$^{-/-}$ mice is strikingly different from both IL-2 or IL-2 receptor deficient mice, which display severe lymphadenopathy, autoimmunity and premature mortality (Schorle, *et al.*, 1991; Sadlack, *et al.*, 1993). Since IL-15 and IL-2 both transduce signals through the common gamma chain, the functional differences between the two cytokines may not only be due to differential tissue distribution but also open the possibility of IL-15 signaling through the cytoplasmic tail of IL-15Rα. Furthermore, the intracellular trafficking of IL-15 due to the usage of different leader sequences and a possible role for IL-15Rα in intracellular signaling and nuclear translocation may be important in the complex regulation of cellular responses to IL-15 *in vivo*.

1.2.2 IL-15 in inflammation and autoimmunity

The complex regulatory mechanisms that influence IL-15 expression have led to the assumption that high levels of IL-15-mRNA present in monocytes in conjunction with low secretion of the mature IL-15-protein may indicate a pool of transcripts enabling rapid secretion of IL-15 following activating stimuli (reviewed by Waldmann and Tagaya, 1999). As mentioned earlier, various bacterial and viral stimuli including herpes-virus (Flamand, et al., 1996) and hepatitis C (Kakumu, et al., 1997), induce IL-15. Also, IL-15 has been shown to enhance immune functions against the human immunodeficiency virus (HIV) *in vitro* (Chehimi, *et al.*, 1997) and has been reported to act as a survival factor for $CD4^+$ and $CD8^+$ cells in HIV-infected individuals (Naora and Gougeon, 1999).

Furthermore, co-expression of IL-15 with an HIV-I DNA vaccine was reported to enhance cell-mediated immunity in BALB/c mice (Xin, *et al.*, 1999). Since the importance of IL-15 for the development of innate immune cells has been clearly demonstrated, these properties highlight the putative therapeutic potential of IL-15 for the treatment of infectious diseases.

However, the expression of IL-15 has also been associated with a number of chronic autoimmune disorders where the activation of T cells by dysregulated IL-15 expression leads to increased pathology. IL-15 has been linked with the development of T cell alveolitis and IFNγ production in the lung of patients with sarcoidosis (Agostini, *et al.*, 1996, 1999). In Ulcerative Colitis and Crohn's diseases, the number of IL-15 expressing monocytes is elevated (Kirman and Nielsen, 1996) and IL-15-activity is enhanced in patients with active inflammatory bowel disease (Sakai, *et al.*, 1998) and post-operational enterocolitis (Mayumi, *et al.*, 1999). Elevated levels of IL-15-mRNA have also been described in human autoimmune thyroid diseases (Ajjan, *et al.*, 1997) and renal allograft rejection (Pavlakis, *et al.*, 1996). Also, elevated serum levels of IL-15 were detected in Systemic Lupus Erythematosus (SLE) patients (Park, *et al.*, 1999).

1.2.3 IL-15 in human Rheumatoid Arthritis

Although the expression of IL-15-mRNA has been reported in a variety of different autoimmune disorders, comparatively little information on the biological role of IL-15 in these diseases is available so far. This however is not the case for Rheumatoid Arthritis (RA), where the effect of IL-15 on the pathology of the disease has been studied in greater detail.

RA is a common human autoimmune disease with a prevalence of approximately 1%, more often occurring in females than in males. The clinical syndrome is characterized by chronic inflammation within the synovial membrane accompanied by infiltration of blood derived T cells, macrophages and plasma cells. All of these cells show signs of activation leading to progressive, erosive destruction of cartilage and underlying bone. Apart from monocytes, T- and B cells, polymorphonuclear cells (especially neutrophils), mast cells, dendritic cells (DC) and fibroblast-like synoviocytes are found in the interstitium (Duke, *et al.*, 1982; Burmester, *et al.*, 1983; Kennedy, *et al.*, 1988; Cush and Lipsky, 1988; Thomas, *et al.*, 1994). The events that initiate and perpetuate RA remain poorly understood. Although RA resembles an ongoing immune response, the antigen initiating inflammatory responses in RA has not yet been characterized.

Cytokines and their receptors as well as high levels of adhesion molecules on endothelial cells and leukocytes are known to play a pivotal role in the pathogenesis of RA (Pitzalis, *et al.*, 1994) by regulating the recruitment and activation of inflammatory cells in the synovial membrane. Within the synovial membrane, several pro-inflammatory cytokines, particularly IL-1β, IL-6, GM-CSF, IL-8 and most importantly TNFα are upregulated, whereas T-cell derived cytokines such as IFNγ, IL-2 and IL-4 are only found at low levels (reviewed by Feldmann, *et al.*, 1996). It remains controversial if the pathology of RA is a macrophage- or T cell mediated process. Therapies that target T cells such as cyclosporin A or antibodies directed against T cell epitopes are clinically beneficial (Horneff, *et al.*, 1991 and 1993). Also, disease severity and prevalence are associated with certain HLA-DR subtypes (Gregersen, *et al.*, 1987). T cells found in the synovial membrane are mostly of the $CD45RB^{dim}CD45R0^{+}CD27^{-}$ phenotype representing a mature, memory T cell population (Thomas, *et al.*, 1992). New data demonstrate that the production of pro-inflammatory cytokines and of tissue-degrading enzymes in rheumatoid

synovitis is T cell dependent and that $CD4^+$ cells are primary regulators in RA. T cells either depleted from or adoptively transferred into NOD-SCID mice engrafted with rheumatoid synovial tissue and subsequent injection of anti-CD2 resulted in the elimination of 80-90% of tissue-infiltrating T cells. A marked decline in the production of IL-1β, TNFα and IL-15 mRNA as well as MMP-1 and MMP-2 could also be observed (Klimiuk, *et al.*, 1999). However so far, no consensus exists over identity of the principal regulatory cell representing an optimal therapeutic target for the treatment of RA.

In the relative absence of IL-2, which is usually found only at mRNA levels and is not produced by synovial T cells in response to exogenous stimuli (Combe, *et al.*, 1985), cytokines that mediate the activation and proliferation of synovial T cells may be of high importance in mediating disease pathology. The biological properties of IL-15 as a macrophage-derived activator of T cells and a potent chemoattractant makes IL-15 an obvious candidate for T cell activation in RA. Interestingly, IL-15 was found in the synovial fluid of approximately 50% of patients with RA and correlated with the presence of TNFα. Immunohistochemical localization of IL-15 identified $CD68^+$ macrophages in the synovial lining layer as well as synoviocytes as the likely source of IL-15 in RA. The chemotactic activity of synovial fluid for peripheral blood lymphocytes could be attributed partially to the presence of IL-15. The responsiveness of T cells to IL-15 could be confirmed by injecting recombinant IL-15 into footpads of DBA/1 mice primed with *Corynebacterium parvum* or type II collagen. This treatment induced swelling and T cell accumulation. Furthermore, PBMC from RA patients responded more vigorously to IL-15 than PBMC from healthy controls (McInnes, *et al.*, 1996; Thurkow, *et al.*, 1997; Harada, *et al.*, 1999). IL-15-activated blood-derived and synovial T cells are able to induce TNFα production from synovium-derived macrophages and macrophage cell lines. For IL-15 to induce TNFα, synovial T cells and macrophages required cell contact, which was mediated

in part by LFA1-ICAM1 and CD69. The ability of synovial T cells to induce TNFα production by macrophages depends on the presence of IL-15. Freshly isolated synovial T cells of the $CD45R0^+$ memory type and IL-15-activated peripheral blood T cells both induce TNFα production. In the absence of IL-15, these cells lose their ability to stimulate TNFα production within 16 hours of culture most likely due to the loss of adhesion molecules such as CD69, which can be upregulated by IL-15 on peripheral blood T cells (McInnes, *et al.*, 1997).

TNFα, mainly produced by macrophages, appears to occupy a pivotal position in the regulation of synovial inflammation (reviewed by Feldmann, *et al.* 1996) by upregulating other pro-inflammatory cytokines. The importance of TNFα as a therapeutic target in RA has also been demonstrated (Elliott, *et al.*, 1993 and 1994). The recruitment and expansion of memory T cells in the synovium by IL-15 may lead to a positive feedback loop, where macrophage or fibroblast-derived IL-15 induces T cell recruitment and TNFα production by synovial macrophages through cell contact. Apart from its central ability to induce TNFα production, IL-15 has also been shown to play a role in the pathological destruction of bone by stimulating osteoclast formation independently from TNFα (Ogata, *et al.*, 1999). Recent reports indicate that IL-15 may work synergistically with IL-18, which is also present in the synovial membrane, in upregulating both TNFα and IFNγ (Gracie, *et al.*, 1999) providing a further possible activation loop in the pathogenesis of RA. Further stimulatory effects of IL-15 on B cell antibody secretion and neutrophils may result in a cytokine-mediated, non-specific activation of polyclonal T cells exacerbating disease pathology in an antigen independent manner. The identification of IL-15 as a monocyte-derived T cell activator, which is found in the synovial membrane of patients with RA and may operate upstream from the effects of TNFα may provide a useful target for therapeutic interventions. One part of this project was designed to

elucidate the therapeutic potential of antagonizing IL-15 in a murine model for RA, Collagen-Induced Arthritis.

1.3 Murine Collagen-Induced Arthritis (CIA) as a model for RA

The establishment of arthritic disease following the immunization with type II collagen has been described in rats, mice and primates (Trentham, *et al.*, 1977; Courtenay, *et al.*, 1980; Yoo, *et al.*, 1988). Murine CIA in DBA/1 mice has widely been regarded as a valuable model for the inflammatory processes observed in RA. Following immunization of mice with type II collagen in CFA, an accumulation of macrophages and CD4+ cells can be observed (Holmdahl, *et al.*, 1988). This stage of accumulation and activation is followed by swelling and infiltration of inflammatory cells into the joint accompanied by pannus formation and erosion of cartilage and bone. The acute phase of CIA resolves after 8-10 days showing reduced inflammatory activity accompanied by fibrous and bony ankylosis (Caulfield, *et al.*, 1982). The susceptibility of DBA/1 mice to CIA is associated with a number of gene loci, most likely genes within the MHC, especially H-2^q and H-2^r. Outside the MHC, the Mcia2 locus located on chromosome 3 was identified as an important genetic factor for susceptibility to CIA (Jirholt, *et al.*, 1998). Although heterologous collagen is used for CIA induction, the development of autoreactive T cells appears to be essential for disease onset (Holmdahl, *et al.*, 1988). However, the development of arthritic lesions following immunization with type II collagen has also been reported in mice lacking both mature T and B cells, suggesting a lymphocyte independent mechanism of disease onset (Plows, *et al.*, 1999). In terms of lymphocyte involvement, pathogenesis and cytokine production, CIA closely resembles human RA.

Although CIA is widely accepted as an experimental model for RA, there are not only similarities but also marked differences between the two diseases. Whereas the inflammatory infiltration in CIA is mainly composed of polymorphonuclear leukocytes (Caulfield, *et al.*, 1982), the RA-infiltrate is dominated by mononuclear cells (Janossy, *et al.*, 1982). A further difference between CIA and RA lies in the prolonged, often fluctuating inflammatory activity of RA, which is not observed in CIA. A number of studies aiming to develop therapeutic strategies have led to the identification of important factors in the pathogenesis of CIA including pro-inflammatory cytokines. The table below summarizes some of the major immunologic approaches as well as some alternative treatments leading to the suppression of CIA by either blocking specific stimulatory pathways or by providing a general anti-inflammatory effect.

Table 1.1 Immunotherapy of CIA

Form of treatment	**Reference**	**Comments**
	Antibodies and recombinant proteins	
Anti-CD4	Ranges *et al.*, 1985 Williams *et al.*, 1994	Only effective when administered at early stage.
Anti-TCRα/β	Yoshino *et al.*, 1991	Variable results in rats and mice.
Anti CD40L	Durie *et al.*, 1993	Suggests the involvement of B cells in CIA.
Anti -IL-6R	Takagi *et al.*, 1998	Single injection ameliorates CIA.
Treatments Antagonizing TNFα	reviewed by Feldmann *et al.*, 1996	Very efficient blockage of CIA, also used in human RA.

Recombinant IL-4 treatment	Horsfall *et al.*, 1997	Requires continuous administration.
Recombinant IL-1R antagonist	Wooley *et al.*, 1993	
anti-IL-1αβ	Van den Berg *et al.*, 1994 Joosten *et al.*, 1996 Joosten *et al.*, 1999	IL-1 is particularly important for cartilage- and bone-destruction.
anti-adhesion-molecule treatment	Kakimoto *et al.*, 1992	
Systemic IFNγ injection	Williams *et al.*, 1993 Vermeire *et al.*, 1997 Kageyama *et al.*, 1998	Moderately beneficial. Anti-IFNR-deficiency exacerbates or inhibits CIA depending on genetic background. Anti-IFNγ-antibody-treatment exacerbates CIA.
anti-Mac-1 (integrinβ_2, CD11b/CD18)	Taylor *et al.*, 1996	Inhibits spleen cell transfer induced CIA into SCID mice.
IL-11	Walmsley, *et al.*, 1998	Reduced clinical severity.
	Studies with knockout and transgenic animals	
B cell deficiency	Svensson *et al.*, 1998	In contrast to Plows *et al* (1999), suggesting that CIA can be induced in mice lacking T and B cells.
GM-CSF deficiency	Campbell *et al.*, 1998	Confirms essential role of GM-CSF, which could not be shown by ab-treatment.
IL-12 deficiency	McIntyre, *et al.*, 1996	Reduced incidence and severity.

IL-6 deficiency	Alonzi *et al.*, 1998 Sasai *et al.*, 1999	Essential role for IL-6 in CIA. Delayed onset in KO mice
TNFα-overexpression	Keffer *et al.*, 1991	Mice develop arthritis without induction.
CD28 deficiency	Tada *et al.*, 1999	Resistance to CIA induction.

Other techniques

IL-10 expression by viral transfer	Ma, *et al.*, 1998 Apparailly, *et al.*, 1998 Whalen, *et al.*, 1999	Viral IL-10 lacks immunostimulatory properties and may be particularly useful for disease suppression.
IL-13	Bessis, *et al.*, 1996	Transplantation of transfected fibroblasts
TNFR	Le, *et al.*, 1997	Adenoviral transfer
IL-1R antagonist secretion	Bakker, *et al.*, 1997	Transplantation of transfected fibroblasts
oral or nasal administration of type II collagen	Trentham, *et al.*, 1993 Garcia, *et al.*, 1999	Tolerization

1.4 Conventional and conditional gene targeting in mice

1.4.1 Historical Perspective

Although the mouse has been well established as a laboratory model since the beginning of the 20th century, techniques allowing the active manipulation of the murine genome have only been developed over the last 20 years. The first report of a direct introduction of foreign DNA into the murine embryo dates from 1974, when Rudolf Jaenisch and Beatrice Mintz found that the injection of purified SV40 DNA into the blastocoel cavity of mouse blastocysts led to the detection of viral DNA in many of the resulting animals. Furthermore, it was demonstrated that the infection of pre-implantation embryos by the Moloney murine leukemia virus resulted in stable germ line transmission of the virus (Jaenisch, 1976).

Studies aiming to elucidate the developmental potential of different parts of the murine post-implantational embryo showed that the early embryonic ectoderm contains cells capable of contributing to all three germ layers of the fetus. These studies were further complemented by the use of transplantable teratocarcinoma cells (Stevens, 1970). Teratocarcinomas consist of a mixture of different cell types derived from gonadal tumors that originate from a population of undifferentiated stem cells known as embryonal carcinoma cells (EC cells). Cells derived from these tumors were shown to be able to contribute to many normal adult tissues when integrated into the blastocyst (Brinster, 1974). Studies conducted on teratocarcinoma cells also led to an advancement of culture techniques enabling *in vitro* experimentation (Skreb and Crnek, 1980). Experiments suggesting that microinjection of DNA into a one-cell embryo might allow the introduction of cloned genes into developing embryos were first conducted in 1980. A cloned HSV-tk gene injected into cultured fibroblasts led to stable integration and expression of the cloned gene in 5-20% of recipient cells (Capecchi, 1980).

The possibilities enabling gene manipulation were revolutionized when blastocyst-derived embryonic stem cell lines (ES cells) were described independently by Gail Martin in San Francisco (Martin, 1981) and Martin Evans and Matt Kaufman in Cambridge (UK) (Evans and Kaufman, 1981). ES cells were demonstrated to be capable of contributing to many tissue types of the adult mouse including the germ line when injected into the blastocyst (Bradley, *et al.* 1984). The first manipulation of a gene and subsequent germ line transmission was demonstrated for the X-chromosome-linked gene encoding *hypoxanthine phosphoribosyl transferase (Hprt)* (Kuehn, *et al.* 1987). Mutant ES cells made *Hprt*-deficient by retroviral infection were selected and used to generated *Hprt*-deficient male mice. Because of differences in purine metabolism, these mice did not show the expected phenotype resembling the Lesch-Nyhan disease caused by *Hprt*-deficiency in humans. However, these experiments showed for the first time that active mutation of a gene and introduction into the mouse embryo could directly result in mice carrying the desired mutation. Gene targeting using homologous recombination was first achieved for *Hprt* (Thomas and Capecchi, 1987) and c-abl (Schwartzberg, *et al*, 1989). Since then, many mouse models have been generated using gene targeting through homologous recombination both to elucidate the function of newly described genes as well as to establish models for human diseases associated with the specific lack of gene function.

1.4.2. Gene targeting through Homologous Recombination

The introduction of a DNA fragment into a mammalian cell can result in the integration of the foreign DNA fragment into the mammalian genome. Instead of integrating randomly as described for the generation of transgenic animals, DNA fragments can recombine with endogenous homologous sequences contained within the genome. Remarkably, despite the extensive use of homologous recombination in gene

targeting, the molecular mechanism underlying recombination events between foreign DNA fragments and endogenous sequences as well as cofactors mediating recombination events remain largely unknown. A positive correlation is known to exist between the frequency at which homologous recombination events occur and the length of homologous sequences contained within the foreign DNA fragment to be integrated into the mammalian genome (Thomas and Capecchi, 1987). However, the amount of non-homologous DNA contained within a targeting construct was not shown to affect the frequency of targeting events (Mansour, *et al.* 1990), nor did the copy number of foreign DNA fragments introduced into the target cell (Thomas and Capecchi, 1986). Homologous recombination was shown to be more frequent in gene loci that are transcriptionally active during transfection time (Nickoloff, et al. 1990).

Since the first demonstration of homologous recombination in a fibroblast cell line (Lin, *et al.*, 1985), the technique based on this phenomenon has been used extensively for gene targeting and has provided major insights into the function of gene products *in vivo* in a variety of disciplines.

1.4.3 Construction of Targeting Vectors

The frequency of gene targeting through homologous recombination in mammalian cells is relatively low. In most cases transfected DNA integrates into a random chromosomal site. The design of the targeting vector is therefore crucial to optimize the chances of a successful gene targeting experiment. A targeting vector is designed to recombine with and mutate a specific chromosomal locus. All targeting vectors therefore consist of sequences homologous to the desired chromosomal integration site and a plasmid backbone as minimal components. The use of isogenic DNA (gDNA originating from the same mouse strain as the ES cells) to construct the targeting vector can result in

20-fold increase of the targeting frequency (te Riele, *et al.*, 1992). The length of the homologous sequences equally affects the efficiency of the targeting experiment. In general, the length of homology should be in the range of 5-8 kb. However gene targeting has successfully been achieved with targeting vectors containing a minimum homology of only 1kb (Hasty, *et al.*, 1991).

To improve the efficiency of targeting experiments, positive and negative selection markers are included in targeting constructs. Positive selection markers primarily serve the purpose of selecting for cells that have integrated the targeting vector after transfection. Cloned into a coding exon of the gene to be targeted, a positive selection marker may also serve as a mutagen. In general, targeting vectors can be classified as either replacement or insertion vectors. Most gene targeting experiments have been conducted using the replacement type vector in which the homologous targeting construct replaces the chromosomal sequence through a double crossover event. A selectable marker, usually neomycin resistance and occasionally hygromycin resistance is placed between two homologous regions replacing crucial coding exon sequence of the targeted gene. Insertion vectors in contrast, integrate via a single crossover event generating a duplication of the target homology separated by the vector backbone (see Figure below). After a successful recombination of the insertion vector with the homologous target sequence, spontaneous intrachromosomal recombination events may occur between the duplicate homologous sequences. This may result in the removal of the plasmid backbone and one complement of the duplication from the target locus. Insertion vectors also have the advantage of elevating the gene targeting frequency by up to 20 fold (Hasty, *et al.*, 1991).

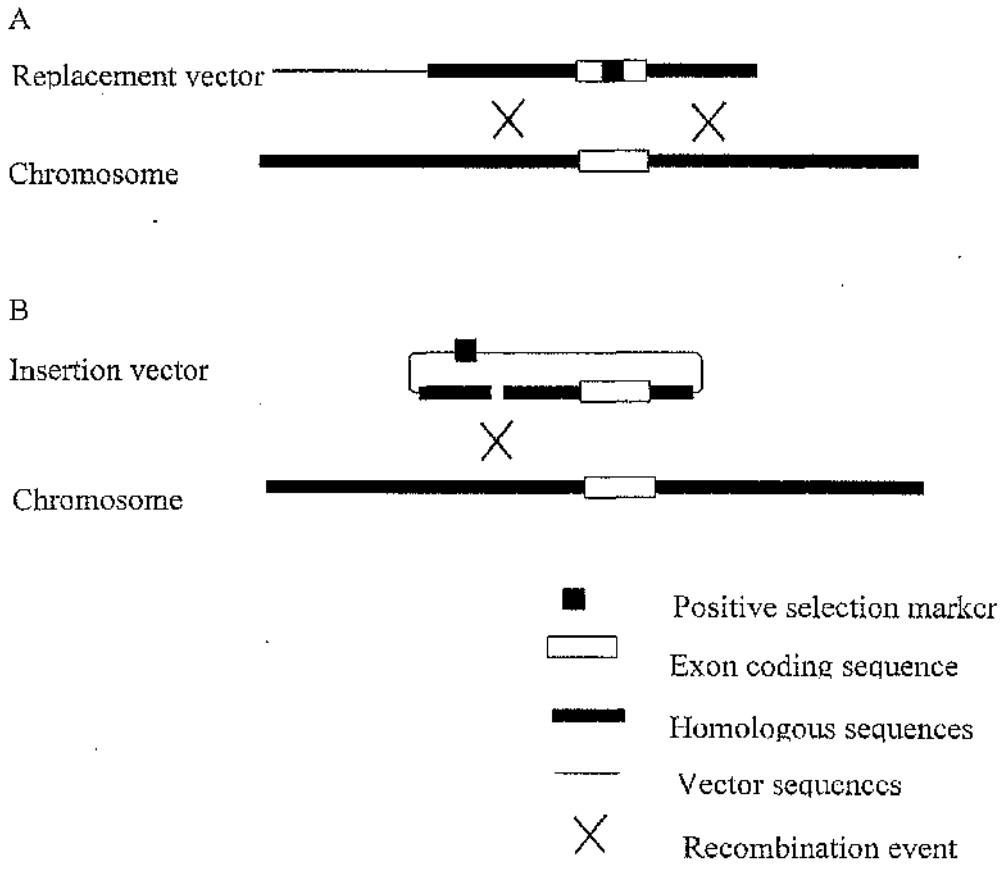


Figure 1.3 Gene targeting vectors can generally be classified as either replacement (A) or insertion (B) vectors. Replacement vectors integrate into the genome via two crossover events and usually contain a positive selectable marker interrupting the target homology and replacing coding sequences of the target allele. Insertion vectors integrate via a single crossover event and result in the duplication of the target homology separated by the vector backbone. Whereas replacement vectors are generally linearized outside the target homology prior to transfection, insertion vectors are lineralized inside the target homology.

Disruption of the coding sequence through replacement with the positive selection marker will in most cases ablate a gene's function. In some cases however, truncated proteins may still be generated from the recombinant allele leading to some biological activity. Null mutations are therefore more likely to occur by recombining the positive selection marker into an upstream coding exon rather than a downstream exon so as to minimize the chances of a functional truncated gene product.

1.4.4 Screening for Targeting Events

A targeting construct can be introduced into the cell by either electroporation or microinjection, electroporation being the simpler method and more widely used. Following transfection of ES cells with the linearized targeting construct, the addition of the respective antibiotic to the culture medium selects for ES cells that have integrated the targeting construct into their genome. Positive selection markers generally confer resistance to an antibiotic, typically neomycin (or G418 as a derivative, respectively). However, positive selection markers do not discriminate between homologous recombination and random integration events of the targeting construct into the genome. In standard targeting experiments with replacement vectors, the vector may also contain a negatively selectable gene at one or both ends of the homologous sequences. In cases where the targeting construct is integrated into the genome by homologous recombination, the negatively selectable marker will typically be lost, whereas random integration will incorporate the negative selection marker. Accordingly, selection against this marker will kill most cells, which have integrated the targeting vector at a random location while correctly targeted cells will survive. A selection marker typically used for negative selection includes the gene encoding *herpes simplex thymidine kinase* (HSV-tk). The nucleoside analog *gancylovir* (1-[2'-deoxy-2'-fluoro-b-D-arabinofuranosyl]-5-iodouracil)

is converted by the HSV-tk gene into a cytotoxic derivative (Mansour, *et al.*, 1988). The enrichment of targeting events achieved by negative selection may reach up to 20 fold.

Following positive and negative selection of transfected ES cells and their culture to obtain ES cell colonies, surviving colonies are clonally isolated and expanded for further analysis of gDNA derived from each ES cell colony obtained. For the verification of targeting events by homologous recombination either screening by Southern blot analysis or PCR can be employed. PCR is a sensitive and fast screening method, which can be used for a large number of clones. However homologous recombination events are normally confirmed by Southern blot. An external probe is used in Southern blot screenings, which is generated from flanking sequences of the homologous region. This external probe therefore does not hybridize with the targeting construct, so that random integration is not detected. Both external probes from 5' and 3' ends are normally used to confirm the gene replacement event.

1.4.5 Embryonic Stem (ES) cells

Together with the discovery that homologous DNA fragments can be inserted into the genome by homologous recombination, the isolation and culture of murine embryonic stem cells has been the major contribution enabling gene-targeting experiments. Homologous recombination allows the generation of any desired mutation within a cloned genomic sequence and in any cell line growing in culture. Prior to the availability of ES cells, gene-targeting approaches were limited by the lack of a suitable *in vitro* system to transfer artificial mutations into the germ line. The isolation and culture conditions to maintain ES cells were first described by Evans and Kaufman (1981) and Martin (1981). ES cells are derived from the inner cell mass of mouse blastocysts. Under stringent culture conditions, these cells can maintain their embryonic developmental potential even after

many passages and after the introduction of a genetic alteration. This way, mutations introduced into ES cells by homologous recombination can be transmitted into the germ line to study the effect of targeted gene products *in vivo*. ES cells can be maintained *in vitro* by culturing in medium supplemented with a soluble factor called leukemia inhibitory factor (LIF) or differentiation inhibiting activity (DIA) (Smith, *et al.*, 1988), or by co-culturing with mitotically inactivated feeder cells, such as embryonic fibroblasts or the fibroblastic cell line STO. Since the first isolation of ES cells, several different permanent ES cell lines have been established and selected for by their ability to transmit genetic alterations into the germ line. Since the isolation of ES cells was a direct extension of previous work on stem cell teratocarcinomas, a germ cell tumor most prevalent in 129/Sc mice, some of the first ES cell lines were isolated from 129/Sv. Since the XY karyotype of ES cells appears to be more stable in culture than the XX karyotype, ES cell line are derived from male mice resulting in a mostly male chimeric offspring after germ line transmission.

1.4.6 The production of chimeric mice

In most cases, only one allele of a gene is targeted by homologous recombination in ES cells. Targeted ES cells are returned to the embryonic environment by injection into a host blastocyst. ES cells can contribute to many kinds of tissues in the resulting chimeras including sperm in the mature animal. This process is called germ-line transmission and can be easily detected in the offspring's coat color. The ES cells are derived from a mouse homozygous for the agouti coat color (129/Sv) allele whereas the recipient blastocyst originates from a black coat color mouse (C57 Bl6). The fur of the resulting chimeras has patches of both colors because the mouse contains cells derived from both genotypes. Breeding of the chimeras to an MF1 albino mouse results in some chinchilla coat color mice among the litter, indicating that the ES cells contributed to the formation of the germ

line. By mating germ-line transmission chimeras with wild type mice, heterozygous offspring can be generated that carry the mutation in one of the two copies of the gene in every cell. These mice are in most instances unaffected by the mutation, because their second, undamaged copy of the gene will still be functional. To identify heterozygous and wild type mice in this stage, DNA from tail tipping is examined by Southern blot or PCR. Mating heterozygous siblings results in offspring having two defective alleles. This process follows Mendelian rules of inheritance. Northern blot, RT-PCR and Western blot can be employed to examine if the targeted gene and the protein it encodes are completely deficient in mice homozygous for the mutation introduced.

1.4.7 Conditional gene targeting using the Cre/lox system

New strategies have been employed recently that allow the *in vivo* manipulation of DNA in ES cells or living animals. These techniques are based on the use of site-specific recombinases interacting with specific sequences ranging from 25 to 150bp in length. The Cre/lox system consists of two components. Cre (*cyclization recombination*) is a 38kD product of bacteriophage P1 that interacts with a 34bp sequence called loxP (*locus of X-over P1*). The loxP site consists of two 13bp inverted repeats flanking an 8bp core sequence. This core sequence is non-palindromic and gives loxP sites directionality. Cre mediated recombination requires four Cre-monomers each of them binding to one of the 13bp inverted repeat regions of the loxP sites. The resulting recombination event between two loxP sites results in the excision of the DNA between them as a covalently closed circle leaving a single loxP site behind. No additional co-factors or topological requirements of the target DNA are needed, which makes the Cre/lox system particularly suitable for targeting experiments in mammalian cells (reviewed by Sauer, 1998).

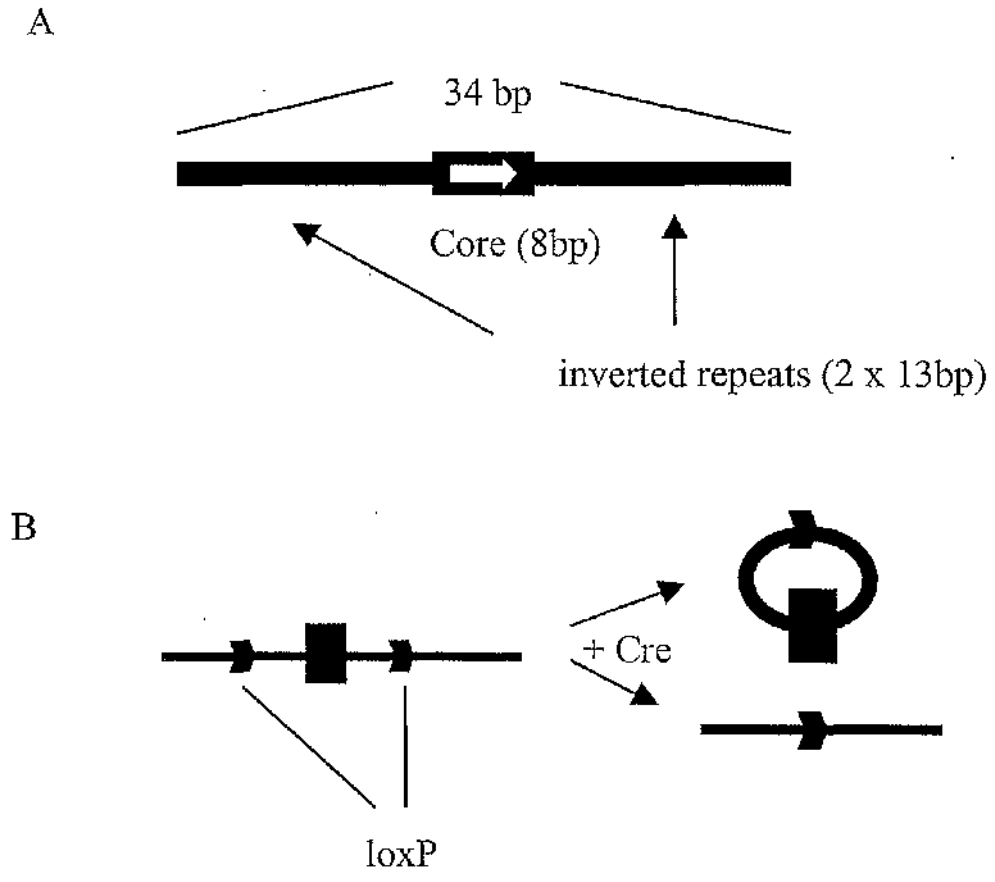


Figure 1.4 Schematic representation of a loxP site consisting of two 13bp inverted repeats flanking a non-palindromic core sequence (A). Cre-mediated recombination between two equally directed loxP sites results in the excision of the DNA between them as a covalently closed circle (B).

a. Tissue specific mutagenesis

The basic strategy used to create tissue specific knockout mice uses conventional gene targeting techniques and homologous recombination to flank crucial parts of one allele of the target gene with loxP sites (Orban, *et al.*, 1992). Modified ES cells are used to generate mice that carry the desired mutation after germ line transmission and homozygous animals are obtained through breeding. Homozygous mice carrying loxP flanked sequences on both alleles of the target gene usually produce normal levels of functional protein as loxP sites can be positioned within intron sequences. Deletion of the gene occurs when mice containing the modified gene are crossed with mice expressing Cre in the desired target tissue under a tissue specific promoter. Cre mediated excision in double transgenic animals then results in tissue specific gene ablation. This approach was first used successfully to generate a T cell specific deficiency for the gene encoding DNA polymerase β (Gu, *et al.*, 1994). To generate T cell specific deletions, Cre recombinase was expressed under the $p56^{lck}$ promoter. No apparent deletion of loxP site flanked sequences was observed in kidney, liver or B lymphocytes. Mating of Cre-transgenic mice with homozygous animals containing the mutated DNA polymerase β gene resulted in the inactivation of the target gene within the T cell population only.

As an alternative to tissue specific expression of Cre recombinase, Cre-transgenic mice have been generated that express Cre in all tissues beginning from the earliest developmental stages. These 'deleter' strains express Cre under constitutive promoters such as the hCMV- (Schwenk, *et al.*, 1995) or adenoviral *EIIa* promoter (Lakso, *et al.*, 1996). These mouse strains are useful tools to derive deletion mutants from chimeric mice carrying a loxP flanked target in their genome and result in offspring with a *null* allele with a similar genotype as conventionally made knockout mice.

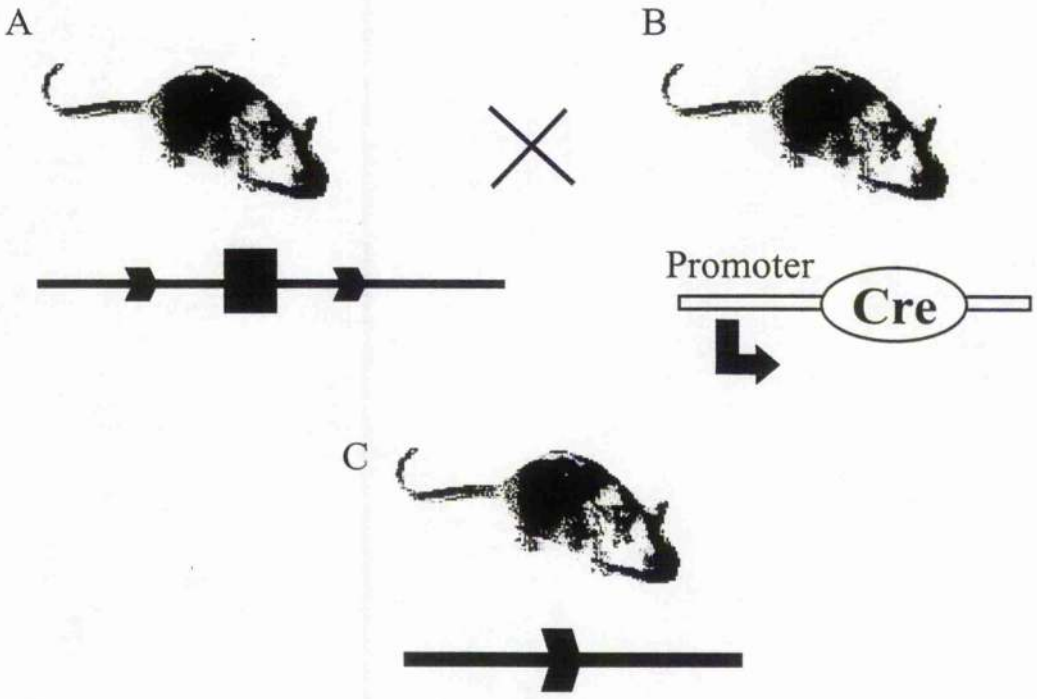


Figure 1.5 Generation of tissue specific knockout mice using the Cre/lox system. Targeting of a gene in ES cells is used to generate mice with loxP sites flanking crucial exons of the target gene (A). A second mouse is mated to these modified animals that is transgenic for Cre recombinase and expresses Cre under a suitable promoter (B). The double transgenic offspring lacks loxP site flanked sequences due to Cre mediated recombination (C).

In bacteriophage P1, Cre recombinase provides a backup mechanism for the cyclization of P1 DNA after infection (Segev and Cohen, 1981). Cre also enhances P1 plasmid stability in bacterial lysogens (Austin, *et al.*, 1981). Although Cre-mediated recombination is very effective, tissue specific mutagenesis of the DNA-polymerase β gene as performed by Gu, *et al.* (1994) resulted in the deletion of the target gene in only 40% of the T cell population when homozygous mice were crossed with transgenic mice expressing Cre under the $p56^{lck}$ promoter. However, in these experiments, wild type Cre recombinase from bacteriophage P1 had been used that is only partially functional in eukaryotic cells. The performance of Cre in eukaryotic cells can be improved by introducing the so-called 'Kozak'-alteration (Gu, *et al.*, 1993) that allows efficient translation in mammalian cells. Thus improved expression of Cre in mammalian cells has been used to generate GalNAc-T- (*UDP-N-acetylgalactosamine-polypeptide-N-acetylgalactosaminyltransferase*) deficient mice lacking GalNAc-T in 100% of the thymocyte population. GalNAc-T catalyzes the transfer of the monosaccharide acetylglucosamine to serine and threonine residues, thereby initiating O-linked oligosaccharide biosynthesis (Hennet, *et al.*, 1995).

b. Marker eviction

As in conventional gene targeting technology, the generation of the desired mutation in ES cells also results in the integration of the positive selection marker gene into the targeted locus. However, the *Neo* gene itself can adversely affect gene expression of neighboring genes (Artelt, *et al.*, 1991) particularly, if the targeted locus is located within a gene cluster. The Cre/lox system offers the possibility of avoiding this potential problem by enabling the removal of the selection marker gene from the targeted locus *in vitro*. Marker eviction can be achieved by including a third directly repeated loxP site in

the original targeting construct. Thus, one loxP-flanked interval is designed for conditional mutagenesis and the other flanked interval is the selectable marker gene itself. Transient expression of Cre in targeted ES cells can lead to partial excision of loxP flanked sequences resulting in the removal of the selectable marker gene only. Complete Cre-mediated recombination would remove both the selectable marker gene and the targeted loxP flanked genomic DNA segment. Cre mediated recombination of a targeted locus containing three loxP sites potentially results in three different mutations. Apart from the excision of either the selectable marker gene alone or both loxP-flanked segments, a third possible recombination event would result in the removal of the target genomic DNA segment leaving the selectable marker gene. For the generation of conditional knockout mice, the removal of the selectable marker gene would be ideal, since Cre-mediated recombination would automatically result in the deletion of the target gene. ES cell clones generated by allowing a complete deletion of loxP-flanked sequences *in vitro* can be used to generate animals carrying a *null* allele of the target gene.

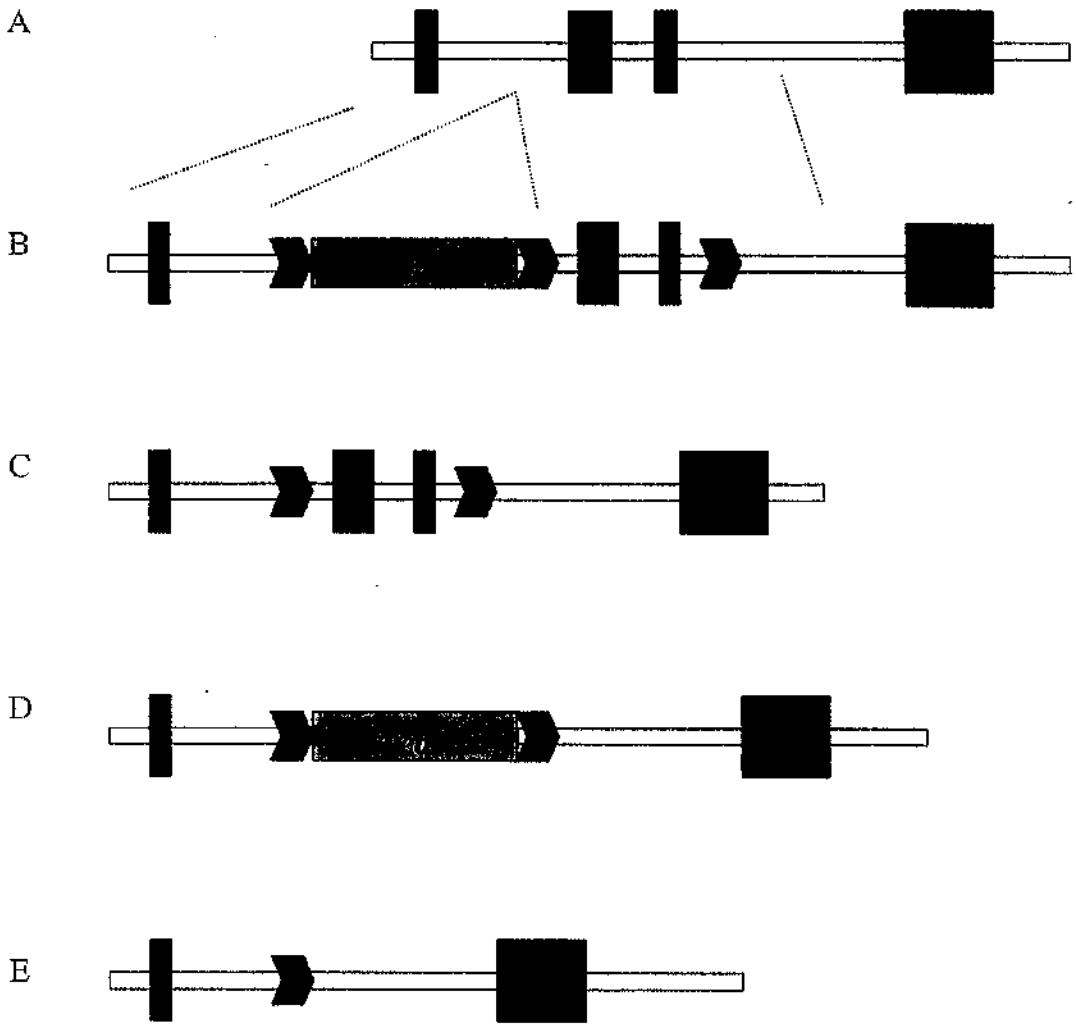


Figure 1.6 Schematic representation of a targeting vector strategy using the Cre/lox system. Homologous recombination of the lineralized vector with the target sequence (A) results in the replacement of the naïve sequence by the targeting construct (B). The construct contains a positive selection marker gene (*Neo*), which itself is flanked by loxP sites. Cre mediated recombination events may result in 3 different genotypes depending on the loxP sites used in the recombination event. (C) Deletion of the selectable marker gene only, (D) deletion of the target sequence not affecting the selectable marker gene or (E) total excision of all loxP flanked sequences.

The efficiency of DNA transfection *in vitro*, which may be below 5% of electroporated ES cells may present a major problem in generating Cre-mediated mutations in targeted ES clones. Since the desired mutation results in the deletion of the selectable marker gene other means of selecting for transfected cells have to be employed. The antibiotic puromycin is capable of killing non-resistant ES cells within 48 hours and can therefore be employed to select for transient transfection events (Taniguchi, *et al.*, 1998). Gu, *et al* (1994) have demonstrated that resistance of transfected cells to gancyclovir can be used to select for Cre-mediated recombination events if the HSV-tk gene is placed within the same gene segment as the positive selection marker cassette. Cre catalyzes recombination at chromosomal loxP sites in 80-100% of cells transiently transfected with the Cre-expression plasmid (Gagneten, *et al.*, 1997). Depending on the promoter used to control Cre expression the generation of partially deleted loxP flanked gene segments may therefore impose difficulties.

c. Inducible mutagenesis

The extent, tissue specificity and time of Cre-mediated recombination in mice that are both Cre-transgenic and contain loxP site flanked target sequences depend on Cre expression. Apart from the possibility of expressing Cre under a tissue specific promoter (e.g. $p56^{lck}$ for T cell-, CD19 for B cell expression), several other designs allow the inducible deletion of target sequences in the adult, double transgenic animal. The temporal activity of Cre can be regulated both at the transcriptional and post-translational level. Cre transcription can be induced by the injection of IFNα/β into mice that express Cre under the control of the Mx promoter. The Mx-gene product is part of the anti-viral defense mechanisms and can be induced by interferon. Administration of IFNα/β or double

stranded RNA (which induces IFNα/β) to double transgenic mice also carrying the previously described loxP-flanked DNA-polymerase β gene (Gu, *et al.*, 1994) resulted in near total deletion of target sequences of liver and spleen lymphocytes (Kuhn, *et al.*, 1995).

Transcriptional control of Cre-recombinase expression can also be achieved by regulating Cre expression via a minimal promoter, which in turn is regulated by transactivating elements. The DNA binding activity of transactivating elements is regulated by the presence or absence of a specific drug, e.g. tetracycline (Gossen, *et al.*, 1995; St-Onge, *et al.*, 1996; Utomo *et al.*, 1999) or ecdysone (No, *et al.*, 1996). Tissue specific expression of transactivating elements under suitable promoters allows the expression of Cre recombinase to be both inducible and tissue specific.

Cre expression can be controlled post-transcriptionally by engineering a fusion protein of the recombinase and the ligand-binding domain of a steroid receptor. In the absence of hormone, Cre recombinase is kept inactive by the binding of heat shock proteins. The addition of hormone results in a sterical change within the ligand-binding domain leading to the release of the heat shock proteins and Cre-activation. To avoid the responsiveness of the system to natural steroids in mice, mutant ligand-binding domains of the estrogen (Zhang, *et al.*, 1996) and progesterone (Kellendonk, *et al.*, 1996) were used. These mutants are unresponsive to their natural ligands but can be activated by synthetic hormone antagonists, e.g. 4-hydroxy-tamoxifen for the mutant estrogen receptor (Feil, *et al.*, 1996).

d. Detection of Cre expression

The success of Cre-mediated recombination events is crucially dependent on the pattern of Cre expression in the transgenic animal. Mosaic expression of Cre in the desired target tissue would result in incomplete gene ablation, whereas unexpected expression of Cre in extraneous tissues would lead to deletion of loxP-flanked sequences in tissues other than the desired cell type. However, mosaic expression is not an uncommon feature in transgenic animals that have been generated by random integration. Several methods have been described that allow the detection of Cre-expression. The existence of both polyclonal and monoclonal antibodies to Cre enables Western blot and immunohistochemical analysis of Cre-expression (Sauer, 1987; Schwenk, *et al.*, 1997). Alternatively, Cre expression can be identified by the expression of a reporter gene. A synthetic STOP sequence flanked by loxP sites was placed in front of the lacZ gene to generate reporter mice. Cre mediated recombination results in the excision of the STOP sequence and subsequent expression of the reporter gene. These mice were then used to identify Cre-transgenic mice with desired expression of Cre in a defined region of the hippocampus (Tsien, *et al.*, 1996).

More recently the generation of a double-reporter mouse has been described that provides a means of monitoring Cre-function both prior to and after its induction. Before Cre-mediated recombination, all cells of the transgenic animal express the lacZ gene. Cre expression leads to the excision of the lacZ gene and activates the transcription of the gene encoding human alkaline-phosphatase, which serves as a second reporter gene (Lobe, *et al.*, 1999). In view of the growing list of Cre-transgenic animals designed to carry out specific genomic alterations these methods greatly contribute to the ongoing research to further develop the Cre/lox system.

e. Further applications of the Cre/lox system

The Cre/lox system has been used for applications other than the generation of tissue specific and inducible knockout mice. The generation of transgenic mice that express a potentially toxic gene product has been facilitated by the generation of dormant transgenes that can be activated after the establishment of the transgenic animal. To inhibit the expression of a potentially toxic gene product, a synthetic STOP sequence derived from SV40 is placed between the transgene and its promoter. The STOP sequence is itself flanked by loxP sites. Cre mediated recombination results in the excision of the STOP sequence and expression of the transgene (Lakso, *et al.*, 1992).

A further application of the Cre/lox system has been demonstrated for the generation of knock-in vectors. The strategy is generally applicable to generate knock-ins at any locus in ES cells providing a useful tool for precise molecular engineering of the mouse genome. Homologous recombination is used to place a loxP site flanked selection marker cassette immediately 5' to the position of the natural ATG start codon for the coding region of the target locus. The flanking loxP sites have been altered in their central spacer region and cannot recombine with each other. However, each of the mutated loxP sites can efficiently recombine with other loxP sites, that have been altered in an identical manner (Hoess, *et al.*, 1986). A circular 'knock-in' vector is then targeted to the modified locus of ES cells by introduction of a gene of interest flanked by mutated loxP sites along with a Cre transient expression vector. Double crossover recombination mediated by Cre replaces the selectable marker gene with the knock-in gene under the control of the promoter at the target locus (Soukharev, *et al.*, 1999).

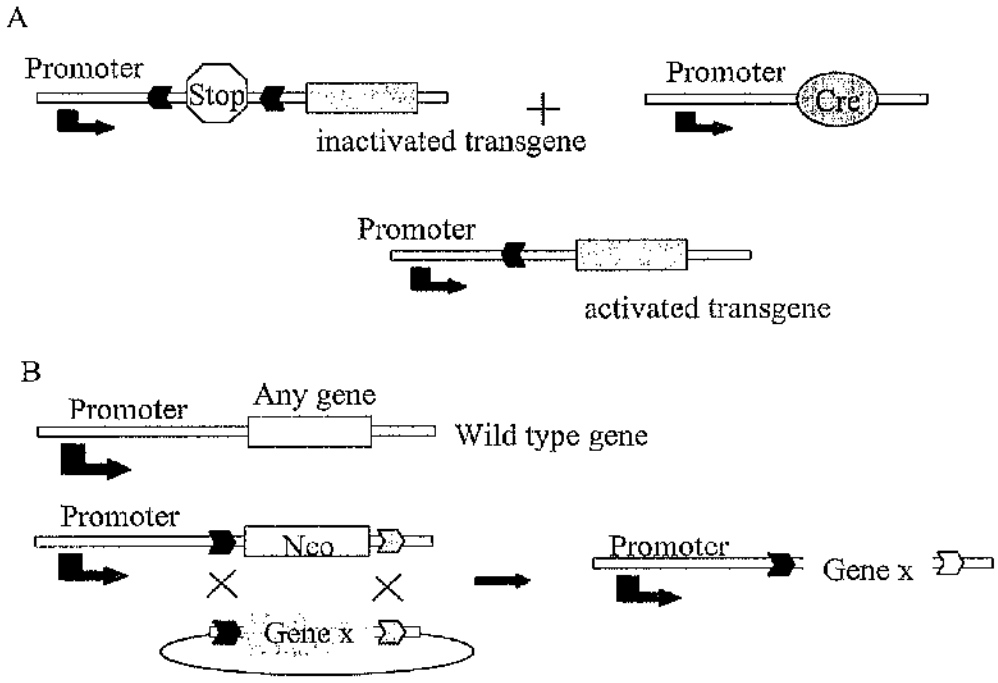


Figure 1.7 The Cre/lox system has been successfully used to generate gene switches (A) and knock-in vectors (B).

(A) To switch on gene expression, the synthetic, SV40 derived STOP sequence is excised from the expression locus using Cre mediated recombination between two loxP sites. These loxP sites are flanking the STOP sequence thus allowing gene expression.

(B) In a first step, a vector containing a selectable marker gene flanked by loxP sites is integrated into the genome by homologous recombination. These two loxP sites flanking the selection marker cassette have been mutated to avoid Cre-mediated recombination events to occur between them. Subsequent transfection of targeted cells with a circular plasmid containing the gene of interest flanked by two equally mutated loxP sites and the addition of Cre results in the Cre-mediated replacement of the selectable marker gene by the gene of interest.

In addition to deletions of DNA segments flanked by equally orientated loxP sites, Cre mediated recombination can be used to generate gene inversions if the two flanking loxP sites are placed in opposite orientation to each other (Abremski, *et al.*, 1986).

Cre is not only capable of mediating intramolecular DNA recombination (excision and inversion) but also catalyses intermolecular recombination events. Cre has therefore been used to generate precise chromosomal translocations in mammalian cells (Smith, *et al*; and van Deursen, *et al.*, 1995). The technique requires the placement of loxP sites at the desired rearrangement endpoints by homologous recombination. As these placements cannot be achieved by a single targeting construct, each mutation requires a different selectable marker gene. The subsequent selection of Cre-mediated recombination is facilitated by using *Hprt*$^-$ ES cells for the two rounds of homologous recombination. Complementary halves of loxP-*Hprt*-fusion gene are placed at each rearrangement endpoint to permit *Hprt*$^+$ selection after a successful rearrangement event.

1.4.8 Application of conditional mutagenesis in Immunology

Recently, the Cre/lox system has been widely used to generate a number of animal models with specific genomic alterations in a variety of research contexts. However, some of the models established can also be placed into an immunological background and have contributed to the current understanding of lymphoid development, tolerance to self antigens, and signaling through cytokine receptors. Some of the major advances using the Cre/lox system in the field of Immunology are highlighted below.

a. Lymphoid development

Lymphotoxin (LT) α, β and TNFα are structurally homologous cytokines. Their genes are closely clustered with the MHC region. LTα and LTβ are produced by activated lymphocytes and NK cells and can either be expressed as a soluble $LT\alpha_3$-homotrimer or as a membrane bound $LT\alpha_1\beta_2$-heterotrimer. Recent reports using gene-targeting techniques suggest an essential role for TNF, LTα and LTβ in secondary lymphoid organ structure and function. Whereas TNF appears to be important for the formation of germinal centers in secondary lymphoid organs, mice lacking LTα exhibit a lack of lymph nodes and splenic disorganization of B and T cell areas. However, it has also been reported that conventional LTα knockout mice show a defect in TNF production. This phenomenon has been attributed to transcriptional interference caused by the retention of the *Neo* selection marker cassette in the targeted LTα locus, which lies in close proximity to the gene encoding TNF. The Cre/lox system has therefore been used to generate a murine model deficient for the LTβ gene. Cre-mediated marker eviction also resulted in deletion of the LTβ gene leaving only a single loxP site behind. These mice show normal levels of LTα and TNF mRNA demonstrating that the Cre/lox system can be used to dissect the functions of genes located in gene clusters. Results obtained indicate a differential role for LTα independently of LTβ in the formation of mesenteric and cervical lymph nodes (Alimzhanov, *et al.*, 1997; Alexopoulou, *et al.*, 1998).

Absence of IL-2Rγ is known to result in abnormal lymphopoiesis and immunodeficiency, as evidenced by X-linked severe combined immunodeficiency (SCIDX1) in man, and in the corresponding canine and murine models of SCIDX1 (Noguchi, *et al.*, 1993). The Cre/lox system has been used to generate ES cell clones *in vitro* that either lack crucial parts of the gene encoding IL-2Rγ or only contain loxP sites in

intron segments. Mice derived from ES cells with mutant IL-2Rγ loci were analyzed for their lymphoid development and compared to mice with only loxP sites located in their IL-2Rγ locus. Lymphoid cells derived from IL-2Rγ $^{-/-}$ ES cells were not detected in peripheral blood of chimeric animals, although control ES cells resulted in normal T-, B-, and NK-cell populations. Mice with germline-deficiency of IL-2Rγ developed some mature splenic B and T cells but the absolute number of lymphocytes was strongly reduced accompanied by a complete depletion of NK cells. Development of gut-associated intraepithelial lymphocytes was also reduced, and Peyer's patches were undetectable. T and B lymphocytes showed reduced responsiveness to mitogen or IL-4, respectively. Using conditional gene targeting, it was concluded that IL-2Rγ plays an important role in B- and T cell generation and that its function is essential for NK-cell development (DiSanto, *et al.*, 1995).

b. Peripheral tolerance

The Cre/lox system has been successfully used to study the fate of auto-reactive $IgM^{+}IgD^{+}$ mature B cells that recognize a self-antigen after their selection into the long-lived peripheral B cell pool. To generate mice whose mature B cells become auto-reactive after their release to the periphery, conditional mutagenesis was applied that takes advantage of the activity of Cre to mediate gene inversions of DNA segments that are flanked by loxP sites with opposing orientations. Prior to Cre mediated recombination these mice express a BCR with non-autoreactive specificity and mature B cells of this specificity were readily detected in peripheral organs. Cre recombinase expressed under the Mx-promoter mediates the inversion of the loxP site flanked DNA segment leading to the expression of a BCR that recognizes MHC class I molecules in mice of a certain MHC haplotype. The results obtained using this method firmly establish, that mature autoreactive

B cells that recognize a membrane antigen with high affinity are rapidly eliminated by clonal deletion (Lam and Rajewsky, 1998).

c. Signaling molecules

A variety of cytokines including IL-15 require the activation of STAT3 for signal transduction following receptor engagement. However targeted mutation of STAT3 by conventional techniques results in early embryonic lethality (Takeda, *et al.*, 1997). The Cre/lox system has been employed to generate mice, in which STAT3 is deficient in a T cell specific manner by crossing mice with a targeted insertion of loxP sites into the gene encoding STAT3 to transgenic mice expressing Cre under the $p56^{lck}$-promoter. In STAT3 deficient T cells, IL-6 induced proliferation was severely impaired leading to increased apoptosis of STAT3 deficient T cells (Takeda, *et al.*, 1998).

Notch proteins are large, conserved cell surface receptors that regulate cell fate specifications and homeostasis of self-renewing tissues (Artavanis-Tsakonas, *et al.*, 1995). The function of *Notch1* was investigated by generating a conditional mutant where an essential portion of the gene was flanked by loxP sites. *Notch1* had been previously shown to play an important part in $CD4^+$ versus $CD8^+$ T cell lineage decision as well as in TCR expression (Robey, *et al.*, 1996; Washburn, *et al.*, 1997). Deletion of *Notch1* by conventional gene targeting resulted in embryonic lethality at approximately day 10 of gestation (Swiatek, *et al.*, 1994). The IFNα/β-inducible Mx-Cre transgenic model was therefore used to study the effect of *Notch1* deletion in the adult mouse. Post-natal deletion of *Notch1* resulted in reduced thymus development and growth retardation. *Notch1* was shown to be important in the development of T cells from the most immature thymocyte $CD44^+CD25^-$ precursor stage (Radtke, *et al.*, 1999).

d. Antigen presentation

MHC class II molecules play a crucial role in the development and function of the immune system by presenting peptides processed from extracellular proteins to $CD4^+$ helper T cells. MHC molecules also direct the process of positive and negative selection during the formation of the T cell repertoire in the thymus. Recently, a murine model has been developed that lacks all classical MHC-II genes by generating a large, 80kb deletion using the Cre/lox system. This murine model may be useful in providing a genetic background to express human HLA genes to investigate the potential role of certain HLA-alleles in autoimmunity by avoiding the possibility of unwanted pairing between human and murine MHC genes in HLA-transgenic mice (Madsen, *et al.*, 1999).

1.4.9 Comparison of conventional and conditional gene targeting

Conventional gene targeting has been widely used and has had an enormous scientific impact on the current knowledge of the function of specific genes and the proteins they are encoding. This is not only the case for advances made in the field of Immunology but can also be extended towards Physiology, Developmental and Behavioral Biology. With many of the techniques involved in the generation of conventional knockout mice being now standardized and commercially available, gene targeting has become a common approach to study the function of newly characterized genes. Conditional mutagenesis using the Cre/lox system requires all the techniques necessary to generate conventional knockout mice. Using the Cre/lox system mice carrying a null mutation can be produced by either *in vitro* transfection of targeted ES cell clones with an expression plasmid delivering Cre-recombinase or by mating of targeted mice with a Cre-transgenic mouse expressing Cre in early development. Both methods have been used successfully to generate null mutations after Cre/lox targeting. Furthermore, the deletion of the *Neo*

selection marker cassette after gene targeting in ES cells minimizes the chances for altered expression of neighboring genes if the targeted locus is located within a gene cluster. The 'recycling' of selectable marker genes by transient expression of Cre in targeted ES cells also provides a useful tool to generate double knockouts in a second targeting round using the same selection marker (Abuin and Bradley, 1996).

The Cre/lox system also offers an approach to bypass the effects of mutations that would otherwise be lethal to the developing homozygous embryo if the targeted gene is crucial for murine development and allows studying the effect of genetic deletion of such genes in the adult animal. Expression of Cre in transgenic animals where Cre is controlled by an inducible promoter (e.g. estrogen, Mx1) offers the opportunity to investigate the effect the lack of a specific gene product may have at defined time points, e.g. after stimulation with specific antigen. A further advantage of using the Cre/lox system is the chance of crossing targeted mice with transgenic animals that express Cre under different promoters thus offering the possibility to create mice lacking the targeted gene function only in defined tissues and cell types. A growing list of Cre-transgenic animals expressing Cre under tissue specific promoters is available offering the chance to limit genetic alterations to defined cell types. Tissue specific gene targeting is a refined targeting technique that may be very beneficial in order to understand the functions of proteins such as cytokines, which often have pleiotropic effects and affect many cell types. One obvious problem with this approach is that regulatory sequences and chromatin structure can influence tissue specific promoters after random integration of the Cre recombinase expression construct. Hence, specific expression of Cre recombinase only in the target cell may be partially compromised. A modified approach to ensure appropriate cell-specific Cre expression is to target the tissue-specific promoter-Cre recombinase construct into the endogenous gene locus. This has been achieved for the CD19-Cre strain deleting loxP-flanked target genes in B cells specifically under the endogenous promoter (Rickert *et al.*,

1997). To monitor the expression of Cre in transgenic animals, several constructs co-expressing reporter constructs as well as both polyclonal and monoclonal antibodies are available. However, tissue specificity depending on promoter specificity and efficiency of Cre-mediated recombination in the intact animal remain two major problems in the application of conditional mutagenesis.

The Cre/lox system has also been used to create chromosomal translocations and deletions that cannot be achieved by conventional gene targeting techniques. The possibility to study the effect of gene products in a tissue specific or inducible manner is widely applicable in Immunology. Redundancy and pleiotropic effects of protein mediators may create situations, in which conventional gene targeting techniques may not be sufficient to elucidate their biological functions in a tissue specific manner.

1.5 Specific aims of this thesis

IL-15 has been characterized as an important activator of T cells and mediator of inflammation. IL-15 expression in the synovial membrane of RA patients accounts for the production of TNFα, a crucial mediator of inflammation in the rheumatoid joint. The fact that IL-15 may act upstream of TNFα, thereby orchestrating inflammatory processes in RA, makes it a potential target for therapeutic approaches.

In lymphocytes, IL-15 acts through a specific receptor. This receptor is composed of the IL-2Rβ and γ chain plus the high affinity IL-15Rα. Gene targeting through homologous recombination in embryonic stem cells is a sophisticated tool to elucidate the function of novel genes and their products. The recently developed technique of conditional mutagenesis using the Cre/lox system not only offers the possibility to generate conventional knockout mice but also the opportunity to study the effects generated by gene targeting in a tissue specific manner. This project was designed to elucidate the possibility of using a soluble form of IL-15Rα as a therapeutic agent in inflammatory diseases which are mediated by IL-15, taking CIA as a model for inflammatory responses closely resembling human RA. Secondly, this project aimed to establish a murine model lacking IL-15Rα in a tissue specific manner to study the effects of IL-15 in inflammatory responses. The objectives of this project where met by two specific aims:

A. To clone and express a soluble, recombinant IL-15Rα in order to antagonize IL-15 *in vivo*.

B. To characterize the gene encoding IL-15Rα and to generate a murine model lacking IL-15Rα by conditional gene targeting in murine embryonic stem cells.

Chapter 2 Materials and Methods

2.1 Molecular Cloning

2.1.1 Buffers and Solutions

Bacterial Lysis buffer:

1%SDS / 200mM NaOH

Denaturing Buffer:

0.5M NaOH / 1.5M NaCl

DNA ligation buffer

50mM Tris HCl, pH 7.6 / 10mM $MgCl_2$ / 1mM ATP / 1mM DTT / 5% w/v polyethylene glycol 8000

DNA loading buffer:

0.25% bromophenol-blue/ 0.25% xylene-cyanole-FF / 150mM Tris.HCl, pH 8.0 / 10mM EDTA / 40% sucrose

GTE buffer:

50mM glucose / 25mM Tris.HCl, pH 8.0 / 1mM EDTA

Hybridization Buffer (Lambda DNA):

6xSSC / 20mM NaH_2PO_4 / 0.1 % SDS and containing 100 µg/ml of freshly denatured salmon sperm DNA (Sigma)

Church-Gilbert-Buffer

7 % SDS / 40mM NaH_2PO_4 / 1mM EDTA, pH 7.5, containing 100 µg/ml of freshly denatured salmon sperm DNA (Sigma)

Neutralizing Buffer:

0.5M Tris.HCl pH 7.4 / 1.5M NaCl

PCR buffer (10x):

200mM Tris.HCl pH 8.4 / 500mM KCl

SM buffer:

100mM NaCl / 10mM $MgSO_4$ / 50mM Tris.HCl, pH 7.4 / 0.01% gelatin

20 x SSC buffer:

3 M NaCl / 420 mM Sodium citrate, pH 7.2

TAE buffer (Freeze and Squeeze):

25mM Tris / 0.3M Sodium Acetate / 1mM EDTA / pH 7.0

TBE buffer (1x):

200mM Tris.HCl, pH 8.0 / 900mM boric acid / 25mM EDTA

TE buffer:

10mM Tris.HCl, pH 7.4 / 1mM EDTA

2.1.2 Total cellular RNA preparation

Total cellular RNA was extracted from J774 cells using the TRIzol Reagent (Gibco, BRL). J774 cells grown to confluence, transferred into an Eppendorf tube were washed twice in PBS and 1ml/10cm^2 of TRIzol was added. After 5 minutes at room temperature, 200µl/ml of chloroform were added and the mixture was shaken by hand for 15 seconds. After incubation at room temperature for 2-3 minutes, the samples were centrifuged in a table microfuge (Hettich, Germany) at 13,000x g for 15 minutes at 4°C and the aqueous phase was transferred into a fresh tube. 500µl 2-propanol per milliliter TRIzol were added, the samples incubated for 10 minutes at room temperature and the RNA precipitated at 13,000x g, 4°C for 10 minutes. The pellet was washed with 75% ethanol, dried at room temperature for 5 minutes and dissolved in 40µl DEPC-treated water. Amount and quality of purified total RNA was determined by measuring the OD_{260} and OD_{280} in a photospectrometer (Pharmacia, Uppsala, Sweden).

2.1.3 Reverse Transcribed Polymerase Chain Reaction (RT-PCR)

Total RNA prepared as described above was subjected to reverse transcription. 5µg of total RNA was mixed with 80ng random hexamers (Roche Biochemicals) and the volume adjusted to 12µl by adding DEPC-treated water. The mixture was heated to 70°C for 10 minutes and put on ice for 1 minute. 2µl 10x PCR buffer, 25mM MgCl, 100mM DTT and 1µl 10mM dNTP were added and the mixture allowed to stand at 28°C for 5 minutes before adding 1µl (200 u) of SuperScipt™ reverse transcriptase (Gibco, BRL). After incubation for 10 minutes at 28°C, the reaction was allowed to proceed at 42°C for 50 minutes and terminated by incubating the samples at 70°C for 15 minutes before placing the samples on ice. 2µl of cDNA synthesized this way were used in subsequent PCR reactions.

2.1.4 Polymerase Chain Reaction (PCR)

For amplification of cDNA fragments or genomic DNA samples, 0.1-50ng of DNA were mixed with 10µl PCR buffer (10x), 4-10µl 50mM $MgCl_2$, 2µl 10mM dNTP and 0.2µg specific 5' and 3' primer-oligonucleotide. 5-10 units of Taq-Polymerase (Gibco BRL) were added and the volume adjusted to 100µl with dH_2O. For genomic DNA amplification, diluted DNA samples were heated to 95°C for 10 minutes prior to adding the DNA to the PCR-reaction. Mixed samples were overlayed with 50µl of mineral oil (Sigma) and amplified in a thermo-cycler (Genius, Techne, Cambridge) under following conditions: denaturing at 95°C for 1 minute, annealing at 54-56°C for 2 minutes and sequence extension at 72°C for 3 minutes. PCR samples were amplified for 30-35 cycles depending on sensitivity of the reaction.

For detection of Cre catalyzed recombination events of loxP site flanked sequences in targeted ES cell clones, 500ng of gDNA were denatured at 94°C for 2minutes. A total of 0.08pg primer (33-mer oligonucleotides), 1.75µl 10mM dNTP, 5µl PCR buffer containing 2.5mM $MgCl_2$ and 15 units of Taq-Polymerase (Roche Biochemicals) were added. Reactions were overlaid with one drop of mineral oil and subjected to the following program in a thermo-cycler (Genius Techne): denaturing at 94°C for 1 minute, annealing at 66°C for 2 minutes and extension at 72°C for 3 minutes for 35 cycles. PCR products were then analyzed by agarose-gel electrophoresis.

DNA oligonucleotides used for cloning of cDNA fragments by PCR are listed in Table 2.1 below.

Table 2.1 List of PCR-oligonucleotides for cloning and screening

Oligonucleotide	**DNA Sequence (5' – 3')**	**Location**
KPN-1	CTCACAGCCAGGGTACC CCTCCCCCTCTGCAA	Subclone 3.2, 3' of Exon 3
KPN-1R	TTGCAGAGGGGGAGGG GTACCCTGGCTGTGAG	Reversed KPN-1
3'wt-1	GTAAGCATTAGTTCTTC ACGTTTTCCCAGGGTTC	Subclone 3.2, 3' of Exon 3
3.5-revR-33	GAAATAGCCAACATTTT GGACCCCTCATCTATT	Subclone 3.5, downstream
IL-15R-anti	GCACCAACCAAGAGGA CCGATGTAGAGATGGC	Murine IL-15Rα-cDNA
IL-15R-sense	TTGCTGCTGCTGCTGTTG CTACTGTTGCTCCC	Murine IL-15Rα-cDNA
IL-15R-3'-*Sal*I	GGTGGGATCCGGCACCA CGTGTCCACCTCCCG	Murine IL-15Rα-cDNA
IL-15R-5'-*Bam*HI	AGATGTCGACTTTCGTC ATTTTGGAACTGTGG	Murine IL-15Rα-cDNA
IL-15R-5'-*Xba*I	CAGGGACCCCAGTCTAG AGTCCCGTTGGGT	Murine IL-15Rα-cDNA
IL-15R-3'-*Xho*I	TTTCGTCATTTTGGAACT CGAGGGAGAAATC	Murine IL-15Rα-cDNA
IgG-Fc-5'-*Xho*I	GCCCAAACTAACTCGAG GGTGACCCTGGGA	Murine IgG_1-Fc-cDNA
IgG-Fc-3-*Eco*RI	AAGGACACTGAATTCAT TTACCAGGAGAGT	Murine IgG_1-Fc-cDNA

2.1.5 Agarose Gel Electrophoresis

Nucleic acid fragments were analyzed by agarose gel electrophoresis. Gel concentration varied according to fragment size or DNA amount, ranging from 0.65 to 2.0%. Plasmid DNA fragments were separated on 1-2 % agarose gels whereas for lambda and genomic DNA 0.6% agarose gels were used. Agarose (Gibco BRL) was dissolved in 0.5 x TBE buffer by boiling and cooled to 50°C before adding ethidium bromide (Sigma) up to a final concentration of 10ng/ml. Gels were allowed to set, cast in tanks and submerged in 0.5 x TBE. DNA samples, mixed 1:10 with DNA loading buffer, were loaded into prepared wells set into the agarose gels using a well-forming comb followed by electrophoresis at 1-5 V/cm and analysis under ultra-violet light.

2.1.6 Endonuclease restriction of DNA

A variety of restriction endonucleases and their corresponding buffer systems were used (all from Roche Biochemicals). Restriction enzyme buffers were used according to the recommendations of the manufacturer in single enzyme digestions. In case of multiple enzyme digests, DNA was first cut with the enzyme requiring lower salt concentration for 1.5 hours at 37°C before adding the second enzyme and raising the salt concentration according to the conditions recommended. For plasmid DNA, 10-20 units of enzyme per μg of DNA were used in a volume of 20-100μl. For genomic DNA derived from ES cells, 60 units of restriction enzyme were added to 10μg of DNA and the digest allowed to proceed for 4 hours before adding another 20 units and incubating the sample at 37°C overnight.

2.1.7 DNA fragment purification

DNA fragments of interest were excised with a clean scalpel and placed into an Eppendorf tube. 1ml of TAE buffer was added and the gel fragment incubated in the dark for 15 minutes. The buffer was then removed and the gel fragment frozen within the tube by placing it into liquid nitrogen for 5 minutes. The frozen gel fragment was then placed into a Spin X centrifuge filter tube (Costar) and centrifuged for 20 minutes at 15,000 x g. The resulting DNA solution was extracted with phenol/chloroform followed by precipitation with 100% ethanol.

2.1.8 Ligation of DNA fragments

To clone DNA fragments in plasmid vectors, vector DNA was cut by restriction endonucleases as described previously. To avoid self-ligation of the plasmid vector, digested vector DNA was subjected to dephosphorylation by adding 1 unit of calf intestine alkaline phosphatase (Roche Biochemicals) and incubating at 37°C for 4 hours. Plasmid DNA vectors were then extracted by phenol:chloroform, precipitated with 100% ethanol, washed with 70% ethanol and resuspended in a suitable volume of dH_2O. Small amounts of cut vector DNA were then analyzed on an agarose gel. DNA fragments with compatible restriction site ends were purified from agarose gels as described before. The amount of vector- and fragment DNA was analyzed by agarose gel electrophoresis prior to ligation. Equimolar amounts of both vector and insert DNA to be ligated, approximately 100-500ng DNA, were mixed and incubated in the presence of 1 unit of T4 ligase (Gibco BRL) in DNA ligation buffer. The volume was adjusted to 10µl with dH_2O and the samples incubated at 15°C overnight.

2.1.9 Cloning and transformation of competent bacteria

Commercially available competent bacteria were used for plasmid transformation. For bacterial expression of soluble IL-15Rα, competent *E. coli* bacteria of the M15 strain (Gibco, BRL) were used. For subcloning of DNA fragments, the E. coli strain DH5a was used. For transformation, 100 µl of competent cell suspension were thawed on ice. Approximately 50ng of plasmid DNA was added and the mixture incubated on ice for 30 minutes. The samples were then heat shocked at 42°C for 90 seconds and put on ice for 5 minutes. 900µl pre-warmed LB medium was added and the samples were incubated with vigorous shaking at 37°C for 1 hour. The cells were centrifuged briefly, the total volume reduced to 100µl and plated on LB-agar plates containing the appropriate antibiotic. The plates were incubated at 37°C overnight to obtain single colonies for further analysis.

2.1.10 Preparation of frozen bacterial stocks

Bacterial cultures grown in LB medium overnight shaking at 37°C were briefly put on ice. One volume of fresh LB medium containing 50% glycerol was added and the culture frozen in 200µl aliquots at –80°C until use.

2.1.11 Preparation of plasmid DNA

Small-scale plasmid DNA from 2ml bacterial overnight cultures in LB medium was prepared using an alkaline lysis protocol. 2ml fresh overnight culture was spun at 3,000 x g in a microfuge (Hettich, Germany) for 5 minutes and the bacterial pellet resuspended in 200µl ice cold GTE buffer followed by alkaline lysis in 400µl bacterial lysis buffer. After incubating 5 minutes at room temperature 300µl 3M potassium acetate, pH 4.8, were added and the lysate mixed gently by inverting the tube several times followed by

incubation on ice for 10 minutes. The samples were then centrifuged at 15,000x g for 10 minutes. The supernatant was recovered and 1 volume of ethanol was added followed by precipitation at 15,000-x g for 10 minutes. The pellet was washed once with 70 % ethanol, dried for 5 minutes at room temperature and resuspended in 50µl TE containing 10 u of RNase (DNase free) (Roche Biochemicals).

Large-scale purification of plasmid DNA for electroporation of cells was purified using a commercially available 'Maxi-Prep' kit (Qiagen) following the protocol provided by the manufacturer.

2.1.12 Double stranded DNA sequencing

Double stranded plasmid DNA fragments were sequenced using a method based on the di-deoxy chain termination method (Sanger, *et al.* 1977). Approximately 10µg of plasmid DNA derived from small-scale plasmid preparations were denatured for 15 minutes at 37°C in 200mM NaOH and a total volume of 10µl. 1µl of specific 17-mer oligonucleotide (Genosys, UK) at 20pmol/µl was added and the DNA precipitated in 3µl 3M potassium acetate, pH4.8 and 75µl ethanol. The samples were centrifuged at 13,000x g for 15 minutes at 4°C and the pellet washed once in 75% ethanol. The precipitated DNA pellet was dried at room temperature for 5 minutes and resuspended in 8µl dH_2O. The sequencing reaction was performed using a commercially available DNA sequencing kit (United States Biochemicals) using the reagents provided within the kit following the protocol recommended by the manufacturer. Radiolabeled $\alpha[^{35}S]$-dATP was obtained from Amersham UK. Electrophoresis of sequencing reactions was performed on 6% polyacrylamide gels containing 8M urea derived from a prepared gel mix (Gibco BRL) in 1xTBE buffer. The samples were run at 60W for 2-4 hours at room temperature until the

bromphenol blue marker provided in the termination mix of the sequencing kit reached the lower end of the sequencing gel. Following electrophoresis, gels were fixed in 20% methanol, 10% glacial acetic acid for 30 minutes and transferred to Whatman 3MM filter paper. The gels were then dried under vacuum at 80°C for 2 hours and subjected to autoradiography (Kodak, BioMax) overnight at room temperature.

Table 2.2 List of PCR oligonucleotides used for DNA sequencing.

Oligonucleotide	**Nucleotide sequence (5'- 3')**	**Binding location**
T 7 primer	AATACGACTCACTATAG	pBluescript, pcDNA3.1, pPNTLoxPNeo (p293)
Reverse primer	AACAGCTATGACCATG	pBluescript, pcDNA3.1 pPNTLoxPNeo (p293)
IL-15R-A	CAAATGTTGCCCACTGG	IL-15Rα cDNA p.161-177
IL-15R-A-reverse	CCAGTGGGCAACATTTG	IL-15Rα cDNA p.161-177
IL-15R-B	CACCATCCCAAACAACT	IL-15Rα cDNA p. 371-387
IL-15R-C	GGAGAGGTATGTCTGTA	IL-15Rα cDNA p. 81-97
IL-15R-D	CTCACAGCCAGAGAGCC	IL-15Rα cDNA p. 261-277
IL-15R-E	ATGACCTTGGAGCCTAC	IL-15Rα cDNA p. 451-467
IL-15R-F	CAGTCACAAGTCCTCCC	IL-15Rα cDNA p. 411-427
3.5-T7-rev	CCTTCAGACTCAGGGAT	Subclone 3.5 near 5' end facing subclone 3.3
3.5-reverse primer-rev	TGGACCCCTCATCTATT	Subclone 3.5 near 3' end facing subclone 3.2
3.3-T7-rev	TCGATTTGTGTAGCCTG	5' end of subclone 3.3
3.3-reverse primer-rev	CGATGGAGGACTCTCTT	3' end of subclone 3.3
3.2-1-rev	TCTGGCTCCCACAGCTC	Subclone 3.2, 41-57bp 3' of exon 2
3.2-2-rev	TGTTGCCTAGGTTTGCA	Subclone 3.2, 90-106bp 3' of exon 3

2.1.13 Southern Blot analysis of lambda and genomic DNA

Lambda or genomic DNA was digested using restriction endonucleases. Approximately 10µg genomic DNA or 1µg lambda DNA were subjected to agarose-gel electrophoresis as described previously. The gel was then photographed under ultra-violet light with a fluorescent ruler set alongside the gel. After washing two times in dH_2O the gel was incubated for 15 minutes in 10 volumes of denaturing buffer. The gel was then washed twice in dH_2O and incubated for 45 minutes in 10 volumes of neutralizing buffer. One sheet of Whatman 3MM of similar width and longer shape was placed on a Perspex block within a bath filled with one liter of 20 x SSC buffer, so that the longer ends of the Whatman 3MM filter paper were in contact with the buffer. The neutralized gel was then washed twice in dH_2O and placed upside down on top of the prepared Whatman 3MM filter paper placed inside the transfer chamber. The Whatman 3MM filter paper not covered by the gel was covered by parafilm to allow capillary transfer through the gel only. One sheet of positively charged nylon membrane (Roche Biochemicals) of the same size as the gel and marked for orientation was briefly soaked in 2 x SSC before placing it on top of the gel. Two additional sheets of filter paper fitting the size of the gel were briefly soaked in 2 x SSC and placed on top of the nylon membrane. Air bubbles between the layers of filter paper were removed and two additional layers of filter paper placed on top. Finally, several layers of paper towel and a 500g weight were positioned on top of the blot assembly. Capillary transfer was allowed to proceed overnight at room temperature. The nylon membrane was then removed, briefly rinsed in 2 x SSC, dried between two layers of Whatman 3MM filter paper and heated at 120°C for 20 minutes.

2.1.14 Radioactive labeling of nucleic acid probes

Nucleic acid probes were purified as described previously. 20-50ng of probe DNA in 25µl dH_2O were denatured at 100°C for 10 minutes and chilled on ice. 4 µl of 10 x concentrated random hexamer mix (Roche Biochemicals) and 6 µl 0.5mM nucleotide mix (dCTP, dGTP, dTTP) and 4µl $\alpha[^{32}P]$-dATP (Amersham) were added to the mixture. The reaction was started by adding 20 u of Klenow enzyme (Roche Biochemicals) and allowed to proceed at 37°C for 1h. To stop the reaction, 60µl TE buffer were added. Radiolabeled DNA probes were purified using Nick columns (Pharmacia) equilibrated with 2ml TE. 100µl reaction mix was applied to the column and the solution allowed to enter the resin following the addition of 400 µl of TE. Radiolabeled probe DNA was eluted by adding an additional 500µl of TE. The purified probe was then denatured for 10 minutes at 100°C prior to usage.

2.1.15 Hybridization of nucleic acids

After transfer of nucleic acids to nitrocellulose filters or charged nylon membrane (both from Roche Biochemicals) as described for Southern blot analysis, pre-hybridization was performed in hybridization buffer for 2-3 hours at 65°C before adding a purified radiolabeled DNA probe. Hybridization was carried out overnight in a hybridization oven (Scotlab) at 65°C. The membrane was then washed twice for 30 minutes in 2xSSC, 0.1% SDS and twice for 30 minutes in 0.2xSDS, 0.1% SDS at 65°C.

Genomic DNA transferred to nylon membrane using the same method was prehybridized in Church-Gilbert buffer at 65°C for 2-3 hours before adding radiolabeled probe DNA. Hybridization was carried out overnight at 65°C. The membrane was washed two times with 5% SDS, 40mM NaH_2PO_4, 1mM EDTA at 65°C for 30 minutes and with

1%SDS, 40mM NaH_2PO_4, 1mM EDTA for another 30 minutes. Autoradiography (Kodak BioMax-MS) was carried out at –80°C for 1-3 days depending on the intensity of the signal.

2.1.16 Preparation of competent bacteria

A frozen stock of the *E. coli* strain XL1-Blue was plated out on LB agar and grown overnight at 37°C until single bacterial colonies formed. A single colony was picked and added to 10ml of LB medium containing 0.2% maltose and 10mM $MgSO_4$. The culture was grown overnight shaking vigorously at 37°C and centrifuged at 2000x g for 10 minutes. The pellet was then resuspended in ice cold 10mM $MgSO_4$, the concentration adjusted to OD600 = 0.5 and the suspension stored at 4°C until use.

2.1.17 Lambda phage library screening by plaque hybridization

In order to characterize the genomic organization of the gene encoding murine IL-15Rα, a λFIX II mouse genomic library (129SV, prepared by Dr. Allen Bradley, Stratagene) was screened using a cloned cDNA fragment encoding sIL-15Rα as a radiolabeled probe.

Titering of the λFIX II mouse genomic library. Two parallel serial dilutions of the λFIX II mouse genomic library were made in SM buffer. 100µl of diluted phage (10^{-3} – 10^{-6}) were used to infect 200µl of competent XL1-Blue prepared as described above. After 30 minutes at 37°C the suspension was added to 3 ml of top-agarose pre-heated to 50°C in a water bath. Samples were mixed carefully and the top-agarose plated on LB-agar in 100mm Petri dishes (Costar). The plates were incubated for 12 –16 hours at 37°C and the

phage-plaques formed in the bacterial layer counted. The concentration was determined by relating the number of plaques per plate to the corresponding dilution thus calculating the number of p.f.u./µl for the λFIX II mouse genomic library.

First screening of the λFIX II mouse genomic library. 500µl of competent XL1-Blue were mixed with 25µl phage library at approximately $4x10^6$ p.f.u./ml to obtain 50,000 plaques per plate. After incubation at 37°C for 30 minutes, the suspension was added to 5ml melted top-agarose at 50°C. The top-agarose was then plated on LB-agar in 150mm Petri-dishes (Costar) and the plates incubated at 37°C for 12-16 hours. The plates were chilled at 4°C for 2 hours and the plaques transferred to nitrocellulose filters (Roche Biochemicals) for 2 minutes. Duplicates were made and the second filter allowed on the plates for 5 minutes. The 150mm plates were then stored at 4°C until needed. Phage DNA transferred to nitrocellulose filters was denatured in 1.5M NaCl, 0.5M NaOH for 5 minutes and neutralized in 1.5M NaCl, 0.5M Tris.HCl, pH8.0 for 7 minutes. The filters were then rinsed briefly in 0.2M Tris.HCl, pH 7.6, 2xSSC, dried on Whatman 3MM filter paper and heated at 80°C for 2 hours. Hybridization with a radiolabeled DNA probe was done as described previously. Following hybridization and washes, filters were subjected to autoradiography at –80°C overnight. Comparing the intensity of autoradiographic signals against background identified putatively positive lambda phage clones.

Groups of lambda phage clones identified were localized on 150mm Petri dishes by comparing their position in relation to the signal produced on film. These clone groups were picked from the LB-agar within a 5mm diameter and incubated in 0.5ml SM buffer at 4°C overnight. The titer of the phage suspension generated that way was determined as described before.

Second screening of λFIX II mouse genomic library. Groups of lambda phage clones identified in the first screening round were subjected to a second screening. Competent XL1-Blue were infected as described before and plated in top-agarose on 100mm Petri dishes to obtain 100-200 plaques per plate. The plaques were then transferred to nitrocellulose membrane and hybridized to IL-15Rα-specific radiolabeled DNA probe as described before. Putatively positive lambda phage clones identified in the second screening were picked as single clones and incubated in 0.5ml SM buffer at 4°C before the titer of the phage suspension was determined. Some clones were subjected to a third screening identical to the second screening round to obtain single clones.

2.1.18 Purification of Lambda DNA

50ml of LB medium containing 10mM $MgSO_4$, 0.2% maltose were inoculated with a single plaque and surrounding XL1-Blue picked from a LB-agar plate prepared as described for the screening process. The culture was grown shaking overnight at 37°C. When the culture became slightly clear 1ml of chloroform was added and the culture continued for one additional hour. The lysate was then centrifuged at 4,500x g for 10 minutes in a JA17 rotor (Beckman) and the supernatant treated with DNase and RNase (10mg/ml each) for 1hour at 37°C. One volume of 20% PEG, 2.5M NaCl was added, mixed well and the samples stored on ice for 1-2 hours before centrifugation at 10,000x g for 30 minutes. The pellet was resuspended in 600µl SM buffer and extracted 3-4 times with chloroform to remove remaining PEG. 15µl 0.5M EDTA and 30µl 5M NaCl were added and the samples extracted with 350µl buffer-saturated phenol. The aqueous phase was recovered and extracted with 350µl chloroform followed by precipitation in 875µl ethanol. The lambda DNA pellet was washed with 75% ethanol, dried at room temperature for 5 minutes and resuspended in 50µl TE buffer. Sometimes, lambda phage DNA was

prepared using a commercially available kit (Lambda Midi Kit, Qiagen) following the procedure recommended by the manufacturer.

2.2 Expression and purification of recombinant IL-15 receptor fusion proteins

2.2.1 Buffers and Solutions

Acrylamide solution

30% w/v acrylamid / bisacrylamide(29:1) in dH_2O

Buffer **B**

8M urea / 0.1M NaH_2PO_4 / 0.01M Tris.HCl, pH 8.0

Buffer **C**

8M urea / 0.1M NaH_2PO_4 / 0.01M Tris.HCl, pH 6.3

Cell lysis buffer

25mM Tris.HCl, pH 7.4 / 150mM NaCl / 1% Nonidet p-40 / 1mM EDTA / 2mM EGTA / 10mM NaF / 1mM DTT / 50μg/ml leupeptin / 50μg/ml aprotinin / 50μg/ml PMSF

Low salt Wash buffer (Western Blot)

0.01M Tris.HCl, pH 7.6 / 0.1M NaCl / 0.1% Tween-20

High salt Wash buffer (Western Blot)

0.01M Tris.HCl, pH 7.6 / 0.5M NaCl / 0.1% Tween-20

Resolving (lower) gel buffer (SDS-PAGE)

1.5M Tris.HCl, pH 8.8 / 1% SDS

Stacking (upper) gel buffer (SDS-PAGE)

0.5m Tris.HCl, pH 6.8 / 1% SDS

TBS

25mM Tris.HCl, pH7.4 / 150mM NaCl / 100μM vanadate

Western Blot Transfer Buffer

20mM Tris / 40mM glycine / 20% v/v methanol

SDS-PAGE buffer

25mM Tris / 250mM glycine / 0.1% SDS

SDS-Sample buffer

1M Tris.HCl, pH6.8 / 200mM DTT / 4% SDS / 0.4% bromophenol blue / 20% glycerol

2.2.2 Cloning of recombinant soluble IL-15Rα

Total RNA was extracted from J774 cells that had been stimulated for 18 h with murine recombinant IFNγ (100 U/ml, a kind gift of Dr. G. Adolf, Bender Wien, Austria). The RNA was then reverse transcribed into cDNA as described above. sIL-15Rα was cloned using nested PCR, and two restriction sites (*Bam*HI and *Sal*I) were introduced during the second amplification. The following primer sets were designed from the published cDNA sequence of murine IL-15Rα: Set I, 5′TTG CTG CTG CTG CTG TTG CTA CTG TTG CTC CC-3′; 5′-GCA CCA ACC AAG AGG ACC GAT GTA GAG ATG GC-3′. Set II, 5′-GGT G*GG ATC C*GG CAC CAC GTG TCC ACC TCC CG-3′; 5′-AGA T*GT CGA C*TT TCG TCA TTT AAC TGT GG3′ (sequences in italics are the *Bam*HI and *Sal*I site, respectively). The PCR product was confirmed by sequencing, cloned into the pQE-30 expression vector (Qiagen, Dorking, U.K.), and then expressed in the *Escherichia coli* M15 strain (Qiagen).

2.2.3 Rapid screening for recombinant sIL-15Rα protein expression

The *E. coli* strain M15 transformed with the pQE30 expression vector carrying an insert encoding sIL-15Rα was used to produce recombinant protein. Single bacterial colonies were obtained by plating transformed bacteria on LB-agar (Gibco BRL) containing 100μg/ml ampicillin and 25μg/ml kanamycin as described before. 12 single colonies were picked and cultured in 2ml LB medium supplemented with 2% glucose shaking at 37°C overnight. Each culture was then duplicated by adding 500μl into 2x1.5ml of fresh, pre-warmed LB (containing ampicillin and kanamycin). The cultures were shaken for another 30 minutes until OD_{600} was between 0.7 and 0.9 when compared to fresh medium. 20μl of 200mM IPTG were added to one set of cultures, the other half serving as non-induced controls. Incubation was continued shaking at 37°C for 3-5 hours after which time cultures were transferred to fresh 2ml Eppendorf tubes. After centrifugation at 3,000x g the supernatant was aspirated and the bacterial pellet resuspended in 200μl buffer **B**. The resuspended bacterial suspension was then frozen at –70°C for 20 minutes, thawed at room temperature whilst inverting several times to allow complete bacterial lysis. The samples were then centrifuged at 15,000x g for 10 minutes and the supernatant transferred to a fresh tube. 40μl of 50% slurry nickel-agarose (Ni-NTA, Qiagen) were added and the samples mixed at room temperature for 30 minutes. The nickel-agarose was then spin-washed 3 times with buffer **C** at 13,000x g before adding 20μl of buffer **C** containing 100mM EDTA. The samples were mixed carefully and spun at 13,000x g to recover the supernatant. 20μl supernatant from IPTG-induced and non-induced samples were mixed with 2x SDS-loading buffer and heated to 95°C for 10 minutes before analysis via SDS-PAGE loading induced and non-induced samples of the same bacterial clones next to each other on the gel for comparison.

2.2.4 Large scale purification of sIL-15Rα

100 ml of 2xYT medium (containing 100μg/ml ampicillin, 25μg/ml kanamycin) were inoculated with a single colony of *E. coli*-M15 transformed with the pQE30 vector carrying an insert encoding sIL-15Rα. The culture was incubated with shaking at 37°C overnight and added to 900ml of fresh 2xYT (Gibco BRL), containing antibiotics, pre-warmed to 37°C. The culture was further incubated with shaking for approximately 2 hours until OD_{600} > 0.7. 10ml of 200mM IPTG was added and incubation with shaking was continued at 37°C for 5 hours. The bacterial suspension was then pelleted by centrifugation in a JA10 rotor (Beckman, USA) at 4000x g, 4°C for 20 minutes. The supernatant was removed and the pellet resuspended in 20ml of buffer **B**. The suspension was incubated with stirring at room temperature overnight followed by centrifugation at 14000x g in a JA17 rotor (Beckman, USA). The supernatant was recovered and 1ml of 50% slurry nickel agarose (Ni-NTA, Qiagen) was added. Recombinant protein carrying a 6xHis tag was allowed to bind to Ni-NTA for one hour by gently shaking the samples at room temperature. The Ni-NTA was then spin-washed 3 times with buffer **B** before loading onto a filter column (Qiagen). The Ni-NTA was allowed to settle by gravity and was washed by applying buffer **B** until the OD_{280} of the flow-through was below 0.001. The Ni-NTA was then washed with buffer **C** until OD_{280} < 0.001. One column volume (approximately 10ml) of buffer **C** containing 20mM imidazole was added to elute nonspecific protein bound to Ni-NTA. Recombinant IL-15Rα bound to Ni-NTA was eluted by adding 2-3ml buffer **C** containing 300mM imidazole and dialyzed in 2000ml PBS containing 2%NaCl at 4°C overnight.

2.2.5 Measurement of protein concentration

The concentration of protein samples was measured using the Coomassie Method. Reagents were purchased from Pierce, Illinois, and the concentration of protein samples measured following the protocol provided by the manufacturer.

2.2.6 Cloning of recombinant IL-15Rα-IgG$_1$-Fc fusion protein

Spleens derived from male DBA/1 mice that had undergone at least 2 weeks of Collagen Induced Arthritis were homogenized and the cells washed two times with PBS. After counting the cells in a Neubauer cell counting chamber, 10^6 cells were used to extract total RNA as described above. RT-PCR was performed using the total RNA as a sample to generate cDNA. 2μl of cDNA were used in a PCR reaction using specific primers (5' Fc-*XhoI*: 5'-GCCCAAACTAACTCGAGGGTGACCCTGGGA-3'and 3' Fc-*EcoRI*: 5'-AAGGACA-CTGAATTCATTTACCAGGAGAGT-3') so as to amplify a 945bp product encoding murine IgG$_1$-Fc. This PCR product was subjected to restriction enzyme digestion using the endonuclease restriction sites contained in the primer sequences. The digested DNA was purified on a 1% agarose gel and cloned into the pcDNA3.1 (Invitrogen, Netherlands) expression vector pre-cut with endonucleases *XhoI* and *EcoRI* using the method described previously.

The cDNA fragment encoding IL-15Rα was cloned using a nested PCR approach as described for cloning the cDNA fragment encoding sIL-15Rα. The first PCR amplification was performed using the same set of primers as for the first PCR reaction used for sIL-15Rα cloning, generating a 723bp DNA fragment. This fragment was used as a template in a second PCR reaction such that two restriction enzyme sites (*XbaI* and *XhoI*)

were introduced, and a 668bp fragment was generated (5'-CAGGGACCCCAGTCTAGA-GTCCCGTTGGGT-3' and 5'-TTTCGTCATTTTGGAACTCGAGGGAGAAATC-3'). This fragment was cut with *XbaI* and *XhoI* and purified via agarose gel electrophoresis before cloning it into the pcDNA3.1 expression vector already containing the DNA fragment encoding IgG_1-Fc. The cloning product was confirmed by DNA sequencing using both external and internal sequencing primers.

2.2.7 Screening for expression of IL-15Rα-IgG_1-Fc fusion protein by DOT blot

COS-7 cells transfected with the pcDNA3.1 plasmid including the DNA insert encoding the IL-15-IgG-Fc fusion protein were serially diluted and grown in 100mm tissue culture dishes for 10 days in the presence of G418 to obtain single colonies. These colonies were picked and transferred to 48 well plates. The cells were then grown in DMEM containing G418 until confluent. 1ml of supernatant from each clone was removed and stored at 4°C until needed. The cells were allowed to grow until clones of high expression were identified by DOT blot. To assess fusion protein expression in COS-7 cell supernatants, Trans-Blot membrane (BioRad, Hempstead, U.K.) was cut to fit into a DOT blot transfer chamber (Hoeffer, USA) and soaked in Western Blot Transfer buffer for 1 minute prior to placing it into the chamber. The chamber was then shut and 1ml of cell culture supernatant from 48 COS-7 cell clones was transferred into the wells of the transfer chamber. Protein within the supernatant was bound to the membrane by connecting the DOT blot chamber to a vacuum pump and allowing the supernatant to be transferred through the membrane for 10 minutes. The membrane was then washed briefly in low salt wash buffer and unspecific binding of antibodies blocked by incubating the membrane in 2% BSA/low salt wash buffer for 1hour at room temperature. 100ng/ml simian IL-15 (a kind gift of the Immunex Corp., USA) in 1% BSA/low salt wash buffer was added for 1hour at room temperature. The membrane was then washed twice with low salt wash

buffer, twice with high salt wash buffer and again twice with low salt wash buffer for 5-10 minutes each. Binding of IL-15 was detected by adding 100ng/ml rat-anti-human IL-15 mAb (biotinolated, R&D systems, USA) in 1% BSA/low salt wash buffer for one hour at room temperature followed by washing the membrane as described before. Avidin-peroxidase, conjugated (1/1000 dilution, Sigma, Poole, U.K.), was added in 1% BSA/low salt wash buffer followed by washing the membrane as described previously before developing by enhanced chemiluminescence (Amersham International, Little Chalfont, U.K.).

2.2.8 Large scale purification of IL-15Rα-IgG$_1$-Fc fusion protein

COS-7 cell clones identified to produce high amounts of the IL-15Rα-IgG$_1$-Fc fusion protein were grown to confluence in 165cm^2 tissue culture flasks (Costar) in medium containing FCS and G418 (see below). When confluent, the medium was aspirated and the cells grown in DMEM in the absence of FCS and G418 for 3 days. The cells were then washed twice with warm PBS and harvested by detaching the cells from the tissue culture flasks with a sterile cell scraper (Costar) followed by centrifugation at 450x g for 5 minutes in a Mirage-2000 centrifuge. The supernatant was discarded and the cells resuspended in 4ml COS-7 cell lysis buffer. Lysis was allowed to proceed on ice for 30 minutes and the lysate transferred to fresh 1.5ml Eppendorf tubes. The lysate was then cleared by centrifugation at 13,000 x g for 10 minutes at 4°C in a table centrifuge (Hettich, Germany). Protein A-coated beads (Amersham, UK) were washed twice in PBS and 1ml per 4ml of lysate were added. Binding of the IL-15Rα-IgG$_1$-Fc fusion protein to Protein A coated beads was done by shaking the suspension gently at 4°C for 2 hours. The beads were then spin washed four times in ice cold PBS before adding 1ml / 4ml lysate of 2xSDS sample buffer (not containing bromophenol blue). The samples were incubated at 100°C

for 10 minutes to elute the fusion protein from the protein A. Eluted protein was then dialyzed in PBS at 4°C overnight before measuring protein concentration.

2.3 Protein analysis and detection

2.3.1. Polyclonal antibody production

300μg of sIL-15Rα were subjected to 10% SDS-PAGE and the gel stained in Coomassie Blue (not containing methanol) for 10 minutes. The gel was then de-stained slowly in water until the protein band of 26kD was visible. This band was excised from the gel, transferred into a fresh 1.5ml tube and frozen in liquid nitrogen. The frozen acrylamide gel was then homogenized and 1ml of PBS was added before freezing in liquid nitrogen for 2 minutes. After incubation at 37°C for 30 minutes the homogenized gel was stored at 4°C overnight and spun in a SpinX filter tube (Costar) in a table centrifuge at 13,000 rpm for 5 minutes. After measuring the concentration of sIL-15Rα in the preparation, 250μl of CFA were added to 250μl containing 100μg of gel-purified sIL-15Rα and passed through a syringe needle several times to obtain a homogenous mixture. 10ml blood was taken from a rabbit as pre-immunization sample, before injecting 500μl subcutaneously to two sites (250μl/site). After 14 days, 100μg of gel-purified sIL-15Rα mixed in IFA were applied to two sites subcutaneously as a boost injection. 24 days after the initial immunization, blood was taken from the rabbit and serum antibody titers against sIL-15Rα determined by ELISA. A third injection similar to the first boost injection was given on day 40 to further increase anti-sIL-15Rα-titer in the serum.

2.3.2 Purification of polyclonal antibodies

Antibodies contained in rabbit and sheep that had been immunized with gel-purified sIL-15Rα were purified to increase specificity and to reduce background in ELISA. The polyclonal antibodies obtained were used to detect sIL-15Rα in serum of injected mice.

a. Purification by affinity to Protein A

Polyclonal antibody derived from a rabbit immunized with sIL-15Rα according to the schedule previously described was purified using a commercially available Avid-Chrome Protein A antibody purification kit (Sigma, USA). Antibodies were purified according to the protocol provided by the manufacturer. In brief, 2ml rabbit serum was filtered through a 0.45μm syringe filter before adding 4ml of binding buffer. The Protein A cartridge provided in the kit was equilibrated by slowly passing 4ml of binding buffer through the cartridge at no more than 1ml/minute flow rate. The sample was then applied to the cartridge at a flow rate of no more than 0.5ml/minute followed by 6ml of binding buffer. The desalting cartridge provided was then attached to the bottom end of the Protein A cartridge and serum antibodies eluted with 5ml of elution buffer at 0.5ml/minute.

b. Purification by precipitation with ammonium-sulfate

Polyclonal anti-sIL-15Rα antibody raised in sheep (SAPU, UK), where purified by sequential precipitation in aqueous saturated $(NH_4)_2SO_4$ to reduce background-binding levels and to raise antibody specificity. Protein contained in 1ml of sheep anti-sIL-15Rα antiserum was precipitated by adding 30% (v/v) aqueous saturated $(NH_4)_2SO_4$ at 4°C and incubation on ice for 30 minutes. Samples were centrifuged at 6000x g in a table centrifuge. The protein pellet was resuspended in 1ml PBS at 4°C. 30% (v/v) of aqueous

saturated $(NH_4)_2SO_4$ was added at 4°C and protein allowed to precipitate on ice for 30 minutes. The supernatant was then discarded and the protein pellet slowly resuspended in PBS. Supernatant and resuspended protein pellets from both precipitation steps were dialyzed in PBS at 4°C overnight and analyzed for binding activity to sIL-15Rα.

2.3.3 Enzyme linked immunosorbent assay (ELISA)

a. General ELISA protocol

96-well ELISA plates (Immulon 4, Dynatech Laboratories, Chantilly, VA) were coated overnight at 4°C with capture antibody or recombinant protein at 2-4μg/ml in 0.1M bicarbonate coating buffer (pH 9.2). Plates were then washed twice with PBS/0.05% Tween20 (PBS/Tween) and blocked by adding 200μl/well 10%FCS/PBS for 2 hours at 37°C. The plates were then washed twice as before and protein-samples were added at graded dilutions or concentrations. After incubation at 37°C for 1-2 hours, the plates were washed four times with PBS/Tween and detecting antibody was added at appropriate dilutions either recommended by the manufacturer or determined experimentally. After incubation for 1hour at 37°C, the plates were washed six times with PBS/Tween. For HRP-conjugated detecting antibodies, 100μl/well TMB microwell peroxidase substrate (Kirkegaard & Perry Laboratories, MA, USA) were added and the optical density measured at 630nm. For biotinylated detecting antibodies, 50μl/well peroxidase-conjugated extravidin (1:1000 Sigma, USA) was added for 30 minutes at 37°C and the plate was washed eight times with PBS/Tween prior to adding TMB substrate. Where indicated, secondary detecting antibodies were used.

b. Binding of recombinant sIL-15Rα to simian IL-15

For ELISA assays, microwell plates were coated with 1 μg/ml of sIL-15Rα in 0.1 M NaH_2CO_3 overnight at 4°C. After blocking with 10% FCS (Life Technologies), graded concentrations of simian rIL-15 or 500 pg/ml mIL-2 (Genzyme, Cambridge, MA) were added. After washing with PBS/Tween, biotin-conjugated anti-murine IL-2 or anti-simian IL-15 (200 ng/ml) was added respectively, followed by avidin-peroxidase (1:1000 dilution, Sigma, Poole, U.K.), and developed with 100μl/well of TMB substrate.

c. Cytokine ELISAs

Murine TNFα, IFNγ, IL-4, IL-6, and IL-10 were assayed using paired antibodies (PharMingen, San Diego, CA) according to the manufacturer's instructions. Lower limits of detection were as follows: IL-4, IL-6, and TNFα were all at 10pg/ml; IL-10 was at 80pg/ml; and IFNγ was at 150 pg/ml. Serum anti-collagen II Ab titers in pooled sera were measured by ELISA. 96-well plates (Immulon 4, Dynatech Laboratories) were coated with 4μg/ml bovine type II collagen in 0.1M NaH_2CO_3 overnight at 4°C, blocked with 10% FCS/PBS, and serial dilutions of sera were added. Bound total IgG was detected with HRP-conjugated goat anti-mouse IgG (Genzyme). IgG1 and IgG2a were detected with biotin-conjugated anti-mouse IgG1 and IgG2a (PharMingen) respectively, and developed as described above. Plates were read at 630nm.

d. Anti-sIL-15Rα antibody titers in serum of immunized rabbits

Polyclonal anti-sIL-15Rα antibody was raised in a rabbit. Antibody titers were determined by ELISA. Plates were coated with 2μg/ml of sIL-15Rα in 0.1M NaH_2CO_3 overnight at 4°C. After blocking at 37°C for 2 hours in 10% FCS/PBS, serial dilutions of anti-serum were added for 1hour at 37°C with rabbit pre-immune serum serving as a

negative control. An HRP-conjugated donkey-anti-rabbit IgG-mAb was used as a detecting antibody.

e. Anti-sIL-15Rα antibody in treated mice

Measurement of anti-sIL-15Rα-antibodies in the serum of mice treated with the sIL-15Rα was determined by ELISA. 96 well ELISA plates were coated with 4μg/ml of sIL-15Rα in 100mM NaH_2CO_3 at 4°C overnight. After blocking with 10% FCS/PBS for 2 hours at 37°C, serum of mice treated with sIL-15Rα as well as serum derived from control mice were added in double dilutions. Following washes with PBS/Tween, anti-sIL-15Rα antibodies were detected by HRP-conjugated goat anti-mouse IgG (Genzyme) and developed using the TMB substrate as described before.

f. Determination of serum sIL-15Rα concentrations

To measure the concentration of sIL-15Rα in murine serum, 4μg/ml of polyclonal sheep anti sIL-15Rα antibody (purified by ammonium-sulfate precipitation) was coated on 96-well plates (Immulon 4, Dynatech Laboratories, Chantilly, VA) in 0.1 M NaH_2CO_3 at 4°C overnight. Serum from mice injected with sIL-15Rα was added and incubated for 2 hours at 37°C. Polyclonal rabbit-anti-sIL-15Rα (Protein A affinity-purified) used as a primary detecting antibody was added at 4μg/ml in 10% FCS/PBS for 1hour at 37°C. A donkey anti-rabbit IgG mAb conjugated with HRP (Genzyme) was used as a secondary detecting antibody. Plates were developed using 100μl/well TMB substrate and read at 630nm as described above.

2.3.4 Preparation of total protein extracts from J774 cells

Treated cells (10^7 cells/sample) were washed two times with ice cold TBS and harvested with a cell scraper (Costar). The cell suspension was then spun at 450x g in a Mirage 2000 centrifuge and the cell pellet resuspended in 0.5ml cell lysis buffer. The cells were lysed on ice for 30 minutes and the lysate transferred to an Eppendorf tube followed by centrifugation at 13,000x g at 4°C for 10 minutes. Supernatants containing solubilized protein were stored at –80°C until use.

2.3.5 Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE ranging from 10-15% acrylamide was performed according to the molecular weight of the protein sampled. Acrylamide was made as a mixture of acrylamide and bis-acrylamide in a ratio of 29:1. Resolving (lower) gels ranging from 10% to 15% were made in resolving gel buffer containing 1% w/v ammonium persulphate (BDH) and 0.1% TEMED (Sigma). The mixed gels were poured between glass plates in a SDS-PAGE gel chamber (BHD) and allowed to polymerize. Stacking (upper) gels were prepared in a similar way using stacking gel buffer containing ammonium persulfate and TEMED. Electrophoresis was performed in SDS-PAGE buffer. Protein samples were mixed with 2x-concentrated SDS-sample buffer and heated to 100°C for 10 minutes prior to loading on gels. Protein molecular weight markers (Rainbow marker, range 14-200 kD, Amersham) were loaded on the gel to compare molecular weight of specific proteins within the samples. Electrophoresis was carried out at 5mA/cm-gel length and allowed to continue until the bromophenol blue dye of the SDS sample buffer reached the bottom end of the gel.

2.3.6 Coomassie Blue staining of SDS-PAGE gels

SDS-PAGE gels were stained in 0.5% Coomassie brilliant blue R250 (Sigma) containing 40% methanol, 10% glacial acetic acid for 1 hour at room temperature. The gels were then destained in 50% methanol, 10% glacial acetic acid for 4-5 hours changing the destaining solution several times until the gel-background was clear and protein bands clearly visible. The stained gels were transferred to filter paper (Whatman 3MM) and dried at 80°C under vacuum. For SDS-PAGE gel purification of sIL-15Rα, gels were stained in a 0.5% aqueous solution of Coomassie brilliant blue for 10 minutes and destained in H_2O until the protein band of interest was clearly visible followed by protein extraction from the gel as described before.

2.3.7 Western blot analysis of proteins

For Western blot analysis, protein samples were electrophoresed in 10-15% SDS-PAGE. A piece of nitrocellulose membrane (BioRad) was cut to fit the SDS-PAGE gel and soaked in Western blot transfer buffer for 10 minutes. Two pieces of filter paper (Whatman 3MM) were cut slightly larger than the gel, soaked in transfer buffer and placed facing the cathode of an electroblotter (Hoeffer, USA). The gel was then placed on top of the pre-soaked filter paper and the membrane placed on top of the gel avoiding air bubbles. Two further pieces of filter paper were cut to cover the membrane lying on top of the gel. Proteins contained in the gel were transferred to the nitrocellulose membrane by applying a constant current of 0.4mA/cm^2 of gel area at 6°C overnight. After the transfer, the nitrocellulose membrane was incubated in 2% BSA/low salt wash buffer for 2 hours at room temperature.

To detect IL-15 binding to sIL-15Rα, recombinant simian IL-15 (a gift of Immunex, Seattle, WA) was added at 20ng/ml in 1%BSA/low salt wash buffer. Binding was allowed to proceed at room temperature for 1 hour. The membrane was then washed for 2x10 minutes in low salt wash buffer, 2x10 minutes in high salt wash buffer and again 2x10 minutes in low salt wash buffer. Binding of IL-15 was detected with mouse anti-human IL-15 mAb (MAB 647, R&D Systems, Oxon, U.K.) at 100ng/ml for 1 hour at room temperature followed by washes as described above. Avidin-peroxidase, (1/1000 dilution in low salt wash buffer) was added and the membrane incubated shaking at room temperature for 30 minutes. After another series of washes the signal was developed by enhanced chemiluminescence (Amersham International, Little Chalfont, U.K.) and exposed to film (Kodak, X-omat).

To detect native IL-15Rα from total protein extracts of J774 cells, polyclonal rabbit anti-sIL-15Rα antiserum was diluted 1:2000 in 1%BSA/low salt wash buffer and applied to the membrane for 1 hour gently shaking at room temperature. Following washes described before, donkey anti-rabbit IgG (HRP conjugated, from SAPU, Carluke, UK) was added for 1 hour at room temperature. After additional washes immuno-reactive bands were visualized using the enhanced chemiluminescence system (ECL, Amersham) described before.

To detect IL-15Rα-IgG$_1$-Fc fusion protein expression from COS-7 cell culture, purified fusion protein was subjected to 10% SDS-PAGE and Western blot. After transfer, the membrane was incubated in 10% w/v milk powder/low salt wash buffer for 2h at room temperature. The membrane was then washed once in low salt wash buffer before adding either anti-c-myc mAb (HRP) (Invitrogen, Netherlands) or goat anti-rabbit IgG (HRP)

(SAPU, Carluke, UK) at 1:2000 dilutions in 5% w/v milk powder in low salt wash buffer followed by shaking at room temperature for 2 hours. The membrane was then washed 2x10 minutes in low salt wash buffer, 2x10 minutes in high salt wash buffer and again 2x10 minutes in low salt wash buffer before enhanced chemiluminescence as described before.

2.4 *In vivo* models

2.4.1 Ethical considerations

All animal experimentation was carried out under a project licence provided by the UK Home Office and was performed to address scientific questions that could not be investigated by *in vitro* studies alone. Mice were obtained from Harlan, UK and kept in designated facilities within the University of Glasgow.

2.4.2 Induction of collagen-induced arthritis in DBA/1 mice

Bovine type II collagen (Sigma) was dissolved at 4mg/ml in 100mM acetic acid/PBS at 4°C overnight. 25mg of H37 mycobacterium TB (Difco, USA) was ground using a pestle and mortar and mixed with 5ml CFA. Dissolved collagen was added to the mix in equal volumes to give a final concentration of 2mg/ml. The mixture was passed through a syringe needle several times to mix. Male DBA/1 mice (6-8-wk-old, Harlan Olac, Bicester, U.K.) received 200 μg of bovine type II collagen, prepared this way, by intra-dermal injection on day 0. After 21 days they received a second injection of collagen (200μg in PBS) intra-peritoneally (i.p.). Daily injections of sIL-15Rα (10 or 40 μg/mouse/day) or human serum albumin (HSA) (40 μg) were administered i.p. for 2 wk starting on day 22. Mice were monitored daily for signs of arthritis, for which severity

scores were derived as follows: 0 = normal, 1 = erythema, 2 = erythema plus swelling, 3 = extension/loss function, and total score = sum of four limbs. Paw thickness was measured with a dial-caliper (Kroeplin, Munich, Germany).

2.5 Tissue culture and *in vitro* assays

2.5.1 Buffers and solutions

ES cell lysis buffer

10mM TrisHCl, pH 7.7 / 10mM EDTA / 10mM NaCl / 0.5% SDS / 100µg/ml proteinase K

Trypsin Solution

1x Trypsin/EDTA (Gibco, BRL) diluted 1:5 in PBS, containing 1% chicken serum

2.5.2 Culture and stimulation of J774 cells

Murine macrophage J774 (American Type Culture Collection (ATCC), USA) cells where grown in RPMI 1640 (supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 25mM HEPES, 10% FCS). 10^7 cells were stimulated for 18 h with recombinant murine IFNγ (100 U/ml, a kind gift of Dr. G. Adolf, Bender Wien, Austria), washed twice with PBS and harvested using a plastic cell scraper. The cell suspension was centrifuged at 450x g and total RNA was prepared as described previously.

2.5.3 Determination of sIL-15Rα bioactivity in CTLL and D10 assay

Cytotoxic T cells CTLL (American Type Culture Collection (ATCC), Rockville, MD) and D10.G4.1 (a T-helper cell line, Kaye *et al.*, 1984 / ATCC) cell lines were grown in RPMI 1640 (supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 25mM HEPES, 10% FCS, 10ng/ml recombinant hIL-2). 24 hours prior to the experiment, recombinant IL-2 was withdrawn from the culture. To block IL-15 activity *in vitro*, cells were cultured in 96-well plates (Nunc, Roskilde, Denmark) with 0.1 ng/ml (for CTLL) or 1.0ng/ml (for D10) of recombinant simian IL-15, which were concentrations that had been previously titrated for optimal proliferation. Recombinant sIL-15Rα (200 ng/ml) was added to triplicate cultures which were then incubated at 37°C in 5% CO_2 for up to 72 h. [^{3}H]-thymidine incorporation over the final 6 h of culture was measured in a beta-plate counter (Wallac Oy, Turku, Finland).

2.5.4 Culture and stimulation of murine spleen cells

Spleen cells were cultured at 2×10^6 cells/ml for up to 96 h in RPMI 1640 (Life Technologies) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 25 mM HEPES buffer, and 10% FCS (all from Life Technologies) at 37°C in 5% CO_2. Cells were stimulated either with graded concentrations of type II collagen or with concanavalin A (ConA) (1μg/ml, Sigma). Proliferation assays were performed in triplicate in U-bottom 96-well plates (Nunc) as previously described. Supernatants from parallel triplicate cultures were stored at -70°C until estimation of cytokine content by ELISA.

2.5.5 Culture and maintenance of COS-7 cells

COS-7 cells (Welcome Research Laboratories, London, UK) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL) containing 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin and 2mM L-glutamine (Gibco BRL).

2.5.6 Stable transfection of COS-7 cells by electroporation

COS-7 cells were grown to confluence in 2x165cm^2 tissue culture flasks (Costar) and washed twice with PBS. Cells were trypsinised to obtain a single cell suspension and counted in a Neubauer cell counting chamber. 10^6 cells were resuspended in 500µl PBS and the cell suspension transferred into an electroporation cuvette. 40µg of *PvuI*-lineralized IL-15Rα-IgG$_1$-Fc expression construct (IL-15Rα-IgG$_1$-Fc in pcDNA3.1 expression vector) was added and the cells transfected by electric pulse (250V / 960µF) in a BioRad gene pulser. Following electroporation, the cells were allowed to stand at room temperature for 10 minutes and transferred to 6ml pre-warmed DMEM. The cells were then equally distributed into 6x100mm tissue culture dishes containing 9ml DMEM and incubated at 37°C / 5% CO_2 for 24 hours.

2.5.7 Selection of G418-resistant COS-7 cells

24 hours after electroporation, cells were grown in DMEM containing 500µg/ml G418 for 14 days. Cell culture medium was replaced every two days. When G418 resistant colonies were visible, cells from each culture dish were treated with 1x trypsin/EDTA to obtain a single cell suspension and washed twice in PBS. After centrifugation at 450x g the cells were resuspended in 5ml DMEM and transferred to a 25cm^2 tissue culture flask.

2.5.8 Transient transfection of COS-7 cells by lipofection

Transient transfection of COS-7 cell was employed to produce and purify recombinant IL-15Rα-IgG$_1$-Fc as an alternative method to stable transfection of COS-7 cells with the IL-15Rα-IgG$_1$-Fc expression construct. The objective of transient protein expression was to obtain a higher yield of recombinant protein due to increased number of plasmid copies per cell compared to cells permanently transfected with the same construct. A commercially available transfection kit was employed to obtain COS cells expressing IL-15Rα-IgG$_1$-Fc (Qiagen, SuperFect). In brief, 2-8x10^5 COS-7 cells were passaged into a 25cm^2 tissue culture flask one day before transfection. On the following day, 5µg non-lineralized plasmid DNA were mixed in 30µl SuperFect solution and complex formation was allowed to occur for 5-10 minutes at room temperature. Cell culture medium was added after this incubation period and the cells were incubated with the transfection mix for 2-3 hours at 37°C and 5% CO_2. The cells were then washed with pre-warmed PBS and cultured in fresh medium for 2-3 days.

2.5.9 Freezing, thawing and long-term storage of COS-7 cells

Cells were allowed to reach confluence in 25cm^2 tissue culture flasks and washed twice in PBS. After treatment with 1x trypsin/EDTA, the cells were centrifuged at 450x g and resuspended in 1.5ml 1x freezing medium (20% FCS / 10%DMSO). The cells were then transferred into 3 freezing vials and placed in a pre-cooled rack at –80°C overnight. The next day, frozen cells were transferred into liquid nitrogen for long-term storage.

2.6 Gene targeting techniques

2.6.1 Culture and maintenance of murine Embryonic Stem (ES) cells

E14/2 ES cells (E14/2-129SV, Center of Genomic Research, Edinburgh, UK) were cultured in BHK21 Glasgow Modified Eagle's Medium (GMEM) (Gibco BRL). The medium was supplemented with 10% ES cell-qualified FCS (Gibco BRL), 1mM sodium pyruvate, 1mM non essential amino-acids, 0.1mM β-mercaptoethanol, 100 U/ml penicillin, 100 U/ml streptomycin and 100U/ml DIA/LIF (Gibco BRL). Tissue culture flasks were coated with 0.1% gelatin / PBS (Sigma) for 10 minutes prior to usage.

2.6.2 Transfection of ES cells by electroporation

E14/2 ES cells were grown to confluence in 4x165cm^2 tissue culture flasks (Costar) and trypsinised to obtain a single cell suspension. Cells were than washed twice in PBS. Cells were counted in a Neubauer cell counting chamber and the cell number adjusted to 10^8 cells in 500µl PBS. 5µl and 20µl of cell suspension respectively were transferred into gelatinized 100mm tissue culture dishes (Costar) containing 10ml of pre-warmed ES cell culture medium as non-transfection controls. The remaining cell suspension was transferred into an electroporation cuvette. 150µg of *NotI*-lineralized targeting construct prepared as described previously were added and the cells electroporated by applying an electric pulse (800V, 3.0µF) in a BioRad gene-pulser. After incubation at room temperature for 10 minutes the cells were transferred into 20ml of pre-warmed ES cell culture medium. The cells were then evenly distributed into 20 gelatinized 100mm cell culture dishes containing 9 ml ES cell culture medium and incubated at 37° / 5% CO_2 for 24 hours.

2.6.3 Selection of geneticin (G418) resistant ES cell colonies

Selection of ES cell colonies resistant to G418 (Sigma) began 24 hours after transfection. Electroporated ES cells were cultured in ES cell culture medium containing 300μg/ml G418 for approximately 10 days until ES cell colonies appeared. The medium was replaced every second day during this period. G418 resistant ES cell colonies were picked into 48 microwell plates (Costar) coated with 0.1% gelatin, expanded and split into one 48- and two 24 microwell plates when confluent. The 48-microwell plate was frozen *in situ* when cells reached confluence while ES cells from 24 microwell plates were used for genomic DNA preparation for Southern analysis and PCR.

2.6.4 Preparation of genomic DNA from ES cells

Genomic DNA from cells grown in 24 microwell plates was prepared by washing cells twice with PBS before adding 300μl ES cell lysis buffer. Plates were sealed with parafilm and incubated at 55°C overnight. The lysate was then transferred to 1.5ml Eppendorf tubes, extracted once with buffer saturated phenol (Gibco BRL) and twice with chloroform. Genomic DNA was precipitated by adding 2 volumes of ethanol and inverting the tube several times before centrifugation at 13,000x g for 10 minutes. DNA pellets were washed with 70% ethanol, dried at room temperature for 5 minutes and resuspended in 100μl TE buffer at 4°C overnight.

2.6.5 Screening for targeting events by Southern hybridization

ES cell clones that had undergone homologous recombination with the targeting construct were identified by Southern hybridization. Genomic DNA derived from

electroporated and selected ES cell clones was subjected to digestion with restriction endonuclease *XhoI*. Restricted gDNA was separated on agarose gels and transferred to nylon membranes as described previously. A 0.9kb DNA fragment, derived from subclone 6.1 by digestion with *Eco*RI and *Xho*I, was used as a radiolabeled external probe to hybridize with a 7kb gDNA fragment corresponding to the wild type IL-15Rα allele. Targeted mutation of the IL-15Rα locus by homologous recombination with the targeting construct was expected to result in the hybridization of the 5' DNA-probe with a 4.2kb fragment due to the insertion of a *Xho*I site into the targeted locus.

2.6.6 Expression of Cre recombinase in targeted ES cells

To delete loxP site flanked sequences in targeted ES cell clones, cells were transiently transfected with the plasmid pCAG-Cre-IP (a kind gift of Dr. A. Smith, Edinburgh). This plasmid expresses Cre recombinase and puromycin acetylase (*pac*). Puromycin is capable of killing non-resistant ES cells within 48 hours of culture and is therefore particularly suitable for selection for transient transfection events. ES cell clones were grown to confluence in 75cm^2 tissue culture flasks. 10^6 cells, trypsinised to obtain a single cell suspension, were subjected to electroporation in a BioRad Gene pulser using 20μg of non-lineralized pCAG-Cre-IP expression plasmid at 800V, 3.0μF.

Electroporated cells were grown in gelatinized 10mm tissue culture dishes in the absence of G418 for 8-10 days replacing. Culture medium was replaced every second day. 1.0μg/ml puromycin (Sigma) was added to the culture medium 24 hours after electroporation for a period of 72 hours.

2.6.7 Detection of Cre-mediated recombination events

Single ES cell colonies where picked, transferred to 48 microwell plates and expanded as described before. Duplicates were made in 48 microwell plates and the cells frozen for short-term storage as described before. Genomic DNA from duplicated clones was prepared as described previously and used in PCR analysis to detect Cre-mediated recombination events of loxP site flanked DNA sequences. PCR oligonucleotides were designed to amplify the loxP-flanked region of the target sequence. The 3'oligonucleotide (3'wt-1) was designed to hybridize outside of the DNA segment covered by the targeting construct. The 5'oligonucleotide was located within the targeted DNA segment (3.5revR-33).

2.6.8 Hybridization, freezing, storage and thawing of ES cells

Short term storage of ES cells in 48 microwell plates. Cells grown to confluence in 48 microwell plates were washed twice with PBS and trypsinised with 200µl 1x trypsin/EDTA (Gibco BRL) to obtain a single cell suspension. One volume of Quench (50% FCS / 50% ES cell culture medium) was added to terminate trypsin digestion. 400µl 2x freezing medium (20% DMSO in ES cell culture medium) were added, the cell suspension mixed well and the plates frozen at –80°C placed in a rack tightly fitted with a filling material to allow the cooling to proceed slowly. Cells frozen in that way were kept for up to 3 weeks.

Long term storage of ES cells in liquid nitrogen. Cells grown to confluence were trypsinised to obtain a single cell suspension and washed twice in PBS. Cells were resuspended in 1x freezing medium containing 20% FCS / 10% DMSO and transferred

into freezing vials. The cell suspensions were then frozen at –80°C in a pre-cooled rack before transferring the vials to liquid nitrogen.

Thawing of ES cells. Cells frozen in 48 microwell plates were thawed by adding 1.5ml pre-warmed ES cell culture medium and gently pipetting up and down. Cells were allowed to attach to the microwell bottom for approximately 6 hours before replacing the medium with fresh ES cell culture medium.

ES cells frozen in liquid nitrogen were thawed by successively adding pre-warmed medium thereby thawing the frozen cells rapidly and transferring them into 10ml of pre-warmed medium. Cells were then pelleted at 450x g in a table centrifuge (Mirage 2000), resuspended in 5ml of fresh ES cell culture medium and transferred to a gelatinized 25cm^2 tissue culture flask.

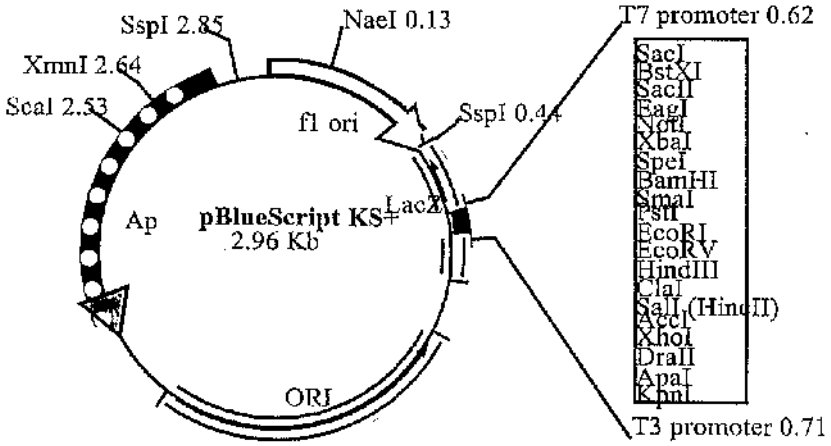
2.7 Statistical analysis

Statistical analysis was performed using Minitab software for Macintosh or PC. The analyses were performed using the log-rank test, Mann-Whitney *U* test, or Student's *t* test as indicated.

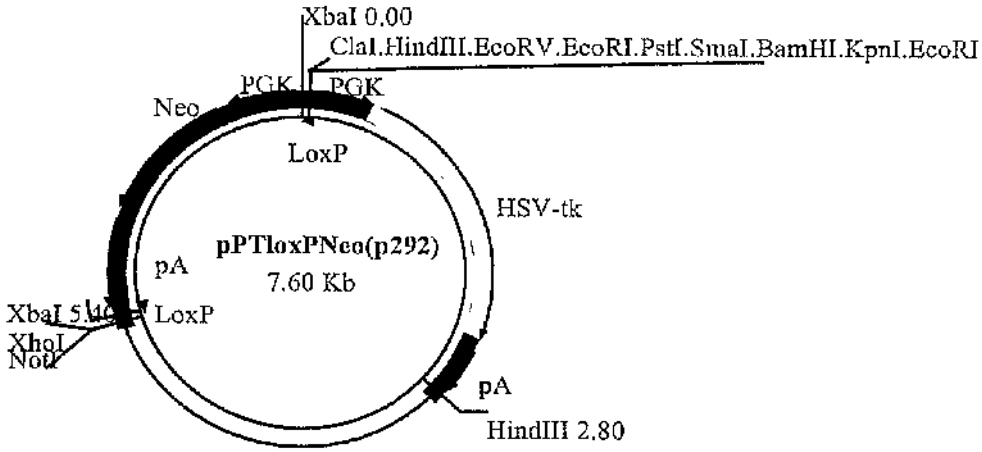
2.8 List of plasmid vectors

Plasmid vectors used for screening, cloning, expression of recombinant proteins or transfection experiments are listed below.

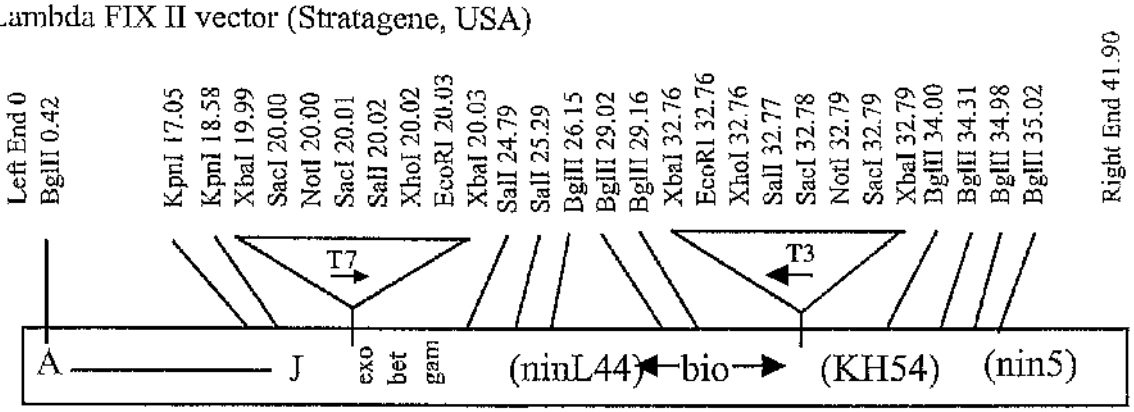
pBlueScript KS+ (Stratagene)



pPTloxPNeo (a kind gift of Dr. Jian Zhong, Wuerzburg)

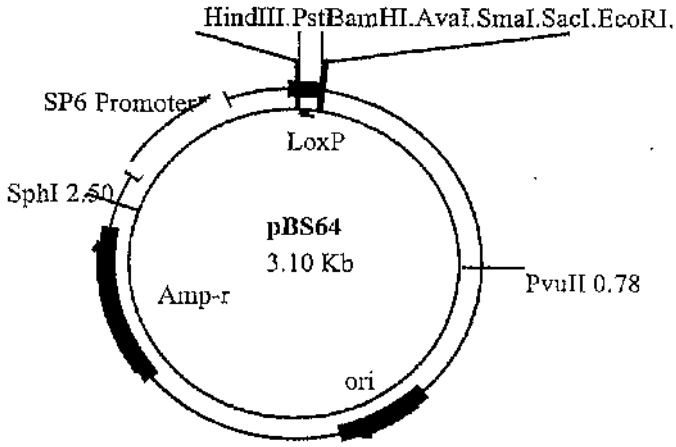


Lambda FIX II vector (Stratagene, USA)

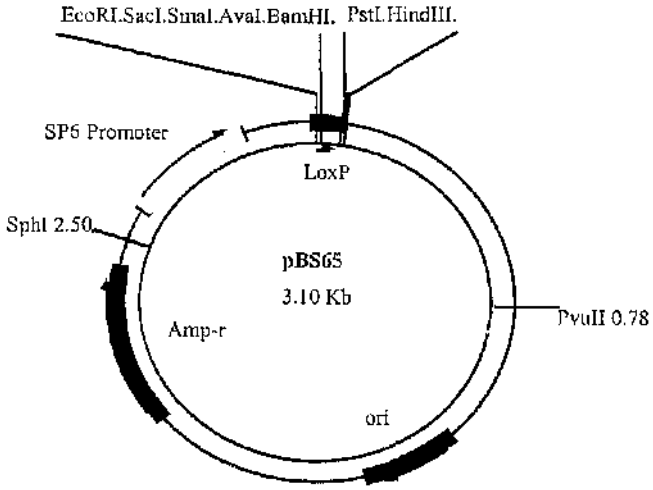


Map of the lambda FIXII vector

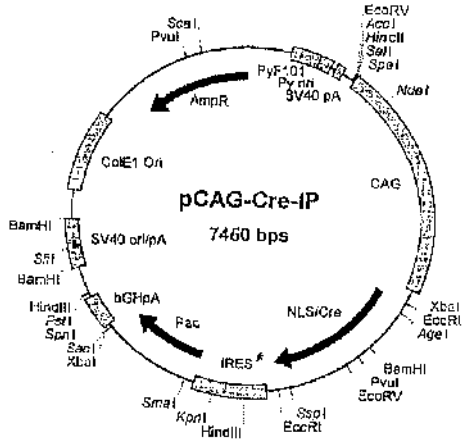
pBS64 (a kind gift of Dr. Jian Zhong, Wuerzburg)



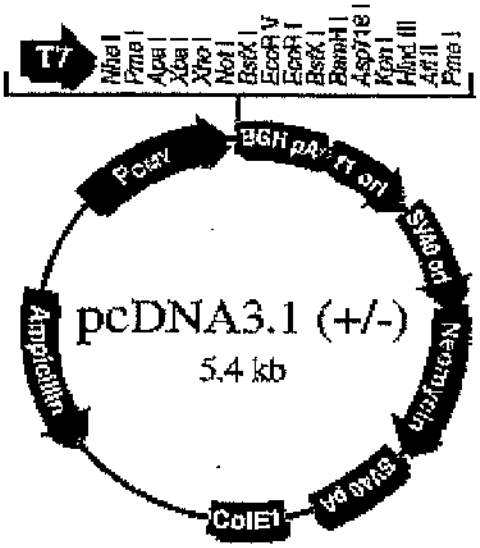
pBS65
(a kind gift of Dr. Jian Zhong, Wuerzburg)



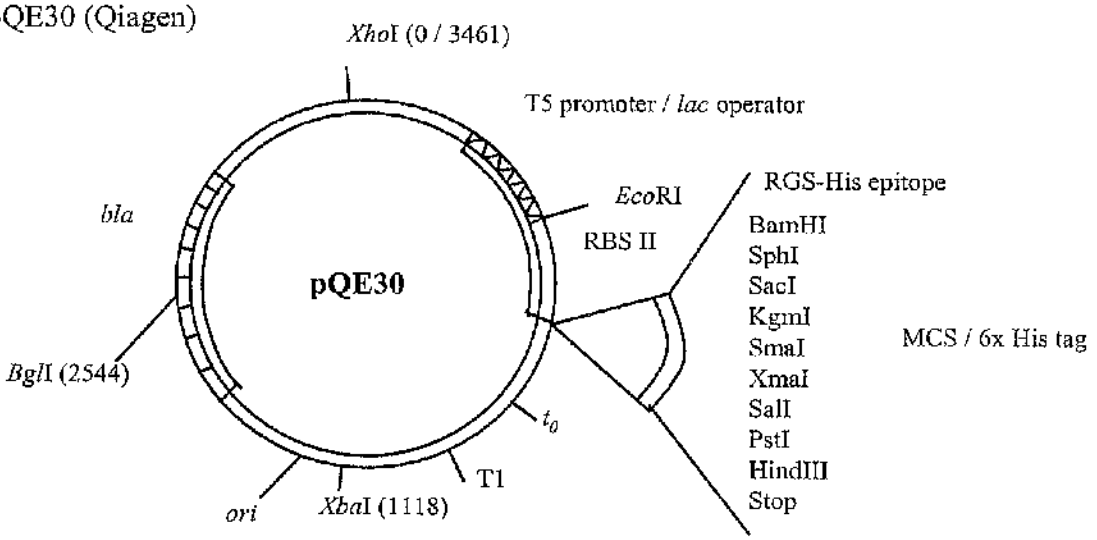
pCAG-Cre-IP
(a kind gift of Dr. A. Smith, Edinburgh)



pcDNA3.1 (Invitrogen)



pQE30 (Qiagen)



Chapter 3

Cloning and expression of recombinant IL-15 receptors

3.1 Cloning and expression of soluble IL-15Rα

3.1.1 Introduction

The general approach underlying the cloning of sIL-15Rα was to express a soluble fragment of the cytokine receptor lacking the native leading sequence, transmembrane region and cytoplasmic tail. The resulting recombinant protein was designed to only contain the protein binding motif and Pro/Thr-rich region of IL-15Rα. A bacterial expression system was chosen to allow inducible, large scale expression under relatively uncomplicated culture conditions. To facilitate purification, a His-tag encoded by the expression vector system (Qiagen, pQE vector) was used. The recombinant protein could therefore be affinity-purified using a metal-agarose chelate system. As sIL-15Rα was found to be best purified under denaturing conditions, refolding of the recombinant protein after purification was done in a high-salt (2% w/v NaCl) PBS buffer.

3.1.2 Cloning of the cDNA insert encoding sIL-15Rα

Murine cDNA was prepared from mRNA extracted from IFNγ-stimulated murine macrophages (J774) by RT-PCR. The nucleotide sequence encoding sIL-15Rα was obtained by nested PCR. The first amplification round yielded a 723bp cDNA fragment but also some unspecific products (Figure 3.1 A). The second amplification round was done using the first PCR product as a template. This second amplification resulted in a PCR product of 541bp (Figure 3.1 B). This band was extracted from the gel and subjected to endonuclease restriction by *Bam*HI and *Sal*I and subsequently cloned both into the pBluescript and pQE30 vector (Figure 3.1 C). Both vectors carrying the DNA insert encoding sIL-15Rα were used to transform competent *E. coli* of the DH5α and M15

strains. Single bacterial colonies derived from these transformations were picked and cultured for small-scale preparations for plasmid DNA. The sequence identity was verified by DNA sequencing. The final product contained 194 amino acid residues, 173 of which encode the "Sushi" domain (also referred to as "short consensus repeat" or GP-I motif) (Davie *et al.*, 1986, Anderson *et al.*, 1995), linker, and Pro/Thr rich region of the native IL-15Rα (see Figure 3.3 for schematic representation).

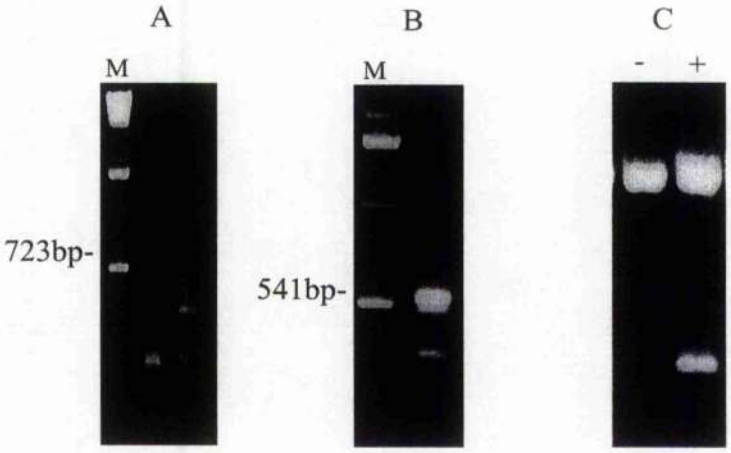


Figure 3.1 A nested PCR approach was employed to clone the cDNA encoding sIL-15Rα. The 1st amplification round yielded a 723bp PCR product but also resulted in unspecific amplification of cDNA resulting in a DNA-smear visible on the agarose-gel (A). The PCR product obtained in the 1st amplification round was used as a template for the 2nd PCR reaction resulting in a 541bp PCR product (B) which, following digestion with *Bam*HI and *Sal*I was purified via agarose gel electrophoresis (C) and cloned in the pQE30 vector (- without; + with insert).

A

1- **ATG**AGAGGAT CGCATCACCA TCACCATCAC GGATCCGGCA CCACGTGTCC

51- ACCTCCCGTA TCTATTGAGC ATGCTGACAT CCGGGTCAAG AATTACAGTG

101- TGAACTCCAG GGAGAGGTAT GTCTGTAACT CTGGCTTTAA GCGGAAAGCT

151- GGAACATCCA CCCTGATTGA GTGTGTGATC AACAAGAACA CAAATGTTGC

201- CCACTGGACA ACTCCCAGCC TCAAGTGCAT CAGAGACCCC TCCCTAGCTC

251 ACTACAGTCC AGTGCCAACA GTAGTGACAC CAAAGGTGAC CTCACAGCCA

301- GAGAGCCCCT CCCCCTCTGC AAAAGAGCCA GAAGCTTTCT CTCCCAAATC

351- AGATACCGCA ATGACCACAG AGACAGCTAT TATGCCTGGC TCCAGGCTGA

401- CACCATCCCA AACAACTTCT GCAGGAACTA CAGGGACAGG CAGTCACAAG

451- TCCTCCCGAG CCCCATCTCT TGCAGCAACA ATGACCTTGG AGCCTACAGC

501- CTCCACCTCC CTCAGGATAA CAGAGATTTC TCCCCACAGT TCCAAAATGA

551- CGAAAGTCGA C CTG CAG CCA AGC TTA ATT AGC **TGA**

B

1- MRGSHHHHHH GTTCPPPVSI EHADIRVKNY SVNSRERYVC NSGFKRKAGT

51- STLIECVINK NTNVAHWTTP SLKCIRDPSL AHYSPVPTVV TPKVTSQFES

101- PSPSAKEPEA FSPKSDTAMT TETAIMPGSR LTPSQTTSAG TTGTGSHKSS

151- RAPSLAATMT LEPTASTSLR ITEISPHSSK MTKVDLQLQP SLIS*

Figure 3.2 Complete cDNA insert nucleotide sequence encoding IL-15Rα (A). Insert sequences are underlined, start and stop codon encoded by pQE30 expression vector are marked in bold. The 5'His tag encoded by vector sequence is double underlined.

Amino acid sequence of sIL-15Rα (B). The complete protein constitutes 194 amino acid residues. Sequences encoded by the cDNA insert are underlined (dashed: Sushi domain; thick: linker/hinge; dotted: Pro/Thr rich region). The N-terminal 6xHis tag encoded by pQE30 is double underlined, * indicates the C-terminus of the amino acid sequence.

3.1.3 Expression of sIL-15Rα in the *E. coli* M15 strain

The pQE30 bacterial expression vector carrying the DNA insert encoding sIL-15Rα was used to transform competent *E. coli* of the M15 strain. 12 single bacterial colonies were picked and subjected to rapid screening for recombinant sIL-15Rα protein expression. Clone Nº 4 was identified to show both maximal expression levels and a protein product of 26kD which was not visible in the unstimulated control (without IPTG) and was subsequently used for all sIL-15Rα preparations.

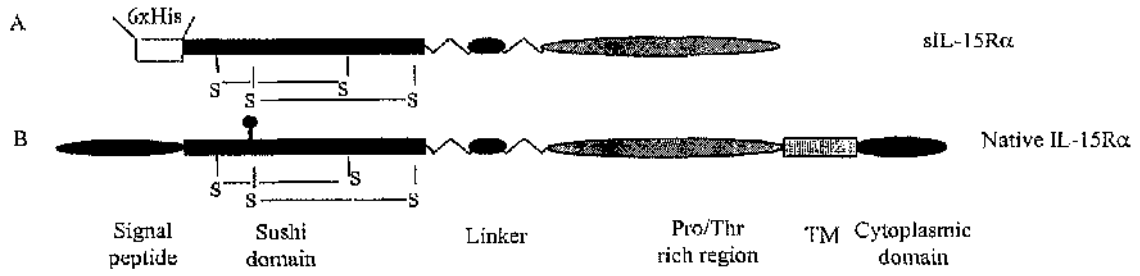


Figure 3.3 Schematic representation of sIL-15Rα (A) compared to native IL-15Rα (B). The recombinant sIL-15Rα includes the protein-binding motif, linker and Pro/Thr-rich region of the native IL-15Rα but lacks its leading sequence, transmembrane region and cytoplasmic domain.

3.1.4 Purification of sIL-15Rα under denaturing conditions

The sIL-15Rα recombinant protein proved to be purified most efficiently under denaturing conditions. Attempts to purify sIL-15Rα under native conditions did not yield satisfactory amounts of recombinant protein. Following purification under denaturing conditions, sIL-15Rα was dialyzed to allow refolding and removal of urea in the preparation. The recombinant protein was insoluble in PBS at concentrations exceeding 250μg/ml. The NaCl concentration of the dialysis buffer was subsequently raised to 2% (w/v) to increase solubility resulting in an optimal concentration of 400μg/ml after dialysis. The yield of recombinant protein ranged between 1.0mg and 1.2mg of sIL-15Rα per liter of bacterial culture. Purity of the protein preparation was assessed by SDS-PAGE followed by Coomassie Blue staining. The possible presence of LPS due to contamination of the recombinant protein derived from the expression culture was measured using the Limulus test (Sigma). Binding of sIL-15Rα to recombinant simian IL-15 was demonstrated in ELISA and Western blot analysis.

3.1.5 Specific binding of sIL-15Rα to IL-15

ELISA and Western blot were employed to demonstrate specific and high affinity binding of sIL-15Rα to IL-15. In ELISA, sIL-15Rα was coated to the ELISA plate at 4°C overnight. Simian IL-15 was added at various concentrations and binding to sIL-15Rα was detected using an anti-human IL-15 mAb. IL-15 was detectable to a minimal concentration of 25pg/ml and bound to sIL-15Rα in a dose dependent manner (Figure 3.4). However, sIL-15Rα did not bind to murine IL-2 when added to the sIL-15Rα-coated plates and developed by anti-murine IL-2 mAb. The recombinant sIL-15Rα was also tested in Western blot analysis. In Western blot, sIL-15Rα was subjected to SDS-PAGE and

transferred on nitrocellulose membrane. Similar to the detection method used in ELISA, simian IL-15 was added and the signal developed using an anti-human IL-15 mAb. A single protein band of approximately 26kD which corresponded to the major band seen on Coomassie Blue stained SDS-PAGE gels was observed (Figure 3.5).

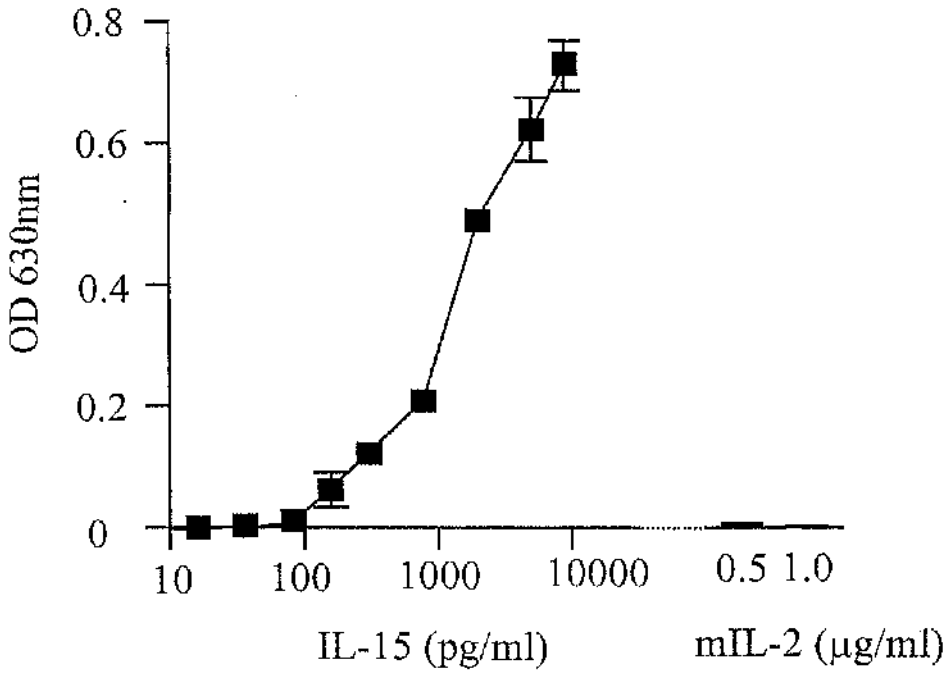


Figure 3.4 Binding of IL-15 to sIL-15Rα in ELISA. 2μg/ml of sIL-15Rα were coated on ELISA plates. Simian IL-15 bound strongly to sIL-15Rα up to a minimal concentration of 25pg/ml. Soluble IL-15Rα did not bind to murine IL-2. Data expressed as mean ± SEM.

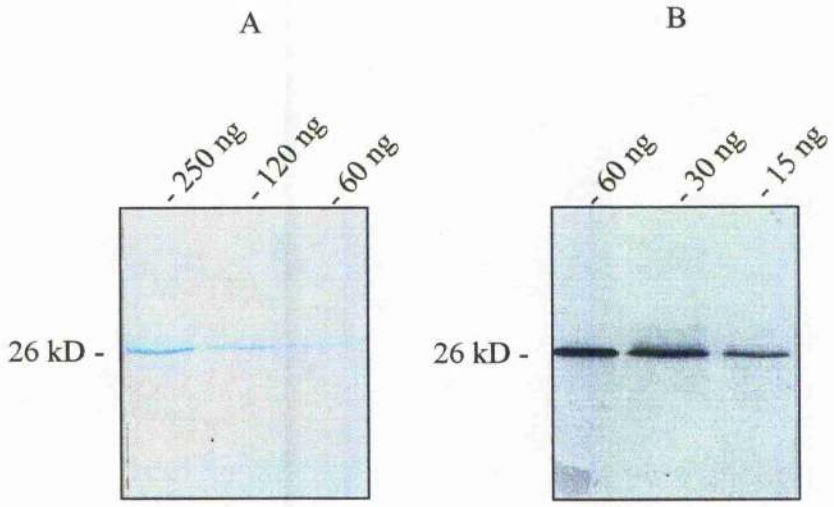


Figure 3.5 Coomassie blue staining (A) and Western blot analysis (B) of sIL-15Rα. Recombinant sIL-15Rα has a molecular weight of 26kD shown in SDS-PAGE and Coomassie Blue staining loading 250, 120 or 60ng of sIL-15Rα (A). The sIL-15Rα bound to simian IL-15 in Western Blot analysis (B). 60, 30 and 15ng of sIL-15Rα were run on SDS-PAGE and subjected to Western blotting. Simian IL-15 was added and binding to a 26kD protein band was detected using anti-human IL-15 mAb.

3.1.6 Bioactivity of sIL-15Rα in CTLL and D10 assays

The recombinant sIL-15Rα was assessed for its ability to inhibit IL-15-induced proliferation of CTLL and D10 cells. IL-15Rα was originally cloned from D10 cells (Giri *et al.*, 1995) and appears to over-express the high affinity IL-15R exhibiting between 5000 and 15000 high affinity binding sites for IL-15 on each cell. Addition of 200ng of sIL-15Rα reduced IL-15 induced proliferation by up to 90% depending on IL-15 concentration (Figure 3.6 A). Optimal inhibition was achieved for CTLL cells stimulated with 0.1ng/ml (B) and D10 cells stimulated with 1.0ng/ml (C) of simian IL-15 for 72h. Addition of sIL-15Rα did not inhibit IL-2 dependent proliferation. Addition of IL-15 over 10ng/ml reversed the inhibitory effect of sIL-15Rα.

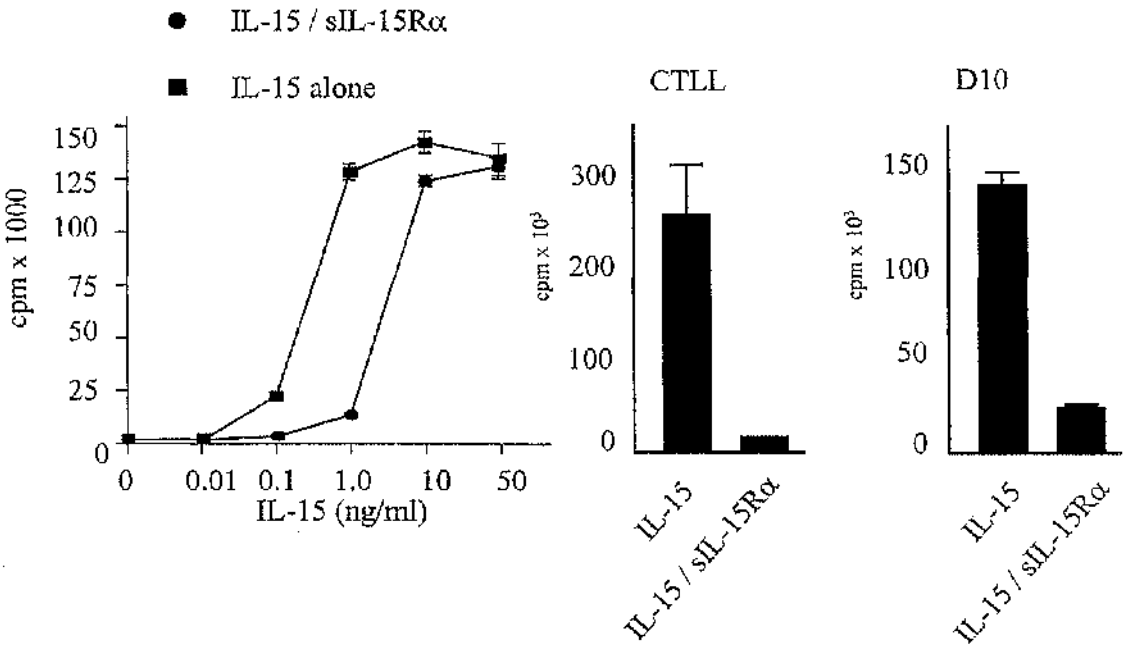


Figure 3.6 (A) Inhibitory effect of sIL-15Rα on IL-15 induced proliferation of D10 cells. Cells were stimulated with increasing concentrations of IL-15 containing 200ng/ml of IL-15Rα or IL-15 alone. (B) Inhibition of CTLL proliferation at 0.1ng/ml of IL-15. (C) Inhibition of D10 proliferation at 1.0ng/ml of IL-15. Data are expressed as mean ± SEM for triplicate cultures.

3.1.7 Gel-purification of sIL-15Rα

Soluble IL-15Rα was purified via SDS-PAGE prior to immunization of animals for polyclonal antibody production. Gel purification removed all additional protein bands previously visible in Coomassie Blue stained acryamide gels when high amounts of sIL-15Rα were run in SDS-PAGE. Approximately 80% of the protein loaded on the gel could be recovered after gel purification.

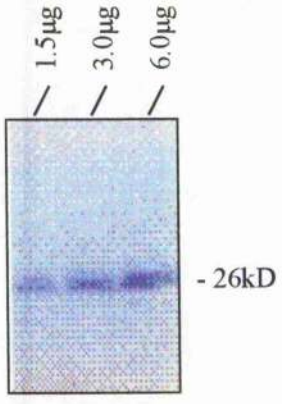


Figure 3.7 Gel purification of sIL-15Rα for rabbit-immunization. Various amounts of gel-purified sIL-15Rα were run on SDS-PAGE to demonstrate purity prior to immunization of rabbits for polyclonal antibody production.

3.1.8 Polyclonal anti-sIL-15Rα-antibody production

Polyclonal antibodies detecting sIL-15Rα were raised in a rabbit following the immunization protocol described in chapter 2. Gel purified recombinant sIL-15Rα was also used to generate sheep-anti-sIL-15Rα-Ab by a commercial company (SAPU, UK). Polyclonal antiserum derived from the sheep was made available to the Dept. of Immunology and purified via sequential precipitation by ammonium-sulfate as described before (see Chapter 2 and below).

Rabbit serum was obtained by bleeding on day 24 p.i. and anti-sIL-15Rα antibody titers compared with titers of pre-immune serum by ELISA. The specificity of raised rabbit-antibody was confirmed by Western blot analysis taking total protein extract of J774 cells stimulated with LPS/IFNγ as a sample. To determine if rabbit-anti-sIL-15Rα antiserum interacts with the 6xHis tag of the recombinant protein, recombinant murine IL-18 (a kind gift of Dr. X.Q. Wei, Dept. of Immunology), purified using the same purification system employed for sIL-15Rα was used as a control. In ELISA rabbit antiserum bound to sIL-15Rα at detectable levels up to a dilution of 1:4000, whereas pre-immune serum did not bind to sIL-15Rα (Figure 3.8 A). In Western Blot analysis rabbit-anti-sIL-15Rα strongly bound to several protein bands ranging between 55kD and 60kD corresponding to the molecular weight of native IL-15Rα (Giri, *et al.*, 1995). Recombinant IL-18 was not detected by rabbit-anti sIL-15Rα-antiserum (Figure 3.8 B). Anti-sIL-15Rα-antibodies from rabbit and sheep were tested for their ability to interfere with the binding of sIL-15Rα to simian IL-15. In CTLL proliferation assays, antiserum derived from sheep but not rabbit-anti-sIL-15Rα antiserum inhibited the bioactivity of sIL-15Rα by restoring IL-15-induced proliferation indicating that sheep anti-sIL-15Rα antiserum detected epitopes contained within the protein binding motif of sIL-15Rα (Figure 3.8 C).

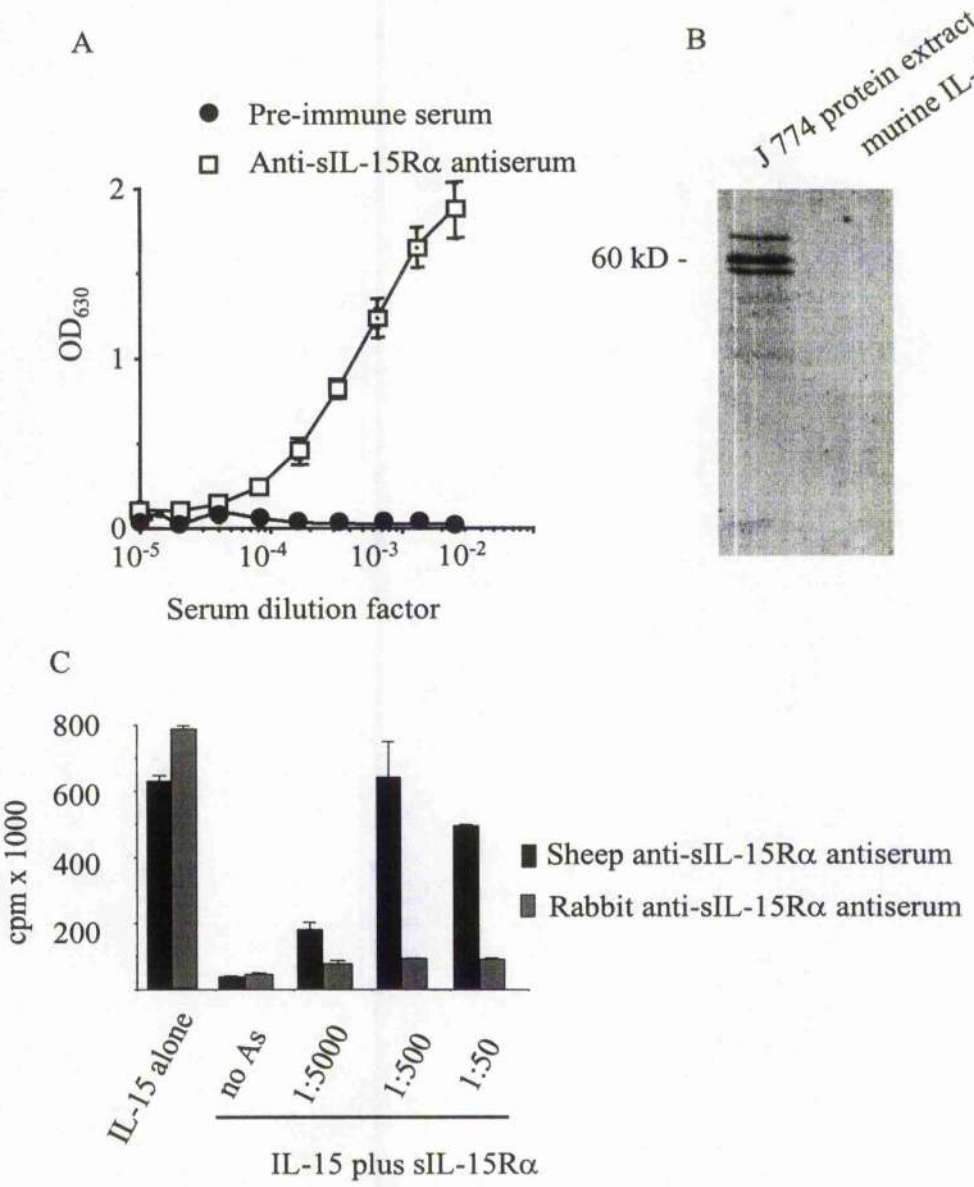


Figure 3.8 (A) ELISA demonstrating the detection of sIL-15Rα coated on the plate by unpurified polyclonal anti-sIL-15Rα-Ab raised in rabbit. Data are presented as mean ± SEM. (B) Western blot analysis using polyclonal anti-sIL-15Rα-Ab to detect native IL-15Rα in protein extracts of J774 cells stimulated with LPS/IFNγ. Murine recombinant IL-18 containing a C-terminal histidine-tag was used as a negative control peptide. (C) CTLL proliferation assay. Sheep but not rabbit anti-sIL-15Rα antiserum inhibited sIL-15Rα-mediated reduction of IL-15-induced proliferation. Data are presented as mean ± SEM.

3.1.9 Purification of polyclonal anti-sIL-15Rα antibodies

Rabbit-anti-sIL-15Rα IgG was purified from serum derived from an immunized rabbit upon completion of the immunization procedure. A commercially available Protein A–IgG purification kit (Sigma, USA) was used. Following the protocol recommended by the manufacturer serum IgG could be purified from crude serum resulting in a final concentration of 1mg/ml.

Sheep-anti-sIL-15Rα antibodies were purified from serum derived from an immunized sheep (SAPU, UK) using sequential precipitation of crude serum with ammonium-sulfate. Four sample fractions were generated corresponding to 50% $(NH_4)_2SO_4$ pellet and supernatant and 30% $(NH_4)_2SO_4$ pellet and supernatant fractions, respectively. The fractions were tested for their ability to serve as capture antibody to detect sIL-15Rα in ELISA. Using sequential precipitation with saturated $(NH_4)_2SO_4$ solution it was concluded that the 30% $(NH_4)_2SO_4$ pellet fraction contained most of the sIL-15Rα binding ability. Coating of a 1:500 dilution of the 30% pellet fraction resulted in a stronger binding signal than coating of crude sheep-anti-sIL-15Rα antiserum, therefore demonstrating an enrichment of anti-sIL-15Rα through the precipitation procedure (Figure 3.9).

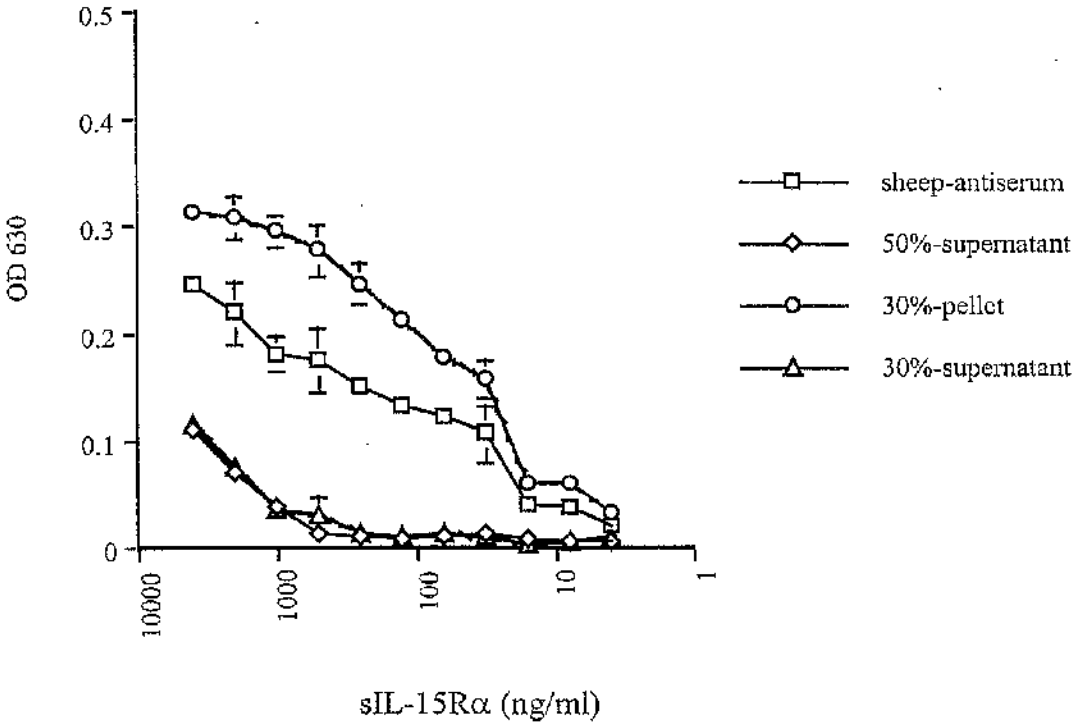


Figure 3.9 ELISA for sIL-15Rα detection using different purification fractions derived from sequential precipitation of sheep-anti-sIL-15Rα antiserum with aqueous, saturated $(NH_4)_2SO_4$. Crude serum was precipitated first with 50% (v/v) $(NH_4)_2SO_4$, centrifuged and resuspended in PBS. 30% (v/v) $(NH_4)_2SO_4$ was added to the resuspended protein and the resulting precipitated fraction was resuspended in PBS. Aliquots of all four fractions were dialyzed in PBS prior to coating on ELISA plates. Amounts of sIL-15Rα added in graded dilution were measured as described before. Data are expressed as mean $OD_{630} \pm$ SEM.

3.1.10 Half-life of sIL-15Rα *in vivo*

To use sIL-15Rα as an anti-inflammatory agent, the time during which sIL-15Rα remains in the blood of an injected individual is an important factor for the design of effective treatment schedules. The biological half-life of sIL-15Rα was measured *in vivo*. Following administration of 80μg of sIL-15Rα into DBA/1 mice by i.p. injection, serum from these mice was taken at 2h, 6h, 12h and 24h post injection (n=3 per time point). The individual serum concentration of sIL-15Rα for each mouse was measured by ELISA and compared to a standard. Figure 3.10 shows that, following a single i.p. injection of sIL-15Rα, the recombinant protein was rapidly cleared from the bloodstream. Two hours after injection, sIL-15Rα-serum levels had declined to approximately 20ng/ml, followed by a 10-fold reduction four hours later. The assay is limited by the sensitivity of the polyclonal antibodies raised in rabbit and sheep with a detection limit of 1ng/ml of sIL-15Rα. Since comparatively low levels of sIL-15Rα are required to block IL-15 mediated proliferation of T cells *in vitro*, low serum concentration of the recombinant protein below 1ng/ml may still block IL-15 function *in vivo*.

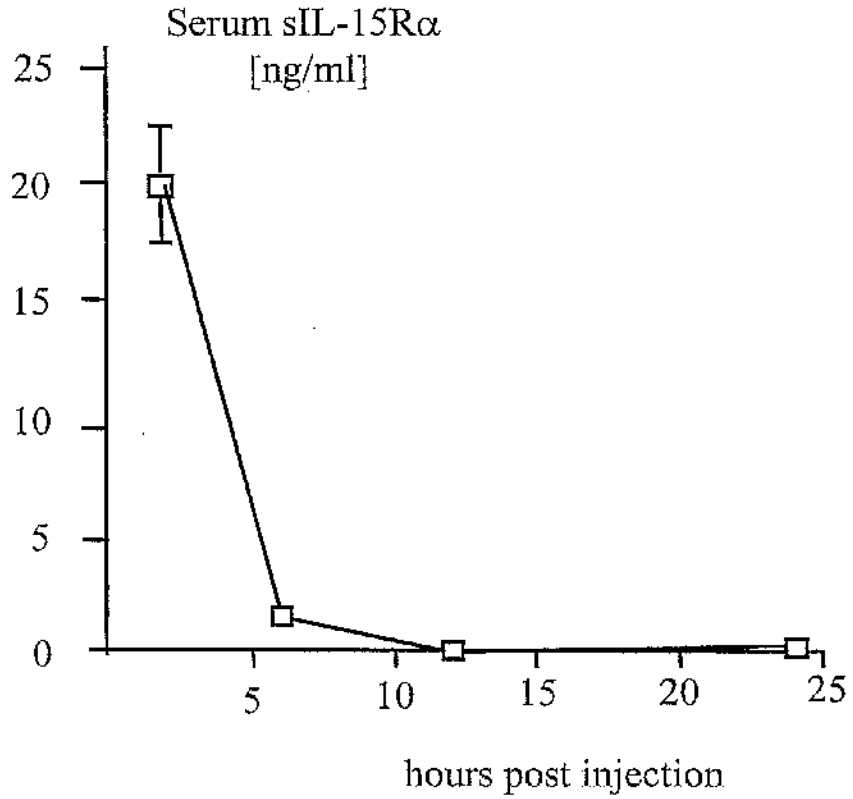


Figure 3.10 Half-life of sIL-15Rα *in vivo*. The highest serum level of sIL-15Rα was detected 2 hours after injection and was followed by a rapid clearance of sIL-15Rα from the circulation. Serum sIL-15Rα levels reached the detection limit of approximately 1ng/ml 5-7 hours after injection.

3.2 Cloning and expression of IL-15Rα-IgG$_1$-Fc

3.2.1 Introduction

A second recombinant IL-15 receptor was obtained by expressing IL-15Rα as an IgG1-Fc fusion protein in a mammalian expression system. This was undertaken under the objective to increase the half-life of recombinant IL-15Rα, which was relatively short for sIL-15Rα reflecting the efficient clearance of a small protein *in vivo*. In comparison to sIL-15Rα, an IL-15Rα-IgG1-Fc fusion protein would be much larger due to the increased number of amino acid residues and the potential for glycosylation on IgG1-Fc in the mammalian expression system. Secondly, any post-translational modifications which are necessary for function, would be more likely to be present in a protein expressed in mammalian cells than in bacterial expression systems. The increased molecular weight and possible interactions of IL-15Rα-IgG1-Fc with endogenous Fc$_\gamma$ receptors was also likely to result in an increased half-life *in vivo*. These reasons make an IL-15Rα-IgG1-Fc fusion protein a desirable reagent as an IL-15 antagonist. Hence, a construct was cloned that expresses the murine IgG1-Fc fused to IL-15Rα under the control of the hCMV promoter.

3.2.2 Cloning of the cDNA insert encoding IL-15Rα-IgG$_1$-Fc

Murine cDNA encoding IgG$_1$-Fc was prepared by RT-PCR from DBA/1 spleens of mice that had undergone collagen-induced arthritis. PCR amplification using specific primers resulted in a DNA fragment of 944bp. This fragment was cut by restriction endonucleases *Xho*I and *Eco*RI using the restriction enzyme sites contained within the primer sequences. The restricted product was cloned into the mammalian expression vector pcDNA3.1. The cDNA encoding the IL-15Rα fragment was cloned using a nested PCR

approach on the same murine cDNA used for IgG_1-Fc cloning. The first amplification round yielded a 723bp fragment, which was used as a template in a second PCR reaction, which resulted in a single PCR product of 668bp. This product was restricted by *Xba*I and *Xho*I endonucleases and cloned into the pcDNA3.1 vector, which already contained the cDNA fragment encoding IgG_1-Fc. The final product contains a 1623bp fragment, which encoded a 541 amino acid residue protein, 37 of which are encoded by vector sequences. The protein product has a predicted molecular weight of 66kD. The sequence identity of the cloned product was confirmed by DNA sequencing and restriction enzyme mapping. (see Figure 3.12 for schematic representation of IL-15Rα-IgG1-Fc)

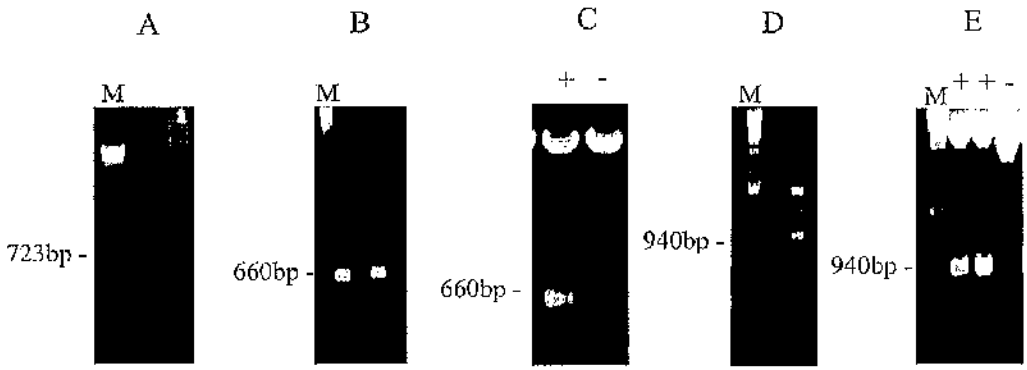


Figure 3.11 Cloning of the IL-15Rα-IgG_1-Fc cDNA. A nested PCR was performed on DBA/1 cDNA derived from spleen cells by RT-PCR to obtain the DNA insert encoding IL-15Rα. A first amplification round yielded a product of 723bp (A) but also resulted in unspecific amplification. This product was used as a template in a second amplification round and resulted in a PCR product of 668bp (B) which was subjected to restriction enzyme digestion by *Xba*I and *Xho*I and cloned into the pcDNA3.1 vector (C). Murine IgG1-Fc was cloned by PCR from DBA/1 spleen cDNA resulting in a product of 944bp (D), which was cut by *Xho*I and *Eco*RI and cloned into pcDNA3.1 already containing IL-15Rα-cDNA (E).

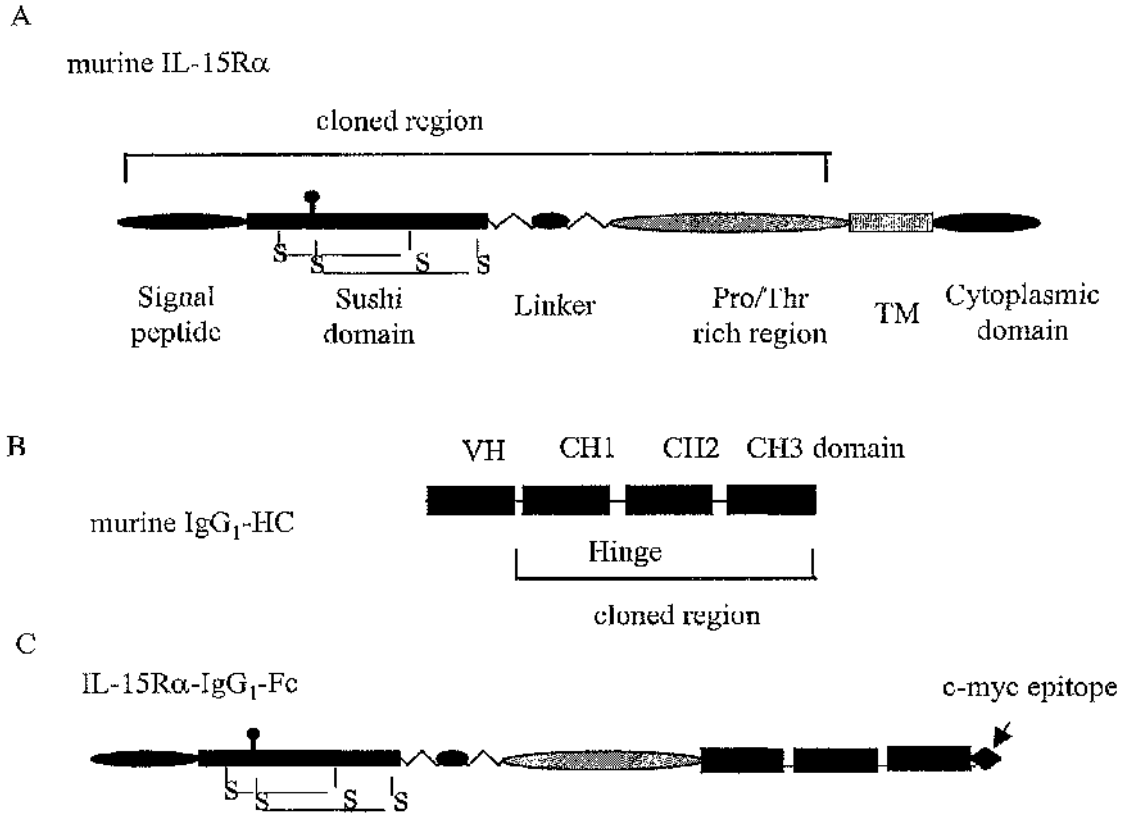


Figure 3.12 Schematic representation of IL-15Rα-IgG$_1$-Fc. The fusion protein includes the leader sequence, binding domain and Pro/Thr rich region of IL-15Rα (A) and the Fc part of murine IgG$_1$ at its C-terminus (B). Fusion of these two components results in the expression of a 66kD protein (C).

A

```
1    -  CGTTGGGTCA CTGCTGGGGA CAATTGGCCA TGGCCTCGCC GCAGCTCCGC
51   -  GGCTATGGAG TCCAGGCCAT TCCTGTGTTG CTGCTGCTGC TGTTGCTACT
101- GTTGCTCCCG CTGAGGGTGA CGCCGGGCAC CACGTGTCCA CCTCCCGTAT
151- CTATTGAGCA TGCTGACATC CGGGTCAAGA ATTACAGTGT GAACTCCAGG
201- GAGAGGTATG TCTGTAACTC TGGCTTTAAG CGGAAAGCTG GAACATCCAC
251- CCTGATTGAG TGTGTGATCA ACAAGAACAC AAATGTTGCC CACTGGACAA
301- CTCCCAGCCT CAAGTGCATC AGAGACCCCT CCCTAGCTCA CTACAGTCCA
351- GTGCCAACAG TAGTGACACC AAAGGTGACC TCACAGCCAG AGAGCCCCTC
401- CCCCTCTGCA AAAGAGCCAG AAGCTTTCTC TCCCAAATCA GATACCGCAA
451- TGACCACAGA GACAGCTATT ATGCCTGGCT CCAGGCTGAC ACCATCCCAA
501- ACAACTTCTG CAGGAACTAC AGCGACAGGC AGTCACAAGT CCTCCCGAGC
551- CCCATCTCTT GCAGCAACAA TGACCTTGGA GCCTACAGCC TCCACCTCCC
601- TCAGGATAAC AGAGATTTCT CCCTCGAGGG TGACCCTGGG ATGCCTGGTC
651- AAGGGCTATT TCCCTGAGCC AGTGACAGTG ACCTGGAACT CTGGATCCCT
701- GTCCAGCGGT GTGCACACCT TCCCAGCTGT CCTGCAGTCT GACCTCTACA
751- CTCTGAGCAG CTCAGTGACT GTCCCCTCCA GCACCTGGCC CAGCGAGACC
801- GTCACCTGCA ACGTTGCCCA CCCGGCCAGC AGCACCAAGG TGGACAAGAA
851- AATTGTGCCC AGGGATTGTG GTTGTAAGCC TTGCATATGT ACAGTCCCAG
901- AAGTATCATC TGTCTTCATC TTCCCCCCAA AGCCCAAGGA TGTGCTCACC
951- ATTACTCTGA CTCCTAAGGT CACGTGTGTT GTGGTAGACA TCAGCAAGGA
1001- TGATCCCGAG GTCCAGTTCA GCTGGTTTGT AGATGATGTG GAGGTGCACA
1051- CAGCTCAGAC GCAACCCCGG GAGGAGCAGT TCAACAGCAC TTTCCGCTCA
1101- GTCAGTGAAC TTCCCATCAT GCACCAGGAC TGGCTCAATG GCAAGGAGTT
1151- CAAATGCAGG GTCAACAGTG CAGCTTTCCC TGCCCCCATC GAGAAAACCA
1201- TCTCCAAAAC CAAAGGCAGA CCGAAGGCTC CACAGGTGTA CACCATTCCA
1251- CCTCCCAAGG AGCAGATGGC CAAGGATAAA GTCAGTCTGA CCTGCATGAT
1301- AACAGACTTC TTCCCTGAAG ACATTACTGT GGAGTGGCAG TGGAATGGGC
1351- AGCCAGCGGA GAACTACAAG AACACTCAGC CCATCATGGA CACAGATGGC
1401- TCTTACTTCG TCTACAGCAA GCTCAATGTG CAGAAGAGCA ACTGGGAGGC
1451- AGGAAATACT TTCACCTGCT CTGTGTTACA TGAGGGCCTG CACAACCACC
1501 ATACTGAGAA GAGCCTCTCC CACTCTCCTG GTAAATGAAT TCCACCACAC
1551 TGGACTAGTG GATCCGAGCT CGGTACCAAG CTTGGGCCCG AACAAAAACT
1601 CATCTCAGAA GAGGATCTGA ATAGCGCCGT CGACCATCAT CATCATCATC
1651 ATTGA
```

Complete nucleotide sequence of IL-15Rα-IgG$_1$-Fc (legend overleaf).

B

1- MASPQLRGYG VQAIPVLLLL LLLLLLPLRV TPGTTCPPPV SIEHADIRVK
51- NYSVNSRERY VCNSGFKRKA GTSTLIECVI NKNTNVAHWT TPSLKCIRDP
101- SLAHYSPVPT VVTPKVTSQP ESPSPSAKEP EAFSPKSDTA MTTETAIMPG
151- SRLTPSQTTS AGTTGTGSHK SSRAPSLAAT MTLEPTASTS LRITEISPSR
201- VTLGCLVKGY FPEPVTVTWN SGSLSSGVHT FPAVLQSDLY TLSSSVTVPS
251- SIWPSETVIC NVAHPASSTK VDKKIVPRDC GCKPCICTVP EVSSVFIFPP
301- KPKDVLTITL TPKVTCVVVD ISKDDPEVQF SWFVDDVEVH TAQTQPREEQ
351- FNSTFRSVSE LPIMHQDWLN GKEFKCRVNS AAFPAPIEKT ISKTKGRPKA
401- PQVYTIPPPK EQMAKDKVSL TCMITDFFPE DITVEWQWNG QPAENYKNTQ
451- PIMDTDGSYF VYSKLNVQKS NWEAGNTFTC SVLHEGLHNH HTEKSLSHSP
501 GKRIPPHWTS GSELGTKLGP EQKLISEEDL NSAVDHHHHH H*

Figure 3.13 (A) Nucleotide sequence of IL-15Rα-IgG$_1$-Fc. Coding nucleotide sequence is underlined. The ATG start and TGA stop codons are in bold. The central *Xho*I and 3' *Eco*RI site are underlined in bold. Vector sequence encoding the *myc* epitope is double underlined. Vector sequence encoding His-tag is underlined dotted.

(B) Predicted amino acid sequence of IL-15Rα-IgG$_1$-Fc. Thick: IL-15Rα signal peptide; single: IL-15Rα domain; dotted: IgG$_1$-Fc domain; double: *myc* epitope; * indicates the C-terminus of the protein.

3.2.3 Expression of IL-15Rα-IgG$_1$-Fc in COS-7 cells

COS-7 cells stably transfected with the IL-15Rα-IgG$_1$-Fc expression construct were separated into single colonies by limiting dilution. 48 colonies were picked and analyzed for recombinant fusion protein expression by DOT blot. Another 48 colonies from non-transfected COS-7 cells were taken as a negative control. A total of 6 COS-7 cell colonies were identified for maximal IL-15 binding capacity and pooled for large-scale expression cultures (Figure 3.14). Using Protein A affinity purification, the recombinant

fusion protein was found to be present in cell lysis preparations rather than in culture supernatants. Approximately 10μg of recombinant protein was obtained per 162cm^2 flask of confluent COS-7 cells (2x10^7).

Since stable transfection of COS-7 cells with the plasmid encoding IL-15Rα-IgG$_1$-Fc resulted in a low yield and poor IL-15 binding capacity of the resulting protein, transient transfection of COS-7 cells using the non-lineralized expression plasmid was performed to increase the copy number of plasmid per cell. Culture supernatant from small-scale transfections in 25cm^2 tissue culture flasks was collected 48 hours after transfection. Anti-IL-15 activity within the supernatant was measured by ELISA and proliferation assay and compared to cell culture supernatant from COS-7 cells transfected with pcDNA3.1 vector alone.

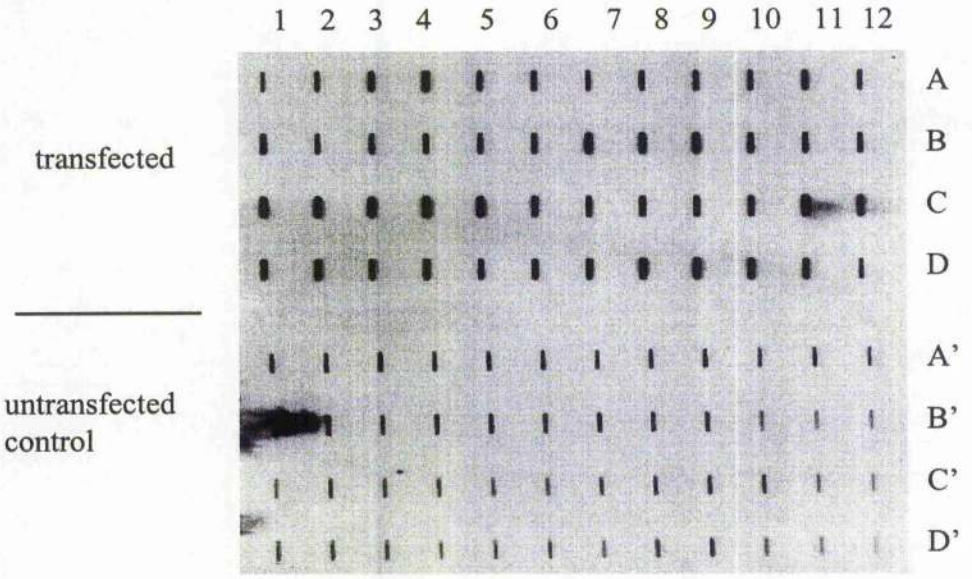


Figure 3.14 DOT blot for the selection of COS-7 colonies expressing maximum quantities of IL-15Rα-IgG_1-Fc. Supernatants from clones of transfected and untransfected COS cells were transferred onto nylon membrane. Expression of recombinant protein was detected by adding simian IL-15 and anti-human-IL-15mAb as a detecting antibody.

3.2.4 Detection of IL-15Rα-IgG$_1$-Fe in Western Blot

Preparations of COS-7 cell lysates and culture supernatants that had undergone Protein A affinity purification were analyzed by Western blot to demonstrate IL-15Rα-IgG$_1$-Fc protein expression. Detection of recombinant protein by a monoclonal antibody recognizing the c-myc epitope contained within the pcDNA3.1 expression vector and a monoclonal antibody directed against murine IgG both resulted in the same protein double band of 66kD (Figure 3.15). No additional bands were visible using these two detection systems. Both antibodies showed a higher expression level of recombinant fusion protein in cell lysates than in culture supernatants. The molecular weight of the COS-7 product was confirmed by Coomassie blue staining of Protein A purified protein resulting in a band of 66kD. No protein band was visible in preparations derived from untransfected COS-7 cells.

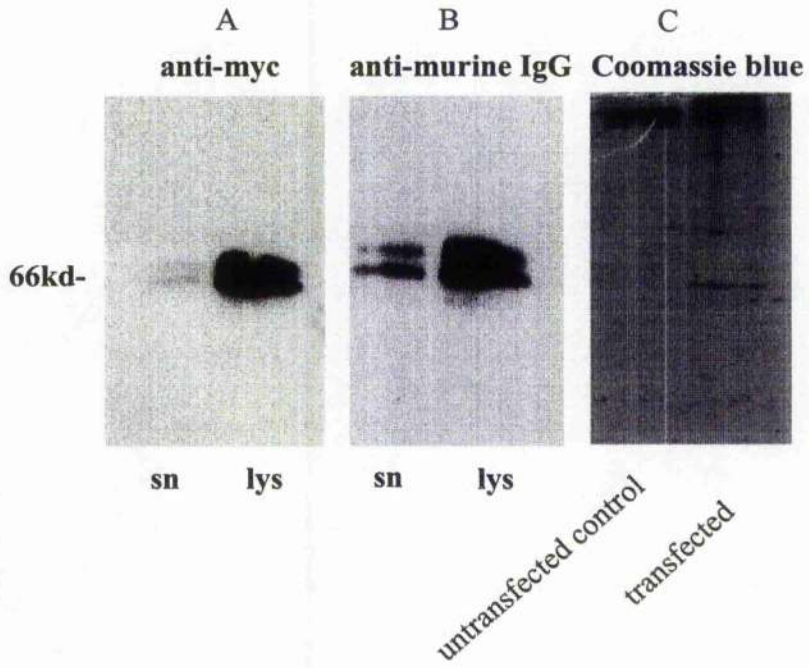


Figure 3.15 Western blot analysis of IL-15Rα-IgG$_1$-Fc prepared from COS-7 cell culture supernatants (sn) and COS-7 cell lysates (lys). The recombinant fusion protein was purified by binding to Protein A coated sephadex beats (Sigma, USA). Two different detecting antibodies were used to show IL-15Rα-IgG$_1$-Fc expression in Western blot analysis. An HRP-conjugated mAb directed against the *myc* epitope (A) binds to a 12 amino acid residue sequence encoded by the 3' nucleotide sequence of the pcDNA3.1 vector. A second HRP conjugated antibody is directed against murine IgG1 (B). Both antibodies detected the same protein band of 66kD. The band intensity suggests that IL-15Rα-IgG$_1$-Fc is only partially secreted in COS-7 cell cultures and preferentially remains within the cells. The molecular weight of the product was reconfirmed by SDS-PAGE followed by Coomassie blue staining (C).

3.2.5 Specific binding of IL-15Rα-IgG$_1$-Fc to IL-15 in ELISA

The specific binding of IL-15Rα-IgG$_1$-Fc to IL-15 was measured by ELISA by its ability to neutralize binding of IL-15 to sIL-15Rα bound to microtiter plates. The cell culture supernatants of COS-7 cells transfected with either pcDNA3.1 alone or the complete IL-15Rα-IgG$_1$-Fc expression construct where collected 48h after transient transfection. Graded dilutions of the different cell culture supernatants were added to microtiter wells that had been previously coated with sIL-15Rα. Simian IL-15 was added to a final concentration of 500 pg/ml. IL-15 that bound to immobilized sIL-15Rα was detected by adding an anti-IL-15 biotinolated mAb. The amount of IL-15 bound to immobilized sIL-15Rα was compared to an IL-15 standard. Cell culture supernatant from COS-7 cells transfected with the IL-15Rα-IgG$_1$-Fc expression construct but not pcDNA3.1 vector neutralized the binding of IL-15 to immobilized sIL-15Rα up to a dilution of approximately 10 fold. The ability of free sIL-15Rα to compete with the binding of IL-15 to immobilized sIL-15Rα was used as a positive assay control. Free sIL-15Rα reduced the binding of IL-15 to immobilized sIL-15Rα at a minimal concentration of 40pg/ml (Figure 3.16). Therefore, these results show the presence of an anti-IL-15 activity in culture supernatant of cells transfected with the IL-15Rα-IgG$_1$-Fc expression plasmid.

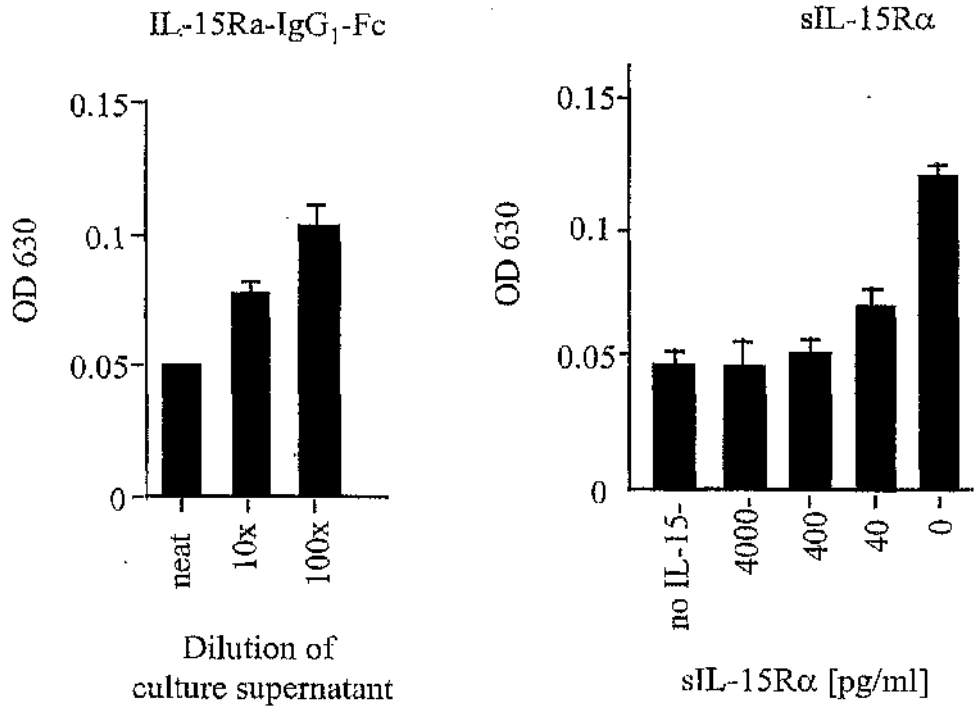


Figure 3.16 Neutralizing effect of cell culture supernatant from IL-15Rα-IgG$_1$-Fc-transfected cells on IL-15 binding by sIL-15Rα. Cell culture supernatant contained an anti-IL-15 activity up to a minimal dilution of approximately 10 fold. Cell culture supernatant from COS-7 cells transfected with pcDNA3.1 alone did not contain anti-IL-15 activity. Free sIL-15Rα competes with immobilized sIL-15Rα at a minimal concentration of 40pg/ml. Data are expressed as means of triplicates ± SEM.

3.3 Summary

The cDNA encoding the extracellular domain of IL-15Rα was cloned from the murine macrophage cell line J774 by RT-PCR. Sequence analysis of the cloned cDNA fragment confirmed 100% sequence identity with murine IL-15Rα. Expression of the peptide encoded by the cDNA fragment as a histidine-tagged recombinant protein in a bacterial expression system resulted in a product of 26kD. This protein bound to simian IL-15 in ELISA and neutralized the IL-15 induced proliferation of the T cell lines CTLL and D10. Polyclonal antibodies against sIL-15Rα have been raised in rabbit and sheep and used to determine the half-life of sIL-15Rα in the murine bloodstream.

A second cDNA fragment encoding IL-15Rα extracellular domain has been cloned from DBA/1 spleen cells by RT-PCR. The cloned cDNA has been linked to a cDNA fragment encoding murine IgG_1-Fc, which has been derived from DBA/1 spleen cells by RT-PCR. The resulting cDNA-construct was cloned into a mammalian expression vector and used to express IL-15Rα-IgG_1-Fc in COS-7 cells. The resulting peptide had a molecular weight of 66kD and could be detected by antibodies against murine IgG1 and the *myc* epitope encoded by the expression vector. Culture supernatant of COS-7 cells transiently transfected with the expression construct contained an anti-IL15 activity, which was not found in culture supernatant derived from cells transfected with the empty vector alone.

Chapter 4

Soluble IL-15Rα in collagen-induced arthritis

4.1 Introduction

The presence of IL-15 in the RA synovium has been previously demonstrated (McInnes *et al.*, 1996). In the synovium, IL-15 may recruit and activate synovial T cells in the relative absence of IL-2. Following IL-15-mediated activation, synovial T cells induce TNFα synthesis by macrophages through cognate interactions indicating an important role for IL-15 in the inflammatory cascade within the synovium (McInnes *et al.*, 1997). Recombinant sIL-15Rα was therefore used to antagonize IL-15 functions in a murine model of RA, collagen-induced arthritis (CIA). The project was carried out in collaboration with Dr. Bernard Leung, Dept. of Immunology. The results were published jointly under shared first authorship (Ruchatz, *et al.*, 1998, reprint attached). In this study it was demonstrated that sIL-15Rα prevented the development of CIA and had a profound effect on the immunological response generated by the disease.

4.2 Administration of sIL-15Rα prevents the development of CIA

The effect of sIL-15Rα administration on CIA in susceptible male DBA/1 mice was monitored since the immunopathogenesis of CIA closely resembles RA. Mice injected intradermally with type II collagen in Freund's complete adjuvant developed severe arthritis when challenged i.p. 21 days later with collagen. The incidence and severity of disease development was markedly suppressed in mice that received daily i.p. injections of 40μg of sIL-15Rα beginning on the day after collagen challenge (day 22) in comparison with controls which received HSA (see Figure 4.1; legend shown on page 147). Histologic examination of the hind limb joints from HSA-treated mice revealed massive mononuclear and polymorphonuclear infiltration of the synovial membrane with synovial hyperplasia and adjacent bone erosion. Administration of sIL-15Rα markedly suppressed each of these parameters (Figure 4.2).

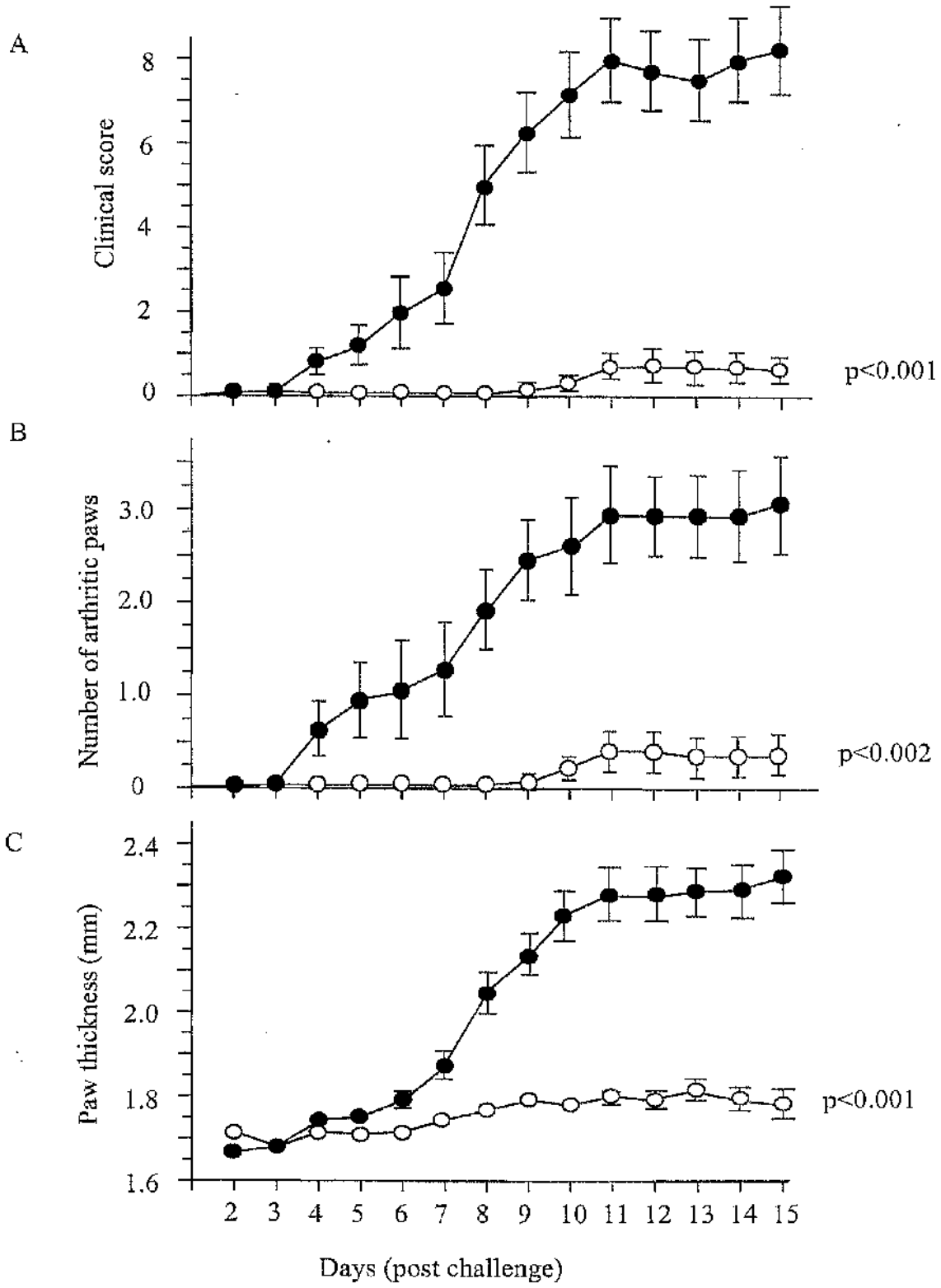

A
Clinical score
0
2
4
6
8
p<0.001
B
Number of arthritic paws
0
1.0
2.0
3.0
p<0.002
C
Paw thickness (mm)
1.6
1.8
2.0
2.2
2.4
p<0.001
2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days (post challenge)

Figure 4.1. (legend for figure on previous page) sIL-15Rα inhibited the development of CIA. Collagen-primed DBA/1 mice were randomly divided into groups of 10, challenged on day 21, and given 14 daily i.p. injections of 40μg of sIL-15Rα (○)or 40μg of HSA (●) starting on day 22. Mice were monitored daily for disease progression, which was quantified as mean clinical score (A), mean number of arthritic paws (B), or mean paw thickness (C). Values are mean ± SEM. At the end of treatment (day 36), 20% of mice developed mild CIA in the sIL-15Rα-treated group compared with 90% which developed severe CIA in the HSA control group ($p < 0.05$). The total number of sIL-15Rα-treated mice was 29, while the total number of HSA control mice was 19.

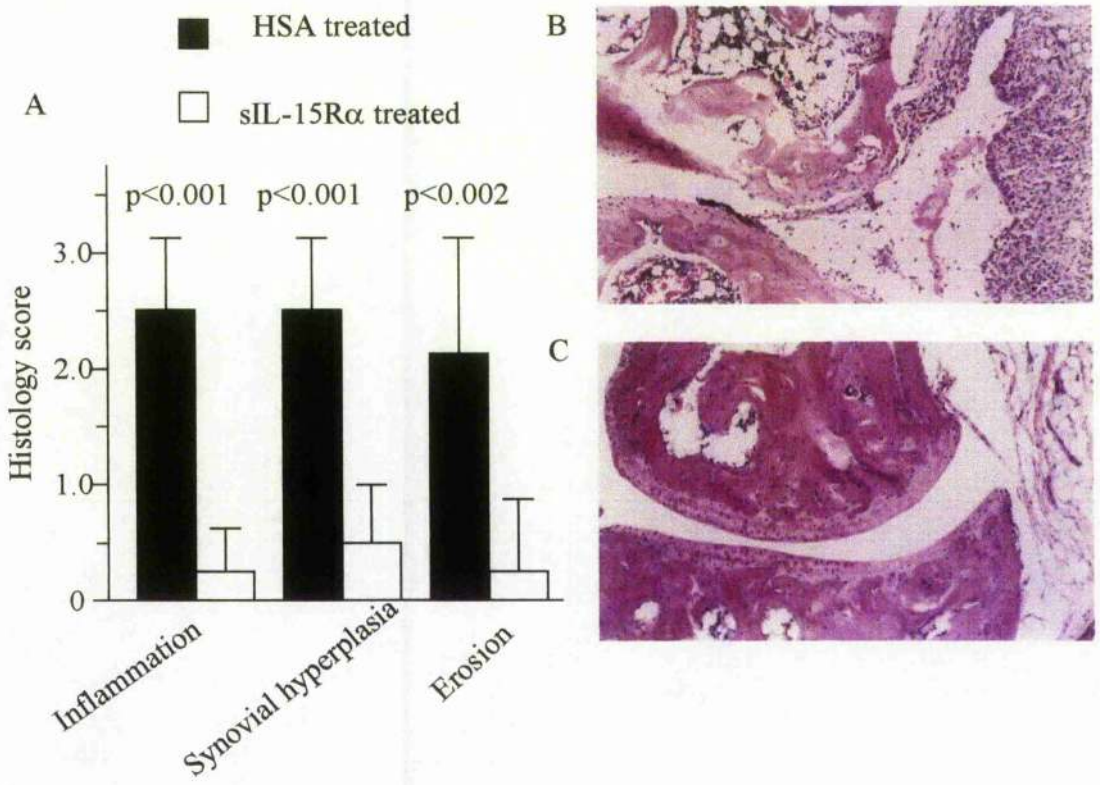


Figure 4.2. Administration of sIL-15Rα significantly reduced articular inflammation and destruction. Immediately following 14 days of sIL-15Rα administration, hind limbs (five mice/group) were removed, formalin-fixed, decalcified, and hematoxylin and eosin sections were prepared (day 36). Histologic appearances in the knee, carpus, and interphalangeal joints in parallel sections (HSA, n = 17; sIL-15Rα, n = 13) were scored independently (0-3) by two treatment-blinded histologists (data are mean ± SD). Extensive erosion ($p < 0.002$), inflammatory infiltration ($p < 0.001$), and synovial hyperplasia ($p < 0.001$) were observed in HSA-treated animals (B) (×40 magnification), but were usually absent in sIL-15Rα recipients (C) (×40 magnification).

4.3 Immunologic consequences of sIL-15Rα administration

To further explore the effect of sIL-15Rα on immunologic responses, spleen cells from mice that had been treated with 40 μg of sIL-15Rα or control HSA were harvested after 2 wk of treatment (day 36) and cultured with type II collagen *in vitro*. Cells from arthritic mice that received control protein proliferated vigorously in response to collagen in a dose-dependent manner. This proliferative response was significantly reduced in cultures of cells from mice treated with sIL-15Rα ($p < 0.001$). Cells from the sIL-15Rα-treated mice produced less IFNγ than cells from control HSA-treated mice, which indicates a suppression of Th1 responses. Moreover, IL-4 was undetectable, and IL-10 levels were low (100-120 pg/ml) and indistinguishable between the groups, suggesting that the mechanism of disease suppression by sIL-15Rα was not by the preferential enhancement of Th2 responses. IL-6 production was significantly suppressed in sIL-15Rα-treated animals compared with HSA controls. TNFα synthesis was not detected, which was consistent with previous observations that the collagen-stimulated expression of TNFα *in vitro* occurs primarily during the early acute phase of CIA (Courtenay *et al.*, 1980). However, the T cell mitogen, Con A, stimulated equally high levels of both proliferation and IFNγ, IL-4, IL-10, and IL-6 production by spleen cells from both groups of mice, indicating that suppression of the immune response was Ag-specific (Figure 4.3).

Commensurate with the above observations, sera from sIL-15Rα-treated mice showed a significant reduction of cytokine levels. Serum samples taken on day 36 were analyzed for the presence of IL-4, IL-5, IL-6, IL-10, TNFα and IFNγ. Whereas IL-4 and IL-5 were undetectable in sera from both treatment groups, TNFα levels were similarly low in both groups. Again, this finding is compatible with earlier reports showing that upregulation of TFNα in CIA occurs only transiently at the early stage of the disease.

However, sera from mice treated with sIL-15Rα contained significantly less IFNγ than those from control mice. Similarly, IL-6 and IL-10 levels were reduced in mice that had received sIL-15Rα when compared with mice treated with HSA (Figure 4.4).

Humoral responses were clearly modified, since serum anti-collagen Ab concentrations were significantly reduced in sIL-15Rα recipients. This was particularly apparent for the IgG2a isotype which is consistent with the preferential suppression of the Th1-type immune response that predominates in CIA (Figure 4.5).

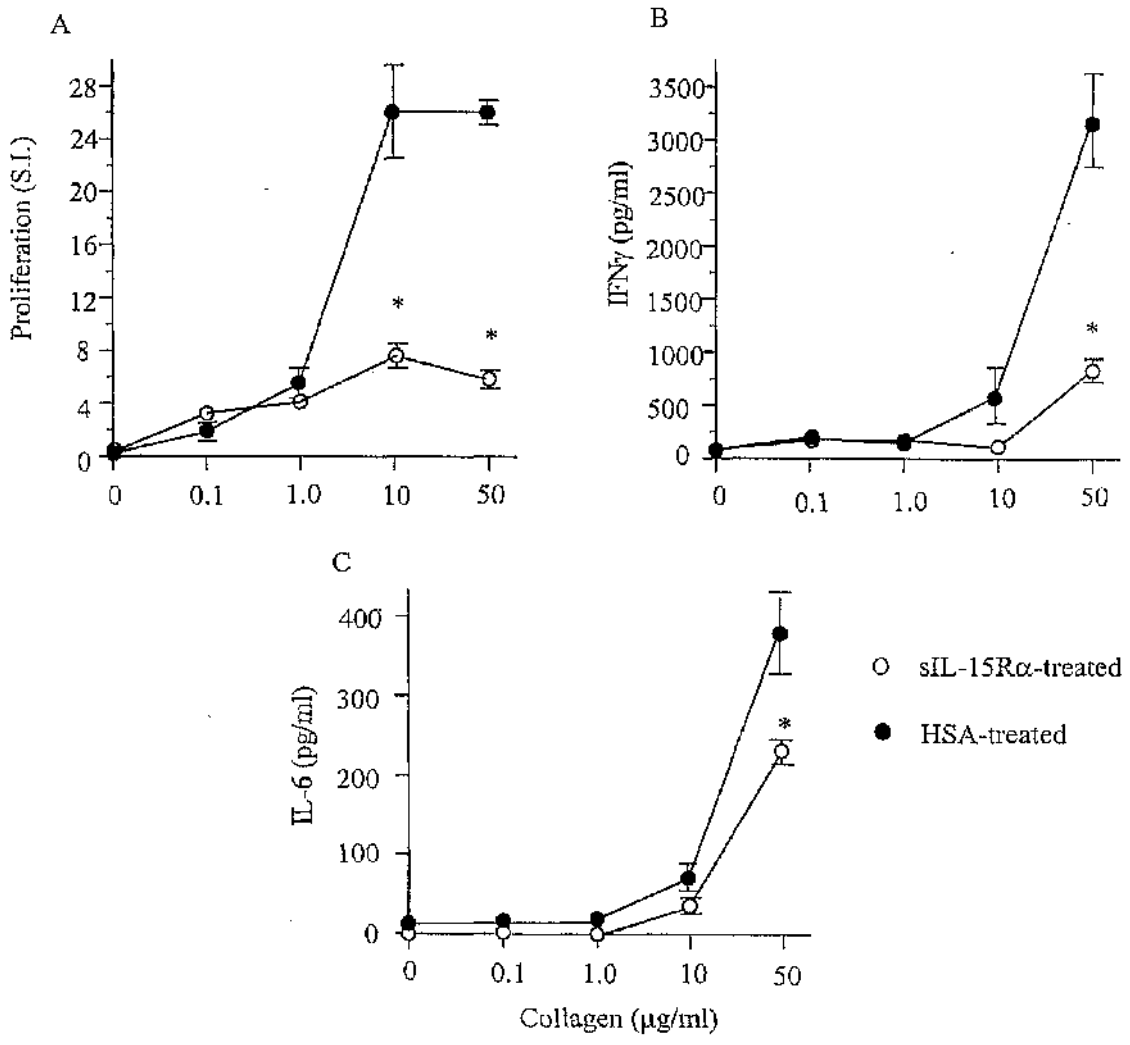


Figure 4.3 Assessment of *in vitro* responses against collagen from mice treated with sIL-15Rα or HSA. Spleen cells (pooled from five mice per group) were collected from sIL-15Rα-treated or HSA-treated mice at the end of the 2-wk treatment phase (day 36) and cultured with graded concentrations of collagen for 96 h. T cell proliferation (A), which was determined by ^{3}[H]thymidine uptake, is expressed as mean stimulation index ± SEM of triplicate cultures (medium control ranged from 3500 to 5000 cpm). Supernatants from parallel cultures were collected after 72 h. IFNγ (B) and IL-6 (C) levels were measured by ELISA and expressed as mean ± SEM. *$p < 0.05$.

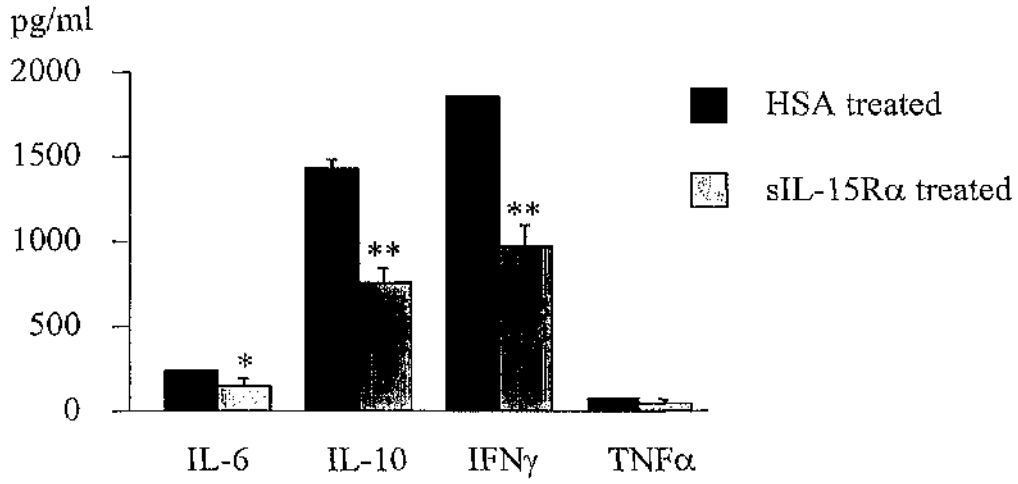


Figure 4.4 Serum cytokine levels in sIL-15Rα and HSA treated mice. Sera from 5 mice were pooled and collected on day 36. Serum cytokine concentrations were measured by ELISA. Data are mean ± SEM. *$p < 0.05$, **$p < 0.005$, Mann-Whitney test.

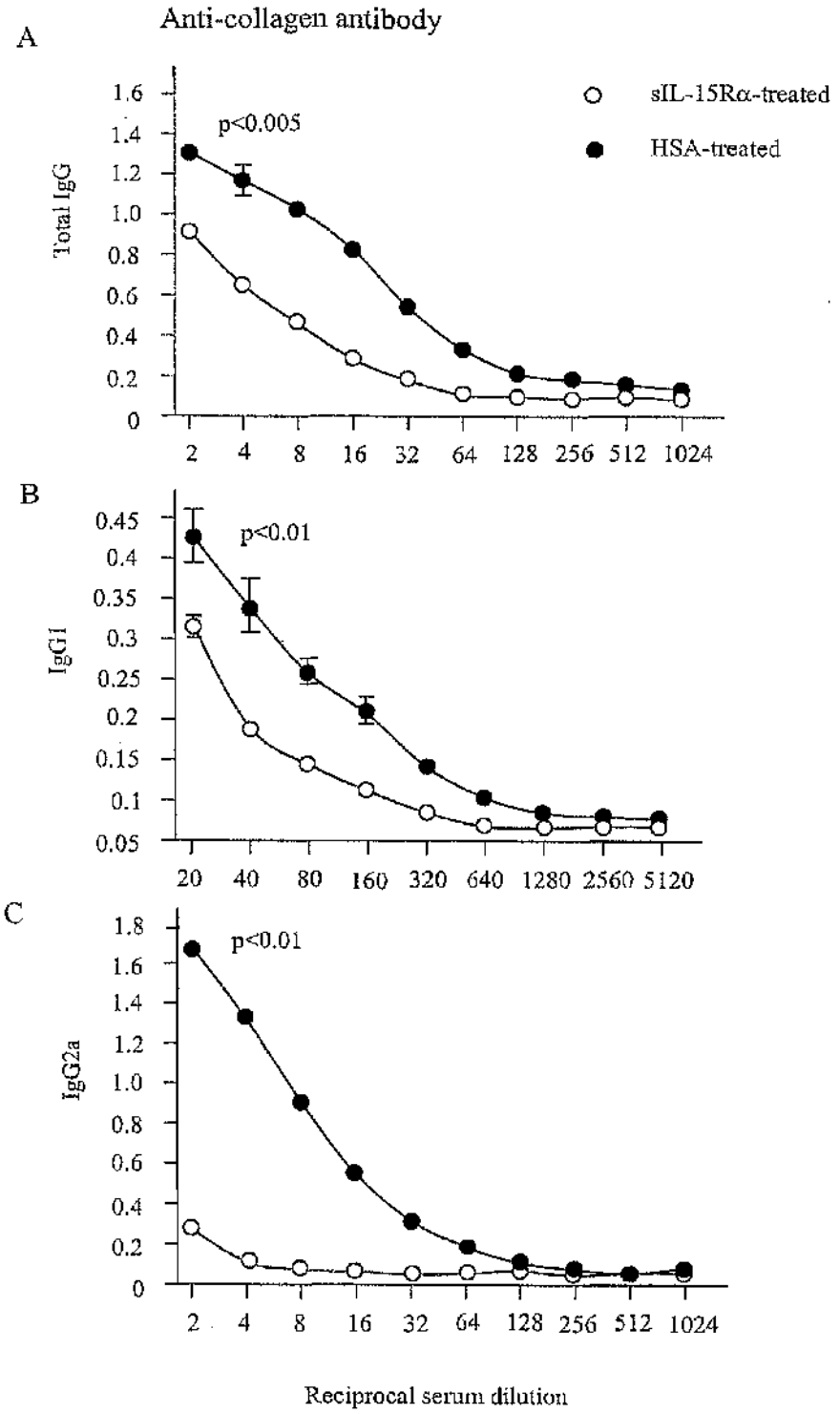


Figure 4.5 Assessment of serum anti-collagen Ab responses in sIL-15Rα-and HSA-treated mice. Anti-collagen Ab titers from mice treated with sIL-15Rα (○) or HSA (●); total IgG (A), IgG1 (B), and IgG2a (C) were measured at the end of treatment (day 36) by ELISA. Data are from pooled serum (five mice per group) and are expressed as mean absorbance (OD 630) from doubling dilutions ± SEM, Wilcoxon rank test.

4.4 Discontinuation of sIL-15Rα administration facilitates disease expression

To determine the duration of disease suppression, sIL-15Rα administration was withdrawn after 14 days. CIA was detected clinically at 5 to 7 days after the cessation of treatment, and 90% of previous sIL-15Rα recipients developed CIA that was indistinguishable from the control HSA-injected group after 10 days. Thus, treated mice developed acute phase CIA soon after the discontinuation of sIL-15Rα injection. This observation was reflected in immune responsiveness *in vitro*. Two weeks after the cessation of treatment (day 50), spleen cells from sIL-15Rα recipients produced higher concentrations of IFNγ and IL-6 in response to collagen, while T cell proliferation in both groups was similar. Spleen cells from sIL-15Rα recipients were compared either with parallel cultures from HSA-treated controls, which were in the chronic phase of CIA at that point, or with spleen cell responses obtained earlier from littermates at the end of sIL-15Rα treatment (day 36). Thus, treatment with sIL-15Rα clearly suppressed the development of acute CIA (Figure 4.6).

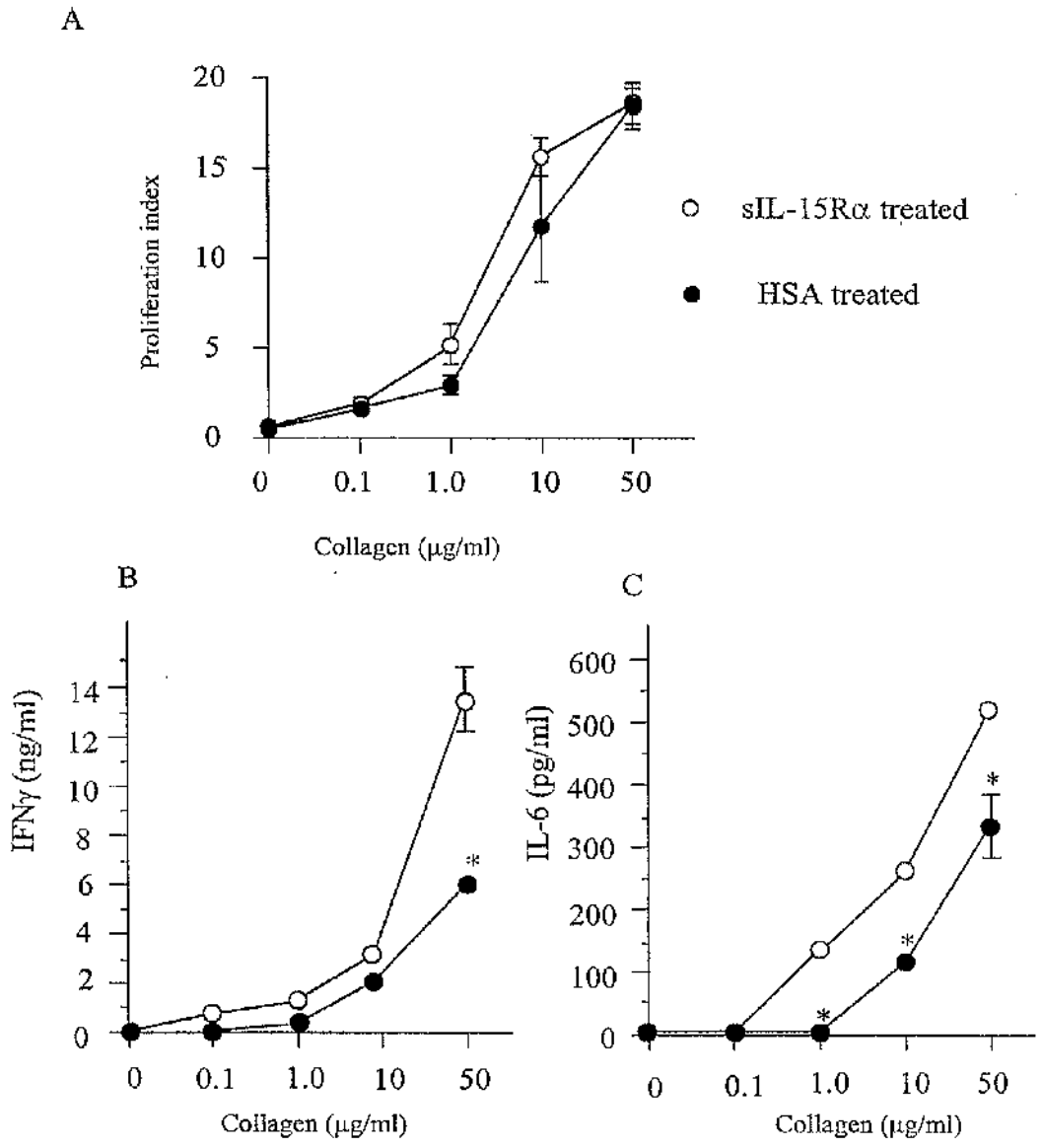


Figure 4.6 At 14 days after the cessation of treatment (day 50), spleen cells (pooled from three mice per group) from sIL-15Rα-treated mice or HSA-treated mice were cultured with collagen as described previously. Withdrawal of sIL-15Rα leads to the loss of suppression of collagen-induced proliferation of spleen cells of mice previously treated with sIL-15Rα (A). IFNγ (B) and IL-6 (C) synthesis in parallel cultures was determined by ELISA, *$p < 0.05$, Mann-Whitney test.

4.5 Injection of sIL-15Rα induces the development of anti-sIL-15Rα antibodies

Continuous sIL-15Rα injection *in vivo* led to the production of anti-sIL-15Rα auto-antibodies in DBA/1 mice. Anti-sIL-15Rα activity was detected in serum of mice injected with 40μg of recombinant protein per day over a period of 14 days. The titer of anti-sIL-15Rα auto-antibodies was measured by ELISA. In serum from DBA/1 mice treated with either high or low dose of sIL-15Rα for over 14 days, an anti-sIL-15Rα antibody activity was detectable up to a serum dilution of 1:60,000. No anti-sIL-15Rα antibody activity was found in serum of mice treated with HSA or in normal serum of control mice that had not undergone CIA (Figure 4.7 A). In Western blot analysis (B), diluted serum from DBA/1 mice continuously injected with sIL-15Rα specifically detected recombinant sIL-15Rα in Western blot. However, anti-sIL-15Rα antibodies contained in this serum also bound to recombinant IL-18 (a kind gift of Dr. X.Q. Wei), which had been used as a control protein due to its similar purification procedure and C-terminal 6x His tag. As the DBA/1 mice, from which the serum was obtained had not been injected with rIL-18, these results indicate that anti-sIL-15Rα auto-antibodies were also directed against the 6x His tag.

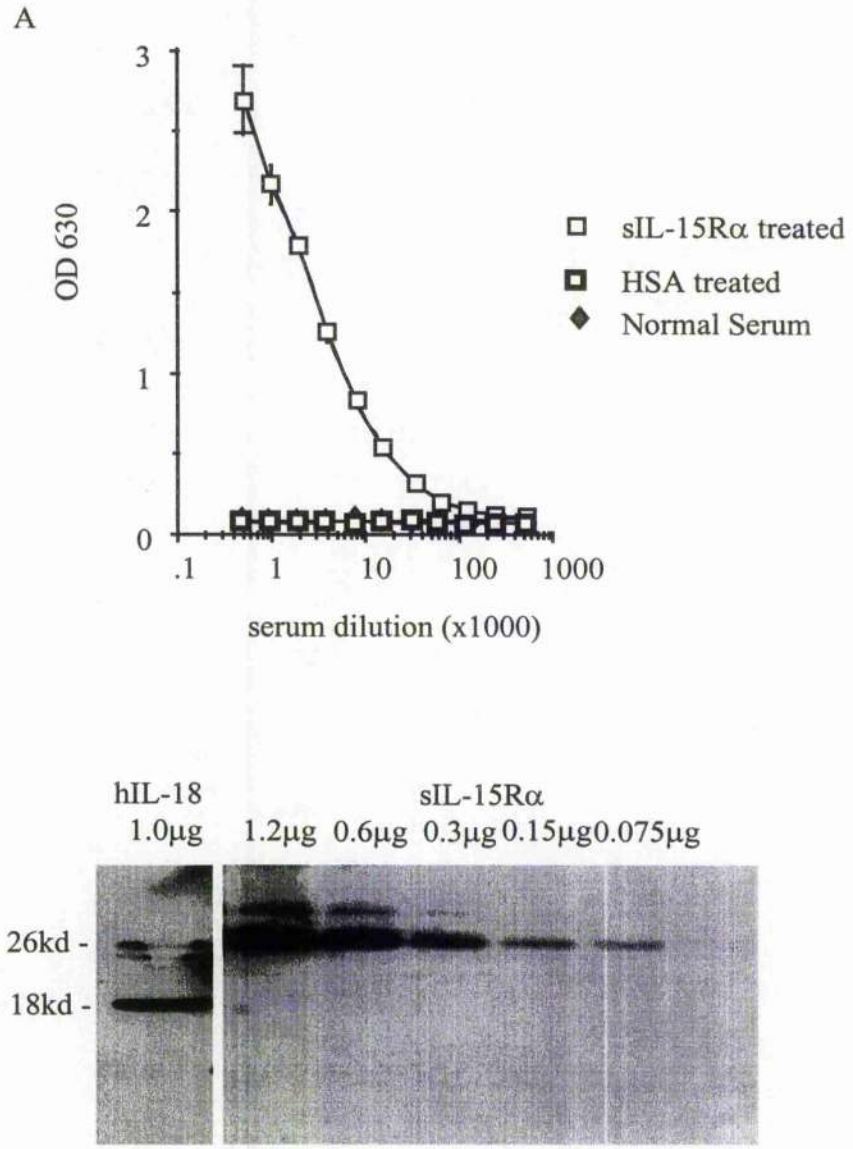


Figure 4.7 (A) ELISA showing anti-sIL-15Rα antibody titers in serum derived from different treatment groups compared to titers in normal serum. Titers where comparable in high and low dose sIL-15Rα treatment group, whereas no anti-sIL-15Rα activity could be detected in normal serum or in serum of mice treated with HSA. Titers are expressed as mean absorbance (OD 630) from doubling dilutions ± SEM.

(B) Western Blot showing anti-IL-15Rα activity of serum derived from DBA/1 mice after 14 days of injection of sIL-15Rα. Anti-sIL-15Rα Ab contained within the serum detected a minimal amount of 0.075μg of sIL-15Rα run on SDS-PAGE. However, human recombinant IL-18, expressed in the pQE30/M15 expression system was also detected, suggesting that the 5'terminal His-tag that is contained in both proteins is the epitope recognized by antibodies within the serum.

To determine if anti-sIL-15Rα antibodies directly inhibited the bioactivity of sIL-15Rα, CTLL cell proliferation assays were performed. IL-15-induced proliferation of CTLL cells was performed as described previously. Serum from DBA/1 mice injected with either HSA (Figure 4.8, A) or sIL-15Rα (B) for 14 days was added to sIL-15Rα in various dilutions prior to adding the recombinant sIL-15Rα to the CTLL cell cultures to block IL-15-induced proliferation. IL-15 strongly stimulated CTLL proliferation as measured by their [^{3}H]-thymidine uptake. Addition of 200ng/ml of sIL-15Rα strongly inhibited proliferation induced by either 0.1 or 1.0ng/ml of simian IL-15. The inhibitory effect of sIL-15Rα could be overcome by adding excess IL-15 (10ng/ml). Murine serum of both treatment groups was added to the cultures containing sIL-15Rα at various dilutions. Neither serum of mice that had received HSA nor serum from sIL-15Rα-treated mice interfered with the inhibitory effect of sIL-15Rα on IL-15-induced CTLL cell proliferation. Serum derived from sIL-15Rα-treated animals was also compared to anti-sIL-15Rα-antisera raised in sheep and rabbit for its capacity to block the inhibitory effect of sIL-15Rα on IL-15-induced proliferation of CTLL cells (C). Using a similar approach, anti-sIL-15Rα-antiserum derived from either sheep, rabbit or DBA/1 mice that had undergone CIA as described before was added to CTLL cells cultured in the presence of 1.0ng/ml IL-15 and 200ng/ml sIL-15Rα. Only sheep-anti-sIL-15Rα was able to restore IL-15-induced proliferation of CTLL cells. Neither murine, nor rabbit-antisera inhibited the bioactivity of sIL-15Rα. Anti-sIL-15Rα antibodies in serum of treated mice did therefore not interfere directly with the IL-15-binding domain of sIL-15Rα but are likely to be directed against the 6xHis tag.

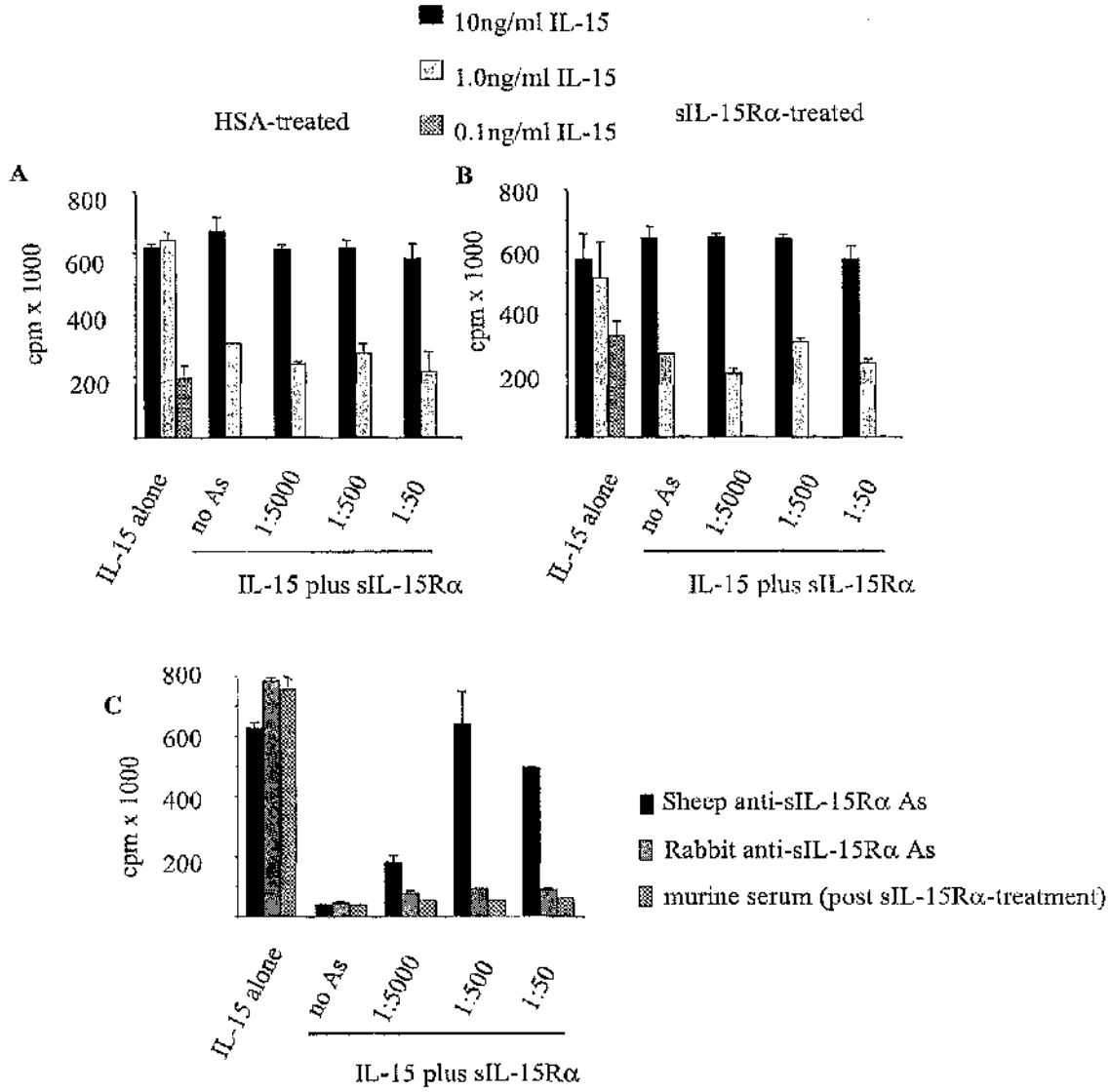


Figure 4.8 CTLL cell proliferation assay showing the effect of murine serum from HSA-treated (A) and sIL-15Rα-treated (B) DBA/1 mice on sIL-15Rα-mediated inhibition of IL-15-induced proliferation. Anti-sIL-15Rα Ab derived from serum of mice treated with sIL-15Rα did not interfere with the inhibitory effect of sIL-15Rα on CTLL cell proliferation. (C) Effects of sheep-, rabbit-, and murine anti-sIL-15Rα-antiserum on sIL-15Rα bioactivity. Only sheep-antiserum restored IL-15-induced proliferation of CTLL cells and thus inhibited sIL-15Rα. Data are means of triplicates ± SEM.

4.6 Summary

The sIL-15Rα was used as a therapeutic agent in murine collagen induced arthritis. Administration of sIL-15Rα prevented the onset of CIA in terms of paw thickness, severity and incidence in male DBA/1 mice. When compared to mice that received human serum albumin, sIL-15Rα-treated mice showed less cell infiltration into the synovium and reduced articular erosion. The inhibitory effect of sIL-15Rα on CIA was reflected by the immunological responses of spleen cells from mice treated with sIL-15Rα in terms of collagen-induced proliferation and cytokine production. Treatment with sIL-15Rα also resulted in a reduction in serum cytokine levels and antigen-specific IgG synthesis. This was particularly pronounced for anti-collagen IgG2a production. Cessation of treatment with sIL-15Rα resulted in the development of acute CIA within 10 days in mice that previously received sIL-15Rα. Administration of sIL-15Rα therefore effectively inhibited the onset and development of acute CIA.

Continuous injection of sIL-15Rα led to the development of anti-sIL-15Rα antibodies in treated mice. These antibodies bound to sIL-15Rα in Western blot analysis and ELISA but also interacted with a control protein also containing a 6x histidine-tag. However, anti-sIL-15Rα antibodies in serum of treated mice did not interfere with the biologic activity of sIL-15Rα to inhibit IL-15-mediated proliferation of CTLL cells.

Chapter 5

Conditional mutagenesis of IL-15Rα in embryonic stem cells

5.1 Introduction

The generation of knockout mice by homologous recombination frequently begins with the cloning of a cDNA fragment encoding the gene-product in question. The cDNA is used as a radiolabeled probe to screen a murine genomic library in order to obtain clones containing at least part of the gene to be mutated through gene targeting. Detailed information on the organization of the gene to be targeted, especially intron/exon organization, restriction enzyme sites and the location of sequences possibly to be used as homologous regions for the targeting construct is required to develop a strategy for the generation of the desired mutation. Once the desired gene is cloned and sufficiently characterized, the second step usually involves cloning of a targeting construct and the development of potential DNA probes or PCR oligonucleotides that can be employed to screen for homologous recombination events. In conventional gene targeting, a targeting construct is made to delete a crucial part of a gene by replacing it with the targeting construct. The created mutation generally leads to the loss of gene function. Once a targeted ES cell clone has been identified among ES cells permanently transfected with the targeting construct, the process of introducing targeted ES cells into the blastocyst and of generating chimeras carrying the desired mutation begins. Homozygous knockout mice can be generated subsequently by breeding the heterozygous offspring of chimeras.

The Cre/loxP system, which allows the conditional mutagenesis of genes targeted by homologous recombination requires a construct different from the ones used in conventional gene targeting approaches. To take advantage of the Cre/loxP system, crucial parts of the gene to be targeted have to be flanked by loxP sites. Consequently, a targeting construct designed to introduce these changes into the genome requires an additional cloning step in which a loxP site has to be placed into the intron sequences surrounding exons crucial for function of the gene product. Further loxP sites that allow the complete

excision of flanked sequences by Cre-mediated recombination can be placed surrounding the *Neo* selection marker cassette. When the Cre/loxP targeting construct is introduced into the ES cell genome by homologous recombination the targeted locus still encodes a fully functional gene product. Deletion of the *Neo* selection marker cassette *in vitro* after gene targeting further reduces the risk of impaired gene function of the targeted locus. *In vitro* deletion of all loxP site flanked sequences by transient transfection of ES cells with a Cre-expressing plasmid vector can also occur. In this case, targeted ES cells will lack both the selection marker cassette and crucial parts of the targeted gene. Mice homozygous for the mutation introduced can be generated similarly to the procedure used in conventional gene targeting.

In this targeting experiment, a lambda phage-mouse genomic library (from 129Sv mice) was screened to obtain clones encoding IL-15Rα. Subclones were made in the pBlueScript vector to characterize the gene by sequencing, restriction enzyme digest and Southern blotting. For the targeting construct, loxP sites were placed around exon 2 of the IL-15Rα gene. This exon encodes the binding domain of the native receptor and thus is particularly suited for gene targeting. The *Neo* selection marker cassette was placed between the 5' and 3' homologous regions of the targeting construct and was also flanked by loxP sites. A 5' external DNA probe was used to screen for targeting events after transfection of ES cells with the targeting construct. ES cells from targeted clones were transfected with a plasmid expressing Cre-recombinase (pCAG-Cre-IP). Cre-recombinase-mediated deletion of the loxP site flanked Exon 2 and the *Neo* selection marker cassette was determined by PCR screening.

5.2 Screening of the λFIX II mouse genomic library

The phage titer of the λFIX II mouse genomic library was determined to be $4x10^6$ p.f.u./ml. Accordingly, 50,000 p.f.u. were used to infect competent *E. coli* XL1Blue cells and plated on 5x150mm Petri dishes containing LB-agar. Using the radiolabeled cDNA encoding sIL-15Rα as a probe to screen for λ-phage clones containing the IL-15Rα gene, 6 putatively positive groups of lambda clones were identified. In a subsequent secondary screening, using the sIL-15Rα-cDNA as a radiolabeled probe, only 3 of these lambda clones showed an amplified positive signal and could be picked as single lambda clones. However, the second screening result was confirmed using a third screening round. During the third screening, nearly all lambda phage plaques showed a strong signal when hybridized with the sIL-15Rα-cDNA radiolabeled probe. As a result of the screening, three lambda clones were identified that strongly hybridized with sIL-15Rα-cDNA.

5.3 Mapping and subcloning of the gene encoding murine IL-15Rα

In order to obtain a restriction map of the λ phage clones derived from library screening, lambda DNA was prepared from these clones. Digestion of lambda DNA with several different restriction endonucleases revealed that two of the three clones obtained were identical and showed the same restriction digest pattern. The two non-identical lambda clones were named clone Nº3 and Nº6 containing DNA inserts of 13.6kb and 14.4kb respectively (Figure 5.1 A). Southern blot analysis of lambda DNA obtained from clone Nº3 and Nº6 was performed using the cDNA encoding sIL-15Rα as a radiolabeled probe to detect restriction fragments containing the gene encoding IL-15Rα (Figure 5.1 B). Southern blot analysis showed that a 4.5kb *Eco*RI / *Sal*I and a 3.5kb *Eco*RI fragment from lambda clone Nº3 contained sequences that hybridized to sIL-15Rα-cDNA. For lambda

clone Nº4, one *Eco*RI / *Sal*I fragment of 2.2kb could be identified. Following this observation, DNA from both lambda clone Nº3 and Nº6 was used for restriction enzyme digestion by *Eco*RI and *Eco*RI / *Sal*I and the resulting fragments were subcloned into the pBlueScript vector. An additional subclone was obtained from lambda clone Nº3 using restriction endonucleases *Bam*HI and *Hind*III resulting in a fragment of 2.4kb. The following pBlueScript clones were obtained and transformed into *E. coli* strain DH5α for bacterial culture and small-scale preparation of plasmid DNA.

Table 5.1 List of subclones obtained from lambda clones 3 and 6.

Lambda clone	**Subclone number**	**Molecular weight**	**Restriction enzymes**
3	3.1	4.5kb	*Eco*RI / *Sal*I
3	3.2	3.8kb	*Eco*RI
3	3.3	2.8kb	*Eco*RI
3	3.4	1.9kb	*Eco*RI / *Sal*I
3	3.5	0.9kb	*Eco*RI
6	6.1	6.5kb	*Eco*RI
6	6.2	2.8kb	*Eco*RI
6	6.3	2.2kb	*Eco*RI / *Sal*I
6	6.4	2.0kb	*Eco*RI / *Sal*I
6	6.5	0.9kb	*Eco*RI
3	3-BH-2.4	2.4kb	*Bam*HI / *Hind*III

Following subcloning of *Eco*RI and *Eco*RI/*Sal*I fragments from lambda clones, double-stranded DNA sequencing was used to sequence all clones using external oligoucleotides T7 and reverse primer whose annealing sites are contained within the pBlueScript vector. Sequencing the 5' and 3' ends of the subcloned inserts showed that the two lambda clones were overlapping in a total of 7.6 kb with lambda clone Nº6 containing 6.8kb of DNA positioned 5' of lambda clone Nº3. Using different combinations of restriction endonucleases, detailed restriction maps were obtained for each subclone (Figure 5.2). The information obtained by restriction enzyme mapping and double stranded DNA sequencing was used to construct a detailed map of the IL-15Rα locus as contained within the lambda clones identified during library screening.

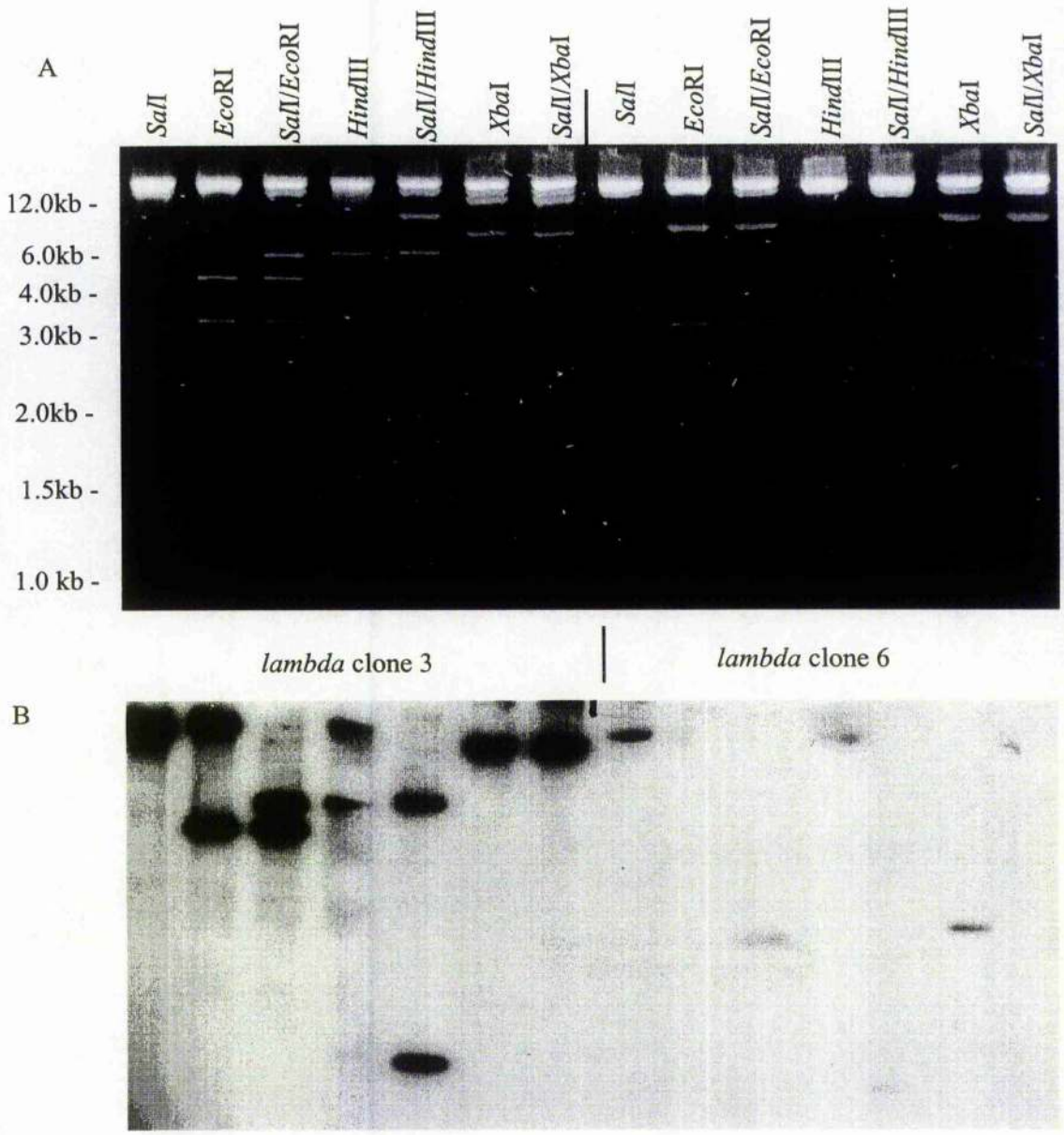


Figure 5.1 (A) Restriction enzyme mapping and subcloning of lambda clones 3 and 6 using a variety of restriction endonucleases.

(B) Southern blot analysis of lambda clone 3 and 6 DNA using radiolabeled sIL-15Rα-cDNA as a probe.

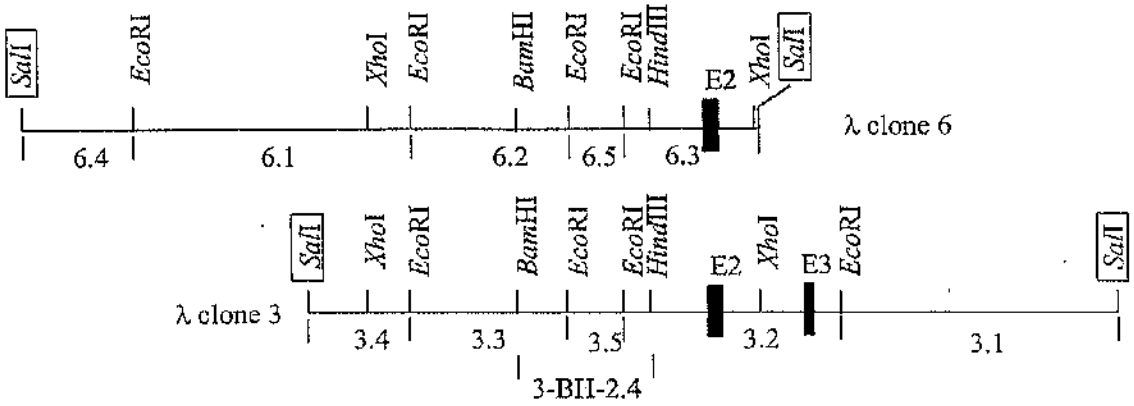


Figure 5.2 Schematic representation of lambda clones 3 and 6. The clones overlapped in a total of 7.6kb. *Eco*RI and *Eco*RI/*Sal*I fragments were subcloned into pBlueScript SK$^+$ as indicated in table 5.1. A further pBlueScript subclone, 3-BH-2.4, was derived from lambda clone 3 by digestion with *Hind*III and *Bam*HI. Subclones 3.2 and 6.3 contain exon DNA encoding IL-15Rα. The *Sal*I sites flanking the inserts originate from the λFIXII library linker sequence.

5.4 Genetic organization of the IL-15Rα locus

Plasmid DNA from pBlueScript subclones containing inserts previously identified in Southern blot analysis using the sIL-15Rα-cDNA as a probe was subjected to double stranded DNA sequencing. In addition to the external sequencing oligonucleotides T7 and Reverse primer, 17-mer oligonucleotides derived from the sIL-15Rα-cDNA sequence were used to identify nucleotide sequences containing IL-15Rα exon DNA. Following this approach, two exons encoding the protein-binding motif, linker and most of the Pro/Thr rich region were identified.

Intron/exon boundaries and nucleotides surrounding exon DNA were sequenced. 17-mer oligonucleotides based intron sequences were designed in order to obtain additional sequence information on the intron/exon organization of the IL-15Rα locus.

In conjunction with the genomic organization of IL-15Rα in both humans and mice, the two exons identified were named exon (E) 2 and 3. E2 contains 197bp encoding 65 amino acids, whereas E3 contains 92bp accounting for 30 amino acid residues. Only one of the four intron-exon boundaries conformed to the known GT/AG donor/acceptor site rule, essentially maintaining the consensus sequence described by Mount (1982). E2 was found in both subclone 3.2 as well as in subclone 6.3, whereas E3 was only contained within subclone 3.2. No further exons encoding IL-15Rα were found in any of the other subclones derived from lambda phage library screening. The distance between the two exons as well as their relative positions within subclones 3.2 and 6.3 were determined by PCR using 17-mer oligonucleotides originally designed for DNA sequencing on plasmid DNA derived from pBlueScript subclones 3.2 and 6.3.

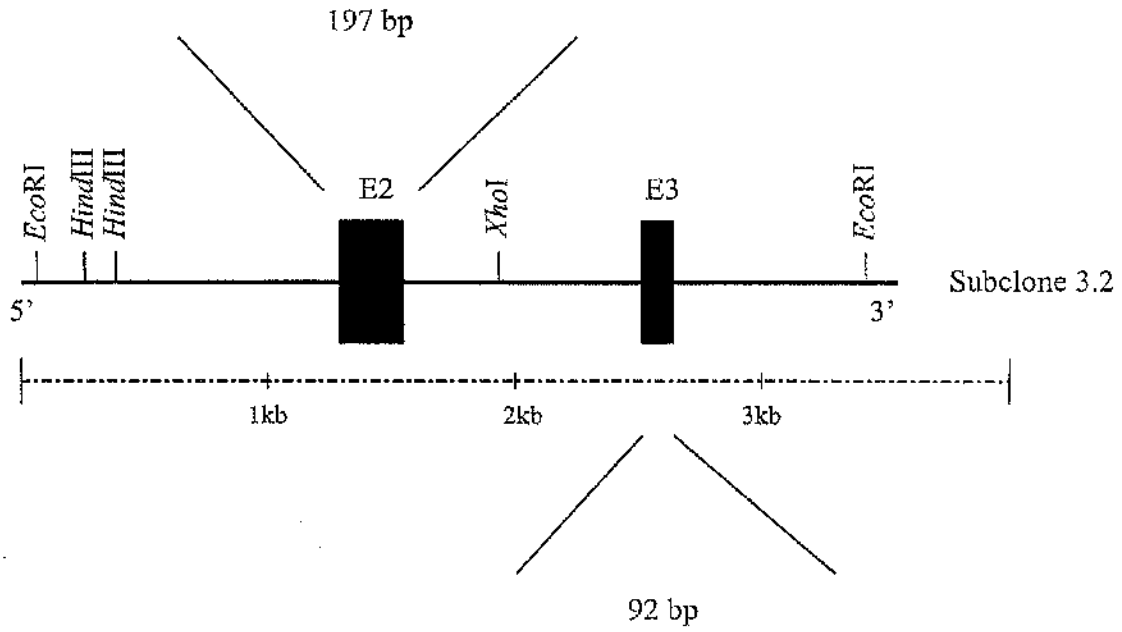


Figure 5.3 Detailed map of exon 2 and 3. Exon 2 contains 197bp, exon 3 contains 92bp. Both exons identified are located within *Eco*RI subclone 3.2. Schematic map drawn to scale.

Table 5.2 Intron-exon boundaries of IL-15Rα exon 2 and exon3.

5' Intron	Exon size	3' Intron
tttgca GGCACC	197bp (Exon2)	CATCAG taagta
tctagg AGACCC	92bp (Exon3)	CAAAAG gtagga

5.5 Construction of the IL-15Rα-targeting construct

To target the IL-15Rα locus in ES cells by homologous recombination, the Cre/loxP system for conditional mutagenesis was employed. In order to use this technology, the targeting construct had to contain loxP sites flanking crucial parts of the IL-15Rα for Cre-mediated recombination. The targeting construct also contained a *Neo* selection cassette mediating G418 resistance, which was also flanked by loxP sites to allow for its removal after targeting. The construct contained a 5' homologous arm of 3.7kb and a 3' homologous arm of 3.4kb with the *Neo* selection cassette cloned between them. The targeting construct also contained a HSV-tk gene located at the 3' end. The 3'homologous arm was prepared by cloning the loxP site of the pBS64 vector into the pBlueScript vector using restriction endonucleases *Pst*I and *Bam*HI. The resulting pBlueScript clone was named pBlueScript-lox. The 3.8kb DNA insert of subclone 3.2 was excised by *Eco*RI and cloned into the pBlueScript-lox. Both the cloning of loxP and the subsequent cloning of the 3.8kb DNA insert was confirmed by DNA sequencing. The resulting pBlueScript clone was named Lox3.2. PCR was performed on Lox3.2 using the T7 and KPN-1 primer, resulting in a 400bp product. This PCR product was cut by restriction endonucleases *Kpn*I (restriction site introduced through PCR with the KPN-1 primer) and *Eco*RI (restriction site contained in the pBlueScript vector) and cloned into the pBlueScript vector. The 400bp DNA-fragment was cloned as a 3'external probe in Southern hybridization to confirm targeting events in ES cells. In a second PCR amplification, primers KPN-1R

(complementary to KPN-1) and Reverse Primer were used to amplify a 3.4kb product. This fragment was cut by restriction enzymes *Bam*HI and *Xho*I to obtain a 2.2kb fragment (=Lox2.2) and *Xho*I/*Kpn*I to obtain a 1.2kb fragment, respectively, which were cloned into pBlueScript using these restriction enzyme sites. To clone the 3'loxP sites of the targeting construct, the pBlueScript clone containing the 1.2kb *Xho*I/*Kpn*I fragment was cloned into pBlueScript-lox. After deletion of the *Xho*I site by nuclease SI digestion and re-ligation, the resulting pBlueScript clone was named Lox1.3. Lox1.3 was cut by *Bam*HI and blunt-ended by nuclease SI digestion. The 1.3kb DNA fragment containing the loxP site was released from pBlueScript by *Kpn*I and purified via agarose-gel electrophoresis. Lox2.2 was cut by *Xho*I and digested with nuclease SI to delete the *Xho*I-site. Following the *Xho*I deletion, Lox2.2 was cut by *Kpn*I and the Lox1.3 fragment purified previously was cloned into Lox2.2 by *Kpn*I and blund-end ligation. The results of the cloning procedure were confirmed by DNA sequencing. The thus finalized 3'homologous arm was excised from pBlueScript and cloned into pPNTLoxPNeo (p293) by *Xba*I and *Kpn*I resulting in a 3.4kb fragment (Figure 5.5).

To clone the 5'homologous arm, the 0.9kb insert of subclone 3.5 was excised by *Eco*RI and purified. Subclone 3.3 was cut by *Eco*RV and dephosphorylated by calf intestine phosphatase (CIP). The purified 0.9kb *Eco*RI fragment of subclone 3.5 was subjected to nuclease SI digestion to remove 3'overlapping nucleotides before cloning it into the *Eco*RV restricted subclone 3.3. The orientation of the 0.9kb insert was verified by DNA sequencing. The resulting 3.7kb insert was excised from the pBlueScript vector by digestion with *Not*I and *Xho*I and cloned into the pPNTLoxPNeo (p293) vector already containing the 3.4kb 3'homologous arm (Figure 5.4). The resulting targeting construct was analyzed by DNA sequencing and restriction enzyme digestion to verify the cloning procedure. The targeting construct was linearized with restriction endonuclease *Not*I prior to transfection of ES cells.

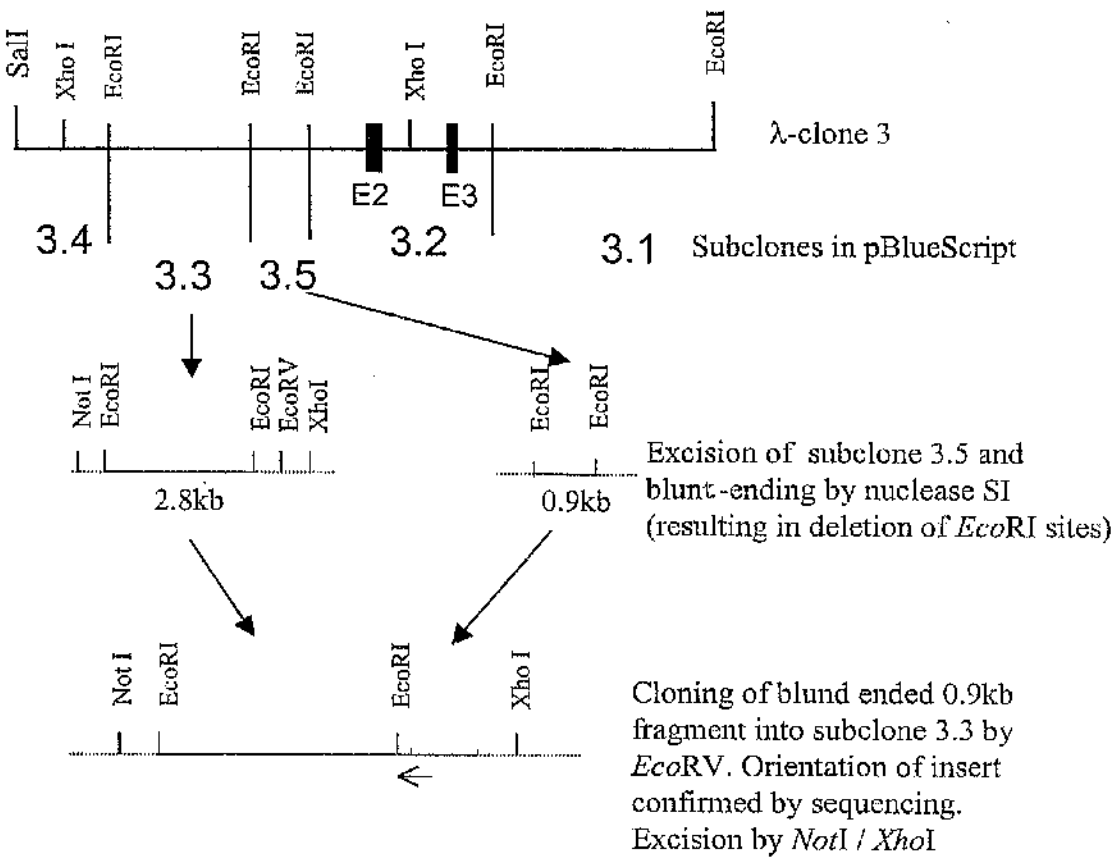


Figure 5.4 Flow-chart describing the cloning strategy for the 5' homologous region of the targeting construct using the pBlueScript subclones of the lambda clone containing part of the gene encoding IL-15Rα.

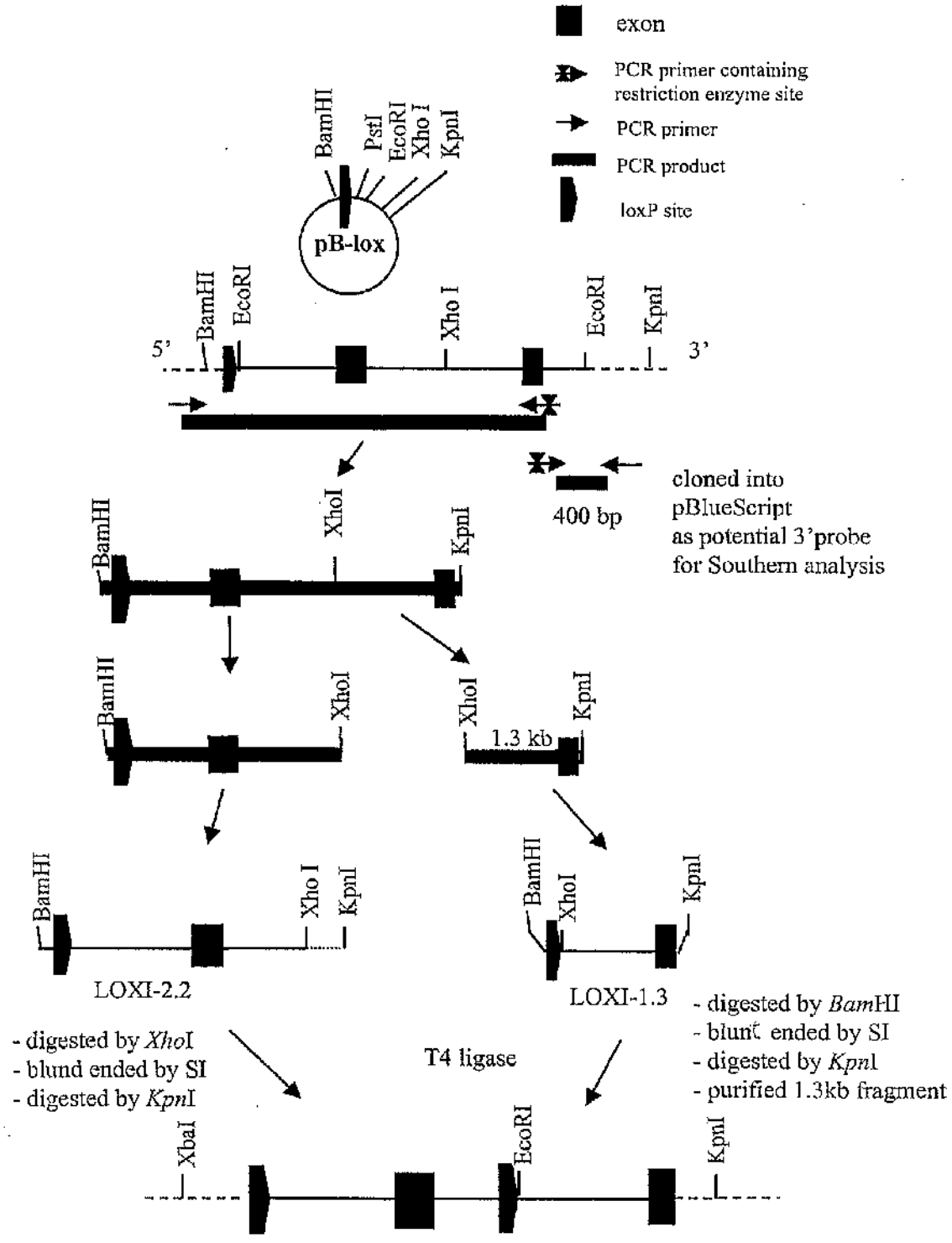


Figure 5.5 Flow-chart describing the cloning strategy for the 3' homologous region of the targeting construct using the pBlueScript subclones of the lambda clone containing part of the gene encoding IL-15Rα. Dotted lines symbolize multiple cloning sequences of the pBlueScript vector.

5.6 Targeting of the IL-15Rα locus in embryonic stem cells

Transfection of ES cells with the lineralized targeting construct by electroporation and subsequent selection with G418 resulted in the generation of G418-resistant ES cell clones. Negative selection by gancyclovir reduced the number of ES cell clones five fold. Four electroporation-experiments yielded a total of 946 ES cell colonies, which were picked and expanded in 48 well tissue culture plates. When confluent, ES cell clones were duplicated for short term freezing and gDNA extraction. Southern blot analysis with the 5' external DNA probe was used to screen for correct integration events through homologous recombination. A total of 4 ES cell colonies were identified as positive in a first screening round showing the wild-type 7kb and additional 4.2kb band in Southern blot hybridization after *XhoI* digest (Figure 5.6). Clones identified were thawed to obtain sufficient cells for long term storage and further analysis. To reconfirm the first screening result, the four ES cell clones obtained were subjected to a second Southern blot hybridization using the 5' external DNA probe. Only one clone, Nº 122-34, was identified as positive in this second screening round (Figure 5.7 A). The 3' external DNA probe which was cloned after PCR amplification of subclone 3.2 (see Figure 5.5) was not suitable for the reconfirmation of 3'targeting events by Southern blot analysis of gDNA derived from clone Nº 122-34 (Figure 5.7 B). This may be due to repetitive intron sequences contained within the probe leading to non-specific hybridization to differentially digested gDNA fragments and resulted in smear bands after Southern blot analysis. ES cell clone Nº 122-34 was subjected to Cre-mediated recombination in order to generate clones suitable for blastocyst injection. The correct 3' integration of the targeting construct was confirmed by PCR analysis of gDNA derived from clone Nº 122-34 after Cre-mediated recombination using a pair of external and internal PCR oligonucleotides (see page 177 and Figure 5.9).

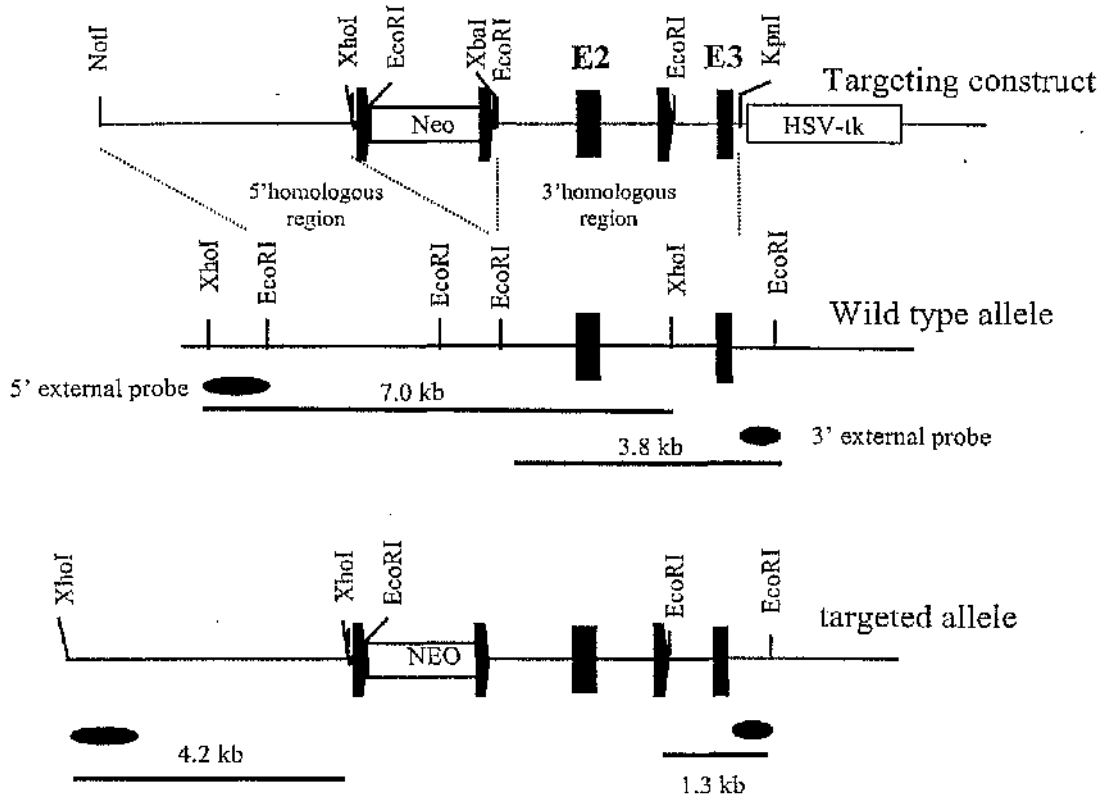


Figure 5.6 Schematic representation of the complete targeting construct. The construct contains a *Neo* selection marker cassette which is flanked by the 5' and 3' homologous regions. The 3'homologous region contains loxP sites flanking exon 2 of the IL-15Rα gene. The 5' external DNA probe hybridized with a 7kb fragment in wild-type DNA in Southern blot analysis. Correct integration of the targeting construct results in the hybridization of the probe with an additional 4.2kb band after digestion with *Xho*I. Hybridization of the 3' external DNA probe with gDNA digested with *EcoR*I results in a 3.8kb band for the wild-type allele. Correct integration of the targeting vector with should lead to the hybridization of the 3' probe with an additional 1.5kb fragment due to the introduction of an *EcoR*I site between exon 2 and exon 3.

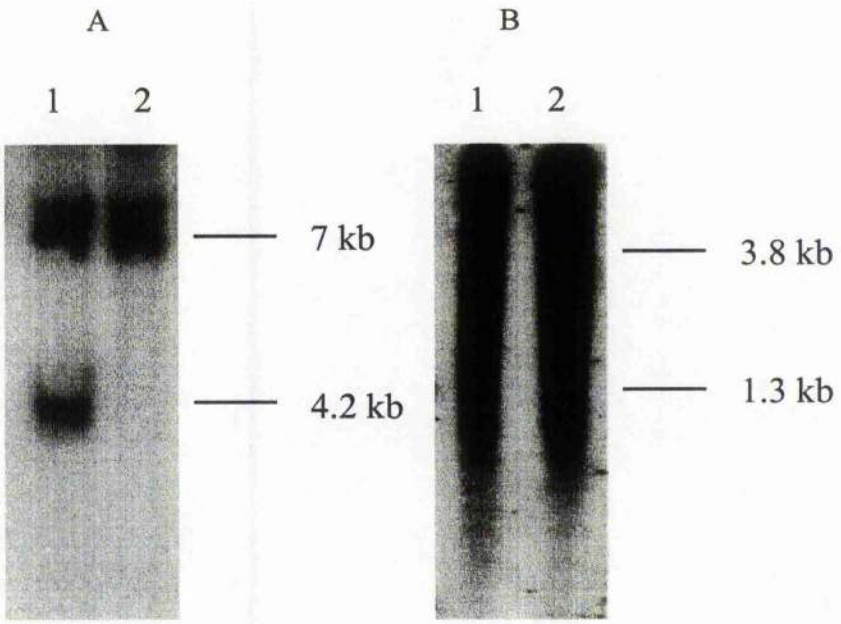


Figure 5.7 (A) Correct integration of the targeting construct within the genome of clone 122-34 was confirmed by Southern blot analysis using the radiolabeled external 5'probe. Genomic DNA obtained from clone 122-34 was digested by restriction endonuclease *Xho*I and separated by agarose-gel electrophoresis. In Southern blot analysis, the radiolabeled 5'external oligonucleotide probe hybridized to a 4.2kb (1) band in addition to the 7kb band representing the wild type allele (2). (B) The 3' external DNA probe cloned after PCR amplification (Figure 5.5) proved to be unsuitable for Southern blot analysis and did not show specific hybridization to gDNA digested with *Eco*RI.

5.7 Cre-recombinase mediated excision of loxP site flanked sequences *in vitro*

Deletion of loxP site flanked sequences was achieved by transient transfection of targeted ES cells with a plasmid expressing Cre and a selection marker gene conferring resistance to puromycin. This plasmid, pCAG-Cre-IP, containes a polycistronic gene encoding Cre recombinase and puromycin acetylase (*Pac*) which are separated by an internal ribosomal entry site (IRES). Twenty four hours after electroporation of the targeted ES cell clone 122-34 with pCAG-Cre-IP, transfected cells were selected by adding 1.0μg/ml puromycin (Sigma) for 72 hours. After this period, resistant cells were allowed to form colonies in GMEM containing neither G418 nor puromycin. Surviving colonies were picked and expanded in 48 well plates. Genomic DNA was prepared and analyzed for Cre-mediated deletion of loxP flanked sequences by PCR using the 3.5rev-R and 3'wt-1 primer pair. Possible Cre-mediated recombination events and their detection by PCR are shown in Figure 5.8.

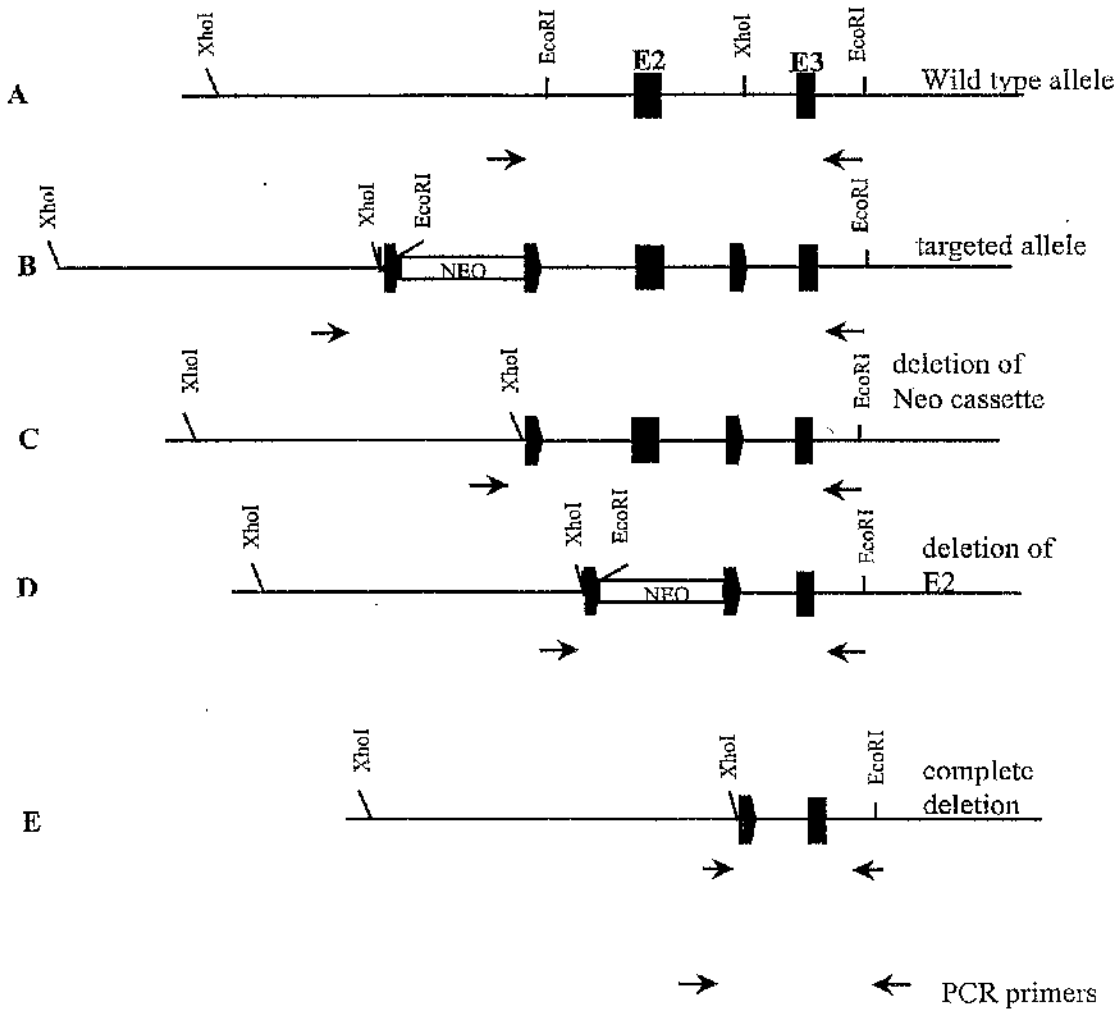


Figure 5.8 Transfection of targeted ES cells with Cre recombinase could theoretically result in several different Cre-mediated recombination events. In comparison to the wild type allele (A) in the targeted allele, both Exon 2 of the IL-15Rα gene as well as the *Neo* selection marker cassette are flanked by loxP sites (B). Cre-mediated excision of the *Neo* selection marker only (C) leads to a PCR product of 3.3kb. This deletion is desired in terms of creating a mouse model capable of expressing native IL-15Rα minimizing possible impairment of expression resulting from the presence of the *Neo* selection marker within the intron sequences separating exon 1 and 2. A further possible Cre-mediated recombination event would excise Exon 2 and surrounding intron sequences resulting in a PCR product of 3.1kb still including the *Neo* selection marker (D). A third possibility of Cre-mediated recombination would result in a PCR product of 0.9kb. This recombination event would excise both exon 2 and *Neo* selection marker cassette creating a 'knockout' allele incapable of expressing a functional IL-15Rα protein (E).

The transfection of ES cell clone 122-34 with pCAG-Cre-IP and subsequent selection with puromycin resulted in a total of 17 ES cell colonies. Genomic DNA from these clones was used in PCR reactions to analyze Cre-mediated recombination events. To detect Cre-mediated recombination events of the targeted allele and to confirm the successful 3'targeting event of the IL-15Rα locus using the targeting construct, specific PCR oligonucleotides 3.5 revR and 3'wt-1 were used, 3'wt-1 being located downstream of the targeting construct (see Figure 5.8). All 17 clones obtained resulted in a PCR product of 0.9kb indicating a complete deletion of the loxP site flanked sequences including both the *Neo* selectable marker and exon 2 (see Figure 5.8 E). Genomic DNA from clone 122-34 prior to transfection with the Cre-expression plasmid resulted in a PCR product of 3.2kb using the same set of oligonucleotides. This band corresponds to the non-targeted wild type allele. However, in the same PCR reaction, the DNA fragment corresponding to the targeted allele of 122-34 prior to Cre-mediated recombination could not be amplified. This is likely to be due to a preferential amplification of the wild type allele, which is 2.0kb smaller than the targeted allele due to the insertion of the *Neo* selectable marker gene inserted into the targeted allele. Furthermore the deletion of the *Neo* selection marker cassette from the targeted allele using Cre-recombinase could not be achieved. All 17 clones obtained after transfection with the Cre-expression plasmid resembled recombination events involving the two most distant loxP sites introduced resulting in a PCR product of 0.9kb (Figure 5.9). The Cre mediated excision of the selectable marker cassette only would have resulted in a PCR product of 3.3kb in addition to the 3.2kb wild type band. This may be due to the strong promotor used to drive Cre-expression and the resulting high efficiency of Cre-mediated recombination within the targeted stem cell allele.

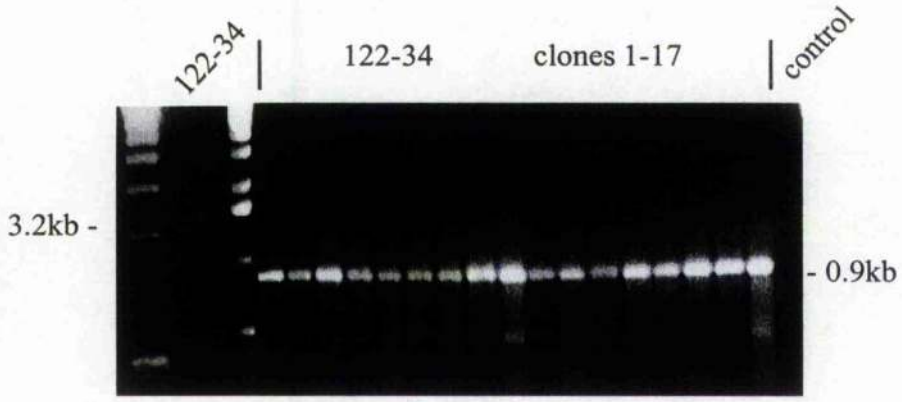


Figure 5.9 Cre mediated recombination on 122-34 clones 1-17. PCR using 3.5revR-33 and 3'wt-1 primer pair results in a single product of 0.9kb indicating the complete deletion of the loxP flanked DNA-segment. Prior to Cre mediated recombination, the same set of PCR oligonucleotides results in a DNA product of 3.2kb corresponding to the wild type allele of IL-15Rα.

5.8 Summary

A cDNA fragment encoding the extracellular domain of murine IL-15Rα was used as a radiolabeled probe to screen a murine λphage genomic library by plaque hybridization. Two λphage clones were found that contained exon 2 and exon 3 of the murine IL-15Rα gene spanning a total of 21.4kb. However, no further exons encoding IL-15Rα were found in these λ-phage clones. Restriction enzyme mapping, Southern blot analysis, subcloning and DNA sequencing was used to obtain a map of the gene segment contained in the λphage clones. A gene-targeting vector, which contains loxP sites on both sides of exon 2 of the IL-15Rα-gene was constructed. The targeting construct also contained a positive (*Neo*) selection marker cassette flanked by loxP sites, and a HSV-*tk* gene at its 3' end. Homologous recombination of the targeting construct with the IL-15Rα gene locus in ES cells resulted in the replacement of the targeted gene segment by the construct in one of 946 G418-resistant ES cell clones screened by Southern blot analysis. Cre mediated recombination in this clone resulted in the complete deletion of all loxP flanked sequences after transient transfection of targeted ES cells by a Cre-expression plasmid *in vitro*.

Chapter 6 General Discussion

6.1 Cloning and expression of recombinant IL-15Rα-fusion proteins

The cDNA encoding sIL-15Rα was cloned from a murine macrophage cell line by RT-PCR. A nested PCR approach was employed to introduce restriction enzyme sites into the cDNA fragment that allowed the cloning into a vector suitable for inducible bacterial expression. Sequence analysis showed that the cloned cDNA fragment had 100% identity with the corresponding sequence published for murine IL-15Rα (Giri, *et al.*, 1994). The bacterial expression system was chosen for its relative simplicity and suitability for high yield production of recombinant protein. Purification of the recombinant protein was facilitated by the N-terminal 6xHis tag allowing the purification of sIL-15Rα by metal-affinity chromatography. The His-tag purification system offers the advantage of conformation-independent purification procedures and mild elution conditions. The 6xHis tag is encoded by the pQE30 expression vector, which was used to express sIL-15Rα. This vector allows a high transcriptional rate of recombinant material under the T5 promotor and confers resistance to ampicillin. To avoid the potentially toxic effects of recombinant material on the *E. coli* host strain and resulting decrease in culture growth and yield of recombinant protein, the bacterial host strain requires the presence of high levels of the *lac* repressor protein. This repressor is encoded by the low copy plasmid pREP4 (Farabaugh, 1978), which also confers resistance to the antibiotic kanamycin. The presence of *lac* repressor inhibits the constitutive expression of recombinant protein. Expression is induced by the addition of IPTG to the culture medium. IPTG binds to the *lac* repressor and inactivates it. The use of two antibiotics in the culture medium ensures that both plasmids (pREP4 and pQE30) are present in the host strain resulting in tight control of recombinant protein expression. For the expression of sIL-15Rα, induction periods of 4-5 hours were found to be optimal for high yield of recombinant protein (1.0-1.2 mg/ liter of culture volume). Small-scale preparations of recombinant protein demonstrated that sIL-15Rα was

contained within the insoluble fraction of the bacterial lysate. Insolubility often occurs as a problem when expressing eukaryotic proteins in a bacterial system, due to the sequestration of highly expressed recombinant protein in insoluble inclusion bodies. It has therefore been necessary to purify sIL-15Rα under denaturing conditions followed by a refolding step to obtain a biologically active protein. This refolding procedure was not found to interfere with the ability of sIL-15Rα to bind to IL-15. To increase solubility and to avoid the unwanted precipitation of purified sIL-15Rα the refolding procedure was performed in a buffer containing high salt (2% w/v NaCl).

The ability of sIL-15Rα to specifically bind IL-15 was demonstrated in Western blot analysis, ELISA and proliferation assays. Furthermore, anti-sIL-15Rα antibodies raised in sheep interfered with the binding of sIL-15Rα to IL-15. Addition of sIL-15Rα did not have any activating effect on any cell type tested (murine spleen, CTLL or D10 cell lines). Intraperitoneal injection of sIL-15Rα up to a maximal dose of 80μg did not have any visible pathogenic effect on mice. Using anti-sIL-15Rα antibodies raised in rabbit and sheep in ELISA, the half-life of sIL-15Rα in the bloodstream of DBA/1 mice was measured. Although the assay sensitivity was limited to approximately 1ng/ml of sIL-15Rα in murine serum, it was clear that sIL-15Rα was very rapidly cleared from the bloodstream. As a consequence of this, a novel IL-15Rα-IgG_1-Fc fusion protein was cloned. It has previously been observed that the fusion of cytokines or their receptors to immunoglobulin heavy chain-constant regions can prolong the biological half-life *in vivo* (Nickerson, *et al.*, 1996; Wooley, *et al*, 1993). The IL-15Rα-IgG_1-Fc fusion protein was cloned from DBA/1 spleen cells by RT-PCR. The cDNA encoding the extracellular domain of IL-15Rα was obtained using a nested PCR approach introducing restriction enzyme sites into the cDNA fragment to facilitate cloning. The cDNA encoding IgG_1-Fc was cloned from reverse transcribed RNA after a single PCR amplification introducing

appropriate restriction endonuclease sites into the cDNA fragment for cloning. Both cDNA fragments were cloned into the pcDNA3.1 vector (Invitrogen) and DNA sequencing was performed to confirm the sequence identity of the fused cDNA inserts. A mammalian expression system was chosen for the production of the recombinant protein enabling post-translational modification to express a functional IgG_1-Fc domain. A functional IgG_1-Fc would retain the ability to bind to endogenous $Fc_\gamma R$ on murine lymphocytes, thereby enhancing the anti-IL-15 effect in the site of inflammation without causing the activation of $Fc_\gamma R$-mediated activation since IL-15Rα-IgG_1-Fc bound to $Fc_\gamma R$ would not lead to the receptor-crosslinking required for activation. The pcDNA3.1 vector contains a gene conferring resistance to G418 and encodes the *myc* epitope allowing detection of recombinant protein by anti-*myc* antibodies (Invitrogen). The recombinant protein is expressed under the control of the hCMV promotor.

Stable transfection of COS-7 cells did not result in high-level recombinant protein expression. Although transfected COS-7 colonies were screened for maximum production of IL-15Rα-IgG_1-Fc fusion protein by DOT blot, large-scale culture and purification of the recombinant protein by affinity to protein A only yielded very small amounts of protein. A potentially toxic effect of IL-15Rα-IgG_1-Fc protein expression in COS-7 cells could also be a reason for the relatively low yield of recombinant protein after stable transfection. In this case, selection of expression colonies by DOT blot may have excluded high-yield colonies due to the toxicity of the protein. However, IL-15Rα-IgG_1-Fc fusion protein could be readily detected in Western blot analysis. COS-7 cells poorly secreted the recombinant protein, which may be due to the use of the native IL-15Rα leader peptide within the IL-15Rα-IgG_1-Fc expression construct. The relatively low yield of recombinant protein in stably transfected COS-7 cells may be partly explained by the downregulation of the

hCMV promotor over time after transfection, which has been demonstrated to occur in many transfected cell types.

To increase the efficiency of recombinant protein expression by increasing the number of plasmid copies per cell, transient transfection of COS-7 cells was performed. Supernatant of transiently transfected COS-7 cells effectively neutralized the binding of sIL-15Rα to IL-15, whereas supernatant from COS-7 cells transfected with pcDNA3.1 alone did not contain anti-IL-15 activity. Taken together, these data indicate that IL-15Rα-IgG_1-Fc fusion protein is expressed in COS-7 cells and is functional in terms of binding to IL-15 and containing an active IgG_1-Fc domain as shown by its interaction with protein A and the anti-murine IgG_1 mAb in Western blot analysis. However, the low yield of IL-15Rα-IgG_1-Fc fusion protein has so far prevented its use *in vivo*. Expression and secretion of IL-15Rα-IgG_1-Fc fusion protein may be increased by employing a different leader peptide, which may prevent the potentially toxic effect of accumulation of the recombinant protein within the host cell or its premature degradation in the cytoplasm. Furthermore, the use of different expression vector systems (e.g. baculovirus) may or different promoters used to drive recombinant protein expression may increase the yield of IL-15Rα-IgG_1-Fc production to enable *in vivo* experimentation.

6.2 Recombinant sIL-15Rα in collagen-induced arthritis

IL-15 mediates pleiotropic effects on a variety of immune cells (Tagaya, *et al.*, 1996). However, unlike IL-2, with which it shares partial functional homology, IL-15 may be generated not only by cells within the immune system but also by cells belonging to other tissues, including keratinocytes and synoviocytes (McInnes, *et al.*, 1996; Thurkow, *et al.*, 1997; Mohamadzadeh, *et al.*, 1995). By this means, host tissues may contribute significantly to the regulation of protective or autoimmune responses. The functional

effects of IL-15 that have been demonstrated thus far include the activation of NK cells and neutrophils and the autocrine regulation of macrophages indicating an important role for IL-15 in early, innate host defense (Carson, *et al.*, 1995; Girard, *et al.*, 1996; Alleva, *et al.*, 1997). However, IL-15 additionally induces the activation of T cell blasts and will support the maturation and isotype switching of B-lymphocytes. Thus, a role in the development of antigen-specific responses might be predicted, although formal evidence of this role *in vivo* has not yet been demonstrated. IL-15 expression has been detected in several human diseases including RA, pulmonary sarcoidosis, inflammatory bowel disease, and chronic active hepatitis, providing circumstantial evidence for a role in chronic immunopathology (McInnes, *et al.*, 1996; Kirman and Nielsen, 1996, Agostini, *et al.*, 1996, Thurkow, *et al.*, 1997). The mechanisms whereby IL-15 could be involved in such inflammatory tissue destruction have not been clearly defined. Results obtained in this thesis indicate that IL-15 expression is required for the induction of erosive inflammatory arthritis following a challenge of collagen-primed DBA/1 mice. Moreover, both the altered serum Ig levels detected and the *in vitro* evidence for suppressed spleen cell proliferation and cytokine production indicate that the collagen-specific response was at least partly dependent on IL-15. Since IL-15 induces T cell chemotaxis *in vivo* and *in vitro* (Wilkinson and Liew, 1995; McInnes, *et al.*, 1996) and induces proliferation, adhesion molecule expression, and cytokine production *in vitro* (Kanegane and Tosato, 1996; McInnes, *et al.*, 1997), it is possible that IL-15 mediates effects in inflammatory arthritis, at least in part, through its activities on antigen-specific T cells. Several recent data have provided suggestive evidence for antigen-driven T cell clonal expansion in patients with long-standing RA (Schmidt, *et al.*, 1996; Scotet, *et al.*, 1996). Further possible explanations include the alteration of antigen-presentation or subsequent T cell costimulation, since both peripheral blood- and skin-derived dendritic cells are known to express IL-15 (Jonuleit, *et al.*, 1997), or the modification of adjuvant activity, since IL-15 up-regulation has been detected during mycobacterial infection (Jullien, *et al.*, 1997).

The CIA model provides an opportunity to study the relative contribution of immune pathways to the development of inflammatory arthritis. Using this approach, previous studies have demonstrated a role for Th1 cells and several proinflammatory cytokines, including TNFα and IL-1 in disease development (van den Berg, *et al.*, 1994; Simon, *et al.*, 1994; Williams, *et al.*, 1994; Mauri, *et al.*, 1996; Mussener, *et al.*, 1997). Such observations in rodents are clearly of relevance, since subsequent clinical trials with neutralizing antibodies against TNFα and soluble TNFR have demonstrated efficacy in human RA (Elliott, *et al.*, 1994; Rankin, *et al.*, 1995) and implied a pivotal role for TNFα in RA pathogenesis. IL-15 is capable of "bystander" activation of RA synovial T cells. After this activation, these cells may produce cytokines, including TNFα, either directly or through cognate interaction with adjacent macrophages (McInnes *et al.*, 1997). Thus, IL-15 could act upstream from TNFα in orchestrating the production of inflammatory cytokines in the chronically inflamed RA synovium. The data presented here provide direct evidence *in vivo* that IL-15 can also play a pivotal role in the development of inflammatory arthritis. Beside its postulated function in innate immunity (Carson, *et al.*, 1995; Girard, *et al.*, 1996), a critical role in the modulation of acquired immunity is suggested for IL-15. IL-15Rα administration effectively suppressed collagen-specific responses that were measured *in vivo* by serum Ig and *in vitro* by spleen cell responses. This finding was unexpected, since the mice used were IL-2 replete. IL-15 and IL-2 share occupancy of the IL-2/15Rβ-chain and the common γ-chain and transduce similar JAK1/3-STAT3/5-dependent pathways thereafter (Johnston, *et al.*, 1995), leading to the suggestion that some functional redundancy might exist. However, the sIL-15Rα chain did not exhibit any binding to IL-2 *in vitro* nor did it inhibit IL-2-mediated CTLL proliferation, making it unlikely that cross-reactivity could explain our observations. Rather, it is likely that early IL-15 production during antigen-challenge is necessary for the normal development of

specific immune responses. The availability of sIL-15Rα and the availability of mice lacking IL-15Rα (Lodolce, *et al.*, 1998) may facilitate future studies to investigate the precise relationship and functional overlap between IL-2 and IL-15.

In DBA/1 mice, the continuous administration of sIL-15Rα on a daily basis led to the development of anti-sIL-15Rα antibodies. This was unexpected since cDNA from a murine cell line was used for the cloning and expression of sIL-15Rα. However, anti-sIL-15Rα antibodies derived from mice treated with sIL-15Rα did not inhibit the neutralizing activity of the recombinant protein on IL-15 *in vitro*. Western blot analysis also indicated, that the immunogenicity of sIL-15Rα may be caused by the non-isogenic 6xHis fusion domain encoded by the pQE30 vector, which was used to purify sIL-15Rα by affinity binding. This is despite the claim that the 6xHis tag confers low immunogenicity (Qiagen). It cannot be excluded that serum anti-sIL-15Rα antibodies facilitated the clearance and neutralization of the sIL-15Rα *in vivo* and may even have had a stimulatory effect on endogenous IL-15Rα. The problem of anti-sIL-15Rα antibodies in treated mice may be overcome by the cloning of a minimal peptide fragment capable of neutralizing IL-15 efficiently without containing 'foreign' peptide sequences. The development of alternative treatment schedules for sIL-15Rα in CIA may also provide a tool to circumvent the development of anti-sIL-15Rα antibodies, which might neutralize the effects of the IL-15 antagonizing agent.

Thus, sIL-15Rα profoundly suppressed the development of CIA and markedly inhibited the onset of the humoral and Th1 cell-mediated anti-collagen response. These results provide important *in vivo* data, which show a role for IL-15 in inflammatory arthritis and suggest that antagonists to this cytokine could be of therapeutic benefit.

6.3 Conditional mutagenesis of IL-15Rα in ES cells

Gene targeting through homologous recombination provides a powerful tool to analyze the function of defined gene products *in vivo*. In the field of Immunology, gene targeting technology has contributed substantially to the current understanding of how cells of the immune system develop, how receptor activation related signals are processed within immune cells and how immune cells respond to activating stimuli. Gene targeting has thus become a common approach in obtaining functional information for newly found cytokines and other immune mediators. The phenotypic observations made from mutant mice that are deficient for a specific cytokine have provided important evidence for the involvement of these cytokines in specific immune responses to pathogens and in autoimmunity. The recently developed Cre/lox system allows the generation of mice that are mutant for specific target genes in a tissue specific and/or inducible manner. One clear advantage of this technique is the possible avoidance of generating mutants that lead to prenatal mortality. However, as previous targeting experiments show, the deficiency for specific immune mediators using conventional gene targeting techniques is unlikely to result in embryonic lethality. On the contrary, the high degree of redundancy observed for the functions of many cytokines may lead to mutant mice that are phenotypically very similar to wild type mice. However this has not been the case where cytokines have been found to mediate developmental processes in the maturation of the immune system (e.g. LTα/b, IL-2, IL-15Rα). In these targeting experiments, the broad effect of the gene product has led to a phenotype in mutants, that could be characterized even without exposing mutant animals to exogenous immunologic stimuli (Korner, *et al.*, 1997; Alimzhanov, *et al.*, 1997, Lodolce, *et al.*, 1998). The Cre/lox system enables the generation of tissue specific mutants where the lack of a specified gene product can be studied in an

otherwise wild-type animal. The system is thus providing the opportunity to obtain a more accurate mutant phenotype where secondary side effects of the generated mutation can be reduced to a minimum. The rapidly growing collection of Cre-transgenic mice that express the recombinase either constitutively or upon induction may make conditional mutagenesis become more suitable for the broad application of gene targeting. In this thesis, conditional mutagenesis of the IL-15Rα locus was used in an attempt to generate ES cell clones that, after germ line transmission, result in either '*null*' mutants or conditional mutants for IL-15Rα when crossed with a Cre-transgenic animal. Unlike IL-2Rα, IL-15Rα is expressed in many types of tissue indicating potential functions for IL-15 outside the immune system. The Cre/lox system has therefore been chosen for the targeting of IL-15Rα so as to allow studies on the effect of IL-15Rα mediated functions in immune cells alone. Although mice lacking IL-15Rα have been generated using conventional gene targeting techniques, this data has not yet been available by the time, the conditional mutagenesis of IL-15Rα was undertaken. As demonstrated for the IL-2/IL-2R system the deletion of a cytokine receptor may result in a more pronounced biological phenotype than the deletion of the corresponding cytokine (Schorle, *et al.*, 1991; van Parjis, *et al.*, 1997, Suzuki, *et al.*, 1997). Therefore the high affinity IL-15Rα rather than IL-15 itself has been selected as a target. The major experimental steps to conditionally target IL-15Rα in ES cells are discussed here.

The gene encoding IL-15Rα has been characterized by using the cDNA encoding the extracellular domain of IL-15Rα as a radiolabeled probe to screen a murine genomic library. Two lambda clones were identified that contained genetic material encoding IL-15Rα. However only two out of seven exons that have been described for the IL-15Rα gene (not including alternative splicing variants) (Anderson, *et al.*, 1995) were contained within these clones. These two exons corresponded to the coding exon/protein domain

structure described for human IL-15Rα and encoded essential parts of the extracellular protein-binding domain of IL-15Rα. No other exon DNA was detected in either of the lambda clones found after library screening. Since exon 2 contains genetic material encoding crucial parts of the protein-binding motif of IL-15Rα most likely to mediate the binding of IL-15, exon 2 was selected as a target for conditional mutagenesis. The targeting vector was designed to flank exon 2 by loxP sites. The targeting construct also contained a positive selection marker cassette placed 5' of exon 2, which was flanked by a 5' loxP site and the loxP site upstream of exon 2.

Integration of transferred DNA into the chromosomes of mammalian cells occurs much more frequently by non-homologous rather than by homologous recombination. This effect impedes the detection and isolation of cells where the rare targeting events have occurred (Capecchi, 1989). Although the exact mechanism by which homologous recombination occurs is not well understood, several factors are known to influence the efficiency of homologous recombination in gene targeting experiments. These factors, most importantly the isogenic nature of the construct, length of the homologous region and transcriptional activity of the target locus therefore influence the design of targeting constructs commonly used to generate genomic alterations.

The frequency of homologous recombination is known to be influenced by the degree of variation between intrinsic genomic DNA segments and the corresponding homologous part of the targeting construct (te Riele, *et al.*, 1992). The number of polymorphic variations in genetic material derived from different mouse strains could therefore lead to a decrease in targeting efficiency if the targeting construct is prepared from DNA derived from a different mouse strain than the ES cells that are used to generate the mutation. Polymorphisms are most likely to occur in non-transcribed introns, rather

than in coding exon sequences. In this targeting experiment, both the E14/2 ES cells and the λFixII genomic library are derived from the 129SV strain. However, the relatively complicated cloning procedure required to generate the 3'homologous region of the targeting construct and the 3'-loxP site may have resulted in a reduction of targeting events due to the insertion of pBlueScript-DNA derived from the MCS of lambda derived subclones.

The length of the DNA segment homologous between targeting vector and target gene is also known to influence the frequency of homologous recombination events (Thomas and Capecchi, 1987; Hasty, *et al.*, 1991). A positive correlation has been shown to exist between the length of the homologous region and the frequency of recombination events. Conventional targeting constructs that contain a selectable marker cassette flanked by two homologous regions usually contain 5-10kb of DNA homologous to the target sequence. In this targeting experiment, the 5' homologous region was composed of 3.7kb, whereas the 3'homologous region contained 3.1kb, resulting in a total of 6.8kb. Asymmetry of homologous regions used to clone the targeting construct may be preferred to allow PCR screening for targeting events. In this targeting experiment, successful PCR screening for genomic alterations generated by the targeting construct could only be employed after Cre-mediated recombination, which caused the shortening of the PCR template compared to the wild-type allele and thus preferential amplification in PCR analysis. Prior to Cre-mediated recombination, PCR screening for 3'targeting events proved difficult. This may have been caused by the relatively long PCR template of the putative targeted allele, which was approximately 2kb longer than the wild type allele (due to the presence of the *Neo* cassette). Amplification could not be achieved even when long range DNA polymerases were used.

Some evidence exists that homologous recombination is more likely to occur in gene loci that are transcriptionally active during the time of the gene targeting experiment (Mansour, *et al.*, 1988; Johnson, *et al.*, 1989; Nickoloff, *et al.*, 1990). Attempts were made to detect the presence of IL-15Rα-mRNA in ES cells by RT-PCR. However, no mRNA encoding IL-15Rα could be detected using this method. The low efficiency of homologous recombination events in IL-15Rα targeting may therefore be due to the fact that the gene encoding IL-15Rα is silent in ES cells. Selectable marker cassettes are frequently used in gene targeting experiments to enrich targeted ES cell clones in culture since their presence within the targeting construct does not appear to negatively influence the frequency of targeting events (Mansour, *et al.*, 1990). In this targeting experiment, a negative selection marker gene (HSV-*tk*) was placed at the 3' end of the targeting construct. The construct also contained a *Neo* selection marker gene placed between the 5' and 3' homologous region. Random integration of the construct should result in ES cell clones that are both G418 resistant and sensitive to gancyclovir since the presence of the HSV-tk gene converts gancyclovir into a toxic derivative (Mansour, *et al.*, 1988). Recombination between the homologous regions of the targeting construct and the endogenous gene locus does not result in the integration of the HSV-tk gene. In this targeting experiment, the use of gancyclovir reduced the number of clones obtained after electroporation by approximately 5 fold. A further enrichment of targeting events by up to 100 fold can be achieved using promotor trap vectors that take advantage of the transcriptional machinery of the endogenous gene locus to drive the expression of the positive selection marker gene following homologous recombination (Mountford, *et al.*, 1994). However, promotor trap vectors require the target gene locus to be transcriptionally active, which is not the case for IL-15Rα.

Southern blot analysis was used to screen for targeting events. Hybridization of the 5' external DNA probe with gDNA derived from ES cell clones resulted in a 7kb band for the wild type, and 4.2 kb band for the targeted IL-15Rα allele due to the insertion of a *Xho*I site 5' of the *Neo* selection marker cassette. The restriction endonuclease *Xho*I was used to fragmentize gDNA prior to Southern blot analysis. This has proved difficult, since the low frequency of *Xho*I sites in murine gDNA may result in poor digestion and fragment separation leading to an insufficient signal in Southern blot analysis. However, it has been necessary to use this approach since the hybridization of a putative 3' external probe (located at the 3' end of subclone 3.2) resulted in unspecific binding to 'smear' bands in Southern blot analysis. This may be due to the presence of large AT-nucleotide repeats within the 3' external probe often found in intron sequences which can lead to nonspecific hybridization. A PCR approach was therefore used to reconfirm the 3' integration event of the targeting construct following Cre-mediated recombination.

Cre-mediated recombination was performed *in vitro* by transiently transfecting targeted ES cells with a Cre-expression plasmid. Initial experiments using the Cre-expression plasmid pBS185 (Gibco, BRL) did not result in clones that had undergone Cre-mediated recombination events, although approximately 200 ES cell clones were analyzed by PCR. This may have been due to the lack of a selectable marker for pBS185-transfected ES cells. Since the desired Cre-mediated recombination in targeted ES cells would result in the deletion of the *Neo* selection marker cassette, G418 was unsuitable for selecting ES cells that had been transfected with Cre-expression plasmid. Using pBS185, ES cells had to be diluted sequentially and cultured in the absence of G418 to obtain ES cell colonies. To select for Cre-transfected ES cells after electroporation, the Cre-expression plasmid pCAG-Cre-IP was used. This plasmid expresses Cre recombinase under the CAG promotor and also contains the gene encoding puromycin-acetylase (*pac*). Puromycin is capable of killing non-resistant ES cells within 48 hours of culture and is therefore particularly

suitable for the selection of transient transfection events (Taniguchi, *et al.*, 1998). Selection with puromycin resulted in only 17 ES cell colonies, indicating that the transfection rate (approximately 10^6 cells used for electroporation) had been very low. All clones obtained exhibited the same Cre-mediated deletion resulting from recombination between the most 5' and 3' loxP sites and subsequent excision of both the loxP flanked exon 2 of the IL-15Rα gene and the selectable marker cassette (*Neo*). This may be due to the fact that Cre-expression under the CAG promotor is very efficient and therefore results in total rather than partial excision of loxP site flanked sequences in transfected cells. PCR analysis using a pair of internal and external oligonucleotides results in a PCR product of 0.9kb.

Conditional mutagenesis of the IL-15Rα gene has therefore led to the generation of two ES cell clones. The first clone contains loxP sites flanking coding exon 2 of the IL-15Rα gene. This exon encodes the major part of the protein binding 'Sushi' domain thought to mediate the binding of IL-15 to its receptor. The clone also contains the *Neo* selectable marker cassette, which is also flanked by loxP sites. It has been reported that the presence of a selectable marker gene may occasionally interfere with gene expression (Artelt, *et al.*, 1991). However, the selectable marker gene in the IL-15Rα locus is positioned within the large intron sequence separating exon 1 and exon 2 and may therefore not have any adverse effect on the expression of a functional IL-15Rα in homozygous mice. This clone may therefore provide the possibility to generate mice that are suitable for conditional targeting experiments by crossing to Cre-transgenic mice, e.g. lck-Cre. However, it may be advantageous to generate ES cell clones lacking the *Neo* cassette by employing a Cre-expression plasmid where Cre is placed under the control of a weak promotor. Also, the optimization of transfection conditions may lead to an increase in Cre-transfected clones providing the possibility to detect *Neo*-deficient ES cell clones by PCR screening. A second ES cell clone results from Cre-mediated recombination of the

targeted ES cell clone. In this clone, the targeted allele is lacking exon 2 and the selectable marker cassette. Germ line transmission and subsequent generation of homozygous mice would result in IL-15Rα-deficient animals.

6.4 Conclusions

The present study is separated into two major components. Firstly, soluble, recombinant IL-15 receptors were cloned and expressed to use as IL-15 antagonists *in vitro* and *in vivo*. The second part of this project has focused on the generation of a targeted ES cell clone to obtain a murine model lacking IL-15Rα by conditional mutagenesis.

Using a bacterial expression system, recombinant sIL-15Rα was successfully produced in suitable quantities. A mammalian expression system was used to produce an IL-15Rα-IgG-Fc fusion protein but this approach did not result in the desired yield although both proteins bound IL-15 specifically. Soluble IL-15Rα was successfully used as a therapeutic agent in murine collagen induced arthritis. These results further support evidence that IL-15 present in the rheumatoid synovium may exacerbate disease pathology, probably by stimulating the production of TNFα. Recombinant antagonists to IL-15 may therefore have a therapeutic potential in human rheumatoid arthritis.

Using the Cre/lox system of conditional mutagenesis, two ES cell clones have been generated. The first clone contains all functional exons encoding IL-15Rα. Exon 2 is flanked by loxP sites. This clone should allow for conditional mutagenesis of the IL-15Rα locus in mice derived from this clone. The second ES cell clone results from Cre mediated deletion of loxP site flanked sequences deleting exon 2 of the IL-15Rα gene and the *Neo* selection marker cassette. Mice derived from this clone should not express a functional IL-15Rα protein. The murine model generated using these clones may provide a useful tool to study the effects of IL-15-mediated immune responses *in vivo*.

6.5 Future studies

The following points provide a possible framework for future studies aiming to further investigate the potential of recombinant IL-15Rα proteins as therapeutic agents *in vivo* as well as for basic investigations of the functions of IL-15 in the murine model.

- Identification of the minimal binding domain of IL-15Rα to specifically interact with IL-15 *in vivo*. Crystal structure analysis of putative binding domains for the design of therapeutic antagonists.

- Optimization of expression of IL-15Rα-IgG_1-Fc fusion protein and subsequent *in vivo* analysis of its therapeutic potential in CIA.

- Identification of therapeutic properties of IL-15 antagonists in murine models of autoimmune disease where IL-15 has been characterized as a putative causative agent.

- Generation of mutant mice using the ES cell clones generated. Analysis of phenotypic results of the deletion in T cell specific and general IL-15Rα knockout mice.

The recombinant sIL-15Rα is currently employed in a number of studies undertaken in different laboratories aiming to elucidate the role of IL-15 in different disease models *in vivo*. The names and addresses of collaborators using the sIL-15Rα are listed below.

The role of IL-15 in cardiac allograft rejection:

Prof. J.A. Bradley, Dept of Surgery, Addensbrooke's Hospital, Cambridge, UK.

Host defense against viral pathogens:

Dr. C. Biron, Division of Biology and Medicine, Brown University, USA.

The role of IL-15 in inflammatory bowel disease:

Dr. F. Powrie, Dept. of Surgery, University of Oxford, Oxford, UK.

The role of IL-15 in HSV infection:

Dr. H. Nishimura, Research Institute for Disease Mechanism and Control, Nagoya, Japan

Bibliography

Internet resources:

Database of Cre-transgenic animals: http://www.mshri.on.ca/develop/nagy/Cre.html

Database of transgenic and knockout mice (TBASE): http://tbase.jax.org

Alphabetic list of knockout genes: http://www.bioscience.org/knockout/alphabet.htm

Journal articles

Abremski, K., Wierzbicki, A., Frommer, B., and Hoess, R. H. (1986). Bacteriophage P1 Cre-loxP site-specific recombination. Site-specific DNA topoisomerase activity of the Cre recombination protein. *J Biol Chem* **261,** 391-6.

Abuin, A., and Bradley, A. (1996). Recycling selectable markers in mouse embryonic stem cells. *Mol Cell Biol* **16,** 1851-6.

Agostini, C., Trentin, L., Facco, M., Sancetta, R., Cerutti, A., Tassinari, C., Cimarosto, L., Adami, F., Cipriani, A., Zambello, R., and Semenzato, G. (1996). Role of IL-15, IL-2, and their receptors in the development of T cell alveolitis in pulmonary sarcoidosis. *J Immunol* **157,** 910-8.

Agostini, C., Trentin, L., Perin, A., Facco, M., Siviero, M., Piazza, F., Basso, U., Adami, F., Zambello, R., and Semenzato, G. (1999). Regulation of alveolar macrophage-T cell interactions during Th1-type sarcoid inflammatory process. *Am J Physiol* **277,** L240-L250.

Ajjan, R. A., Watson, P. F., and Weetman, A. P. (1997). Detection of IL-12, IL-13, and IL-15 messenger ribonucleic acid in the thyroid of patients with autoimmune thyroid disease. *J Clin Endocrinol Metab* **82,** 666-9.

Alexopoulou, L., Pasparakis, M., and Kollias, G. (1998). Complementation of lymphotoxin alpha knockout mice with tumor necrosis factor-expressing transgenes rectifies defective splenic structure and function. *J Exp Med* **188,** 745-54.

Alimzhanov, M. B., Kuprash, D. V., Kosco-Vilbois, M. H., Luz, A., Turetskaya, R. L., Tarakhovsky, A., Rajewsky, K., Nedospasov, S. A., and Pfeffer, K. (1997). Abnormal development of secondary lymphoid tissues in lymphotoxin beta-deficient mice. *Proc Natl Acad Sci U S A* **94,** 9302-7.

Allavena, P., Giardina, G., Bianchi, G., and Mantovani, A. (1997). IL-15 is chemotactic for natural killer cells and stimulates their adhesion to vascular endothelium. *J Leukoc Biol* **61,** 729-35.

Alleva, D. G., Kaser, S. B., Monroy, M. A., Fenton, M. J., and Beller, D. I. (1997). IL-15 functions as a potent autocrine regulator of macrophage proinflammatory cytokine production: evidence for differential receptor subunit utilization associated with stimulation or inhibition. *J Immunol* **159,** 2941-51.

Alonzi, T., Fattori, E., Lazzaro, D., Costa, P., Probert, L., Kollias, G., De Benedetti, F., Poli, V., and Ciliberto, G. (1998). Interleukin 6 is required for the development of collagen-induced arthritis. *J Exp Med* **187,** 461-8.

Anderson, D. M., Johnson, L., Glaccum, M. B., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Valentine, V., Kirstein, M. N., Shapiro, D. N., Morris, S. W., and et al. (1995). Chromosomal assignment and genomic structure of Il15. *Genomics* **25,** 701-6.

Anderson, D. M., Kumaki, S., Ahdieh, M., Bertles, J., Tometsko, M., Loomis, A., Giri, J., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and et al. (1995). Functional characterization of the human interleukin-15 receptor alpha chain and close linkage of IL15RA and IL2RA genes. *J Biol Chem* **270,** 29862-9.

Angiolillo, A. L., Kanegane, H., Sgadari, C., Reaman, G. H., and Tosato, G. (1997). Interleukin-15 promotes angiogenesis in vivo. *Biochem Biophys Res Commun* **233,** 231-7.

Apparailly, F., Verwaerde, C., Jacquet, C., Auriault, C., Sany, J., and Jorgensen, C. (1998). Adenovirus-mediated transfer of viral IL-10 gene inhibits murine collagen-induced arthritis. *J Immunol* **160,** 5213-20.

Armitage, R. J., Macduff, B. M., Eisenman, J., Paxton, R., and Grabstein, K. H. (1995). IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation. *J Immunol* **154,** 483-90.

Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M. E. (1995). Notch signaling. *Science* **268,** 225-32.

Artelt, P., Grannemann, R., Stocking, C., Friel, J., Bartsch, J., and Hauser, H. (1991). The prokaryotic neomycin-resistance-encoding gene acts as a transcriptional silencer in eukaryotic cells. *Gene* **99,** 249-54.

Atedzoe, B. N., Ahmad, A., and Menezes, J. (1997). Enhancement of natural killer cell cytotoxicity by the human herpesvirus-7 via IL-15 induction. *J Immunol* **159,** 4966-72.

Austin, S., Ziese, M., and Sternberg, N. (1981). A novel role for site-specific recombination in maintenance of bacterial replicons. *Cell* **25,** 729-36.

Azimi, N., Brown, K., Bamford, R. N., Tagaya, Y., Siebenlist, U., and Waldmann, T. A. (1998). Human T cell lymphotropic virus type I Tax protein trans-activates interleukin 15 gene transcription through an NF-kappaB site. *Proc Natl Acad Sci U S A* **95,** 2452-7.

Badolato, R., Ponzi, A. N., Millesimo, M., Notarangelo, L. D., and Musso, T. (1997). Interleukin-15 (IL-15) induces IL-8 and monocyte chemotactic protein 1 production in human monocytes. *Blood* **90,** 2804-9.

Bakker, A. C., Joosten, L. A., Arntz, O. J., Helsen, M. M., Bendele, A. M., van de Loo, F. A., and van den Berg, W. B. (1997). Prevention of murine collagen-induced arthritis in the knee and ipsilateral paw by local expression of human interleukin-1 receptor antagonist protein in the knee. *Arthritis Rheum* **40,** 893-900.

Bamford, R. N., Grant, A. J., Burton, J. D., Peters, C., Kurys, G., Goldman, C. K., Brennan, J., Roessler, E., and Waldmann, T. A. (1994). The interleukin (IL) 2 receptor beta 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 cells. *Proc Natl Acad Sci U S A* **91,** 4940-4.

Bamford, R. N., Battiata, A. P., Burton, J. D., Sharma, H., and Waldmann, T. A. (1996). Interleukin (IL) 15/IL-T production by the adult T-cell leukemia cell line HuT-102 is associated with a human T-cell lymphotrophic virus type I region /IL-15 fusion message that lacks many upstream AUGs that normally attenuates IL-15 mRNA translation. *Proc Natl Acad Sci U S A* **93,** 2897-902.

Bamford, R. N., DeFilippis, A. P., Azimi, N., Kurys, G., and Waldmann, T. A. (1998). The 5' untranslated region, signal peptide, and the coding sequence of the carboxyl terminus of IL-15 participate in its multifaceted translational control. *J Immunol* **160,** 4418-26.

Bessis, N., Boissier, M. C., Ferrara, P., Blankenstein, T., Fradelizi, D., and Fournier, C. (1996). Attenuation of collagen-induced arthritis in mice by treatment with vector cells engineered to secrete interleukin-13. *Eur J Immunol* **26,** 2399-403.

Borger, P., Kauffman, H. F., Postma, D. S., Esselink, M. T., and Vellenga, E. (1999). Interleukin-15 differentially enhances the expression of interferon-gamma and interleukin-4 in activated human (CD4+) T lymphocytes. *Immunology* **96,** 207-14.

Bulfone-Paus, S., Ungureanu, D., Pohl, T., Lindner, G., Paus, R., Ruckert, R., Krause, H., and Kunzendorf, U. (1997). Interleukin-15 protects from lethal apoptosis in vivo. *Nat Med* **3,** 1124-8.

Bulfone-Paus, S., Bulanova, E., Pohl, T., Budagian, V., Durkop, H., Ruckert, R., Kunzendorf, U., Paus, R., and Krause, H. (1999). Death deflected: IL-15 inhibits TNF-alpha-mediated apoptosis in fibroblasts by TRAF2 recruitment to the IL-15Ra chain. *Faseb J* **13,** 1575-85.

Burmester, G. R., Locher, P., Koch, B., Winchester, R. J., Dimitriu-Bona, A., Kalden, J. R., and Mohr, W. (1983). The tissue architecture of synovial membranes in inflammatory and non-inflammatory joint diseases. I. The localization of the major synovial cell populations as detected by monoclonal reagents directed towards Ia and monocyte-macrophage antigens. *Rheumatol Int* **3,** 173-81.

Burton, J. D., Bamford, R. N., Peters, C., Grant, A. J., Kurys, G., Goldman, C. K., Brennan, J., Roessler, E., and Waldmann, T. A. (1994). 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. *Proc Natl Acad Sci U S A* **91,** 4935-9.

Bradley, A., Evans, M., Kaufman, M. H., and Robertson, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309,** 255-6.

Brinster, R. L. (1974). The effect of cells transferred into the mouse blastocyst on subsequent development. *J Exp Med* **140,** 1049-56.

Brown, S. J., Becherer, K. A., Blumeyer, K., Kautzer, C., Axelrod, F., Le, H., McConnell, S. J., Whalley, A., and Spinella, D. G. (1998). Expression and ligand binding assays of soluble cytokine receptor-immunoglobulin fusion proteins. *Protein Expr Purif* **14,** 120-4.

Campbell, I. K., Rich, M. J., Bischof, R. J., Dunn, A. R., Grail, D., and Hamilton, J. A. (1998). Protection from collagen-induced arthritis in granulocyte-macrophage colony-stimulating factor-deficient mice. *J Immunol* **161,** 3639-44.

Capecchi, M. R. (1980). High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* **22,** 479-88.

Capecchi, M. R. (1989). Altering the genome by homologous recombination. *Science* **244,** 1288-92.

Carson, W. E., Fehniger, T. A., Haldar, S., Eckhert, K., Lindemann, M. J., Lai, C. F., Croce, C. M., Baumann, H., and Caligiuri, M. A. (1997). A potential role for interleukin-15 in the regulation of human natural killer cell survival. *J Clin Invest* **99,** 937-43.

Caulfield, J. P., Hein, A., Dynesius-Trentham, R., and Trentham, D. E. (1982). Morphologic demonstration of two stages in the development of type II collagen-induced arthritis. *Lab Invest* **46,** 321-43.

Chae, D. W., Nosaka, Y., Strom, T. B., and Maslinski, W. (1996). Distribution of IL-15 receptor alpha-chains on human peripheral blood mononuclear cells and effect of immunosuppressive drugs on receptor expression. *J Immunol* **157,** 2813-9.

Chehimi, J., Marshall, J. D., Salvucci, O., Frank, I., Chehimi, S., Kawecki, S., Bacheller, D., Rifat, S., and Chouaib, S. (1997). IL-15 enhances immune functions during HIV infection. *J Immunol* **158,** 5978-87.

Combe, B., Pope, R. M., Fischbach, M., Darnell, B., Baron, S., and Talal, N. (1985). Interleukin-2 in rheumatoid arthritis: production of and response to interleukin-2 in rheumatoid synovial fluid, synovial tissue and peripheral blood. *Clin Exp Immunol* **59,** 520-8.

Courtenay, J. S., Dallman, M. J., Dayan, A. D., Martin, A., and Mosedale, B. (1980). Immunisation against heterologous type II collagen induces arthritis in mice. *Nature* **283,** 666-8.

Cullen, B. R., Podlaski, F. J., Peffer, N. J., Hosking, J. B., and Greene, W. C. (1988). Sequence requirements for ligand binding and cell surface expression of the Tac antigen, a human interleukin-2 receptor. *J Biol Chem* **263,** 4900-6.

Cush, J. J., and Lipsky, P. E. (1988). Phenotypic analysis of synovial tissue and peripheral blood lymphocytes isolated from patients with rheumatoid arthritis. *Arthritis Rheum* **31,** 1230-8.

D'Andrea, A., Fasman, G. D., and Lodish, H. F. (1989). Erythropoietin receptor and interleukin-2 receptor beta chain: a new. *Cell* **58,** 1023-4.

Davie, E. W., Ichinose, A., and Leytus, S. P. (1986). Structural features of the proteins participating in blood coagulation and fibrinolysis. *Cold Spring Harb Symp Quant Biol* **51 Pt 1,** 509-14.

DiSanto, J. P., Muller, W., Guy-Grand, D., Fischer, A., and Rajewsky, K. (1995b). Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. *Proc Natl Acad Sci U S A* **92,** 377-81.

Doherty, T. M., Seder, R. A., and Sher, A. (1996). Induction and regulation of IL-15 expression in murine macrophages. *J Immunol* **156,** 735-41.

Dubois, S., Magrangeas, F., Lehours, P., Raher, S., Bernard, J., Boisteau, O., Leroy, S., Minvielle, S., Godard, A., and Jacques, Y. (1999). Natural splicing of exon 2 of human interleukin-15 receptor alpha-chain mRNA results in a shortened form with a distinct pattern of expression. *J Biol Chem* **274,** 26978-84.

Duke, O., Panayi, G. S., Janossy, G., and Poulter, L. W. (1982). An immunohistological analysis of lymphocyte subpopulations and their microenvironment in the synovial membranes of patients with rheumatoid arthritis using monoclonal antibodies. *Clin Exp Immunol* **49,** 22-30.

Durie, F. H., Fava, R. A., Foy, T. M., Aruffo, A., Ledbetter, J. A., and Noelle, R. J. (1993). Prevention of collagen-induced arthritis with an antibody to gp39, the ligand for CD40. *Science* **261,** 1328-30.

Elliott, M. J., Maini, R. N., Feldmann, M., Long-Fox, A., Charles, P., Bijl, H., and Woody, J. N. (1994). Repeated therapy with monoclonal antibody to tumour necrosis factor alpha (cA2) in patients with rheumatoid arthritis. *Lancet* **344,** 1125-7.

Elliott, M. J., Maini, R. N., Feldmann, M., Long-Fox, A., Charles, P., Katsikis, P., Brennan, F. M., Walker, J., Bijl, H., Ghrayeb, J., and et al. (1993). Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha. *Arthritis Rheum* **36,** 1681-90.

Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292,** 154-6.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., and Chambon, P. (1996). Ligand-activated site-specific recombination in mice. *Proc Natl Acad Sci U S A* **93,** 10887-90.

Feldmann, M., Brennan, F. M., and Maini, R. N. (1996). Role of cytokines in rheumatoid arthritis. *Annu Rev Immunol* **14,** 397-440.

Flamand, L., Stefanescu, I., and Menezes, J. (1996). Human herpesvirus-6 enhances natural killer cell cytotoxicity via IL-15. *J Clin Invest* **97,** 1373-81.

Fleming, S. D., and Campbell, P. A. (1996). Macrophages have cell surface IL-10 that regulates macrophage bactericidal activity. *J Immunol* **156,** 1143-50.

Gaggero, A., Azzarone, B., Andrei, C., Mishal, Z., Meazza, R., Zappia, E., Rubartelli, A., and Ferrini, S. (1999). Differential intracellular trafficking, secretion and endosomal localization of two IL-15 isoforms. *Eur J Immunol* **29,** 1265-74.

Gagneten, S., Le, Y., Miller, J., and Sauer, B. (1997). Brief expression of a GFP cre fusion gene in embryonic stem cells allows rapid retrieval of site-specific genomic deletions. *Nucleic Acids Res* **25,** 3326-31.

Garcia, G., Komagata, Y., Slavin, A. J., Maron, R., and Weiner, H. L. (1999). Suppression of Collagen-induced Arthritis by Oral or Nasal Administration of Type II Collagen. *J Autoimmun* **13,** 315-324.

Garcia, V. E., Jullien, D., Song, M., Uyemura, K., Shuai, K., Morita, C. T., and Modlin, R. L. (1998). IL-15 enhances the response of human gamma delta T cells to nonpeptide microbial antigens. *J Immunol* **160,** 4322-9.

Gillis, S., and Smith, K. A. (1977). In vitro generation of tumor-specific cytotoxic lymphocytes. *J Exp Med* **146,** 468-82.

Girard, D., Paquet, M. E., Paquin, R., and Beaulieu, A. D. (1996). Differential effects of interleukin-15 (IL-15) and IL-2 on human neutrophils: modulation of phagocytosis, cytoskeleton rearrangement, gene expression, and apoptosis by IL-15. *Blood* **88,** 3176-84.

Giri, J. G., Ahdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L. S., Cosman, D., and Anderson, D. (1994). Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *Embo J* **13,** 2822-30.

Giri, J. G., Kumaki, S., Ahdieh, M., Friend, D. J., Loomis, A., Shanebeck, K., DuBose, R., Cosman, D., Park, L. S., and Anderson, D. M. (1995). Identification and cloning of a novel IL-15 binding protein that is structurally related to the alpha chain of the IL-2 receptor. *Embo J* **14,** 3654-63.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. *Science* **268,** 1766-9.

Grabstein, K. H., Eisenman, J., Shanebeck, K., Rauch, C., Srinivasan, S., Fung, V., Beers, C., Richardson, J., Schoenborn, M. A., Ahdieh, M., and et al. (1994). Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science* **264,** 965-8.

Gracie, J. A., Forsey, R. J., Chan, W. L., Gilmour, A., Leung, B. P., Greer, M. R., Kennedy, K., Carter, R., Wei, X. Q., Xu, D., Field, M., Foulis, A., Liew, F. Y., and McInnes, I. B. (1999). A proinflammatory role for IL-18 in rheumatoid arthritis. *J Clin Invest* **104,** 1393-1401.

Gregersen, P. K., Silver, J., and Winchester, R. J. (1987). The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum* **30,** 1205-13.

Gu, H., Marth, J. D., Orban, P. C., Mossmann, H., and Rajewsky, K. (1994). Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* **265,** 103-6.

Gu, H., Zou, Y. R., and Rajewsky, K. (1993). Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* **73,** 1155-64.

Hanisch, U. K., Lyons, S. A., Prinz, M., Nolte, C., Weber, J. R., Kettenmann, H., and Kirchhoff, F. (1997). Mouse brain microglia express interleukin-15 and its multimeric receptor complex functionally coupled to Janus kinase activity. *J Biol Chem* **272,** 28853-60.

Harada, S., Yamamura, M., Okamoto, H., Morita, Y., Kawashima, M., Aita, T., and Makino, H. (1999). Production of interleukin-7 and interleukin-15 by fibroblast-like synoviocytes from patients with rheumatoid arthritis. *Arthritis Rheum* **42,** 1508-16.

Hasty, P., Rivera-Perez, J., and Bradley, A. (1991). The length of homology required for gene targeting in embryonic stem cells. *Mol Cell Biol* **11,** 5586-91.

Hasty, P., Rivera-Perez, J., Chang, C., and Bradley, A. (1991). Target frequency and integration pattern for insertion and replacement vectors in embryonic stem cells. *Mol Cell Biol* **11,** 4509-17.

Hatakeyama, M., Mori, H., Doi, T., and Taniguchi, T. (1989). A restricted cytoplasmic region of IL-2 receptor beta chain is essential. *Cell* **59,** 837-45.

Hennet, T., Hagen, F. K., Tabak, L. A., and Marth, J. D. (1995). T-cell-specific deletion of a polypeptide N-acetylgalactosaminyl- transferase gene by site-directed recombination. *Proc Natl Acad Sci U S A* **92,** 12070-4.

Hoess, R. H., Wierzbicki, A., and Abremski, K. (1986). The role of the loxP spacer region in P1 site-specific recombination. *Nucleic Acids Res* **14,** 2287-300.

Holmdahl, R., Jansson, L., Andersson, M., and Larsson, E. (1988). Immunogenetics of type II collagen autoimmunity and susceptibility to collagen arthritis. *Immunology* **65,** 305-10.

Holmdahl, R., Jonsson, R., Larsson, P., and Klareskog, L. (1988). Early appearance of activated CD4+ T lymphocytes and class II antigen-expressing cells in joints of DBA/1 mice immunized with type II collagen. *Lab Invest* **58,** 53-60.

Horneff, G., Emmrich, F., and Burmester, G. R. (1993). Advances in immunotherapy of rheumatoid arthritis: clinical and immunological findings following treatment with anti-CD4 antibodies. *Br J Rheumatol* **32 Suppl 4,** 39-47.

Horneff, G., Winkler, T., Kalden, J. R., Emmrich, F., and Burmester, G. R. (1991). Human anti-mouse antibody response induced by anti-CD4 monoclonal antibody therapy in patients with rheumatoid arthritis. *Clin Immunol Immunopathol* **59,** 89-103.

Horsfall, A. C., Butler, D. M., Marinova, L., Warden, P. J., Williams, R. O., Maini, R. N., and Feldmann, M. (1997). Suppression of collagen-induced arthritis by continuous administration of IL-4. *J Immunol* **159,** 5687-96.

Jaenisch, R. (1976). Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus. *Proc Natl Acad Sci U S A* **73,** 1260-4.

Jaenisch, R., and Mintz, B. (1974). Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. *Proc Natl Acad Sci U S A* **71,** 1250-4.

Janossy, G., Montano, L., Selby, W. S., Duke, O., Panayi, G., Lampert, I., Thomas, J. A., Granger, S., Bofill, M., Tidman, N., Thomas, H. C., and Goldstein, G. (1982). T cell subset abnormalities in tissue lesions developing during autoimmune disorders, viral infection, and graft-vs.-host disease. *J Clin Immunol* **2,** 42S-56S.

Jirholt, J., Cook, A., Emahazion, T., Sundvall, M., Jansson, L., Nordquist, N., Pettersson, U., and Holmdahl, R. (1998). Genetic linkage analysis of collagen-induced arthritis in the mouse. *Eur J Immunol* **28,** 3321-8.

Johnson, H. M., Torres, B. A., Green, M. M., Szente, B. E., Siler, K. I., Larkin, J., 3rd, and Subramaniam, P. S. (1998). Cytokine-receptor complexes as chaperones for nuclear translocation of signal transducers. *Biochem Biophys Res Commun* **244,** 607-14.

Johnston, J. A., Bacon, C. M., Finbloom, D. S., Rees, R. C., Kaplan, D., Shibuya, K., Ortaldo, J. R., Gupta, S., Chen, Y. Q., and Giri, J. D. (1995). Tyrosine phosphorylation and activation of STAT5, STAT3, and Janus kinases by interleukins 2 and 15. *Proc Natl Acad Sci U S A* **92,** 8705-9.

Jonuleit, H., Wiedemann, K., Muller, G., Degwert, J., Hoppe, U., Knop, J., and Enk, A. H. (1997). Induction of IL-15 messenger RNA and protein in human blood-derived dendritic cells: a role for IL-15 in attraction of T cells. *J Immunol* **158,** 2610-5.

Joosten, L. A., Helsen, M. M., Saxne, T., van De Loo, F. A., Heinegard, D., and van Den Berg, W. B. (1999). IL-1 alpha beta blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-alpha blockade only ameliorates joint inflammation. *J Immunol* **163,** 5049-55.

Joosten, L. A., Helsen, M. M., van de Loo, F. A., and van den Berg, W. B. (1996). Anticytokine treatment of established type II collagen-induced arthritis in DBA/1 mice. A comparative study using anti-TNF alpha, anti-IL-1 alpha/beta, and IL-1Ra. *Arthritis Rheum* **39,** 797-809.

Jullien, D., Sieling, P. A., Uyemura, K., Mar, N. D., Rea, T. H., and Modlin, R. L. (1997). IL-15, an immunomodulator of T cell responses in intracellular infection. *J Immunol* **158,** 800-6.

Kageyama, Y., Koide, Y., Yoshida, A., Uchijima, M., Arai, T., Miyamoto, S., Ozeki, T., Hiyoshi, M., Kushida, K., and Inoue, T. (1998). Reduced susceptibility to collagen-induced arthritis in mice deficient in IFN-gamma receptor. *J Immunol* **161,** 1542-8.

Kakimoto, K., Nakamura, T., Ishii, K., Takashi, T., Iigou, H., Yagita, H., Okumura, K., and Onoue, K. (1992). The effect of anti-adhesion molecule antibody on the development of collagen-induced arthritis. *Cell Immunol* **142,** 326-37.

Kakumu, S., Okumura, A., Ishikawa, T., Yano, M., Enomoto, A., Nishimura, H., Yoshioka, K., and Yoshika, Y. (1997). Serum levels of IL-10, IL-15 and soluble tumour necrosis factor-alpha (TNF-alpha) receptors in type C chronic liver disease. *Clin Exp Immunol* **109,** 458-63.

Kanegane, H., and Tosato, G. (1996). Activation of naive and memory T cells by interleukin-15. *Blood* **88,** 230-5.

Kaye, J., Gillis, S., Mizel, S. B., Shevach, E. M., Malek, T. R., Dinarello, C. A., Lachman, L. B., and Janeway, C. A., Jr. (1984). Growth of a cloned helper T cell line induced by a monoclonal antibody specific for the antigen receptor: interleukin 1 is required for the expression of receptors for interleukin 2. *J Immunol* **133,** 1339-45.

Keffer, J., Probert, L., Cazlaris, H., Georgopoulos, S., Kaslaris, E., Kioussis, D., and Kollias, G. (1991). Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. *Embo J* **10,** 4025-31.

Kellendonk, C., Tronche, F., Monaghan, A. P., Angrand, P. O., Stewart, F., and Schutz, G. (1996). Regulation of Cre recombinase activity by the synthetic steroid RU 486. *Nucleic Acids Res* **24,** 1404-11.

Kennedy, T. D., Plater-Zyberk, C., Partridge, T. A., Woodrow, D. F., and Maini, R. N. (1988). Representative sample of rheumatoid synovium: a morphometric study. *J Clin Pathol* **41,** 841-6.

Kirman, I., and Nielsen, O. H. (1996). Increased numbers of interleukin-15-expressing cells in active ulcerative colitis. *Am J Gastroenterol* **91,** 1789-94.

Klimiuk, P. A., Yang, H., Goronzy, J. J., and Weyand, C. M. (1999). Production of cytokines and metalloproteinases in rheumatoid synovitis is T cell dependent. *Clin Immunol* **90,** 65-78.

Kono, T., Doi, T., Yamada, G., Hatakeyama, M., Minamoto, S., Tsudo, M., Miyasaka, M., Miyata, T., and Taniguchi, T. (1990). Murine interleukin 2 receptor beta chain: dysregulated gene expression in. *Proc Natl Acad Sci U S A* **87,** 1806-10.

Korner, H., Cook, M., Riminton, D. S., Lemckert, F. A., Hoek, R. M., Ledermann, B., Kontgen, F., Fazekas de St Groth, B., and Sedgwick, J. D. (1997). Distinct roles for lymphotoxin-alpha and tumor necrosis factor in organogenesis and spatial organization of lymphoid tissue. *Eur J Immunol* **27,** 2600-9.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44,** 283-92.

Kozak, M. (1987). At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J Mol Biol* **196,** 947-50.

Kozak, M. (1989). Context effects and inefficient initiation at non-AUG codons in eucaryotic cell-free translation systems. *Mol Cell Biol* **9,** 5073-80.

Kozak, M. (1991). A short leader sequence impairs the fidelity of initiation by eukaryotic ribosomes. *Gene Expr* **1,** 111-5.

Krause, H., Jandrig, B., Wernicke, C., Bulfone-Paus, S., Pohl, T., and Diamantstein, T. (1996). Genomic structure and chromosomal localization of the human interleukin 15 gene (IL-15). *Cytokine* **8,** 667-74.

Kuehn, M. R., Bradley, A., Robertson, E. J., and Evans, M. J. (1987). A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. *Nature* **326,** 295-8.

Kuhn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995). Inducible gene targeting in mice. *Science* **269,** 1427-9.

Kumaki, S., Armitage, R., Ahdieh, M., Park, L., and Cosman, D. (1996). Interleukin-15 up-regulates interleukin-2 receptor alpha chain but down- regulates its own high-affinity binding sites on human T and B cells. *Eur J Immunol* **26,** 1235-9.

Kurt-Jones, E. A., Beller, D. I., Mizel, S. B., and Unanue, E. R. (1985). Identification of a membrane-associated interleukin 1 in macrophages. *Proc Natl Acad Sci U S A* **82,** 1204-8.

Lakso, M., Pichel, J. G., Gorman, J. R., Sauer, B., Okamoto, Y., Lee, E., Alt, F. W., and Westphal, H. (1996). Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. *Proc Natl Acad Sci U S A* **93,** 5860-5.

Lakso, M., Sauer, B., Mosinger, B., Jr., Lee, E. J., Manning, R. W., Yu, S. H., Mulder, K. L., and Westphal, H. (1992). Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc Natl Acad Sci U S A* **89,** 6232-6.

Lam, K. P., and Rajewsky, K. (1998). Rapid elimination of mature autoreactive B cells demonstrated by cre- induced change in B cell antigen receptor specificity in vivo [In Process Citation]. *Proc Natl Acad Sci U S A* **95,** 13171-5.

Le, C. H., Nicolson, A. G., Morales, A., and Sewell, K. L. (1997). Suppression of collagen-induced arthritis through adenovirus-mediated transfer of a modified tumor necrosis factor alpha receptor gene. *Arthritis Rheum* **40,** 1662-9.

Lee, Y. B., Satoh, J., Walker, D. G., and Kim, S. U. (1996). Interleukin-15 gene expression in human astrocytes and microglia in culture. *Neuroreport* **7,** 1062-6.

Leonard, W. J., Depper, J. M., Kanehisa, M., Kronke, M., Peffer, N. J., Svetlik, P. B., Sullivan, M., and Greene, W. C. (1985). Structure of the human interleukin-2 receptor gene. *Science* **230,** 633-9.

Lin, F. L., Sperle, K., and Sternberg, N. (1985). Recombination in mouse L cells between DNA introduced into cells and homologous chromosomal sequences. *Proc Natl Acad Sci U S A* **82,** 1391-5.

Lindner, G., Ruckert, R., Bulfone-Paus, S., and Paus, R. (1998). Inhibition of chemotherapy-induced keratinocyte apoptosis in vivo by an interleukin-15-IgG fusion protein [letter]. *J Invest Dermatol* **110,** 457-8.

Lobe, C. G., Koop, K. E., Kreppner, W., Lomeli, H., Gertsenstein, M., and Nagy, A. (1999). Z/AP, a double reporter for cre-mediated recombination. *Dev Biol* **208,** 281-92.

Lodolce, J. P., Boone, D. L., Chai, S., Swain, R. E., Dassopoulos, T., Trettin, S., and Ma, A. (1998). IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* **9,** 669-76.

Ma, Y., Thornton, S., Duwel, L. E., Boivin, G. P., Giannini, E. H., Leiden, J. M., Bluestone, J. A., and Hirsch, R. (1998). Inhibition of collagen-induced arthritis in mice by viral IL-10 gene transfer. *J Immunol* **161,** 1516-24.

Madsen, L., Labrecque, N., Engberg, J., Dierich, A., Svejgaard, A., Benoist, C., Mathis, D., and Fugger, L. (1999). Mice lacking all conventional MHC class II genes. *Proc Natl Acad Sci U S A* **96,** 10338-43.

Mansour, S. L., Thomas, K. R., and Capecchi, M. R. (1988). Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* **336,** 348-52.

Mansour, S. L., Thomas, K. R., Deng, C. X., and Capecchi, M. R. (1990). Introduction of a lacZ reporter gene into the mouse int-2 locus by homologous recombination. *Proc Natl Acad Sci U S A* **87,** 7688-92.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* **78,** 7634-8.

Mauri, C., Williams, R. O., Walmsley, M., and Feldmann, M. (1996). Relationship between Th1/Th2 cytokine patterns and the arthritogenic response in collagen-induced arthritis. *Eur J Immunol* **26,** 1511-8.

Mayumi, T., Takezawa, J., Takahashi, H., Yamaguchi, H., Nishimura, H., Enomoto, A., Ichiyama, S., and Yoshikai, Y. (1999). Il-15 Is Elevated in the Patients of Postoperative Enterocolitis. *Cytokine* **11,** 888-893.

McInnes, I. B., al-Mughales, J., Field, M., Leung, B. P., Huang, F. P., Dixon, R., Sturrock, R. D., Wilkinson, P. C., and Liew, F. Y. (1996). The role of interleukin-15 in T-cell migration and activation in rheumatoid arthritis. *Nat Med* **2,** 175-82.

McInnes, I. B., Leung, B. P., Sturrock, R. D., Field, M., and Liew, F. Y. (1997). Interleukin-15 mediates T cell-dependent regulation of tumor necrosis factor-alpha production in rheumatoid arthritis. *Nat Med* **3,** 189-95.

McIntyre, K. W., Shuster, D. J., Gillooly, K. M., Warrier, R. R., Connaughton, S. E., Hall, L. B., Arp, L. H., Gately, M. K., and Magram, J. (1996). Reduced incidence and severity of collagen-induced arthritis in interleukin-12-deficient mice. *Eur J Immunol* **26,** 2933-8.

Miyazaki, T., Liu, Z. J., Kawahara, A., Minami, Y., Yamada, K., Tsujimoto, Y., Barsoumian, E. L., Permutter, R. M., and Taniguchi, T. (1995). Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation. *Cell* **81,** 223-31.

Mohamadzadeh, M., McGuire, M. J., Dougherty, I., and Cruz, P. D., Jr. (1996). Interleukin-15 expression by human endothelial cells: up-regulation by ultraviolet B and psoralen plus ultraviolet A treatment. *Photodermatol Photoimmunol Photomed* **12,** 17-21.

Mount, S. M. (1982). A catalogue of splice junction sequences. *Nucleic Acids Res* **10,** 459-72.

Mountford, P., Zevnik, B., Duwel, A., Nichols, J., Li, M., Dani, C., Robertson, M., Chambers, I., and Smith, A. (1994). Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression. *Proc Natl Acad Sci U S A* **91,** 4303-7.

Mussener, A., Litton, M. J., Lindroos, E., and Klareskog, L. (1997). Cytokine production in synovial tissue of mice with collagen-induced arthritis (CIA). *Clin Exp Immunol* **107,** 485-93.

Musso, T., Calosso, L., Zucca, M., Millesimo, M., Ravarino, D., Giovarelli, M., Malavasi, F., Ponzi, A. N., Paus, R., and Bulfone-Paus, S. (1999). Human monocytes constitutively express membrane-bound, biologically active, and interferon-gamma-upregulated interleukin-15. *Blood* **93,** 3531-9.

Naora, H., and Gougeon, M. L. (1999). Enhanced survival and potent expansion of the natural killer cell population of HIV-infected individuals by exogenous interleukin-15. *Immunol Lett* **68,** 359-67.

Nickerson, P., Zheng, X. X., Steiger, J., Steele, A. W., Steurer, W., Roy-Chaudhury, P., Muller, W., and Strom, T. B. (1996). Prolonged islet allograft acceptance in the absence of interleukin 4 expression. *Transpl Immunol* **4,** 81-5.

Nickoloff, J. A., and Reynolds, R. J. (1990). Transcription stimulates homologous recombination in mammalian cells. *Mol Cell Biol* **10,** 4837-45.

Nishimura, H., Washizu, J., Nakamura, N., Enomoto, A., and Yoshikai, Y. (1998). Translational efficiency is up-regulated by alternative exon in murine IL-15 mRNA. *J Immunol* **160,** 936-42.

No, D., Yao, T. P., and Evans, R. M. (1996). Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc Natl Acad Sci U S A* **93,** 3346-51.

Noguchi, M., Yi, H., Rosenblatt, H. M., Filipovich, A. H., Adelstein, S., Modi, W. S., McBride, O. W., and Leonard, W. J. (1993). Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency. *Cell* **73,** 147-57.

Ogasawara, K., Hida, S., Azimi, N., Tagaya, Y., Sato, T., Yokochi-Fukuda, T., Waldmann, T. A., Taniguchi, T., and Taki, S. (1998). Requirement for IRF-1 in the microenvironment supporting development of natural killer cells [published erratum appears in Nature 1998 Apr 23;392(6678):843. *Nature* **391,** 700-3.

Ogata, Y., Kukita, A., Kukita, T., Komine, M., Miyahara, A., Miyazaki, S., and Kohashi, O. (1999). A novel role of IL-15 in the development of osteoclasts: inability to replace its activity with IL-2. *J Immunol* **162,** 2754-60.

Ohteki, T., Ho, S., Suzuki, H., Mak, T. W., and Ohashi, P. S. (1997). Role for IL-15/IL-15 receptor beta-chain in natural killer 1.1+ T cell receptor-alpha beta+ cell development. *J Immunol* **159,** 5931-5.

Ohteki, T., Yoshida, H., Matsuyama, T., Duncan, G. S., Mak, T. W., and Ohashi, P. S. (1998). The transcription factor interferon regulatory factor 1 (IRF-1) is important during the maturation of natural killer 1.1+ T cell receptor- alpha/beta+ (NK1+ T) cells, natural killer cells, and intestinal intraepithelial T cells. *J Exp Med* **187,** 967-72.

Onu, A., Pohl, T., Krause, H., and Bulfone-Paus, S. (1997). Regulation of IL-15 secretion via the leader peptide of two IL-15 isoforms. *J Immunol* **158,** 255-62.

Orban, P. C., Chui, D., and Marth, J. D. (1992). Tissue- and site-specific DNA recombination in transgenic mice. *Proc Natl Acad Sci U S A* **89,** 6861-5.

Park, Y. B., Kim, D. S., Lee, W. K., Suh, C. H., and Lee, S. K. (1999). Elevated serum interleukin-15 levels in systemic lupus erythematosus. *Yonsei Med J* **40,** 343-8.

Pavlakis, M., Strehlau, J., Lipman, M., Shapiro, M., Maslinski, W., and Strom, T. B. (1996). Intragraft IL-15 transcripts are increased in human renal allograft rejection. *Transplantation* **62,** 543-5.

Perera, L. P., Goldman, C. K., and Waldmann, T. A. (1999). IL-15 induces the expression of chemokines and their receptors in T lymphocytes. *J Immunol* **162,** 2606-12.

Perez, C., Albert, I., DeFay, K., Zachariades, N., Gooding, L., and Kriegler, M. (1990). A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. *Cell* **63,** 251-8.

Perkins, S. J., Haris, P. I., Sim, R. B., and Chapman, D. (1988). A study of the structure of human complement component factor H by Fourier transform infrared spectroscopy and secondary structure averaging methods. *Biochemistry* **27,** 4004-12.

Pitzalis, C., Kingsley, G., and Panayi, G. (1994). Adhesion molecules in rheumatoid arthritis: role in the pathogenesis and prospects for therapy. *Ann Rheum Dis* **53,** 287-8.

Plows, D., Kontogeorgos, G., and Kollias, G. (1999). Mice lacking mature T and B lymphocytes develop arthritic lesions after immunization with type II collagen. *J Immunol* **162,** 1018-23.

Prinz, M., Hanisch, U. K., Kettenmann, H., and Kirchhoff, F. (1998). Alternative splicing of mouse IL-15 is due to the use of an internal splice site in exon 5. *Brain Res Mol Brain Res* **63,** 155-62.

Quinn, L. S., Haugk, K. L., and Damon, S. E. (1997). Interleukin-15 stimulates C2 skeletal myoblast differentiation. *Biochem Biophys Res Commun* **239,** 6-10.

Quinn, L. S., Haugk, K. L., and Grabstein, K. H. (1995). Interleukin-15: a novel anabolic cytokine for skeletal muscle. *Endocrinology* **136,** 3669-72.

Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H. R., and Aguet, M. (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10,** 547-58.

Ranges, G. E., Sriram, S., and Cooper, S. M. (1985). Prevention of type II collagen-induced arthritis by in vivo treatment with anti-L3T4. *J Exp Med* **162,** 1105-10.

Rankin, E. C., Choy, E. H., Kassimos, D., Kingsley, G. H., Sopwith, A. M., Isenberg, D. A., and Panayi, G. S. (1995). The therapeutic effects of an engineered human anti-tumour necrosis factor alpha antibody (CDP571) in rheumatoid arthritis. *Br J Rheumatol* **34,** 334-42.

Rickert, R. C., Roes, J., and Rajewsky, K. (1997). B lymphocyte-specific, Cre-mediated mutagenesis in mice. *Nucleic Acids Res* **25,** 1317-8.

Robb, R. J., and Greene, W. C. (1987). Internalization of interleukin 2 is mediated by the beta chain of the. *J Exp Med* **165,** 1201-6.

Robey, E., Chang, D., Itano, A., Cado, D., Alexander, H., Lans, D., Weinmaster, G., and Salmon, P. (1996). An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. *Cell* **87,** 483-92.

Ruchatz, H., Leung, B. P., Wei, X. Q., McInnes, I. B., and Liew, F. Y. (1998). Soluble IL-15 receptor alpha-chain administration prevents murine collagen-induced arthritis: a role for IL-15 in development of antigen- induced immunopathology. *J Immunol* **160,** 5654-60.

Sadlack, B., Merz, H., Schorle, H., Schimpl, A., Feller, A. C., and Horak, I. (1993). Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene [see comments]. *Cell* **75,** 253-61.

Sakai, T., Kusugami, K., Nishimura, H., Ando, T., Yamaguchi, T., Ohsuga, M., Ina, K., Enomoto, A., Kimura, Y., and Yoshikai, Y. (1998). Interleukin 15 activity in the rectal mucosa of inflammatory bowel disease. *Gastroenterology* **114,** 1237-43.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74,** 5463-7.

Sasai, M., Saeki, Y., Ohshima, S., Nishioka, K., Mima, T., Tanaka, T., Katada, Y., Yoshizaki, K., Suemura, M., and Kishimoto, T. (1999). Delayed onset and reduced severity of collagen-induced arthritis in interleukin-6-deficient mice. *Arthritis Rheum* **42,** 1635-43.

Satoh, J., Kurohara, K., Yukitake, M., and Kuroda, Y. (1998). Interleukin-15, a T-cell growth factor, is expressed in human neural cell lines and tissues. *J Neurol Sci* **155,** 170-7.

Sauer, B. (1987). Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae. *Mol Cell Biol* **7,** 2087-96.

Sauer, B. (1998). Inducible gene targeting in mice using the Cre/lox system. *Methods* **14,** 381-92.

Schmidt, D., Goronzy, J. J., and Weyand, C. M. (1996). CD4+ CD7- CD28- T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. *J Clin Invest* **97,** 2027-37.

Schorle, H., Holtschke, T., Hunig, T., Schimpl, A., and Horak, I. (1991). Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature* **352,** 621-4.

Schwartzberg, P. L., Goff, S. P., and Robertson, E. J. (1989). Germ-line transmission of a c-abl mutation produced by targeted gene disruption in ES cells. *Science* **246,** 799-803.

Schwenk, F., Baron, U., and Rajewsky, K. (1995). A cre-transgenic mouse strain for the ubiquitous deletion of loxP- flanked gene segments including deletion in germ cells. *Nucleic Acids Res* **23,** 5080-1.

Schwenk, F., Sauer, B., Kukoc, N., Hoess, R., Muller, W., Kocks, C., Kuhn, R., and Rajewsky, K. (1997). Generation of Cre recombinase-specific monoclonal antibodies, able to characterize the pattern of Cre expression in cre-transgenic mouse strains. *J Immunol Methods* **207,** 203-12.

Scotet, E., David-Ameline, J., Peyrat, M. A., Moreau-Aubry, A., Pinczon, D., Lim, A., Even, J., Semana, G., Berthelot, J. M., Breathnach, R., Bonneville, M., and Houssaint, E. (1996). T cell response to Epstein-Barr virus transactivators in chronic rheumatoid arthritis. *J Exp Med* **184,** 1791-800.

Sebbag, M., Parry, S. L., Brennan, F. M., and Feldmann, M. (1997). Cytokine stimulation of T lymphocytes regulates their capacity to induce monocyte production of tumor necrosis factor-alpha, but not interleukin-10: possible relevance to pathophysiology of rheumatoid arthritis. *Eur J Immunol* **27,** 624-32.

Seder, R. A. (1996). High-dose IL-2 and IL-15 enhance the in vitro priming of naive CD4+ T cells for IFN-gamma but have differential effects on priming for IL-4. *J Immunol* **156,** 2413-22.

Segev, N., and Cohen, G. (1981). Control of circularization of bacteriophage P1 DNA in Escherichia coli. *Virology* **114,** 333-42.

Simon, A. K., Seipelt, E., and Sieper, J. (1994). Divergent T-cell cytokine patterns in inflammatory arthritis. *Proc Natl Acad Sci U S A* **91,** 8562-6.

Skreb, N., and Crnek, V. (1980). Development of embryo-derived teratomas in vitro. *Results Probl Cell Differ* **11,** 283-9.

Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M., and Rogers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336,** 688-90.

Smith, A. J., De Sousa, M. A., Kwabi-Addo, B., Heppell-Parton, A., Impey, H., and Rabbitts, P. (1995). A site-directed chromosomal translocation induced in embryonic stem cells by Cre-loxP recombination. *Nat Genet* **9,** 376-85.

Soukharev, S., Miller, J. L., and Sauer, B. (1999). Segmental genomic replacement in embryonic stem cells by double lox targeting. *Nucleic Acids Res* **27,** e21.

St-Onge, L., Furth, P. A., and Gruss, P. (1996). Temporal control of the Cre recombinase in transgenic mice by a tetracycline responsive promoter. *Nucleic Acids Res* **24,** 3875-7.

Stevens, L. C. (1970). The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. *Dev Biol* **21,** 364-82.

Strehlow, I., and Schindler, C. (1998). Amino-terminal signal transducer and activator of transcription (STAT) domains regulate nuclear translocation and STAT deactivation. *J Biol Chem* **273,** 28049-56.

Suzuki, H., Duncan, G. S., Takimoto, H., and Mak, T. W. (1997). Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor beta chain. *J Exp Med* **185,** 499-505.

Svensson, L., Jirholt, J., Holmdahl, R., and Jansson, L. (1998). B cell-deficient mice do not develop type II collagen-induced arthritis (CIA). *Clin Exp Immunol* **111,** 521-6.

Swain, S. L. (1991). Lymphokines and the immune response: the central role of interleukin-2. *Curr Opin Immunol* **3,** 304-10.

Swiatek, P. J., Lindsell, C. E., del Amo, F. F., Weinmaster, G., and Gridley, T. (1994). Notch1 is essential for postimplantation development in mice. *Genes Dev* **8,** 707-19.

Tada, Y., Nagasawa, K., Ho, A., Morito, F., Ushiyama, O., Suzuki, N., Ohta, H., and Mak, T. W. (1999). CD28-deficient mice are highly resistant to collagen-induced arthritis. *J Immunol* **162,** 203-8.

Tagaya, Y., Burton, J. D., Miyamoto, Y., and Waldmann, T. A. (1996). Identification of a novel receptor/signal transduction pathway for IL- 15/T in mast cells. *Embo J* **15,** 4928-39.

Tagaya, Y., Kurys, G., Thies, T. A., Losi, J. M., Azimi, N., Hanover, J. A., Bamford, R. N., and Waldmann, T. A. (1997). Generation of secretable and nonsecretable interleukin 15 isoforms through alternate usage of signal peptides. *Proc Natl Acad Sci U S A* **94,** 14444-9.

Takagi, N., Mihara, M., Moriya, Y., Nishimoto, N., Yoshizaki, K., Kishimoto, T., Takeda, Y., and Ohsugi, Y. (1998). Blockage of interleukin-6 receptor ameliorates joint disease in murine collagen-induced arthritis. *Arthritis Rheum* **41,** 2117-21.

Takeda, K., Noguchi, K., Shi, W., Tanaka, T., Matsumoto, M., Yoshida, N., Kishimoto, T., and Akira, S. (1997). Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci U S A* **94,** 3801-4.

Takeda, T., Kurachi, H., Yamamoto, T., Nishio, Y., Nakatsuji, Y., Morishige, K., Miyake, A., and Murata, Y. (1998). Crosstalk between the interleukin-6 (IL-6)-JAK-STAT and the glucocorticoid-nuclear receptor pathway: synergistic activation of IL-6 response element by IL-6 and glucocorticoid. *J Endocrinol* **159,** 323-30.

Taniguchi, M., Sanbo, M., Watanabe, S., Naruse, I., Mishina, M., and Yagi, T. (1998). Efficient production of Cre-mediated site-directed recombinants through the utilization of the puromycin resistance gene, pac: a transient gene-integration marker for ES cells. *Nucleic Acids Res* **26,** 679-80.

Taylor, P. C., Chu, C. Q., Plater-Zyberk, C., and Maini, R. N. (1996). Transfer of type II collagen-induced arthritis from DBA/1 to severe combined immunodeficiency mice can be prevented by blockade of Mac-1. *Immunology* **88,** 315-21.

te Riele, H., Maandag, E. R., and Berns, A. (1992). Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proc Natl Acad Sci U S A* **89,** 5128-32.

Thomas, K. R., and Capecchi, M. R. (1986). Introduction of homologous DNA sequences into mammalian cells induces mutations in the cognate gene. *Nature* **324,** 34-8.

Thomas, K. R., and Capecchi, M. R. (1987). Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51,** 503-12.

Thomas, R., Davis, L. S., and Lipsky, P. E. (1994). Rheumatoid synovium is enriched in mature antigen-presenting dendritic cells. *J Immunol* **152,** 2613-23.

Thomas, R., McIlraith, M., Davis, L. S., and Lipsky, P. E. (1992). Rheumatoid synovium is enriched in CD45RBdim mature memory T cells that are potent helpers for B cell differentiation. *Arthritis Rheum* **35,** 1455-65.

Thurkow, E. W., van der Heijden, I. M., Breedveld, F. C., Smeets, T. J., Daha, M. R., Kluin, P. M., Meinders, A. E., and Tak, P. P. (1997). Increased expression of IL-15 in the synovium of patients with rheumatoid arthritis compared with patients with Yersinia-induced arthritis and osteoarthritis. *J Pathol* **181,** 444-50.

Trentham, D. E., Dynesius-Trentham, R. A., Orav, E. J., Combitchi, D., Lorenzo, C., Sewell, K. L., Hafler, D. A., and Weiner, H. L. (1993). Effects of oral administration of type II collagen on rheumatoid arthritis. *Science* **261,** 1727-30.

Trentham, D. E., Townes, A. S., and Kang, A. H. (1977). Autoimmunity to type II collagen an experimental model of arthritis. *J Exp Med* **146,** 857-68.

Tsien, J. Z., Chen, D. F., Gerber, D., Tom, C., Mercer, E. H., Anderson, D. J., Mayford, M., Kandel, E. R., and Tonegawa, S. (1996). Subregion- and cell type-restricted gene knockout in mouse brain [see comments]. *Cell* **87,** 1317-26.

Utomo, A. R., Nikitin, A. Y., and Lee, W. H. (1999). Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nat Biotechnol* **17,** 1091-1096.

Van den Berg, W. B., Joosten, L. A., Helsen, M., and van de Loo, F. A. (1994). Amelioration of established murine collagen-induced arthritis with anti-IL-1 treatment. *Clin Exp Immunol* **95,** 237-43.

Van Deursen, J., Fornerod, M., Van Rees, B., and Grosveld, G. (1995). Cre-mediated site-specific translocation between nonhomologous mouse chromosomes. *Proc Natl Acad Sci U S A* **92,** 7376-80.

Van Parijs, L., Biuckians, A., Ibraginov, A., Alt, F. W., Willerford, D. M., and Abbas, A. K. (1997). Functional responses and apoptosis of CD25 (IL-2R alpha)-deficient T cells expressing a transgenic antigen receptor. *J Immunol* **158,** 3738-45.

Vermeire, K., Heremans, H., Vandeputte, M., Huang, S., Billiau, A., and Matthys, P. (1997). Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. *J Immunol* **158,** 5507-13.

Waldmann, T. A., and Tagaya, Y. (1999). The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annu Rev Immunol* **17,** 19-49.

Walmsley, M., Butler, D. M., Marinova-Mutafchieva, L., and Feldmann, M. (1998). An anti-inflammatory role for interleukin-11 in established murine collagen-induced arthritis. *Immunology* **95,** 31-7.

Washburn, T., Schweighoffer, E., Gridley, T., Chang, D., Fowlkes, B. J., Cado, D., and Robey, E. (1997). Notch activity influences the alphabeta versus gammadelta T cell lineage decision. *Cell* **88,** 833-43.

Whalen, J. D., Lechman, E. L., Carlos, C. A., Weiss, K., Kovesdi, I., Glorioso, J. C., Robbins, P. D., and Evans, C. H. (1999). Adenoviral transfer of the viral IL-10 gene periarticularly to mouse paws suppresses development of collagen-induced arthritis in both injected and uninjected paws. *J Immunol* **162,** 3625-32.

Wilkinson, P. C., and Liew, F. Y. (1995). Chemoattraction of human blood T lymphocytes by interleukin-15. *J Exp Med* **181,** 1255-9.

Williams, R. O., Mason, L. J., Feldmann, M., and Maini, R. N. (1994). Synergy between anti-CD4 and anti-tumor necrosis factor in the amelioration of established collagen-induced arthritis. *Proc Natl Acad Sci U S A* **91,** 2762-6.

Williams, R. O., Williams, D. G., Feldmann, M., and Maini, R. N. (1993). Increased limb involvement in murine collagen-induced arthritis following treatment with anti-interferon-gamma. *Clin Exp Immunol* **92,** 323-7.

Witthuhn, B. A., Silvennoinen, O., Miura, O., Lai, K. S., Cwik, C., Liu, E. T., and Ihle, J. N. (1994). Involvement of the Jak-3 Janus kinase in signalling by interleukins 2 and 4 in lymphoid and myeloid cells. *Nature* **370,** 153-7.

Wooley, P. H., Dutcher, J., Widmer, M. B., and Gillis, S. (1993). Influence of a recombinant human soluble tumor necrosis factor receptor FC fusion protein on type II collagen-induced arthritis in mice. *J Immunol* **151,** 6602-7.

Wooley, P. H., Whalen, J. D., Chapman, D. L., Berger, A. E., Richard, K. A., Aspar, D. G., and Staite, N. D. (1993). The effect of an interleukin-1 receptor antagonist protein on type II collagen-induced arthritis and antigen-induced arthritis in mice. *Arthritis Rheum* **36,** 1305-14.

Xin, K. Q., Hamajima, K., Sasaki, S., Tsuji, T., Watabe, S., Okada, E., and Okuda, K. (1999). IL-15 expression plasmid enhances cell-mediated immunity induced by an HIV-1 DNA vaccine. *Vaccine* **17,** 858-66.

Yoo, T. J., Kim, S. Y., Stuart, J. M., Floyd, R. A., Olson, G. A., Cremer, M. A., and Kang, A. H. (1988). Induction of arthritis in monkeys by immunization with type II collagen. *J Exp Med* **168,** 777-82.

Yoshino, S., Cleland, L. G., and Mayrhofer, G. (1991). Treatment of collagen-induced arthritis in rats with a monoclonal antibody against the alpha/beta T cell antigen receptor. *Arthritis Rheum* **34,** 1039-47.

Zhang, X., Sun, S., Hwang, I., Tough, D. F., and Sprent, J. (1998). Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. *Immunity* **8,** 591-9.

Zhang, Y., Riesterer, C., Ayrall, A. M., Sablitzky, F., Littlewood, T. D., and Reth, M. (1996). Inducible site-directed recombination in mouse embryonic stem cells. *Nucleic Acids Res* **24,** 543-8.