THE ROLE OF B7-1 MOLECULES IN THE ACTIVATION OF $CD4^+$ T CELLS FROM IL-2 DEFICIENT MICE

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ABSTRACT

IL-2 null mice provide a good model of generalised autoimmunity. Mice carrying a targeted mutation of both alleles of the IL-2 gene develop haemolytic anaemia and inflammatory bowel disease (IBD). C57BL/6 IL-$2^{-/-}$ mice die of a $CD4^+$ T cell dependent IBD. Based on the critical role of $CD4^+$ T cells in the pathogenesis of disease, the activation requirements of these cells were investigated. Importantly, whether IL-$2^{-/-}$ $CD4^+$ T lymphocytes are intrinsically different from IL-$2^{+/+}$ $CD4^+$ T lymphocytes, in that IL-$2^{-/-}$ $CD4^+$ T cells do not appear to be using distinct cytokines to substitute for IL-2.

Functional analyses of IL-$2^{-/-}$ $CD4^+$ T cells were undertaken using different experimental approaches. FACS studies were used to characterise the distinctive splenomegaly, which is characteristic of autoimmune disease in this model, and the Vβ repertoire of IL-$2^{-/-}$ mice. These studies indicated an accumulation of macrophages and $CD38^+$ $CD4^+$ T cells together with up-regulation of costimulatory molecules and markers of activation, such as LFA-1 and CD44, in IL-$2^{-/-}$ spleens. Interestingly, the FACS analyses did not reveal abnormalities in the Vβ repertoire of IL-$2^{-/-}$ mice.

The cytokine profile of activated IL-$2^{-/-}$ $CD4^+$ T cells was examined in the presence of exogenous recombinant cytokines, mainly the common γ chain receptor cytokines and anti-cytokine mAb in short-term cultures. These studies did not reveal a differential effect of a specific cytokine or anti-cytokine mAb on IL-$2^{-/-}$ $CD4^+$ T cells. In addition, ELISA studies demonstrated an enhanced production of IL-10 by activated IL-$2^{-/-}$ $CD4^+$ T cells.

At the mRNA level, RNase protection assays revealed a differential up-regulation of IL-10 mRNA by IL-$2^{-/-}$ spleen cells at early time points *ex vivo*. RT-PCR analysis of activated IL-$2^{-/-}$ $CD4^+$ T cells demonstrated a cytokine profile which correlated with cytokines produced by human IBD patients, where IFN-γ is produced in Crohn's disease and IL-5 is indicative of ulcerative colitis. Moreover, cDNA gene arrays revealed a differential up-regulation of costimulatory molecules such as LFA-1 and

CD6 in activated IL-$2^{-/-}$ $CD4^+$ T cells. Surprisingly, cDNA gene arrays demonstrated differential expression of metabolic enzymes and housekeeping genes depending on the activation stimulus used.

Finally, preliminary hCTLA-4Ig blocking studies (blockade of the B7: CD28/CTLA-4 pathway) suggest a nonessential role for the B7: CD28/CTLA-4 pathway in the pathogenesis of IBD disease.

Collectively, these data do not illustrate that IL-$2^{-/-}$ $CD4^+$ T cells are intrinsically different to IL-$2^{+/+}$ $CD4^+$ T cells. Using cDNA gene arrays to compare activated IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells a number of genes were found to be differentially regulated. The potential role of some of these genes is discussed.

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CHAPTER 1

INTRODUCTION

1-Brief overview of the immune system

A successful immune response must achieve a balance whereby it specifically recognises and eliminates foreign invaders but abstains from damaging the host. In part, this is achieved by the production of T lymphocytes, each bearing a different antigen receptor to counter the diverse variety of antigens present in the microbial and viral worlds. Most immune responses are dependent on the activation of T cells. This group of lymphocytes consists of phenotypically and functionally diverse subsets of cells, the principal division being into cytotoxic T lymphocytes (CTLs) and helper T lymphocytes (Th). Distinct molecules are expressed on the cell surface of these cellular subsets: CD4 for T helper cells and CD8 for cytotoxic T cells. CD4 and CD8 T cells recognise antigens through their antigen receptor, the TCR, which is composed of an α and a β chain.

The T helper population can be further subdivided into TH_1 or TH_2 cells. This depends on the cytokine profile produced. TH_1 cells mainly synthesise interleukin-2 and interferon-γ, whereas TH_2 cells produce interleukin-4, interleukin-5 and interleukin-10. Furthermore, the two T helper subsets appear to serve distinct functions. TH_1 cells activate antigen presenting cells, such as macrophages, mediate delayed hypersensitivity and promote antibody class switching from IgM to IgG2a. Conversely, TH_2 cells provide help for B cells and promote class switching from IgM to IgG1 and IgE.

Importantly, the pathogenesis of certain diseases is dependent on the T helper subset involved. For instance, in leishmaniasis a predisposition to a TH_1 immune response is essential for successful immune defence, whereas the development of TH_2 is ineffective at eliminating the pathogen and damaging to the host. TH_2 polarised immune responses are advantageous in the case of infection with nematodes.

The physiological activation of T lymphocytes comprises at least two signals, both of which are provided by the APCs. The first involves the presentation of an immunogenic peptide bound to a major histocompatibility complex (MHC class II)

protein to T cells; the second involves costimulatory signals, which initiate, maintain and regulate the activation of genes encoding cytokines and other growth factors.

1.1-The Role of Interleukin 2 (IL-2) in the immune response:

During an immune response, crosslinking of the T cell receptor (TCR) and costimulatory signals result in the cell undergoing a series of biochemical events. The earliest events include those which lead to the transcription of activation-associated genes encoding cytokines and other growth factors. One of the earliest consequences of T cell activation is the production of IL-2. IL-2 is a powerful growth factor which acts on a variety of immune cells with diverse actions such as inducing the proliferative response of T cells, stimulating the production of immunoglobulins by B cells and augmenting the cytolytic response of lymphocytes.

IL-2 was originally described as T cell growth factor and reported as an activity present in the supernatants of human mitogen-activated peripheral blood lymphocytes which stimulated lymphocyte proliferation *in vitro* (1). This allowed the culture of T lymphocytes for longer than previously had been possible by using conditioned medium. It also provided a model for a number of cytokine/cytokine receptor systems. IL-2 is of particular historical significance as it was the first growth factor to be purified, cloned and its receptor was the first to be biochemically characterised (2).

IL-2 has a dual role in the immune system. It is a major growth and survival factor but it also sensitises cells to Fas-mediated activation-induced cell death (AICD) (3). In short, IL-2 is largely responsible for proliferation, survival and apoptosis responses in the immune system. This is particularly interesting because some of these functions oppose one another.

1.2-The IL-2/IL-2R system:

The high affinity IL-2 receptor is composed of three distinct subunits: IL-2Rα, IL-2Rβ and IL-2Rγ or (γ_c). Two major advances were crucial in allowing the biochemical characterisation of IL-2 receptors. The first was the isolation of a monoclonal antibody named anti-Tac (4) which was subsequently identified to be an anti IL-2 receptor antibody (5). The second was the discovery that certain T cell lines, primarily those infected with human retrovirus T lymphotropic virus I (HTLV-1) expressed high levels of IL-2 receptors. HTLV-1 cells provided a source of receptor protein for purification and characterisation. The anti-Tac antibody, because of its ability to block IL-2/IL-2 receptors signals, was instrumental in elucidating the role of IL-2 signalling in different immune responses. The anti-Tac antibody was found to bind specifically to a 55 kDa glycoprotein named IL-2Rα (5). IL-2Rα is expressed exclusively on activated T lymphocytes (6,7). The IL-2Rα does not generate intracellular signals but, in association with the β and γ_c components of the receptor, constitutes the IL-2 high affinity receptor (8,9). A recent study by Eicher et al. has demonstrated that IL-2Rα plays a critical role in enhancing IL-2 signalling. This group showed that IL-2Rα on one cell can present IL-2 to IL-2R βγ on another cell, thereby augmenting IL-2 signalling (10). This implies that the primary function of IL-2Rα is to sensitise activated cells to IL-2 signals (11).

IL-2 Rβ was discovered by Sharon et al. using ^{125}I-IL-2 in affinity labelling studies (12). Radiolabelled IL-2 was bound to cells and cross-linked with disuccinimidylsuberate (DSS). Analysis on SDS polyacrylamide gels indicated, in addition to the IL-2/IL-2Rα complex, a second higher molecular weight complex in association with the high affinity but not the low affinity receptors. This led to the conclusion that IL-2Rα constituted the low affinity receptor, whereas the higher affinity receptors consisted of IL-2Rα, a second protein named as IL-2Rβ and finally IL-2Rγ. Subsequent studies revealed that IL-2Rβ has a similarly sized extracellular domain to the IL-2Rα chain but an extensive cytoplasmic tail not present in the α

subunit. cDNA expression studies has revealed an instrumental role for IL-2Rβ, but not IL-2Rα, in IL-2 receptor signalling (13).

The main signal transducing regions of the IL-2Rβ chain are the "serine rich" and the "acidic" regions of its cytoplasmic tail. Van Parijs et al. have recently dissected regions of the IL-2Rβ chain which are responsible for the different effector functions of IL-2 signalling using mutation studies. The authors used a retrovirus-based approach to express wild type or mutant forms of IL-2Rβ in IL-2Rβ deficient T cells. They chose to use two mutant forms that were defective in Stat5 or Akt activation. These molecules were chosen on the basis of their function in IL-2 induced proliferative responses (14). Akt is a target of phosphatidylinositol-3 kinase (PI-3K), which is an important member of the phosphoinositide kinases responsible for multiple cellular functions. Activation of Akt transduces signals that regulate a variety of biological functions, including apoptosis, through regulating the phosphorylation of BAD and inhibiting its pro-apoptotic function (15), gene expression and cellular proliferation (16).

Signal transducers and activators of transcription (STATs) and Janus kinases (JAKs) are part of the intracellular signalling molecules involved in cytokine signal transduction (17). IL-2 mediates its signalling through the phosphorylation of JAK1, JAK3 and STAT1, STAT3 and STAT5 (18). The activation of STAT5 increases Fas L expression by activated T cells (19), a potential mechanism by which IL-2 induces its apoptotic function. Therefore, STAT5 potentially regulates IL-2-induced AICD, whereas Akt seems to play an important role in stimulating IL-2-induced proliferative responses. Using mutants which failed to activate Akt, Van Parijs et al showed that Akt signalling is essential for T cell survival, whereas Stat5 is involved in promoting AICD in activated T cells. The role of Stat5 in the maintenance of tolerance is supported by the phenotype of mice which are deficient in STAT5a and STAT5b.

These mice accumulate activated T cells in the spleen, indicative of a defect in elimination of activated peripheral T cells (20).

IL-2Rγ or the γ chain (γ_c) constitutes the third component of the IL-2 receptor. The γ chain was found to be critical for the formation of high and intermediate affinity receptors, as well as for the internalization of the IL-2 receptor (21). This chain is also a component of the IL-4 (22), IL-7 (23), IL-9 (24) and IL-15 (25) receptors. These cytokines are collectively known as the common γ chain cytokines. The common γ_c of the IL-2, IL-4, IL-7, IL-9 and IL-15 receptors is defective in humans with X-linked severe combined immunodeficiency (X-SCID); a disease characterised by significantly diminished cell-mediated and humoral immunity (26) (27). The clinical manifestations of X-SCID patients can therefore be partly attributed to the impaired function of five cytokine systems, the common γ_c cytokines. Moreover, γ_c associates with and mediates the activation of JAK3 and signals through the γ_c are instrumental in preventing T cell anergy (24,28).

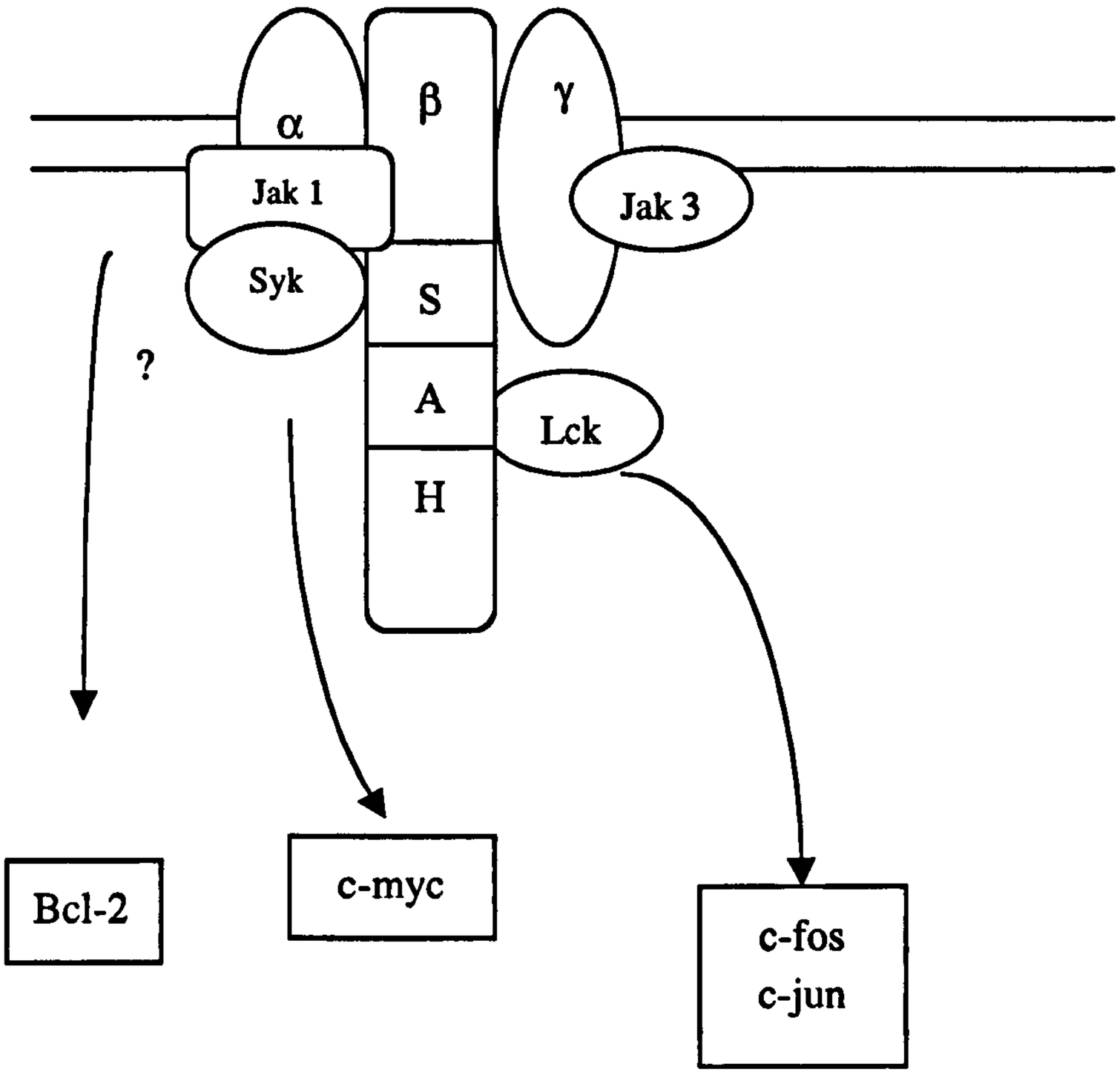


Figure 1.1 Schematic diagram of the IL-2 receptor complex and signalling model.

This figure summarises three IL-2-induced distinct signalling pathways: the p56lck activation pathway, leading to c-fos and c-jun induction, the c-myc and the bcl-2 pathways. As depicted in the diagram, the serine rich region "S" of the IL-2Rβ associates with two protein tyrosine kinases (PTKs) Jak1 of the Janus PTKs family and Syk of the Syk/ZAP-70 PTKs family. Syk activation leads to the induction of the c-myc gene. The S region of the IL-2Rβ is instrumental in activating bcl-2 and c-myc. The acidic region "A" of the IL-2Rβ is necessary for the physical association of p56 lck leading to the activation of c-fos and c-jun. The proline rich "H" region is critical for the activation of Stat5. With regard to the γ_c, the carboxy-terminal region is important for the association of Jak3 (29).

1.3-Positive regulatory role of IL-2/Role of IL-2 in proliferation:

IL-2 mediates its positive regulatory role as a growth factor by activating several proteins representing proto-oncogenes, PTKs, JAKs and STATs.

1.3.1-c-fos/c-jun and c-myc:

These immediate early genes are transcribed immediately following activation through ligation of the TCR, generate activating protein-1 (AP-1) activity in cells through homodimerization and heterodimerization (30). The Jun/Fos proteins have T cell specific functions and induce their effects through association with the transcription factor nuclear factor of activated T cells (NF-AT) (31). Fos/Jun heterodimers and NF-AT form a transcriptional activation complex that binds to specific DNA regulatory sites of many cytokine genes, thereby activating their transcription and inducing proliferation.

1.3.2-Akt:

Activation of Akt by IL-2 inhibits apoptosis by the induction of c-myc and bcl-2 (16). Akt transduces PI-3K-dependent IL-2 signals that phosphorylate Rb and promote the activation of E2F (32). E2F/DP heterodimers induce the expression of several genes that regulate S-phase entry, including c-myc (33).

1.3.3-PTKs:

IL-2 stimulation results in the phosphorylation of several PTKs. These include Raf-1 kinase (34), mitogen-activated kinase (MAP-kinase), p70 S6 kinase (35), $p33^{cdk2}$ (cdk2) and $p34^{cdc2}$ (36). Other PTKs associate constitutively with IL-2Rβ and γ_c. The *src*-family PTK $p56^{lck}$ associates with the A region of IL-2Rβ whereas Syk PTK associates with the S region.

In addition to the PTKs mentioned above, IL-2 stimulation results in the activation of the lipid kinase PI-3K. IL-2 signalling results in a rapid increase in the activity of PI-3K possibly via the association between the tyrosine-phosphorylated IL-2Rß and the *src* homology 2 (SH2) domains of the regulatory subunit of PI-3K (37). Moreover, IL-2 signalling results in the activation of $P21^{ras}$ (Ras) (38). $P21^{ras}$ controls cell growth and was shown to regulate the MAP kinases ERK1 and ERKII (39).

1.3.4-JAK1 and JAK3:

As mentioned previously, Jak1 associates with the S region of IL-2Rβ and Jak3 with the carboxy-terminal region of γ_c. The activation of these Jaks via the IL-2 signal results in the phosphorylation of STATs and cytokine gene transcription.

1.3.5-STAT5:

STAT5 plays a role in both positive and negative aspects of IL-2 signalling. STAT5α/β mutant peripheral T cells are severely deficient in proliferation and fail to undergo cell cycle progression (20). Moreover, STAT5$\alpha^{-/-}$ splenocytes failed to up-regulate IL-2Rα following activation, which in turn resulted in decreased proliferation to IL-2 (40). Both these studies point to the positive regulatory role of Stat5 in IL-2 signalling. On the other hand, STAT5 promotes AICD by allowing the expression of Fas L following T cell activation (19).

1.3.6-Cell cycle regulation:

Following stimulation, quiescent cells (G0 state) undergo a series of cell cycle events enabling them to proliferate. There are four cell cycle phases: G1, the first gap; S, DNA synthesis; G2, the second gap and M, mitosis. These events are tightly controlled by positive regulators such as cyclins and cyclin dependent kinases (CDK) and negative regulators such as CDK inhibitors, which prevent the cell from entering S phase. One group of inhibitors are the Cip/Kip family composed of p21, p27 and p57; these inhibitors prevent the G1/S transition. IL-2 contributes to cell cycle regulation by stimulating loss of p27 in activated T cells, allowing CDK activation and progress from quiescence to G1 (41). Conversely, IL-2 up-regulates the inhibitor p21 thereby controlling CDK activity during cycling (42). Therefore, one of the mechanisms by which IL-2 controls proliferation is via regulation of p21 and p27.

1.4-Negative regulatory role of IL-2/role in AICD:

Lenardo was the first to demonstrate the role of IL-2 in cell death. The author demonstrated that mature $CD4^+$ and $CD8^+$ T cells undergo programmed cell death when repeatedly stimulated and exposed to IL-2 (3). Further studies to investigate the role of IL-2 in promoting proliferation and/or cell death led to the discovery of two distinct mechanisms of apoptosis: AICD and passive cell death (PCD). PCD was the term used to define naïve T cells undergoing cell death as a result of inadequate stimulation signals or the withdrawal of growth factors. Passive cell death is not Fas mediated and is regulated by costimulatory molecules and bcl-2 (43) (44).

AICD is a mechanism by which activated T cells are restimulated with high concentrations of cognate antigen or antibodies to the TCR and undergo cell death. In $CD4^+$ T lymphocytes this form of cell death is mainly mediated by the co-expression of Fas and Fas L; the engagement of these receptors results in signals leading to cell death (45) (46). This mechanism is critical for the maintenance of self-tolerance and does not function to end responses to foreign antigen. This concept is further supported by the Fas and Fas L deficient mice which despite developing autoimmune disease, do not manifest aberrant and prolonged immune responses to foreign antigen. Fas-mediated apoptosis results in the termination of T cells responding to a systemic self antigen and preventing autoreactive T cell proliferation (47) (44). The importance of this pathway is seen in mice and humans with functional defects in Fas or Fas L. Mutations in Fas or its ligand Fas L result in dysregulated lymphocyte homeostasis and autoimmune disease (48) (49) (50). It is important to distinguish AICD from passive cell death (PCD). Growth factors, anti-apoptotic genes; bcl-2, bcl-x_L and IL-2 can protect T cells from PCD but not AICD.

In conclusion, the death regulatory role of IL-2 relies on its ability to induce IL-2Rα and increase the surface expression of Fas L (51) (19) (52).

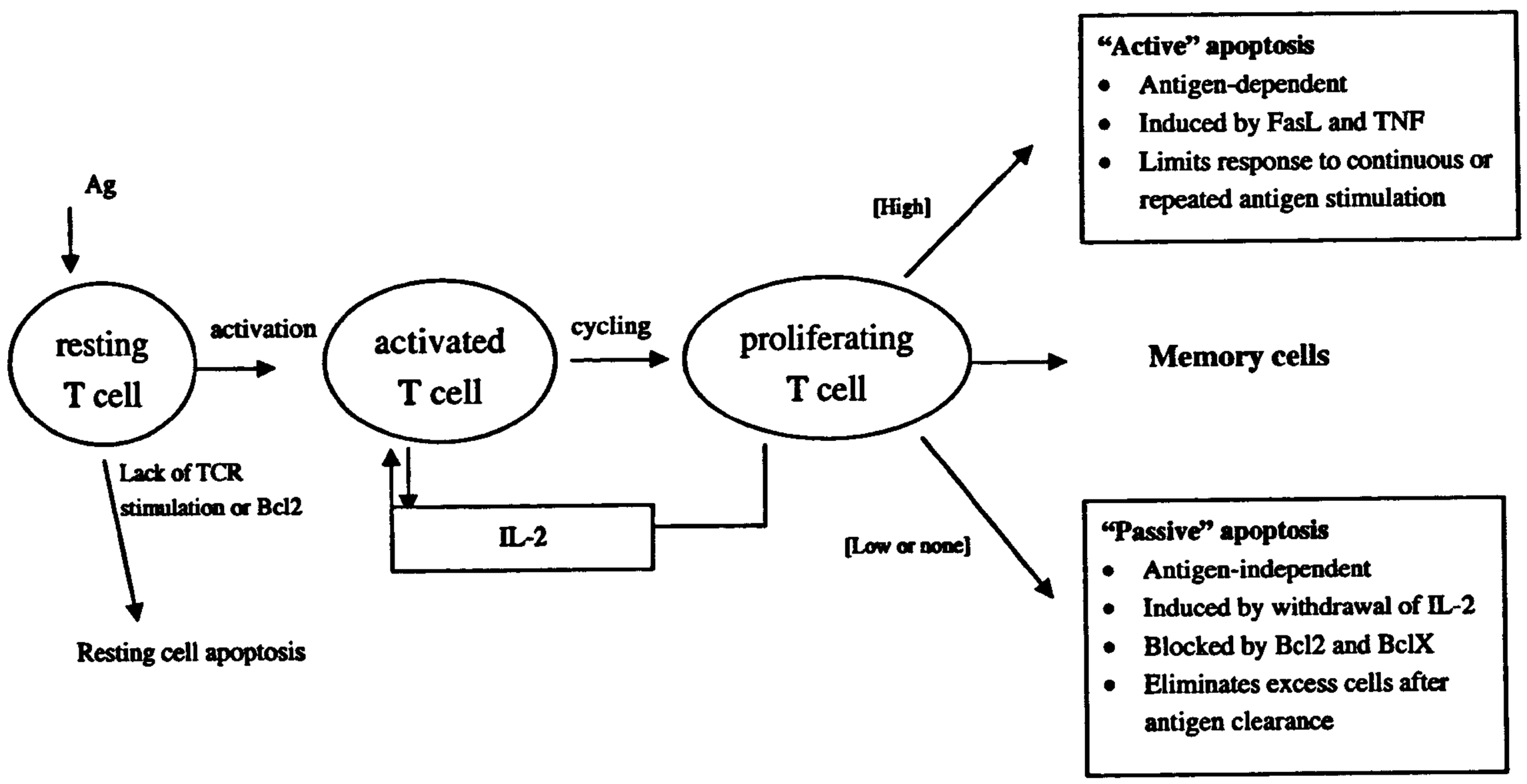


Figure 1.2 Diagram illustrating the two mechanisms of cell death: active and passive cell death.

T cell responses to antigen occur in two distinct phases, an activation phase whereby IL-2 is produced and a proliferative phase where IL-2-induced cell cycling occurs (G1-S). Active apoptosis occurs when high doses of antigen are present, whereas passive apoptosis as result of inadequate supply of antigen or after termination of antigen supply and IL-2.

2-The IL-2 deficient model (IL-2 KO)

Targeted mutagenesis into the mouse IL-2 gene by Schorle et al. was used to inactivate the IL-2 gene; a neomycin-resistance (neo^r) gene was inserted in the reverse orientation into the third exon of the IL-2 gene. This insertion resulted in the introduction of several stop codons in the IL-2 reading frame rendering the gene non-functional. The critical role of IL-2 in T cell activation/expansion *in vitro* led to the anticipation of a chronic immunodeficient phenotype. Contrary to expectations, initial studies on 4 week old C57BL/6 mice reported normal thymic development. FACS analysis on spleen and lymph node cells did not reveal major differences between knockout (KO) and wild-type (WT) littermates. These findings illustrated that IL-2 is not vital for thymocyte maturation or seeding of the periphery with mature lymphocytes. Functional studies on cells from thymus, spleen and lymph nodes revealed defects in polyclonal activation *in vitro*. Another remarkable defect was the immunoglobulin isotype distribution in non-immunised WT and KO littermates. IL-2 KO mice had significantly higher levels of IgG2a and 2b but levels of IgM were comparable (53).

Successive functional studies using anti-viral T cell responses revealed that *in vivo* primary and secondary cytotoxic T cell responses (CTLs) against vaccinia and lymphocytic choriomeningitis (LCMV) were comparable between WT and KO mice. Vesicular stomatitis virus (VSV) was used to assess the function of T_H and B cells in KO mice. The IgM response to VSV is independent of T_H cells, whereas the isotype switch from IgM to IgG is T_H-dependent (54) (55,56). KO IgM responses against intravenously injected VSV were comparable to WT but there was a delay in the IgG response. The delayed IgG response in KO mice was indicative of an impaired but nevertheless effective $CD4^+$ T_H response. These observations illustrated that *in vivo* CTL responses against vaccinia virus, LCMV and VSV were functional in IL-2 KO mice. Conversely, *in vitro* stimulation of primed vaccinia-specific memory CTLs from IL-2 KO mice was severely defective. This emphasised the idea that IL-2 plays a significant and central role in immunoregulation *in vitro* but not *in vivo* (57). This

study by Kundig et al. was instrumental in questioning the *in vivo* role of IL-2 as had been previously assumed by *in vitro* studies.

2.1-IL-2 deficient mice: a model of autoimmunity:

Sadlack et al. were the first group to describe autoimmune disease in the IL-2 KO mouse. This study examined C57BL/6 IL-2 KO mice and showed that IL-$2^{-/-}$ mice develop normally for the first 3-4 weeks of age but subsequently become extremely compromised and 50% of the animals die between 4-9 weeks of age. All remaining mice develop inflammatory bowel disease (IBD) with significant resemblance to human ulcerative colitis (58). Human IBD is a chronic inflammatory condition characterised by two overlapping phenotypes-ulcerative colitis (UC) and Crohn's disease (CD). IBD predominantly affects the colon (UC and CD) and/or the distal small intestine (CD). The kinetics with which pathological alteration develops depends on the genetic background into which the IL-2 null mutation was crossed. This was manifested by differences in disease kinetics, pathology and the primary causes of death. IL-2 deficient mice on a BALB/c genetic background develop lymphoproliferative disease and die within 5 weeks from autoimmune haemolytic anaemia (AHA) (59). Mice of C57BL/6 genetic background die of colitis between 4-9 weeks of age. Although colitis and AHA seem to be the primary causes of death, IL-2 KO mice represent a generalised inflammatory disease with mononuclear infiltrate into multiple organs including the heart, lungs, blood vessels, liver and pancreas; as well as generalised amyloidosis and autoantibodies with multiple organ specificities (58) (60).

The first apparent immune abnormalities in the BALB/c IL-2 KO model are the activation of $CD4^+$ T cells. Activation markers such as CD69 (61), CD44 (62), and IL-2 Rα (4) are up-regulated in IL-$2^{-/-}$ $CD4^+$ T cells. Activation of $CD4^+$ T cells was followed by activation of B and $CD8^+$ T cells (58). At this stage, spleen and lymph nodes are hypercellular and eventually lose their architecture. The activation of lymphocytes leads to augmented proliferation of T and B cells, as illustrated by BrdU

incorporation studies (58) (59).

To summarise, IL-2 deficient mice represent a model of generalised autoimmunity, with particular focus on IBD and AHA. As mentioned previously, the kinetics and severity of disease is dependent on the genetic background of the mice, with fulminant progression in BALB/c mice and slow progression in the C57BL/6 strain. Finally, the colitis syndrome is not specific to IL-2 deficient mice. In fact, there are several mouse models where colitis is induced by genetic manipulation, drugs or transfer of a specific cell subset into $Rag^{-/-}$ or SCID immunodeficient mice. Each of these animal models gives insight into different factors contributing to disease pathogenesis.

2.1.1-Functionally important cell subsets in disease induction in IL-2 KO mice:

$CD4^+$ T cells play a key role in the immunopathology of this model. This is also true for other animal experimental colitis models. Several experiments have illustrated the importance of $CD4^+$ T cells in disease pathogenesis; they are as follows:

- IL-2 deficient mice that also lack the recombination activation gene (*RAG-2*), $RAG\text{-}2^{-/-}$ mice lack B and T cells, do not develop autoimmune disease (63). Moreover, transfer of $IL\text{-}2^{-/-}$ $RAG\text{-}2^{+/+}$ bone marrow into RAG-2 deficient mice resulted in IBD (64). These findings illustrated that cells of haematopoietic origin, in particular those with rearranging receptors, mediate the IL-2 deficiency syndrome.
- Introduction of the IL-2 mutation into athymic mice (*nu/nu*) does not result in disease despite the presence of an intact B cell compartment; indicating a key role for thymus-derived T cells in disease pathogenesis and not B cells (64).
- $IL\text{-}2^{-/-}$ $JH^{-/-}$ double mutant mice, which lack B cells and IL-2, develop IBD with the same kinetics as $IL\text{-}2^{-/-}$ mice (63). Nevertheless, these double mutants did not develop anaemia. This demonstrated that T cells are required for colitis whereas B cells are required for autoimmune haemolytic anaemia in IL-2 deficient mice.

- IL-2$^{-/-}$ β_2m$^{-/-}$ double mutant mice develop colitis. This demonstrated that CD4$^+$ T cells alone can mediate the development of mucosal inflammation (65). Nonetheless, other studies have shown that CD8$^+$ T cells may contribute to immunopathology. Simpson et al. have demonstrated that transfer of bone marrow cells from donor mice lacking perforin (PFP null mice) to reconstitute Tgε26 mice, mice which are transgenic for a high copy number of human CD3ε, resulted in reduced wasting and colitis. Tgε26 mice lack T cells and natural killer cells and develop colitis when reconstituted with syngeneic WT bone marrow (66). In conclusion, although cytotoxic T cells may contribute to mucosal inflammation, their presence is not fundamental to inflammation.

The above evidence emphasises the fundamental role of CD4$^+$ T cells in disease pathogenesis in IL-2 deficient mice: indeed CD4$^+$ T cells proved to be the mediators of immunopathology in several other experimental IBD models. A good example is the 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model where the direct inhibition of CD4$^+$ T lymphocytes using either CD4 mimetics or anti-CD4 monoclonal antibody (mAb) impeded disease (67).

In view of the fundamental role of CD4$^+$ T cells in disease pathogenesis, it is important to note that germ-free IL-2 deficient mice do not develop colitis (58) (68). This suggests that colitis is a dysregulated immune response to normal enteric bacteria. Moreover, HLA-B27 transgenic rats, which develop an illness resembling the B27-associated human disease ankylosing spondylitis, with intestinal, joint and skin lesions, do not develop mucosal inflammation when housed under germ-free conditions, implicating the commensal gut flora (69).

2.2-Animal models of IBD:

There are four major classifications of IBD animal models as outlined below:

- Genetic susceptibility IBD models such as the C3H-HeJBir mouse strain. C3H-HeJBir mice show greater sensitivity to dextran sulfate sodium (DSS)-induced colitis than NOD/LεJ, which in turn are more susceptible than C57BL/6 mice. Invariably, the genetic makeup of a mouse affects the way its immune system responds to external factors (70) (71).
- Drug-induced colitis models: here colitis is generated by reagents which induce an immune response in otherwise normal mice. Examples of external agents which induce colitis are TNBS and Oxazolone (72) (73).
- An important category is one of experimental colitis induced by genetic manipulation of cytokine/cytokine receptors, intracellular signalling machinery, mucosal barrier function and/or TCR. This group is the largest and provides insight into the role of different components of the immune system in mucosal inflammation. It can be subdivided into three groups: cytokine function, T cell function and IEC barrier function.

2.2.1-Table 1. IBD in genetically engineered (transgenic and knockout) animals: cytokine function. Adapted from (74).

Reference	Mutant	Abnormality	Organs involved
(58) (11)	IL-2$^{-/-}$ and IL-2Rα$^{-/-}$	Immunoregulation, thymic selection	Colon
(75)	IL-10$^{-/-}$	Immunoregulation, ↑Th1 activity	Colon, small intestine
(76)	TGFβ$^{-/-}$	Immune dysfunction	Gastrointestinal tract and other organs
(77)	TNFΔARE	↑TNF activity	Small intestine
(78)	IL-7 transgenic	Lympho-haematopoietic alterations	Colon
(79)	Stat3$^{-/-}$ (Mø and neutrophils)	↑Th1 activity	Colon
(80)	Stat4 transgenic	↑Th1 activity	Colon
(81)	*CRFB4*$^{-/-}$	Recapitulate IL-10$^{-/-}$ phenotype	Colon, small intestine

2.2.2-Table 2. IBD in genetically engineered animals: T cell function.

Reference	Mutant	Abnormality	Organs involved
(82)	TCR$\alpha^{-/-}$	Immunoregulation	Colon
(82)	TCR$\beta^{-/-}$	Immunoregulation	Colon
(82)	MHC class $II^{-/-}$	Immunoregulation	Colon
(83)	HLA-B27-transgenic rat	Antigen presentation	Colon, small intestine, joints, skin, male genitals
(84)	Gαi2$^{-/-}$	Defects in thymocyte maturation and function	Colon

2.2.3-Table 3. IBD in genetically engineered animals: IEC barrier function.

Reference	Mutant	Abnormality	Organs involved
(85)	N-cadherin "dominant negative"	Disruption of epithelial barrier	Small intestine
(86)	*mdr1a*$^{-/-}$	Disruption of epithelial barrier	Colon
(87)	Keratin $8^{-/-}$	Epithelial barrier	Colon

- The fourth, highly interesting category, are the "transfer models" where transfer of cell subsets into immunodeficient hosts results in colitis depending on the cell population transferred. This approach is fundamental in defining which cell population is specifically involved in immune pathogenesis. Although some $CD4^+$ T cells are responsible for the initiation of colitis, others can also regulate inflammation. This was first described in the SCID transfer model where SCID mice were reconstituted with different $CD4^+$ T cell subsets, some of which prevented while others promoted disease. Two groups demonstrated that pathogenic cells were contained in the $CD4^+CD45RB^{hi}$ (naïve) T cell subset whereas inhibitory or regulatory T cells were contained within the $CD4^+CD45RB^{lo}$ (memory) T cell population. Thus, transfer of IL-$2^{+/+}$ $CD4^+CD45RB^{hi}$ into SCID mice resulted in colitis whereas co-transfer of $CD4^+CD45RB^{hi}$ and $CD4^+CD45RB^{lo}$ resulted in no colitis (88) (89).

The protective quality of the $CD4^+CD45RB^{lo}$ subset was shown to be dependent on TGF-β but independent of IL-4; this suggested that this regulatory role was not Th2 mediated. Furthermore, it indicated that TGF-β production is independent of IL-4 (90). More recently, this simple dichotomy was further complicated by evidence that the $CD4^+CD45RB^{lo}$ subset contains cells capable of inducing progressive colitis. However, the $CD4^+CD45RB^{lo}$ subset delayed the kinetics of disease when compared to that induced by $CD4^+CD45RB^{hi}$ cells. This suggested that in effect the $CD4^+CD45RB^{lo}$ subset contains a mixture of autoaggressive and counter-regulatory cells and that disease-inducing potency was dependent on their activation state and IL-12 responsiveness. In a study by Claesson et al., SCID mice were reconstituted with syngeneic resting or activated $CD4^+$ T cells that were further subdivided into $CD45RB^{hi}$ or $CD45RB^{lo}$ subsets. Additionally, they used STAT-$4^{-/-}$ $CD4^+$ T cells, which are unresponsive to IL-12, to reconstitute SCID mice and monitored disease progression (91). In general, autoaggressive $CD4^+$ T cells in most mucosal inflammation models are IL-12 driven. Conversely, the regulatory T cell subset produces suppressor cytokines such as TGF-β and/or IL-10 (90) (92) (93).

2.3-Effector mechanisms in mucosal inflammation models:

In all experimental mucosal inflammation models, the major effector cells are $CD4^+$ T cells which either over-produce Th1 cytokines (TNF, IFN-γ) or Th2 cytokines (IL-4, IL-5 and IL-13). The pattern of cytokines produced depends on the model. Moreover, the histopathology of inflammation is different depending on the pattern of cytokines produced. The general consensus is that Th1-associated immunopathology is associated with transmural inflammation, of granulomatous character and containing few acute inflammatory cells such as neutrophils and eosinophils. Th2 immunopathology is associated with a more superficial inflammation but with acute inflammatory cell exudates. Generally, a Th1 immune pathology is associated with Crohn's disease-like inflammation and Th2 with that of ulcerative colitis (94).

2.3.1-IL-12/IFN-γ-mediated pathways:

The Th1 cytokine pathway is induced by IL-12. IL-12 signalling occurs via the heterodimeric IL-12 receptor, which when triggered leads to the activation of Jak2 and Tyk2 succeeded by STAT3 and STAT4 and finally the induction of IFN-γ mRNA synthesis (reviewed in (95) (96)). Several models of mucosal inflammation are a result of dysregulation of the Th1 response (97) (98). Most notable are the SCID transfer model (99), TNBS-induced models (100) and the IL-$2^{-/-}$ TNP-KLH (TNP-conjugated keyhole limpet hemocyanin)-induced model (101). In all the above models, administration of anti-IL-12 or anti-IFN-γ prevented or alleviated disease. However, IFN-$\gamma^{-/-}$ mice can be induced to develop TNBS-induced colitis. Moreover, in the Tgε26-transfer model (Tgε26 mice lack T cells and natural killer cells and develop colitis when reconstituted with syngeneic WT bone marrow), transfer of bone marrow cells from IFN-$\gamma^{-/-}$ mice results in colitis, whereas IL-$12^{-/-}$ bone marrow transfer prevents development of colitis (102). These findings suggest that IL-12 functions in both an IFN-γ-dependent and independent manner. One factor that may explain the IFN-γ discrepancy is that anti-IFN-γ antibodies would annul only the function of IFN-γ, whereas, anti-IL-12 would affect both IFN-γ and TNF functions. In support of this,

is the finding that TNF overproduction results in CD-like syndrome which is probably orchestrated by IL-12 (77).

2.3.2-TNF-mediated pathways:

Th1 inflammation results in both IFN-γ and TNF production. IL-12 acts on T cells and induces the production of these two cytokines, which in turn positively regulate IL-12 production by APCs. TNF mediates signalling events through its receptors TNFRI and TNFRII. These two receptors are part of the TNFR family of molecules. An important member of this family is CD40, a receptor present on APCs and B cells and which interacts with its ligand CD40L (gp39) which is expressed on activated T cells. The CD40/CD40L pathway is instrumental in T-B interactions and initiation of Th1 differentiation via stimulation of APCs and IL-12 production. Indeed, anti-CD40-ligand antibody prevents the development of disease in TNBS-induced colitis, possibly by abolishing signalling by APCs needed for IL-12 production (72).

Over-expression of TNF in a recent experimental model (TNFΔARE) resulted in mucosal inflammation. The disease in this model is specific to the small intestine and had granulmatous histology that is similar to that in CD.

To summarise, several experimental mucosal inflammation models are characterised by dysregulation of a Th1 cytokine pattern, where the overproduction of TNF/IFN-γ mediates immune pathology in these models. This overproduction is mainly driven by IL-12 and is illustrated by a model where crosses between IL-12$^{-/-}$ and TNFΔARE resulted in the abrogation of disease. The above finding together with the preventive administration of anti-IL-12 in several colitis models suggests that IL-12 is the main cytokine necessary for initiation of a dysregulated Th1 immune response.

2.3.3-Th2-mediated pathways:

Interestingly, not all mucosal inflammation models are Th1-mediated. Indeed, a dysregulated Th2 immune response is implicated in a few models. Most notably, the TCRα$^{-/-}$ colitis model. TCRα$^{-/-}$ mice develop spontaneous chronic colitis, resembling

human UC, a condition which is IL-4 dependent. In fact, administration of anti-IL-4 antibodies in TCR$\alpha^{-/-}$ mice inhibits the Th2 cell-dominated IBD in this model (103). In a similar model, administration of TNBS to IFN-$\gamma^{+/+}$ or IFN-$\gamma^{-/-}$ BALB/c mice resulted in colitis. Indeed, crypt inflammation was greater in IFN-$\gamma^{-/-}$ BALB/c (a Th2-predisposed strain) than their IFN-$\gamma^{+/+}$ counterparts (94). This study provided evidence in support of a pathogenic role for both Th1 and Th2 cytokine patterns and the cytokine pattern produced dictates the different types of pathology with varying kinetics.

Another Th2-mediated colitis model is that by induced Oxazolone. Oxazolone administered to SJL/J mice results in a UC-like pathology that is mediated by IL-4 producing cells. Administration of anti-IL-4 in this model impeded disease (73).

2.4-Anti-inflammatory pathways: how pathogenesis is controlled:

Direct evidence of a T cell population that regulates mucosal inflammation came from $CD4^{+}CD45RB^{lo}$, which when co-transferred with $CD4^{+}CD45RB^{hi}$ T cells prevents disease. This raised questions about the mechanism(s) by which these regulatory T cells act. Powrie et al. showed that the ability of $CD4^{+}CD45RB^{lo}$ T cells to protect SCID mice from $CD4^{+}CD45RB^{hi}$ T cell-Th1-induced colitis is associated with TGF-β production. Administration of anti-TGF-β abrogated the protective effect of $CD4^{+}CD45RB^{lo}$ T cells (90). Therefore, TGF-β seems to play an important role in regulating mucosal inflammation. Indeed, IL-$2^{+/+}$ mice immunised with TNP-KLH produce significant amounts of TGF-β, whereas their IL-$2^{-/-}$ counterparts fail to produce TGF-β following immunisation. Interestingly, TNP-KLH-induced colitis in IL-$2^{-/-}$ mice was prevented by administration of anti-CD3, which in turn induced the production of TGF-β. Co-administration of anti-TGF-β with anti-CD3 resulted in disease (104). This study emphasised the role of suppressor cytokines and suggested that an impaired TGF-β response induced autoimmunity in IL-$2^{-/-}$ mice.

Another study implicating the importance of suppressor cytokines in immune regulation is the IL-10$^{-/-}$ mouse model. IL-10$^{-/-}$ mice develop a Th1-mediated mucosal inflammation that involves both the colon and small intestine (75). Moreover, *CRFB4*$^{-/-}$ mice (*CRFB4* is a gene which encodes a component of the IL-10 receptor) also develop colitis (81). These studies emphasised the role of IL-10 signalling in immune regulation. Recently, a study by Asseman et al. showed that CD4^{+}CD45RB^{lo} T cells from IL-10$^{-/-}$ mice were not able to inhibit disease (93).

In fact, Groux et al. were the first group to describe an IL-10 dependent CD4^{+} T cell subset, designated Tr1, which suppresses antigen-specific responses and inhibits colitis induced in SCID mice by the pathogenic CD4^{+}CD45RB^{hi} T cell subset *in vivo* (92). [More details on the role of regulatory T cell subsets in IL-2$^{-/-}$ mice will be included under the peripheral tolerance mechanisms section].

3-IL-2 Receptor Deficient models: IL-2Rα$^{-/-}$, IL-2Rβ$^{-/-}$ and IL-2Rγ$^{-/-}$

3.1-The IL-Rα deficient model:

IL-2Rα expression is induced during different stages of early T and B lymphocyte development. The α chain is expressed transiently on pro-B cells and on an immature subset of thymocytes which are in an intermediate stage between $CD3^-CD4^-CD8^-$ and the double positive $CD4^+CD8^+$ T cell compartment (105). Mice deficient for IL-2Rα have phenotypically normal T and B cell development when young. As adults, IL-2Rα KO mice develop immunoproliferative disease characterised by enlarged peripheral lymphoid organs, haemolytic anaemia and inflammatory bowel disease (11), similar to the IL-2 deficient model. The IL-2Rα chain does not independently mediate signal transduction (9), its association with the β and γ chains results in the high affinity receptor. It is also induced on mature activated lymphocytes implying that it also functions to increase the sensitivity of activated lymphocytes to IL-2.

3.2-The IL-2Rβ deficient model:

The IL-2Rβ chain is expressed on a variety of cells including B cells, NK cells, neutrophils, foetal and adult γ/δ dendritic epidermal T cells (106) and a small subset of phenotypically mature αβ T cells which express autoreactive receptors (107). Its expression on resting T lymphocytes is low and is up-regulated upon activation. This chain is also shared by the IL-15 receptor. Mice deficient in IL-2Rβ develop autoimmunity mainly marked by granulocytopoiesis and granulocyte tissue infiltrates but do not develop inflammatory bowel disease (IBD) (108). This implies that IL-2Rβ is important in exacerbating immune responses and specifically that IL-15 may play a role in IBD.

3.3-The IL-2Rγ deficient model:

Mice lacking the IL-2Rγ chain develop hypoplastic thymi and are compromised in their ability to respond to γ_c dependent cytokines (109). In addition, there is absence of NK, γ/δ intestinal intraepithelial cells, dendritic epidermal T lymphocytes, peripheral

lymph nodes and gut-associated lymphoid tissue (109). The γ chain deficient mouse model reflects the X-linked severe combined immune deficiency (SCID) human condition (110).

4-IL-2 KO mice: a model of central tolerance failure?

Central tolerance is the process by which developing thymocytes are screened for MHC restriction and their level of interaction with self-antigens in the thymus. It comprises two forms of selection process: positive and negative selection. Positive selection refers to the mechanism of rescuing MHC-restricted thymocytes from programmed cell death; this means that cells which express a MHC class II-restricted receptor are positively selected to the CD4 lineage, whereas cells expressing MHC class I-restricted receptors are positively selected to the CD8 lineage. Negative selection refers to the functional elimination or clonal deletion of potentially autoreactive cells; these are cells expressing receptors that react strongly with thymic self-antigens. Positive and negative selection are fundamental mechanisms for T cell education ensuring the integrity of the repertoire. Despite the initial studies illustrating normal distribution of CD4/8 T cells in the IL-2 KO repertoire (53), IL-2 may play a role in the negative/positive selection mechanisms that may not be apparent from the CD4/8 distribution pattern. In support of this are the activated phenotype of $CD4^+$ T cells in the periphery and the development of autoimmunity in IL-2 KO mice.

Kramer et al. were the first to describe the role of IL-2 KO thymocytes in disease induction (64). This study showed that athymic $IL\text{-}2^{-/-}$ mice did not develop autoimmune disease, implicating thymus-derived T cells in disease pathogenesis.

Other studies have investigated intrathymic T cell development in IL-2 KO mice (111). $IL\text{-}2^{-/-}$ mice immunised with TNP-KLH exhibited changes in thymocyte populations. These changes were characterised by decreased numbers of double positive thymocytes ($CD4^+CD8^+$) and increased numbers of $CD4^+$ single positive thymocytes and to a lesser extent $CD8^+$ single positive thymocytes. Interestingly, the

single positive thymocytes expressed activation markers. Moreover, thymocytes from immunised IL-2 null mice produced more IFN-γ and less IL-4 than immunised IL-$2^{+/+}$ mice. These defects were IL-12-dependent because co-administration of anti-IL-12 with TNP-KLH abolished changes in thymocyte populations and production of IFN-γ. Consequently, to investigate whether these changes in thymocyte subpopulations and IFN-γ production are directly associated with the development of colitis, a small number of purified single positive IL-$2^{-/-}$ thymocytes (previously immunised with TNP-KLH) were injected into IL-$2^{+/+}$ mice. This resulted in colitis in IL-$2^{+/+}$ mice and provided direct evidence that IL-$2^{-/-}$ thymocytes are implicated in the development of colitis.

Most interestingly, this study by Luviksson et al. demonstrated a defect in thymic selection. Injection of anti-CD3 intraperitoneally (i.p.) induces cortical intrathymic cell death in IL-$2^{+/+}$ mice (as detected by in situ apoptosis), with a 78% decrease in the total number of thymocytes. Injection of anti-CD3 i.p. in IL-$2^{-/-}$ mice resulted in a 34% decrease in total thymocyte numbers. This indicated that IL-$2^{-/-}$ mice exhibit decreased susceptibility to cortical apoptosis. To conclude, this study illustrated that in the absence of IL-2, thymocyte maturation is directed towards a TH_1 pathogenic T cell response, orchestrated by IL-12 (111).

It is noteworthy that initial reports did not demonstrate a change in IL-$2^{-/-}$ thymocyte subsets (53) (112). However, this discrepancy can be a result of differences in experimental models. Schorle et al. used unstimulated thymocytes, whereas TNP-KLH stimulated thymocytes were used by Ludviksson et al. Furthermore, Kramer et al. studied CD8 IL-$2^{-/-}$ F5 [TCR specific for influenza nucleoprotein (NP) 366-374 + H-$2D^b$] transgenic mice (112); as described in the TNP-KLH immunised model, most of the characteristic defects were associated with $CD4^+$ T cells (111). In fact, another discrepancy was found in the IL-$2^{-/-}$ F5 transgenic mice (112). Initial studies by Kundig et al. showed that *in vivo* CTL responses against vaccinia virus and LCMV were functional in IL-2 KO mice (57). In the IL-$2^{-/-}$ F5 $CD8^+$ transgenic model, CTL

responses were dysfunctional. The use of peptide immunisation and activation of most of the CD8 peripheral population, without the participation of $CD4^+$ T cell responses, may explain the difference in IL-$2^{-/-}$ CTL activity between the transgenic and non-transgenic systems. Indeed, following peptide immunisation, the transgenic CTL activity *in vivo* was quite low, given the high number of potential CTL precursors. Therefore, additional signals such as $CD4^+$ T cell-derived cytokines may enhance the CTL activity or perhaps other cell interaction molecules, up-regulated as a result of virus infection, may be absent in the transgenic system (112).

A recent study has examined the role of IL-2 in regulating the thymic microenvironment. Immunohistochemical staining of thymic sections from IL-$2^{-/-}$ mice and WT counterparts of different ages revealed loss of cortical epithelial cells, MHC class II-expressing cells, monocytes and macrophages (113). To investigate the role of the thymic microenvironment, thymocyte progenitor populations ($CD3^-CD4^-CD8^-$) from 4 week old IL-$2^{-/-}$ mice were injected into the thymus of sublethally irradiated IL-$2^{+/+}$ mice. At this age, the thymic composition of IL-$2^{-/-}$ mice is similar to their WT counterparts. 4-5 weeks after transfer, animals that were injected with IL-$2^{-/-}$ thymocytes into their thymic lobes did not exhibit any signs of disease and the IL-$2^{-/-}$ thymocytes were able to expand normally when compared to IL-$2^{+/+}$ injected thymocytes. This emphasised that the aberrant T cell development in IL-$2^{-/-}$ does not occur in normal and intact thymic microenvironment, which in turn suggested that these defects are driven by an aberrant thymic microenvironment in IL-$2^{-/-}$ mice. Moreover, it suggested that IL-$2^{-/-}$ T cells do not have an intrinsic developmental defect since they can expand normally in IL-$2^{+/+}$ thymuses and do not promote disease (113). Of particular interest is the study by Kramer et al. where RAG-$2^{-/-}$ mice were reconstituted with *nu/nu* IL-$2^{-/-}$ bone marrow cells. Six weeks after transfer, all of the chimaeras that had received *nu/nu* IL-$2^{-/-}$ bone marrow cells exhibited signs of disease (64). This indicated that, despite the functional thymus in RAG-$2^{-/-}$ mice, IL-$2^{-/-}$ bone marrow cells were able to induce disease in reconstituted RAG-$2^{-/-}$ mice. Taken

together, these two studies suggest that IL-$2^{-/-}$ APCs are needed for the development of the IL-2 syndrome, since IL-$2^{-/-}$ thymocytes alone can not induce disease.

To conclude, the above studies focussing on central tolerance point to a regulatory role for IL-2 in central tolerance, possibly through regulating the thymic microenvironment and/or generating a non-pathogenic/non-T_H1 biased T cell repertoire.

5-IL-2 KO mice: a model of peripheral tolerance failure?

Peripheral tolerance is a mechanism by which autoreactive T cells that escape central deletion in the thymus are eliminated or controlled in the periphery. These include cells that escape central deletion because they recognise a self-antigen that was not present in sufficient concentration to cause deletion; or they recognise a self-antigen that is not present in the thymus, i.e., an antigen specific to an organ such as the pancreas. Moreover, it is also a mechanism by which an immune response is shut down when obsolete and prevented when harmful. In the same way as activation and expansion of immune cells are critical for fighting infections, it is also important for immune responses to contract once the infection is over. There are several mechanisms by which peripheral tolerance is achieved; namely anergy, clonal deletion or exhaustion and suppression of immune responses. These mechanisms will be discussed in detail in the sections below.

5.1-Anergy:

Anergy is a cellular state in which a lymphocyte fails to respond to TCR signalling. This results in no proliferation or IL-2 synthesis. The term was initially used by Nossal and Pike to describe an unresponsive state generated by the injection of soluble protein antigen *in vivo* (114). In this model, antigen-specific B cells were detected in the animal but could not be reactivated by antigen or mitogen to make immunoglobulin. Subsequently, studies on murine $CD4^+$ T cell clones led to the discovery of several methods by which this unresponsive state can be induced. These include presentation of peptide antigens on chemically fixed APCs (115) or in planar lipid membranes

containing MHC class II molecules (116), stimulation of highly purified T cells with plate-bound anti-CD3 (117) or with concanavalin A (118).

These findings suggested that occupancy of the TCR alone, in the absence of other signals, was responsible for inducing anergy or unresponsiveness. This deduction was further supported by a study by Jenkins et al; various costimulators (including cytokines such as IL-1) were added to T cells at the time of exposure to antigen on chemically modified APCs. None was able to influence the unresponsive state of the T cells with the exception of allogeneic spleen cells. Addition of allogeneic non-T spleen cells resulted in a proliferative response. Further analysis of the splenic non-T population revealed that low density B cells and macrophages were the best source of costimulatory activity. This study was instrumental in elucidating the dependence of IL-2 production and proliferation of T cell clones on costimulatory signals provided by non-T accessory cells in addition to the TCR signals (119).

In vitro, anergy can be induced by stimulation through the TCR in the absence of costimulatory signals as mentioned previously; by altered peptide ligands, even in the presence of costimulatory molecules (120); by cytokines such as IL-10 (121); or by antigen presentation on non-professional APCs, for example T:T antigen presentation (122).

In vivo, anergy can be induced by ligation of CTLA-4 (123), the inhibitory T cell receptor of B7 costimulatory molecules. CTLA-4 (CD152) is a homologue of CD28 that binds B7-1 (CD80) and B7-2 (CD86) and functions as a negative regulator of the immune response (124) (125). Perez et al. used adoptive transfer of TCR transgenic (Tg) T cells, specific for the ovalbumin peptide $(OVA)_{323-339}$ in the context of $I\text{-}A^d$, into syngeneic WT recipients and a tolerizing mode of antigen application (large dose of antigen injected intraperitoneally). When anti-CTLA-4 blocking antibody was co-administered with antigen, Tg T cells exhibited potent proliferative responses and secreted large amounts of IL-2. This revealed that the induction of T cell anergy *in*

vivo requires CTLA-4 engagement (123). However, it is important to distinguish between cytokine-mediated immunosuppression and anergy *in vivo*.

Schwartz et al. described an *in vivo* tolerance model, which involved both clonal anergy and cytokine-mediated immunosuppression (126). In this model, $CD4^+$ T cells from RAG-$2^{-/-}$ Tg mice fail to produce IL-2, IL-4 and IFN-γ upon antigenic rechallenge after multiple immunisations with staphylococcal enterotoxin A (SEA). The two mechanisms (cytokine-mediated suppression and anergy) were distinct at cellular and molecular levels. The suppressive effect of SEA-treated spleens involved both IL-10 and TGF-β but additionally appeared to involve unidentified factor(s). Also, suppressive supernatants did not cause a block in early response kinases (ERKs), as does anergy. Moreover, anti-TGF-β was able to block the cytokine-mediated suppressive effect of TGF-β but not anergy (126).

Finally, although anergy can be beneficial via immunological self-tolerance, it may also be harmful to the immune system if induced in regulatory T cells. This is illustrated in the non-obese diabetic (NOD) mouse model. This is a model of autoimmune insulin-dependent diabetes mellitus (IDDM), which results from immune dysregulation culminating in pancreatic islet β-cell destruction (reviewed in (127)) (128). NOD thymocytes and peripheral T cells exhibit hyporesponsiveness (129) and produce small amounts of IL-2 and virtually no IL-4 (130). The authors hypothesized that TH_2 cells may be anergic in NOD mice and that the administration of IL-4 may reverse anergy and prevent IDDM. Indeed, exogenous IL-4 reversed the unresponsiveness of both thymic and peripheral T cells from NOD mice as well as restored the production of IL-2. Moreover, *in vivo* administration of rIL-4 to prediabetic NOD mice prevented the onset of disease in these animals (130). The contribution of anergy to autoimmune disease development has been fully reviewed by Salojin et al. and sheds a new light on this so called "peripheral tolerance" mechanism (131).

5.1.1-Anergy in relation to IL-2:

As mentioned previously, the most prominent outcome of TCR engagement and costimulation is the production of IL-2, the driving force behind immune proliferative responses. In the case of anergy, T cells which are stimulated through the TCR in the absence of costimulation fail to proliferate if restimulated in the presence of costimulation and antigen. In $CD4^+$ TH_1 and $CD8^+$ CTL clones, this "unresponsiveness" was found to be a result of a block in IL-2 production (132) (133). However, IL-2 is not the only cytokine whose production is blocked as a result of anergy. IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF) production is also decreased, to a lesser extent, in anergic T cells (134). *In vitro*, however, IL-2 production remains a major indicator of T cell anergy.

Recent studies have highlighted the role of IL-2 in anergy regulation. One of the mechanisms by which IL-2 controls cellular proliferation is through regulating the cell cycle inhibitors, p21 and p27. In activated T cells, IL-2 promotes the loss of p27, allowing exit from quiescence and progress through the G1 phase (41). Moreover, Boussiotis et al. described a model whereby stimulation of T cells in the absence of costimulation results in anergy induction and the overexpression of p27. In this study a direct relationship was established between T cell unresponsiveness and p27 expression levels in p27-transfected T cells (135). Likewise, P21 has been implicated in tolerance and autoimmune disease. $P21^{-/-}$ mice lose tolerance to self-antigens and develop an autoimmune disease similar to systemic lupus erythematosous (136). Although p21 and p27 cell cycle regulatory molecules influence both cell proliferation and T cell tolerance, the exact mechanism(s) by which these molecules mediate their effects are unknown.

5.2-Suppressor T cells:

T suppressor cells, designated T regulatory cells (Tr) are now, once again after a period of neglect, accepted as one of the essential arms of peripheral tolerance. While anergy is defined as a failure to proliferate or produce IL-2, suppression has been considered an "active" process which involves the activation of T regulatory cells and can be adoptively transferred. As mentioned by Schwartz et al, anergy and suppression are distinct at cellular and molecular levels but are linked mechanisms *in vivo* (126).

Recently, self-tolerance at the T cell level has been ascribed to three mechanisms, namely non-responsiveness (anergy), clonal deletion (activation-induced cell death) and active T-cell-mediated suppression. There are several studies and animal models illustrating the role of suppression in T cell tolerance and in the prevention of autoimmunity. Tr cells can be divided into $CD4^+CD25^+$, T_H3, Tr1, and $CD4^+CD45RB^{low}$ subsets. The different Tr subsets will be discussed below with specific emphasis on Tr subsets involved in the prevention of colitis, which is the primary cause of death in C57BL/6 IL-$2^{-/-}$ mice.

5.2.1-$CD4^+CD25^+$ Tr cells:

Cell surface markers and functional differences were first used by Powrie and Mason to describe regulatory and effector T cell subsets (137). Two other seminal models referred to a regulatory $CD4^+$ T cell subset. In the first model, genetically autoimmune susceptible mice were thymectomized (Tx) at 3 days of age (d3Tx) and developed organ-specific autoimmune disease (138). Later studies demonstrated that the transfer of $CD4^+$ T cells (by day 14 of life) from adult mice could prevent disease in d3Tx mice. In particular, this preventive effect was mediated by a minor subset of $CD4^+$ that co-expressed CD25 (139). In the second model, the $CD4^+CD25^+$ subset was depleted from $CD4^+$ T cells isolated from peripheral lymphoid tissue of normal mice. The remaining $CD4^+CD25^-$ T population was then injected into *nu/nu* mice and organ-specific autoimmune disease developed (140). Most importantly, cotransfer of

$CD4^+CD25^+$ T cells within 10 days of the $CD4^+CD25^-$ subset prevented disease. These studies pointed to a regulatory function of the $CD4^+CD25^+$ T cell subset.

Subsequent studies have established that $CD25^+$ Tr cells comprise 5-10% of the peripheral $CD4^+$ T cells in normal mice (140) (141). Moreover, $CD4^+CD25^+$ Tr cells are anergic, resistant to superantigen-mediated deletion and are dependent on IL-2 on their generation and survival *in vitro* and *in vivo* (142). In fact, these cells are absent in $IL\text{-}2^{-/-}$ mice.

Other studies have focussed on the mechanism by which these Tr cells are activated and mediate their suppressive effect. Thornton and Shevach demonstrated that $CD4^+CD25^+$ Tr cells required activation via the TCR to exert their suppressive function, are potent inhibitors of polyclonal activation *in vitro* and mediate their regulatory role by inhibiting the induction of IL-2 in the responder $CD4^+CD25^-$ T cell subset. Suppression was mediated via a cytokine-independent, cell-cell contact-dependent manner (141). A recent study demonstrated that $CD4^+CD25^+$ T cells required activation via the TCR to become suppressive, but once activated, their regulatory function is completely non-specific (143). Furthermore, this suppressive role was abrogated by the addition of exogenous IL-2 (141) or anti-CTLA-4 mAb (144) (145). Notably, on activation, most $CD4^+$ T cells can acquire CD25 without becoming regulatory; by contrast, $CD4^+$ $CD25^+$ Tr acquire these properties as they develop in the thymus (146). To date, the cell surface molecules involved in T-T cell contact and induction of suppression have not been identified.

5.2.2-T_H3 Tr cells:

T_H3 cells secrete high levels of TGF-β but lower levels of IL-4 and IL-10 (147). Evidence for the critical role of TGF-β in the induction of oral tolerance and prevention of colitis came from multiple studies. In the $IL\text{-}2^{-/-}$ model, TNP-KLH-immunised mice failed to produce TGF-β, whereas their WT counterparts produced

significant amounts of TGF-β (104). In fact, Neurath et al. have demonstrated that TGF-β can abolish experimental granulomatous colitis even after it is established (148).

It has been suggested that the differentiation of T_H1 versus Tr cells is dependent on the balance between IL-12 and TGF-β in the intestine (149). To date, the major effector mechanism by which some Tr cells exert their regulatory function is via the production of IL-10 and TGF-β. Both IL-10 and TGF-β have immunosuppressive effects on APCs and T cells (reviewed in (150)). Mice which express a dominant negative TGF-β receptor II subunit (this is the subunit which blocks the function of all TGF-β isoforms on T cells) under a T-cell specific promoter exhibit autoimmune disease. Other seminal studies demonstrating the regulatory role of TGF−β are adoptive transfer models, where cotransfer of a Tr subset with the pathogenic $CD4^+CD45RB^{hi}$ subset into SCID mice resulted in the prevention of disease. Mechanistic studies revealed that the regulatory function of the Tr subset was TGF-β dependent. Most recently, studies have revealed that the ligation of CTLA-4 and TGF-β production are obligatory for the function of $CD4^+CD25^+$ Tr. Most interestingly, Chen et al. demonstrated that engagement of CTLA-4 in the presence of TCR ligation results in the production of TGF-β in unfractionated $CD4^+$ T cells (151). This implies that CTLA-4-mediated TGF-β production may be one of the mechanisms by which CTLA-4 exerts its suppressive effect.

Briefly, TGF-β plays a major role in oral tolerance and regulation of pathogenic T cells. It is therefore not surprising that administration of anti-TGF-β mAb in colitis adoptive transfer models abolishes the regulatory function of Tr cells.

5.2.3-Tr1 cells:

Tr1 cells consistently produce high levels of IL-10 and low levels of TGF-β. IL-10 is critical for the maintenance of T cell tolerance in the gut, since $IL\text{-}10^{-/-}$ mice develop

severe enterocolitis (75). Tr1 cells were able to prevent colitis induced by the autoaggressive $CD4^{+}CD45RB^{hi}$ in SCID mice *in vivo*. Co-transfer of antigen-specific Tr1 clones (with a TCR Tg specific for OVA) with $CD4^{+}CD45RB^{hi}$ into SCID mice resulted in the prevention of mucosal inflammation. However, suppression was only mediated when the Tr1 cells were activated, that is, disease was only inhibited in recipients fed with OVA in their drinking water (92).

Moreover, systemic administration of recombinant IL-10 in SCID mice reconstituted with the pathogenic $CD4^{+}CD45RB^{hi}$ subset abrogates mucosal inflammation in these animals (99). Recently, a study by Asseman et al. demonstrated that $CD4^{+}CD45RB^{lo}$ cells from IL-$10^{-/-}$ mice were not able to prevent IBD in the SCID adoptive transfer model. Moreover, treatment with anti-IL-10 receptor mAb ablated the protective effects of WT $CD4^{+}CD45RB^{lo}$ co-transferred with IL-$10^{-/-}$ $CD4^{+}CD45RB^{hi}$ to RAG-$2^{-/-}$ mice (93). This finding demonstrated that the protective effect of $CD4^{+}CD45RB^{lo}$ cells is not dependent on the differentiation of $CD4^{+}CD45RB^{hi}$ progeny into IL-10-producing cells.

The exact mechanism(s) by which the protective IL-10-secreting cells prevent mucosal inflammation are not identified as yet. However, the immunosuppressive effects of IL-10 on T cells and APCs are well documented (152) (153). Its immunosuppressive effects are mediated via multiple mechanisms ranging from downregulating costimulatory molecules on APCs (154) to the recently described generation of functional chemokine decoy receptors on APCs (155).

Despite the success of IL-10 in abrogating mucosal inflammation in murine models, human clinical trials with IL-10 have been unsuccessful. This brings into question the aptitude of the clinical trials on which the efficacy of IL-10 is judged and the validity of the animal models in relation to human disease (156).

5.2.4-$CD4^+CD45RB^{lo}$ Tr cells:

Powrie and Mason were the first to describe a rat model in which $CD4^+$ T cells can be divided into two subpopulations, depending on their expression of the CD45RB isoforms. Athymic rats injected with $CD4^+CD45RB^{hi}$ T cells from euthymic donors developed a chronic wasting disorder. Conversely, recipients of $CD4^+CD45RB^{lo}$ T cells did not develop any disease (137). Separation of mouse $CD4^+$ T cells followed and murine $CD4^+CD45RB^{lo}$ and $CD4^+CD45RB^{hi}$ subsets were injected into immunodeficient SCID or RAG-$2^{-/-}$ mice as mentioned previously (88). These studies emphasised the role of TGF-β and IL-10 production by $CD4^+CD45RB^{lo}$ cells in the prevention of mucosal inflammation.

Attempts have been made to further characterise the regulatory $CD4^+CD45RB^{lo}$ subset Recently, $CD4^+CD45RB^{lo}$ cells were subdivided into $CD25^+$ and $CD25^-$ fractions and tested for their ability to inhibit colitis induced by $CD4^+CD45RB^{hi}$ cells. Inhibition of colitis was found to be dependent on the $CD25^+$ fraction. Interestingly, the $CD4^+CD45RB^{lo}$ $CD25^-$ subpopulation was incapable of fully inhibiting colitis induced by transfer of $CD4^+CD45RB^{hi}$ cells. Although $CD4^+CD45RB^{lo}$ $CD25^-$ were incapable of inhibiting colitis, transfer of this subpopulation to SCID mice did not result in transfer of disease. This finding demonstrated that the autoaggressive effector cells were not present in this subpopulation and did not cause a lack of regulatory function (144).

Another report subdivided the $CD4^+CD45RB^{lo}$ T cells subset into $CD38^+$ and $CD38^-$ fractions and tested for their ability to respond to recall antigens, proliferate *in vitro* and produce cytokines. $CD4^+CD45RB^{lo}$ $CD38^-$ T cells responded to polyclonal stimulation and produced high levels of cytokines, whereas the $CD4^+CD45RB^{lo}$ $CD38^+$ cells were incapable of responding to anti-CD3 and producing detectable levels of cytokines (157). Although $CD4^+CD45RB^{lo}$ $CD38^+$ cells failed to proliferate or produce detectable levels of cytokines, co-culture of this subpopulation with

$CD4^+CD45RB^{lo}$ $CD38^-$ resulted in the inhibition of anti-CD3-induced proliferation and cytokine production by $CD4^+CD45RB^{lo}$ $CD38^-$ T cells.

Despite the *in vitro* immunosuppressive effect of the $CD4^+CD45RB^{lo}$ $CD38^+$ subpopulation, there is no direct association between *in vivo* suppression mediated by $CD4^+CD45RB^{lo}$ T cells and the *in vitro* regulatory role of $CD4^+CD45RB^{lo}$ $CD38^+$ T cells.

More importantly, it is not yet known whether some of the T regulatory cells mentioned previously are derived from one progenitor population that expresses different phenotypic markers during different stages of development, or perhaps the differences reported are a result of different disease models.

5.3-Activation-induced cell death (AICD):

Activation-induced cell death (AICD) is the principle mechanism by which cells encountering repeated antigen stimulation die. This process is Fas-Fas L dependent. Fas (CD95) is expressed on a variety of cells, including epithelial cells. The expression of Fas on B and T cells is up-regulated upon activation (158). However, the expression of Fas L is more restricted and is induced on activated $CD4^+$ and $CD8^+$ T cells (159). The engagement of Fas on $CD4^+$ T cells induces cell death signals; the binding of Fas L to Fas induces the trimerization of the Fas receptor, which recruits caspase 8 via the FADD/MORT 1 (Fas associating protein with death domain). FADD binds to Fas via interactions between their death domains (160). Caspase 8, initially termed FLICE (161), is related to the interleukin 1β converting enzyme (ICE) protease family. The ICE family are cysteine proteases causing apoptosis when overexpressed in cells (162). Activation of caspase 8 may result in the self-activation of proteolytic activity that triggers the ICE protease cascade. The activated ICE caspases then cleave various proteins such as poly (ADP) ribose polymerase and actin and cause the morphological changes to cells and nuclei characteristic of apoptosis (163).

5.3.1-The CD95/CD95L apoptotic pathway:

Singer et al. were the first to demonstrate that Fas-Fas L interactions are required for peripheral but not central tolerance (50). In this study, T cell development and responses to antigen were analysed by breeding a T cell transgenic receptor into Fas-defective MRL-*lpr/lpr* and control MRL$^{+/+}$ mice. Mature CD4^{+} T cells from the MRL-*lpr/lpr* are resistant to apoptotic cell death when stimulated with high doses of antigen. *In vivo* administration of high doses of peptide causes deletion of thymic T cells in both MRL-*lpr/lpr* and MRL$^{+/+}$ control mice. Conversely, antigen-induced T cell deletion of peripheral T lymphocytes occurs in MRL$^{+/+}$ but not MRL-*lpr/lpr* mice. This study emphasised the role of Fas in peripheral T cell tolerance, activation-induced cell death, and not in central tolerance (50). However, other studies have illustrated a role for Fas in central tolerance (164); moreover, the authors suggested that administration of high doses of peptide antigens may not mimic the continuous presence of self-antigen *in vivo*. In order to mimic the presence of self-antigen *in vivo*, a hen egg lysozyme (HEL)-specific TCR (3A9) was bred into WT or *lpr/lpr* mice and subsequently crossed with mice expressing high serum levels of soluble HEL (165), also on WT or *lpr/lpr* backgrounds. This meant that these mice expressed a TCR Tg specific for HEL as well as HEL expressed systemically as "self" antigen (166) (44). In this model, HEL is effectively a self-protein and the majority of TCR-specific thymocytes cells will undergo deletion, that is, negative selection. This study revealed that both 3A9 x sHEL/*lpr* and 3A9 x sHEL/WT T cells undergo thymic deletion (166). This is in agreement with other studies confirming that Fas is not required for negative selection (50) (43).

5.3.2-CD95/CD95L apoptotic pathway and IL-2:

IL-2 can both promote cell proliferation and induce cell death. The first report illustrating this possibility suggested that the administration of IL-2 following antigen stimulation resulted in a proliferative effect, whereas IL-2 before antigen stimulation led to cell death (3). Other studies have shown that IL-2$^{-/-}$ and IL-2R$\alpha^{-/-}$ mice failed to

undergo AICD (167) (52). The latter models emphasised the importance of IL-2 in the regulation of immune responses by illustrating that normal expression of Fas and Fas L in IL-$2^{-/-}$ and IL-$2R\alpha^{-/-}$ mice does not lead to AICD in the absence of IL-2. Therefore, the autoimmunity in IL-$2^{-/-}$ and IL-$2R\alpha^{-/-}$ mice can be regarded as a cumulative effect of activated or autoreactive T cells, which under normal conditions are deleted via the Fas/Fas L pathway. However, a defect in the Fas/Fas L pathway can only partly explain the phenotype of the IL-2 deficient mouse. This is because Fas/Fas L and IL-2 deficient mice develop different forms of autoimmune disease, lymphoadenopathy and nephritis in Fas/Fas L deficient mice, whereas IL-2 KO mice develop haemolytic anaemia and IBD. An important point arising from these findings is whether IL-2 regulates activated $CD4^+$ T lymphocytes directly or through a regulatory T cell subset.

5.3.3-CTLA-4 and AICD:

Initial studies to investigate the role of CTLA-4 revealed a costimulatory function. Addition of CTLA-4 antibodies to activated human T cell cultures resulted in enhanced proliferation and led to a postulated costimulatory function (168). However, subsequent studies using soluble forms of CTLA-4 and CTLA-4 mAb that were cross-linked resulted in inhibition of immune responses (169) (170) (124).

Finally, the generation of CTLA-$4^{-/-}$ mice emphasised the pivotal role of this molecule in peripheral tolerance. Mice which are deficient for CTLA-4 have uncontrolled lymphoproliferative disorders and die by 3-4 weeks of age (171) (172). Several lymphoproliferative disorders have recently been associated with the dysregulation of apoptosis in activated T lymphocytes (173). In support of this is a study by Gribben et al. which suggested that CTLA-4 promotes its immunosuppressive function through mediating T cell apoptosis (174). However, other studies have not been able to reproduce this effect and have demonstrated that CTLA-4 mediates cell cycle arrest and inhibition of IL-2 production rather than induction of apoptosis (175) (125). Therefore, CTLA-4-induced apoptosis can be a by-product of depriving an IL-2

dependent clone of IL-2. Moreover, another group has reported that CTLA-4-induced apoptosis in activated T cells is independent of Fas. Cross-linking of CTLA-4 on activated murine $CD4^+$ T cells resulted in apoptosis in WT and Fas deficient *lpr/lpr* mice (176).

Collectively, these studies point to CTLA-4 mediating different functions depending on the activation status of the T cell. Moreover, the last study points to a Fas-independent CTLA-4 apoptotic pathway. Despite the complexity with regard to the molecular mechanisms by which CTLA-4 mediates its immunosuppressive effects, it is clear that this molecule is critical for the maintenance of peripheral tolerance.

6-Costimulatory molecules

B7/CD28/CTLA-4 are the best characterised costimulatory molecules to date. CD28 is the primary costimulatory receptor for inducing survival of naïve $CD4^+$ T lymphocytes and the production of high levels of IL-2 (177). CD28 also functions to prevent T cell death or anergy that may occur as a result of TCR engagement alone. Other costimulatory molecules include the LFA-1/ICAM-1 integrin family (178) (179) (180), ICOS (181), OX-40/OX-40 L (182) (183) and the CD27/ CD70L TNF families (184).

6.1-The B7-1 (CD80) and B7-2 (CD86) costimulatory molecules:

B7-1 was first described by Yokochi et al. as a B cell activation molecule (185) and subsequently identified to be a ligand for CD28 (186) (187). Despite initial studies describing the critical role of B7-1 in T cell proliferation and IL-2 production, the use of B7-1 mAb to inhibit T cell proliferation proved inefficient, whereas CTLA-4Ig was extremely efficient (188). Moreover, B7-$1^{-/-}$ mice were able to generate immune responses and APCs isolated from these animals could be stained with labelled hCTLA-4Ig (189). This pointed to the existence of an alternative ligand for CTLA-4 and subsequently to the cloning of B7-2 (190).

B7-1 and B7-2 expression is differentially regulated on APCs. For example, B7-1 is not expressed on resting cells and is up-regulated only after 3-4 days following activation, whereas B7-2 is constitutively expressed and is up-regulated within hours of activation on macrophages, dendritic and B cells (189). Similarly, the affinities of B7-1 and B7-2 and their interactions with CD28 and CTLA-4 differ greatly. B7-2 binds to CD28 with the lowest affinity, whereas B7-1 binds to CTLA-4 with the highest affinity. Linsley et al demonstrated that CTLA-4Ig binds to B7-1 with a 20-50 higher affinity than CD28Ig (191). These findings led to the speculation that B7-2 may be involved in the initiation of immune responses, whereas B7-1 is more involved in sustaining and perhaps terminating immune responses. This remains to be validated.

To date, there have been no clear functional differences demonstrated between B7-1 and B7-2 (192) (193).

Recently, new members of the B7 family have been discovered. These include B7h (194), B7RP-1 (195) and others. The identification of these novel B7 or B7 related molecules within the last 2 years has raised more questions than answers. This is mainly because they provide additional complexity to our present understanding of the role of costimulatory molecules in immune responses.

6.2-The CD28 and CTLA-4 cell surface molecules:

CD28 emerged as a prominent player in immune responses when several groups discovered the ability of CD28 to reverse antigen-specific unresponsiveness in TH_1 $CD4^+$ T cell clones (196) (197). CD28 is constitutively expressed on 100% of murine T cells (reviewed in (198)); however, its expression is up-regulated following activation (199). On the other hand, the expression pattern of CTLA-4 is more puzzling. Interestingly, there is discordance between CTLA-4 mRNA transcription and cell surface expression. Indeed, in both mouse and humans, cell surface expression of CTLA-4 is up-regulated after 48 hrs of activation (168) (170). Furthermore, the expression of CTLA-4 appears to be dependent on CD28 signals. Activated $CD28^{-/-}$ murine T cells were incapable of up-regulating CTLA-4 unless exogenous IL-2 was added. Finally and most importantly is the difference in roles. In contrast with CD28, CTLA-4 has been shown to deliver negative signals to activated T cells (170). The CTLA-4 null mouse model best exemplifies CTLA-4's regulatory role. As mentioned previously, $CTLA\text{-}4^{-/-}$ mice have significant lymphoproliferative disease and die by 4 weeks of age (172).

6.3-Regulation of T cell responses by CD28 and CTLA-4:

Direct evidence for the negative regulatory role of CTLA-4 came from CTLA-4 null mice. These mice develop a spontaneous autoimmune disease with lymphoproliferation and massive lymphoadenopathy, splenomegaly, myocarditis and

pancreatitis, and die by 4 weeks of age. This emphasised the critical role of CTLA-4 in downregulating immune responses. The exact mechanism(s) by which CTLA-4 functions is yet to be deciphered. There are reports describing the induction of apoptosis on human T cells by crosslinking CTLA-4 (174), and the blocking of proliferative responses by cell cycle arrest (175) by inhibiting the production of cyclin D3, cyclin-dependent kinases (cdk) 4 and 6 (200).

With regard to CD28, following the TCR signal, ligation of CD28 results in up-regulation of IL-2Rα, β and γ chains (201), increased IL-2 mRNA transcription (202), up-regulation of CTLA-4 mRNA (203) and CD40 ligand (204). CD28 signalling also results in the up-regulation of survival genes such as bcl-x_L (205). In this context, B7/CD28 signals are clearly critical in the activation of immune responses. Moreover, this activation is largely dependent on the production of several cytokines, mainly IL-2, and survival genes. The IL-2 null mouse is a model of spontaneous autoimmunity that provides a vehicle to investigate secondary pathway(s) responsible for *in vivo* cellular proliferation and homeostasis in the absence of IL-2.

6.4-B7: CD28/CTLA-4 molecules in autoimmunity:

The expression of B7 molecules has been studied in various autoimmune models, some of which are systemic lupus erythematosus (SLE) and experimentally induced allergic encephalomyelitis (EAE).

SLE is a multi-organ autoimmune disease that occurs spontaneously in various inbred or cross-bred murine strains. The best characterised is the NZB/NZW F1 model. Cross-breeding New Zealand black mice with New Zealand white mice results in an F1 generation that is predisposed to SLE, with full clinical manifestation of disease. This includes anti-DNA antibodies in the serum and chronic glomerulonephritis, a result of immunoglobulin desposition in the kidney.

Importantly, treatment of NZB/W F1 mice with CTLA-4Ig, which blocks both B7-1 and B7-2, results in complete abrogation of the disease. Indeed, there was no production of anti-DNA antibodies and disease was abrogated at advanced stages as well (206). This study was pivotal in illustrating the potential therapeutic benefits in manipulating costimulatory molecules and provided rationale for using CTLA-4Ig, anti-B7-1 or anti-B7-2 for disease prevention.

Experimentally induced murine allergic encephalomyelitis can be induced in susceptible strains of mice, such as SJL, by injection of the dominant encephalitogenic epitope of myelin proteolipid protein. Indeed, anti-B7-1 mAb prevented disease and epitope spreading (207).

Thus far, the exact role of B7 molecules in several autoimmune models is not known. Inflammatory bowel disease has been described in several genetically manipulated murine models, such as IL-2 null mice. Furthermore, a study by Razi-Wolf et al. has described that IL-$2^{-/-}$ $CD4^{+}$ T cells are responsive to costimulatory signals (208). This suggests the therapeutic potential of B7 antagonists in the prevention or treatment of inflammatory bowel disease.

6.5-Aims:

Previous studies by Razi-Wolf et al. have shown that the activation of IL-$2^{-/-}$ $CD4^+$ T cells, like their IL-$2^{+/+}$ counterparts, requires stimulation through both the TCR and CD28 *in vitro* (208). Moreover, given that IL-$2^{-/-}$ $CD4^+$ T lymphocytes are responsible for disease pathogenesis *in vivo*, their activation requirements are of profound interest (65). The results presented in this thesis describe investigations carried out in an attempt to further our understanding of the role of B7 molecules in the activation of IL-$2^{-/-}$ $CD4^+$ T lymphocytes *in vitro* and give insight into secondary growth mechanism(s) that are possibly masked by the prominent IL-2 pathway. More specifically, whether there are distinct mechanisms by which IL-$2^{-/-}$ $CD4^+$ T lymphocytes are activated in comparison to IL-$2^{+/+}$ $CD4^+$ T lymphocytes. This involved the study of differential expression of cytokine or other mRNA species in activated IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes, using RNase protection assays and cDNA gene expression arrays techniques. The role of IL-2 in endogenous SAg deletion was investigated to give insight into the IL-$2^{-/-}$ Vβ repertoire. Finally, the role of B7/CD28 costimulatory pathway in the pathogenesis of inflammatory bowel disease was investigated *in vivo* using CTLA-4Ig.

CHAPTER 2

MATERIALS AND METHODS

2.1 Mice

IL-$2^{+/-}$ mice on a C57BL/6 background were provided by Dr. David Gray (purchased from the Jackson laboratories, USA), transgenic IL-$2^{+/-}$ DO11-10 TCR Tg on a BALB/c background were provided by Professor Hans Reiser, and NOD mice were provided by Dr. Julian Dyson. All mice were bred and maintained at the Biological Services Unit, Hammersmith Hospital, Du Cane Road, London. All mice were cared for within the guidelines of the Biological Services Unit. 6-8 week old mice were used in all experiments, unless otherwise stated in the text.

2.2 Isolation of genomic DNA from tail/ear

The tip of the tail was removed under ethyl chloride anaesthesia. Each sample was incubated in 550µl tail buffer (100mM Tris pH 8.0, 5mM EDTA, 0.5% SDS, 100mM NaCl, proteinase K 1mg/ml) at 56˚C for 12 hours. 50µl of saturated NaCl was added, followed by 600µl of phenol (Gibco Ultra Pure Buffer Saturated phenol). Samples were mixed and centrifuged for 6 min.

The upper aqueous phased was subsequently transferred to a fresh 1.5 eppendorf tube and 600µl of phenol: chloroform: isoamyl alcohol 25:24:1 (Gibco Ultra Pure) was added, mixed and centrifuged for 6 min. Again, the upper aqueous phase was transferred to a new tube, ensuring that no material from the interface was removed. Subsequently, 600µl of isopropanol was added, mixed by inverting the tube several times and incubated at room temperature for 20 min.

Following incubation, the supernatant was removed and DNA precipitates were washed with 80% ethanol. Ethanol was discarded and DNA pellets were air-dried for 2-4 min on the bench. Finally DNA was dissolved in TE buffer (pH 8.0).

At a later stage in the project, a different technique was employed. Ear snaps were incubated in 20µl of tail buffer and 1mg/ml proteinase K. Samples were incubated for 30 min at 56˚C. Following incubation, samples were mixed and 180µl of distilled water was added to each sample. This mixture was boiled at 100˚C for 5 min. The resultant "ear juice" mix was used in PCR analysis.

2.3 Genotyping of mice by PCR

Tail/ear snaps of litters were collected and digested for DNA extraction. Mice were typed by amplification of the IL-2 gene by polymerase chain reaction (PCR) at 4 weeks of age. PCR products were electrophoresed in 1.5-% agarose gels in 1x Tris-acetate-EDTA buffer (TAE). Bands of 324 base pair (bp) and 500 bp represent the WT and KO alleles respectively. IL-$2^{+/-}$ DO11-10 mice were genotyped for both IL-2 and the transgene (which produced a band of 300 bp).

- ***IL-2 PCR protocol***

1 x 94˚C for 5 min, 35 x 94˚C for 1 min, 35 x 65˚C for 1 min, 35 x 72˚C for 1 min and 1 x 72˚C for 10 min. Reaction mixtures (100µl) contained 1µg of each primer, 2 µl of 20mM dNTP (Pharamcia Biotech), 10 µl of Buffer (Roche Diagnostics), 0.5µl of 5U/µl Taq DNA polymerase (Roche Diagnostics), 1µl of template DNA.

IL-2 Primer sequences:

001: 5'-TCG AAT TCG CCA ATG ACA AGA CGC T-3'

002: 5'-CTA GGC CAC AGA ATT GAA AGA TCT-3'

003: 5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3'

- ***DO11-10 PCR protocol***

1 x 94˚C for 5 min, 30 x 94˚C for 1 min, 30 x 55˚C for 1 min, 30 x 72˚C for 1 min and 1 x 72˚C for 10 min. Reaction mixtures (50µl) contained 0.5-1µg of primers, 0.5µl of 20mM dNTPS (Pharamcia Biotech), 0.5µl of 5U/µl Taq polymerase (Roche Diagnostics), 5µl of buffer (Roche Diagnostics) and 1µl of DNA.

DO11-10 primer sequence:

001: 5'-CAG GAG GGA TCC AGT GCC AGC-3'

002: 5'-TGG CTC TAC AGT GAG TTT GGT-3'

2.4 Media and buffers

Tris NH_4Cl: 0.83% NH_4Cl was prepared. Tris-HCl (pH7.65) was prepared. To prepare Tris NH_4Cl, 90ml of 0.83% NH_4Cl was added to 10ml of Tris-HCl, mixed and filtered.

2% paraformaldehyde: 10g of paraformaldehyde (Sigma) were added to 400ml H_2O. pH was adjusted to 10. When paraformaldehyde was dissolved, 50ml of 10x PBS were added and pH was adjusted to 7.2. Volume was adjusted to 500ml with H_2O and the solution was sterile filtered.

Media for cell lines: 250 ml of DME high glucose (Gibco/BRL) were added to 250 ml of 1 x F12 Nutrient Mixture-HAM (Gibco/BRL) and supplemented with 10% FCS (Harlan), 4mM L-glutamine (Gibco/BRL), 100U/ml penicillin, 100µg/ml streptomycin, 250ng/ml amphotericin B (Gibco/BRL), and 10mM Hepes (Gibco/BRL). 400µg/ml Geneticin (Gibco/BRL) was used to select for CHO B7-1 transfectant cells and 400µg/ml of Geneticin and Hygromycin (Boeringher Mannheim) were used to select for CHO I-A^d B7-1 transfectant cells.

Media for hybridomas: RPMI 1640 (Gibco/BRL) was supplemented with 5-10% FCS (Harlan), 4mM L-glutamine (Gibco/BRL), 100U/ml penicillin,100µg/ml streptomycin, 250ng/ml amphotericin B (Gibco/BRL).

2.5 Cell lines and antibodies

All antibodies were purchased from PharMingen, unless stated otherwise in the text. PE or FITC-conjugated anti-mouse CD4 mAb (clone GK1.5), FITC-conjugated anti-mouse CD8 mAb (clone 53.6.7), FITC-conjugated anti CD3 (clone 145-2C11), FITC-conjugated anti-mouse CD45R/B220 mAb (clone RA3-6B2), Biotin or FITC-conjugated anti-mouse CD11b/MAC-1 mAb (clone M1/70), PE-conjugated anti-mouse mAb CD11c (clone HL3), FITC-conjugated/unconjugated anti-mouse Fas mAb (clone Jo2), FITC-conjugated anti-mouse B7-1 (clone16-10A1), PE or FITC-conjugated Vβ3 TCR (clone JK25), Vβ4, (clone KT4) Vβ5.1/2 (clone MR9-4), Vβ6 (clone RR4-7), Vβ8.1/2 (clone MR5-2), Vβ8.3 (clone 1B3.3), Vβ10 (clone B21.5), Vβ12(clone MR11-1), Vβ13 (clone MR12-3) and Vβ14(clone 14-2).

The anti-H2-$A^{b,d,q}$, anti H2-$E^{d,k}$ hybridoma M5/114 (209), the anti-CD8.2 hybridoma ADH4 (210) and the anti-CD3 (clone 145-2C11) were obtained from the American Type Culture Collection. The CHO ø and CHO B7-1 have been described previously (201). CHO H2-A^d and CHO H2-A^d cells were also described previously (211).

2.6 Flow cytometry

Spleens or mesenteric lymph nodes were harvested and a single cell suspension was treated with 2.4G2 (anti-FcγII/II receptor) hybridoma supernatant for 30 min on ice to minimise non-specific binding. Spleen suspensions were washed three times with phosphate buffered saline (PBS), 1% bovine serum albumin (BSA) and centrifuged at 1200 rpm for 7 min. 1-3x10^6 cells were stained per sample. Spleens were stained for a selection of markers, some of which are indicated in chapter 3. Antibody solutions were made up as appropriate from the listed antibodies above, diluted in PBS/azide. Splenocytes were incubated with conjugated antibodies in the dark for 30 min on ice. Cells were washed three times and secondary antibodies, where required, were applied for another 30 min. After the final antibody incubation, cells were washed three times, fixed in 1% paraformaldehyde, and analysed on a FACScan (Becton Dickinson, CA, USA). The data were then analysed with CELLQuest Software (Becton Dickinson,

CA, USA). 50-80 thousand cells were analysed per sample. CHO transfectants were analysed for surface expression of B7-1 and H2-A^d molecules periodically to maintain expression levels.

2.7 $CD4^+$ T cell purification

$CD4^+$ splenocytes from BALB/c or C57BL/6 were purified as described previously (212). Briefly, mice were killed by CO_2 narcosis and cervical dislocation. Spleens were harvested and resuspended. Splenocytes were treated with Tris-NH_4Cl for 5 min at 37°C to lyse red blood cells. T cells were washed with complete culture medium (CCM). CCM consisted of RPMI 1640 medium (Gibco/BRL), 10 mM Hepes (Gibco/BRL), 4 mM L-glutamine (Gibco/BRL), non-essential amino acids (Sigma), 100U/ml penicillin G/100μg/ml streptomycin sulphate/250ng/ml amphotericin B (Gibco/BRL), 10%FCS and 50μM 2-mercaptoethanol (Sigma). Splenocytes were then incubated in a warm nylon wool column for 30 min to enrich for T cells. Columns were washed with medium and the collected cell suspension was incubated with mAb M5-114 (anti-class II) and ADH4 (anti-CD8.2) for 30 min on ice. Cells were washed and incubated with a suspension of goat anti-mouse Fc-coupled magnetic beads (PerSeptive Biosystems) for another 30 min on ice. The beads were removed from the suspension by a magnet and washed. Another round of supernatant treatment and bead incubation was applied. This procedure typically resulted in 92-95% pure $CD4^+$ T cells.

2.8 Proliferation assays

200μl microcultures were set up in round 96 well plates in triplicates with $4x10^6$cells/ml $CD4^+$ T cells, $4x10^5$cells/ml 1% paraformaldehyde-fixed CHO B7-1/CHO ø, anti-CD3 supernatant at varying concentrations and, where indicated, culture media supplemented with one or more of the following additives: anti-IL-2 ascites (S4B6), anti-IL-4 mAb (11B11), anti-IL-10 mAb (R and D systems), anti-IL-15 mAb (R and D), sIL-15Rα (kindly provided by Prof. Eddy Liew, Glasgow) and recombinant IL-2, IL-4, IL-10 and IL-15 cytokines (R and D). Cultures were incubated for 72 hrs and pulsed with 1μCi of [^{3}H] thymidine per well for the last 12

hrs. All CHO cells were fixed with 1% paraformaldehyde before addition to the culture. For paraformaldehyde treatment, CHO cells were washed in PBS three times and resuspended in 2ml of 1% paraformaldehyde for 20 min at RT. CHO cells were then washed with PBS twice and in CCM once. The sequence of addition is as follows: CHO transfectants, $CD4^+$ T cells, soluble anti-CD3, and where indicated, an anti-cytokine mAb or recombinant cyokines. For Elisa assays, supernatants were harvested at 60 hrs before pulsing.

2.9 ELISA

Whole spleen suspensions or purified $CD4^+$ T cell were stimulated using either the CHO B7-1/anti-CD3 or the PMA plus ionomycin activation systems. Supernatants were collected at the indicated time-points. Elisa assays were used according to manufacturer's instructions (R and D). IL-4, IL-10 and IFN-γ were detected by the relevant specific ELISA, measuring against linear standard curves using IL-4, IL-10 and IFN-γ specific kits from R & D systems, Abingdon, UK.

2.10 RNA isolation

Two methods were utilised for RNA isolation, depending on the ensuing experimental technique. For the purpose of RNAse protection assays, supernatant was harvested from culture wells and 400µl of TRIzol Reagent (Gibco/BRL) was added to each well. Using RNA only pipetman, the TRIzol and cell mix was transferred to diethylpyrocarbonate (DEPC)-treated (Sigma) and autoclaved microcentrifuge tubes. Samples were incubated at room temperature for 5 min. 100µl of chloroform was added, the tubes were mixed vigorously for 10 min and microcentrifuged at 12000g for 15 min at 4°C.

The upper aqueous layer was removed from the sample and 200µl of isopropanol was added to each sample. This mix was incubated at room temperature for 10 min and microcentrifuged for 12 min at 12000g at 4°C. Subsequently, the isopropanol was removed using RNase free aerosol tips and 400µl of ice cold 75% ethanol was added to each sample. Each sample was incubated for 12 hrs at –20°C. When the ethanol was

removed, the tubes were air-dried for 5 min and the RNA pellets were resuspended in 10µl DEPC water.

For Clontech cDNA gene arrays, total RNA was isolated according to the manufacturer's instructions using the spin protocol of the SV Total RNA isolation system (Promega, Southampton, UK). The yield and purity of RNA was determined by measuring the OD_{260} to OD_{280} ratio (Pharmacia Biotech Ultraspec 1000). In addition, the RiboGreen® RNA quantitation kit was used (Molecular Probes, Cambridge Bioscience, UK).

2.11 RNase protection assays (RPA)

Following isolation of RNA from activated splenocytes or purified $CD4^+$ T lymphocytes (indicated in text), the RiboQuant® mCK-1 probe was synthesised and hybridised to RNA samples according to manufacturer's instructions (PharMingen, BD, UK). Following RNase treatment to remove free probe and other single stranded RNA, the remaining "RNase-protected" probes are purified and resolved on denaturing polyacrylamide gels. The undigested probe was used as a marker and migration distance versus log nucleotide length were plotted as a standard curve on semi log graph paper. This curve was used to establish the identity of "RNase-protected" bands in the experimental samples [Multi-Probe RNase Protection Assay System, instruction manual, 5th edition, 1998]. The Novex™ QuickPoint rapid nucleic acid separation system was used for resolving the protected probes. This system comprises pre-Cast gels, Quickpoint buffers and an electrophoresis cell. RPA samples were electrophoresed according to manufacturer's instructions (NOVEX, UK).

2.12 Production of cDNA/TH_1: TH_2 PCR

cDNA was produced using the BRL Superscript Reverse transcriptase kit (InVitrogen, UK). cDNA was "cleaned" from the reverse transcriptase mix, which can interfere with the multiplex TH_1: TH_2 PCR. To clean cDNA, 1µl of 0.5M EDTA, pH8.0 was added to each sample, followed by 20µl of phenol: chloroform mix (Gibco/BRL). This mixture was microcentrifuged for 10 min and the upper aqueous layer was transferred to a fresh tube. An equal amount of 4M ammonium acetate and twice the volume of

ice cold 100% ethanol were added. This mixture was incubated at –20°C for 30 min and subsequently microcentrifuged for 15 min at 4°C. The supernatant was removed and the cDNA pellet was resuspended in 10-20µl distilled water.

Following the isolation of cDNA, the mTH12-Multiplex PCR set 2 amplification kit was used according to manufacturer's instructions (Biosource International, Belgium).

2.13 Clontech cDNA gene array

Initially the Atlas^{TM} pure Total RNA isolation system was used (Clontech, UK). However, this system resulted in poor RNA yield. As a result, the Promega SV Total RNA isolation system was used as described in section 2.10. Following total RNA isolation, poly A^+ RNA enrichment and cDNA probe synthesis was completed using the Atlas^{TM} pure RNA labelling system (Clontech, UK). Subsequently, labeled cDNA was purified from unincorporated ^{32}P using column chromatography as described in the Atlas^{TM} cDNA expression arrays user manual, Clontech, 1999. Finally, labelled probes are hybridised to the Atlas^{TM} mouse 1.2 II membrane arrays overnight. Following hybridisation, the membranes are washed stringently according to manufacturer's instructions [Atlas^{TM} cDNA expression arrays user manual, Clontech, 1999]. The hybridisation patterns are detected using x-ray film and phosphorimaging (PhosphorImager 445 SI, Molecular Dynamics). Finally, Phosphorimaging scans are analysed using the AtlasImage Software system (Clontech, UK).

2.14 Human CTLA-4Ig (hCTLA-4Ig) and hIgG1 treatment regimen

Thirty breeding pairs of heterozygous IL-2 mice were set up on the same day. The litters, comprising approximately one hundred mice, were genotyped at 3 weeks of age using PCR. hCTLA-4Ig was kindly provided by Dr. Neil Burden (GlaxoSmithKline). At four weeks of age, the first treatment was administered. Mice were injected twice weekly with 100µg/ml (100µl total volume) hCTLA-4Ig i.v. Two weeks after the initial treatment (mice were now 6 weeks old), group 1 (consisting of 3 IL-$2^{-/-}$ mice treated with hCTLA-4Ig, 3 IL-$2^{-/-}$ mice treated with hIgG1 and 3 IL-$2^{+/+}$ mice treated with IgG1) was culled to collect spleen and colon tissue for FACS analysis and histology. Group 2 consisting of the same genotypes as mice in group 1, continued to

receive hCTLA-4Ig or hIgG1. This group was due to be culled 2-3 weeks later (mice at 8-9 weeks old). However, as a result of disease development in IL-$2^{-/-}$ mice, they were culled by week 7 of age and spleen and colon tissues were collected.

2.15 Histology

The colon was excised from IL-$2^{-/-}$ and IL-$2^{+/+}$ mice. All fat and blood vessels were removed from the outside of the colon. The colon was then flushed with PBS to clean up the lumen. 5mm sections of the colon were cut and then opened up longitudinally. These sections were rolled up and snap-frozen. 4-6μm tissue sections were cut and air-dried for 10 min on glass slides, and fixed in acetone for 10 min before staining with hematoxylin and eosin (H & E). When the acetone evaporated, slides were immersed in hematoxylin for 40 sec and washed in water. The slides were quickly immersed in eosin and washed with acid-alcohol (10mM HCL in 62% EtOH in water). Subsequently, the slides were washed in increasing concentrations of EtOH, starting from 70%, 90% to 100% and finally histoclear (National Diagnostics). Cover slips with DPX mountant (BDH) were applied on to the slides.

CHAPTER 3

CHARACTERISATION OF IL-$2^{+/+}$ AND IL-$2^{-/-}$ $CD4^+$ T CELL Vβ REPERTOIRES AND SPLEEN COMPOSITION.

INTRODUCTION

The IL-2 deficient model:

When IL-2 was discovered, it was described as T cell growth factor (TCGF), indicative of its growth-promoting abilities on lymphocytes *in vitro*. Subsequently, when the IL-2 null mouse was made, it was expected to have an immunodeficient phenotype. Contrary to expectations, it was found to be a model of systemic autoimmunity. This underlined the role of IL-2 in mediating biological functions other than proliferation and growth promotion, which were unforeseen. It also emphasised the redundancy of the cytokine network *in vivo*, in that, in the absence of IL-2 other cytokines appeared to promote growth and proliferation of cells.

The prominent autoimmune manifestations in C57BL/6 IL-2 deficient mice are the development of inflammatory bowel disease (IBD) accompanied by hyperplasia of the spleen and lymph nodes and generalised amyloidosis (58) (60). Despite generalised autoimmune disease, the primary cause of death in C57BL/6 IL-2 deficient mice is IBD. IBD is a chronic inflammation limited to the large bowel (ulcerative colitis) or anywhere along the gastrointestinal tract (Crohn's disease). The current dogma with regard to the aetiology of this disease suggests that it is the result of an abnormal immune response to the presence of normal gut-residing enteric bacteria. In effect, a breakdown in the mucosal immune homeostasis which is dependent on the fragile balance between reacting to pathogens invading the gut and continuous bombardment of the mucosa with antigens from food proteins and enteric bacteria. This notion is further enforced by the absence of IBD in germ-free IL-$2^{-/-}$ mice.

This chapter will focus on the characterisation of the splenomegaly observed in IL-$2^{-/-}$ mice and analysis of cell populations accumulating in the spleen. Futhermore, the Vβ repertoire of IL-$2^{-/-}$ mice was examined in an attempt to investigate whether IL-$2^{-/-}$ mice have a skewed Vβ repertoire or possibly an oligoclonal expansion of a pathogenic T cell subset driven by gut-bacterial antigens. Finally, the role of IL-2 in mediating endogenous superantigen deletion has been examined.

Superantigens, Vß repertoire and immune disorders:

Superantigens (SAgs) are so named because of their ability to activate large numbers of T cells irrespective of their antigen specificity. SAgs are a class of immunostimulatory and disease-causing proteins of bacterial or viral origin (213) (214). Activation primarily involves the simultaneous interaction of the SAg with the variable region of the T cell receptor beta chain (Vβ) and MHC class II molecules on antigen presenting cells. The strong influence of SAgs on the immune system has led to speculation that they may be implicated in autoimmune disease, either by initiating immune disorders or inducing relapse in individuals harbouring autoreactive T cells. SAgs may possibly activate immune responses through activation of autoreactive T cells which subsequently migrate to tissue and mediate tissue damage and destruction. Alternatively, SAgs may activate autoreactive B cells (215) (216). Indeed, the T cells need not recognise a specific antigen to provide help for the B cells as the SAg will provide a bridge between the T and B cells (217). Finally, SAgs may activate antigen presenting cells such as macrophages; this in turn results in the release of cytokines and other proinflammatory mediators (218), possibly leading to modifications in antigen processing and culminating in presentation of autoantigens or cryptic epitopes to crossreactive and autoreactive T lymphocytes.

Among the viral superantigenic proteins, only the mouse mammary tumour virus (MMTV) has been thoroughly defined. MMTV is a milk-transmitted retrovirus that encodes a SAg in its 3' terminal repeat (reviewed in (219)). MMTV proviruses originate through the integration of infectious MMTV into the host germline cells. In contrast, infectious MMTV is milk-transmitted from mother to offspring. Upon infection with MMTV, B cells present SAg to T cells and a strong T:B interaction ensues. This leads to the preferential expansion of infected B cells and amplification of integrated virus by lymphocyte division and finally to infection of host mammary glands after puberty ensuring a complete viral life cycle. Most pertinent to the questions addressed in this chapter is the role of endogenous SAgs, originally named minor lymphocyte stimulating (Mls) antigens (220), in shaping the immune repertoire.

Indeed, because MMTV infection occurs in neonatal life and because the infection is persistent, there is a gradual deletion or functional silencing of SAg-reactive T cells (221). Most importantly, it is this clonal anergy and gradual deletion of SAg-reactive T cells that shapes the immune repertoire. The specificity of interactions of SAgs with the Vβ domain of the TCR has permitted the study of SAg-reactive T cells *in vivo*, independently of functional assays (222) (223) (224).

In this context, questions addressing the role of IL-2 in mediating clonal anergy and thymic/peripheral deletion of SAg-reactive T cells were investigated, using two models: the first involves the peripheral deletion of C57BL/6 $CD4^+V\beta5^+$ T cells. Since $CD4^+V\beta5^+$ T cells are chronically deleted in C57BL/6 mice (225), the deletion of $CD4^+V\beta5^+$ T cells was used as an indicator to investigate the effect of IL-2 absence in this class of peripheral deletion event. This deleting effect has been attributed to Mtv-8, Mtv-9 and Mtv-30 SAg (226). The second model involves thymic deletion of $V\beta3^+$ T cells in the non-obese diabetic mouse, which is a model for human autoimmune diabetes. Vβ3 thymic deletion was compared in (C57BL/6 $IL\text{-}2^{+/-}$ x NOD) F1 mice giving rise to NOD/$IL\text{-}2^{+/+}$ F1 and NOD/$IL\text{-}2^{+/-}$ F1 littermates. The offspring of the C57BL/6 $IL\text{-}2^{+/-}$ x NOD cross will inherit Mtv-3 from the NOD parent. Earlier studies revealed a profound deletion of $V\beta3^+$ T cells in NOD mice and linked it to Mtv-3 insertion in chromosome 11 (227). If IL-2 was critical to SAg-mediated thymic deletion, then the IL-2 deficit in $IL\text{-}2^{+/-}$ heterozygotes may lead to defective or partial deletion of Vβ3 $CD4^+$ T cells.

Finally, the Vβ repertoires of C57BL/6 $IL\text{-}2^{+/+}$ and $IL\text{-}2^{-/-}$ mice were compared to investigate the potential skewing of the repertoire. The rationale behind this is that the deficit in IL-2 may influence the repertoire at 2 levels:

- The deletion of endogenous SAg-reactive cells may be mediated by an IL-2 dependent mechanism. Since this form of deletion has been linked to peripheral tolerance, the autoimmunity in IL-2 null mice may be due to a deficit in deletion of enodgenous SAg autoreactive T cells.

- If a complex array of antigens is produced by gut bacteria, multiple Vβ families would expand and skewing would not be expected. However, if the antigenic complexity were highly restricted, perhaps involving SAgs, skewing of the Vβ repertoire might be expected. Similar to endogenous SAgs, IL-2-mediated deletion may be required for the exogenous SAg (produced by gut bacteria) driven T cells. The production of SAg by gut bacteria may lead to a skewed repertoire depending on the antigen(s) array produced.

Recently the role of SAgs in autoimmune disorders has been highlighted in animal models of experimental allergic encephalomyelitis and rheumatoid arthritis. There are several mechanisms by which SAgs may contribute to the breakdown of self-tolerance and the generation of immune responses against specific organ(s) and antigens(s). One mechanism involves the activation of autoreactive T lymphocytes by microbial antigens that cross-react with antigens on target organs. Other mechanisms include models where the SAg does not necessarily initiate the immune response but may modulate disease pathogenesis. For example, the presence of autoreactive T cells in susceptible individuals, although necessary, is not sufficient for the development of autoimmune disease. Development of autoimmune disease in these individuals requires the expansion of these autoreactive T lymphocytes and SAg activation would promote clonal expansion of such autoreactive cells. The collagen-induced arthritis mouse model exemplifies the mechanism discussed above. In this model, the *Mycoplasma arthritidis* SAg MAM appeared to exacerbate collagen-induced arthritis (CIA). Moreover, mice receiving suboptimal doses of collagen, with no clinical manifestations of disease, developed CIA when challenged with MAM (228).

Finally, with regard to the oligoclonal expansion of autoreactive T cell subsets, several studies have reported the expansion of specific T cell subpopulations in autoimmune models. For example, an analysis of the TCR β chain repertoire of synovial T cells from RA patients revealed preferential expansion of $V\beta14^+$ T lymphocytes when compared to peripheral blood T cells in the same individuals (229). Therefore, if the $CD4^+$ T cell expansion in IL-$2^{-/-}$ mice was driven by a small set of microbial products,

a skewed repertoire would be a possibility. Conversely, if the expansion was driven by a complex set of antigens, the overall repertoire would not be expected to be greatly skewed. It would also be interesting to look into whether IL-2 is required for SAg-driven Vβ repertoire deletions. If so, a skewed repertoire might be expected, thereby associating SAg with disease pathogenesis in IL-2 null mice. If IL-2 is not required for the deletion of SAg-reactive T cells, then SAg may not be implicated in disease pathogenesis.

RESULTS

3.2-Genotyping of IL-2$^{-/-}$, IL-2$^{+/-}$ and IL-2$^{+/+}$ mice:

IL-2$^{-/-}$ mice cannot be bred as homozygotes because IL-2$^{-/-}$ males have severe orchitis (invasion of the gonads by lymphocytic infiltrates) and females have abnormal ovarian development (personal communication, The Jackson Laboratory). This necessitated crossing heterozygotes and screening litters using PCR as described in experimental procedures. PCR products were electrophoresed in 1.5% agarose gels. Figure 3.1 depicts bands of 324bp representing IL-2$^{+/+}$ mice and 500bp represent IL-2$^{-/-}$ mice; both bands represent IL-2$^{+/-}$ mice. All mice were typed at 4weeks of age.

3.3-Splenomegaly in IL-2$^{-/-}$ mice:

As mentioned previously, the principal autoimmune manifestations in C57BL/6 IL-2$^{-/-}$ mice are the development of IBD, splenomegaly and lymphoadenopathy. Figure 3.2 illustrates the splenic hyperplasia of 12 week old IL-2$^{-/-}$ mice and IL-2$^{+/+}$ littermates. The typical cellularity of an IL-2$^{-/-}$ spleen ($25.9x10^7$) is approximately four times that of an IL-2$^{+/+}$ spleen ($6.7x10^7$). The splenic cellular composition of IL-2$^{+/+}$ and IL-2$^{-/-}$ mice was analysed by FACS as described in experimental procedures.

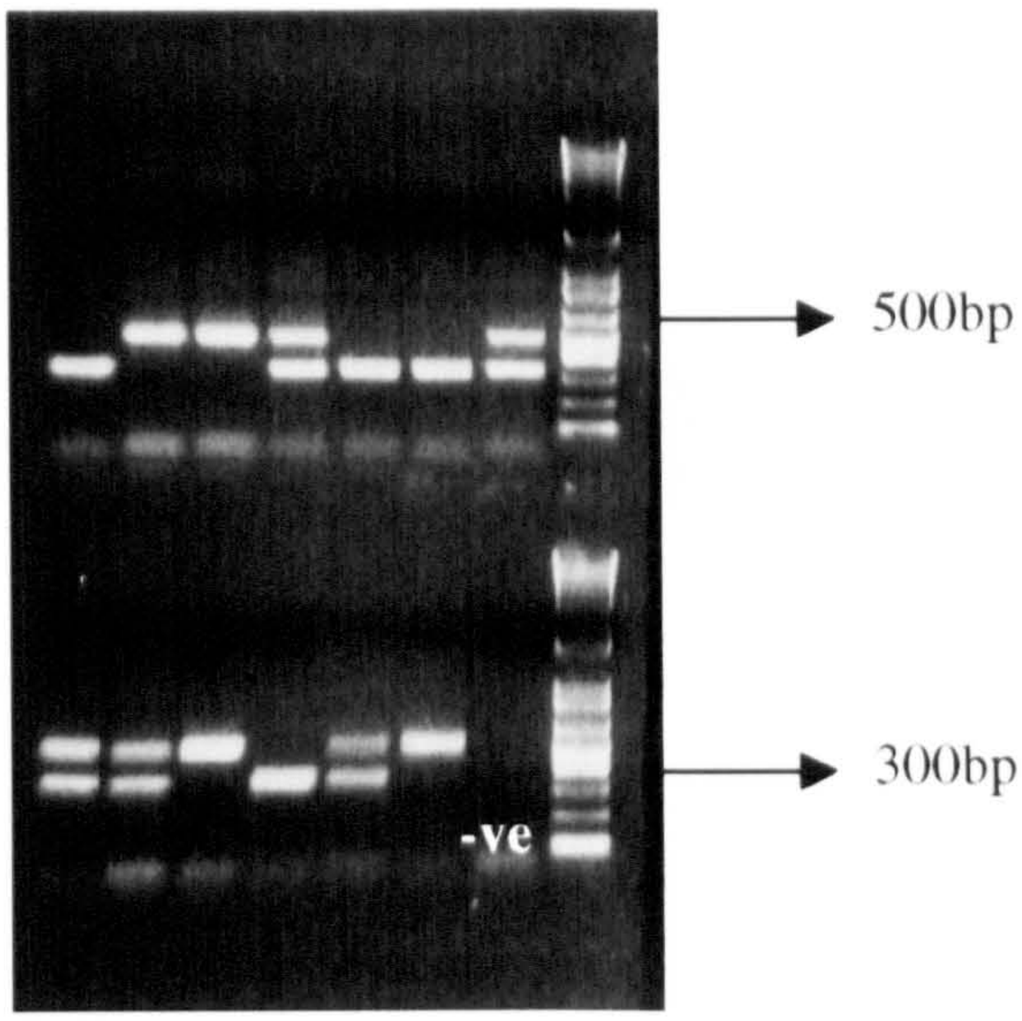


Figure 3.1 **Genotyping of IL-2 mice.**

IL-2 mice are bred as heterozyogtes and all litters are screened using PCR. IL-2 deficient mice cannot breed because KO males have severe orchitis (invasion of the gonads by lymphocytic infiltrates) and females have abnormal ovarian development. PCR products are electrophoresed in 1.5% gels, bands of 324 bp for IL-$2^{+/+}$ mice, 500 bp for homozygous IL-$2^{-/-}$ mice and both bands for heterozygote mice.



Figure 3.2 **Splenomegaly of IL-2 deficient mice.**

Demonstration of the difference in spleen sizes of 12 week old IL-2 deficient mice and their WT littermates. The typical cellularity of an IL-$2^{-/-}$ spleen ($26x10^7$) is approximately four times that of IL-$2^{+/+}$ spleens ($7x10^7$). The splenomegaly in IL-$2^{-/-}$ mice is a sign of an uncontrolled proliferative response.

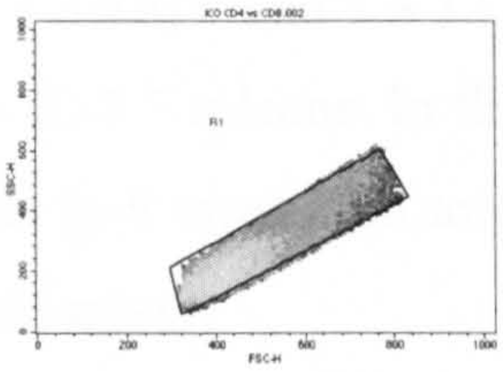
3.4-FACS analysis of IL-2$^{+/+}$ and IL-2$^{-/-}$ spleen cells:

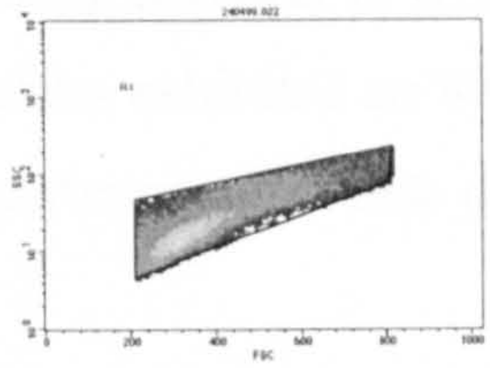
Figure 3.3.1A demonstrates the expansion of $CD4^+$ T cells in IL-$2^{-/-}$ spleens, a 33% increase in $CD4^+$ T lymphocytes when compared to IL-$2^{+/+}$ splenocytes (Fig. 3.3.1B). This increase can range from 10% to approximately 40% as depicted in figures 3.3.2A and B, which demonstrate the co-expression of CD3 and CD4 on T helper lymphocytes; the residual $CD3^+$ cell population representing $CD8^+$ T cells in IL-$2^{+/+}$ and IL-$2^{-/-}$ spleens. Interestingly, concomitant with the splenic hyperplasia of IL-$2^{-/-}$ animals was a substantial decrease in the percentage of B cells (Fig. 3.3.3A). IL-$2^{-/-}$ spleens exhibited around an 80% decrease in $B220^+$ cells, which is exacerbated as disease progresses. Occasionally, during the last stages of disease, IL-$2^{-/-}$ mice develop prolapse; figure 3.3.3.1A shows the extreme deficiency of splenic $B220^+$ cells in prolapsing animals (14 weeks old). Given the increased cellularity of the IL-$2^{-/-}$ spleens, the B cell compartment appears not to have changed in absolute size.

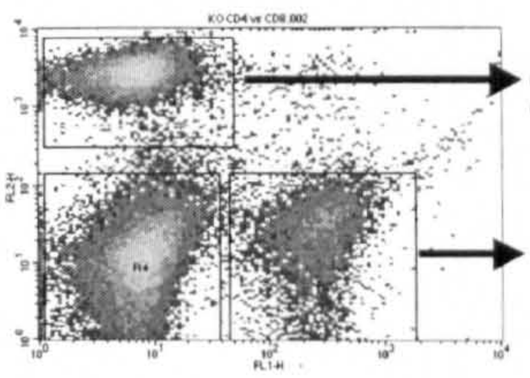
Figures 3.3.1A and B demonstrate the percentage change in $CD4^+$ and $CD8^+$ T lymphocytes in IL-$2^{-/-}$ spleens (6-8 weeks old). FACS analysis of IL-$2^{+/+}$ and IL-$2^{-/-}$ spleens *ex vivo* indicates that in this example, IL-$2^{-/-}$ $CD4^+$ T cells represent 28% of the splenic population, whereas IL-$2^{+/+}$ $CD4^+$ T cells represent 21% of the total splenic population. This represents a 33% increase in the $CD4^+$ T cell population.

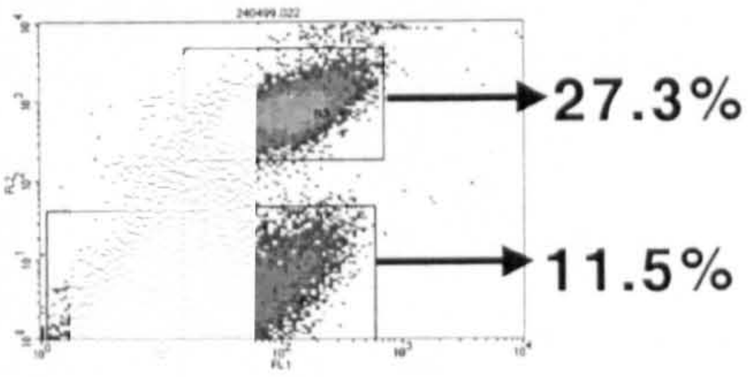
Figures 3.3.2A and B demonstrate the co-expression of CD3 and CD4 on T helper lymphocytes. The residual $CD3^+$ cell population represents $CD8^+$ T lymphocytes. In this example, there is a 10% increase in the $CD4^+$ T cell population in IL-$2^{-/-}$ spleens.

KO

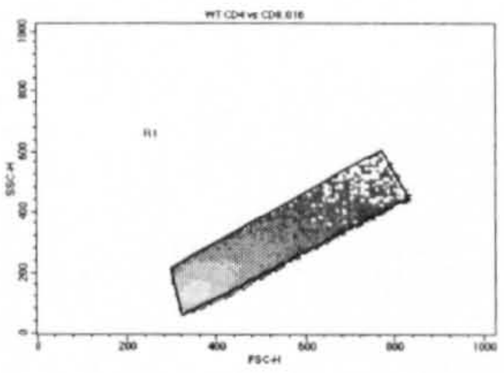


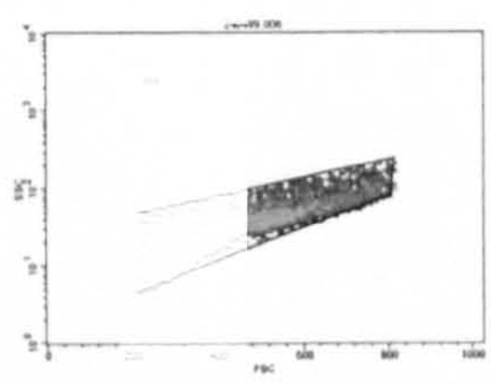


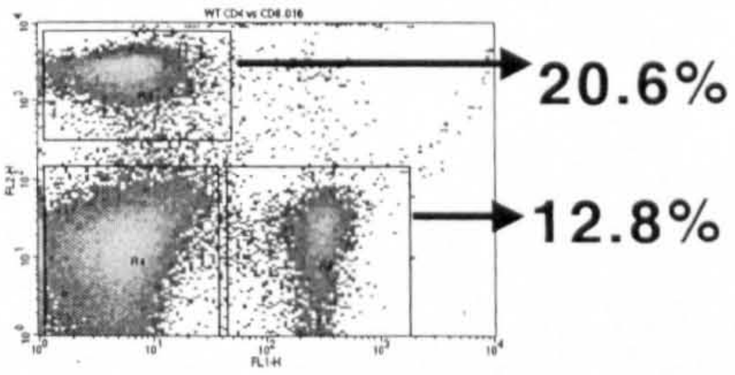


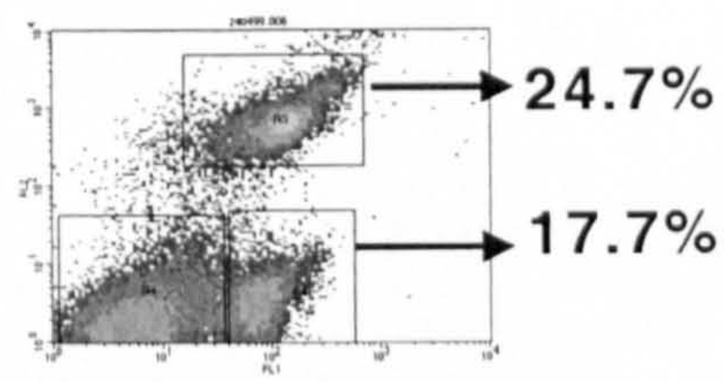


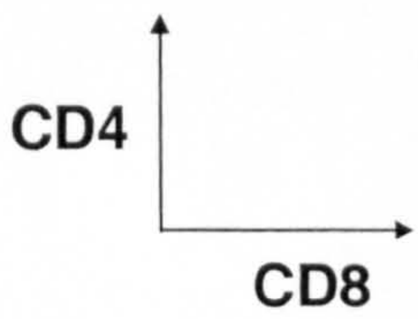
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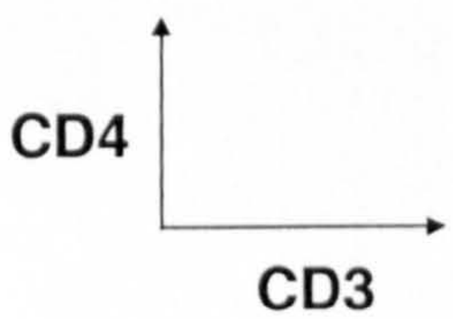






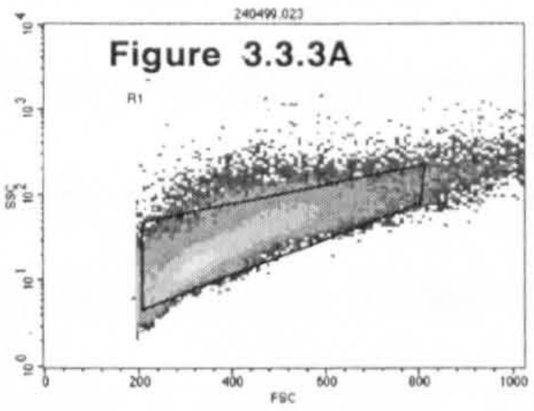


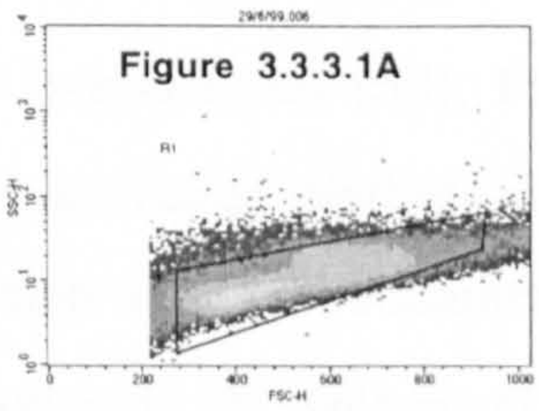


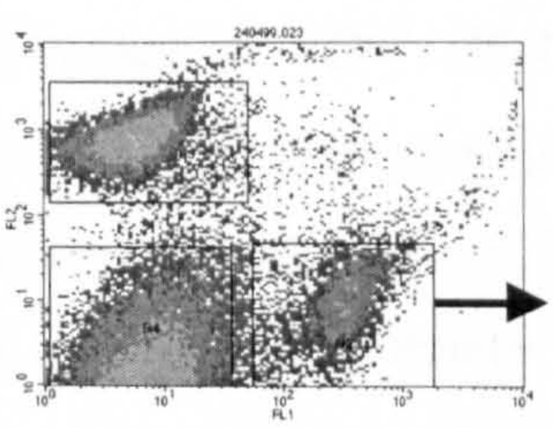


Figures 3.3.3A/B and 3.3.3.1A/B illustrate the percentage decrease in $B220^{+}$ cells (B cells) in IL-$2^{-/-}$ spleens. In this example, the IL-$2^{-/-}$ spleen exhibited an 80% decrease in B cells (6-8 weeks old mice), which is further exacerbated as disease develops (14 weeks old mice).

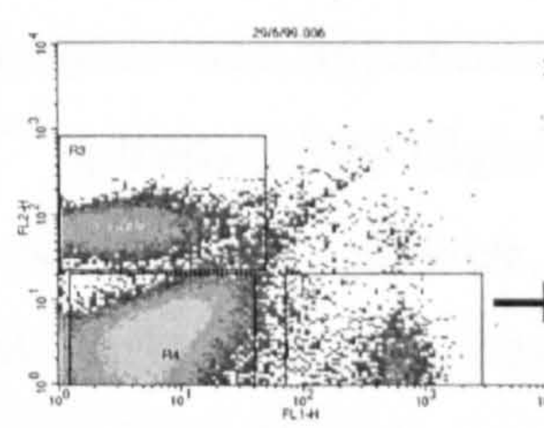
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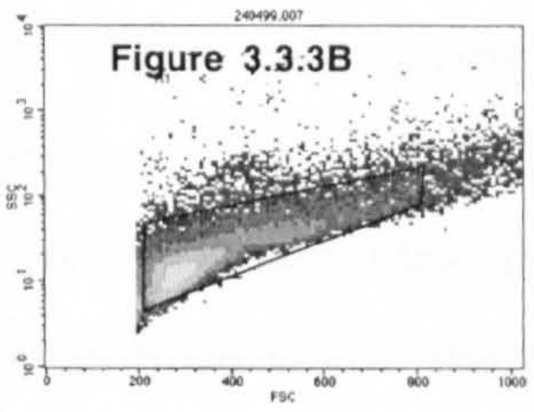


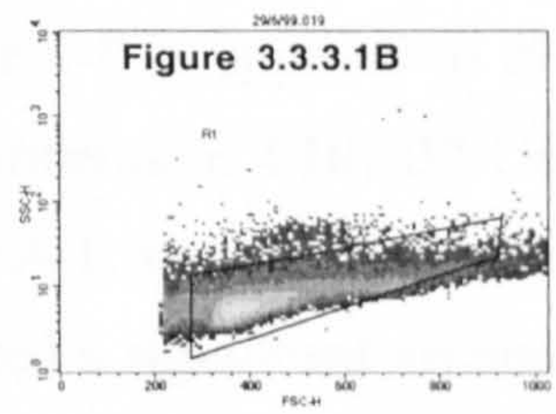
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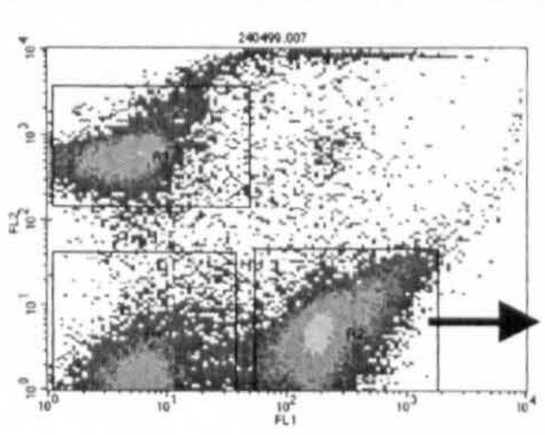


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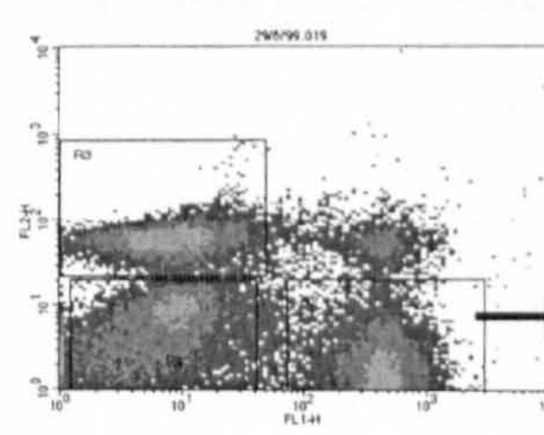
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48.4%



47.5%

CD4

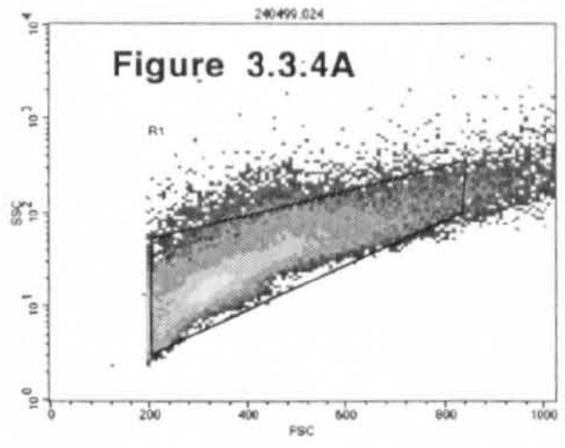
B220

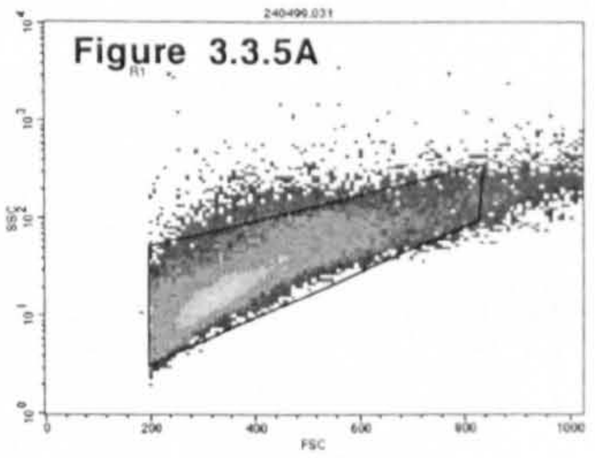
3.5-The expression of activation markers on IL-2$^{-/-}$ spleen cells:

Contrary to data from IL-2$^{-/-}$ colonic lymphocytes (58), FACS analyses of IL-2$^{-/-}$ spleens did not indicate an increase in activation markers such as CD25 and CD69 (data not shown). However, the expression of CD44, a marker of previously activated memory T cells (62), was up-regulated in IL-2$^{-/-}$ spleens (fig. 3.3.4A). Indeed, the percentage of CD4^{+} CD44^{+} T lymphocytes was approximately doubled in IL-2$^{-/-}$ spleens (60%) when compared to IL-2$^{+/+}$ spleens (33%) (figures 3.3.4A and B). These data are representative of five separate experiments. Likewise, activation markers such as LFA-1, a costimulatory molecule that is critical in the adhesion stage of lymphocyte activation, were significantly up-regulated in IL-2$^{-/-}$ spleens; in this example 85% of IL-2$^{-/-}$ CD4^{+} splenocytes expressed LFA-1 as opposed to 29% of the IL-2$^{+/+}$ CD4^{+} splenocytes (Figures 3.3.5A and B). Furthermore, CHO B7-1/anti-CD3-activated IL-2$^{-/-}$ CD4^{+} T lymphocytes up-regulated LFA-1, as confirmed in chapter 5 (Table 5.2). Spleens from IL-2$^{-/-}$ mice also exhibited a significant accumulation of macrophages (Mac-1^{+} cells) when compared to spleens from IL-2$^{+/+}$ littermates (figures 3.3.6A-3.3.6.3A and 3.3.6B-3.3.6.1B).

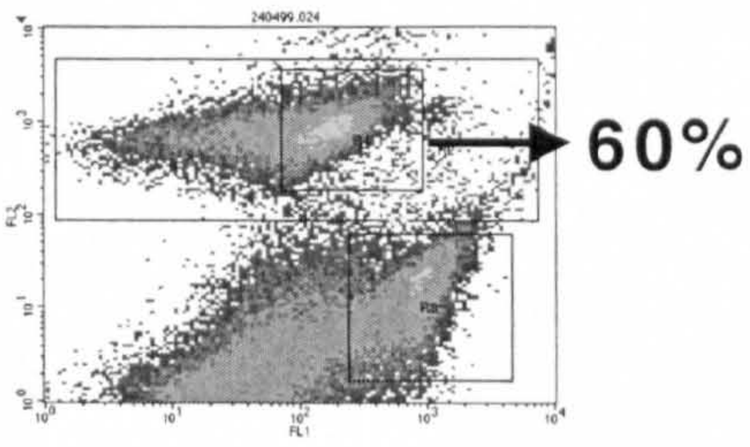
Figures 3.3.4A and B demonstrate the expression of the activation marker CD44 in IL-$2^{-/-}$ splenocytes. CD44 is a marker of previously activated T cells. In this example, the expression of CD44 by IL-$2^{-/-}$ $CD4^+$ T cells is approximately doubled when compared to IL-$2^{+/+}$ $CD4^+$ T cells. This finding concurs with the uncontrolled activation of T cells observed in other autoimmune models.

Figures 3.3.5A and B illustrate the up-regulation of the costimulatory molecule LFA-1 in IL-$2^{-/-}$ spleen cells. LFA-1 costimulatory molecules were significantly up-regulated in IL-$2^{-/-}$ splenocytes. 85% of IL-$2^{-/-}$ $CD4^+$ splenocytes expressed LFA-1, whereas 29% of IL-$2^{+/+}$ splenocytes expressed LFA-1. This represents a 193% increase in LFA-1 expression on IL-$2^{-/-}$ spleen cells.

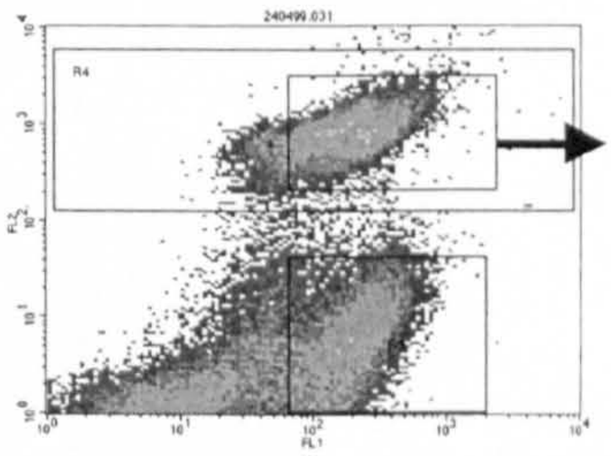




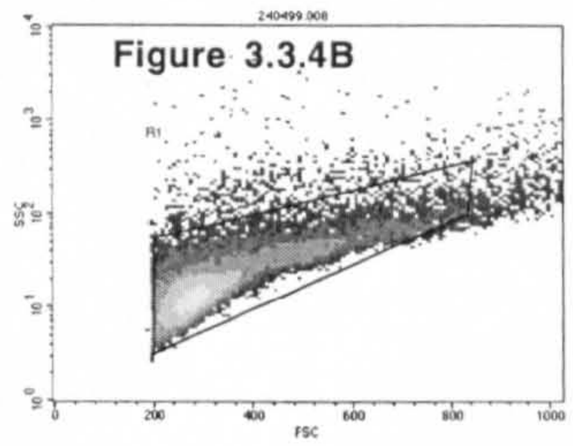
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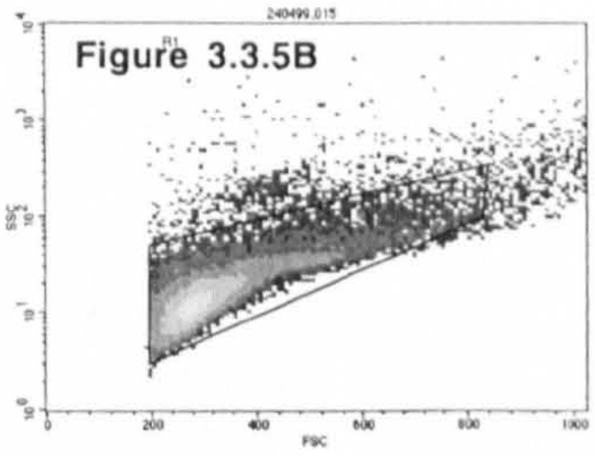


60%



85%

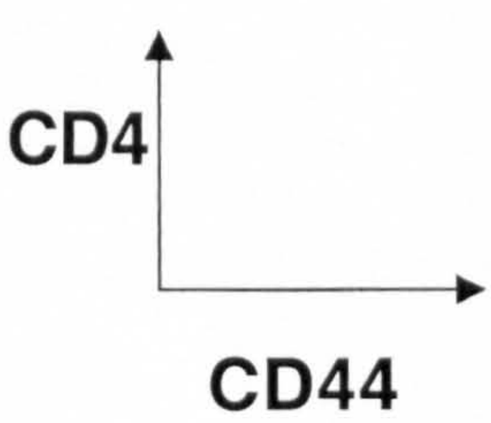


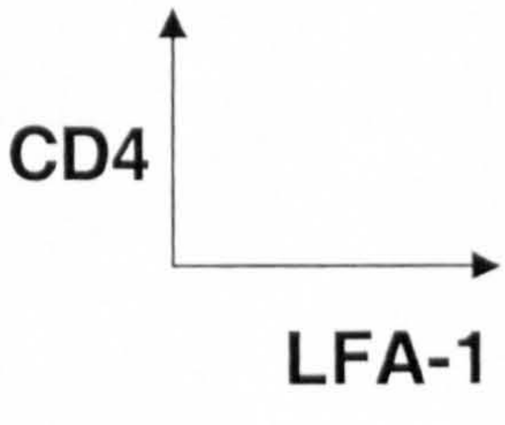


WT

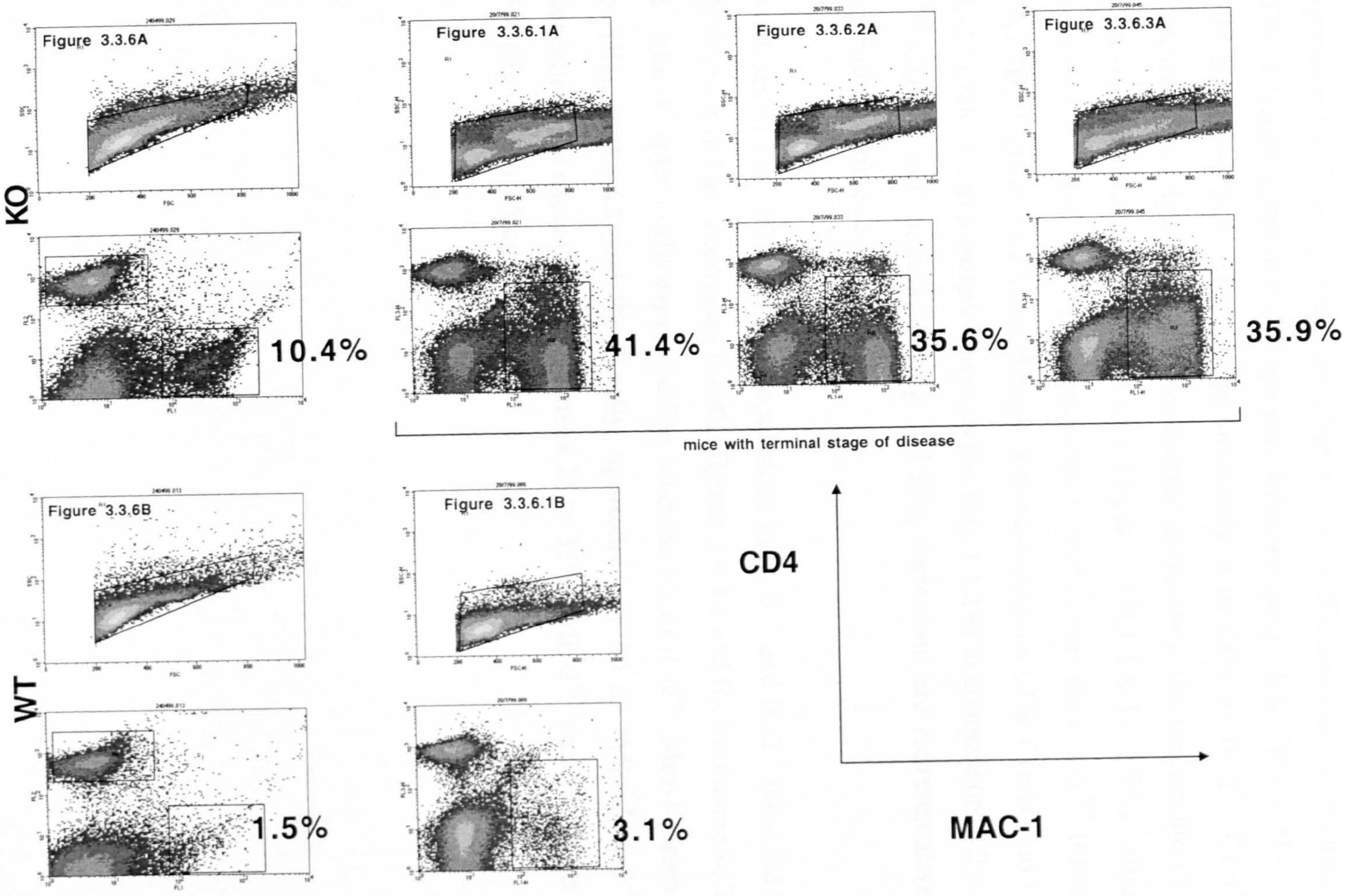
33%

29%





Figures 3.3.6A and B show the typical accumulation of macrophage cells (Mac-1^{+} cells) in IL-$2^{-/-}$ spleens. Mac-1^{+} cells in IL-$2^{-/-}$ spleens represent 10% of the total cellular composition, whereas in IL-$2^{+/+}$ spleens, Mac-1^{+} cells represent approximately 2% of the total cellular composition. This accumulation is exacerbated in the terminal stage of disease as demonstrated in figures 3.3.6.1A-3.3.6.3A (14 weeks old) when compared to the IL-$2^{+/+}$ counterpart of the diseased spleens (fig. 3.3.6.1B).


Figure 3.3.6A
Figure 3.3.6.1A
Figure 3.3.6.2A
Figure 3.3.6.3A
KO
10.4%
41.4%
35.6%
35.9%
mice with terminal stage of disease
Figure 3.3.6B
Figure 3.3.6.1B
WT
1.5%
3.1%
CD4
MAC-1

3.6-FACS analysis of Fas expression in IL-2$^{-/-}$ spleen cells.

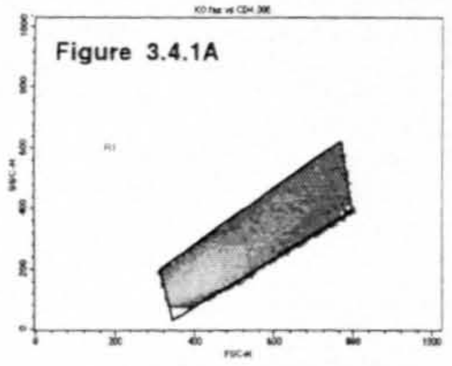
As described previously in chapter1, section 5.3, activation-induced cell death is the principle mechanism by which cells encountering repeated antigen stimulation die. Importantly, this mechanism is thoroughly Fas-dependent. One study has revealed that lymphocytes from IL-2$^{-/-}$ mice fail to undergo activation-induced cell death, despite normal expression of Fas on IL-2$^{-/-}$ CD4^{+} T cell blasts (167). However, this study used anti-CD3 or SAg-activated lymphocytes. Based on this study, splenocyte Fas expression was investigated *ex vivo*. Figure 3.4.1A illustrates the accumulation of Fas^{+} CD4^{+} T lymphocytes in IL-2$^{-/-}$ spleens. Approximately 10 to 50% of IL-2$^{-/-}$ CD4^{+} T cells expressed Fas, whereas approximately 2 to 18% of IL-2$^{+/+}$ CD4^{+} T cells expressed Fas (figure 3.4.1B). As disease progresses, the percentage of cells co-expressing Fas and CD4 decreases (figures 3.4.1.1A-3.4.1.3A), although the proportion of CD4^{+} Fas^{+} T lymphocytes is still higher than IL-2$^{+/+}$ littermate (fig. 3.4.1.1B). Figures 3.4.2A and B depict Fas expression on CD8 T cells. 6 to 16% of the IL-2$^{-/-}$ CD8^{+} T cell population express Fas (fig. 3.4.2A) in comparison to 2% of the IL-2$^{+/+}$ CD8^{+} T cell population (fig. 3.4.2B), these data are representative of three separate experiments.

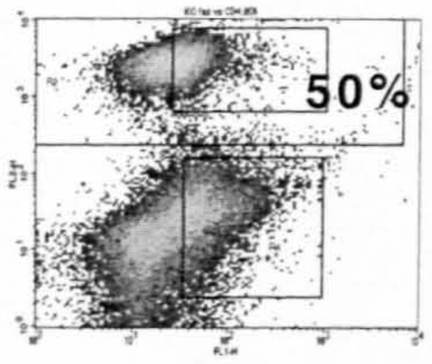
Analyses of Fas^{+} B220^{+} B cell populations in IL-2$^{+/+}$ and IL-2$^{-/-}$ mice did not reveal differences in Fas^{+} expressing B cells (figures 3.4.3A and B). Furthermore, 28% of IL-2$^{-/-}$ Mac-1^{+} spleen cells expressed Fas, whereas 5% of IL-2$^{+/+}$ Mac-1^{+} splenocytes did so (figures 3.4.4A and B). Finally, approximately 50 to 76% of IL-2$^{-/-}$ CD11c^{+} dendritic cells expressed Fas, whereas 26 to 35% of IL-2$^{+/+}$ dendritic cells expressed Fas (figures 3.4.5A and B).

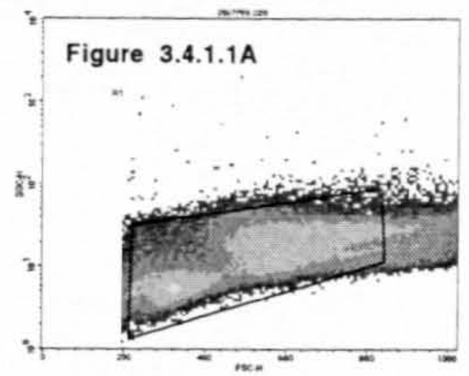
Figures 3.4.1A and B demonstrates the accumulation of Fas^+ $CD4^+$ T cells in IL-$2^{-/-}$ spleens (6-8 weeks old mice). Approximately 50% of the IL-$2^{-/-}$ $CD4^+$ T lymphocyte population expressed Fas, whereas 18% of IL-$2^{+/+}$ T cells express Fas.

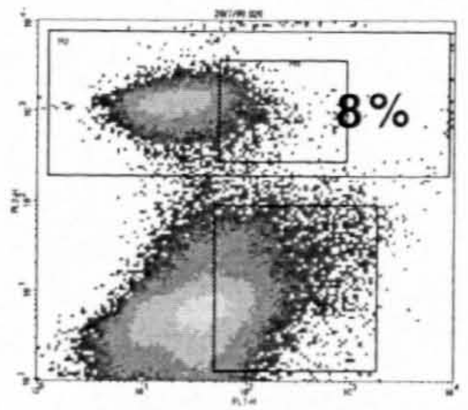
Figures 3.4.1.1A-3.4.1.3A and 3.4.1.1B represent the percentage of cells co-expressing Fas and CD4 in the terminal stage of disease. Although the percentage of Fas^+ $CD4^+$ T cells decreases as disease develops (14 weeks old), the proportion of IL-$2^{-/-}$ Fas^+ $CD4^+$ T lymphocytes (approximately 10%) remains higher than IL-$2^{+/+}$ Fas^+ $CD4^+$ T lymphocytes

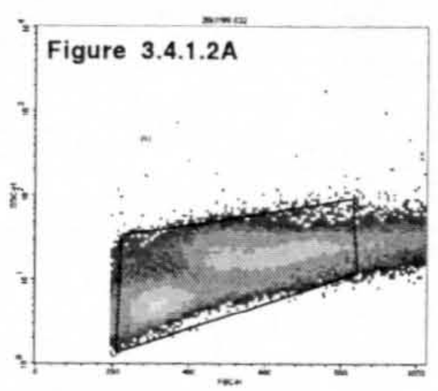
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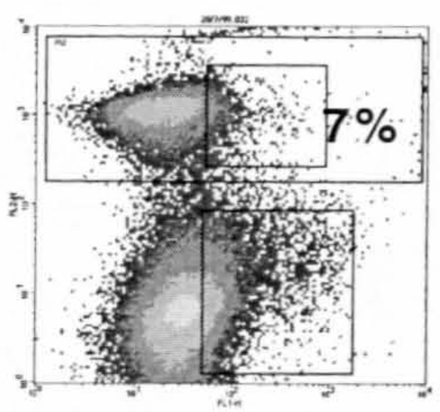


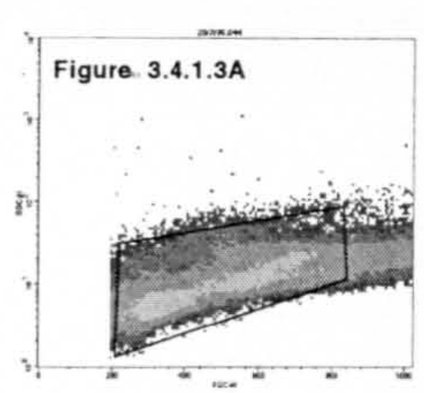


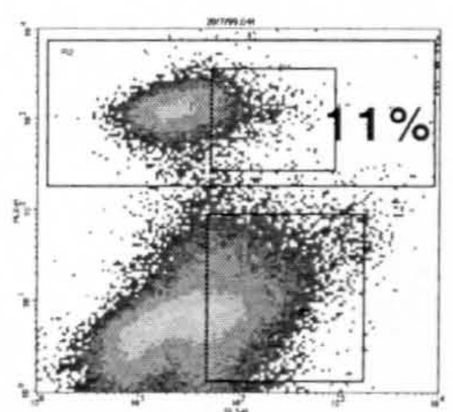






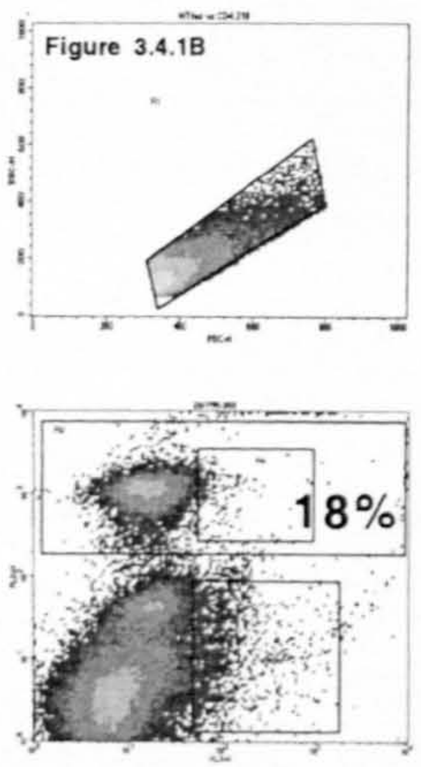


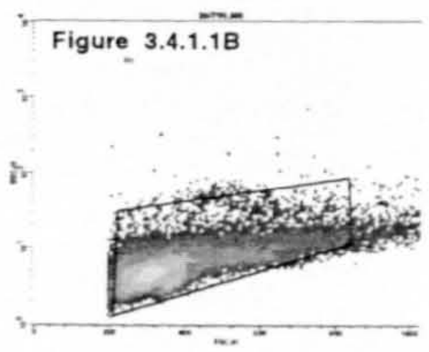


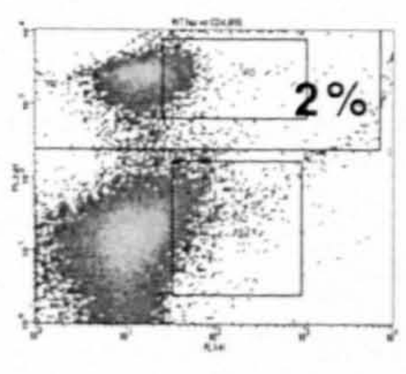


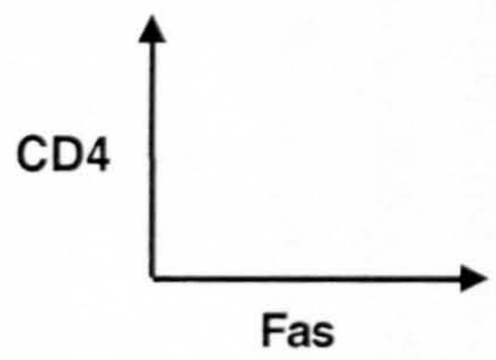
mice with terminal stage of disease

WT



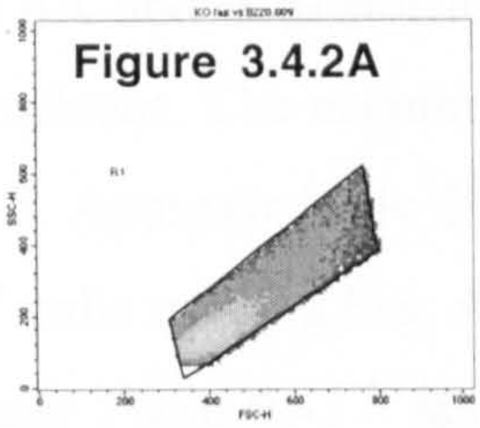


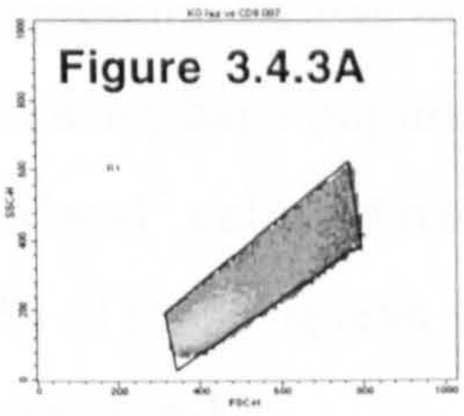




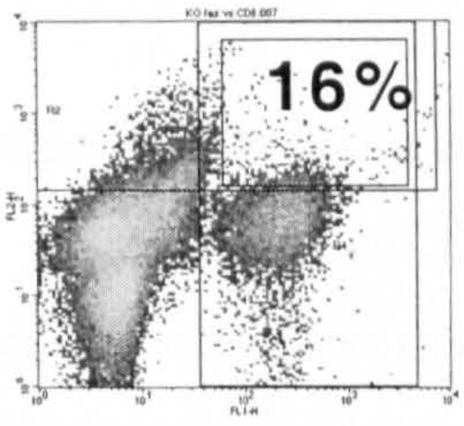
Figures 3.4.2A and B demonstrate the percentage increase in Fas^+ $CD8^+$ T lymphocytes in IL-$2^{-/-}$ spleens. In this example, 16% of IL-$2^{-/-}$ $CD8^+$ T cells co-express Fas, whereas 2% of IL-$2^{+/+}$ $CD8^+$ T cells co-express Fas.

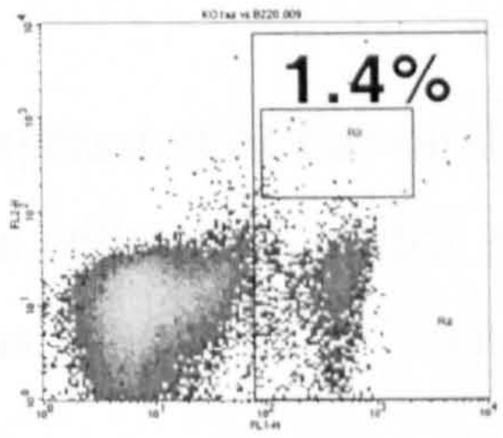
Figures 3.4.3A and B illustrate the analysis of Fas^+ $B220^+$ B cell populations in IL-$2^{+/+}$ and IL-$2^{-/-}$ spleens. There is no difference in percentage of B cells co-expressing Fas molecules in IL-$2^{+/+}$ and IL-$2^{-/-}$ spleens. Importantly, the percentage decrease of $B220^+$ B cells in IL-$2^{-/-}$ spleens is recapitulated. Approximately, 58% of IL-$2^{+/+}$ spleen cells express B220, whereas 7% of IL-$2^{-/-}$ spleen cells express B220.

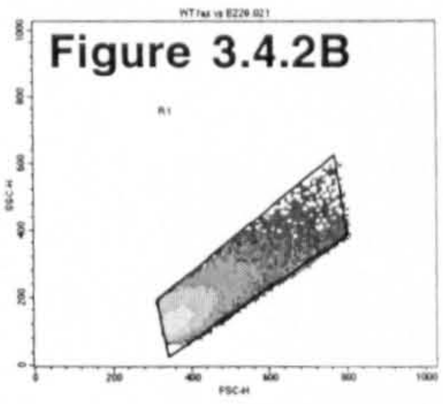

Figure 3.4.2A

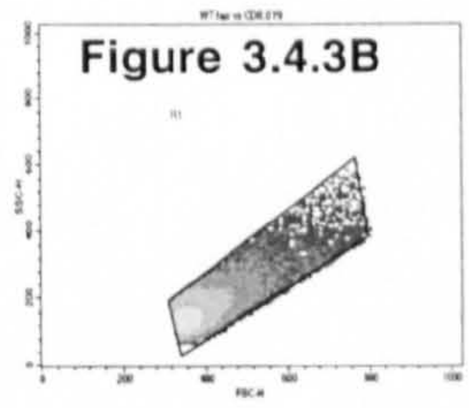

Figure 3.4.3A

KO

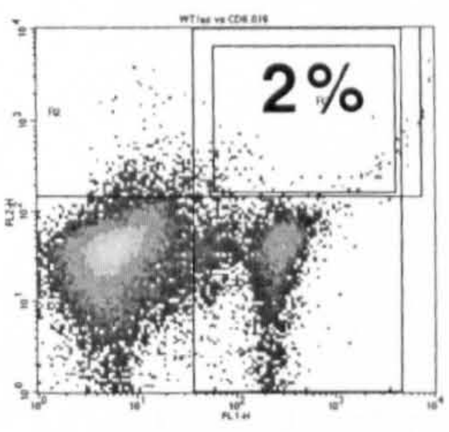

16%

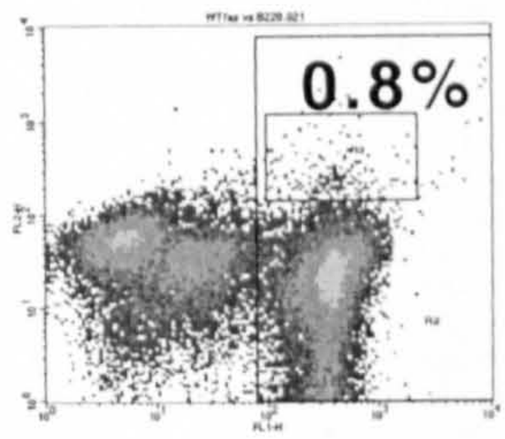

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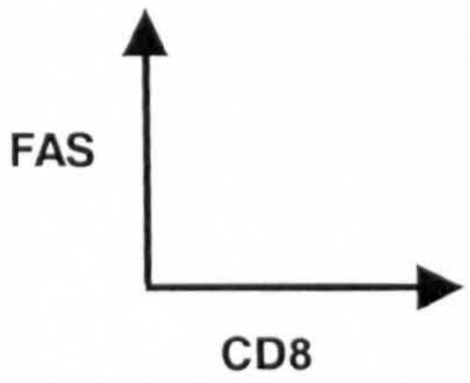

Figure 3.4.2B

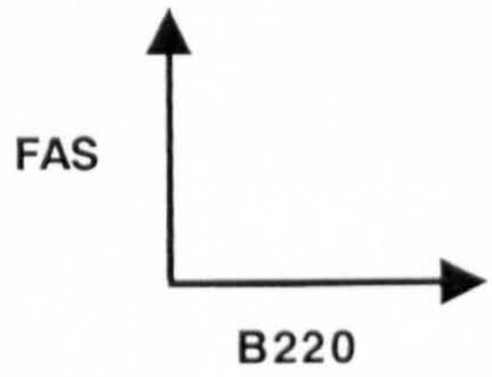

Figure 3.4.3B

WT


2%


0.8%

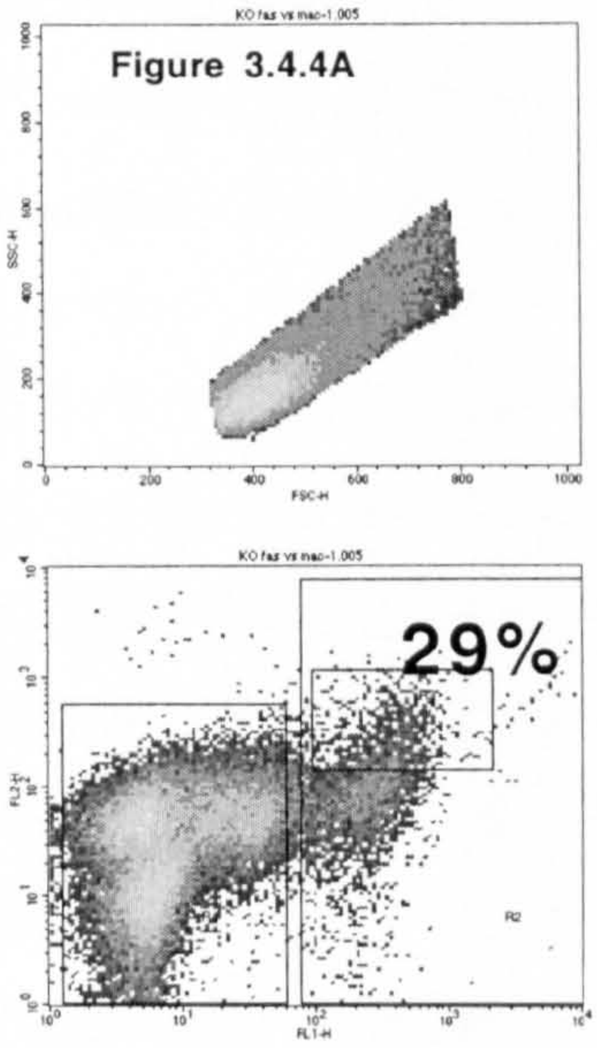

FAS
CD8

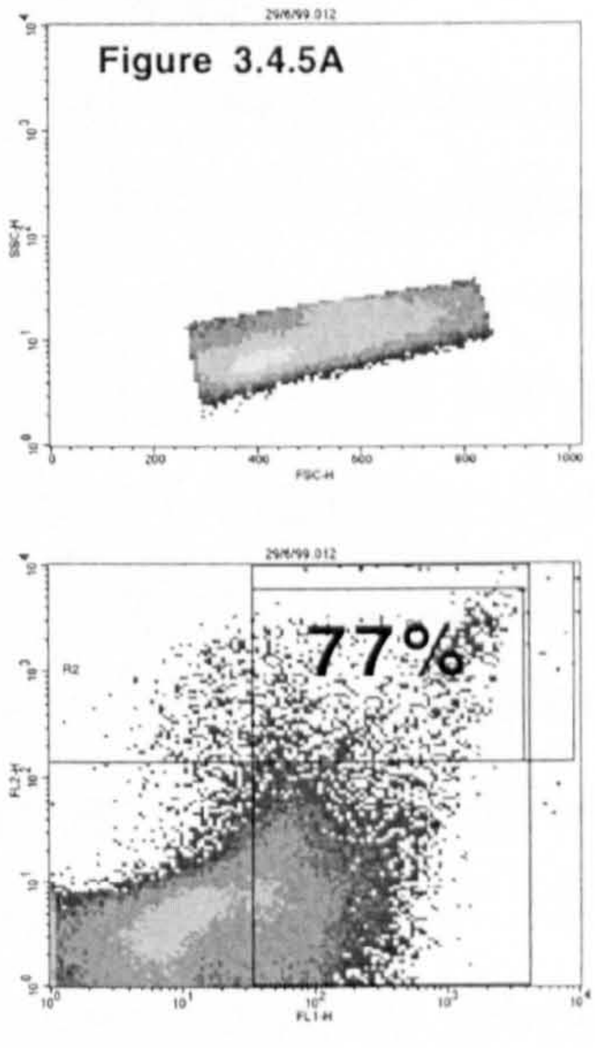

FAS
B220

Figures 3.4.4A and B demonstrate the expression of Fas on macrophages in IL-$2^{+/+}$ and IL-$2^{-/-}$ spleens. The accumulation of macrophage populations in IL-$2^{-/-}$ spleens is recapitulated. Approximately 29% IL-$2^{-/-}$ Mac-1^{+} cells express Fas, whereas 4% of IL-$2^{+/+}$ Mac-1^{+} cells express Fas. Around 12% of IL-$2^{-/-}$ spleen cells are Mac-1^{+}, whereas approximately 3% of IL-$2^{+/+}$ spleen cells are Mac-1^{+}.

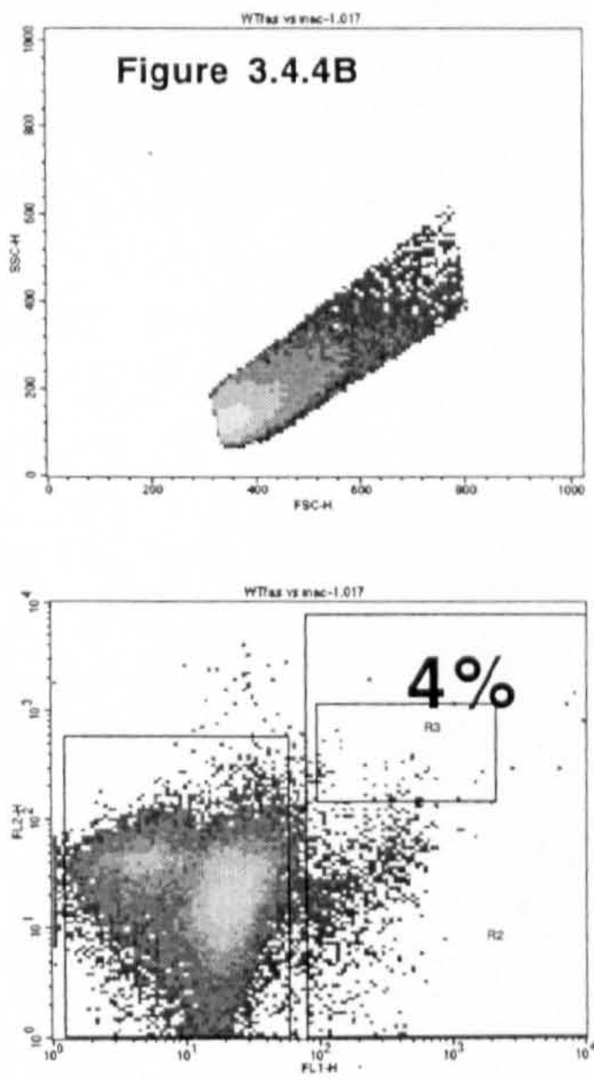
Figures 3.4.5A and B demonstrate the expression of Fas on dendritic cells in IL-$2^{+/+}$ and IL-$2^{-/-}$ spleens. 2.2% of IL-$2^{-/-}$ spleen cells are dendritic cells (CD11c^{+}). 77% of IL-$2^{-/-}$ dendritic cells express Fas, whereas 26% of IL-$2^{+/+}$ dendritic cells express Fas. This is representative of four experiments.

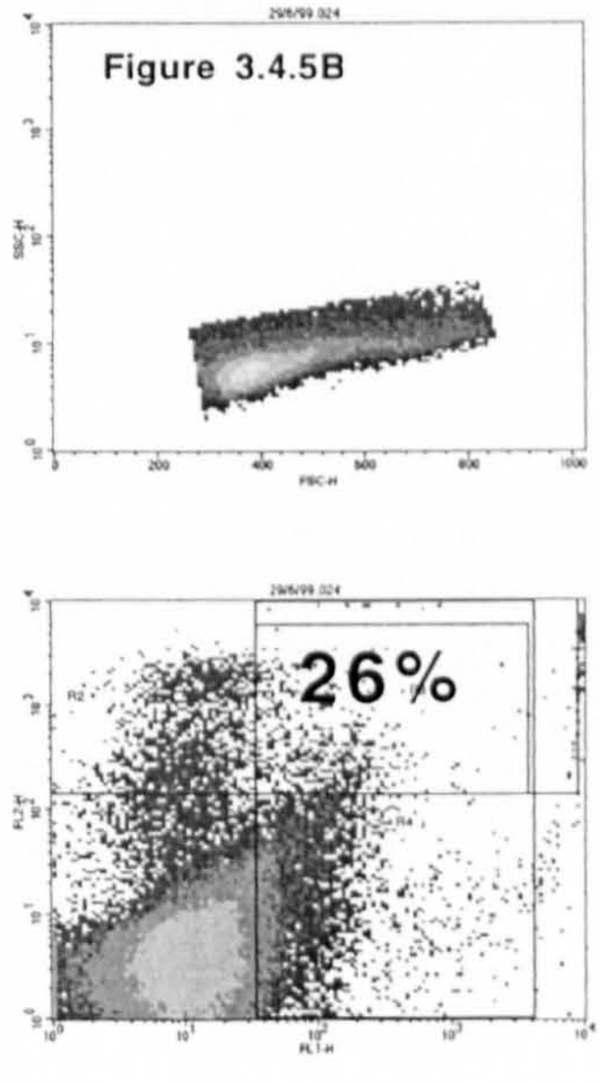
KO


Figure 3.4.4A
KO fas vs mac-1.005
SSC-H
FSC-H
29%
R2
FL2-H
FL1-H

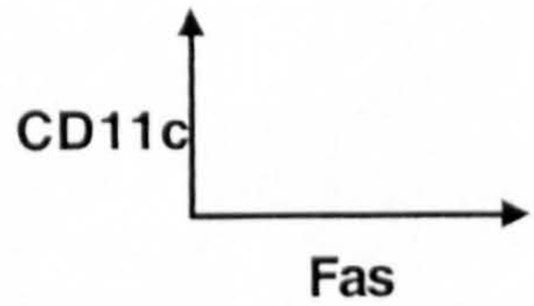

Figure 3.4.5A
29/6/99.012
SSC-H
FSC-H
77%
R2
FL2-H
FL1-H

WT


Figure 3.4.4B
WTfas vs mac-1.017
SSC-H
FSC-H
4%
R3
R2
FL2-H
FL1-H


Figure 3.4.5B
29/6/99.024
SSC-H
FSC-H
26%
R2
R4
FL2-H
FL1-H


Fas
Mac-1

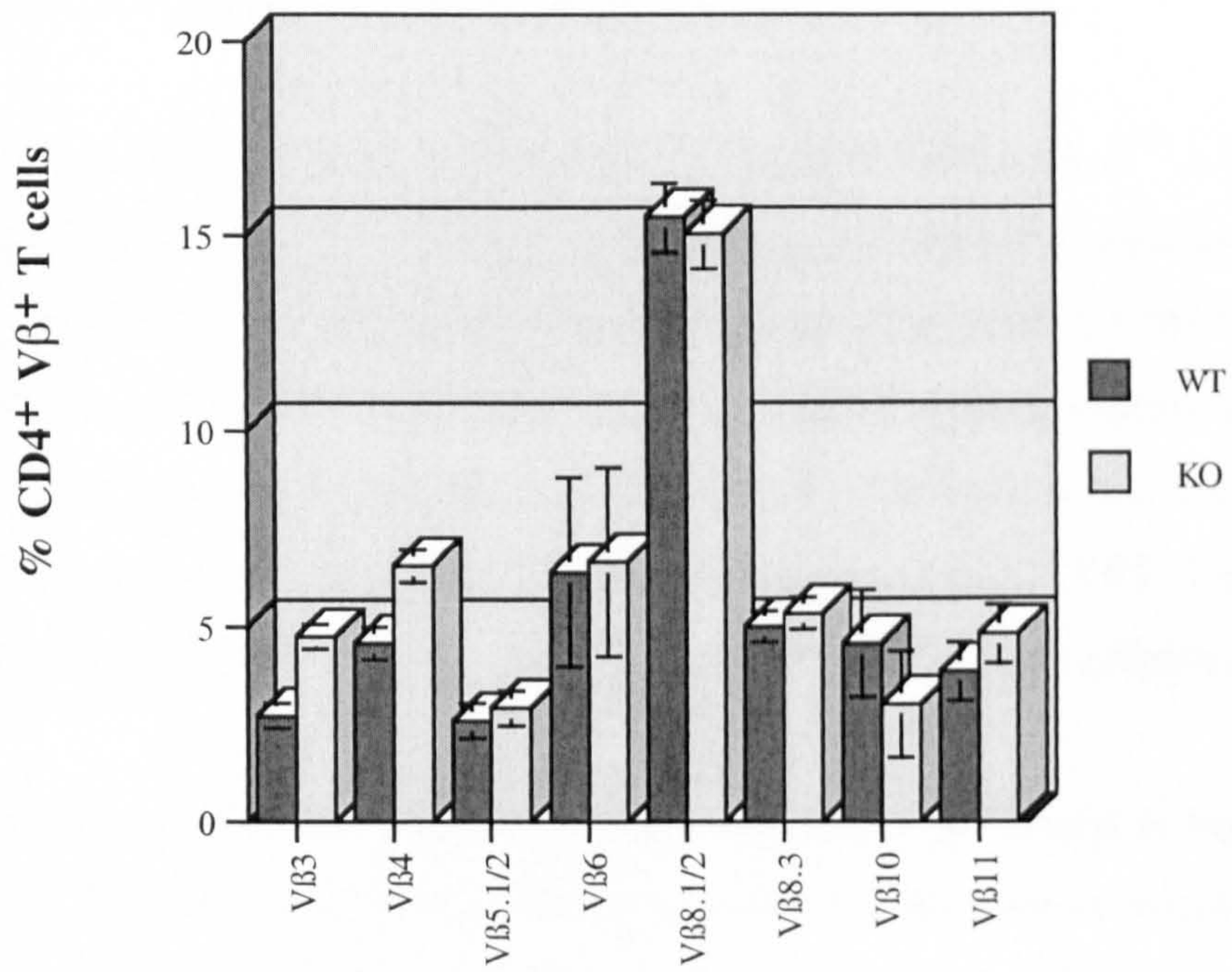

CD11c
Fas

3.7-Analysis of C57BL/6 IL-2$^{+/+}$ and IL-2$^{-/-}$ Vβ repertoire:

Several studies suggest that IBD in IL-2$^{-/-}$ mice is an abnormal immune response to antigens produced by the resident gut bacteria. If this abnormal immune response is directed against a specific antigen or a limited set of antigens, an oligoclonal expansion of the antigen-specific T cells, which are of a limited Vβ repertoire may ensue. It would be reasonable to predict that the IL-2$^{-/-}$ repertoire may be different (perhaps not dramatically) if the expansion were driven by a relatively limited set of antigens. Conversely, a response to a wide range of antigens, perhaps associated with bystander activation, would not result in skewing of the IL-2$^{-/-}$ Vβ repertoire. As illustrated in figure 3.5, the Vβ repertoires of both IL-2$^{+/+}$ and IL-2$^{-/-}$ mice are similar. This suggests that the antigenic complexity stimulating the abnormal immune response in IL-2$^{-/-}$ mice is not likely to be highly restricted, rather it is more consistent with a complex antigen load.

Figure 3.5 Analysis of C57BL/6 IL-$2^{+/+}$ and IL-$2^{-/-}$ Vβ repertoires did not reveal differences between the two. Moreover, the peripheral deletion of lymph node $V\beta5^{+}$ $CD4^{+}$ T cells is functional in the absence of IL-2. The results shown are the mean and SD of 3 separate experiments comprising 3 mice each.

Analysis of C57BL/6 IL-$2^{+/+}$ and IL-$2^{-/-}$ Vβ repertoire



3.8-The role of IL-2 in thymic and peripheral deletion:

IL-$2^{+/+}$ C57BL/6 mice chronically delete $CD4^+$ $V\beta5^+$ T cells from the peripheral repertoire (225), whereas $CD4^+$ $V\beta3^+$ T cells are thymically deleted in IL-$2^{+/+}$ NOD F1 mice (227). The role of IL-2 in mediating thymic and peripheral deletion of SAg-reactive T cells was investigated using the two models described above.

In the case of C57BL/6 mice, peripheral deletion of $CD4^+$ $V\beta5^+$ T cells was compared in IL-$2^{+/+}$ and IL-$2^{-/-}$ mice. Figure 3.5 depicts equal representation of $CD4^+$ $V\beta5^+$ T cells in the $CD4^+$ repertoires of IL-$2^{+/+}$ and IL-$2^{-/-}$ mice.

For the Mtv-3 deletion of Vβ3 $CD4^+$ T cells, (C57BL/6 IL-$2^{+/-}$ x NOD)$_{F1}$ mice were typed for IL-$2^{+/-}$ or IL-$2^{+/+}$ and the consequence of the 50% reduction of IL-2 assessed. Figure 3.6A demonstrates that the Vβ repertoires of both (C57BL/6 IL-$2^{+/-}$ x NOD)$_{F1}$ and (C57BL/6 IL-$2^{+/+}$ x NOD)$_{F1}$ mice are similar. Since complete absence of IL-2 did not affect the peripheral deletion of $CD4^+$ Vβ5 T cells, it is not surprising that half the IL2 gene dosage had no effect on thymic deletion of $CD4^+$ $V\beta3^+$ T cells either. Indeed, (C57BL/6 IL-$2^{+/-}$ x NOD)$_{F1}$ mice delete $CD4^+$ $V\beta3^+$ T cells effectively (fig. 3.6B).

In both of these models of SAg-mediated deletion, peripheral or thymic, manipulation of IL-2 did not affect the efficacy of deletion. For the peripheral deletion of Vβ5 $CD4^+$ T cells, deletion occurred as seen in fig. 3.5, in which Vβ5 $CD4^+$ T cells are equally represented in both repertoires. It is surprising that despite the absence of IL-2, $V\beta5^+$ IL-$2^{-/-}$ $CD4^+$ T cells expanded and also deleted in the "right" amounts. Likewise, the thymic deletion of $CD4^+$ $V\beta3^+$ T cells in (C57BL/6 IL-$2^{+/-}$ x NOD)$_{F1}$ mice is equivalent to that of (C57BL/6 x NOD IL-$2^{+/+}$)$_{F1}$ mice (fig. 3.6B).

Figure 3.6A shows that lymph node analysis of the (C57BL/6 IL-$2^{+/-}$ x NOD)$_{F1}$ repertoire did not reveal differences between the (C57BL/6 IL-$2^{+/+}$ x NOD)$_{F1}$ and (C57BL/6 IL-$2^{+/-}$ x NOD)$_{F1}$ repertoires. This concurs with data from the C57BL/6 IL-$2^{+/+}$ and IL-$2^{-/-}$ repertoires (fig. 3.5). The results shown are the mean and SD of 3 separate experiments comprising 3 mice each.

(C57BL/6 IL-$2^{+/+}$ x NOD)$_{F1}$ and (C57BL/6 IL-$2^{+/-}$ x NOD)$_{F1}$ Vβ repertoires

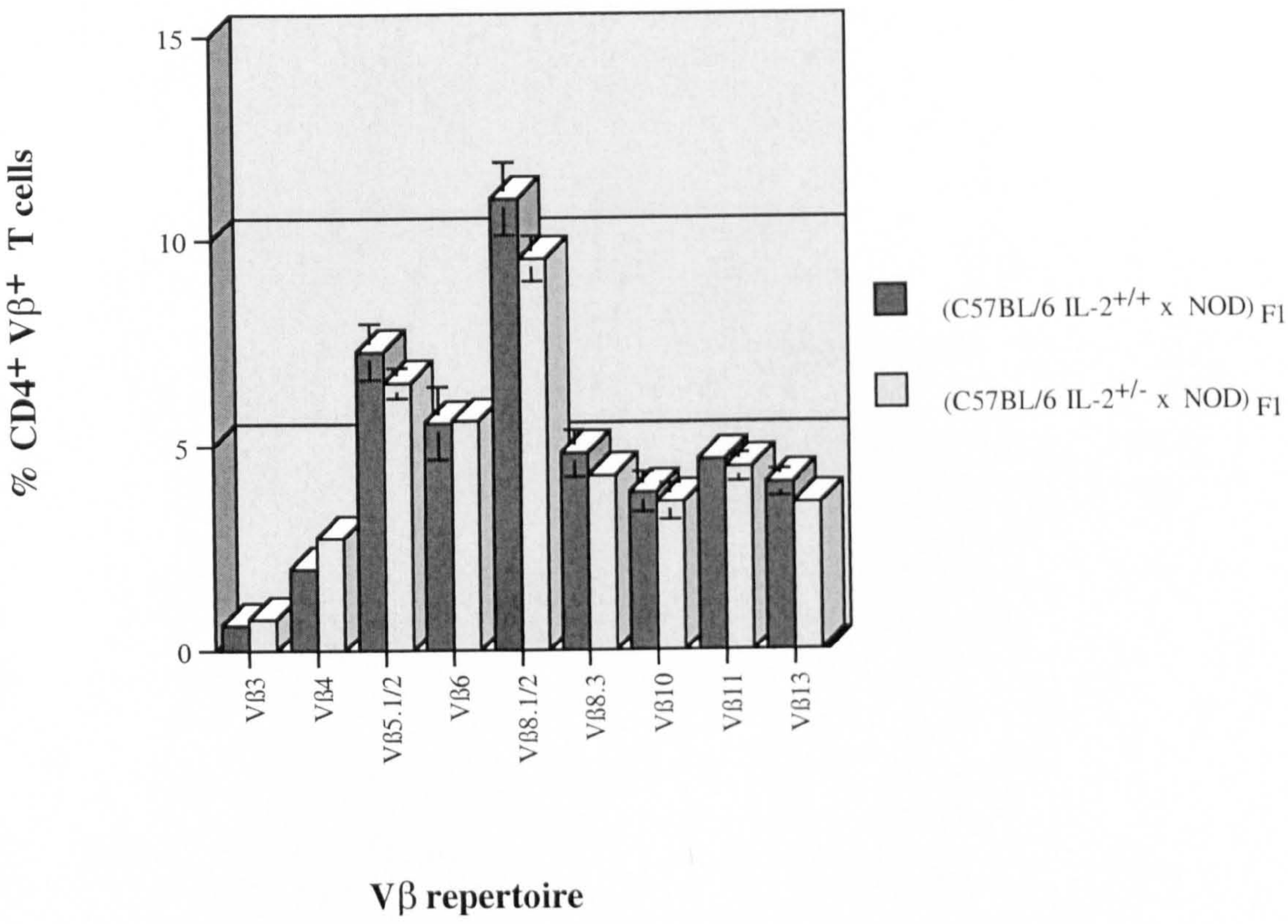
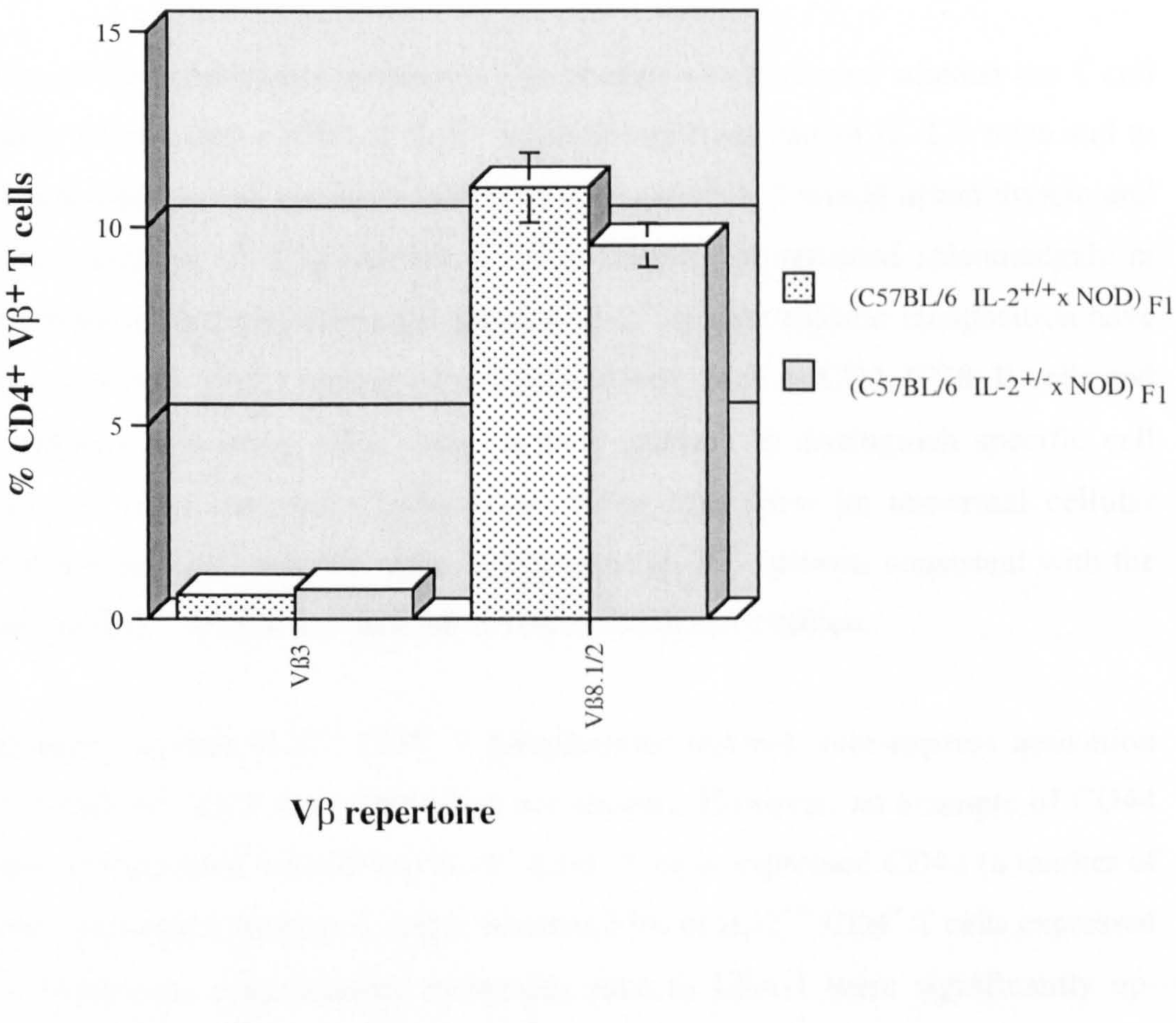


Figure 3.6B The thymic deletion of $V\beta3^+$ $CD4^+$ T cells in (C57BL/6 IL-$2^{+/+}$ x $NOD)_{F1}$ and (C57BL/6 IL-$2^{+/-}$ x $NOD)_{F1}$ mice is similar. Therefore, half the dosage of IL-2 does not affect the thymic deletion of Mtv-3 reactive T cells.

Deletion of Vβ3 CD4$^+$ T lymphocytes in (C57BL/6 IL-2$^{+/-}$ x NOD)$_{F1}$ mice



DISCUSSION

A study by Luviksson et al. demonstrated a defect in thymic selection in IL-$2^{-/-}$ mice. In that study injection of anti-CD3 intraperitoneally (i.p.) induced cortical intrathymic cell death in IL-$2^{+/+}$ mice, with a 78% decrease in the total number of thymocytes. Injection of anti-CD3 i.p. in IL-$2^{-/-}$ mice resulted in a 34% decrease in total thymocyte numbers. This indicated that IL-$2^{-/-}$ mice exhibit decreased susceptibility to cortical apoptosis (111). This finding by Luviksson et al. suggests that defective thymic negative selection of T cells may influence the T cell repertoire in IL-$2^{-/-}$ mice.

The aim of the experiments reported in this chapter was to dissect whether the T cell repertoire of diseased C57BL/6 IL-$2^{-/-}$ mice differs from that of IL-$2^{+/+}$ mice and to investigate whether the partial or complete absence of IL-2 would affect thymic and peripheral deletion of SAg-reactive T cells. Despite the reported splenomegaly in various studies, data describing the detailed IL-$2^{-/-}$ splenic cellular composition have not been reported. FACS studies of cell populations, such as CD4, CD8, B cells and other antigen presenting cells, using surface markers to distinguish specific cell populations were analysed. Collectively, these data show an abnormal cellular composition in IL-$2^{-/-}$ spleens when compared to IL-$2^{+/+}$ spleens, consistent with the increase in IL-$2^{-/-}$ splenic size and the *in vivo* inflammatory milieu.

Interestingly, splenic IL-$2^{-/-}$ $CD4^+$ T lymphocytes did not over-express activation markers such as CD25 nor CD69 (data not shown). However, an example of CD44 staining demonstrated that 60% of IL-$2^{-/-}$ $CD4^+$ T cells expressed CD44 (a marker of previously activated memory T cells), whereas 33% of IL-$2^{+/+}$ $CD4^+$ T cells expressed CD44. Moreover, costimulatory molecules such as LFA-1 were significantly up-regulated in IL-$2^{-/-}$ $CD4^+$ splenocytes. This finding is recapitulated later on in chapter 5 (Table 5.2). Other important differences between IL-$2^{+/+}$ and IL-$2^{-/-}$ spleens are the accumulation of Mac-1^+ cells (macrophages) and increased expression of Fas on several cell types in IL-$2^{-/-}$ spleens. Macrophages are commonly described as scavenger cells because they phagocytose and process protein antigens. Upon

activation, they secrete several bactericidal and microbicidal reagents and other pro-inflammatory monokines, as well as up-regulating MHC class II and costimulatory molecules on their cell surface. This contributes to the activation of neighbouring T lymphocytes and mediates immune damage through the release of superoxides. Thus, the accumulation of macrophages in IL-$2^{-/-}$ spleens corresponds well with the general inflammatory microenvironment in IL-$2^{-/-}$ peripheral organs. Indeed, patients with CD and UC overproduce monocytes, possibly as a consequence of increased demand for macrophages in the inflamed gut (230). In the case of IL-$2^{-/-}$ mice, overproduction of pro-inflammatory cytokines (IFN-γ, TNF-α) by autoreactive T cells could result in augmentation of macrophage activity and subsequent recruitment to secondary lymphoid organs such as the spleen.

Surprisingly, Fas expression on a number of cell types was increased in IL-$2^{-/-}$ spleens. As described in the literature (167), the Fas-Fas L apoptotic pathway is defective in IL-$2^{-/-}$ mice. This suggests that the intracellular programmes resulting in surface expression of Fas and Fas L molecules are functional in IL-$2^{-/-}$ lymphocytes. Rather, the implementation of the death programme is defective. This raises the question of whether the increase in Fas expression in IL-$2^{-/-}$ spleens is a result of an accumulation of activated cells that express Fas, which cannot be eliminated. Further analyses of Fas^{+} expressing splenic populations revealed a higher percentage of different cell subsets expressing Fas in IL-$2^{-/-}$ spleens. This is apparent in dendritic cells ($CD11c^{+}$), with 50 to 76% of IL-$2^{-/-}$ DCs expressing Fas in comparison to 26 to 35% of IL-$2^{+/+}$ DCs.

Recent data have revealed a new role of Fas in dendritic cell (DC) physiology. Engagement of Fas on immature DCs by anti-Fas antibodies or Fas L does not induce cell death (231). Rather, it leads to phenotypic and functional maturation of primary DCs (232). This suggests that the Fas/Fas L pathway can mediate different effector mechanisms, depending on the secondary machinery involved. Presumably, there are different secondary pathways leading to different effector functions, either cell death

or proliferation, operating in different cell types. Indeed, a recent report has described a role of Fas in T cell activation depending on the current activation status of the T cell (233). Thus, IL-$2^{-/-}$ mice may lack the secondary signalling pathways which transmit Fas-Fas L apoptotic signal but may have functional secondary pathways for Fas-Fas L DC-mediated proliferation. Alternatively, Fas expression may be an outcome of the activation of DCs.

Finally, analysis of the T cell repertoire of IL-$2^{-/-}$ mice did not reveal an abnormal T cell repertoire when compared to IL-$2^{+/+}$ mice. Moreover, the role of IL-2 in thymic and peripheral T cell deletion was studied using two different models of SAg-reactive T cell deletion. Peripheral deletion of $CD4^+$ $V\beta5^+$ T cells was functional in the absence of IL-2. Furthermore, thymic deletion of $CD4^+$ $V\beta3^+$ T cells was operational despite the partial absence of IL-2 in (C57BL/6 IL-$2^{+/-}$ x NOD)$_{F1}$ mice. These findings suggest that IL-2 is not involved in SAg-mediated deletion.

Despite the well-documented involvement of IL-2 in several mechanisms of immune response termination, these data suggest that the deletion of SAg-reactive T cells does not appear to involve IL-2 or the Fas/Fas L pathway. Indeed, in T cells, the Fas/Fas L apoptotic pathway is potentiated by IL-2 and has been implicated in the elimination of T cells that repeatedly encounter antigens, such as self-antigen. Given that the expression of endogenous SAg can probably be regarded as a source of persistent antigen, the involvement of Fas/Fas L pathway, hence IL-2, was expected.

A potential source of SAg relevant to the phenotype of IL-$2^{-/-}$ mice, are the gut-residing bacteria. In this regard, germ-free IL-$2^{-/-}$ mice do not develop IBD. This points to a potential role of bacterial SAg in intestinal inflammation. SAg may mediate intestinal inflammation by activation of T cells that cause direct tissue injury through the release of pro-inflammatory cytokines or the expansion of autoreactive T cell clones. Alternatively, SAg may eliminate regulatory T cell clones that prevent intestinal inflammation. If this were the case, the Vβ repertoire of IL-$2^{-/-}$ mice should have been skewed.

Evidence supporting the possible involvement of SAg in intestinal inflammation includes the following observations:

- Aisenberg et al. reported that intestinal epithelial cells are exposed to a variety of bacterial superantigens passing through the intestinal lumen and can present exogenous SAg to mucosal T cells. In addition, human mucosal T cells proliferated in response to SAg *in vitro* (234).
- The involvement of SAg in several chronic inflammatory disease models. For instance, in collagen-induced arthritis, the *M. arthritidis* SAg exacerbates disease.

However, another observation does not support this hypothesis:

- Despite the chronic intestinal inflammation in IL-$2^{-/-}$ mice, the Vβ repertoire was not skewed when compared to IL-$2^{+/+}$ littermates. This thesis suggests that SAg do not play a role in this intestinal inflammatory model.

To summarise, the role of IL-2 in termination of T cell responses has been well-documented as is exemplified by the uncontrolled accumulation of activated T cells in IL-$2^{-/-}$, IL-2Rα$^{-/-}$ and IL-2Rβ$^{-/-}$ mice. These models suggest that IL-2 is critical for the termination of T cell responses and its role in T cell expansion can be replaced by other growth factors. However, in endogenous SAg-mediated thymic and peripheral deletions, mechanisms other than the IL-2-dependent Fas/Fas L-mediated deletion of SAg-reactive T cell mediate deletion.

CHAPTER 4

IN VITRO CYTOKINE ANALYSIS OF B7-1/ANTI-CD3 INDUCED PROLIFERATION OF IL-$2^{+/+}$ AND IL-$2^{-/-}$ $CD4^+$ T LYMPHOCYTES.

INTRODUCTION

CHO B7-1/anti-CD3-mediated immune responses:

While the participation of the B7/CD28 pathway in the activation of T cells by alloantigen and foreign antigen has been extensively studied (235) (236), its potential role in the induction and maintenance of autoimmune disease and inflammatory responses is less certain. The IL-2 deficient mouse is a model of spontaneous autoimmunity which can be used to elucidate secondary pathway(s) responsible for *in vivo* cellular proliferation and homeostasis in the absence of regulation by IL-2. Previous studies by Razi-Wolf et al. have shown that the activation of IL-$2^{-/-}$ $CD4^+$ T lymphocytes, like their WT counterparts, requires stimulation through the TCR and CD28 *in vitro*. Given that IL-$2^{-/-}$ $CD4^+$ T cells are responsible for disease pathogenesis *in vivo*, their activation requirements are of profound interest (65). Therefore, the *in vitro* TCR/CD28 stimulation system can be used to investigate the activation requirements of IL-$2^{-/-}$ $CD4^+$ T cells.

For WT, IL-$2^{+/+}$ $CD4^+$ T cells, CHO B7-1 and Con A or anti-CD3 synergize to induce proliferation of $CD4^+$ T cells. This costimulatory effect is mediated via the induction of IL-2 production and IL-2 receptor expression. Indeed, previous studies have illustrated that the murine B7-1 (mB7-1) molecule, when expressed in Chinese hamster ovary cells in a stable manner, can deliver powerful costimulatory signals for the TCR-mediated activation of $CD4^+$ T cells by anti-CD3 or the lectin Con A (201).

This chapter will focus on the *in vitro* cytokine analysis of B7-1/anti-CD3 induced proliferation of IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells. Several approaches were used to investigate cytokine profiles produced by IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes. Using anti-cytokine mAbs, recombinant cytokines and ELISA studies, the role of several cytokines in B7-1/anti-CD3 proliferative responses was investigated.

Postulated cytokines in CHO B7-1/anti-CD3-mediated IL-2$^{-/-}$ CD4^{+} T cell activation: In IL-2$^{+/+}$ CD4^{+} T cells the ligation of the TCR and B7-1/CD28 results in IL-2 mRNA transcription (187). As shown by Razi-wolf et al., activation of IL-2$^{-/-}$ CD4^{+} T cells is also dependent on TCR and B7-1 signalling. Consequently, proliferative responses of IL-2$^{-/-}$ CD4^{+} T cells were hypothesized to be mediated by one or more cytokine(s). The principal candidate cytokines are members of the common γ chain family, which share their IL-2Rγ component with IL-2 receptor and have overlapping downstream effects. IL-15 was the primary postulated candidate in IL-2 deficient T cell activation because it shares two of its receptor components, β and γ chains, with the IL-2 receptor (25). Moreover, IL-15 uses the same JAK/STAT signalling pathway as IL-2 and shares a number of biological activities with IL-2, including stimulating the proliferation of activated T cells (237). Furthermore, patients with active ulcerative colitis have a higher percentage of PBMC expressing IL-15 and the number of IL-15 expressing cells in patients with active IBD decreased within two weeks of treatment with 5-amino-salicylic acid (238). Finally, IL-2Rβ deficient mice lack IL-2 and IL-15 receptor signals and develop granulocytosis but not IBD (108), implying that IL-15 may play a role in the activation of colitis-inducing IL-2$^{-/-}$ CD4^{+} T cells.

As mentioned previously, IL-15 is a pleiotropic cytokine which is structurally similar to IL-2. Firstly, the heterotrimeric receptors for IL-2 and IL-15 share two receptor components, namely IL-2Rβ and γc. Hence, they also share downstream cytoplasmic signals. Moreover, their private receptor chains, IL-2Rα and IL-15Rα, are structurally similar (reviewed in (239)).

Despite the biological similarities of IL-2 and IL-15, there remain several important differences. The consequence of these differences has been illuminated recently through the generation of IL-15Rα$^{-/-}$ and IL-15$^{-/-}$ mice. One of the prominent differences between the two cytokines is the complex control of IL-15 expression. In addition to control at the transcriptional level, IL-15 expression is controlled posttrancriptionally. Posttranscriptional control is mediated by several elements that

hinder translation including multiple upstream AUGs and two unusual signal peptides (240) (reviewed in (241)). These controls are perhaps a necessity because of the diverse array of cells that respond to and produce IL-15. This is in contrast to IL-2, which is mainly produced by activated T cells, and IL-2Rα expression, which is expressed on activated monocytes, T, B and NK cells. IL-15Rα is expressed on monocytes, T, B, NK, stromal cell lines from the thymus and bone marrow and in tissues such as liver, skeletal muscle, spleen and heart. Thus far, there have been no reports on the co-regulation of IL-2 and IL-15 in T cells. Indeed, in view of the different distribution patterns of IL-2 and IL-15, co-regulation of these two cytokines is not expected despite their shared biological functions.

Recently, the generation of IL-15Rα$^{-/-}$, IL-15$^{-/-}$ and IL-15 transgenic murine models has shed new lights on the unique biological functions of IL-15. In contrast to IL-2$^{-/-}$ and IL-2Rα$^{-/-}$ mice, mice with targeted inactivation of IL-15Rα and IL-15 mice do not develop autoimmune disease. In fact, IL-15Rα$^{-/-}$ and IL-15$^{-/-}$ mice are lymphopoenic and defective in NK cells, NK T cells, intestinal intraepithelial T lymphocytes and activated/memory CD8^{+} T lymphocytes (242) (243). IL-15 transgenic mice exhibited increased numbers of both NK cells and memory CD8^{+} T lymphocytes. Most interestingly, IL-2-mediated AICD was dysfunctional in IL-15 transgenic mice (244).

The study by Marks-Konczalik et al. revealed that, despite sharing multiple biological functions, IL-2 and IL-15 essentially mediate different roles in adaptive T cell immunity. Indeed, IL-2 serves to maintain peripheral tolerance through potentiating AICD of CD4^{+} T helper lymphocytes and elimination of autoreactive T cells. By contrast IL-15, through its inhibition of IL-2-mediated AICD, maintenance of CD8 memory T cell responses and NK cells, serves to propagate memory immune responses against foreign pathogens.

RESULTS

Requirement of B7-1 signals for the activation of IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes.

4.2-CON A-stimulated immune responses:

Figure 4.1A depicts the stimulation of WT C57BL/6 $CD4^+$ T lymphocytes with Con A and CHO B7-1 or vector transfected control CHO ø. The CHO transfectants were fixed with 1% paraformaldehyde; hence the proliferative response derives from the $CD4^+$ T cells. Different concentrations of CHO B7-1 and CHO ø were used to provide costimulatory signals. The proliferative responses, particularly with limited Con A, were dependent on mB7-1 signals. The next obvious point was to test whether IL-$2^{-/-}$ $CD4^+$ T cells responded to B7-1/Con A in a similar fashion to IL-$2^{+/+}$ $CD4^+$ T cells. Moreover, since the proliferative response induced in IL-$2^{-/-}$ $CD4^+$ T cells is IL-2 independent, are cytokines being produced which substitute for IL-2? Mice 6-8weeks of age (before overt onset of clinical disease) were used for all the proliferation assays, unless stated otherwise in the text.

As figure 4.1B shows, CHO B7-1 and Con A synergized to induce the proliferation of IL-$2^{-/-}$ $CD4^+$ T lymphocytes. However, in these experiments, vector-transfected CHO ø cells induced a proliferative response, albeit less than that induced by CHO B7-1. This may be a result of incomplete fixation of CHO transfectants, or alternatively, insufficient purification of $CD4^+$ T cells. T cells cultured alone with Con A resulted in low background levels of proliferation, indicating that the purity of $CD4^+$ T cells was not leading to apparent costimulation independent proliferation. Therefore, optimisation of the assay was required to reduce background proliferation.

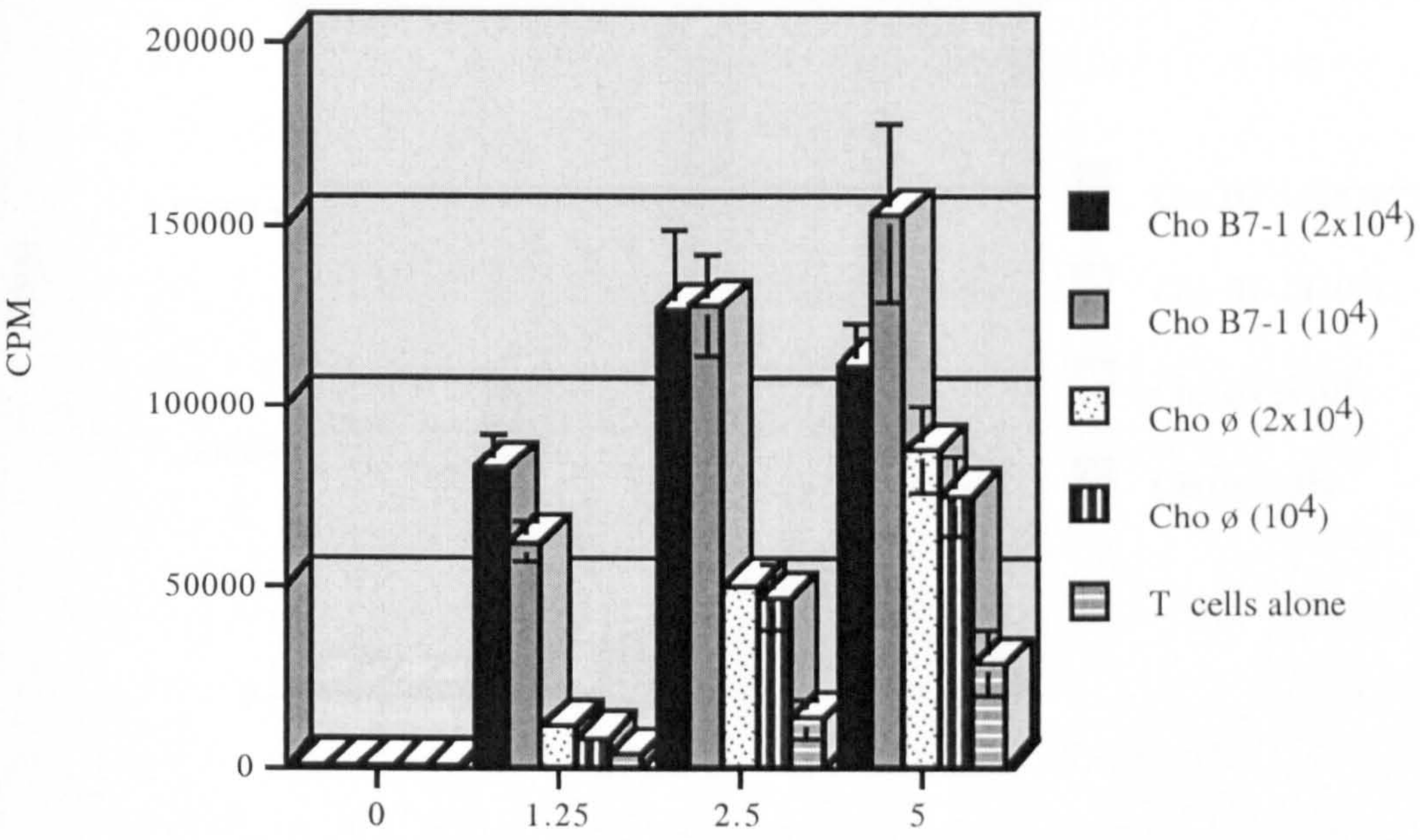


Figure 4.1A **Response of WT C57BL/6 CD4⁺ T cells to the lectin concanavalin A (Con A) and B7-1 signals.**

$CD4^+$ T cells were purified and set up in mirocultures at a concentration of $2x10^5$/well C57BL/6 T cells. CHO transfectants expressing the costimulatory molecule B7-1 and vector-transfected CHO ø were fixed with 1% paraformaldehyde and co-cultured at varying concentrations ($2x10^4$ or 10^4 cells per well) with the purified $CD4^+$ T cells and different concentrations of Con A.

Cultures were pulsed after 60 hrs with 1μCi of [^{3}H] thymidine per well for the last 12 hrs of incubation to assay for T cell proliferation. The results shown are the mean and SD of triplicate cultures.

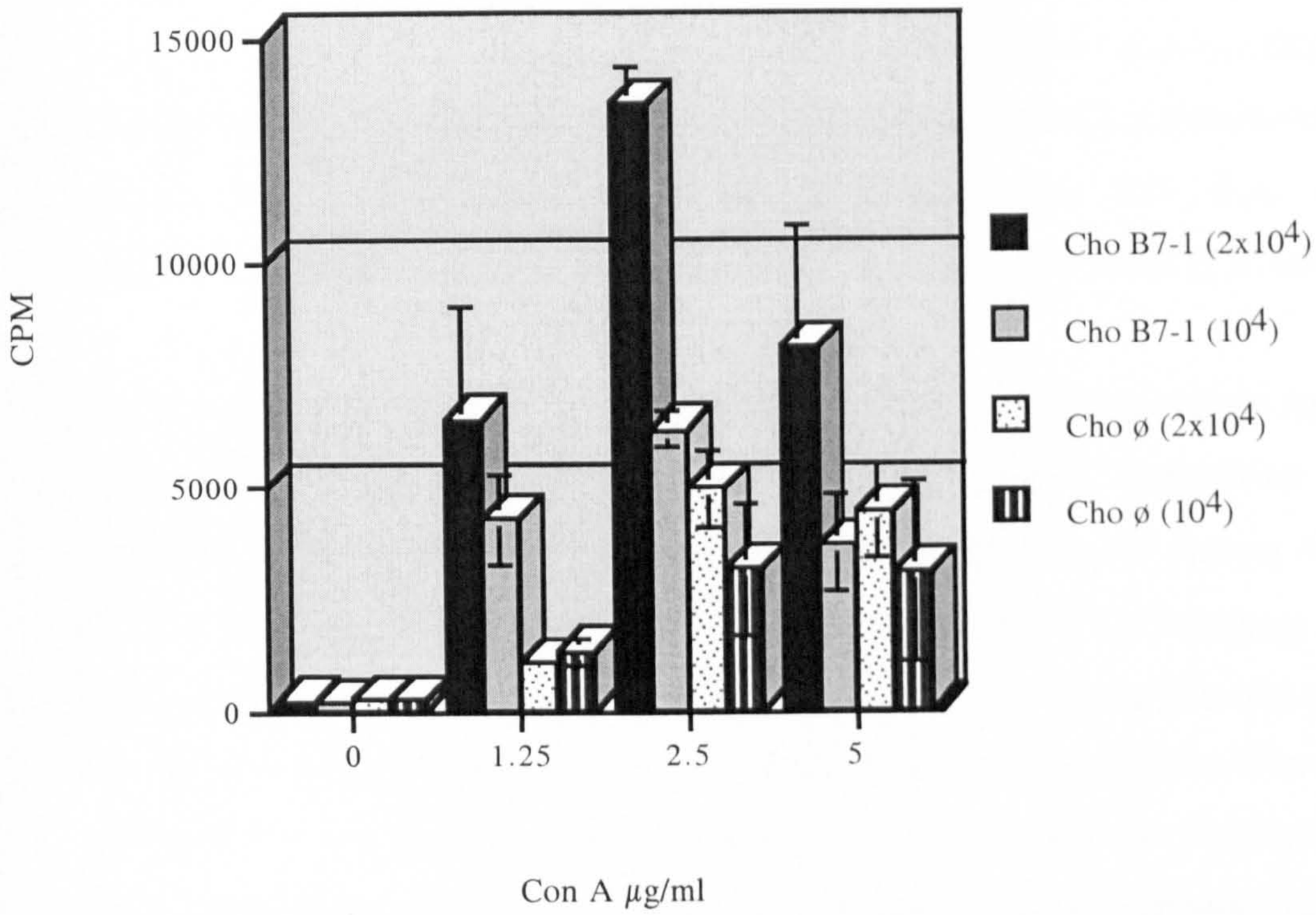


Figure 4.1B **Response of IL-2 deficient C57BL/6 CD4$^+$ T cells to the lectin Con A and B7-1 signals.**

IL-2$^{-/-}$ CD4$^+$ T cells were set up as described in figure 4.1A. Cultures were pulsed after 60 hrs with 1μCi of [^{3}H] thymidine per well for the last 12 hrs of incubation to assay for T cell proliferation. The results shown are the mean and SD of triplicate cultures.

Figure 4.2A depicts the activation of IL-$2^{+/+}$ $CD4^+$ T cells with Con A and B7-1 costimulatory molecules. CHO B7-1 cells were highly efficient at augmenting T cell proliferation induced by Con A and vector-transfected CHO ø, again, served as negative control in this experiment. Moreover, the addition of anti-CD25 (anti-IL-2Rα) blocking antibody abrogated proliferation and reduced counts to background levels. This confirmed previous data illustrating that B7-1/Con A-induced proliferation is dependent on the production of IL-2 and expression of IL-2Rα.

T cells can be triggered to proliferate, in the absence of costimulatory signals, by a combination of pharmacological reagents such as phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore, ionomycin (245). Figure 4.2B depicts the response of IL-2 deficient C57BL/6 $CD4^+$ T cells to Con A and B7-1 costimulatory signals. In this experiment, three negative controls were used. T cells alone stimulated with Con A, Con A and PMA and finally Con A with vector-transfected CHO ø. The former two controls show that costimulatory signals are absolutely critical for T cell proliferation. The latter clarifies whether any background response is due to improper fixation of the CHO transfectants. As depicted in figure 4.2B all negative controls resulted in low proliferation while CHO B7-1 augmented proliferation. Notably, the WT cells display approximately 4 to 8 fold higher proliferative responses than the KO cells.

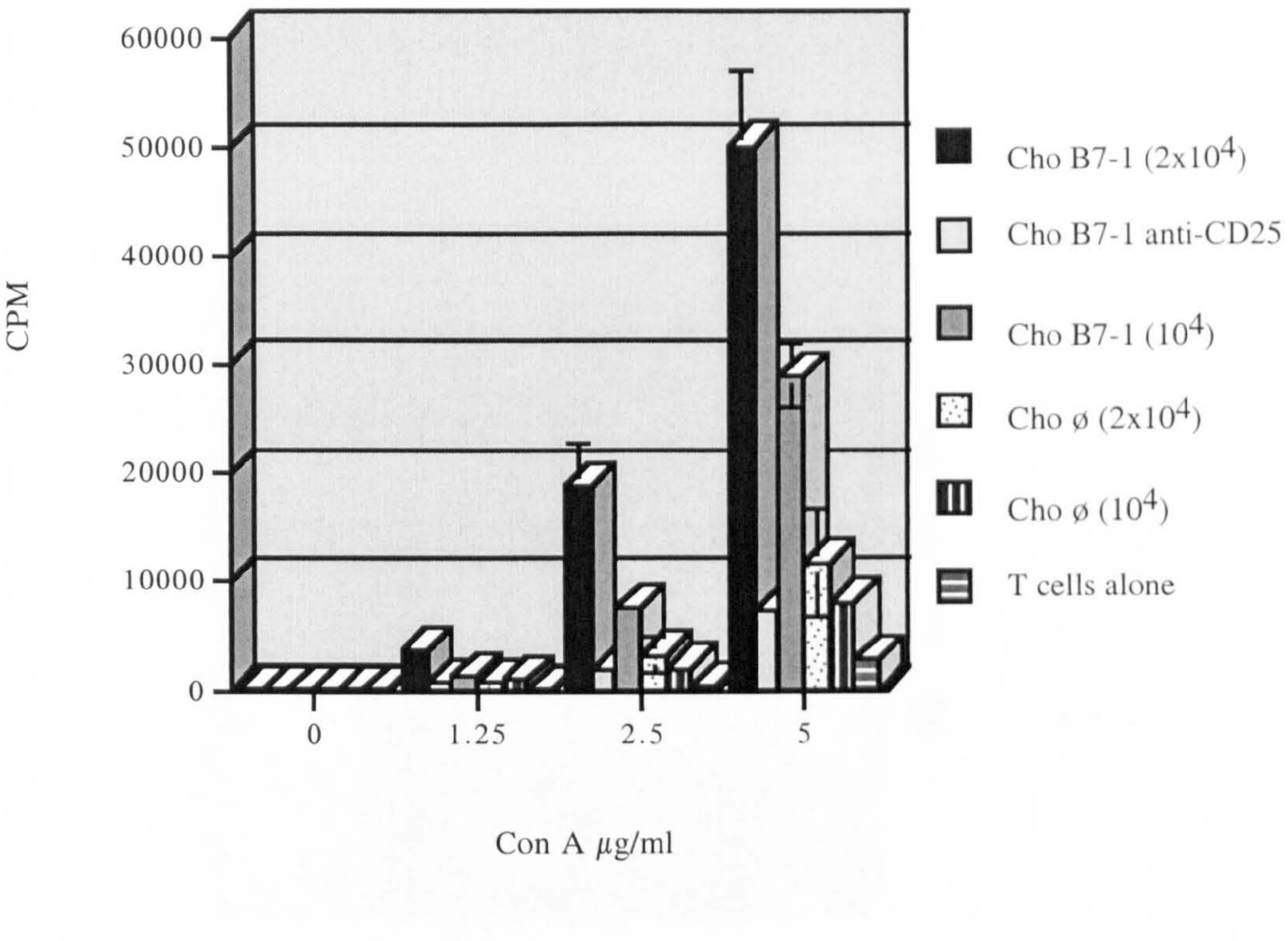


Figure 4.2A **Response of WT C57BL/6 CD4⁺ T cells to Con A and B7-1 costimulatory signals.**

$CD4^+$ T cells were purified and set up in microcultures at a concentration of $2x10^5$ C57BL/6 T cells. CHO transfectants expressing the costimulatory molecule B7-1 or vector transfected CHO ø were fixed with 1% paraformaldehyde and co-cultured at varying concentrations ($2x10^4$ or 10^4 cells per well) with the purified $CD4^+$ T cells and different concentrations of Con A.

Cultures were pulsed after 60 hrs with 1μCi of [^{3}H] thymidine per well for the last 12 hrs of incubation to assay for T cell proliferation. The results shown are the mean and SD of triplicate cultures.

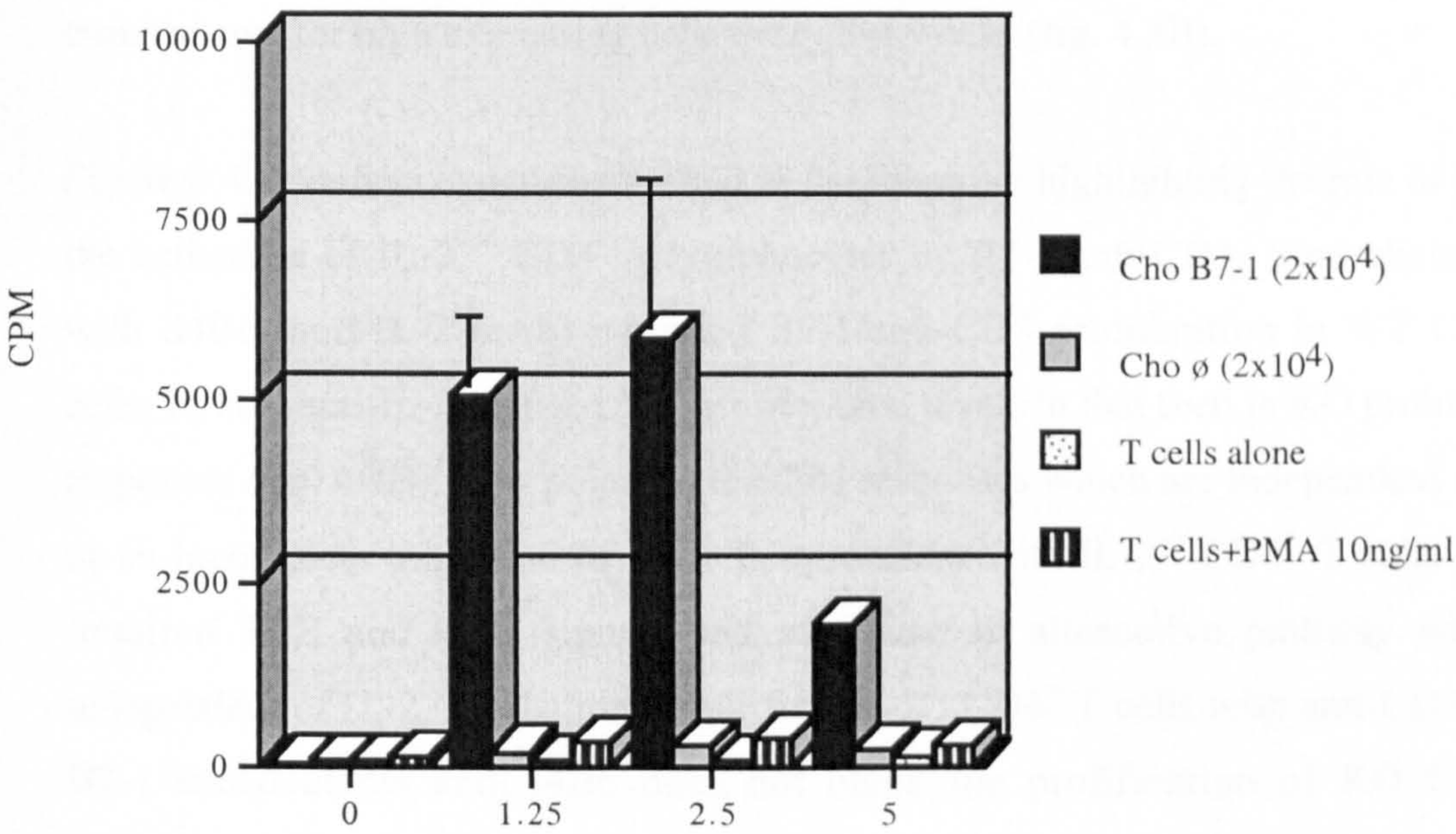


Figure 4.2B **Response of IL-2 deficient C57BL/6 CD4$^+$ T cells to Con A and B7-1 costimulatory signals.**

IL-2$^{-/-}$ CD4$^+$ T cells were purified and set up in microcultures at a concentration of $2x10^5$ C57BL/6 T cells. CHO transfectants expressing the costimulatory molecule B7-1 or vector transfected CHO ø were fixed with 1% parafomaldehyde and co-cultured at $2x10^4$ cells per well with the purified CD4$^+$ T cells and different concentrations of Con A. Cultures were pulsed after 60 hrs with 1μCi of [^{3}H] thymidine per well for the last 12 hrs of incubation to assay for T cell proliferation. The results shown are the mean and SD of triplicate cultures.

4.3-CHO B7-1/anti-CD3-mediated immune responses:

Figure 4.3A depicts the effect of B7-1 expression level on proliferation of IL-$2^{-/-}$ $CD4^+$ T cells stimulated with CHO B7-1 and anti-CD3. This experiment indicates that mB7-1 transfectant Chinese hamster ovary cells, but not vector transfected controls, synergize with anti-CD3 mAb to induce proliferation. Furthermore, this proliferation was dependent on the level of B7-1 expression on CHO transfectants. The CHO transfectants were moderately stable; nevertheless, it was decided to sort B7-1 transfectants for high expressing cells every 3-4 weeks (fig. 4.3B).

Figure 4.4A confirm experiments cited in the literature highlighting the role of IL-2 in the activation of IL-$2^{+/+}$ $CD4^+$ T lymphocytes by B7-1/anti-CD3. Neutralizing IL-2 with S4B6 (anti-IL-2 mAb) inhibited B7-1/anti-CD3 proliferation in WT $CD4^+$ T cells. In fact, anti-IL-2 reduced WT proliferation levels to that seen in KO proliferative responses (fig. 4.4B). This points to residual responses which are independent of IL-2 or an incomplete inhibition of IL-2. In agreement with IL-$2^{-/-}$ $CD4^+$ T cells clearly required TCR and B7-1 signals and must use an alternative pathway which is independent of IL-2. Incubation of purified IL-$2^{-/-}$ $CD4^+$ T cells with anti-CD3, CHO B7-1 transfectants and S4B6 does not block the proliferation of KO $CD4^+$ T lymphocytes (fig. 4.4B).

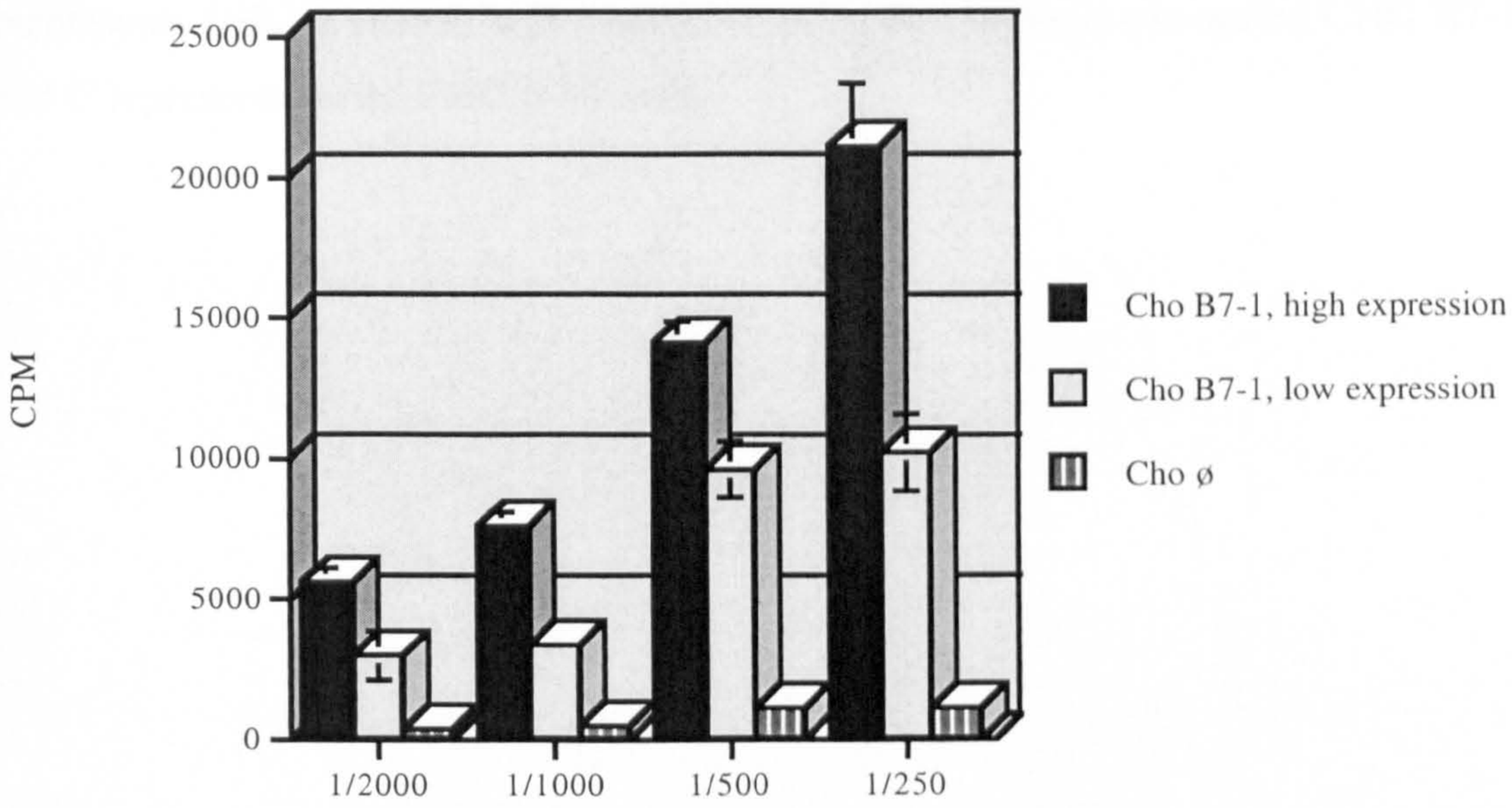
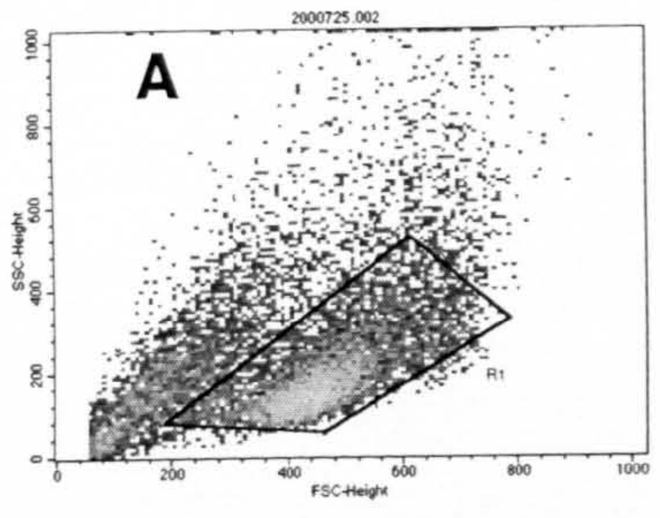


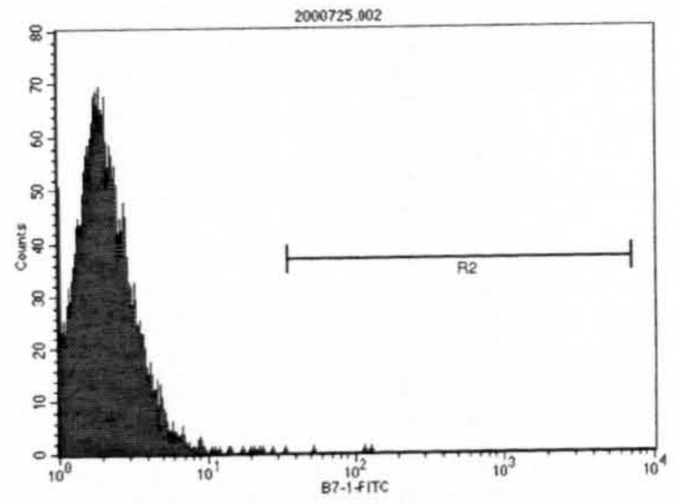
Figure 4.3A **Activation of T cells from IL-2 deficient mice with anti-CD3 requires B7-1 costimulation.**

Microcultures were set up with $2x10^5$ purified $CD4^+$ T cells from IL-$2^{-/-}$ mice. CHO transfectants expressing different levels of B7-1 or vector transfected CHO ø were fixed with 1% paraformaldehyde and co-cultured with the purified $CD4^+$ T cells with varying concentrations of soluble anti-CD3 mAb, 145-2C11 hybridoma culture supernatant. Cultures were pulsed after 60 hrs with 1μCi of [^{3}H] thymidine per well for the last 12 hrs of incubation to assay for T cell proliferation. The results shown are the mean and SD of triplicate cultures.

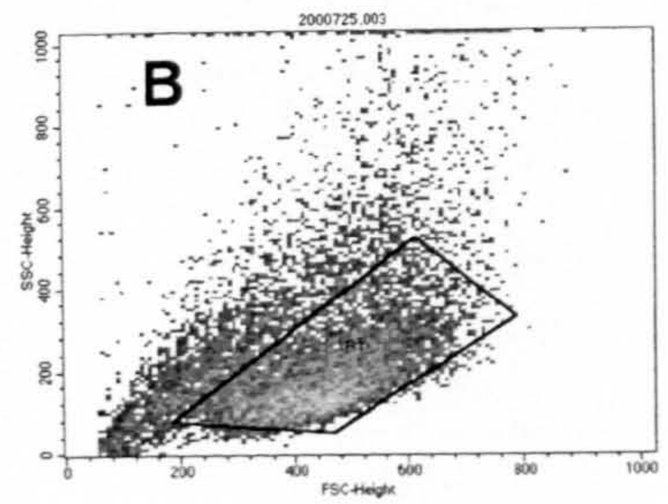
Figure 4.3B **CHO B7-1 sort for high expressing cells.**

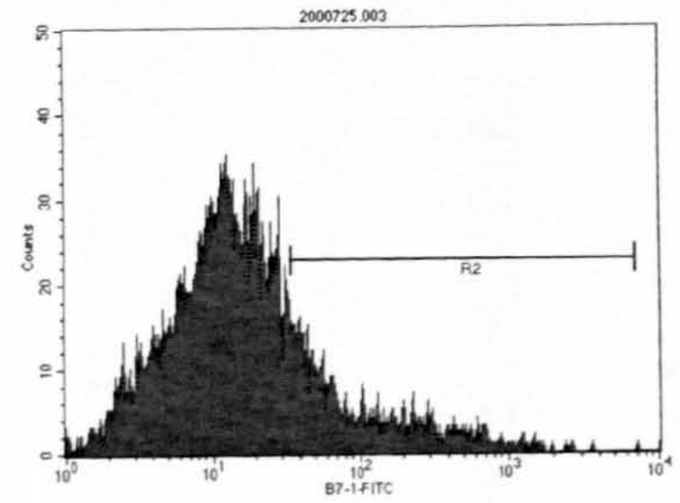
CHO B7-1 transfectants were cultured as described in materials and methods. CHO B7-1 cells were washed 3 times in PBS post-culture before staining with anti-B7-1 FITC at 1/100 and sorting on the Becton Dickinson FACS Vantage. In this example, **A** respresents CHO ø stained with anti-B7-1 mAb, **B** represents pre-sorted CHO B7-1 and **C** represents sorted CHO B7-1 cells.



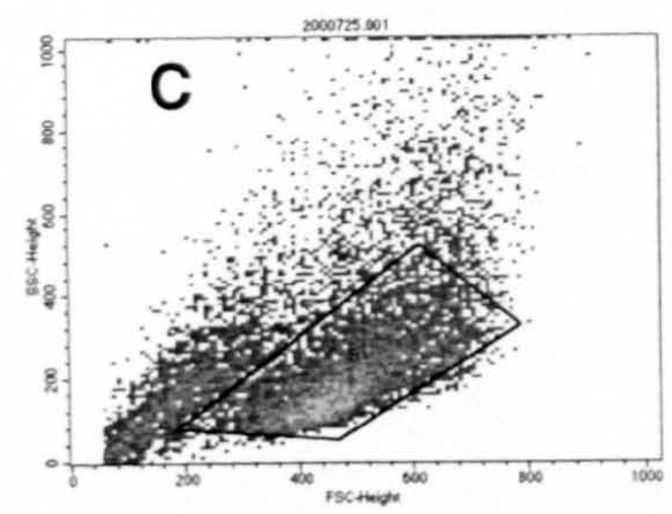


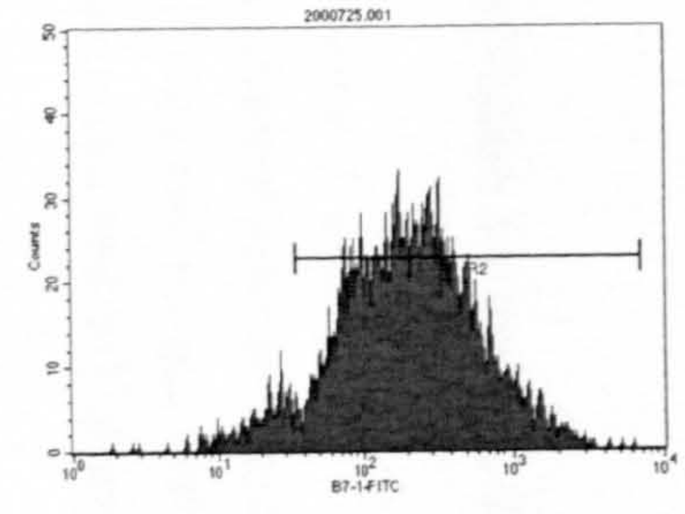
Negative control





Pre-sort





Post-sort

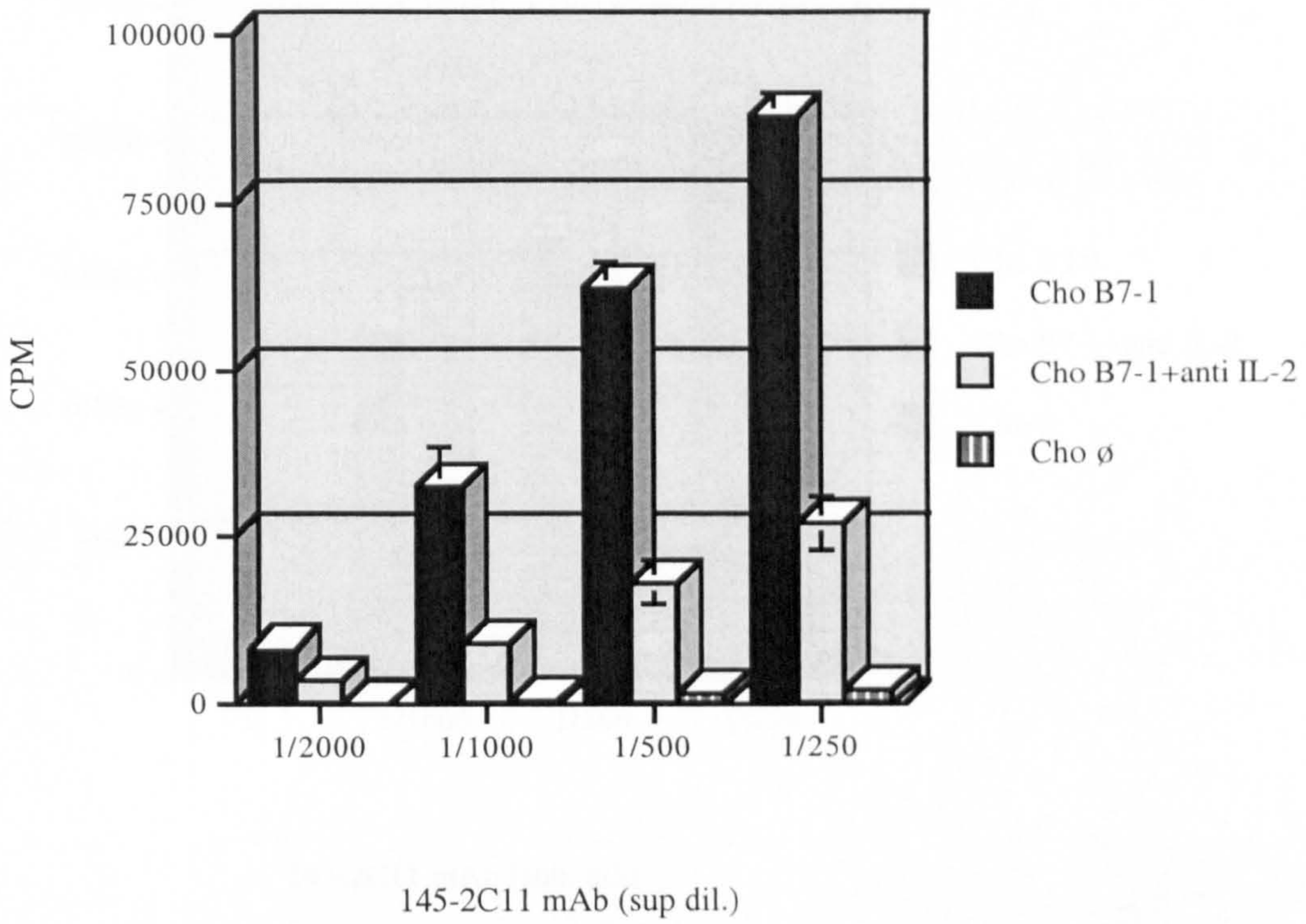


Figure 4.4A **Inhibition of WT C57BL/6 CD4$^+$ T cell activation by anti-IL-2 mAb (1:1600 dilution of S4B6 ascites).**

T cell cultures were set up as described in the legend to figure 3 with $2x10^5$ CD4$^+$ T cells from mice that were genotyped as WT, $2x10^4$ of paraformaldehyde-fixed CHO B7-1 or CHO ø as indicated. Cultures were incubated for 72 hrs and pulsed with 1μCi [^{3}H] thymidine for the last 12 hrs. The results shown are the mean and SD of triplicate cultures.

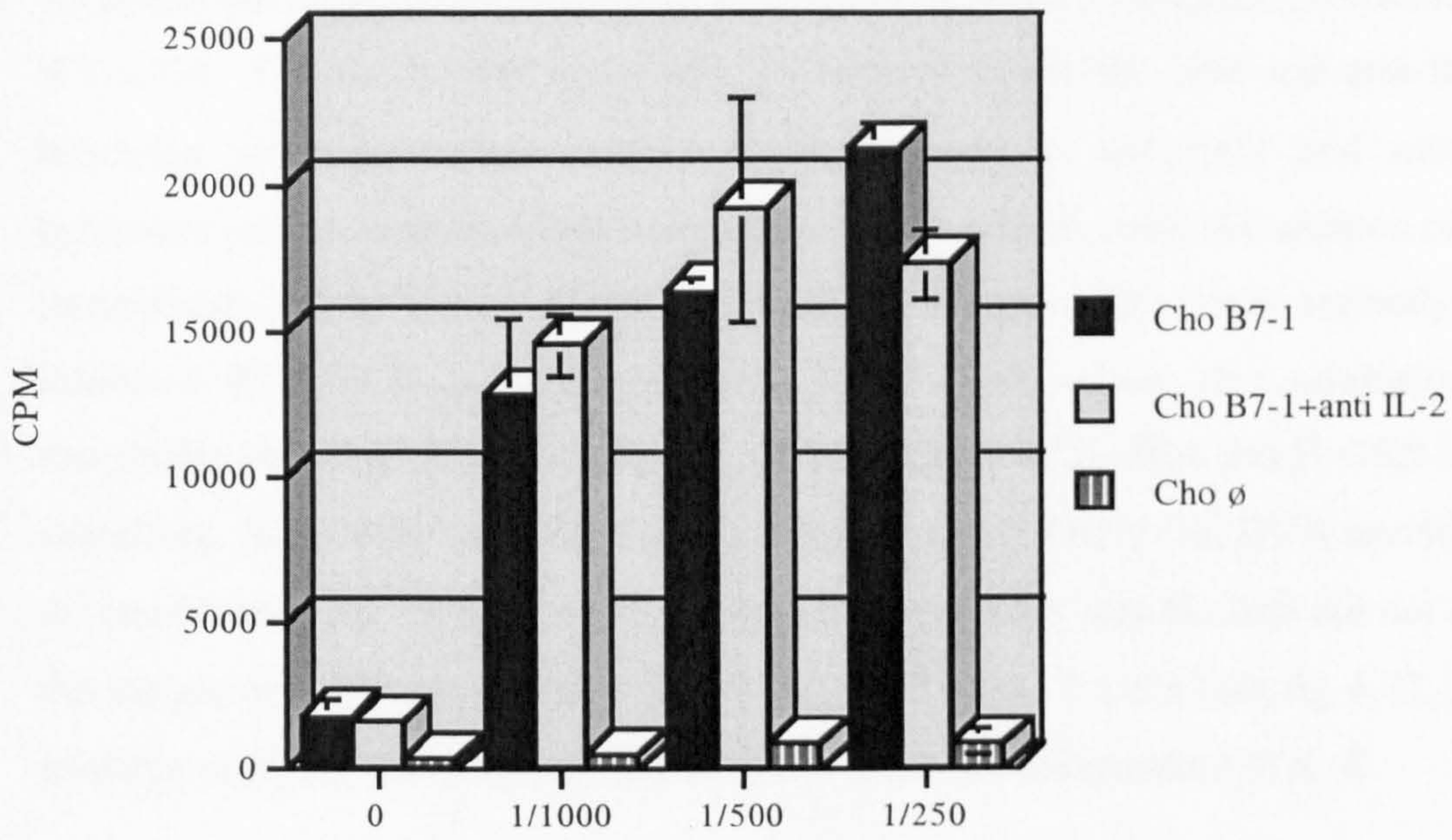


Figure 4.4B **Anti-IL-2 mAb (1:1600 dilution of S4B6 ascites) does not inhibit IL-2 deficient C57BL/6 CD4$^+$ T cell activation by TCR/B7-1 signals**.
Microcultures were set up with $2x10^5$ purified CD4$^+$ T cells from mice that were genotyped as KO, $2x10^4$ paraformaldehyde-fixed CHO B7-1 or CHO ø transfectant cells. Cultures were pulsed after 60 hrs with 1μCi [^{3}H] thymidine per well for the last 12 hrs of incubation. The results shown are the mean and SD of triplicate cultures.

4.3.1-The significance of IL-2 in IL-2$^{+/+}$ CD4^{+} T cells CHO-B7-1/anti-CD3-induced proliferative responses:

To assess the importance of the IL-2 receptor in TCR/B7-1 mediated proliferation of WT CD4^{+} T cells, I have examined the effects of anti-IL-2Rα and anti-IL-2Rβ blocking antibodies (fig. 4.4C). As mentioned in materials and methods, cytokine/cyotkine receptor mAb were added last to cultures, after the addition of CHO transfectants, CD4^{+} T cells and soluble anti-CD3 respectively. Each antibody alone inhibited B7-1/TCR-induced proliferation. In combination, this inhibition was marginally enhanced. This underscores the importance of IL-2Rα and IL-2Rβ in IL-2 signalling. In addition, anti-IL-2Rβ was incubated with DO11-10, OVA specific H2-A^{d}-restricted, IL-2$^{-/-}$ CD4^{+} T cells. As expected, however, anti-IL-2Rβ did not inhibit the antigen-specific activation of DO11-10 IL-2$^{-/-}$ CD4^{+} T cells (see fig 4.12, p147), possibly implying that they use an alternative pathway, independent of IL-2.

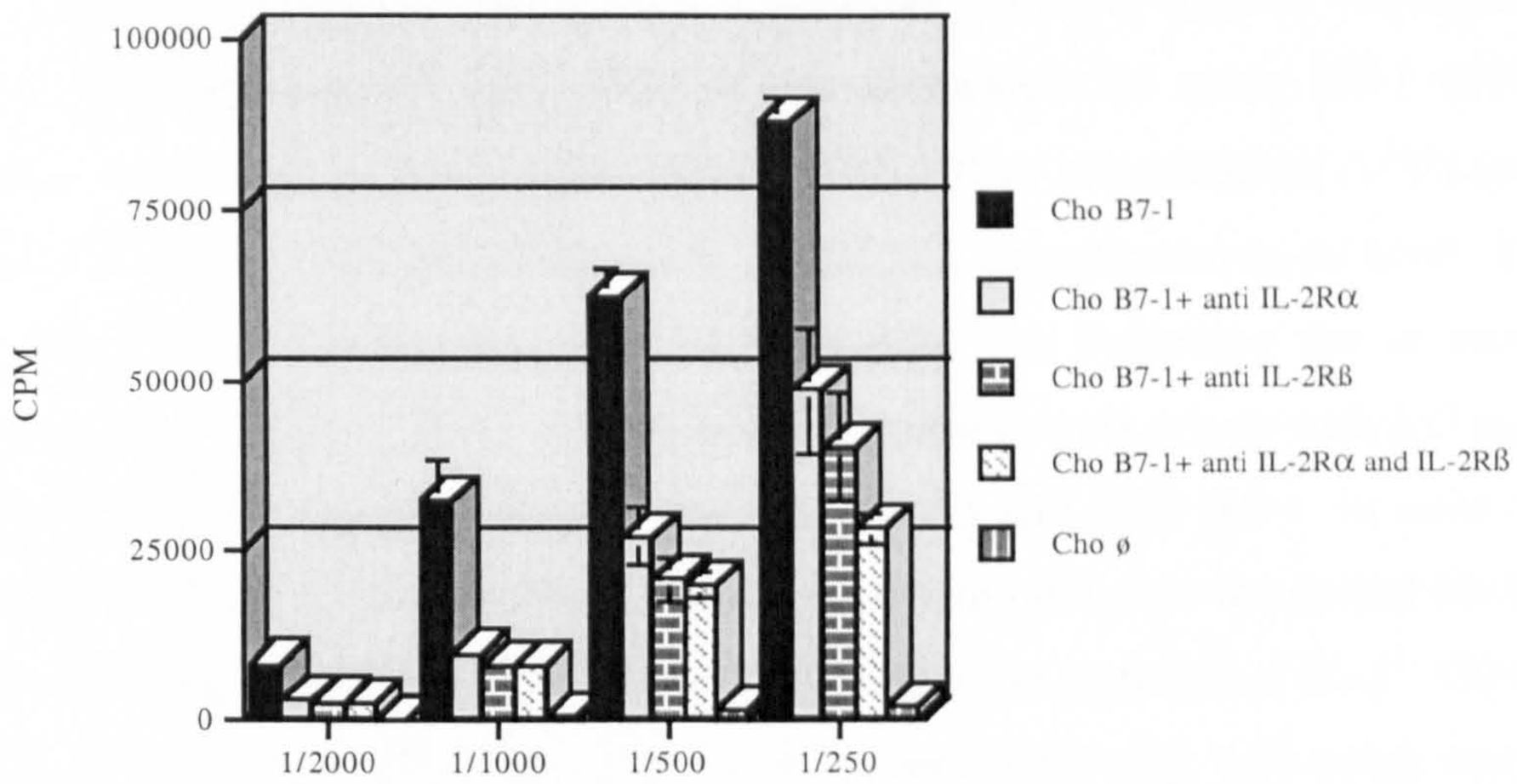


Figure 4.4C **Inhibition of WT C57BL/6 CD4⁺ T cells responses using IL-2 receptor subunit blocking antibodies.**

$2x10^5$ purified $CD4^+$ T cells were set up with paraformaldehyde-fixed CHO B7-1 or CHO ø and 145-2C11. Anti IL-2Rα and anti IL-2Rß mAb were added independently or combined at 5 μg/ml as indicated. Cultures were set up for 72 hrs and pulsed with 1μCi [^{3}H] for the last 12 hrs of incubation. The results shown are the mean and SD of triplicate cultures.

4.3.2-Comparison of IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4^{+} T cells CHO B7-1/anti-CD3-induced proliferative responses:

Previous studies have demonstrated that IL-2 deficient mice had reduced polyclonal immune T cell responses *in vitro*. One of the advantages of using B7-1 CHO transfectants is that they express higher levels of B7-1 than physiological APCs and can therefore deliver a defined costimulatory signal at a supraphysiological level. To examine whether the high levels of B7-1 molecules can overcome the *in vitro* hyporesponsiveness of IL-2$^{-/-}$CD4^{+} T lymphocytes, cultures were set up with KO and WT purified CD4^{+} T cells and stimulated with anti-CD3 and CHO B7-1. In most of the *in vitro* proliferation assays, DNA synthesis was measured over the period 60-72 hrs by the incorporation of [^{3}H] thymidine. The proliferative response of IL-2$^{-/-}$ CD4^{+} T cells was at best 2 fold lower than values of WT counterparts (fig. 4.5) and at worst 4 to 8 fold lower than WT counterparts. Furthermore, the KO response to 5µg/ml Con A was lower than to 2.5µg/ml unlike the WT response (figures 4.1A/4.2A and 4.1B/4.2B). This indicates that, although responsive to B7-1/TCR activation signals, IL-2$^{-/-}$ CD4^{+} T cells are hyporesponsive *in vitro*. The addition of exogenous IL-2 to IL-2$^{-/-}$ CD4^{+} T cells restored their proliferative responses towards WT CD4^{+} T cell levels (fig. 4.6). Therefore, when given IL-2, IL-2$^{-/-}$ CD4^{+} T lymphocytes are able to respond efficiently and utilise their IL-2 receptor. Moreover, despite the absence of anti-CD3 signalling, exogenous IL-2 synergized with B7-1 costimulatory signals to induce low levels of proliferation in IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4^{+} T cells (fig. 4.6).

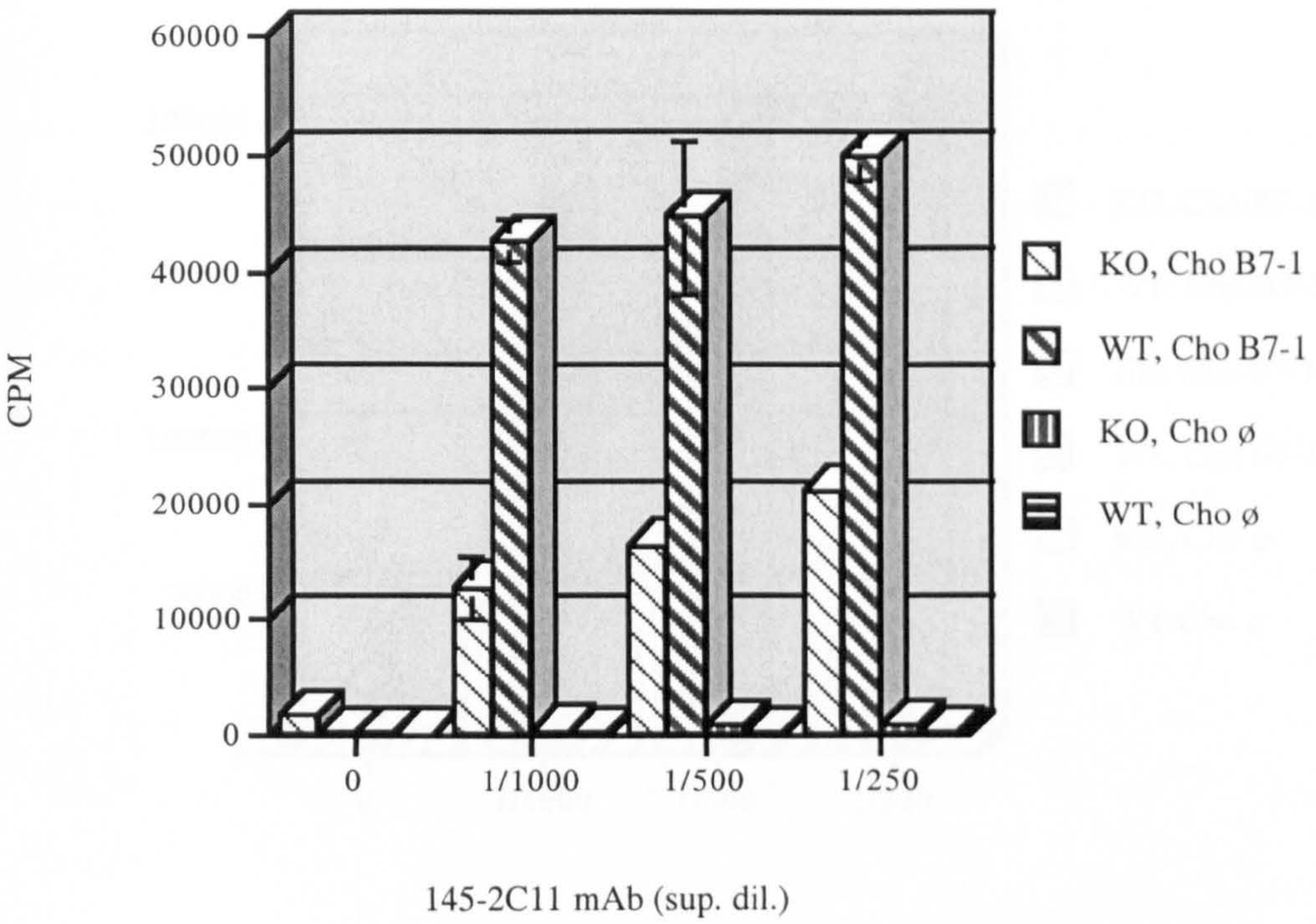


Figure 4.5 **Costimulation of C57BL/6 IL-2 KO versus WT CD4⁺ T lymphocytes by B7-1.**

Microcultures were set up with $2x10^5$ purified cells from IL-$2^{-/-}$ and IL-$2^{+/+}$ mice, $2x10^4$ paraformaldehyde-fixed CHO B7-1 or CHO ø. The x-axis represent the concentration of anti-CD3 mAb 145-2C11 added to the culture. 1μCi of [^{3}H] thymidine per well was added to the culture for the last 12 hrs of the total 72 hrs culture time. The results represent the mean and SD of triplicate cultures. This figure represents the hyporesponsiveness of IL-$2^{-/-}$ CD4⁺ T cells in comparison to IL-$2^{+/+}$ CD4⁺ T cells *in vitro*.

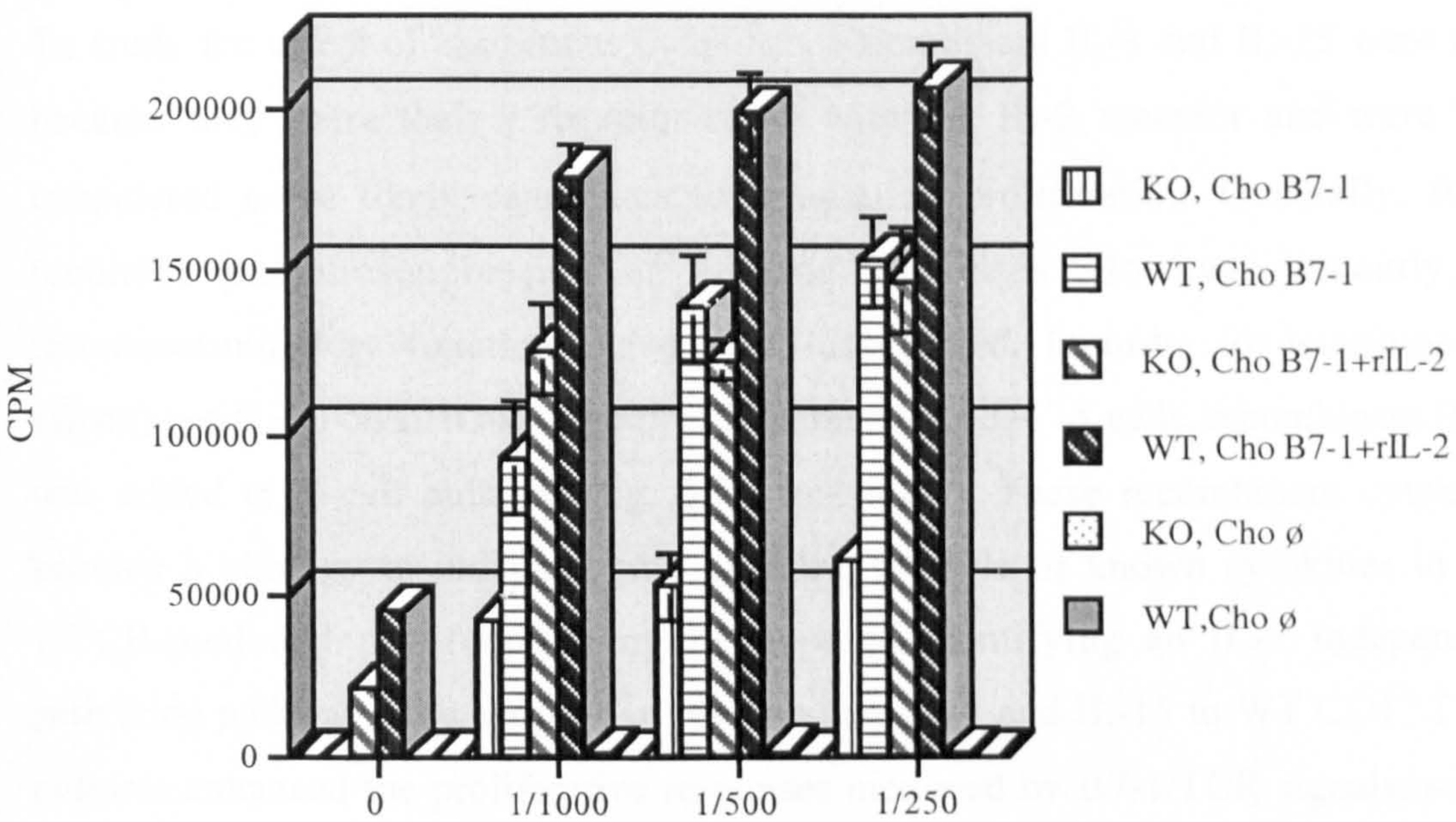


Figure 4.6 **Enhancement of KO and WT CD4⁺ T lymphocytes responses to exogenous IL-2.**

To assay for the effect of exogenous IL-2 on IL-2$^{-/-}$ CD4^{+} T lymphocytes, cultures were set up with $2x10^5$ purified CD4^{+} T cells from WT and KO mice, $2x10^4$ 1% paraformaldehyde-fixed CHO B7-1 or CHO ø, exogenous IL-2 at a concentration of 100 U/ml and anti-CD3 mAb. Culture wells were pulsed after 60 hrs with 1μCi of [^{3}H] thymidine per well for the last 12 hrs of incubation. The results indicated are the mean and SD of triplicate cultures.

4.4-Effect of recombinant cytokines, representing the common γ chain receptor cytokines, on B7-1/TCR induced proliferation of IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4^{+} T lymphocytes.

To study the effect of exogenous cytokines, recombinant IL-4 and IL-15 were used because they share their γ receptor chain with the IL-2 receptor and were thus considered to be likely candidates for mediating proliferation. Generally, IL-10 mediates immunosuppression of immune responses. However, recently, its immunostimulatory functions have been highlighted. In order to determine the effect(s) of IL-10 on CHO B7-1/anti-CD3-stimulated CD4^{+} T cells, recombinant IL-10 was added to T cell cultures (fig. 4.7A and 4.7B). These recombinant cytokines provide a strategy to independently examine the role of known cytokines in B7-1/TCR-mediated proliferation, in the hope of identifying an IL-2 independent activation pathway. The addition of recombinant IL-4 and IL-15 to WT CD4^{+} T cell cultures enhanced the proliferative responses mediated by B7-1/TCR signals; so did IL-10 (fig. 4.7A).

The addition of rIL-4 to IL-2$^{-/-}$ CD4^{+} T lymphocytes enhanced proliferation at low concentrations of anti-CD3 mAb, which is absent at higher concentrations of anti-CD3 (fig. 4.7B). Likewise, rIL-15 only enhanced proliferation of KO CD4^{+} T cells at low concentrations of anti-CD3. Recombinant cytokines may only enhance proliferative responses of KO CD4^{+} T cells at low concentrations of anti-CD3. At higher concentrations of anti-CD3, the system appeared to get saturated and reached a plateau. Recombinant IL-10 did not have an effect on IL-2$^{-/-}$ CD4^{+} T cells at any concentration of anti-CD3 (fig. 4.7B). This may be a result of the deficiency in IL-2 production by KO CD4^{+} T cells, since IL-10 has been reported to enhance IL-2/IL-4-driven immune responses.

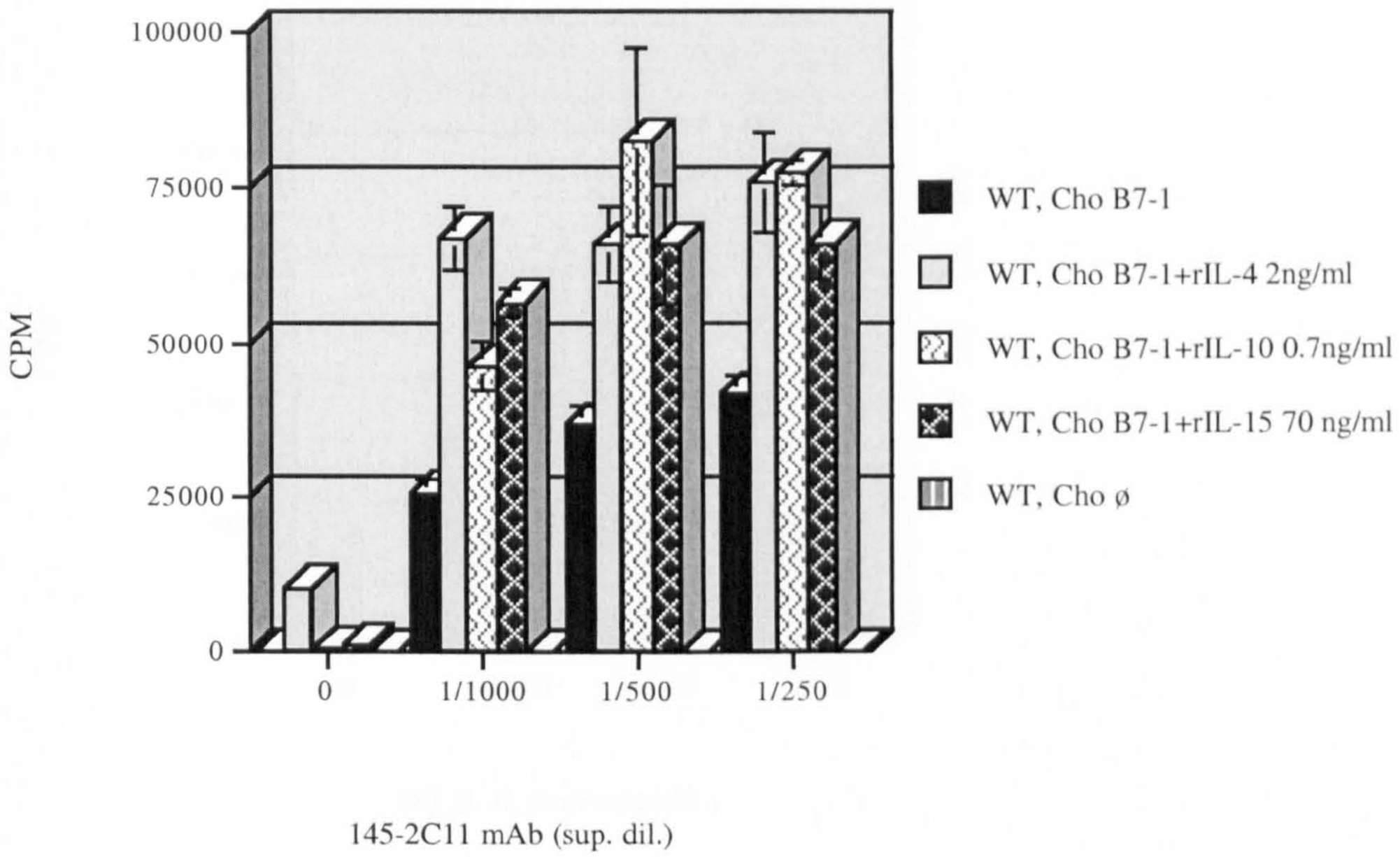


Figure 4.7A **Enhancement of WT CD4$^+$ T lymphocyte proliferation by B7-1 molecules and recombinant cytokines representing the common γ chain cytokine family.**

Microcultures were set up with $2x10^5$ purified CD4$^+$ T lymphocytes, $2x10^4$ paraformaldehyde-fixed transfectant cells and recombinant cytokines; 2ng/ml of rIL-4, 0.7ng/ml of rIL-10 and 70ng/ml of rIL-15. Recombinant IL-4 and IL-15 are ligands for the common γ chain receptor cytokine family. Concentrations of recombinant cytokines were chosen to be marginally above their ED50 (as described by product description information). The results shown represent the mean and SD of triplicate cultures.

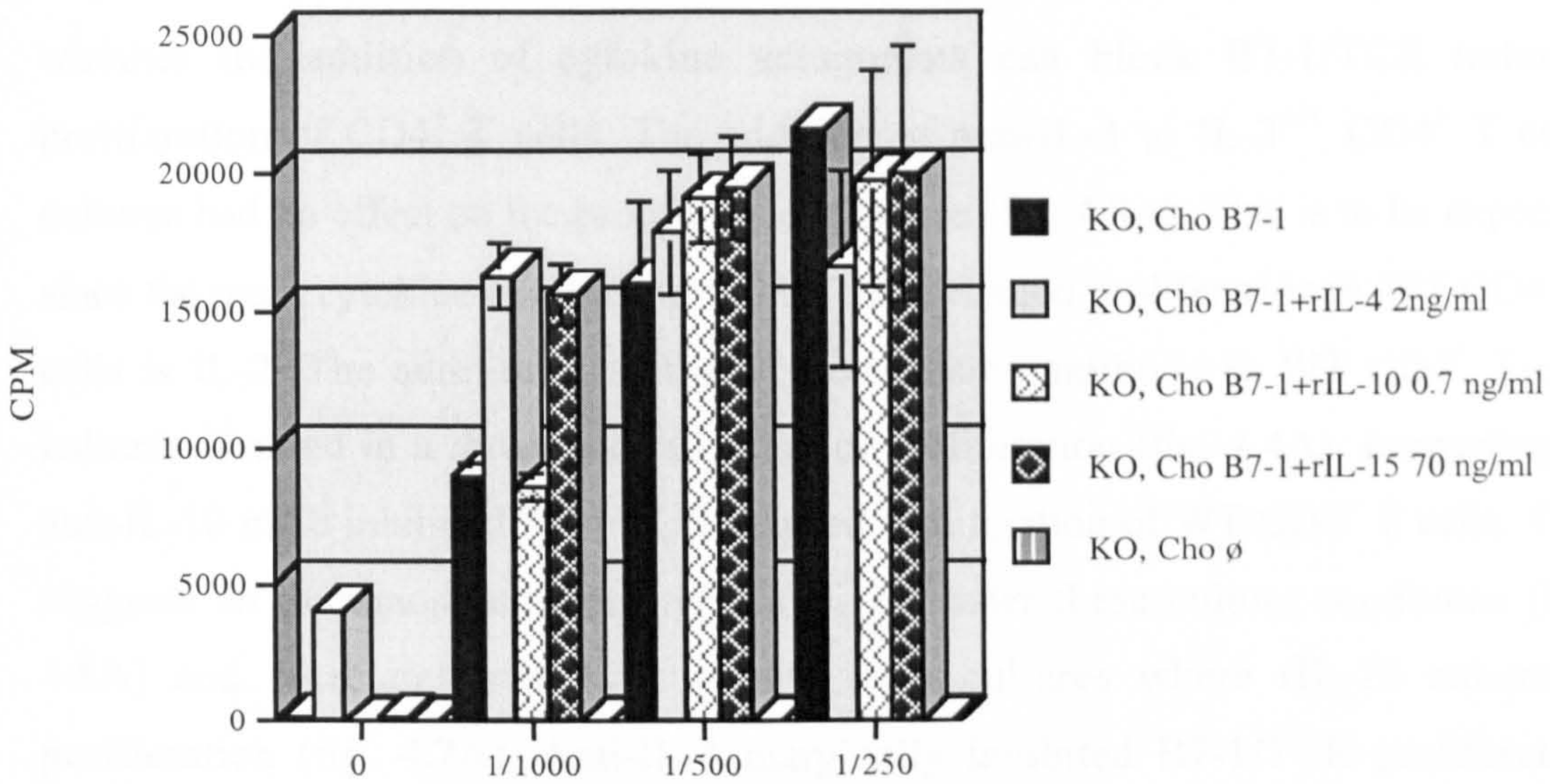


Figure 4.7B **Enhancement of KO CD4⁺ T lymphocytes proliferation by B7-1 molecules and recombinant cytokines representing the common γ chain cytokine family.**

Microcultures were set up as described in the legend 4.7A. The results indicated are the mean and SD of triplicate cultures. This figure depicts the effect of exogenous recombinant cytokines, representing the common γ chain cytokine family with the exception of IL-10, on IL-$2^{-/-}$ $CD4^+$ T cells.

4.5-Effect of anti-IL-4 and anti-IL-10 mAbs on the B7-1/anti-CD3 induced proliferation of IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4^{+} T cells.

In parallel to recombinant cytokine studies, anti-cytokine antibodies were used to test whether the addition of cytokine antagonists can block B7-1/TCR induced proliferation of CD4^{+} T cells. The addition of anti-IL-4 to IL-2$^{+/+}$ CD4^{+} T cells cultures had no effect on the proliferative response (fig. 4.8A). This is to be expected since the main cytokine involved in B7-1/TCR mediated proliferation in WT CD4^{+} T cells is IL-2. The addition of anti-IL-2 neutralising antibody to WT CD4^{+} T cell cultures resulted in a three fold reduction of proliferation (fig. 4.4A). Interestingly, anti-IL-10 mAb inhibited B7-1/TCR induced proliferation of WT CD4^{+} T cells. This suggests an immunostimulatory role of IL-10 under these culture conditions (fig. 4.8A) and correlates well with results from cultures where rIL-10 enhanced proliferation (fig. 4.7A). Anti-IL-4 marginally inhibited B7-1/TCR proliferative responses in IL-2$^{-/-}$ CD4^{+} T cells (fig. 4.8B). This indicates that IL-4 may play a role in IL-2$^{-/-}$ CD4^{+} T cell proliferation. Anti-IL-10 inhibited B7-1/TCR proliferation of IL-2$^{-/-}$ CD4^{+} T lymphocytes, suggesting that IL-10 produced in cultures may mediate proliferation (fig. 4.8B). In separate experiments with WT CD4^{+} T cells, anti-IL-4 ascites (11B11) at a concentration of 1:1000 did not inhibit IL-2$^{+/+}$ CD4^{+} T cells (fig. 4.8C). This confirms results where anti-IL-4 purified mAb was used to treat WT CD4^{+} T cells (fig. 4.8A). In fact, anti-IL-4 ascites marginally enhanced the proliferative responses of WT CD4^{+} T cells; this is probably due to other growth factors present in the ascites fluid.

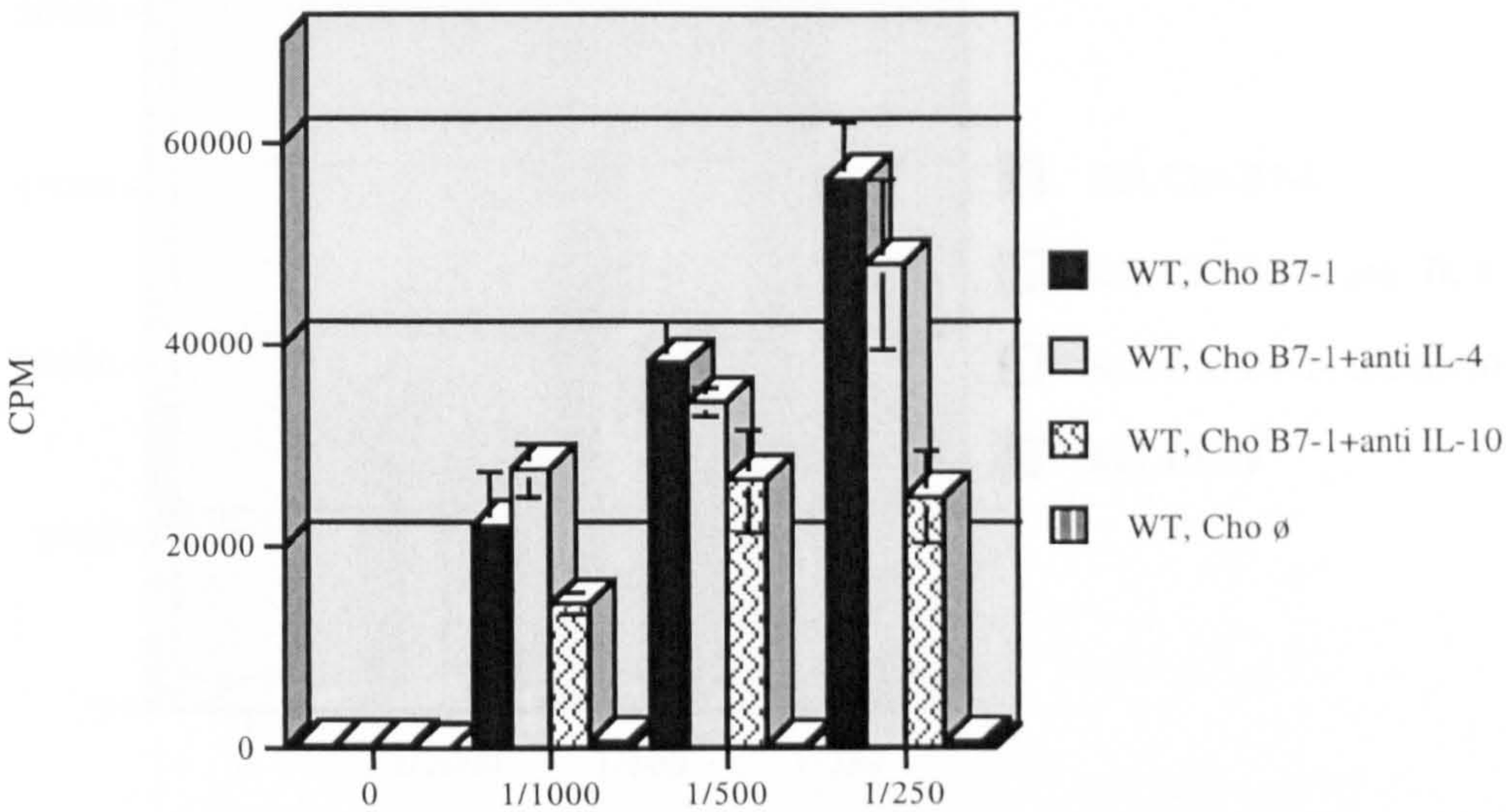


Figure 4.8A **WT C57BL/6 CD4$^+$ T cells stimulated with 145-2C11 and different cytokine blocking antibodies.**

Microcultures were set up using $2x10^5$ purified CD4$^+$ T cells, $2x10^4$ 1% paraformaldehyde-fixed CHO B7-1 or CHO ø, anti CD3 mAb at varying concentrations and anti-IL-4 at 1μg/ml or anti-IL-10 at 1μg/ml as indicated. Cultures were incubated for 72 hrs and pulsed with 1μCi [^{3}H] thymidine per well for the last 12 hrs of culture to assay for CD4$^+$ T cell proliferation. The results shown are the mean and SD of triplicate cultures.

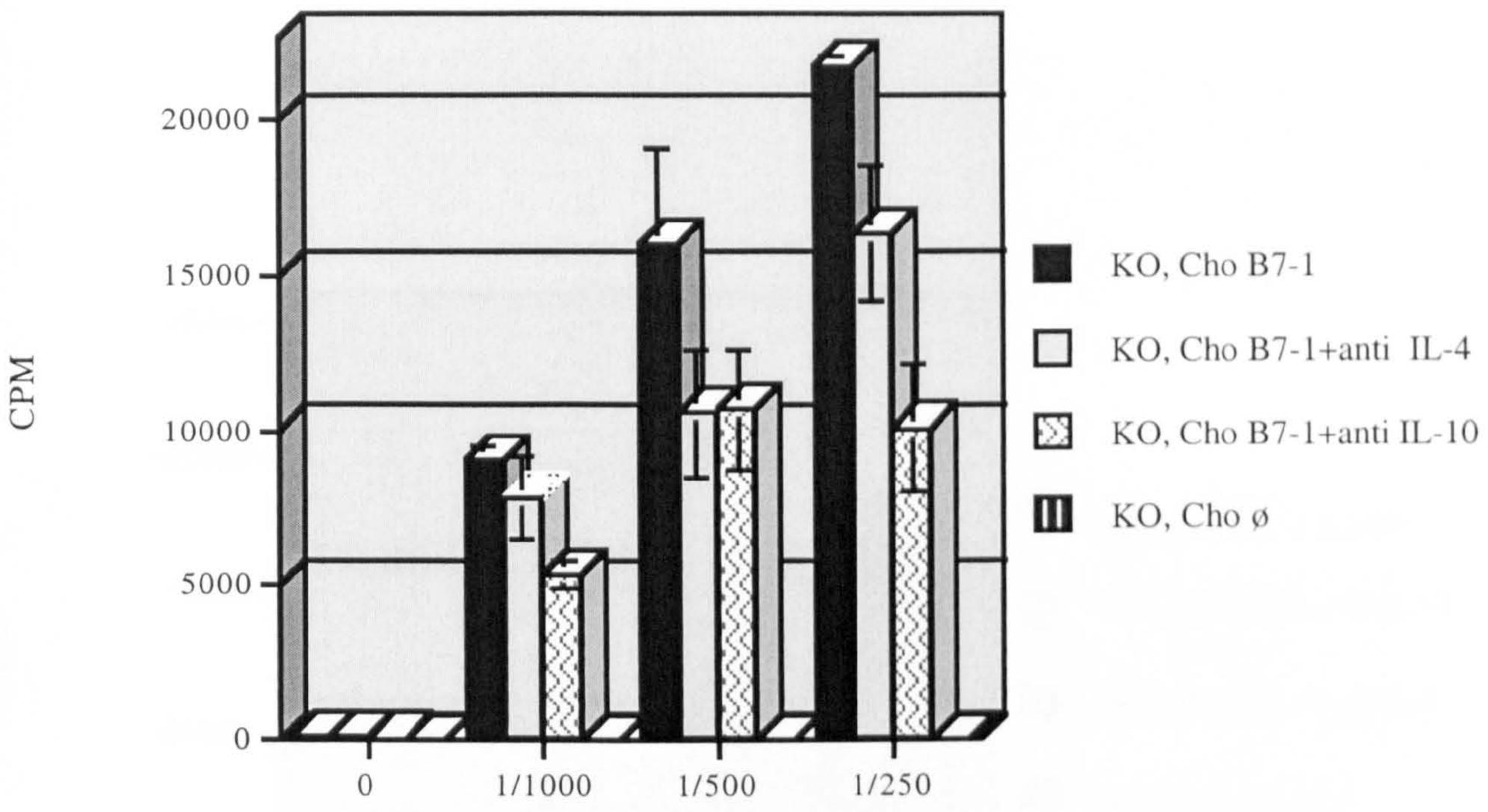


Figure 4.8B **The inhibitory effect of anti IL-4 and anti IL-10 on C57BL/6 KO $CD4^+$ T cells.**

Microcultures were set up as described in figure 4.8A. Cultures were incubated for 72 hrs and pulsed with 1μCi [^{3}H] thymidine per well for the last 12 hrs of culture to assay for $CD4^+$ T cell proliferation.

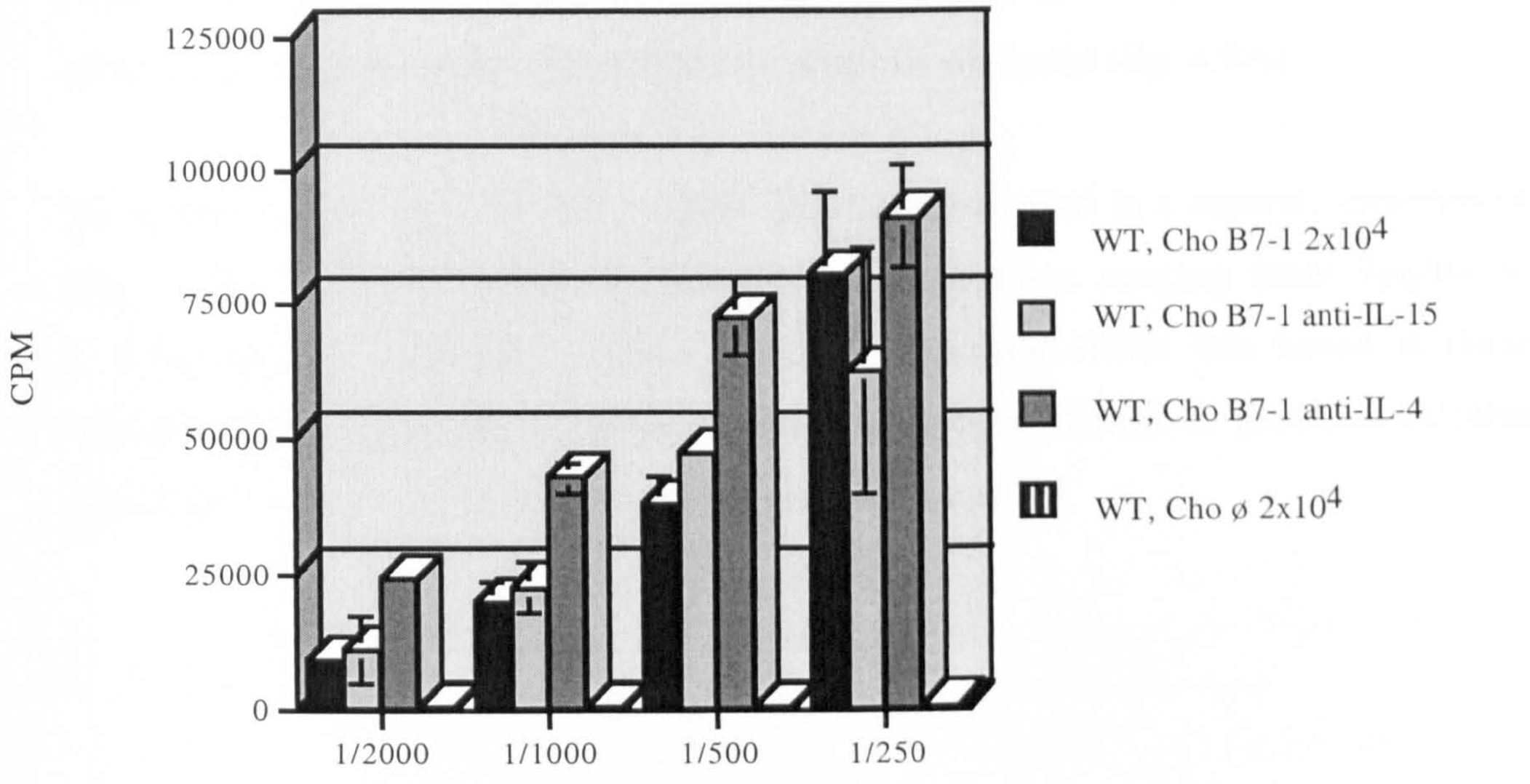


Figure 4.8C **WT C57BL/6 CD4⁺ T cells stimulated with 145-2C11 and different cytokine blocking antibodies.**

Microcultures were set up using $2x10^5$ purified $CD4^+$ T cells, $2x10^4$ 1% paraformaldehyde-fixed CHO B7-1 or CHO ø, anti-CD3 mAb at varying concentrations and anti-IL-4 at 1:1000 diluted ascites and anti-IL-15 mAb at 5 μg/ml.

Cultures were incubated for 72 hrs and pulsed with 1 μCi [^{3}H] thymidine per well for the last 12 hrs of culture to assay for DNA replication.

4.6-The role of IL-15 in B7-1/TCR induced proliferation of IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4^{+} T lymphocytes in vitro.

IL-15 shares many biological activities with IL-2 as well as utilising the β and γ chains of the IL-2 receptor in association with its specific α chain. Consequently, IL-15 was postulated to play a significant role in B7-1/TCR-induced proliferation of IL-2$^{-/-}$ CD4^{+} T cells. Figures 4.8C and 4.9A indicate that anti-IL-15 mAb did not inhibit IL-2$^{+/+}$ CD4^{+} T cell activation. However, a partial block to proliferation of IL-2$^{-/-}$ CD4^{+} T cells at low concentrations of anti-CD3 *in vitro* was observed (fig. 4.9A).

To further clarify these results, purified anti-CD3 was used in a separate experiment (fig. 4.9B). Anti-CD3 mAb was used at concentrations ranging from 3μg/ml to 0.375μg/ml. As depicted in figure 4.9B, no blocking effect was noted at these concentrations of anti-CD3; these results were similar results to previous cultures stimulated with anti-CD3 supernatant (figures 4.9A and B).

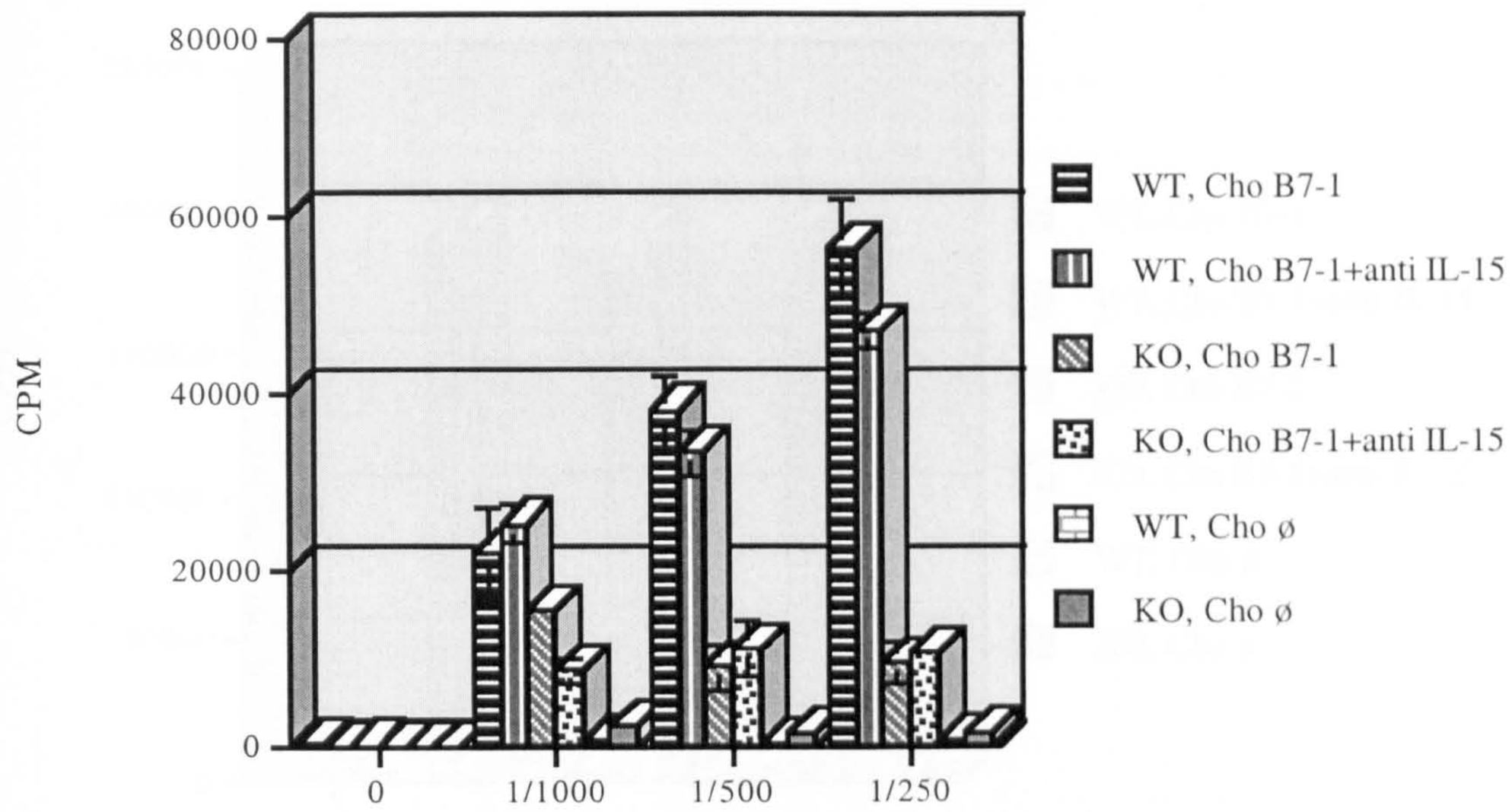


Figure 4.9A **Anti-mIL-15 blocking antibody does not strongly inhibit the proliferation of purified IL-2$^{-/-}$ and IL-2$^{+/+}$ CD4$^+$ T lymphocytes *in vitro*.**

Cultures were set up with $2x10^5$ purified CD4$^+$ T cells, $2x10^4$ 1% paraformaldehyde-fixed CHO B7-1 and CHO ø transfectant cells, anti-CD3 mAb (145-2C11) and anti IL-15 mAb at 5μg/ml.

Cultures were incubated for 72 hrs and pulsed with 1μCi [^{3}H] for the last 12 hrs to assay for T cell proliferation in the presence of anti IL-15 mAb. The results shown are the mean and SD of triplicate cultures.

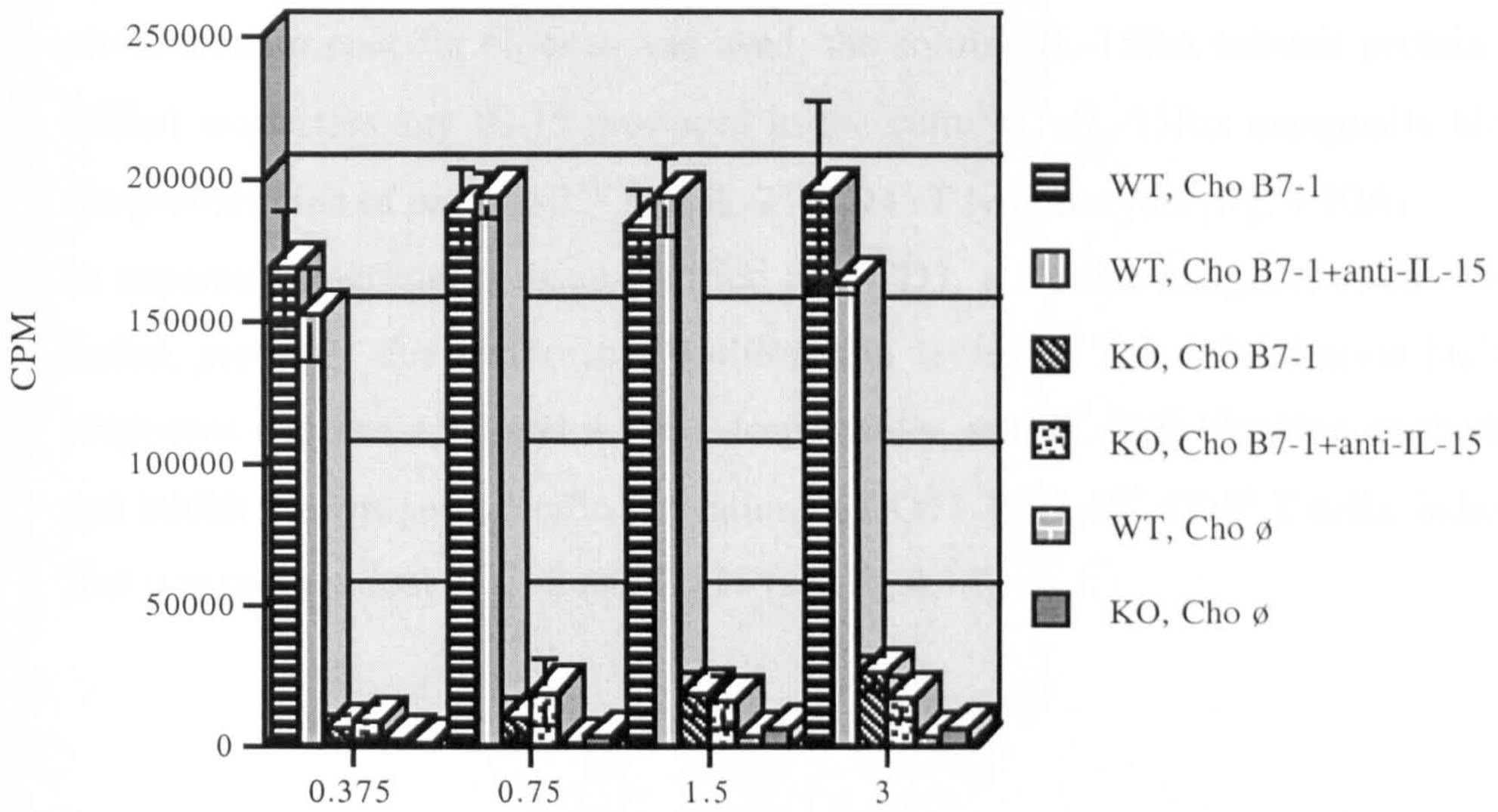


Figure 4.9B **Anti-mIL-15 blocking antibody does not strongly inhibit the proliferation of purified IL-2$^{-/-}$ and IL-2$^{+/+}$ CD4^{+} T lymphocytes *in vitro*.**
Cultures were set up as described in figure 4.9A. Cultures were incubated for 72 hrs and pulsed with 1μCi [^{3}H] for the last 12 hrs to assay for T cell proliferation in the presence of anti IL-15 mAb. The results shown are the mean and SD of triplicate cultures.

4.6.1-Neutralisation of IL-15 using sIL-15Rα:

To look further into the potential role of IL-15 in B7-1/TCR-induced proliferation *in vitro*, another specific blocker was used; the soluble IL-15Rα subunit protein (sIL-15Rα) sequesters any IL-15 produced in the cultures. sIL-15Rα marginally blocked the proliferation of both IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes (fig. 4.10A).

In separate experiments using purified anti-CD3, a similar marginal effect was not noted, probably due to the low proliferation levels of KO cells despite high WT responses (figures 4.9B and 4.10B). Importantly, anti-IL-2Rβ blocking antibody did not inhibit the antigen-specific activation of DO11-10 IL-$2^{-/-}$ $CD4^+$ T cells, indicating that it is independent of IL-2 and IL-15 (see fig.4.12).

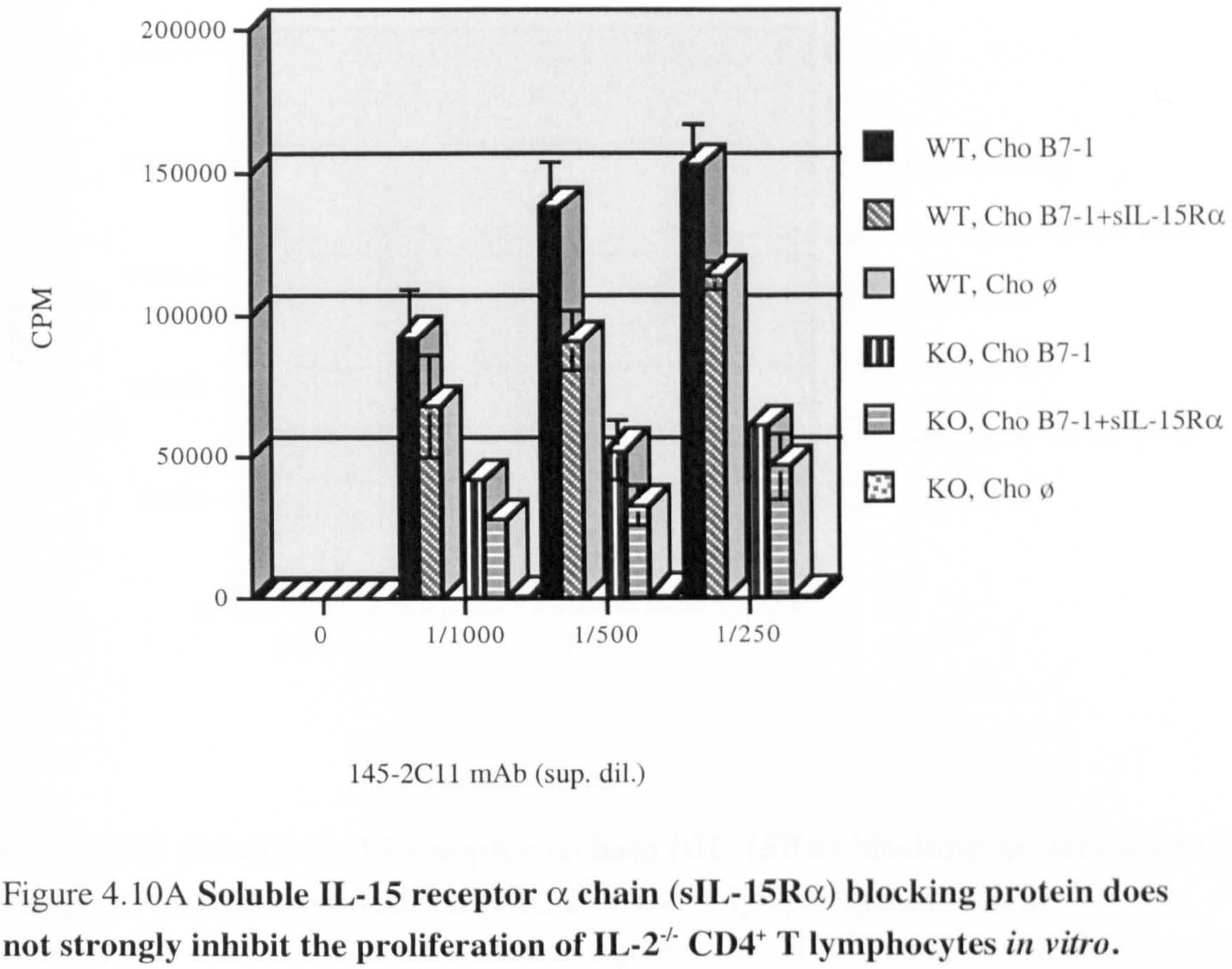


Figure 4.10A **Soluble IL-15 receptor α chain (sIL-15Rα) blocking protein does not strongly inhibit the proliferation of IL-2$^{-/-}$ CD4^{+} T lymphocytes *in vitro*.**
Microcultures were set up with $2x10^5$ CD4^{+} T cells, $2x10^4$ paraformaldehyde-fixed CHO B7-1 or CHO ø transfectant cells, soluble anti-CD3 mAb and murine IL-15Rα fragment at 200 ng/ml. To assay T cell proliferation, cultures were pulsed after 60 hrs with 1μCi [^{3}H] thymidine for the last 12 hrs of incubation. The results shown represent the mean and SD of triplicate cultures.

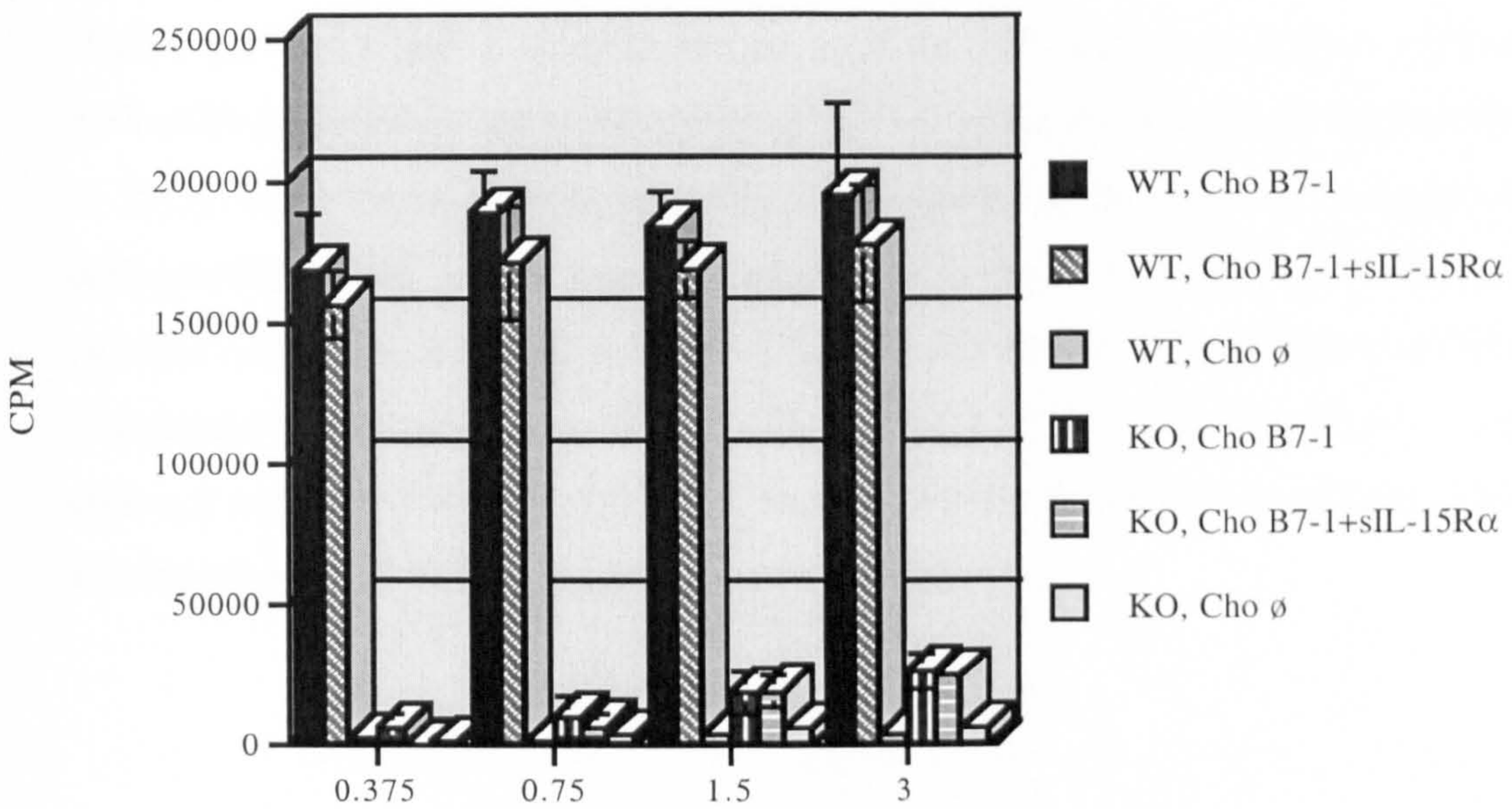


Figure 4.10B **Soluble IL-15 receptor α chain (sIL-15Rα) blocking protein does not strongly inhibit the proliferation of IL-2$^{-/-}$ CD4^{+} T lymphocytes in vitro.**
Microcultures were set up as described in figure 4.10A. The results shown represent the mean and SD of triplicate cultures.

4.7-Effect of bypassing the costimulatory signal(s) provided by CHO B7-1 on IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4^{+} T cells.

Highly purified CD4^{+} T cells do not respond to TCR signals without costimulatory molecules provided by antigen presenting cells or other pharmacological reagents such as PMA. In order to examine the effects of bypassing the B7-1/TCR signal on KO cells, purified CD4^{+} T cells from KO and WT mice were stimulated with PMA and the calcium ionophore, ionomycin. IL-2$^{-/-}$ CD4^{+} T cells were hyporesponsive to PMA and ionomycin in comparison to IL-2$^{+/+}$ CD4^{+} T cells (fig. 4.11). Thus, IL-2$^{-/-}$ CD4^{+} T cells appear to have an intrinsic defect in their *in vitro* proliferative capacity. This was also true for IL-2$^{-/-}$ CD4^{+} antigen specific T cells.

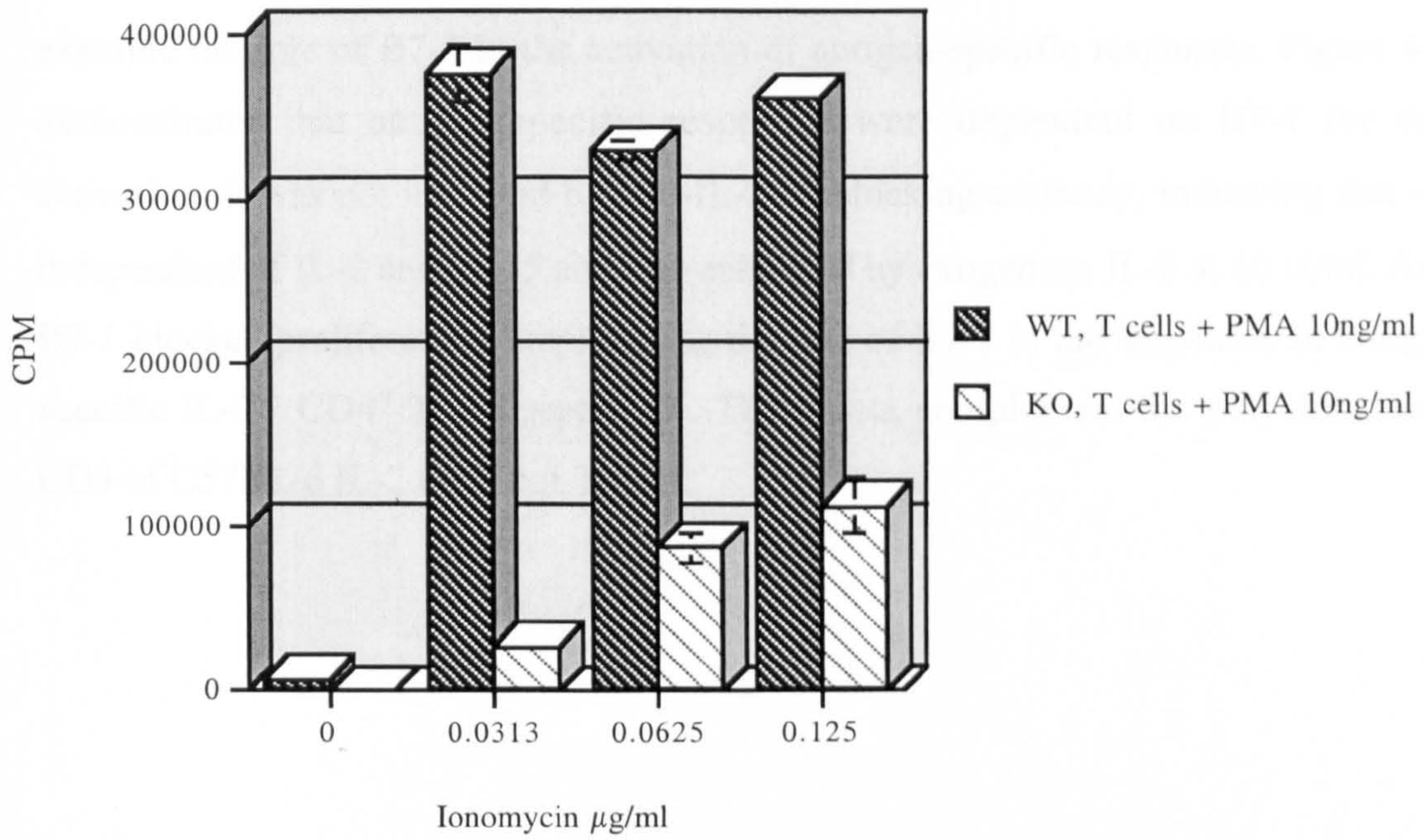


Figure 4.11 **Effect of bypassing the costimulatory signal provided by CHO B7-1 on KO and WT CD4⁺ T lymphocytes.**
T cell cultures were set up with $2x10^5$ purified $CD4^+$ T cells, 10ng/ml phorbol 12-myristate 13-acetate (PMA), and varying concentrations of calcium ionophore. To assay for T cell proliferation, cultures were pulsed after 60 h with 1 μCi of [^{3}H] thymidine per well for the last 12 hrs of incubation. Displayed on the x-axis are the concentrations of ionomycin μg/ml. The results shown are the mean and SD of triplicate cultures.

4.8-B7-1-induced proliferation of antigen-specific DO11-10 IL-2$^{-/-}$ CD4^{+} T lymphocytes.

BALB/c OVA-specific H2-A^{d}-restricted DO11-10 IL-2$^{-/-}$ CD4^{+} T cells were used to examine the role of B7-1 in the activation of antigen-specific responses. Figure 4.12 demonstrates that antigen-specific responses were dependent on B7-1 for their activation; it was not inhibited by anti-IL-2Rβ blocking antibody, indicating that it is independent of IL-2 and IL-15 and was enhanced by exogenous IL-2 at 10 U/ml. Anti-B7-1 blocked proliferation, emphasising the role of B7-1 in the activation of antigen-specific IL-2$^{-/-}$ CD4^{+} T cell responses. These data complement the polyclonal anti-CD3 of C57BL/6 IL-2 deficient T cells.

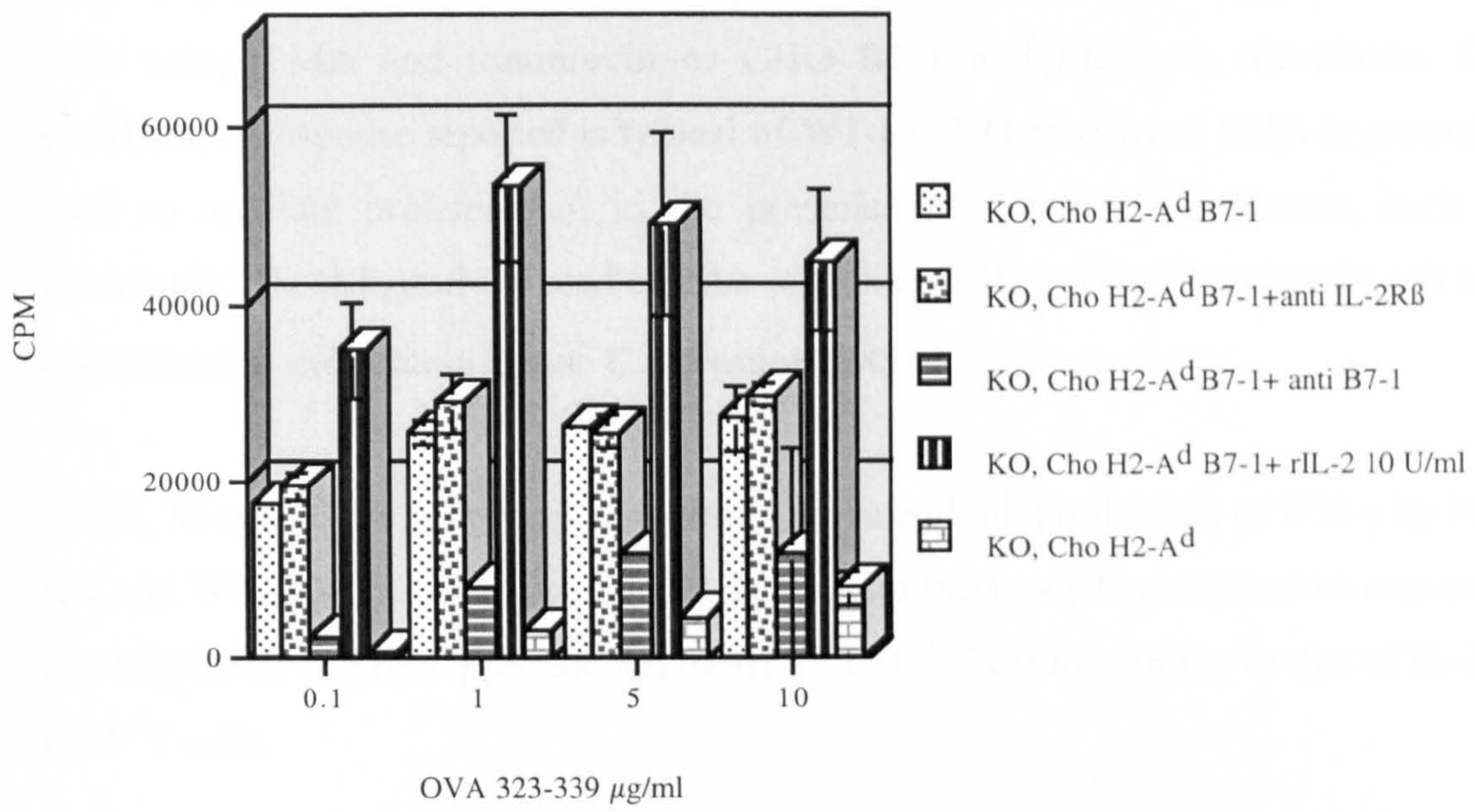


Figure 4.12 **Enhancement of BALB/c antigen-specific IL-2$^{-/-}$ CD4$^+$ T cell activation by B7-1.**

OVA-specific H2A^d-restricted DO11.10 IL-2$^{-/-}$ CD4$^+$ T cells ($2x10^5$) were cultured with varying amounts of OVA peptide 323-339 and $2x10^4$ paraformaldehyde-fixed CHO transfectants expressing H2A^d or H2A^d and B7-1 as indicated. Anti IL-2Rß (5μg/ml), anti B7-1 (2μg/ml) and IL-2 (10U/ml) were added as indicated. Cultures were pulsed after 60 hrs with 1 μCi [^{3}H] thymidine/well for the final 12 hrs of incubation. The results shown are the mean and SD of triplicate cultures.

4.9-Analysis of cytokine production by ELISA.

ELISA assays were used to gain insight into cytokine protein production by activated $CD4^+$ T lymphocytes. Figure 4.13A depicts the activation of WT and KO $CD4^+$ T cells using PMA and ionomycin or CHO B7-1 and PMA as stimulants. The proliferative response reported is typical of WT and KO responses. PMA is generally used to mediate proliferation in the presence of calcium ionophores, such as ionomycin. These ligand-induced cellular responses follow a rise in cytosolic calcium concentration and protein kinase C activation (245).

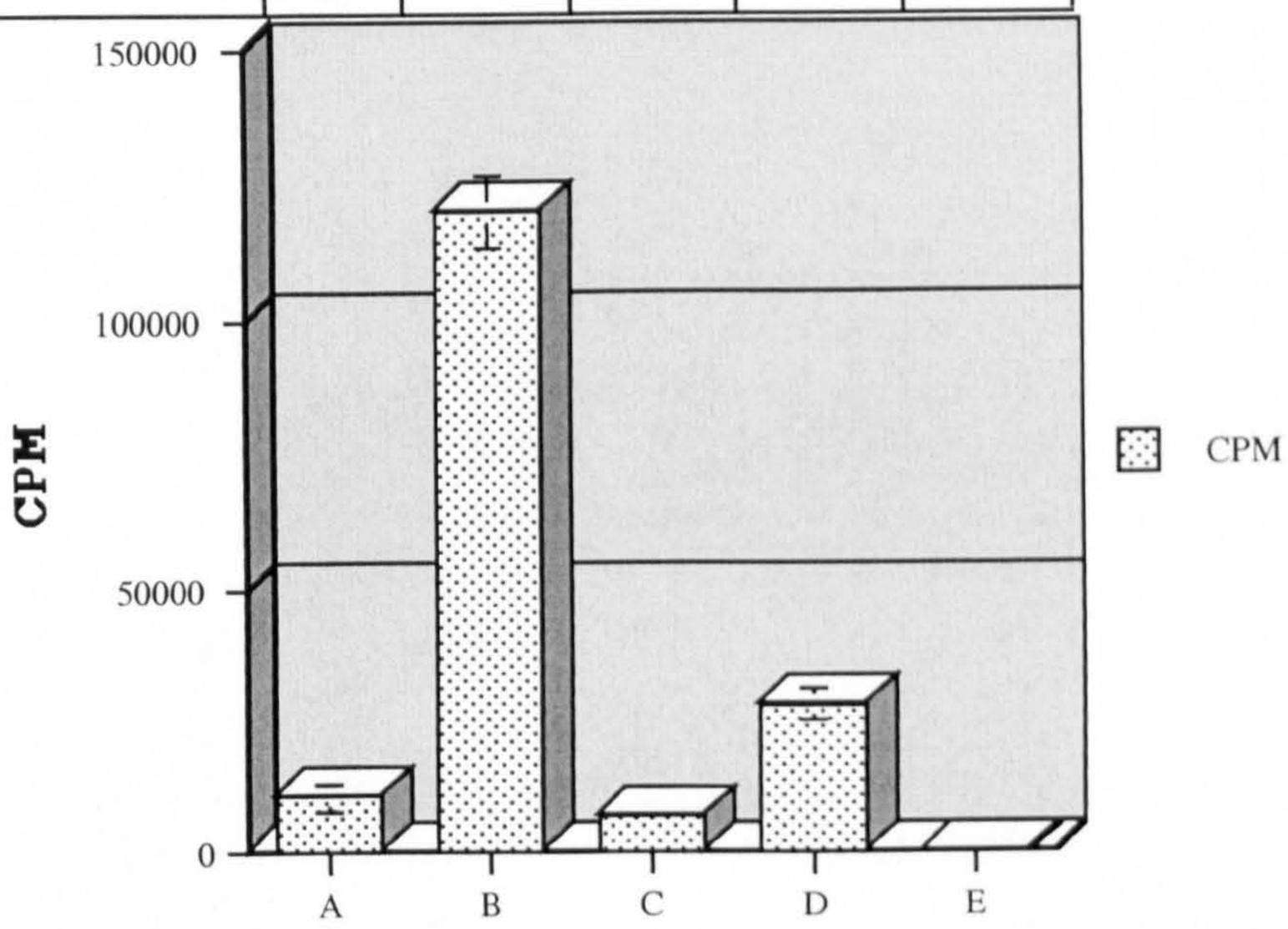
At 60, 72 and 96 hrs following stimulation revealed high production of IFN-γ by both KO and WT $CD4^+$ T cells (fig. 4.13D). In view of the low KO proliferative response, it is surprising that IFN-γ production by IL-$2^{-/-}$ $CD4^+$ T cells is similar to that of IL-$2^{+/+}$ $CD4^+$ T cells.

IL-4 production at 60 hrs was higher in WT $CD4^+$ T cells than KO counterparts, albeit lower than 100pg/ml (fig. 4.13B). IL-4 production values below 100pg/ml may be artefactual. At all three time points, IL-10 production was higher in KO $CD4^+$ T cells, especially with PMA and ionomycin stimulation, than IL-$2^{+/+}$ $CD4^+$ T cells. The difference in IL-10 production between KO and WT cells becomes less clear under CHO B7-1 and PMA stimulation conditions (fig. 4.13C). These ELISA results comprised two separate experiments for each individual time point.

Figure 4.13A **Proliferative responses of IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells stimulated with PMA and ionomycin or CHO B7-1 and PMA.**

Cultures were purified and set up in microcultures at a concentration of $2x10^5$ per well C57BL/6 IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells. CHO transfectants expressing the costimulatory molecule B7-1 and vector-transfected controls were fixed with 1% paraformaldehyde and co-cultured at $2x10^4$ with the purified $CD4^+$ T cells and 10ng/ml PMA. Alternatively, $CD4^+$ T cells were set up with PMA 10ng/ml and ionomycin at 250ng/ml. $CD4^+$ T cells were set up with B7-1 only as a negative control.

PMA 10ng/ml+IONO 250ng/ml	+	+			
PMA 10ng/ml+CHO B7-1			+	+	
CHO B7-1 only					+
WT		+		+	
KO	+		+		+

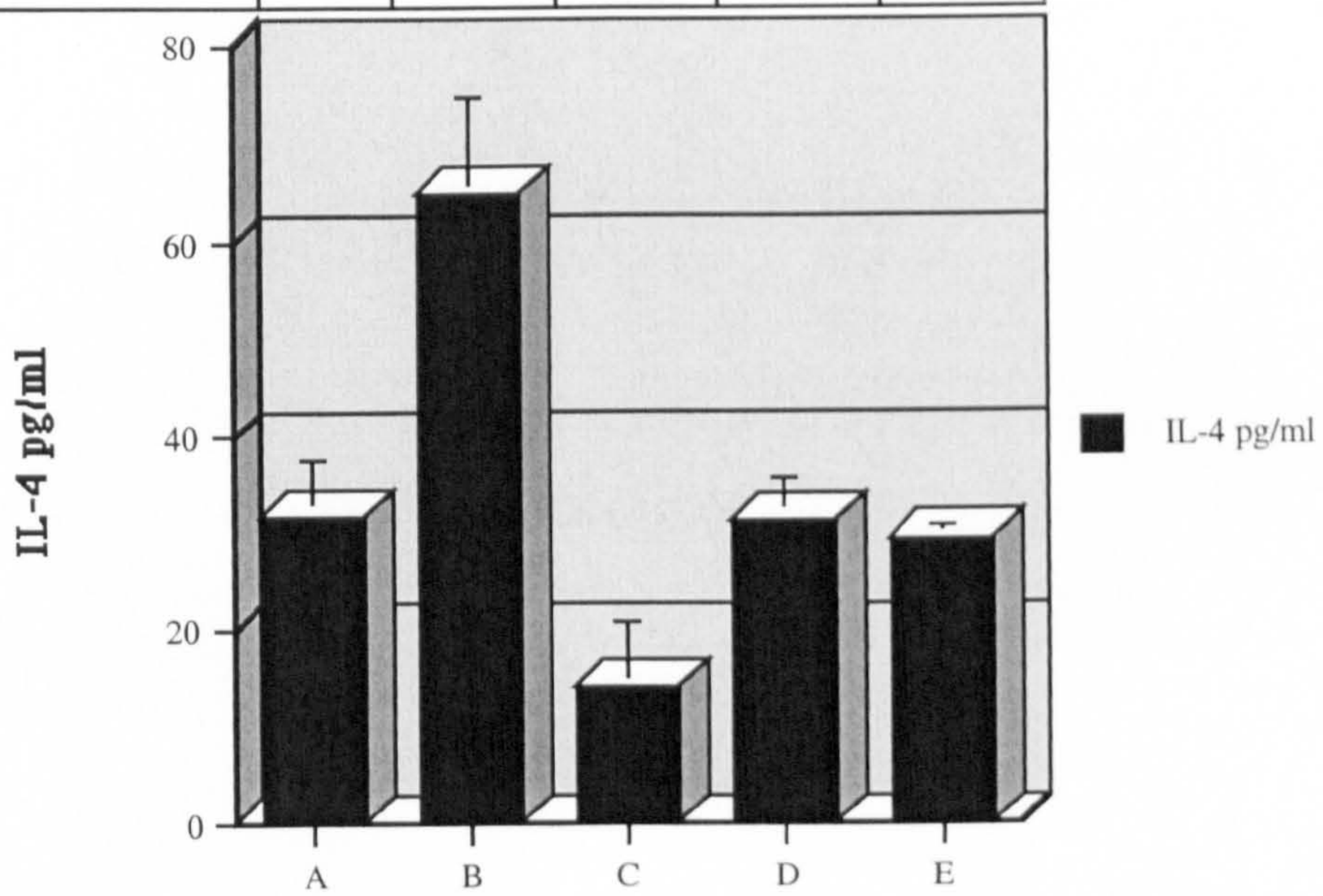


Cell Culture Conditions

Figure 4.13B **IL-4 production by IL-2$^{-/-}$ and IL-2$^{+/+}$ CD4^{+} T lymphocytes stimulated with PMA and ionomycin or CHO B7-1 and PMA at 60 hrs.**

Cultures were set up as described previously and supernatants were harvested and assayed for IL-4 at 60 hrs.

PMA 10ng/ml+IONO 250ng/ml	+	+			
PMA 10ng/ml+CHO B7-1			+	+	
CHO B7-1 only					+
WT		+		+	
KO	+		+		+

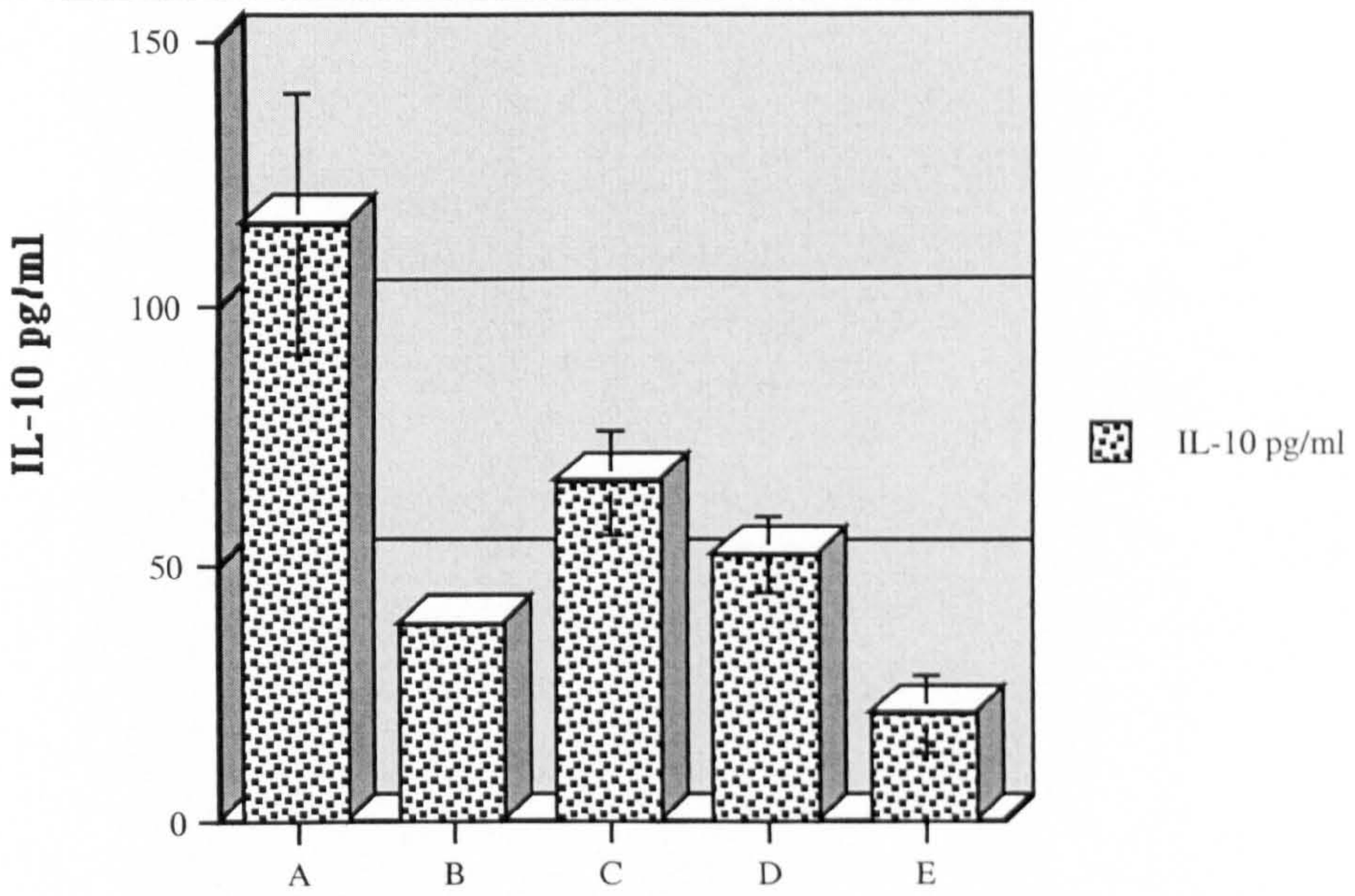


Cell Culture Conditions

Figure 4.13C **IL-10 production by IL-2$^{-/-}$ and IL-2$^{+/+}$ CD4^{+} T lymphocytes stimulated with PMA and ionomycin or CHO B7-1 and PMA at 60 hrs.**

Cultures were set up as described previously and supernatants were harvested and assayed for IL-10 at 60 hrs.

PMA 10ng/ml+IONO 250ng/ml	+	+			
PMA 10ng/ml+CHO B7-1			+	+	
CHO B7-1 only					+
WT		+		+	
KO	+		+		+

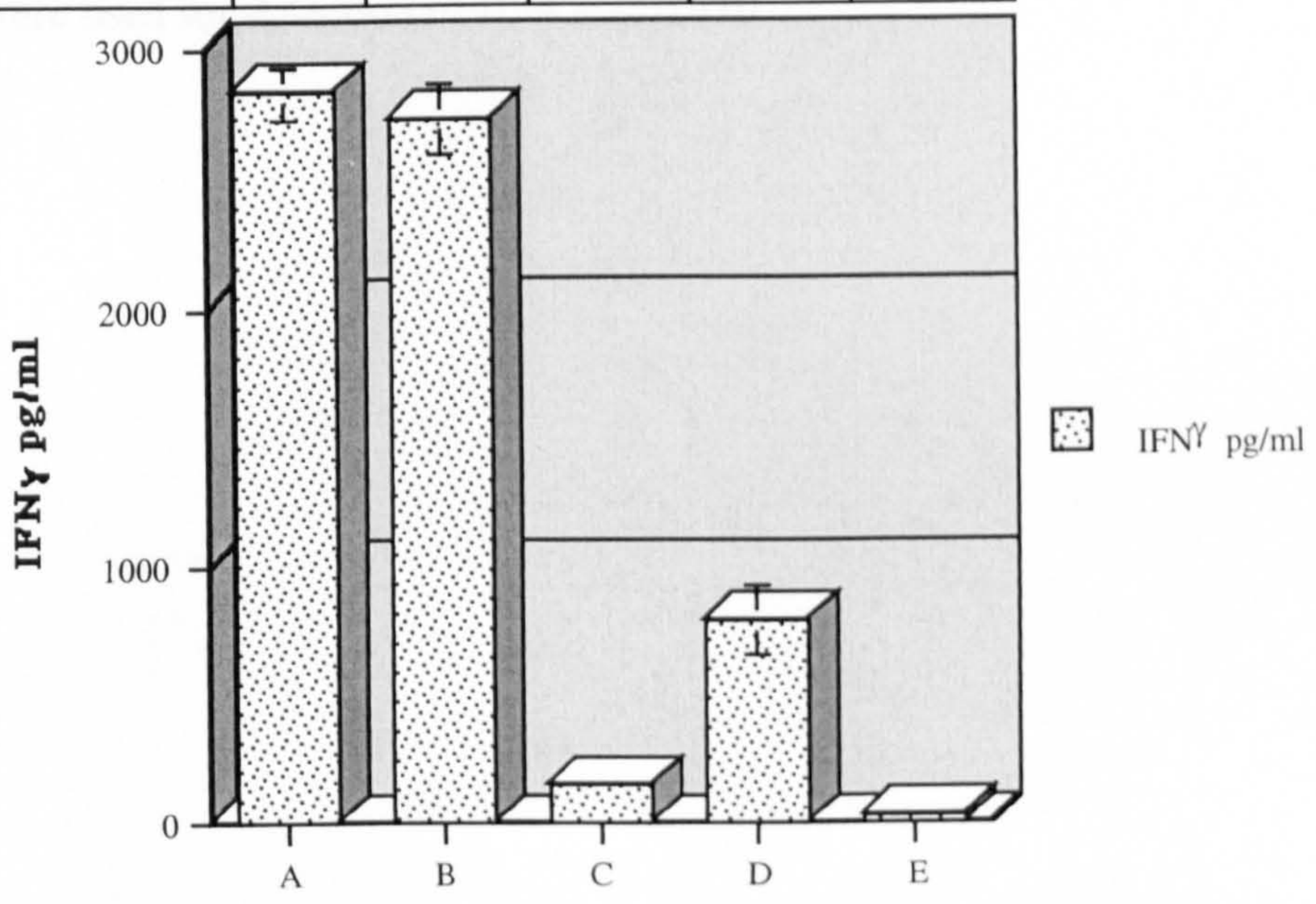


Cell Culture Conditions

Figure 4.13D IFN-γ production by IL-2$^{-/-}$ and IL-2$^{+/+}$ CD4^{+} T lymphocytes stimulated with PMA and ionomycin or CHO B7-1 and PMA at 60 hrs.

T cell cultures were set up as described previously and supernatants were harvested and assayed for IFN-γ at 60 hrs.

PMA 10ng/ml+IONO 250ng/ml	+	+			
PMA 10ng/ml+CHO B7-1			+	+	
CHO B7-1 only					+
WT		+		+	
KO	+		+		+



Cell Culture Conditions

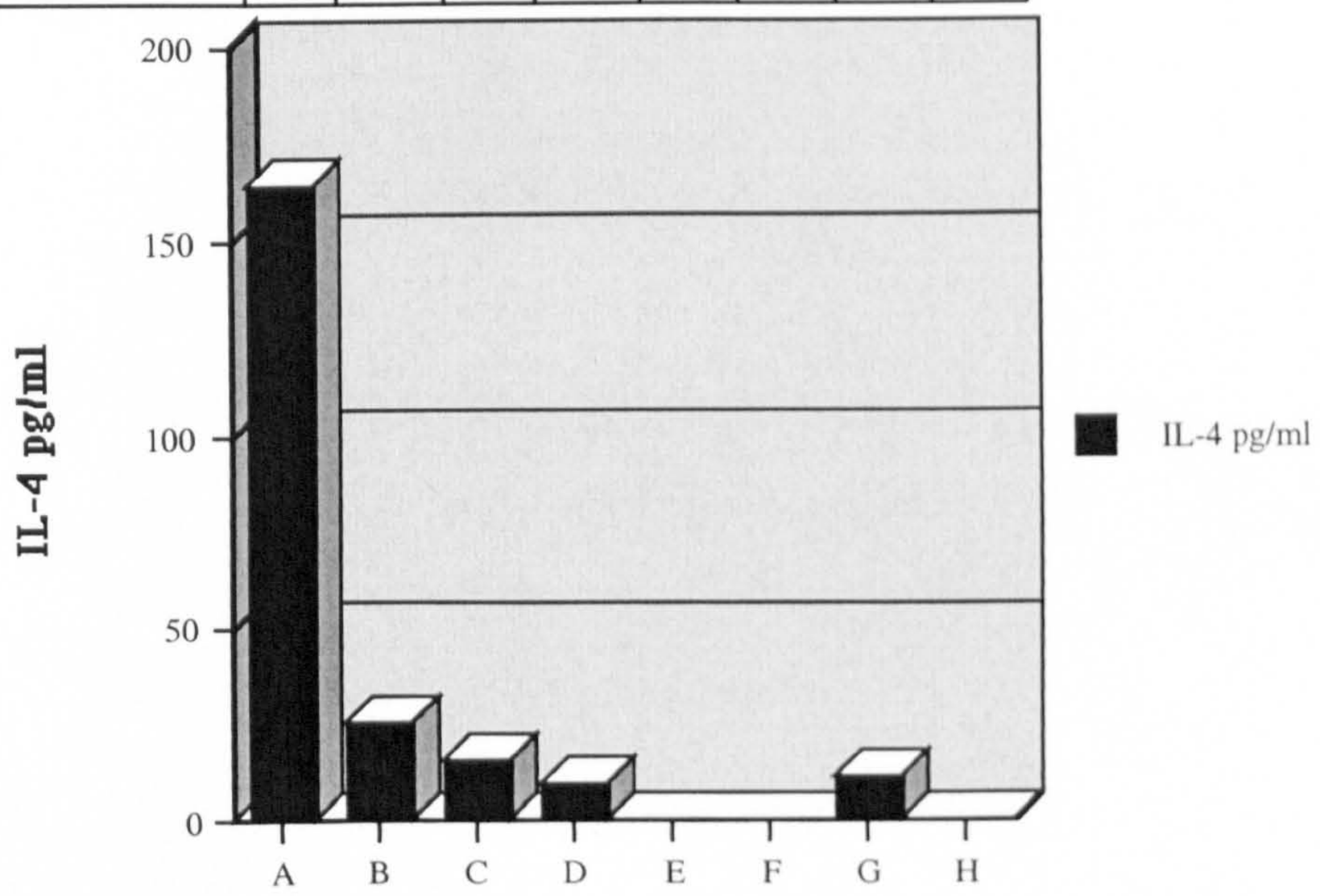
4.9.1-Cytokine production by purified $CD4^+$ T cells at 72 hrs:

At 72 hrs, IL-4 production was dramatically higher in IL-$2^{-/-}$ $CD4^+$ T cells than IL-$2^{+/+}$ $CD4^+$ T cells when stimulated with PMA and ionomycin. Interestingly, IL-4 production by IL-$2^{+/+}$ $CD4^+$ T cells with PMA and ionomycin is halved, in comparison to the 60 hrs time point (fig. 4.14A and 4.13B); this may be an outcome of consumption by the cells during the assay period. At 72 hrs, IL-10 production is further enhanced in IL-$2^{-/-}$ $CD4^+$ T cells under the stimulants PMA and ionomycin, with no significant changes for other stimulation conditions (fig. 4.14B). IFN-γ production, however, remains stable and as seen at the earlier time point is similar in IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells (fig. 4.14C). It is noteworthy that supernatants had to be diluted for both WT and KO $CD4^+$ T cells for the detection of IFN-γ production. Neat supernatants were used for detection of IL-4 and IL-10.

Figure 4.14A **IL-4 production by IL-2$^{-/-}$ and IL-2$^{+/+}$ CD4^{+} T lymphocytes stimulated with PMA and ionomycin or CHO B7-1 and PMA at 72 hrs.**

T cell cultures were set up as described previously and supernatants were harvested and assayed for IL-4 at 72 hrs.

PMA 10ng/ml+IONO 250ng/ml	+	+						
PMA 10ng/ml+CHO B7-1			+	+				
PMA 10ng/ml only					+	+		
CHO B7-1 only							+	+
WT		+		+		+		+
KO	+		+		+		+	



Cell Culture Conditions

Figure 4.14B **IL-10 production by IL-2$^{-/-}$ and IL-2$^{+/+}$ CD4^{+} T lymphocytes stimulated with PMA and ionomycin or CHO B7-1 and PMA at 72 hrs.**

T cell cultures were set up as described previously and supernatants were harvested and assayed for IL-10 at 72 hrs.

PMA 10ng/ml+IONO 250ng/ml	+	+						
PMA 10ng/ml+CHO B7-1			+	+				
PMA 10ng/ml only					+	+		
CHO B7-1 only							+	+
WT		+		+		+		+
KO	+		+		+		+	

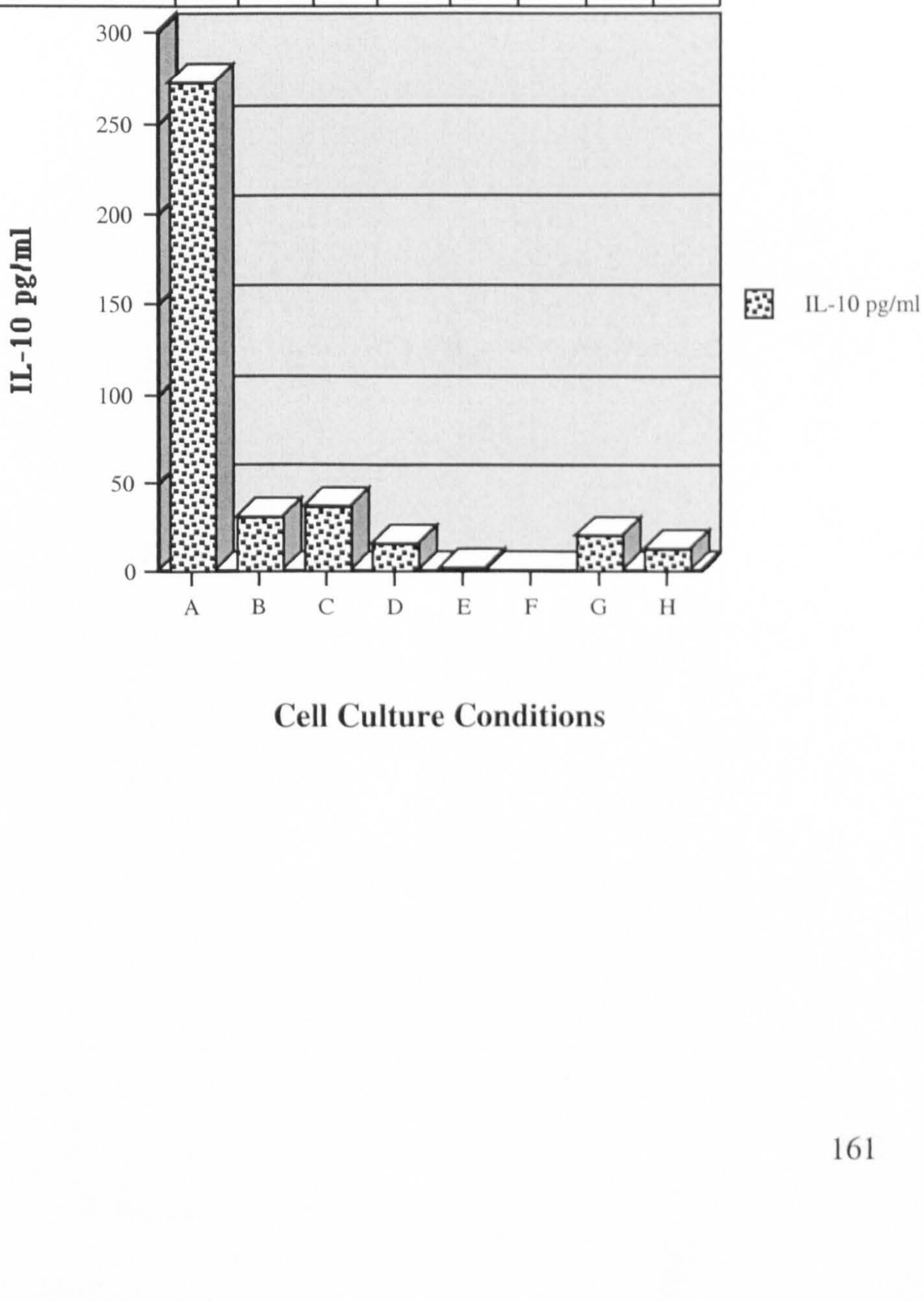
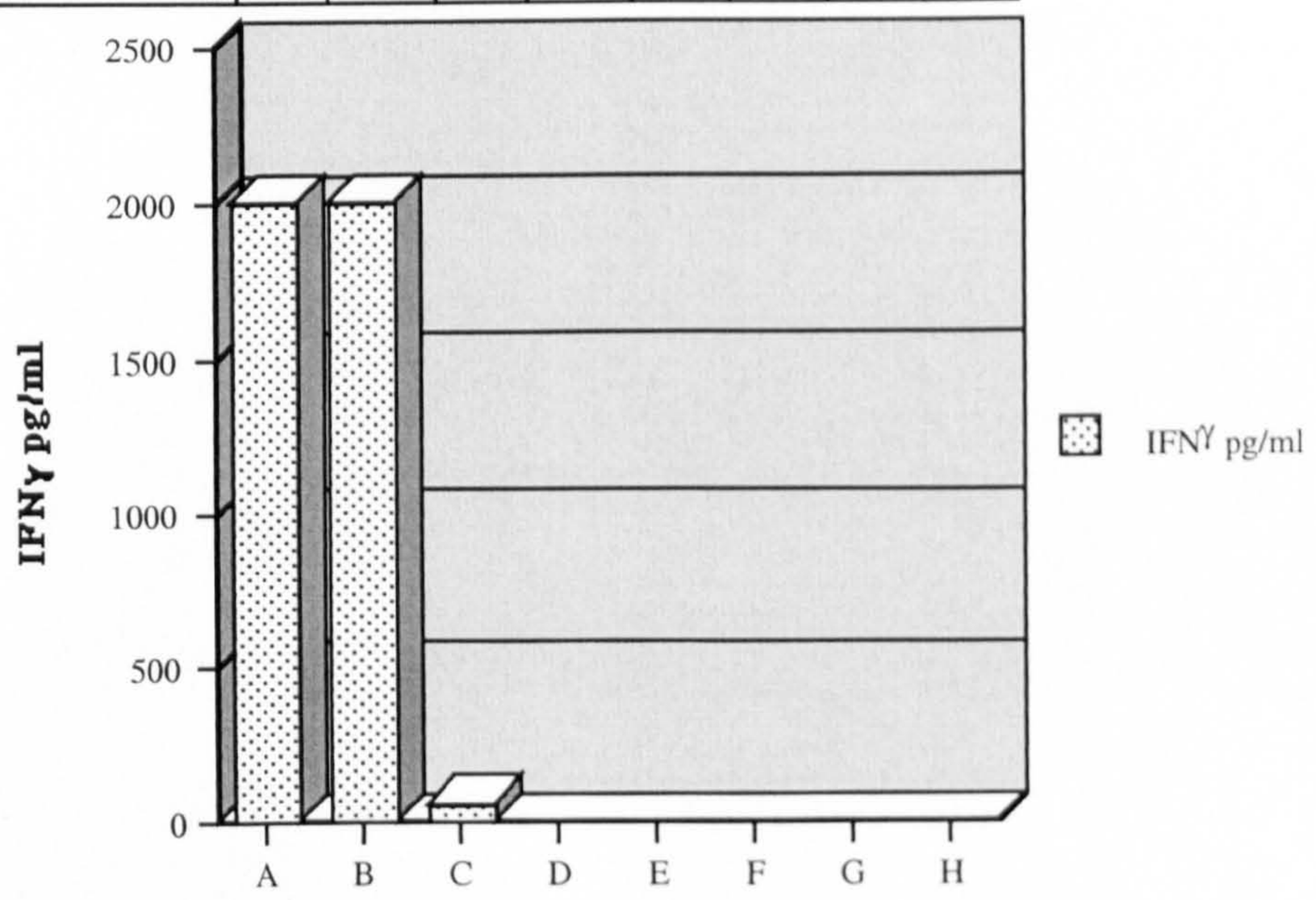


Figure 4.14C **IFN-γ production by IL-2$^{-/-}$ and IL-2$^{+/+}$ CD4^{+} T lymphocytes stimulated with PMA and ionomycin or CHO B7-1 and PMA at 72 hrs.**

T cell cultures were set up as described previously and supernatants were harvested and assayed for IFN-γ at 72 hrs.

PMA 10ng/ml+IONO 250ng/ml	+	+						
PMA 10ng/ml+CHO B7-1			+	+				
PMA 10ng/ml only					+	+		
CHO B7-1 only							+	+
WT		+		+		+		+
KO	+		+		+		+	



Cell Culture Conditions

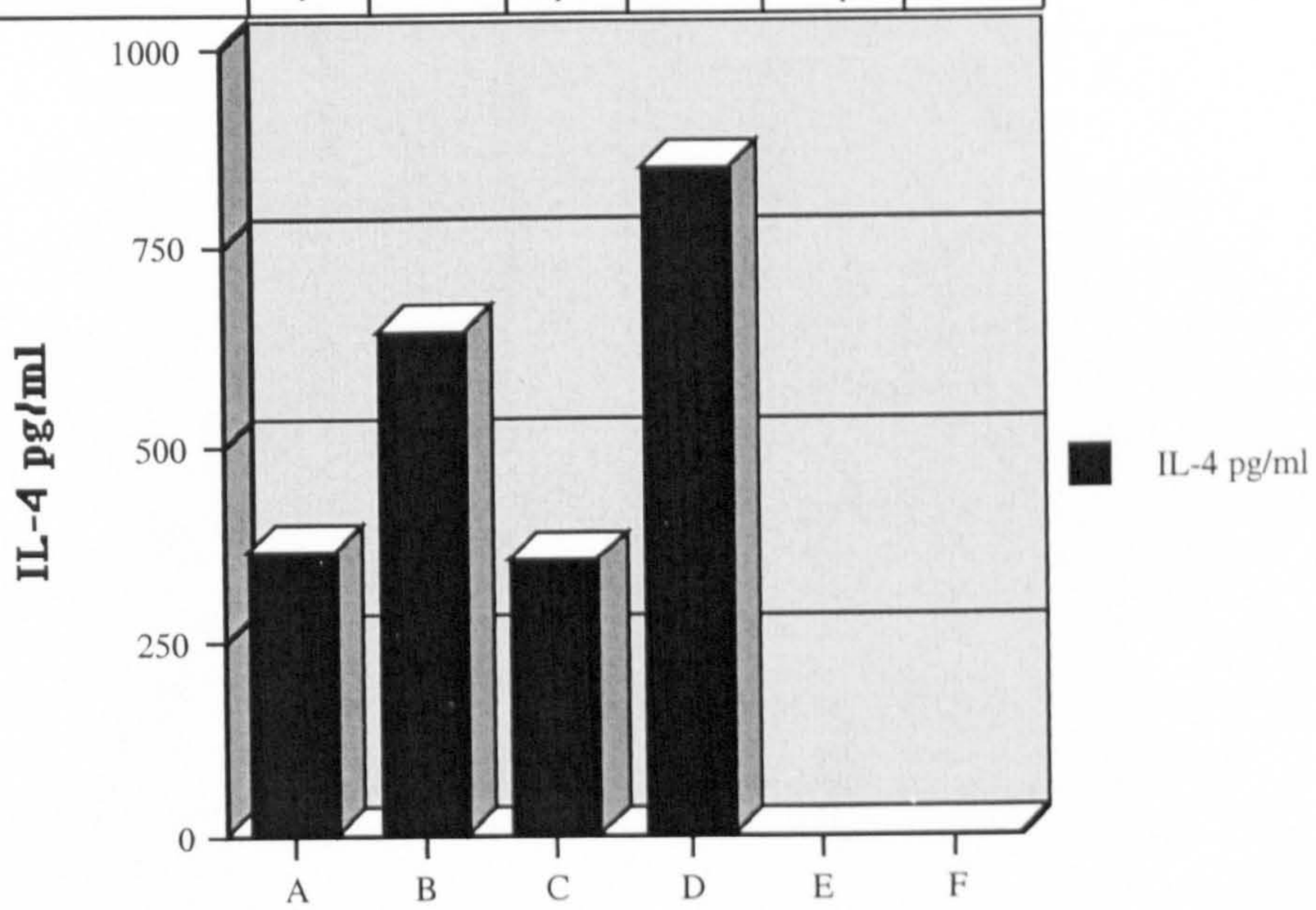
4.9.2-Cytokine production by purified CD4$^+$ T cells at 96 hrs:

At 96 hrs following stimulation, IL-4 production was significantly increased in both WT and KO, although more notable in IL-2$^{+/+}$ CD4$^+$ T cells. This data supplements results cited in the literature, indicating that IL-4 production peaks at later stages of cellular differentiation (fig. 4.15A). Moreover, IL-10 production was significantly augmented at this time point in IL-2$^{-/-}$ CD4$^+$ T cells and is particular to KO CD4$^+$ T cells. In fact, at 96 hrs IL-10 production by IL-2$^{-/-}$ CD4$^+$ T cells reaches values above 1000pg/ml, the highest value on the IL-10 standard curve (fig. 4.15B). IFN-γ production is also up-regulated under all stimulation conditions and reaches O.D. values above the highest detectable concentration point on the standard curve. O.D. values of 2.3 are equivalent to 3000pg/ml. As depicted in figure 4.15C, IFN-γ O.D. values extend to values of 4.

Figure 4.15A **IL-4 production by IL-2$^{-/-}$ and IL-2$^{+/+}$ CD4^{+} T lymphocytes stimulated with PMA and ionomycin or CHO B7-1 and PMA at 96 hrs.**

T cell cultures were set up as described previously and supernatants were harvested and assayed for IL-4 at 96 hrs.

PMA 20ng/ml+IONO 250ng/ml	+	+				
PMA 10ng/ml+IONO 250ng/ml			+	+		
PMA 10ng/ml only					+	+
WT		+		+		+
KO	+		+		+	

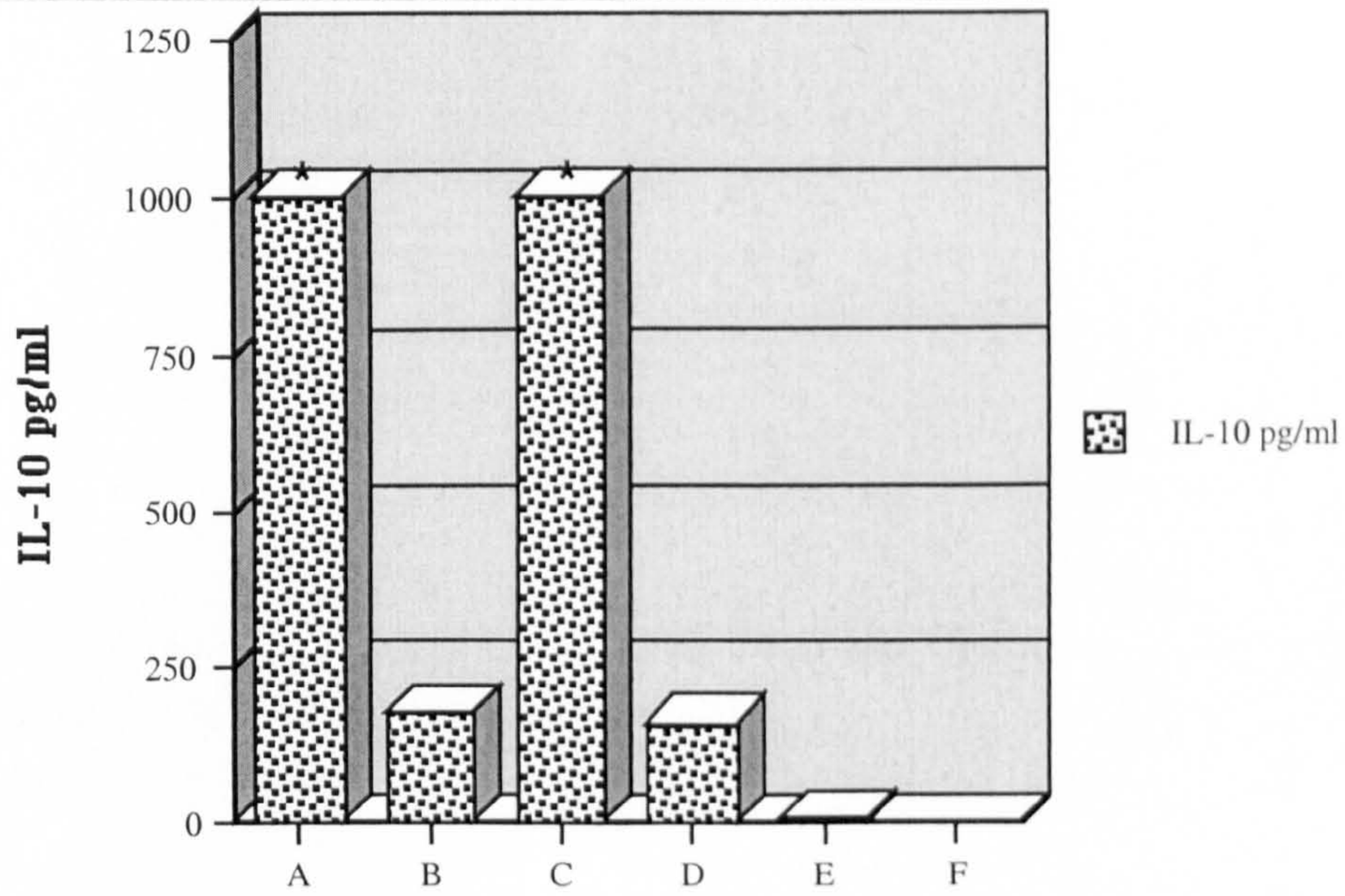


Cell Culture Conditions

Figure 4.15B **IL-10 production by IL-2$^{-/-}$ and IL-2$^{+/+}$ CD4^{+} T lymphocytes stimulated with PMA and ionomycin or CHO B7-1 and PMA at 96 hrs.**

T cell cultures were set up as described previously and supernatants were harvested and assayed for IL-10 at 96 hrs.

PMA 20ng/ml+IONO 250ng/ml	+	+				
PMA 10ng/ml+IONO 250ng/ml			+	+		
PMA 10ng/ml only					+	+
WT		+		+		+
KO	+		+		+	



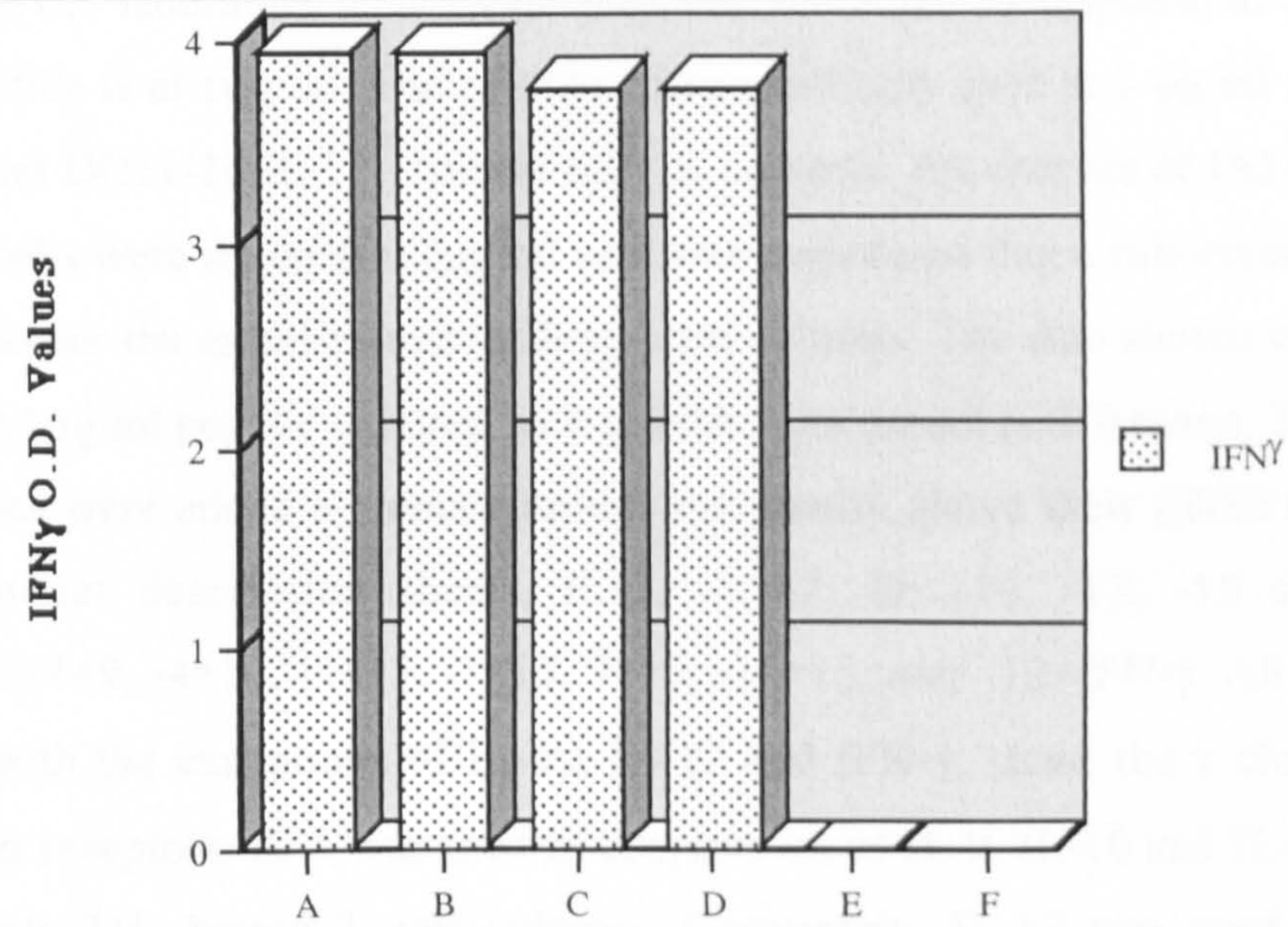
Cell Culture Conditions

* Represents values greater than 1000 pg/ml

Figure 4.15C **IFN-γ production by IL-2$^{-/-}$ and IL-2$^{+/+}$ CD4^{+} T lymphocytes stimulated with PMA and ionomycin or CHO B7-1 and PMA at 96 hrs.**

T cell cultures were set up as described previously and supernatants were harvested and assayed for IFN-γ at 96 hrs.

PMA 20ng/ml+IONO 250ng/ml	+	+				
PMA 10ng/ml+IONO 250ng/ml			+	+		
PMA 10ng/ml only					+	+
WT		+		+		+
KO	+		+		+	



Cell Culture Conditions

* 2.3 O.D. value represents 3000pg/ml

4.10-DO11-10 IL-2$^{-/-}$ long-term CD4^{+} T cell cultures.

All of the data discussed above derive from *in vitro* short-term cultures. T cell survival in the peripheral pool is an active process requiring MHC-peptide/TCR engagements and the response to environmental cytokines. To investigate which candidate cytokine (s) may play a role in promoting the survival of IL-2$^{-/-}$ CD4^{+} T cells *in vivo*, different cytokine/cytokine combinations were used in long-term T cell cultures in an attempt to elucidate the *in vivo* cytokine milieu. Previous reports have indicated that IL-2, -4, -7, and –15 can prevent the death of activated T cells *in vitro*. Therefore, the DO11-10 IL-2$^{-/-}$ CD4^{+} T cell model, which is specific for OVA (323-339) in the context of I-A^{d}, was used for the production of long-term cell cultures.

Previous studies in our laboratory have established that the maximal response to the OVA 323-339 peptide is at 1μg/ml. The peptide was accordingly used at 1 μg/ml for DO11-10 IL-2$^{-/-}$ and DO11-10 IL-2$^{+/+}$ splenic cells as controls. Another set of DO11-10 IL-2$^{-/-}$ splenic cells were set up at 0.2μg/ml as it was considered that a sub-optimal stimulation may favour the establishment of long-term cultures. The data shown will be results from the 1μg/ml peptide cultures, as it provided maximum proliferation. The following cytokines were added at concentrations marginally above their ED50 (as described by product description sheet), IL-2, -4, -7, -9, -10, -12, -15 and combinations of IL-4+9, -4+10, -4+15, -7+15, 9+15, -12+15, and –12+IFN-γ. All of these cytokines, with the exceptions of IL-10, IL-12 and IFN-γ, share the γ chain component of their receptors. IL-4 was used in conjunction of IL-9, IL-10 and IL-15 to possibly mediate TH_2 biased T cell cultures. Conversely, IL-12 was used in combination with other cytokines to induce TH_1 biased cultures. 1.5x10^{6} whole splenic DO11-10 IL-2$^{+/+}$ and DO11-10 IL-2$^{-/-}$ cells were set up with CHO H2-A^{d} B7-1 transfectants, the appropriate cytokine/cytokine combinations and peptide concentration.

Following this primary set up, the cultures were harvested, counted and restimulated every 10 days. Some cultures were viable for nine weeks and endured six rounds of re-stimulation. Figures 4.16A and B show cultures stimulated with rIL-4 or rIL-7; neither

IL-4 nor IL-7 supported the proliferation of DO11-10 IL-$2^{+/+}$ and DO11-10 IL-$2^{-/-}$ T cells after the second round of restimulation. In fig. 4.16, 100% survival rate represents no change in the absolute cell numbers between rounds of restimulations. Figures 4.16C and D represent cultures stimulated with IL-9 or IL-10. Under these conditions DO11-10 IL-$2^{-/-}$ T cells died by the second round of restimulation, whilst DO11-10 IL-$2^{+/+}$ T cells survived until the third round of restimulation. In fact, DO11-10 IL-$2^{+/+}$ T cell proliferation peaked in the second round of restimulation.

Figure 4.16E represents cultures stimulated with rIL-12. DO11-10 IL-$2^{-/-}$ T lymphocytes survived well under this condition. However, despite the presence of viable cells, the cultures were not expanding and were terminated after the sixth round of restimulation. Conversely, DO11-10 IL-$2^{+/+}$ T cells were defunct by the second round of restimulation. KO $CD4^+$ T cell cultures stimulated with rIL-15 survived until the fourth round of restimulation. In contrast, WT $CD4^+$ T cells declined by the second round of restimulation (fig. 4.16F). Most interestingly, under the IL-12 or the IL-15 activation conditions, DO11-10 IL-$2^{-/-}$ T cells flourished during the second round of restimulation but then levelled in the following round. Conversely, WT $CD4^+$ T cells died by the second round of stimulation. Intriguingly, when IL-12+15 were combined in a single culture, DO11-10 IL-$2^{-/-}$ T cells deteriorated faster than in cultures where these cytokines were added individually (fig. 4.16M).

Figure 4.16G represents the addition of exogenous IL-2 to both KO and WT $CD4^+$ T cell cultures. Under this condition, both DO11-10 IL-$2^{+/+}$ and DO11-10 IL-$2^{-/-}$ declined by the fourth round of restimulation. Exogenous IL-2 did not expand DO11-10 IL-$2^{-/-}$ T cells but maintained them in a uniform growth plane, until the sudden decline by round 4 of restimulation. However, DO11-10 IL-$2^{+/+}$ T cells had expanded briefly by the second round of restimulation and subsequently declined by the fourth round.

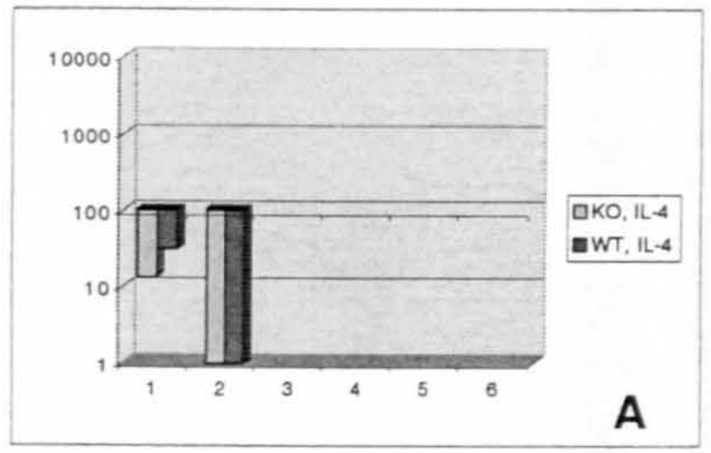
Under TH_2 conditioning cultures, IL-4+9, -4+10 and –4+15, both KO and WT $CD4^+$ T cells died by the second round of restimulation (fig. 4.16H, I and J).

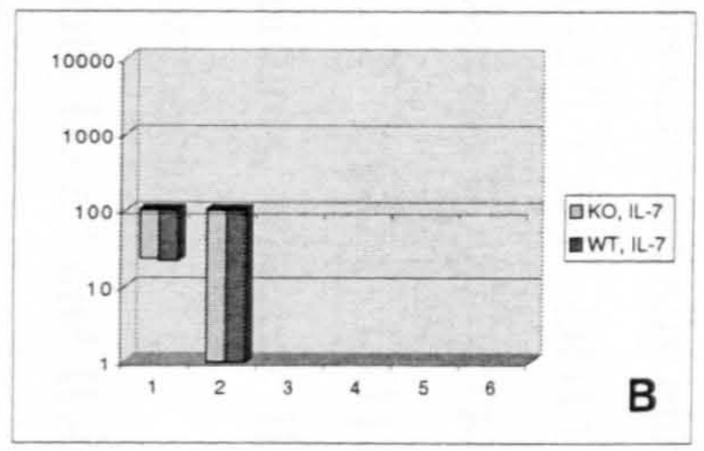
Figure 4.16K depicts T cell cultures stimulated with IL-7+15. Under these conditions, both KO and WT $CD4^+$ T cells survived until the sixth round of stimulation, although the cells were continuously declining after the second round of stimulation.

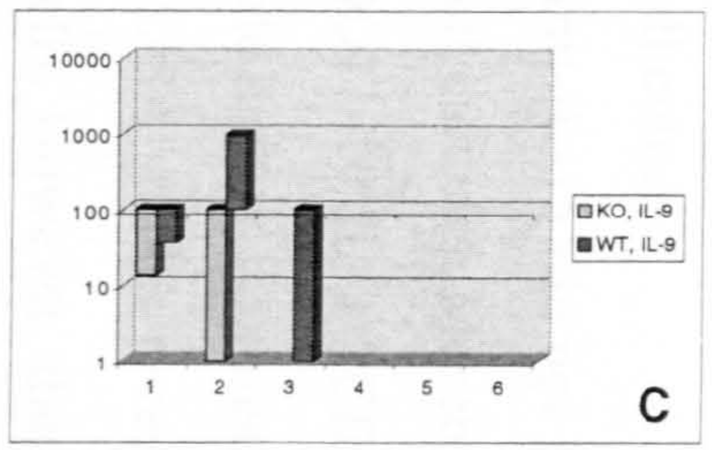
The IL-9+15 conditioning culture promoted proliferation in round two within the DO11-10 IL-$2^{+/+}$ T cells. However, in subsequent rounds these cells were viable but did not expand (fig. 4.16L). Finally under TH_1 conditioning cultures, IL-12+IFN-γ, KO $CD4^+$ T cells initially expanded, subsequently deteriorated and died by the fifth round of restimulation. Interestingly, WT $CD4^+$ T cells followed the same deterioration pattern but remained viable for several rounds (fig. 4.16N).

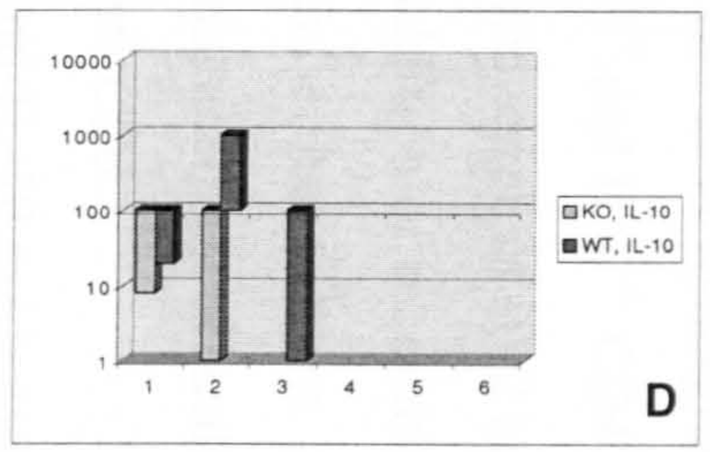
Figure 4.16 DO11-10 IL-2$^{-/-}$ long-term CD4^{+} T cell cultures.

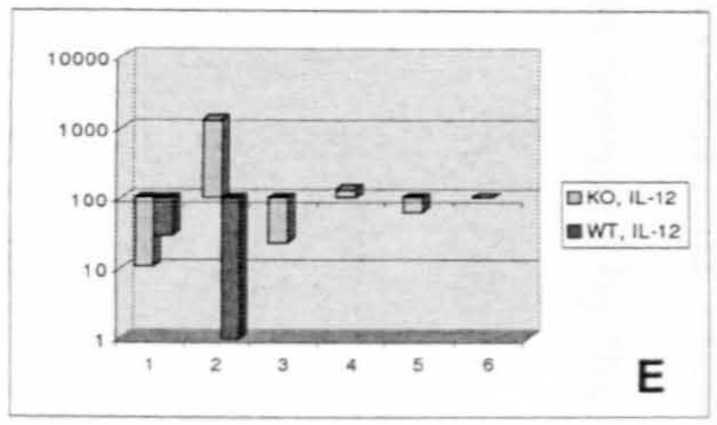
DO11-10 antigen specific IL-2$^{-/-}$ CD4^{+} T cells were stimulated with 1 μg/ml of the OVA 323-339 peptide and co-cultured with 1% paraformaldehyde fixed CHO I-A^{d} B7-1 transfectants. Different recombinant cytokines or cytokine combinations were added to the cultures as described on figures 4.16A-N. Cultures were restimulated every 10 days with the peptide and fixed CHO I-A^{d} B7-1 transfectants. Displayed on the x-axis is the number of restimulations and the y-axis the percentage increase/decrease of the previous stimulation.

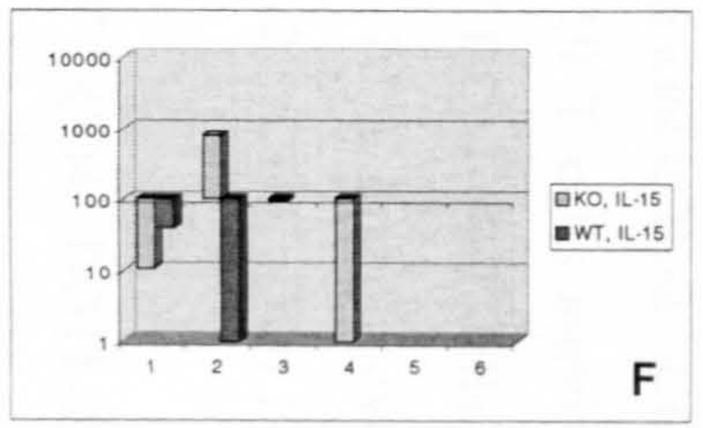

10000
1000
100
10
1
1 2 3 4 5 6
KO, IL-4
WT, IL-4
A

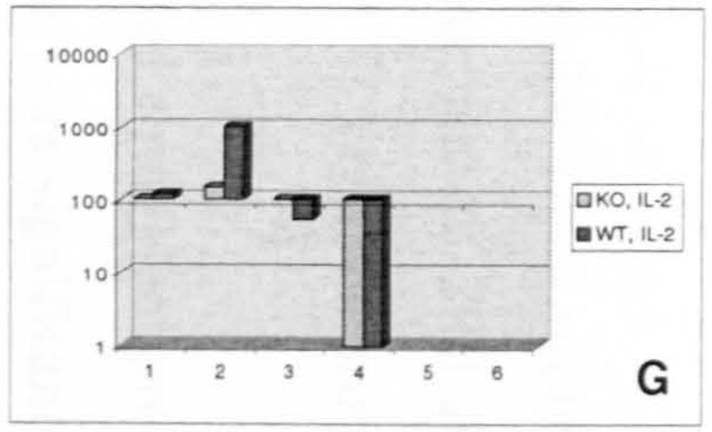

10000
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10
1
1 2 3 4 5 6
KO, IL-7
WT, IL-7
B

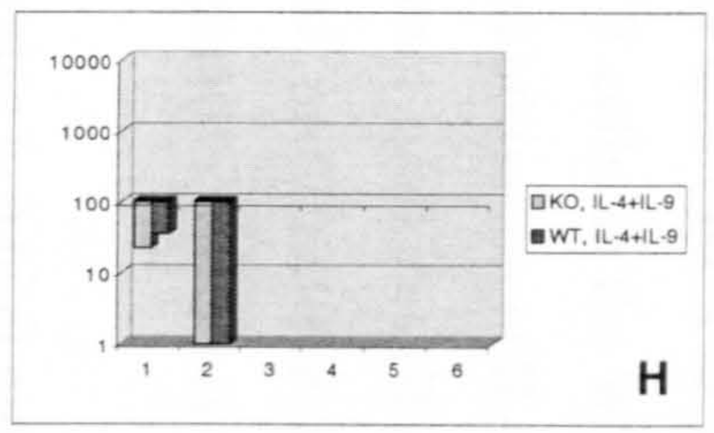

10000
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1 2 3 4 5 6
KO, IL-9
WT, IL-9
C

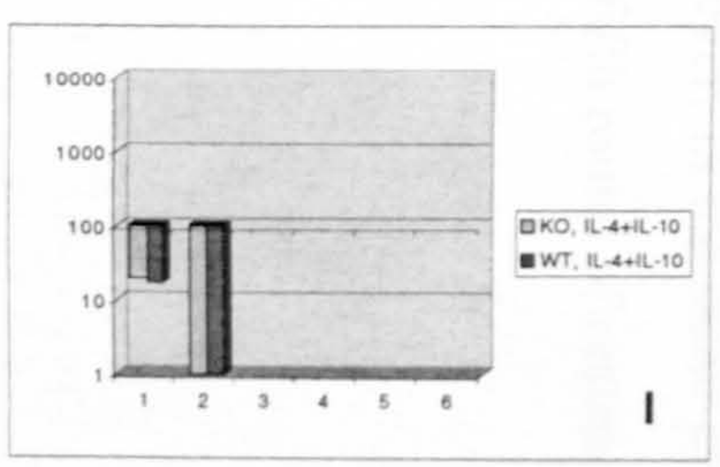

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10
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1 2 3 4 5 6
KO, IL-10
WT, IL-10
D

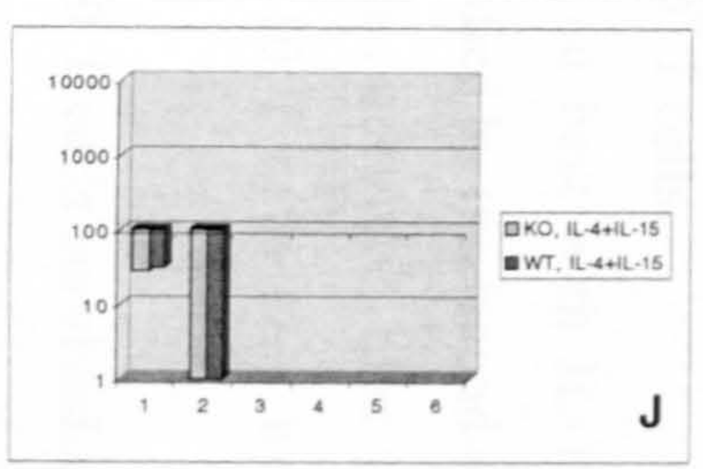

10000
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1
1 2 3 4 5 6
KO, IL-12
WT, IL-12
E

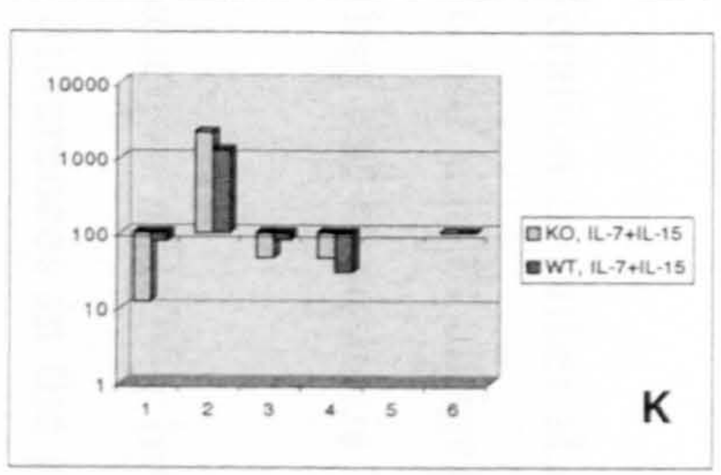

10000
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10
1
1 2 3 4 5 6
KO, IL-15
WT, IL-15
F

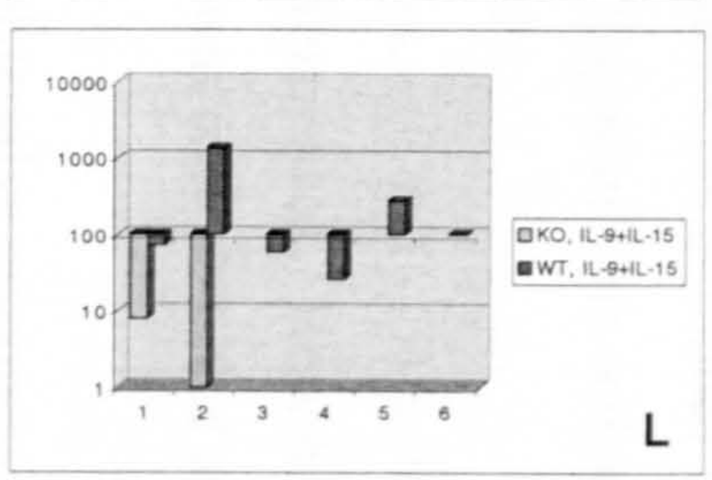

10000
1000
100
10
1
1 2 3 4 5 6
KO, IL-2
WT, IL-2
G

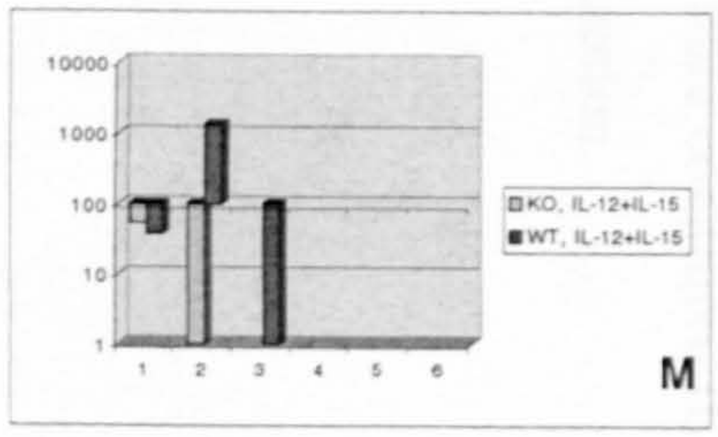

10000
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10
1
1 2 3 4 5 6
KO, IL-4+IL-9
WT, IL-4+IL-9
H

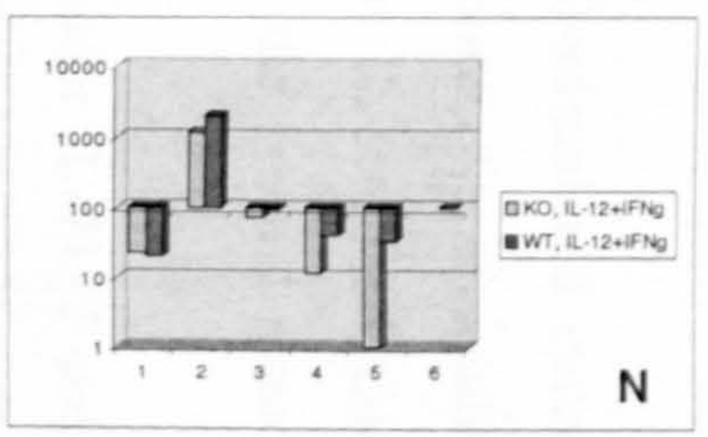

10000
1000
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10
1
1 2 3 4 5 6
KO, IL-4+IL-10
WT, IL-4+IL-10
I

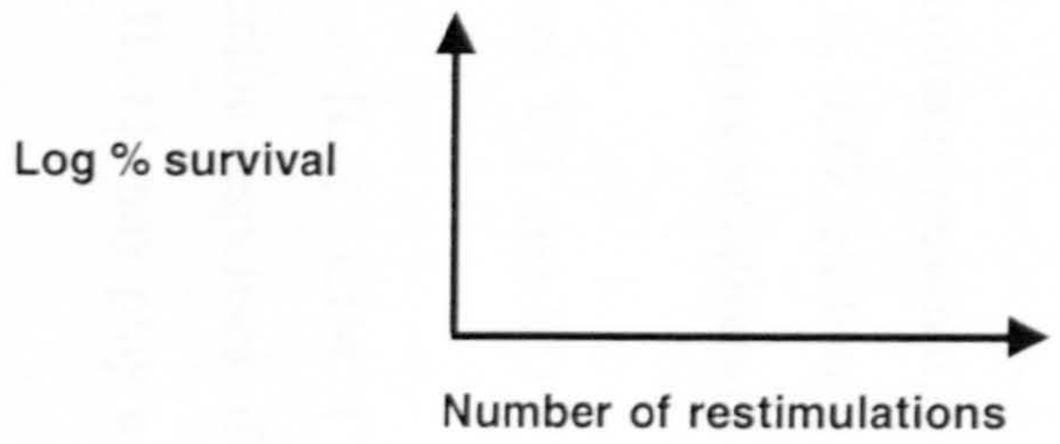

10000
1000
100
10
1
1 2 3 4 5 6
KO, IL-4+IL-15
WT, IL-4+IL-15
J


10000
1000
100
10
1
1 2 3 4 5 6
KO, IL-7+IL-15
WT, IL-7+IL-15
K


10000
1000
100
10
1
1 2 3 4 5 6
KO, IL-9+IL-15
WT, IL-9+IL-15
L


10000
1000
100
10
1
1 2 3 4 5 6
KO, IL-12+IL-15
WT, IL-12+IL-15
M


10000
1000
100
10
1
1 2 3 4 5 6
KO, IL-12+IFNg
WT, IL-12+IFNg
N


Log % survival
Number of restimulations

4.11-Summary:

The data presented in this chapter support the following conclusions:

- CHO B7-1 synergizes with Con A or anti-CD3 to induce the proliferation of IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells. In the WT system, this activation is exerted via the production of IL-2 and utilisation of IL-2.
- Addition of recombinant cytokines (IL-4, -10 and -15) to WT cultures resulted in the enhancement of proliferative responses. However, in KO cultures, enhancement of proliferative responses was only observed at low concentrations of anti-CD3.
- Anti-IL-10 mAb was able to inhibit proliferative responses in both WT and KO T cell cultures.
- Anti-IL-15 did not inhibit T cell proliferation in either IL-$2^{+/+}$ or IL-$2^{-/-}$ $CD4^+$ T cell cultures. Moreover, anti-IL-2Rβ did not inhibit antigen-specific responses of DO11-10 IL-$2^{-/-}$ $CD4^+$ T cells, disproving the hypothesis that IL-15 may play a role in their responses.
- ELISA studies revealed equivalent production of IFN-γ by KO and WT $CD4^+$ T cells. However, there were significant differences in the production of IL-10; it was substantially enhanced in IL-$2^{-/-}$ CD^+ T cells.
- IL-$2^{-/-}$ $CD4^+$ DO11-10 T cells survived best with IL-12-stimulated cultures, whereas IL-$2^{+/+}$ $CD4^+$ DO11-10 T cells died by the second round of restimulation.
- IL-$2^{+/+}$ $CD4^+$ DO11-10 T lymphocytes survived best in IL-9+15-stimulated cultures, whereas IL-$2^{-/-}$ $CD4^+$ DO11-10 T cells died by the second round of stimulation.
- Both IL-$2^{-/-}$ and IL-$2^{+/+}$ $CD4^+$ DO11-10 T cells survived under IL-7+15 and IL-12+IFN-γ stimulation conditions. However, in the latter culture condition, IL-$2^{-/-}$ $CD4^+$ DO11-10 T cells declined by the fifth round of restimulation.

DISCUSSION

Previous studies by Razi-Wolf et al. have demonstrated that IL-2 deficient mice are responsive to and dependent on B7-1/TCR signals (208). Recombinant cytokines and anti-cytokine blocking mAbs studies were applied to look at the role of cytokines in the proliferation of IL-$2^{-/-}$ $CD4^{+}$ T lymphocytes. These studies have revealed that the proliferation of IL-$2^{-/-}$ $CD4^{+}$ T cells induced by B7-1/TCR signals is marginally dependent on IL-4 and IL-10 (fig. 4.8B). This indicates that IL-4 and IL-10 may play a role in IL-$2^{-/-}$ $CD4^{+}$ T lymphocyte proliferative responses. In this context, it is noteworthy that IL-2, IL-4 double knockout mice (IL-$2^{-/-}$/IL-$4^{-/-}$) do not develop colitis but still develop the generalised lesions present in the IL-2 KO model such as those in the pancreas, lung and heart (246). This implies that IL-4 specifically may play a role in the immunopathogenesis of colitis. In fact, a recent study reported that anti-IL-4 mAb treated T cell receptor alpha chain-deficient mice (mice which develop inflammatory bowel disease) exhibited no clinical or histological signs of disease when compared to control mAb treated counterparts (103).

Surprisingly, anti-IL-10 mAb partially inhibited proliferative responses in both WT and KO T cell cultures. This could be the outcome of different phenomena. Firstly, IL-10 is a pleiotropic cytokine, secreted by a wide variety of cells and can mediate immunosuppressive or immunostimulatory activities. Indeed, recent reports have highlighted the immunostimulatory role of IL-10 in $CD8^{+}$ T cells (247) (248). Moreover, IL-10 can exacerbate certain autoimmune diseases such as diabetes (249) (250) and in certain cases its administration either at the same time or after the bone marrow transplant accelerated graft-vs-host disease (251). This demonstrates that IL-10 can mediate pathogenic effects as well as protective activities. Interestingly, ELISA studies have demonstrated that IL-$2^{-/-}$ $CD4^{+}$ T cells produce significantly more IL-10 than their WT counterparts. This is not a consequence of overproduction of IL-10 by IL-$2^{-/-}$ macrophages as means to combat an inflammatory milieu, since purified IL-$2^{-/-}$ $CD4^{+}$ T cells were used in ELISA studies.

Secondly, the overproduction of IL-10 by IL-$2^{-/-}$ $CD4^+$ T lymphocytes maybe an outcome of the expansion of $CD4^+$ $CD38^+$ T cells in KO spleens (fig. 6.8). This population, which in WT mice represents a regulatory T cell subset, is significantly augmented in IL-2 deficient spleens, possibly to compensate for the absence of the $CD4^+CD25^+$ T cell subset in IL-2 KO mice (142).

Finally, IL-10 mediates inhibition of cytokine synthesis via a variety of mechanisms. The best characterised is the inhibition of inflammatory cytokine production by blocking macrophage accessory cell function (252). This study by Macatonia et al. reported that dendritic cell-induced TH_1 proliferation was unaffected by IL-10, whereas macrophage-stimulated proliferation was inhibited in the same system. This demonstrates clearly that IL-10 immunosuppressive activities are dependent on the nature of the antigen-presenting cell. In this context, it is noteworthy that CHO B7-1 transfectants were used as APCs in the short–term cultures. It is unlikely that IL-10 could mediate an immunosuppressive effect under these culture conditions. Hence, recombinant IL-10 was able to enhance proliferation in T cell cultures and anti-IL-10 inhibited proliferative responses.

As mentioned previously, initially IL-15 was the postulated candidate for mediating B7-1/TCR proliferation of IL-$2^{-/-}$ $CD4^+$ T cells. Anti-IL-15 and sIL-15Rα marginally inhibited T cell proliferation in both IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells (fig. 4.9A and 4.10A). There are several explanations for these results:

Firstly, among cells of the immune system, IL-15 appears to be primarily produced by monocyte/macrophages (253). Therefore, T cells may not be the best choice to look for IL-15 overproduction in IL-2 deficient mice.

Secondly, despite sharing two receptor subunits and sharing a common JAK3/STAT5 signalling pathway, IL-2 and IL-15 appear to manifest some contrasting roles such as promotion and prevention of AICD respectively. This has been attributed to production by different cell types and complex secretion control mechanisms

(reviewed in (241)). Moreover, their private receptor subunits, IL-2Rα and IL-15Rα, differ greatly, with IL-15Rα having a more diverse distribution pattern. These differences emphasise an underlying pleiotropy of IL-15 and may contribute to the differences in IL-15 and IL-2 action. Hence, despite the similarities, IL-15 may not necessarily substitute for IL-2.

Thirdly, other unknown or newly identified cytokines may be the mediators of B7-1/TCR proliferation of $IL2^{-/-}$ $CD4^+$ T lymphocytes. One such candidate is IL-21. This newly identified interleukin is most closely related to IL-2 and IL-15. Furthermore, i*n vitro* assays demonstrate that IL-21 has a role in the proliferation and maturation of NK cells, B cell populations costimulated with CD40, and T cells stimulated with anti-CD3. Indeed, C57BL/6 splenic and lymph node cells were depleted of $CD19^+$ B cells and assayed for proliferation in response to plate-bound anti-CD3 mAb in the presence or absence of increasing concentrations of IL-21. T cells that were costimulated with high concentrations of IL-21 proliferated most efficiently. Using quantitative RT-PCR, IL-21 was found to be expressed by $CD4^+$ T cells that have been activated using stimuli that mimic an antigen encounter (254). Finally, the recently characterised IL-21R was found to be structurally similar to IL-2Rβ and like IL-2Rβ associated with JAK 1 and STAT 5 (255). This makes the newly identified IL-21 a good candidate for promoting B7-1/TCR-induced proliferation of $IL\text{-}2^{-/-}$ $CD4^+$ T lymphocytes.

IL-4 production was measured at 60, 72 and 96 hrs. Figure 4.13B depicts IL-4 production at 60 hrs. $IL\text{-}2^{+/+}$ and $IL\text{-}2^{-/-}$ $CD4^+$ T cells were stimulated with PMA 10ng/ml and ionomycin 250ng/ml or CHO B7-1 plus PMA 10ng/ml. At this time point, $IL\text{-}2^{+/+}$ $CD4^+$ T lymphocytes produced the highest quantity of IL-4, albeit lower than 100pg/ml. PMA plus ionomycin cell evoked higher quantities of IL-4 than CHO B7-1 plus PMA for both KO and WT cells. At 72 hrs, the amount of IL-4 produced by PMA plus ionomycin-stimulated $IL\text{-}2^{+/+}$ $CD4^+$ T cells was reduced, whereas production by $IL\text{-}2^{-/-}$ $CD4^+$ T lymphocytes was up-regulated (fig. 4.14A). The reduction in IL-4 quantity by $IL\text{-}2^{+/+}$ $CD4^+$ T cells is probably due to IL-4 being

utilised by T cultures at that time point. This time point is probably the optimum for proliferation, hence [^{3}H] thymidine DNA incorporation measurements in short-term cultures are taken are this time-point. Since by IL-2$^{-/-}$ CD4$^+$ T lymphocytes have different growth potential *in vitro*, the kinetics of IL-4 production may differ as a result. At 96 hrs. production of IL-4 by IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4$^+$ T cells was up-regulated, albeit more so for WT cells in comparison to the 72 hrs time point (fig. 4.15A).

IFN-γ production by KO and WT T cells was equivalent at each time point. However, production was reduced at the 72 hrs time point (fig. 4.14C), suggesting consumption by T cell cultures at that time point, and was further up-regulated at 96 hrs of culture (fig. 4.15C). It is noteworthy that supernatants for IFN-γ detection were diluted 1:5, 1:10 and 1:20. Despite this, levels were still substantially high; dilutions of 1:50 and 1:100 would have been more appropriate. Interestingly, Both IL-4 and IFN-γ production by IL-2+/+ CD4+ T cells at 72 hrs was reduced in comparison to 60 hrs (figures 4.14A and C), suggesting the optimum proliferation of T cells and hence growth factor consumption.

The relationship between IL-4 and IFN-γ production was studied in a report by Kamogawa et al. on transgenic mice whose IL-4-producing cells expressed herpes simplex virus 1 thymidine kinase. These cells were eliminated by ganciclovir (GANC). Activation of transgenic T cells in the presence of GANC resulted in the elimination of both IL-4 and IFN-γ production. This suggested that IFN-γ-producing cells express or have expressed IL-4 at some stage (256). This concurs with my ELISA data, showing that IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4$^+$ T cells produce both IL-4 and IFN-γ. Overall, the reduced production of IL-4 by KO T cells could be a result of a TH_1 dominant milieu in vivo in IL-2 deficient mice. Several reports have illustrated that TH_1 and TH_2 lineages are not pre-programmed in the thymus. Nevertheless, they become committed post-thymically to a developmental pathway that is dependent on several factors, one of which is the condition of their microenvironment or cytokine milieu (reviewed in (257)). The TH_1-biased cytokine milieu in IL-2 deficient mice in

vivo may influence the propensity of $CD4^+$ T cells to produce IL-4 (111), hence the reduced production of IL-4 by IL-$2^{-/-}$ $CD4^+$ T lymphocytes.

Further analysis of cytokines that may promote long-term survival and proliferation in IL-$2^{-/-}$ transgenic T cells revealed that IL-$2^{-/-}$ DO11-10 $CD4^+$ T cells survived best in IL-12 or IL-7+IL-15 stimulation cultures. Major differences were observed with IL-12, where KO T cells survived six rounds of restimulation and WT T cells died by the second round of restimulation. The aim of these long-term cultures was to develop an IL-$2^{-/-}$ T cell line that could be cloned and analysed for growth factors produced in the medium. So far, no IL-$2^{-/-}$ cell line has been reported. The fact that T cell survival in IL-12-stimulated cultures differed significantly between KO and WT T cells is perhaps a reflection of the TH_1 propensity of IL-$2^{-/-}$ $CD4^+$ T lymphocytes (fig. 4.16E). This finding complements studies in the literature demonstrating TH_1 bias-IL-$2^{-/-}$ $CD4^+$ T cells. Ludviksson et al. reported that in the absence of IL-2, thymocyte maturation is abnormally directed by IL-12 toward the development of mature and activated TH_1-type thymocytes that are capable of inducing colitis (111).

Interestingly, in both IL-12- and IL-15-stimulated cultures, IL-$2^{-/-}$ $CD4^+$ T cells performed better than IL-$2^{+/+}$ cells. In IL-15-stimulated cultures, KO T cells survived until the fourth round of restimulation, whereas WT T cells died by the second round of restimulation (fig. 4.16F). Moreover, IL-$2^{-/-}$ $CD4^+$ T cells had actually expanded in the second round of restimulation. However, a combination of IL-12+IL-15 did not promote KO T cell survival (fig. 4.16M). Rather, IL-$2^{-/-}$ $CD4^+$ T cells died by the second round and IL-$2^{+/+}$ T cells survived only until the third. KO IL-12+IL-15-stimulated cultures were perhaps exhausted by the presence of both cytokines. This implies that *in vitro* IL-$2^{-/-}$ $CD4^+$ T cells appear to have a low proliferation propensity and reach a plateau faster than IL-$2^{+/+}$ $CD4^+$ T cells. This is also reflected in the recombinant cytokine short-term cultures, where rIL-4 and rIL-15 enhanced proliferation of IL-$2^{-/-}$ $CD4^+$ T lymphocytes at low concentrations of anti-CD3 only (fig. 4.7B). At higher concentrations of anti-CD3, there was no increase in proliferative responses; this is indicative of saturation. By contrast, the addition of

recombinant cytokines to IL-$2^{+/+}$ $CD4^+$ T cell cultures enhanced proliferation at all concentrations of anti-CD3 (fig. 4.7A).

Other major differences were observed in the IL-9+IL-15-stimulated cultures, where IL-$2^{+/+}$ $CD4^+$ T lymphocytes survived until the sixth round of restimulation and IL-$2^{-/-}$ $CD4^+$ T cells died by the second round of restimulation (fig. 4.16L). Recently, the role of IL-15 in T cell survival has come to light. Despite their biological similarities, IL-2 and IL-15 mediate distinct actions in activation-induced cell death (AICD). Although IL-2 is an important growth and survival factor, it also plays a pivotal role in Fas-mediated AICD of $CD4^+$ T cells (3) (19) (14). In contrast, IL-15 acts to extend the survival of lymphocytes by acting as a growth factor and by inhibiting IL-2-mediated AICD. However, the mechanism underlying this inhibition of AICD has not yet been identified (244). In this study, IL-15 transgenic mice demonstrated an inhibition of IL-2-mediated AICD and increased numbers of NK cells and $CD8^+$ memory T cells. Furthermore, that was overcome by the addition of anti-IL-15 blocking antibody to ex-vivo cultures (244).

All of the above studies point to a role of IL-15 in promoting T cell survival. More importantly, in a study by Vella et al., IL-4 and IL-15 were reported to prevent activated T cell death *in vitro*. In this study, mice transgenic for a TCR bearing Vβ3 and specific for moth cytochrome *c* were injected *in vivo* with the Vβ3-reactive staphylococcal enterotoxin A (SEA). Two days later, lymph node T cells were removed and activated *in vitro* for 24 or 48 hrs with exogenous cytokines. Cells were then harvested and percentages of apoptotic cells were measured. Of the cytokines tested, only IL-2, IL-4, IL-7 and IL-15 prevented the *in vitro* death of the *in vivo* pre-activated T cells (258). Figure 4.16L depicts the survival of WT T cells in IL-9+IL-15-stimulated cultures. IL-9 was not reported to prevent T cell death in the study by Vella et al. However, high-affinity receptors for IL-9 have not been found on T cells activated for short time periods (259). In the case of DO11-10 IL-$2^{-/-}$/IL-$2^{+/+}$ $CD4^+$ T cell cultures, T cells were activated for a period of nine weeks and endured six rounds

of restimulation, as opposed to the four-five days activation protocol used in the study by Vella et al. Therefore, it is feasible that IL-9 high-affinity receptors on T cells were up-regulated as a result of this long-term activation protocol.

Finally, the comparable survival of KO and WT IL-7+IL-15 stimulated T cell cultures complements other reports in the literature (fig. 4.16K) that IL-7 maybe closely involved with T cell survival *in vivo* (260). Thymocyte development in IL-7 or IL-7 receptor component-deficient animals is severely impaired (261) (262). In addition, T cells that mature in such animals have abnormal phenotypes, shortened life span and are functionally impaired (263). These defects were rescued in IL-7 receptor-deficient mice by overexpression of the anti-apoptotic protein bcl-2 (264) (265). As mentioned previously, IL-15 has been reported to contribute to enhanced immune memory by impeding IL-2-mediated AICD and generating memory T cells (244). It is therefore not surprising that both KO and WT T cells survived under IL-7+IL-15 culture conditions. It is noteworthy that IL-$2^{-/-}$ and IL-$2^{+/+}$ $CD4^+$ T cell survival was relatively similar in IL-2-stimulated cultures. Both died by the fourth round of restimulation; however, IL-$2^{+/+}$ $CD4^+$ T cells had actually expanded at the second round of restimulation when compared to KO cells. The death mechanism involved in the fourth round of restimulation may possibly be AICD since the cells were repeatedly activated in the presence of IL-2 for six weeks (fig. 4.16G).

CHAPTER 5

mRNA ANALYSIS OF MITOGEN AND B7-1/TCR-INDUCED PROLIFERATION OF IL-2$^{-/-}$ CD4^{+} T LYMPHOCYTES.

INTRODUCTION

To extend the recombinant cytokine and anti-cytokine blocking studies, cytokine mRNA levels were investigated. Several techniques were used to analyse mRNA levels in activated IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes. Initially, the multi-probe RNase protection assay system (PharMingen, BD) was used. This system is highly sensitive and is based on the use of DNA-dependent RNA polymerases from SP6, T7 and T3 bacteriophage and their cognate promoter sequences. The high specificity of these RNA polymerases for their promoters rendered them ideal for the synthesis of high specific activity RNA probes from DNA templates. Moreover, these DNA-dependent RNA polymerases transcribe long segments of RNA at high rates. Consequently, the cDNA of interest can be subcloned into a plasmid that contains bacteriophage promoters. This construct can then be used to synthesise radiolabelled anti-sense RNA probes.

The multi-probe RNase protection assay system has the advantage of generating a series of such templates, each distinct in length and representing the specific mRNA antisense sequence of a cytokine. The probe set is hybridised in excess to target mRNA in solution. Subsequently, any free single-stranded RNA and free probe are digested using RNases. Finally, the remaining double-stranded RNase-protected probe segments are purified, resolved on denaturing polyacrylamide gels and quantified by autoradiography or phosphoimaging. The quantity of each mRNA species in the original mRNA sample can be quantified based on the intensity of the appropriately sized protected probe fragment in relation to probes representing housekeeping genes also included in the set. In addition, individual mRNA species can be compared between different samples as well as within the same sample [RiboQuant® Multi-Probe RNase Protection System, instruction manual, 5th edition, April 1998].

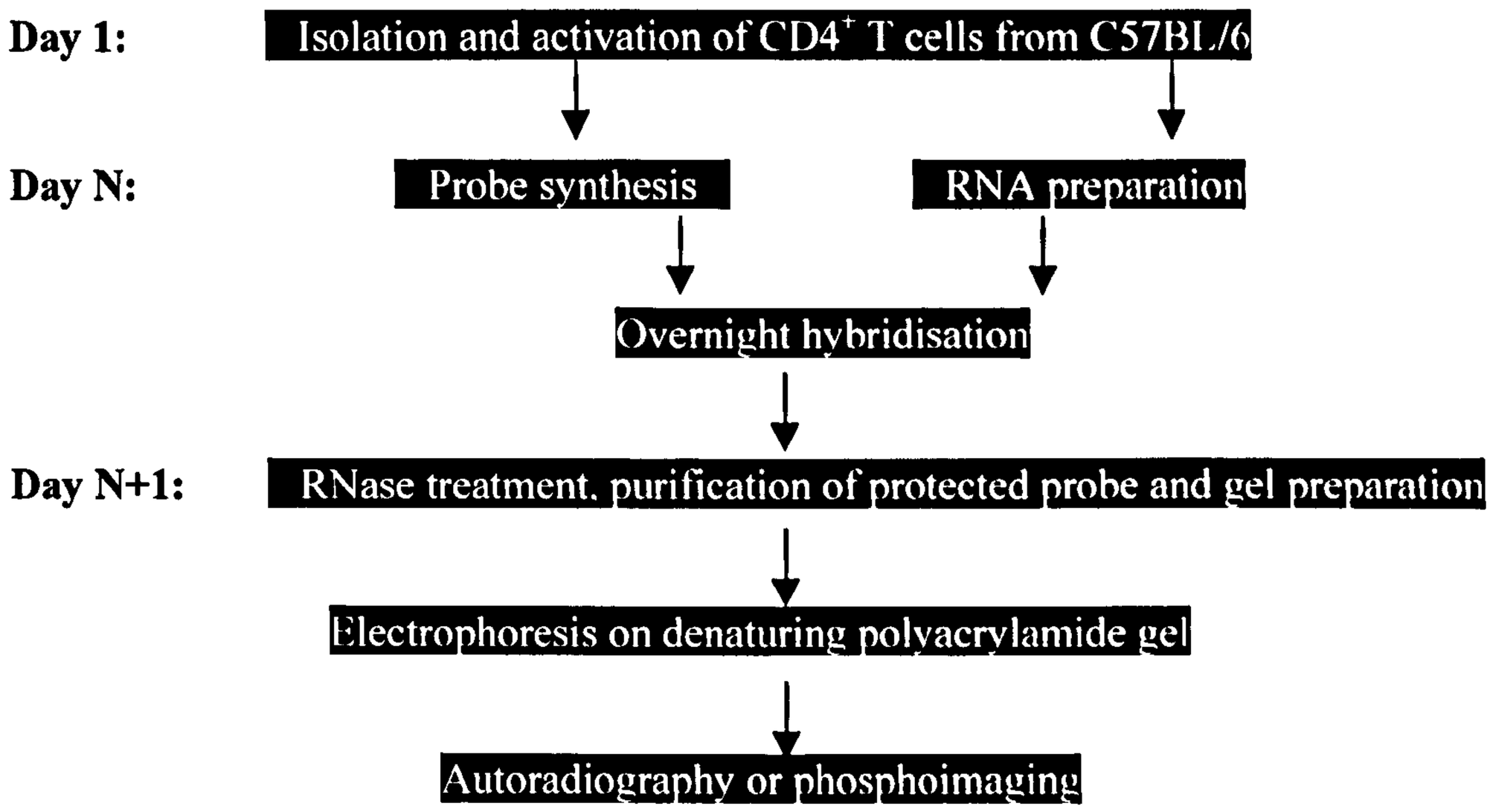


Figure 5.1 **Schematic representation of the RPA protocol.** Adapted from RiboQuant® Multi-Probe RNase Protection System, instruction manual, 5th edition, April 1998. N represents day number.

In a parallel approach, CLONTECH's Atlas™ cDNA expression arrays were used. Nucleic acid arrays and their applications have been reported in several studies (266) (267). This technique has diverse applications, including investigating normal biological and disease processes and gene expression in different tissues. CLONTECH's Atlas™ cDNA expression arrays include hundreds of cDNAs spotted on positively charged nylon membranes. The Atlas™ 1.2 mouse array II was used to detect the profile of genes up-regulated in activated IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells. The Atlas™ 1.2 mouse array II includes 1176 mouse cDNAs, nine housekeeping cDNAs as positive controls for quantitation of mRNA species as well as plasmid and bacteriophage DNAs as negative controls to confirm hybridisation specificity. In this context, detecting differential gene expression between KO and WT activated $CD4^+$ T lymphocytes would be advantageous and would give insight into the activation of IL-$2^{-/-}$ $CD4^+$ T cells at the molecular level. In particular, this gene set is not limited to cytokine genes.

Finally, RT-PCR was also used to detect the abundance of a specific mRNA in poly (A) RNA samples. Using Biosource International's mouse TH_1/TH_2 cytokine set, the expression of IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12p40 and IL-13 was analysed in activated IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes.

As described in chapter 4, significant differences were observed in IL-10 production by activated IL-$2^{-/-}$ $CD4^+$ T lymphocytes when compared to IL-$2^{+/+}$ $CD4^+$ T cells. To further investigate the profile of cytokines produced by KO T cells, cytokine mRNA levels were studied using RNase protection assays, CLONTECH's cDNA expression arrays and RT-PCR. The aim of the experiments reported in this chapter was to gain insight into the mechanism resulting in the activation of IL-$2^{-/-}$ $CD4^+$ T cells by B7-1/TCR or by mitogen, PMA and ionomycin. More importantly, to conclude whether there is differential expression of cytokine or other mRNA species in IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes.

RESULTS

5.2-RNase protection assays:

IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells were stimulated with PMA 10ng and ionomycin 250ng/ml or PMA 10ng/ml only as a negative control for the T cells. Alternatively, T cells were stimulated with CHO B7-1 and anti-CD3 (3μg/ml), CHO B7-1 alone stimulated with anti-CD3 was used as a negative control for the APC. Subsequently, $CD4^+$ T cells were harvested at 18 and 48 hrs, RNA was extracted and radiolabelled probe was synthesised as described in Materials and Methods. Initially, the PMA and ionomycin stimulation was only used for the 18-hrs time point. As depicted in figure 5.2, CHO B7-1 cells treated with anti-CD3 did not manifest any mRNA expression, illustrating that cytokine mRNA expression will be specific to $CD4^+$ T cells. At 18 hrs, the CHO B7-1/anti-CD3 stimulated IL-$2^{-/-}$ $CD4^+$ T cells sample failed and IL-$2^{+/+}$ $CD4^+$ T cells did not express cytokine mRNA despite display of mRNA for the housekeeping genes L32 and GAPDH. At 48 hrs, both IL-$2^{+/+}$ and IL-$2^{-/-}$ B7-1/TCR-activated $CD4^+$ T cells exhibited IL-13, IL-10 (traces) and IFN-γ mRNA.

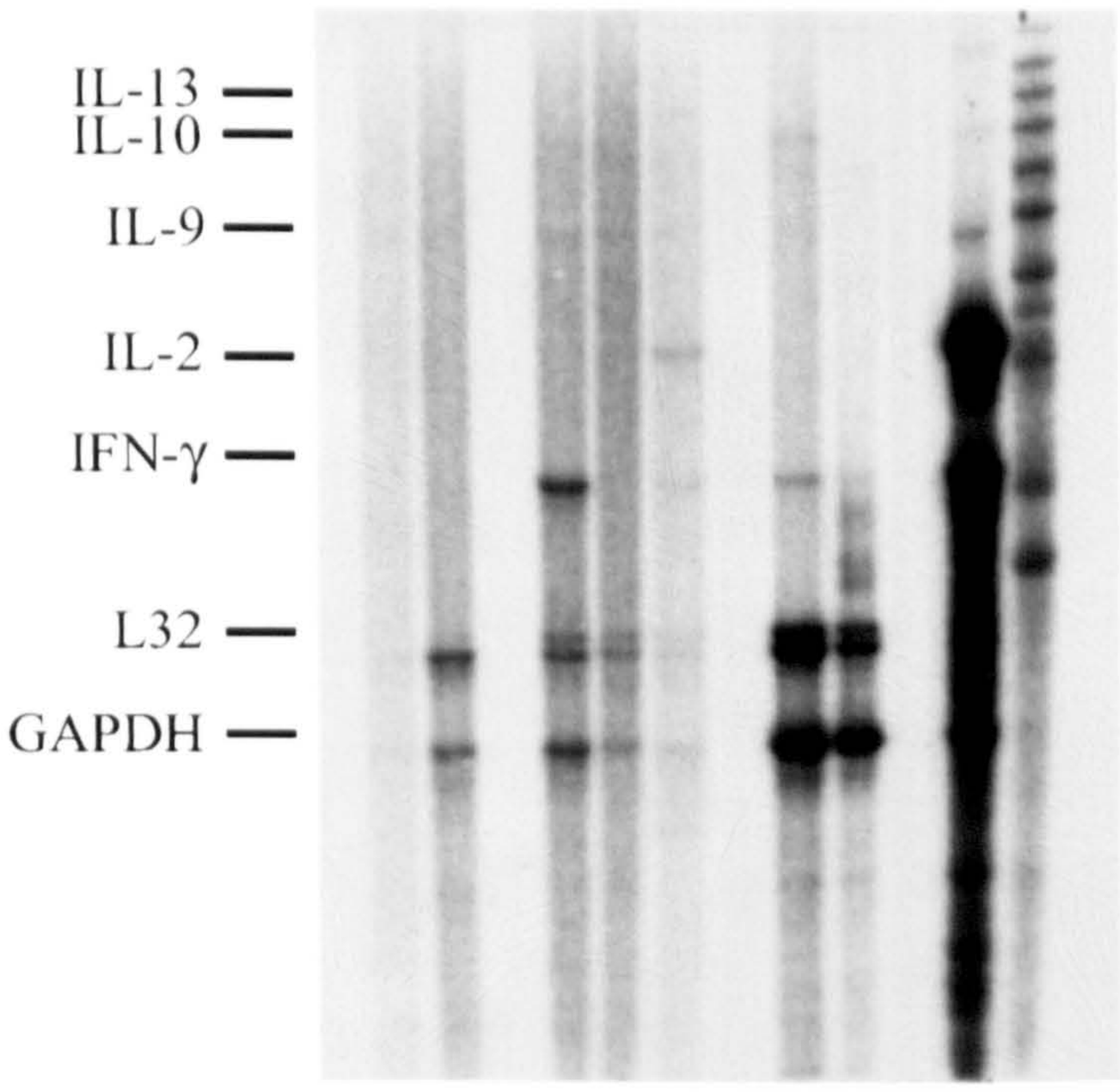
PMA and ionomycin were used to provide maximum stimulation since IL-$2^{-/-}$ $CD4^+$ T cells are hyporesponsive to physiological signals *in vitro*. At 18 hrs, IL-$2^{-/-}$ $CD4^+$ T cells expressed IL-13, IL-6 (traces) and IFN-γmRNA, whereas IL-$2^{+/+}$ $CD4^+$ T lymphocytes expressed IL-2 and IFN-γ mRNA. The expression of IL-2 mRNA, despite low loads, by IL-$2^{+/+}$ $CD4^+$ T cells is a good internal control for the assay.

In general, signals in RPA experiments with B7-1/anti-CD3-stimulated $CD4^+$ T cells were weak as a result of high background smears, indicating that, although CHO B7-1 mRNA did not bind to the radioactive probe, it produced smearing. To circumvent this problem, PMA and ionomycin activation or whole splenocytes stimulated with anti-CD3 were used. The stronger activation signal delivered by PMA plus ionomycin may help to increase the signal to noise ratio. Each RNase protection assay was a separate experiment. This also applies the ELISA, Clontech gene arrays and RT-PCRs described in this chapter. The mice used in these assays were aged 6-8 weeks.

Figure 5.2 **$CD4^+$ T cells stimulated with PMA and ionomycin or CHO B7-1/anti-CD3 at 18 and 48 hours.**

$CD4^+$ T lymphocytes were stimulated with PMA and ionomycin or CHO B7-1/anti-CD3 for 18 hrs and with CHO B7-1/anti-CD3 for 48 hrs. RNA was isolated and hybridised to mCK-1 (BD PharMingen) as described in Materials and Methods.

	18 hours							48 hours				
PMA 10ng/ml+Iono				+		+						
PMA 10ng/ml					+		+					
CHO B7-1+anti-CD3	+	+						+	+			
CHO B7-1control			+									
KO	+			+	+			+				
WT		+				+	+		+			
-VE										+		
+VE											+	
Marker												+



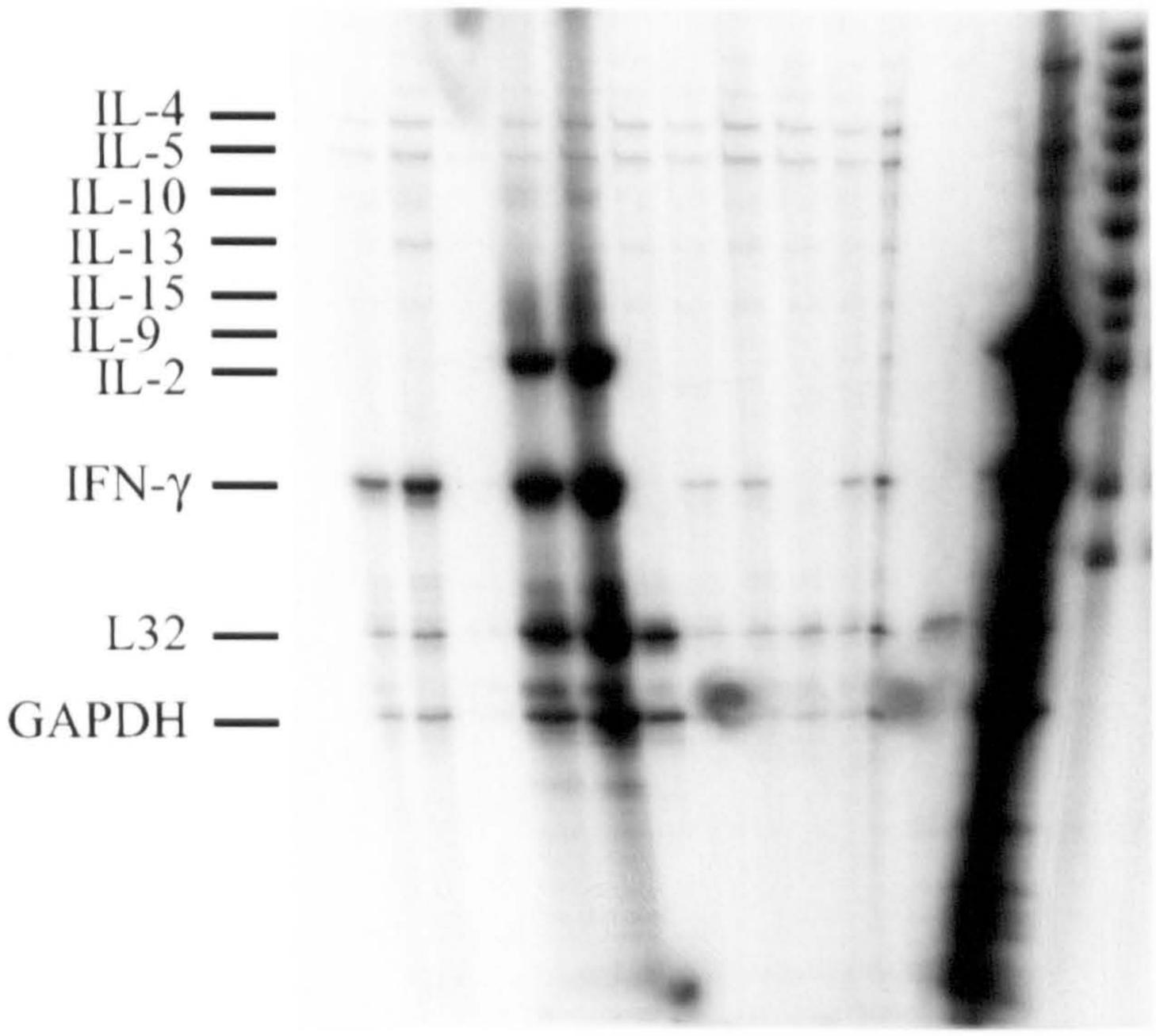
5.2.1-Cytokine mRNA expression by PMA plus ionomycin-activated CD4$^+$ T cells at 18 and 72 hrs:

Figure 5.3 depicts IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4$^+$ T cell activation using PMA and ionomycin at 18 and 72 hrs. In this experiment, both IL-2$^{-/-}$ and IL-2$^{+/+}$ CD4$^+$ T cells expressed IL-4, -5, -10, -13, -15, and IFN-γ mRNA. However, at 18 hrs IL-2$^{-/-}$ CD4$^+$ T cells express IL-9, whereas it was less apparent in IL-2$^{+/+}$ CD4$^+$ T cells, possibly because of high background from the IL-2 band specific to IL-2$^{+/+}$ CD4$^+$ T lymphocytes. Subsequently, the high levels of certain mRNA species such as IL-2 and IFN-γ can obscure the detection of rarer mRNA species. At 72 hrs, both KO and WT CD4$^+$ T cells express IL-4, -5, -10, -13, -15, -9 and IFN-γ. Only IL-2 was exclusively expressed in IL-2$^{+/+}$ CD4$^+$ T lymphocytes, serving as a good internal control.

Briefly, these data do not demonstrate significant differences in cytokine mRNA expression between IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4$^+$ T lymphocytes, apart from IL-2 mRNA in IL-2$^{+/+}$ CD4$^+$ T cells (figures 5.2 and 5.3). This result could be influenced by the protocol used to isolate the CD4$^+$ T cells. Splenocytes are isolated and CD4$^+$ T cells are purified using an extensive method of antibody depletion that lasts six hours at 4°C. During this time, the cells may down-regulate their metabolism or change their phenotype *in vitro*. Possibly also, optimal induction of cytokine genes may require professional APCs to deliver additional costimulatory signals that are not provided by CHO B7-1 transfectants. Alternatively, it is possible that there are no major differences in cytokine mRNA expression between IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4$^+$ T lymphocytes. To test whether IL-2$^{-/-}$ CD4$^+$ T cells behave differently if stimulated in a more physiological context, whole splenocytes were activated with anti-CD3 or PMA and ionomycin. These stimulation conditions may be closer to those *in vivo* and also avoid the purification process.

Figure 5.3 $CD4^+$ T cells stimulated with PMA and ionomycin at 18 and 72 hours. $CD4^+$ T lymphocytes were stimulated with PMA and ionomycin for 18 hrs and 72 hrs. RNA was isolated and hybridised to mCK-1 (BD PharMingen) as described in Materials and Methods.

	18 hours						72 hours								
PMA 20ng/ml+Iono	+			+			+			+					
PMA 10ng/ml+Iono		+			+			+			+				
PMA 10ng/ml			+			+			+			+			
KO	+	+	+				+	+	+						
WT				+	+	+				+	+	+			
-VE													+		
+VE														+	
Marker															+



5.2.2-Cytokine mRNA expression by activated whole spleen cells:

Whole splenocyte cultures were stimulated with anti-CD3 (resident APCs would provide costimulatory signals in this case), or PMA plus ionomycin, and RNA was isolated after 6 or 18 hrs in culture. The rationale behind the 6 and 18 hrs time points is that IL-2 mRNA has been reported to accumulate by 6-8 hrs of culture (268). Also, after 18 hrs in culture, up-regulation of other cytokine mRNAs, which are produced at later time points than IL-2, would be expected. As expected, anti-CD3 supernatant at a concentration of 1/125 was a weaker stimulus than PMA and ionomycin (fig. 5.4). It is important to note that these will stimulate cell subsets other than $CD4^+$ T cells. Indeed, murine primary B cells were reported to be activated by phorbol esters such as PMA (269). Therefore, in view of the different splenic composition of IL-$2^{-/-}$ spleens (chapter 3), it is important to consider cytokine production by splenocytes other than $CD4^+$ T cells. At 6 hrs, both KO and WT PMA and ionomycin-stimulated splenocytes expressed IL-4, -10, -13, -15, -2, -6 and IFN-γ. Interestingly, KO splenocytes stimulated with PMA and ionomycin express higher levels of IL-10 and IFN-γ than WT splenocytes, as well as trace quantities of IL-2 mRNA.

In view of the previous ELISA results (figure 4.13), it seems likely that IL-10 mRNA overproduction in IL-$2^{-/-}$ spleens was by $CD4^+$ T lymphocytes. With regard to trace IL-2mRNA levels in IL-$2^{-/-}$ spleens, IL-2 deficient mice were produced by the insertion of a neo-resistance gene into the third exon of the IL-2 genomic clone. This insertion introduces stop codons in all reading frames of the IL-2 genes and abolishes the biological activity of the IL-2 protein completely (270) (53). Therefore, depending on the IL-2 cDNA fragment used to synthesise the probe, it is possible that the RPA cDNA probe is binding to a truncated transcript which would produce biologically inactive protein.

At 18 hrs, IL-$2^{+/+}$ splenocytes expressed IL-4, -10, -13 (trace levels), -15, -2, -6 and IFN–γ. Conversely, IL-$2^{-/-}$ splenocytes expressed IL-9, -6 (traces) and IFN-γ only.

Despite the much-reduced expression of L32 and GAPDH, it appears that at 18 hrs, IL-10 and IFN-γ are no longer overexpressed in KO splenocytes (fig. 5.4).

Earlier time points were therefore selected to analyse IL-10 mRNA expression in IL-$2^{-/-}$ $CD4^+$ T cells. Figure 5.5 depicts IL-$2^{-/-}$, IL-$2^{+/-}$ and IL-$2^{+/+}$ splenocytes stimulated with anti-CD3 supernatant at a concentration of 1/62.5 or PMA and ionomycin at 3, 6 and 18 hrs. At 3 hrs PMA and ionomycin-stimulated IL-$2^{+/+}$ and IL-$2^{+/-}$ splenocytes expressed IL-4, -10, -15, -2 and IFN-γ; while IL-$2^{-/-}$ splenocytes expressed IL-10, -15 and IFN-γ. It is noteworthy that IL-10 mRNA expression was significantly higher in IL-$2^{-/-}$ than IL-$2^{+/+}$ and IL-$2^{+/-}$ splenocytes. Trace quantities of IL-2 mRNA were expressed in IL-$2^{-/-}$ splenocytes, whereas IL-2 mRNA expression was comparable in IL-$2^{+/+}$ and IL-$2^{+/-}$ splenocytes. This complements the data reported in the previous experiment and also serves as an internal control for the assay (fig. 5.4).

At 6 hrs, IL-$2^{+/+}$ splenocytes express IL-4, -10, -13, -15, 2 and IFN-γ: of these, IL-$2^{+/-}$ splenocytes faild to express IL-13 (fig. 5.5). For IL-$2^{-/-}$ splenocytes at 6 hrs, the sample failed, as the control is very weak, apart from a band representing IFN-γ. At 18 hrs, IL-$2^{+/+}$ splenocytes expressed IL-13 (low levels), -2 and IFN-γ, whereas IL-$2^{-/-}$ splenocytes expressed IL-13, -9 (low levels) and IFN-γ. In figure 5.4, at 18 hrs IL-$2^{+/+}$ splenocytes expressed IL-4, -10, -13, -15 (at low levels), -2, -6 and IFN-γ whereas in fig. 5.5, IL-$2^{+/+}$ splenocytes expressed IL-13, -2 and IFN-γ at 18 hrs. This discrepancy is possibly a result of trace mRNA expression levels of the missing cytokines in the latter experiment; the RNA polymerase had started to perform less efficiently here too, producing non-specific products above the bands representing housekeeping genes (fig. 5.5).

At 18 hrs IL-$2^{+/-}$ splenocytes express IL-4, -5, -10, -13, -15, -9, -2 and IFN-γ. Thus, the kinetics of cytokine mRNA expression in activated splenocytes is possibly dependent on the expression status of the IL-2 alleles. This is also manifested in the early expression of IL-10 mRNA in IL-$2^{-/-}$ splenocytes which is down-regulated after 6 hrs in culture.

To conclude, the only marked difference in the behaviour of IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells is the expression of IL-10 and IFN-γ in IL-$2^{-/-}$ $CD4^+$ T cells at the early time point (6 hrs) seen most clearly in figure 5.5.

Figure 5.4 **RPA of whole splenocyte cytokine mRNA expression**

IL-$2^{+/+}$ and IL-$2^{-/-}$ splenocytes stimulated with PMA and ionomycin or 145-2C11 (anti-CD3ε) supernatant at 1/125 at 6 and 18 hrs. RNA was isolated and hybridised to mCK-1 (BD PharMingen) as described in Materials and Methods.

	6 hours						18 hours								
PMA 20ng/ml+Iono	+			+			+			+					
Anti-CD3 mAb		+			+			+			+				
PMA 10ng/ml			+			+			+			+			
KO				+	+	+				+	+	+			
WT	+	+	+				+	+	+						
-VE														+	
+VE													+		
Marker															+

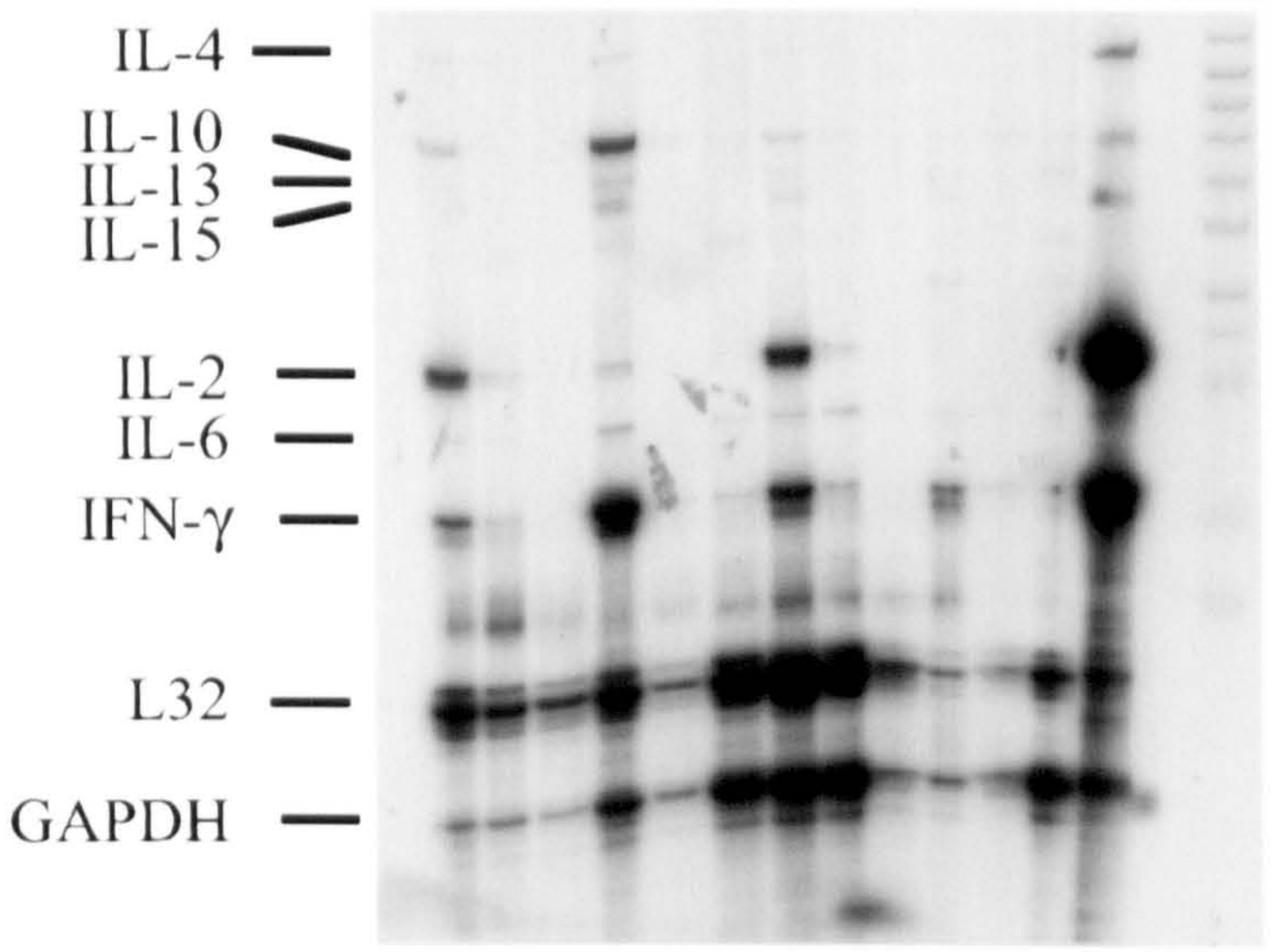
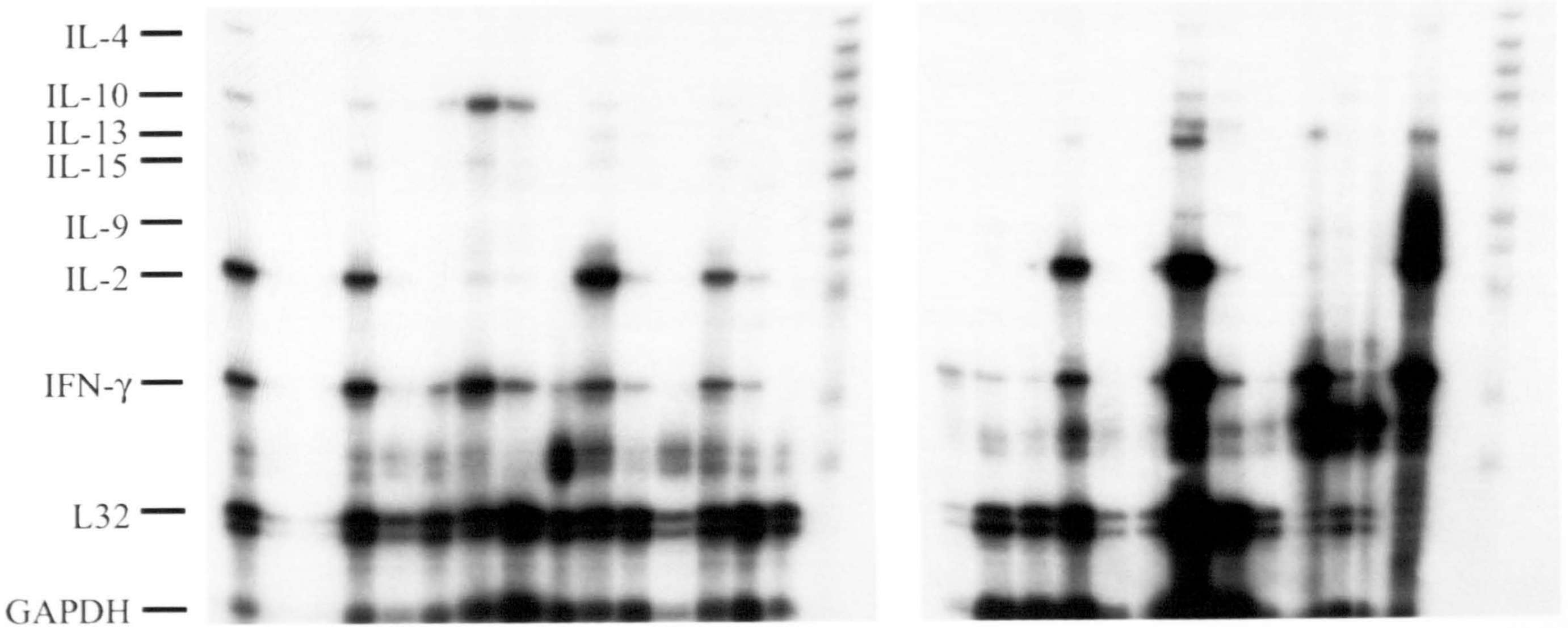


Figure 5.5 **RPA of splenocyte cytokine mRNA expression**

IL-$2^{+/+}$, IL-$2^{+/-}$ and IL-$2^{-/-}$ splenocytes stimulated with PMA and ionomycin or 145-2C11 (anti-CD3ε) supernatant at 1/62.5 at 3, 6 and 18 hrs. RNA was isolated and hybridised to mCK-1 (BD PharMingen) as described in Materials and Methods.

	3 hours									6 hours							18 hours											
	WT			HET			KO			WT			HET				KO			WT			HET			KO			
PMA 20ng/ml+Iono	+			+			+			+			+				+			+			+			+			
Anti-CD3 mAb		+			+			+			+			+				+			+			+					
splenocytes only			+			+			+			+			+				+			+			+				
-VE																											+		
+VE																										+			
Marker																+													+



5.3-Analysis of IL-2$^{+/+}$ and IL-2$^{-/-}$ splenocyte-derived cytokine production by ELISA.

In parallel to splenocyte cytokine mRNA analysis, protein levels were studied using ELISA. Unfractionated spleens were stimulated with anti-CD3 mAb and supernatants were harvested at 96 hrs. IL-2$^{-/-}$ spleens proliferated poorly, in agreement with studies with Con A(53). Based on the appearance of spleen cells under the microscope and the low counts reported above, cultures were harvested at 96 hrs. Figure 5.6.1A depicts IL-4 production by IL-2$^{+/+}$ and IL-2$^{-/-}$ spleen cells stimulated with PMA 20ng/ml and ionomycin 250ng/ml as positive controls. IL-2$^{+/+}$ splenocytes produced 470pg/ml of IL-4, whereas IL-2$^{-/-}$ spleen cells produced trace levels of IL-4. Low production of IL-4 by IL-2$^{-/-}$ splenocytes correlates with human studies of IL-4 production by lamina propria mononuclear cells (LPMCs) from normal and inflamed intestines. Human ELISA studies demonstrated significantly lower production of IL-4 from diseased LPMCs than control cells (271). Figure 5.6.1B illustrates IL-4 production by KO and WT spleen cells stimulated with anti-CD3 supernatant at 1/250. Under these conditions, both KO and WT splenocytes produce trace levels of IL-4.

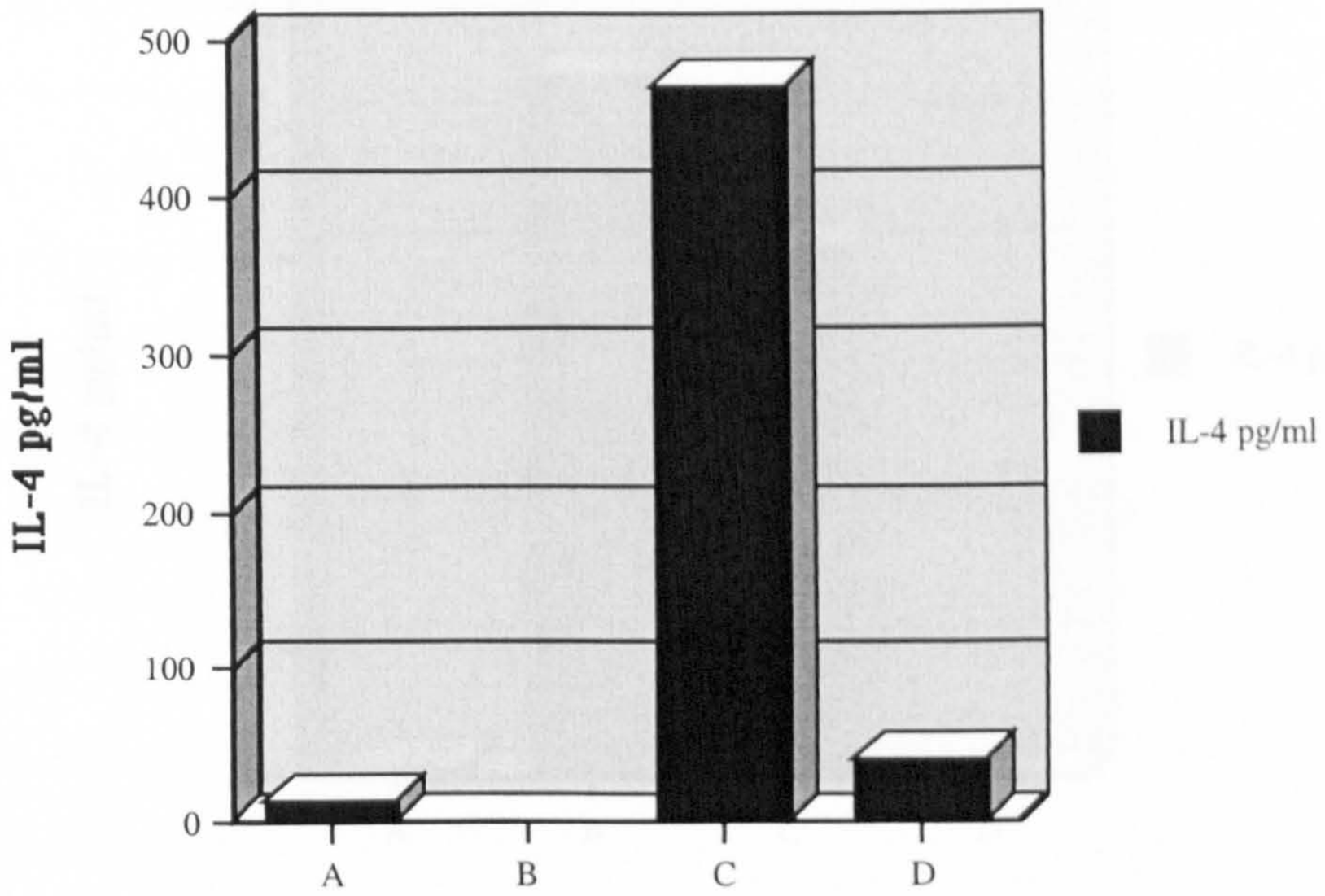
Figure 5.6.1A **IL-4 production at 96 hrs by IL-2$^{+/+}$ and IL-2$^{-/-}$ spleen cultures stimulated with PMA plus ionomycin.**

Splenocytes cultures were set up with PMA plus ionomycin or PMA only. Supernatants were harvested from triplicate wells, pooled and assayed for IL-4.

Figure 5.6.1B **IL-4 production at 96 hrs by IL-2$^{+/+}$ and IL-2$^{-/-}$ spleen cultures stimulated with anti-CD3 mAb.**

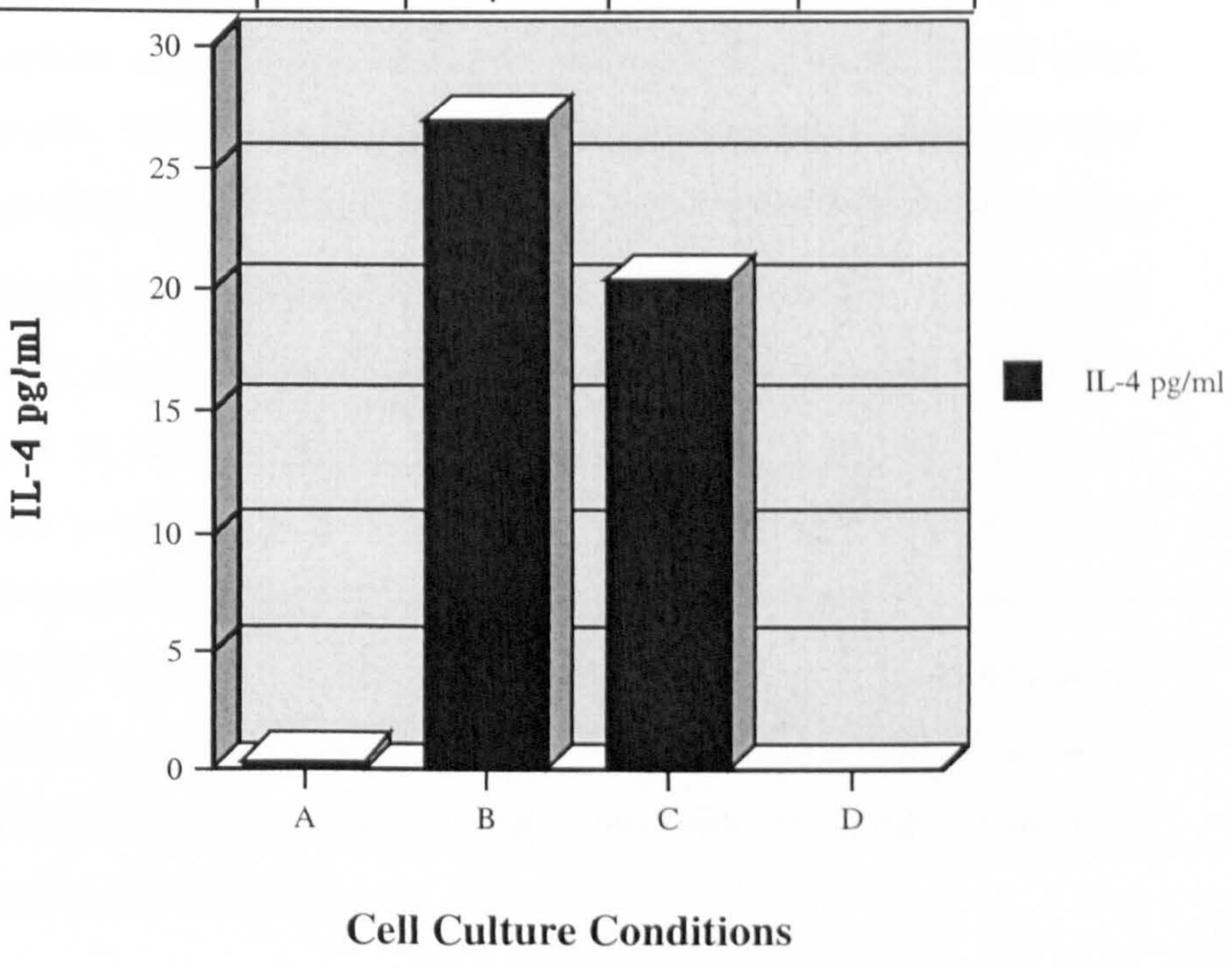
Splenocytes cultures were set up with anti-CD3 mAb (145-2C11 supernatant at 1/250). Supernatants were harvested from triplicate wells, pooled and assayed for IL-4.

PMA 20ng/ml+IONO 250ng/ml	+		+	
PMA 20ng/ml only		+		+
WT			+	+
KO	+	+		



Cell Culture Conditions

Anti-CD3 supernatant 1/250		+	+	
Splenocytes only	+			+
WT			+	+
KO	+	+		



5.3.1-IL-10 production by activated whole spleen cells:

IL-10 production by PMA and ionomycin-stimulated IL-$2^{+/+}$ spleen cells was higher than IL-$2^{-/-}$ splenocytes (fig. 5.6.2A). This is in contrast to purified $CD4^+$ T cells. At 96 hrs, IL-10 production by IL-$2^{-/-}$ $CD4^+$ T lymphocytes was significantly augmented and reached values above 1000pg/ml (fig. 4.15B). This discrepancy is perhaps an outcome of the presence of physiological APCs, such as macrophages, in splenocyte cultures. This implies that in the presence of physiological APCs, the IL-10 produced is either consumed by monocytes or production is inhibited.

Interestingly, FACS analysis of IL-$2^{-/-}$ spleens illustrates an expansion of macrophages that express Fas molecules on their cell surface (fig. 3.4.4A). A recent report has revealed that Fas receptor engagement results in the induction of the IL-10 gene, particularly in monocytes. Monocyte-derived IL-10 was reported to suppress anti-CD3-induced T cell proliferation without modifying CD3 transmembrane signalling (152). This study suggests that induction of IL-10 via the Fas receptor in monocytes may possibly represent an important anti-inflammatory mechanism. This has significant implications in IL-$2^{-/-}$ spleens. As mentioned previously, the Fas-Fas L receptor pathway is non-functional in IL-2 deficient mice. This implies that this novel anti-inflammatory mechanism is possibly defective in IL-$2^{-/-}$ macrophages. Hence, the low production of IL-10 by IL-$2^{-/-}$ spleens in comparison to IL-$2^{+/+}$ spleen cells. Moreover, Fas receptor engagement results in the induction of the IL-10 gene in monocytes, but not in lymphocytes or dendritic cells. Therefore, IL-10 production by IL-$2^{-/-}$ $CD4^+$ T cells would still be intact.

Alternatively, PMA and ionomycin presumably activate other cells such as B cells in splenocyte cultures. Therefore, the interaction of other cells with T cells in splenocyte cultures may result in the down-regulation of IL-10 production. It is noteworthy that under anti-CD3 stimulation, the difference in IL-10 production between IL-$2^{+/+}$ and IL-$2^{-/-}$ spleen cells was significantly reduced, albeit IL-10 production was marginally higher in WT splenocyte cultures (fig. 5.6.2B).

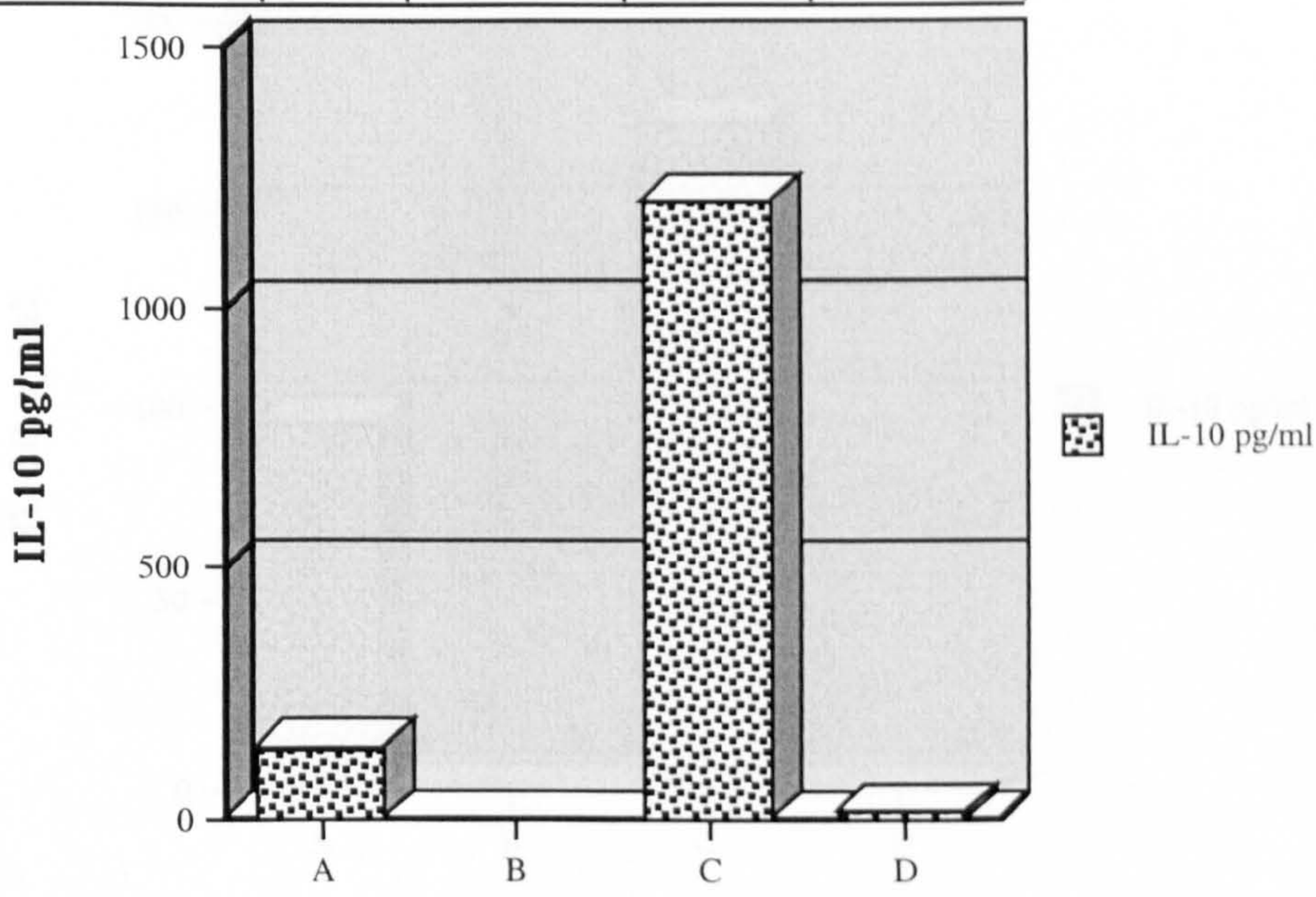
Figure 5.6.2A **IL-10 production at 96 hrs by IL-2$^{+/+}$ and IL-2$^{-/-}$ spleen cultures stimulated with PMA plus ionomycin.**

Splenocytes cultures were set up with PMA plus ionomycin or PMA only. Supernatants were harvested from triplicate wells, pooled and assayed for IL-10.

Figure 5.6.2B **IL-10 production at 96 hrs by IL-2$^{+/+}$ and IL-2$^{-/-}$ spleen cultures stimulated with anti-CD3 mAb.**

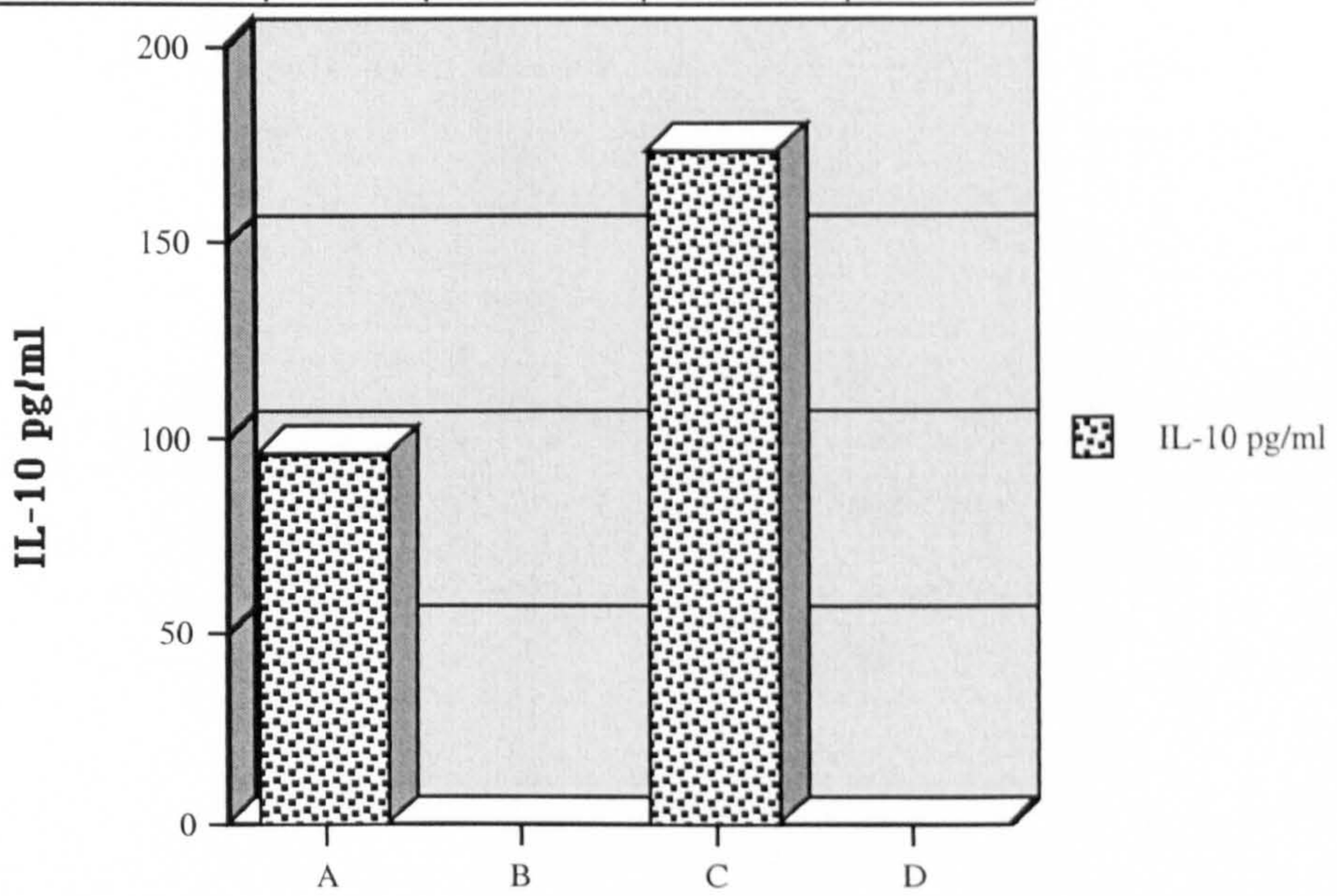
Splenocytes cultures were set up with anti-CD3 mAb (145-2C11 supernatant at 1/250). Supernatants were harvested from triplicate wells, pooled and assayed for IL-10.

PMA 20ng/ml+IONO 250ng/ml	+		+	
PMA 20ng/ml only		+		+
WT			+	+
KO	+	+		



Cell Culture Conditions

Anti-CD3 supernatant 1/250	+		+	
Splenocytes only		+		+
WT			+	+
KO	+	+		



5.3.2-IFN-γ production by activated splenocytes:

Finally, IFN-γ production was marginally higher in PMA and ionomycin-stimulated IL-$2^{-/-}$ spleen cell cultures (fig. 5.6.3A). This was reversed with anti-CD3 stimulation (fig. 5.6.3B). This is probably because anti-CD3 is a weaker stimulus than PMA and ionomycin. Since IL-$2^{-/-}$ splenocytes are hyporesponsive *in vitro*, production of IFN-γ is expected to be minimal. However, due to the TH_1 propensity of KO splenocytes, when the cells are driven with a strong stimulus such as PMA and ionomycin, they produce a high level of IFN-γ, as demonstrated in figure 5.5.

Figure 5.6.3A **IFN-γ production at 96 hrs by IL-2$^{+/+}$ and IL-2$^{-/-}$ spleen cultures stimulated with PMA plus ionomycin.**

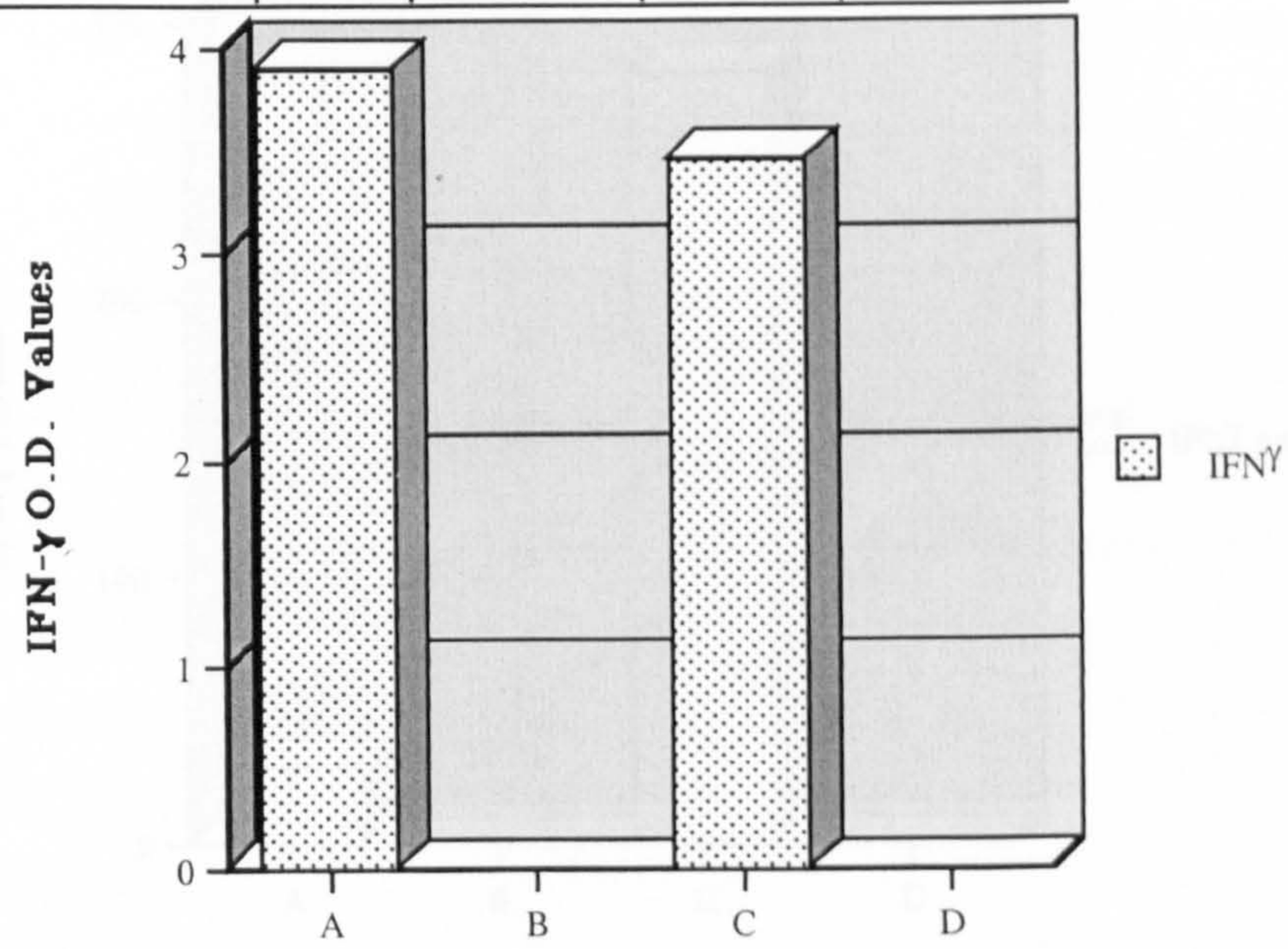
Splenocytes cultures were set up with PMA plus ionomycin or PMA only. Supernatants were harvested from triplicate wells, pooled and assayed for IFN-γ.

* O.D. value represents 3000 pg/ml. Supernatants were diluted 1/10.

Figure 5.6.3B **IFN-γ production at 96 hrs by IL-2$^{+/+}$ and IL-2$^{-/-}$ spleen cultures stimulated with anti-CD3 mAb.**

Splenocytes cultures were set up with anti-CD3 mAb (145-2C11 supernatant at 1/250). Supernatants were harvested from triplicate wells, pooled and assayed for IFN-γ.

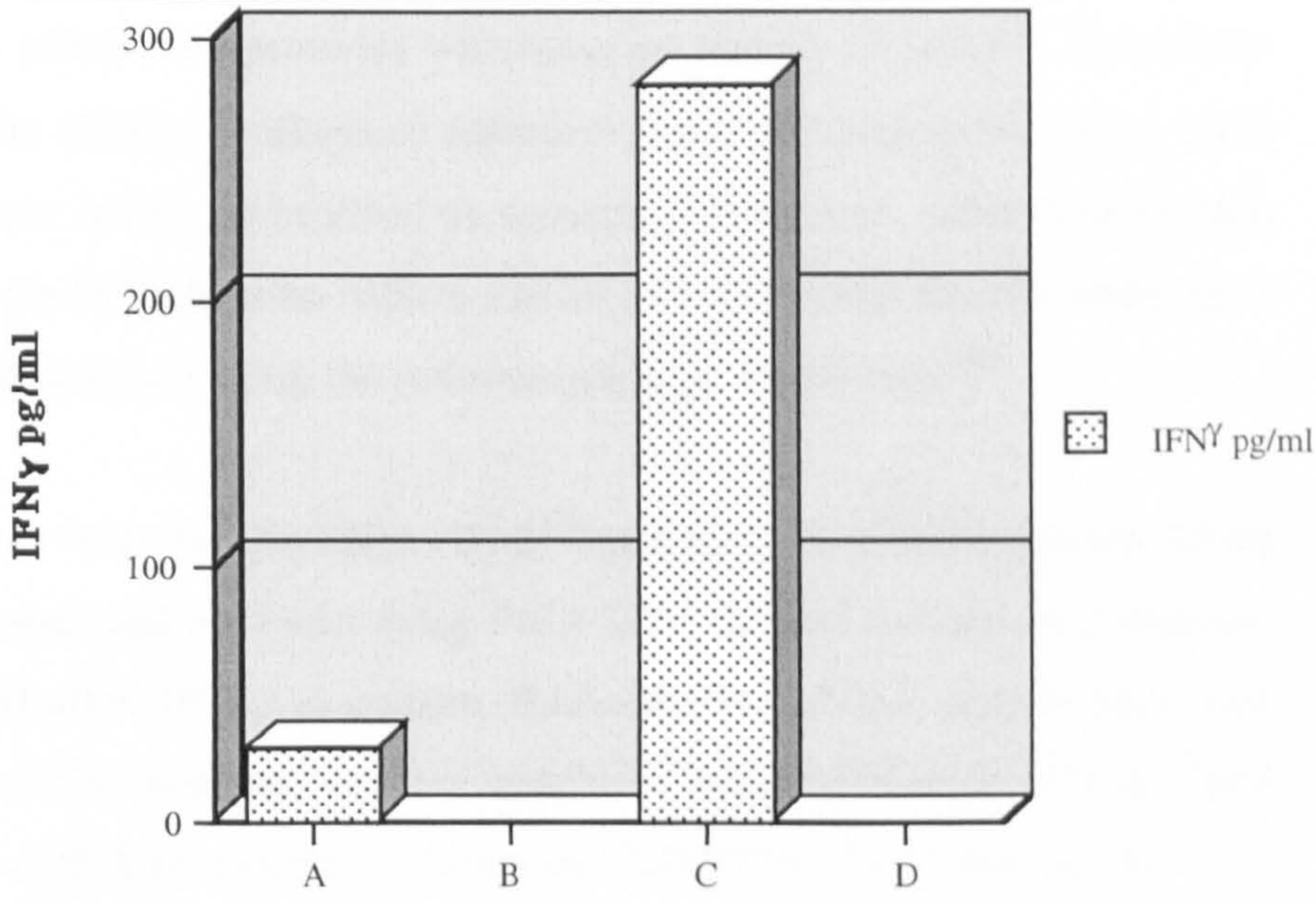
PMA 20ng/ml+IONO 250ng/ml	+		+	
PMA 20ng/ml only		+		+
WT			+	+
KO	+	+		



Cell Culture Conditions

* O.D. value of 2.3 represents 3000 pg/ml.

Anti-CD3 supernatant 1/250	+		+	
Splenocytes only		+		+
WT			+	+
KO	+	+		



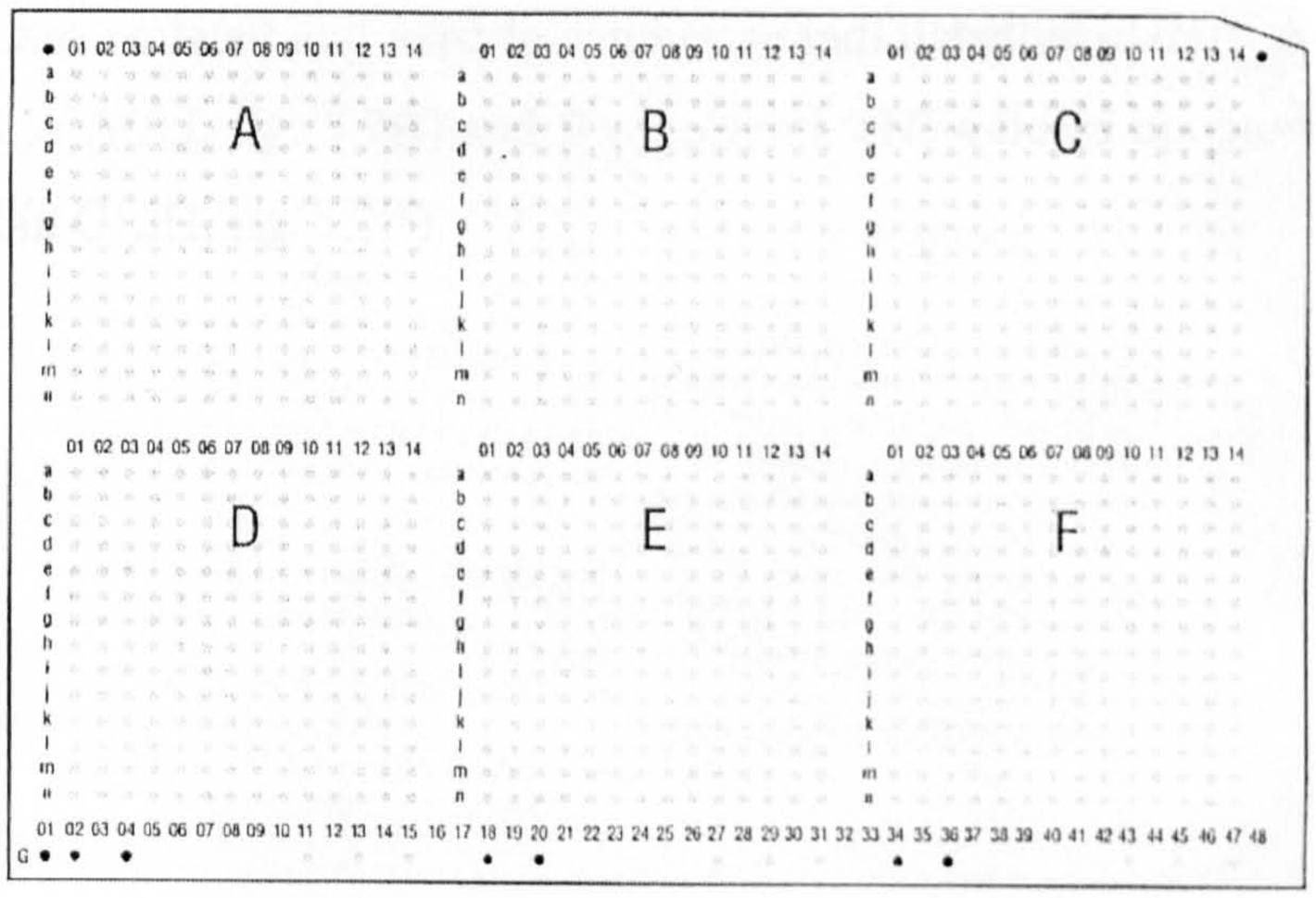
Cell Culture Conditions

5.4-CLONTECH's cDNA gene arrays.

The Clontech Atlas procedure is used to compare the gene expression profiles of a panel of cell surface antigens, adhesion molecules, transcription factors, protein kinase receptors, cell cycle proteins, oncogenes and tumour suppressors, enzymes of metabolism, growth factor and chemokine receptors and many others. The Clontech Mouse 1.2 Array II includes 1176 mouse cDNA and nine housekeeping control cDNAs and comprises two identical membranes on which hundreds of cDNAs are spotted. Genes of immunological relevance are located in box "D" on the membrane and include genes for cytokines/cytokine receptors and chemokines (see p214) The first step involves the isolation of mRNA samples from activated IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes. Subsequently, the mRNA samples are reverse-transcribed to probes using cDNA primer mix provided with the array and [α-^{32}P] dATP. The primer mixes ensure that the cDNAs synthesised represent genes included in the array. Each radioactive probe mix is then hybridised to separate membranes. After several high stringency washes, the hybridisation pattern can be detected using phosphoimaging or autoradiography and analysed using the software package AtlasImage™.

$CD4^+$ T lymphocytes were purified from IL-$2^{+/+}$ and IL-$2^{-/-}$ C57BL/6 spleens (from mice 6-8 weeks of age) and activated using PMA 20ng/ml and ionomycin 250ng/ml. mRNA was isolated after 48 hrs in culture. Radiolabelled cDNA probes were then synthesised. Figure 5.7A illustrates autoradiographs of the hybridisation of IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cell cDNA to membrane filters of CLONTECH's cDNA Mouse 1.2II array. KO and WT filters were also scanned using a phosphoimager. AtlasImage, the software for analysing Atlas™ arrays, was used to align both membranes and compare gene expression profiles. Following alignment, arrays are normalised according to housekeeping genes in both arrays and a normalisation coefficient is used to normalise array 2 to array 1. Figure 5.7B illustrates a colour schematic comparison of the two arrays, where array 1 represents hybridised WT cDNA and array 2 represents hybridised KO cDNA. This schematic comparison consists of an array of boxes, each representing an Atlas array gene. Each box is divided in half, horizontally: the upper half describes the ratio of the signal between the arrays (after normalisation) and the

lower half describes the difference in signal between the arrays (after normalisation) [AtlasImage^{TM} 1.5 User manual].



A membrane is divided into six major boxes, named A-F. Each box comprises 14 columns and 14 rows labelled as a-n. For example, the gene co-ordinate (C02m) means that the gene is located in box C, column 2, row m. The grid position of the nine housekeeping control cDNAs which were used for normalisation are: G11, G13, G15, G27, G29, G31, G43, G45, G47.

Definition of colours in the Array Comparison scheme:

	DEFINITION Array 2 vs. Array 1	RATIO (upper half of box)	DIFFERENCE (lower half of box)
ORANGE	Up-regulation	A2/A1 >X	A2-A1 >Y
PURPLE	Down-regulation	A2/A1 <1/X	A2-A1 <-Y
GREEN	Equal expression	1/X <A2/A1 <X	-Y <A2-A1 <Y
GREY	Background level	One or both arrays below signal threshold*	Both arrays are below signal threshold✶

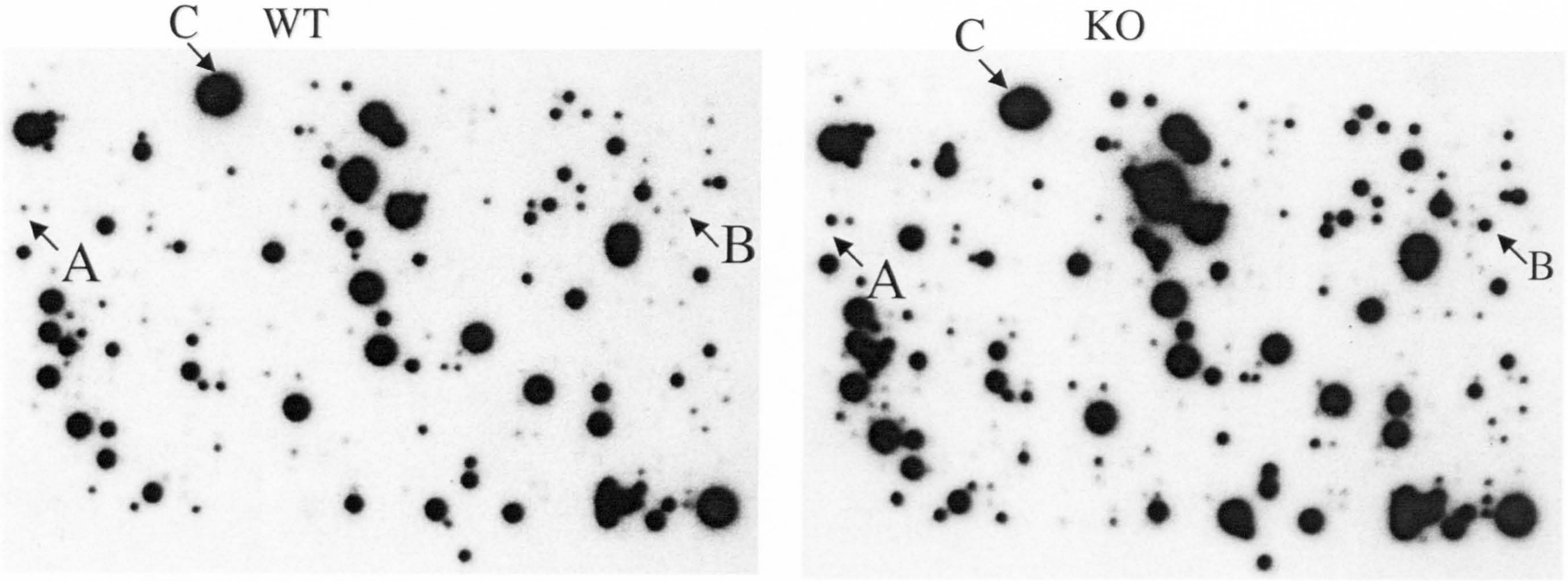
*Represents values of >2 or <0.5. Adapted from [AtlasImage^{TM} 1.5 User manual].

✶Represents values of 10 or 12 depending on method of activation.

Figure 5.7A **CLONTECH'S cDNA membrane analysis of mRNA from IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4^{+} T lymphocytes stimulated with PMA plus ionomycin at 48 hours.**

CD4^{+} T lymphocytes were purified and stimulated with PMA plus ionomycin for 48 hours. mRNA was isolated and used to synthesise radiolabelled cDNA. A represents CD37 (located in A01J, fig. 5.7B) and B represents TNF-related apoptosis-inducing ligand (located in C12H, fig. 5.7B).

Figure 5.7A $CD4^+$ T lymphocytes stimulated with PMA plus ionomycin at 48 hours.



A = CD37
B = TNF-related apoptosis-inducing ligand
C = Apolipoprotein CII

As shown by the complete boxes in figure 5.7B, approximately 11 genes out of the 1176 mouse cDNAs were found to be significantly differentially expressed in the two types of $CD4^+$ T lymphocytes. The two genes with immune involvement were selected for further study, mainly CD37 (located in A01j) and TNF-related apoptosis-inducing ligand (TRAIL), located in C12h. Table 5.1 demonstrates the ratio and difference reports generated on the AtlasImage Software from the stimulated IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes. The expression of CD37 and TRAIL was 2 fold and 5 fold higher respectively in IL-$2^{-/-}$ $CD4^+$ T cells.

Figure 5.7B **Colour schematic Atlas™ array comparison of IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells stimulated with PMA plus ionomycin at 48 hours.**

AtlasImage, the software for analysing Atlas™ arrays was used to align both membranes, shown in fig. 5.7A. Following alignment, arrays were normalised according to housekeeping genes. This schematic comparison consists of an array of boxes, each representing an Atlas array gene. Arrays are numbered alphabetically in rows and numerically in columns; with 6 main boxes labelled within the array, top three labelled A, B, C respectively and bottom three representing D, E and F respectively. Orange represents up-regulation in IL-$2^{-/-}$ $CD4^+$ T lymphocytes, purple: down-regulation in IL-$2^{-/-}$ $CD4^+$ T lymphocytes, green: equal expression in both IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells and grey: background levels.

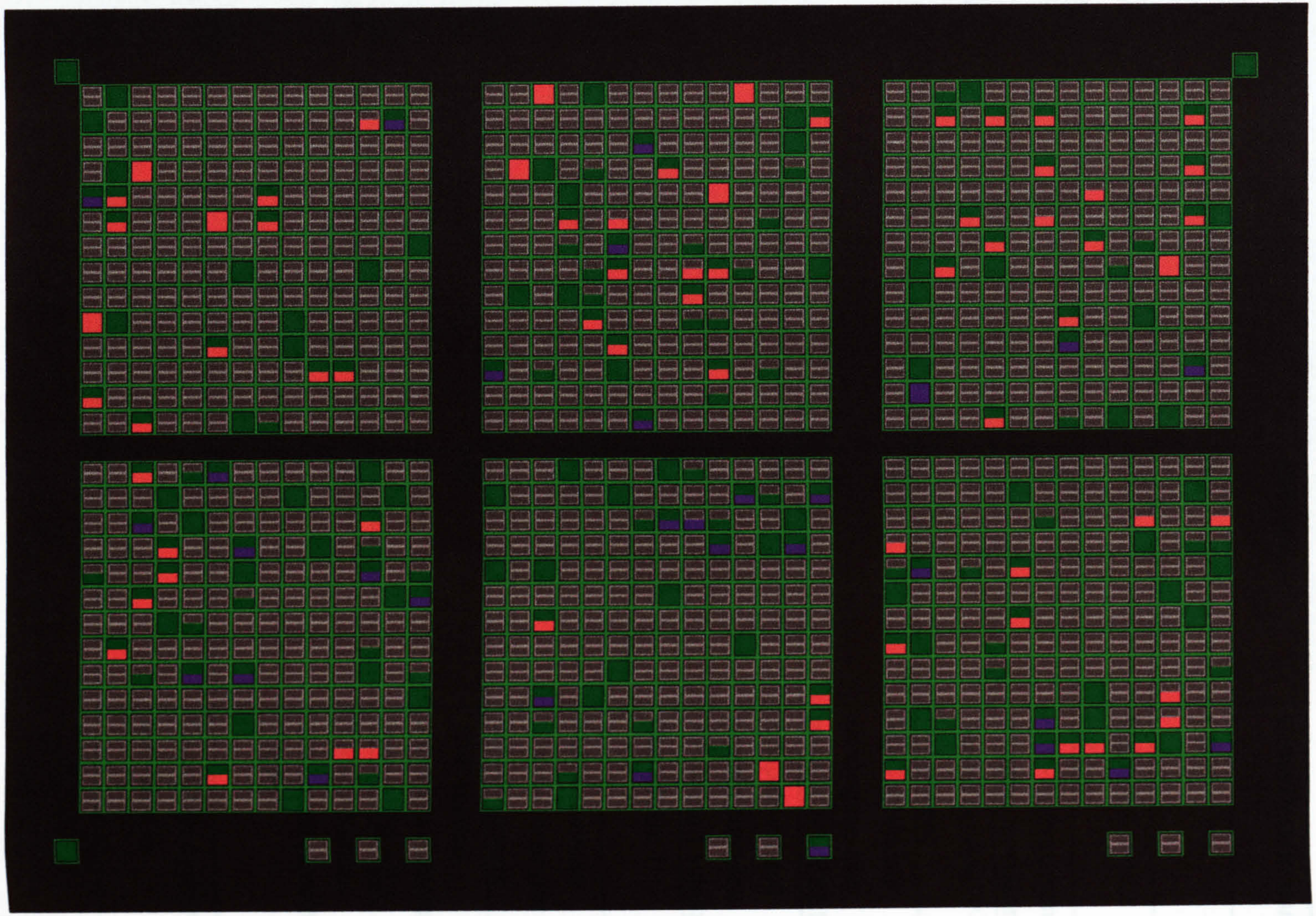


Table 5.1 Differential gene expression by PMA and ionomycin-stimulated IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4^{+} T lymphocytes.

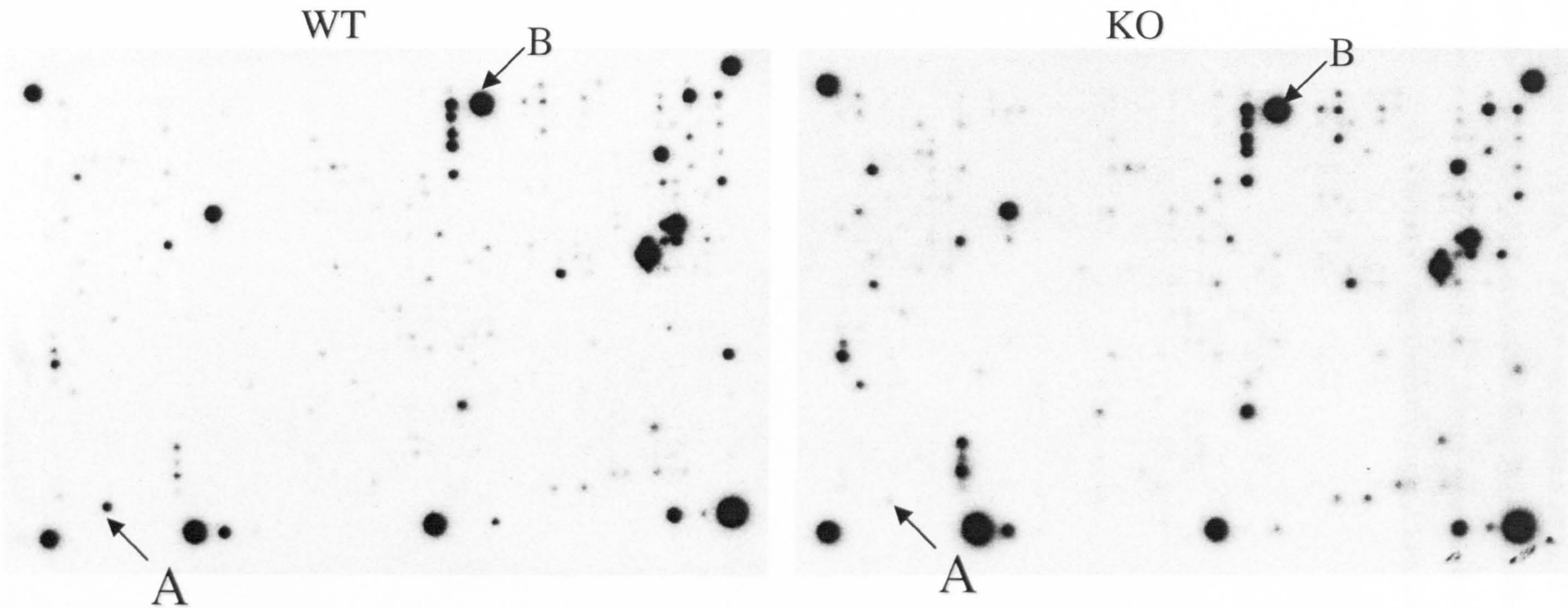
Gene co-ordinate	Ratio	Difference	Gene/Protein name
A01J	2.09	36	CD37
A03d	2.14	47	Epithelial V-like antigen
A06f	2.75	28	Aryl-hydrocarbon receptor repressor
B02d	2.35	77	Serum amyloid P-component
B03a	2.96	106	Myeloblastosis onocogene-like protein 1(MYBL1)
B10e	2.00	16	Adaptor-related protein complex AP-4beta 1 subunit
B11a	2.30	26	Complexin 1
C02m	0.47	-59	Medium-chain acetyl-Coenzyme A dehydrogenase
C12h	5.47	67	TNF-related apoptosis inducing ligand (TRAIL)
E12m	2.99	1759	S phase kinase-associated protein 2 (SKP2)
E13n	2.13	72	Guanylate cyclase activator 1a

Figure 5.8A demonstrates membrane filters of IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes stimulated with CHO B7-1/anti-CD3 for 48 hrs. Figure 5.8B represents the schematic comparison of IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells. Under these conditions, more genes were differentially expressed. Genes such as CD6 (A02b), CD82 (A02i), LFA-1 (A03f) and CD3δ (D09j), ε (D09k) and γ (D09l) were up-regulated in IL-$2^{-/-}$ $CD4^+$ T lymphocytes, whereas IL-17R was down-regulated (D04m) (fig. 5.8B). Table 5.2 demonstrates the ratio and difference reports generated on the AtlasImage Software from CHO B7-1/anti-CD3-stimulated IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes.

Figure 5.8A CLONTECH'S cDNA membrane analysis of mRNA from IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4^{+} T lymphocytes stimulated with CHO B7-1 and anti-CD3ε 3μg/ml at 48 hours.

CD4^{+} T lymphocytes were purified and stimulated with CHO B7-1 and anti-CD3ε 3μg/ml for 48 hours. mRNA was isolated and used to synthesise radiolabelled cDNA.

Figure 5.8A CD4+ T lymphocytes stimulated with CHO B7-1/anti-CD3 at 48 hours.



A = IL-17 receptor

B = Glyceraldehyde-3-phosphate dehydrogenase

Figure 5.8B **Colour schematic Atlas™ array comparison of IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4^{+} T cells stimulated with CHO B7-1 and anti-CD3ε 3μg/ml at 48 hours.**
AtlasImage, the software for analysing Atlas™ arrays was used to align both membranes, shown in fig. 5.8A. Details of the schematic comparison are as described in fig 5.7B.

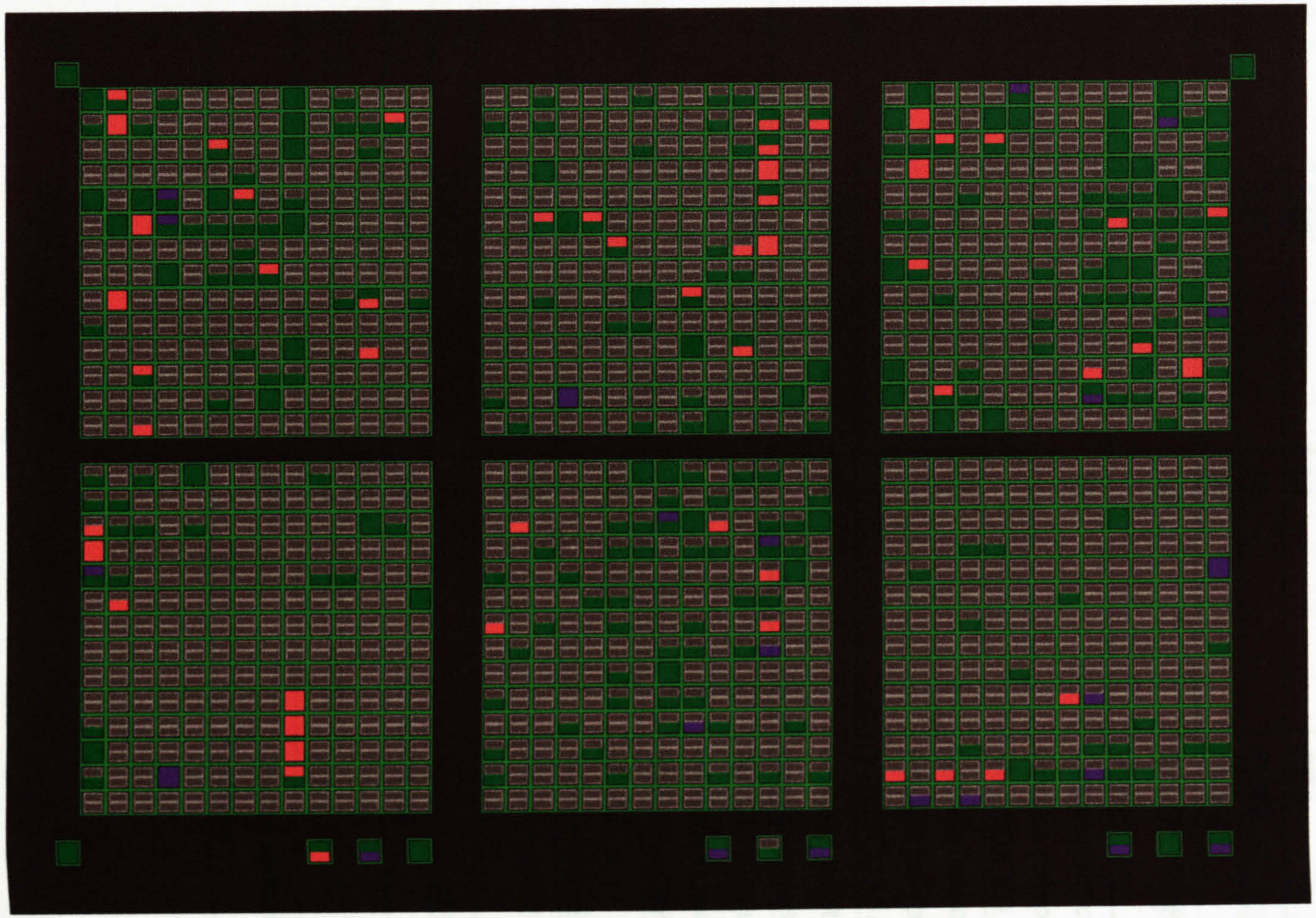


Table 5.2 Differential gene expression by CHO B7-1/anti-CD3-stimulated IL-2$^{+/+}$ and IL-2$^{-/-}$ CD4^{+} T lymphocytes.

Gene Co-ordinate	Ratio	Difference	Gene/Protein name
A02b	6.00	10	CD6
A02i	5.67	14	CD82
A03f	3.77	36	LFA-1
B04m	0.29	-10	4F2/CD98 light chain
B12d	2.16	43	Cytochrome c oxidase polypeptide Vb (COX5B)
B12g	2.64	46	Cytochrome c oxidase polypeptide VIIIa (COX8A)
C02b	2.29	18	Soluble malate dehydrogenase
C02d	5.00	28	NADH-ubiquinone oxidoreductase MWFE subunit
C13l	2.86	26	DEAD box polypeptide 5
D01d	2.36	30	High mobility group protein 2
D04m	0.07	-39	IL-17 receptor
D09j	2.94	33	CD3δ
D09k	3.00	20	CD3ε
D09l	4.05	67	CD3γ
F14e	0.18	-47	Sperm specific antigen

Figure 5.9 illustrates the schematic comparison of IL-$2^{-/-}$ $CD4^{+}$ T lymphocytes stimulated with CHO B7-1/anti-CD3 after 8 and 48 hrs in culture. Genes such as the 47kDa-interferon gamma-inducible protein (located in E14c) were up-regulated in the 8-hrs cultures, whereas genes such as CD3ε were equally expressed at both time points. Table 5.3 demonstrates the ratio and difference reports generated on the AtlasImage Software from CHO B7-1/anti-CD3-stimulated IL-$2^{-/-}$ at 8 and 48 hrs.

Figure 5.9 Colour schematic Atlas™ array comparison of IL-2$^{-/-}$ CD4^{+} T cells stimulated with CHO B7-1 and anti-CD3ε 3μg/ml at 8 and 48 hours.

AtlasImage, the software for analysing Atlas™ arrays was used to align both membranes. Following alignment, arrays were normalised according to housekeeping genes. Details of the schematic comparison are as described in fig 5.7B.

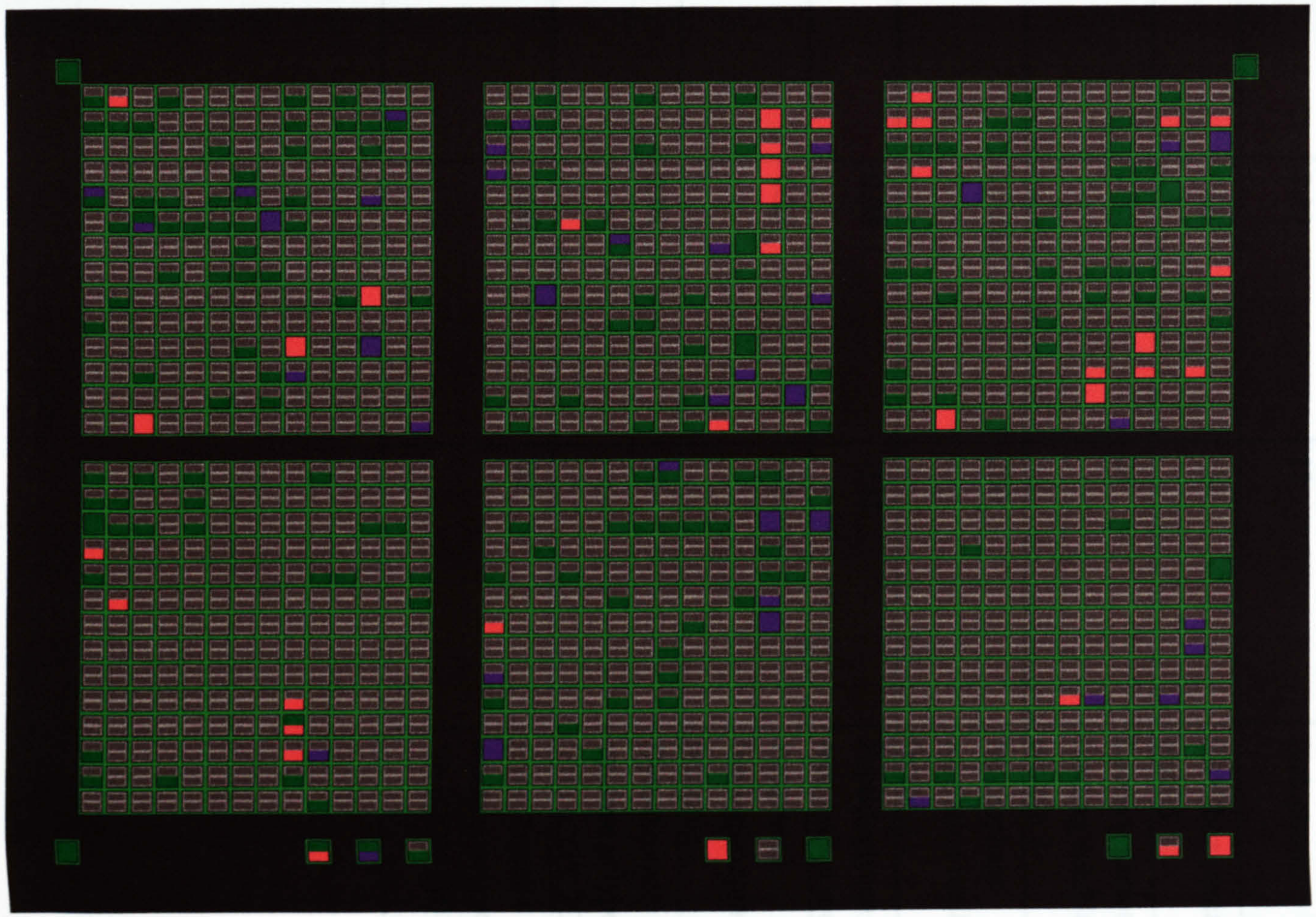


Table 5.3 Differential gene expression by CHO B7-1/anti-CD3-stimulated IL-2$^{-/-}$ CD4^{+} T lymphocytes at 8 and 48 hrs.

Gene co-ordinate	Ratio	Difference	Gene/Protein name
A03n	2.80	18	Sialophorin
A08f	0.04	-24	SRY-box containing gene 6
A09k	2.41	24	Lectin galactose-binding soluble protein 3
A12i	16.38	200	Macrophage migration inhibitory factor
A12k	0.08	-143	Peptidoglycan recognition protein
B03i	0.27	-22	Peripheral benzodiazepine receptor
B12b	2.91	63	Cytochrome c oxidase polypeptide VIIc (COX 7C)
B12d	5.00	60	Cytochrome c oxidase polypeptide Vb (COX 5B)

Table 5.3 continued:

Gene co-ordinate	Ratio	Difference	Gene/Protein name
B12e	2.70	46	Cytochrome c oxidase polypeptide VI a1 (COX 6A1)
B13m	0.24	-16	X-linked glucose-6-phosphate
C03n	3.09	23	Phosphoglycerate kinase 1
C04e	0.22	-29	Prosaposin
C09m	2.54	156	Peptidylprolyl isomerase A
C11k	4.81	259	Ribosomal protein L6
C14c	0.17	-24	RNA binding motif protein
E011	0.07	-69	Monokine induced by IFN-γ (MIG)
E12c	0.24	-13	Lipocortin 1
E12g	0.07	-851	S100 calcium-binding protein A9
E14c	0.32	-13	47KDa interferon gamma-inducible protein

5.4.1-Comparison of the CHO B7-1/anti-CD3 and PMA plus ionomycin activation systems:

Surprisingly the pattern of gene expression generated by the 2 activation systems (Figures 5.7A and 5.8A) were found to markedly differ with a number of genes showing extreme bias in their expression. While the differences between the WT and KO comparisons were in the 2-6 range, genes such as CD12d and TNF-induced protein 1 showed up to 100 fold differences between the two stimuli (table 5.4). Figure 5.10 represents the schematic comparison for IL-$2^{+/+}$ $CD4^{+}$ T lymphocytes which are summarised in table 5.4. The two activation systems appear to regulate differential gene expression of several metabolic proteins and intracellular kinase members. Most notable was the expression of apolipoprotein CII (A13b), specific to PMA plus ionomycin stimulation and glyceraldehyde-3-phosphate dehydrogenase (B14b), specific to CHO B7-1/anti-CD3-mediated activation. Although the two activation systems lead to T cell proliferation, at the molecular level there are surprising differences in the programme of gene activation.

Figure 5.10 Colour schematic Atlas™ array comparison of IL-2$^{+/+}$ CD4^{+} T cells stimulated with CHO B7-1/anti-CD3 and PMA plus ionomycin at 48 hours.

AtlasImage, the software for analysing Atlas™ arrays was used to align both membranes. Following alignment, arrays were normalised according to housekeeping genes. Details of the schematic comparison are as described in fig 5.7B. Two prominent examples of differential gene expression are labelled C (fig. 5.7A), located in A13b (fig. 5.10), specific to PMA plus ionomycin stimulation and B (fig.5.8A), located in B14b (fig. 5.10), specific to CHO B7-1/anti-CD3 stimulation.

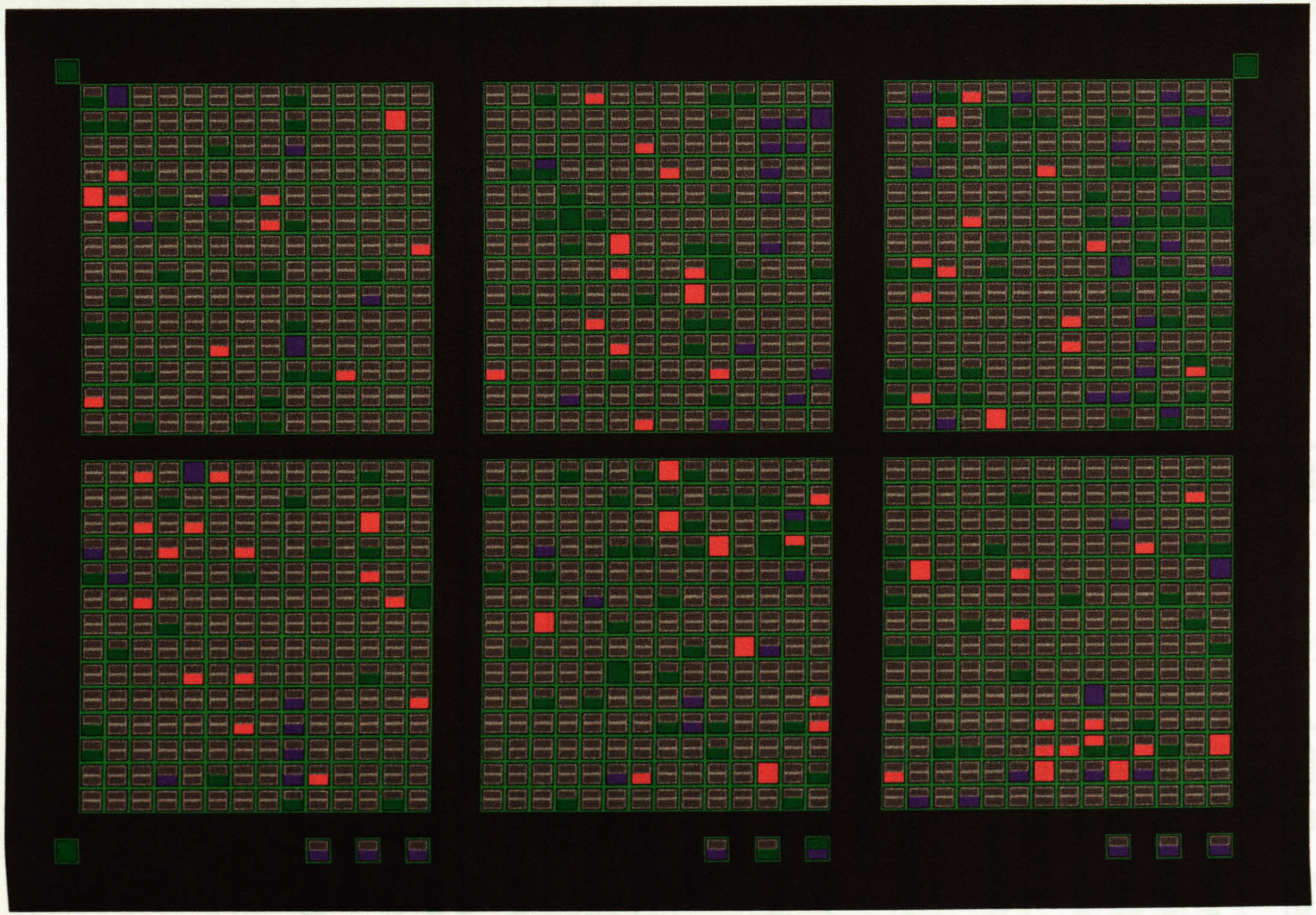


Table 5.4 Differential gene expression by CHO B7-1/anti-CD3 and PMA plus ionomycin-stimulated IL-2$^{+/+}$ CD4^{+} T lymphocytes.

Gene co-ordinate	Ratio	Difference	Gene/Protein name
A01e	99.00	294	CD1d2 antigen
A02a	0.14	-6	CD48
A09k	0.09	-31	Lectin galactose-binding protein 3
A13b	95.75	379	Apolipoprotein CII
B06g	259.50	517	Organic cation transporter 3
B09i	45.88	359	Brain α-spectrin 2
B14b	0.01	-485	Glyceraldehyde-3-phosphate deydrogenase
C05n	11.80	54	Mitochondrial glutamate oxaloacetate transaminase 2
C10h	0.20	-8	HSP 60
D05a	0.14	-6	IL- 2Rβ
D12c	3.75	11	Olfactory receptor C6
E03g	83.00	164	Wingless-related MMTV integrated site 1 protein precursor (WNT 1)

Table 5.4 continued:

Gene co-ordinate	Ratio	difference	Gene/protein name
E08a	3.11	19	MAPKAP kinase 2
E08c	61.75	243	MAP/ERK kinase 3
E10d	2.20	12	Cell line NK14 derived transforming oncogene
E11h	4.00	6	cGMP phosphodiesterase 6D rod delta
E12m	28.00	81	S phase kinase-associated protein 2 (SKP2)
F02e	100.50	199	Mannan-binding lectin serine protease 2 (MASP2)
F07m	9.86	62	Drebrin-like
F09j	0.09	-30	Vimentin (VIM)
F10m	12.33	34	DNA primase p49 subunit
F14e	0.04	-55	Sperm specific antigen 1
F14l	134.00	399	Tumour necrosis factor induced protein 1

5.5-Cytokine mRNA detection using RT-PCR.

In a parallel approach, RT-PCR was used to analyse IFN-γ, IL-2, -4, -5, -10, -12p40 and IL-13 cytokine expression by activated IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells-stimulated with CHO B7-1/anti-CD3 or PMA plus ionomycin at 48 hrs. RT-PCR analysis of IL-$2^{+/+}$ $CD4^+$ T cells-stimulated with CHO B7-1/anti-CD3, despite weak GAPDH bands, revealed expression of IL-2, -4, -5 and –13, whereas IL-$2^{-/-}$ $CD4^+$ T lymphocytes expressed IL-4, -5, IFN-γ and IL-13. Using PMA and ionomycin, at 48 hrs, IL-$2^{+/+}$ $CD4^+$ T lymphocytes expressed IL-2, -5 and -13. IL-$2^{-/-}$ $CD4^+$ T cells expressed IL-5 and –13 only (fig. 5.11).

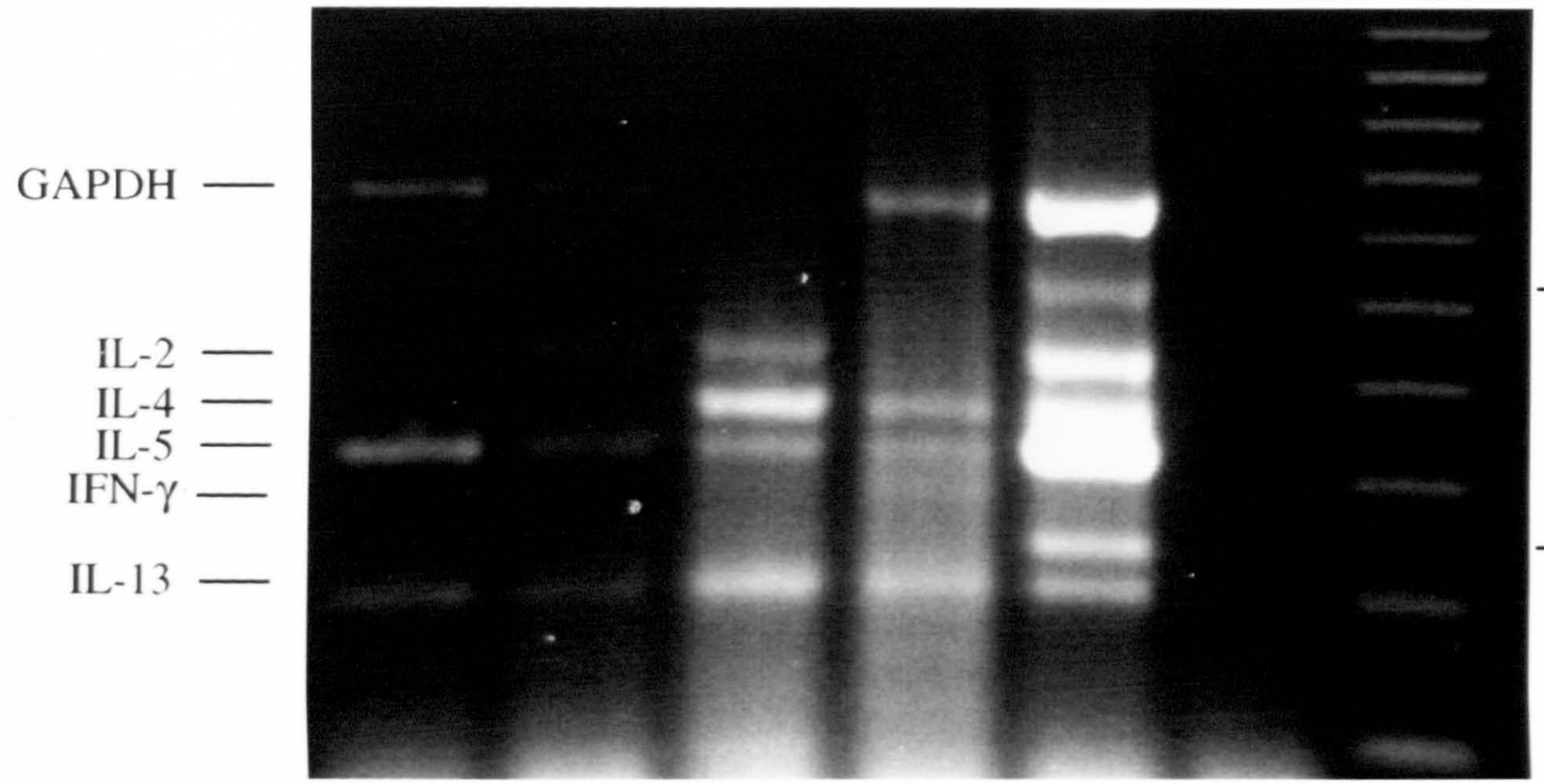
Collectively, these data do not demonstrate significant differential expression of cytokines between IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes. Rather, they point to some differences such as high production of IL-10 by IL-$2^{-/-}$ $CD4^+$ T cells and the up-regulation of genes such as LFA-1 and CD82 (involved in immune regulation) in these cells.

Figure 5.11 RT-PCR of IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells stimulated with CHO B7-1 and anti-CD3ε 3μg/ml or PMA plus ionomycin at 48 hours.

$CD4^+$ T lymphocytes were purified and stimulated with CHO B7-1 and anti-CD3ε 3μg/ml or PMA 20ng/ml plus ionomycin 250ng/ml for 48 hours. RNA was isolated after 48 hrs in culture and cDNA was synthesised. RT-PCR was performed using primers from the CytoXpress multiplex PCR (Biosource International).

Figure 5.11 RT-PCR on WT and KO CD4+ T lymphocytes at 48 hours.

PMA + Iono	+	+					
B7-1 + anti-CD3			+	+			
KO	+			+			
WT		+	+				
-VE						+	
+VE					+		
Marker							+



DISCUSSION

RNase protection assays (RPAs) provide a specific method for the simultaneous detection of several mRNA species in a single sample of RNA. In general, CHO B7-1/anti-CD3 stimulation did not produce distinct bands due to high background smearing unlike PMA and ionomycin-stimulation, which provided a stronger stimulus. Two time points were selected, 18 and 72 hrs, because cytokine mRNA was expected to be up-regulated by 18 hrs and potentially down-regulated at the late time point as confirmed in figure 5.3. These data did not reveal significant differences in cytokine mRNA expression between IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes. As described previously in the results section, this may be a consequence of the protocol used. The cell purification process is time consuming and may modify the cells' phenotype from the starting *ex vivo* phenotype.

Alternatively, real differences in cytokine profiles may only be observed when physiological APCs are used instead of CHO B7-1 transfectants. This is possible because:

Firstly, IL-2 is a pleiotropic cytokine and its deficiency may affect other cell subsets such as APCs. If this is the case, due to the interaction between APCs and T cells *in vivo*, APCs may regulate the cytokine profile produced by $CD4^+$ T cells. Indeed, the cytokine microenvironment produced collectively by APCs and activated T cells in the *in vivo* milieu plays a critical role in T helper cell differentiation towards TH_1 or TH_2 cell types during an immune response (272) (257) (273).

Secondly, physiological APCs may deliver additional costimulatory signals, not provided by CHO B7-1 transfectants that are needed by IL-$2^{-/-}$ $CD4^+$ T lymphocytes for optimal activation and cytokine production *in vitro*. To test these hypotheses, IL-$2^{+/+}$ and IL-$2^{-/-}$ splenocytes were stimulated with PMA and ionomycin or anti-CD3 mAb. At early time points such as 3 and 6 hrs, PMA and ionomycin-stimulated IL-$2^{-/-}$

spleen cells expressed higher levels of IL-10 mRNA that subsided at later time points (fig. 5.4 and 5.5).

Collectively, RPA splenocyte data do not reveal any significant differences in cytokine mRNA expression between IL-$2^{+/+}$ and IL-$2^{-/-}$ spleen cell, apart from IL-10 mRNA over-expression in IL-$2^{-/-}$ $CD4^+$ T cells at early time points. These data point to differences in the kinetic regulation of cytokine mRNA depending on the expression status of the IL-2 alleles. This is emphasised with regard to the higher IL-10 mRNA expression at 3 and 6 hrs in IL-$2^{-/-}$ spleen cells (figures 4.13C, 4.14B and 4.15B).

IL-10 is a well-known anti-inflammatory and immunosuppressive cytokine. Its immunosuppressive activities inhibit the synthesis of T helper TH_1 cytokines (153) and macrophage-derived cytokines such as TNF-α, IL-12 (274). Moreover, it is associated with down-regulation of costimulatory molecules (275) (276), MHC class II expression on APCs (277) and effector functions of macrophages such as nitric oxide production (278). It is therefore tempting to suggest that IL-10 overproduction by IL-$2^{-/-}$ $CD4^+$ T cells is a result of an anti-inflammatory response aimed at targeting the inflammatory milieu in IBD. In support of this is the IL-$10^{-/-}$ mouse model which develops a spontaneous colitis (75). Spontaneous chronic colitis in IL-$10^{-/-}$ mice was suggested to be an outcome of nitric oxide (NO) overproduction, because of lack of NO regulation due to absence of IL-10, and an uncontrolled TH_1 cytokine response by $CD4^+$ T cells (279). Likewise, recent studies have highlighted the role of inducible nitric oxide synthase (iNOS) in IBD (280) (281). Surprisingly, mice deficient in both iNOS and IL-10 (iNOS$^{-/-}$/IL-$10^{-/-}$) developed chronic colitis. This suggested that iNOS does not play a major role in the development or severity of inflammation in IL-$10^{-/-}$ mice (282).

On the other hand, IL-10 has recently been described to mediate immunostimulatory activities such as stimulation of activated $CD8^+$ T cells (247). Moreover, IL-10 has

been implicated in the immunopathology of other autoimmune models such as systemic lupus erythematosus (SLE) (283) (284) and diabetes in non-obese mice (250) (285) (286). In this context, it is noteworthy to remark that IL-10 overproduction by IL-$2^{-/-}$ $CD4^+$ T cells may be pathogenic. Perhaps the presence of IL-2 is required for exertion of IL-10 immunosuppressive activities. As yet, this notion has not been investigated. Administration of anti-IL-10 blocking mAb in IL-$2^{-/-}$ mice or crossing IL-2 deficient mice with IL-$10^{-/-}$ mice (IL-$2^{-/-}$/IL-$10^{-/-}$) will clarify this question. As there is no evidence in support of this notion, it is fair to state that the general consensus in the literature points to a possible regulatory role of IL-10 in IBD. Interestingly, administration of rIL-10 in IL-$10^{-/-}$ young mice prevents disease. However, administration of IL-10 in adult IL-$10^{-/-}$ mice did not reverse the disease process but did ameliorate disease progression (279).

In addition to RPA studies, cDNA gene microarrays were used to investigate comparative gene regulation in IL-$2^{-/-}$ and IL-$2^{+/+}$ $CD4^+$ T lymphocytes. This provides the means to study the expression profile of hundreds of genes in IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes. Figure 5.7B and table 5.1 illustrates the relative over-expression of CD37 (A01j) and TNF-related apoptosis-inducing ligand (C12h) in PMA and ionomycin-stimulated IL-$2^{-/-}$ $CD4^+$ T cells in comparison to IL-$2^{+/+}$ $CD4^+$ T cells.

CD37 is a member of the tetraspanin superfamily, which includes CD9, CD53, CD63, CD81 and CD82 (287). All members encode cell surface proteins that span the lipid bilayer four times, hence the name. Still awaiting definitive functional studies, these molecules have been detected in complexes with integrins, MHC class II molecules and other tetraspanins. Specifically, CD37 was shown to be enriched in exosomes and associated with the costimulatory molecule B7-2 and MHC class II (288). More importantly, CD37 was reported to be expressed mainly on B cells and at low levels on T cells and monocytes (289). Recently, a CD37 deficient mouse model was generated in an attempt to gain insight into its functional role *in vivo*. Knobeloch et al. reported that CD37 was not critical for B cell development. Furthermore, under

suboptimal conditions, such as administration of antigen in the absence of adjuvant, antibody responses were poor in CD37$^{-/-}$ mice. Thus, T cell-dependent B cell responses were defective in CD37$^{-/-}$ mice. T cell-independent B cell responses, such as IgG specific antibody responses to TNP-Ficoll (which cross-links B cell membrane immunoglobulins resulting in B cells activation) were elevated in CD37$^{-/-}$ mice. This suggests that CD37 mediates an immunoregulatory role in T cell-independent B cell humoral responses (290).

To date, the above are all the data reported about CD37. It is most interesting that CD37 is up-regulated on IL-2$^{-/-}$ CD4^{+} T lymphocytes as demonstrated in figure 5.7B, specifically as it is expressed at low levels on T cells under normal circumstances. Interestingly, the proliferative responses of anti-CD3-stimulated IL-2$^{-/-}$ unfractionated lymph node cells were blocked more effectively by anti-B7-2 than anti-B7-1 (208). This suggests that B7-2 costimulatory molecules may play a pivotal role in the physiological activation of IL-2$^{-/-}$ CD4^{+} T lymphocytes. Furthermore, as mentioned previously, CD37 was reported to be enriched in exosomes and associate with the costimulatory molecule B7-2 and MHC class II (288). Therefore, it is possible that the up-regulation of CD37 in IL-2$^{-/-}$ CD4^{+} T cells may lower the activation threshold of the T cells by facilitating their interaction with APCs, thereby contributing to the exaggerated proliferative responses of IL-2$^{-/-}$ T cells *in vivo*.

Other up-regulated molecules in IL-2$^{-/-}$ CD4^{+} T lymphocytes include TNF-related apoptosis-inducing ligand (TRAIL). The cell surface protein TRAIL is a TNF family member and a potent mediator of apoptosis (291). Most interesting is the promiscuous nature of TRAIL and also its significant (6-fold) up-regulation in IL-2$^{-/-}$ CD4^{+} T cells. TRAIL was reported to bind to five receptors, two of which induce apoptosis: TRAIL-R1 and TRAIL-R2. The remaining three receptors, TRAIL-R3, TRAIL-R4 and the soluble receptor osteoprotegerin (OPG) have not been shown to transmit apoptotic signals (reviewed in (292)). Moreover, TRAIL appeared to specifically kill transformed cell lines but not normal cells. With regard to T lymphocytes, TRAIL was

not able to induce significant levels of apoptosis in human peripheral T cells regardless of their activation state (reviewed in (293)). As mentioned previously, the Fas/Fas L pathway is defective in IL-$2^{-/-}$ mice. In this context, it is most intriguing that PMA and ionomycin-stimulated IL-$2^{-/-}$ $CD4^+$ T lymphocytes up-regulate TRAIL expression. This is possibly to compensate for the non-functional Fas/Fas L pathway. Mechanistic studies to analyse whether TRAIL on IL-$2^{-/-}$ $CD4^+$ T cells is operative will be most informative and would give insight into potential alternative mechanisms to a dysfunctional APO-1/Fas apoptotic pathway.

CHO B7-1/anti-CD3-activated IL-$2^{-/-}$ $CD4^+$ T lymphocytes up-regulated costimulatory molecules such as CD6 (A02b), CD82 (A02i) and LFA-1 (A03f) and down-regulated IL-17 receptor (D04m) (fig. 5.8B and table 5.2). Recently, the importance of immunological synapses has been highlighted in several studies and the role of adhesion molecules in stabilising immunological synapses has been acknowledged (reviewed in (294)).

Looking at the up-regulated costimulatory molecules listed above, it is important to note that CD82 is another member of the tetraspanin family whose precise role has not been identified as yet. However, like CD37, CD82 has been associated with adhesion and facilitation of immune responses (287).

LFA-1 is a member of the integrin superfamily and binds to its ligand ICAM-1. Initial studies suggested that adhesion receptor functions may be regulated by TCR signals (295). The idea was that signals from the cytoplasm would directly regulate integrin affinity through a mechanism of "inside-out" signalling. The current dogma suggests that, on resting lymphocytes, LFA-1 is restrained on the cell surface via an active mechanism by the lymphocyte cytoskeleton. When LFA-1 is held on the lymphocyte surface, it is not capable of forming clusters and mediating adhesion. Following lymphocyte activation, LFA-1 is uncoupled from the actin cytoskeleton, as measured by its diffusion rate. This induces clustering of LFA-1 and subsequently, enhances

binding to ICAM-1 via an "inside-out" signalling mechanism (296) (297). Binding to ICAM-1 introduces conformational changes in LFA-1 that initiate a poorly defined signalling process, an "outside-in" signal that enhances the cytoskeletal changes and strengthens adhesion (298). These studies indicate that LFA-1 is a major player in the adhesion stage of lymphocyte activation, a crucial step in bringing the TCR and MHC into close proximity. Clearly, if overexpression of LFA-1 mRNA in B7-1/anti-CD3-stimulated IL-$2^{-/-}$ $CD4^+$ T cells results in enhanced surface expression and subsequent release of LFA-1, it would facilitate antigen-specific adhesion to APCs and formation of immunological synapses, finally culminating in T cell activation.

Surprisingly, IL-17R was down-regulated in B7-1/anti-CD3-activated IL-$2^{-/-}$ $CD4^+$ T cells. IL-17 is produced by a restricted set of cells, human activated memory $CD4^+$ T cells (299) and mainly murine activated alpha beta $TCR^+CD4^-CD8^-$ thymocytes (300). Despite the restricted production of IL-17, IL-17R is ubiquitously expressed. Human IL-17 was designated as an inflammatory cytokine, implicated in autoimmune diseases such as rheumatoid arthritis (301). Murine recombinant soluble IL-17 receptor was shown to inhibit T cell proliferation and IL-2 production induced by mitogen stimulation (Con A or PHA) or anti-TCR mAb (302). This study emphasises the role of IL-17 in T cell proliferation. Based on the reported inflammatory role of IL-17, an increase in IL-17 production by IL-$2^{-/-}$ $CD4^+$ T lymphocytes might have been expected. The converse was, however, seen suggesting a potential regulatory role for IL-2 in IL-17R expression.

An unexpected finding from these studies was that different activation systems result in a striking disparity in gene expression. As illustrated in table 5.4, the two activation systems appear to include distinct sets of genes. For example, apolipoprotein CII (A13b) induction was specific to PMA plus ionomycin stimulation, while glyceraldehyde-3-phosphate dehydrogenase (B14b) induction was specific to CHO B7-1/anti-CD3-mediated activation. Apolipoprotein CII and glyceraldehyde-3-

phosphate play key roles in energy metabolism. Therefore, different activation system appear to elicit different metabolic pathways.

Finally, RT-PCR analysis of the cytokine profile of IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes did not reveal significant differences (fig. 5.11). However, it demonstrated that the cytokine profile produced by B7-1/anti-CD3-activated IL-$2^{-/-}$ $CD4^+$ T cells (IL-4, -5, -IFN-γ and IL-13) correlated with that produced by human IBD patients, where IFN-γ is produced in Crohn's disease and IL-5 is indicative of ulcerative colitis (303).

Collectively, the data presented in this chapter provide evidence that strongly suggests minor differences in cytokine expression between activated IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T lymphocytes. Perhaps most interesting are the costimulatory and adhesion genes; CD6, CD37, and LFA-1; up-regulated in IL-$2^{-/-}$ $CD4^+$ T cells. These findings do not point to major quantum shifts, but rather to subtle differences, whose molecular basis and relevance remain obscure.

CHAPTER 6

THE ROLE OF B7: CD28/CTLA-4 BLOCKADE IN THE PATHOGENESIS OF IBD IN IL-2$^{-/-}$ MICE.

INTRODUCTION

The engagement of a T cell antigen receptor is important, but not sufficient, to mediate complete activation, especially of naïve T cells. In fact, it can result in a state of T cell unresponsiveness named anergy. Anergy is a cellular status in which the T cell fails to respond to TCR signalling, to proliferate, and to synthesise the primary growth factor IL-2. Complete T cell activation requires a second signal, a "costimulatory signal" provided by secreted soluble factors, cytokines and growth factors, or cell-surface molecules normally on professional APCs. The requirement for two distinct signals for the complete activation of T cells provides a system to maintain the fidelity of the activation and a regulated immune response, which is in turn translates to the maintenance of tolerance and the prevention of unwanted immune responses.

As mentioned previously, the best-characterised pathway is via B7: CD28/CTLA-4. As a direct consequence its critical role in T cell activation, one can envisage its potential role(s) in mediating disease. More importantly, these molecules offer potential opportunities for clinical intervention as the effects of costimulation blockade would be specific to T cells that have already received signal 1. This approach constitutes a more specific intervention in comparison to methods promoting generalised immune suppression, such as the use of corticosteroids.

The effectiveness of costimulatory blockade in immune intervention was first demonstrated in the early 1990s using mouse CTLA-4Ig (169). CTLA-4Ig is a fusion protein consisting of the murine CTLA-4 extracellular domain and the Fc portion of human IgG1 that binds to the B7 molecules, B7-1 and B7-2, thereby blocking interactions with B7 and its ligands CD28/CTLA-4. Despite the discovery of additional costimulatory pathways, such as CD40L (CD154)/CD40, LFA-1 (CD18)/ICAM-1 (CD54) and CD2/CD58, the B7: CD28/CTLA-4 pathway remains the principal target for the treatment of autoimmune disease in mice.

The role of B7: CD28/CTLA-4 blockade in experimental models of autoimmune diseases:

The critical role of the B7: CD28/CTLA-4 pathway in mediating disease has been investigated using experimental models of autoimmune diseases in rodents. In this context, spontaneuos autoimmune diabetes and experimental autoimmune encephalomyelitis (EAE), which is a model for human multiple sclerosis will be discussed.

EAE is a demyelinating disease of the central nervous system (CNS) that can be induced in susceptible strains of mice by immunisation with different immunodominant peptides derived from myelin proteins, such as myelin basic protein (MBP) and proteolipoprotein (PLP). EAE is characterised by an initial phase of paralysis followed by recovery and subsequent relapses. Blockade of the costimulatory B7: CD28/CTLA-4 pathway was found to be an effective intervention in both the initial phase of disease onset (304) as well as in recurrent relapses (207). In the context of disease initiation, spontaneous development of EAE was observed in RAG-$1^{-/-}$ MBP-specific TCR-transgenic mice (TgMBP^{+}/RAG-$1^{-/-}$). The deletion of CD28 in TgMBP^{+}/RAG-$1^{-/-}$ mice ($CD28^{-/-}$ TgMBP^{+}/RAG-$1^{-/-}$) abrogated disease and provided evidence that CD28 is critical for the initiation of spontaneous EAE (304). In the context of recurrent relapses, anti-B7-1 F(ab) fragment treatment of mice immunised with an immunodominant epitope from PLP (PLP 139-151) resulted in abrogation of clinical relapses and amelioration of CNS pathology (207). In another study, the immunisation of susceptible female Lewis rats with guinea pig spinal cord homogenate in CFA resulted in severe EAE with 100% mortality by day 17 post-immunisation. However, treatment with hCTLA-4Ig dramatically abrogated disease severity and prevented fatal EAE (305).

The role of the B7: CD28/CTLA-4 pathway has also been investigated in the spontaneous NOD mouse diabetes model. In contrast to EAE and the other

autoimmune experimental models, such as autoimmune thyroiditis or collagen-induced arthritis, blockade of the B7: CD28/CTLA-4 pathway in NOD mice appears to accelerate autoimmune diabetes. This finding was first reported in a study of CD28$^{-/-}$/NOD and CTLA-4Ig transgenic NOD (CTLA-4Ig Tg/NOD) mice (306). Surprisingly, CD28$^{-/-}$/NODs (mice that are defective in the B7: CD28 pathway) and CTLA-4Ig Tg/NODs (mice that are defective in the B7: CD28/CTLA-4 pathway) had exacerbated autoimmune diabetes. Evidently there are basic differences between the NOD model of spontaneous diabetes and other experimental autoimmune models.

Firstly, the diabetes observed in NOD mice is spontaneous and has been attributed to chronic exposure to pathogenic self-antigens (307), whereas other experimental autoimmune models have to be induced using immunisation with the relevant self-antigen(s) in pro-inflammatory adjuvant. In this context, CD28 has been reported to be non-essential in chronic long–term stimulation conditions, when compared to short-term stimulation conditions. This is manifested in studies by Kundig et al. in which the repeated stimulation of CD28$^{-/-}$ mice with peptides *in vivo* resulted in functional T cell responses, whereas transient T cell stimulation with peptides *in vivo* resulted in anergy (308). This emphasises the differential role of CD28 depending on the activation status of the T cell and the circumstances of activation. Memory T cells were found to be less dependent than naïve cells on CD28 costimulation. Also, T cells stimulated with high doses of antigen (309) and strong peptide agonists (308) were independent of CD28. The independence of long-term responses of CD28 signals may elucidate the non-essential role of CD28 in spontaneous autoimmune models such as diabetes. However, it does not explain the acceleration of diabetes in the absence of CD28.

Secondly, a TH_2 immune response appears to be protective in NOD autoimmune diabetes. Indeed, treatment of young NOD mice with anti-CD28 mAb at 2 weeks of age inhibited destructive insulitis (T cells infiltrating the pancreatic islets of Langerhans) and protected against diabetes by an IL-4 dependent mechanism (310). Arreaza et al. reported that CD28 stimulation during weeks 2-4 of age is essential for

the prevention of NOD diabetes; it was mediated by the generation of a TH_2 cell-enriched non-destructive environment in the pancreatic islets of treated NOD mice (310). However, this effect was not observed when treating older mice. These results highlight the complex effects of blocking the B7: CD28 costimulatory pathway and bring to attention that effective blockade of this pathway depends on the nature and stage of the disease process involved and the T cell populations mediating it. Thus, the balance between TH_1 and TH_2 differentiation is critical for a successful blockade. In the case of young NODs, CD28 signals are necessary for TH_2 differentiation (311). Therefore, inhibition of TH_2 differentiation (which is a result of B7: CD28 blockade) can lead to an exacerbated TH_1 immune response, as in this attack on pancreatic β cells, whereas TH_2-mediated diseases such as lupus are ameliorated. This is exemplified by the NZB/NZW F1 mouse strain, which is an antibody-dependent model of systemic lupus erythematosus. As mentioned previously in chapter 1, section 6.4, CTLA-4Ig treatment of NZB/NZW F1 mice results in complete abrogation of disease.

Finally, CD28 signals have been shown to be critical in up-regulating the negative regulator CTLA-4 (312) (313). Thus, blocking B7: CTLA-4 signals will prevent their limiting influence on T cell proliferation and lead to the exacerbation of disease.

To summarise, effective blockade of the B7/CD28 is dependent on the nature of disease as B7/CD28 signals regulate the TH_1/TH_2 balance. Since the differentiation of TH_P cells toward TH_2 is strictly dependent on B7/CD28 signals (314) (311), it is not surprising that blockade of B7/CD28 signals in the $CD28^{-/-}$ NODs leads to exacerbation of diabetes. In addition, Lenschow et al. reported that the increase of diabetes in $CD28^{-/-}$ NODs was a result of absence of TH_2 T lymphocytes (306). Importantly, in some cases, the inhibition of TH_2 responses following B7/CD28 blockade may exacerbate TH_1 pathogenic responses, as there would not be a counter-balance to the TH_1 immune response. The differential effects of B7 signalling on naïve

versus primed T cells and why B7 signalling is critical for TH_2 but not TH_1 responses are yet to be clarified.

Blockade of the B7: CD28/CTLA-4 pathway and control of regulatory T cells:

As mentioned previously in chapter 1, section 5.2, T regulatory cells (Tr) are now accepted as one of the essential arms of peripheral tolerance. Suppression is an "active" process which involves the activation of Tr cells and can be adoptively transferred. In a recent study on B7-1/B7-$2^{-/-}$ NOD mice, a model of accelerated diabetes, Salomon et al. reported a dramatic decrease of the immunoregulatory $CD4^+$ $CD25^+$ Tr cells. Indeed, $CD4^+$ $CD25^+$ Tr were also found to be absent in $CD28^{-/-}$ NODs and transfer of the Tr from NOD mice into $CD28^{-/-}$ NOD littermates prevents disease (307). This study highlighted a previously unforeseen role for B7: CD28/CTLA-4 signalling in the homeostasis of regulatory T cells.

Summary of how blockade of B7: CD28/CTLA-4 signals modulate immune responses:

Blockade of the costimulatory B7: CD28/CTLA-4 pathway presents a unique and promising therapy and has proven to be effective in certain experimental autoimmune models. However, recently, unforeseen consequences of this approach have been discovered. It is now recognized that the complex outcome of this approach must be monitored. Factors such as:

- the nature of the disease;
- ability of autoreactive cells to proliferate in the absence of costimulatory molecules;
- the prevalence of T reg,

have to be considered before application of this approach.

Thus far, the blockade of the B7: CD28/CTLA-4 pathway has been discovered to cause a defect in the homeostasis of regulatory T cells, possibly through an IL-2 dependent mechanism (IL-$2^{-/-}$ mice do not have $CD4^+$ $CD25^+$ T cells). Likewise, it

results in selective inhibition of TH_2 immune responses, which may result in exacerbation of the TH_1 pathogenic immune response. Finally, depending on the stage of disease, the blockade of B7: CTLA-4 responses as a consequence of CTLA-4Ig treatment may result in an enhanced proliferation of autoreactive T cells.

6.2-hCTLA-4Ig treatment of IL-2 deficient mice.

Blockade of the B7: CD28/CTLA-4 costimulatory pathway has so far proven to be a promising alternative therapy to corticosteroid immune suppression. Inflammatory bowel disease (IBD) has been described in many genetically modified mouse strains (reviewed in (315)). T cells from one of these strains, the C57BL/6 IL-$2^{-/-}$ mouse, respond to B7-1 and B7-2 costimulatory signals (208). However, the exact role of these costimulatory molecules in IBD pathogenesis *in vivo* has not been determined. In order to address this point, hCTLA-4Ig (consisting of the human CTLA-4 extracellualr domain and the Fc portion of human IgG1) was administered in healthy 4 week old C57BL/6 IL-$2^{-/-}$ mice (hCTLA-4Ig was kindly provided by Dr. Neil Burden, GlaxoSmithKline).

The treatment protocol was as follows (fig. 6.1):

Type of treatment	**Number of mice/group**
hCTLA-4Ig	3/time point
hIgG1 control protein	3/time point
Untreated	3/time point

Each type of treatment was applied to groups of three IL-$2^{+/+}$ and three IL-$2^{-/-}$ mice. Mice were injected twice weekly with 100μg hCTLA-4Ig i.v. (total volume 100μl). This dosage was used in another study by Khattri et al. where autoimmune disease in CTLA-$4^{-/-}$ mice was abolished using mCTLA-4Ig (316). Previously, Chambers et al. had shown that lymphoproliferation in CTLA-$4^{-/-}$ mice was mediated by costimulation-dependent $CD4^+$ T lymphocytes (317).

Two weeks after the initial immunization, spleen and large bowel tissues were collected for histology and FACS analysis. Since diseased IL-$2^{-/-}$ mice develop IBD characterised by abnormalities in gut architecture, cellular infiltrates and splenomegaly, FACS analysis of CTLA-4Ig-treated IL-$2^{-/-}$ splenocytes may give

insight into whether the treatment abolished the IL-$2^{-/-}$ splenic abnormalities reported in chapter 3.

				Planned protocol
Age of mice (weeks)	4	6	7	*8-9*
Procedure	Weekly i.v. injection hCTLA-4Ig or hIgG1, as indicated, commences	Sacrifice 3 mice per group (*). Collect samples of spleen and colon for FACS and histology	Remaining mice culled due to illness	*Planned: Sacrifice remaining 3 mice per group. Collect samples for FACS and histology*

Groups of mice (*)

Mice	Treatment	No of mice
1) IL-2 KO	hCTLA-4Ig	6
2) IL-2 KO	hIgG1	6
3) WT	hIgG1	6

Figure 6.1 Schematic diagram of the hCTLA-4Ig and hIgG1 treatment regimen

RESULTS

6.3-Hematoxylin and eosin staining of control spleens and colons from 8 week old IL-2$^{+/+}$ and IL-2$^{-/-}$ littermates.

Under microscopic examination, the spleen is composed of discrete white nodules known as white pulp consisting of lymphoid tissue, surrounded by red pulp which constitutes most of the tissue and is highly vascularised (fig. 6.2A). This discrete architecture is less prominent in IL-2$^{-/-}$ spleens (fig. 6.2B). Overproduction of lymphocytes in IL-2$^{-/-}$ mice is reflected morphologically by prominent hyperplasia of the spleen (fig. 3.2) and lymph nodes (personal observations).

Microscopic examination of IL-2$^{-/-}$ colons illustrates crypt hyperplasia and destruction of the crypt architecture (figures 6.4A and B). In this example, there are minimal infiltrating immune cells thay may have previously caused inflammation. The absence of mucus-secreting goblet cells and the deformation of crypts imply an earlier presence of cellular infiltrate. In this context, it is important to note that UC is episodic.

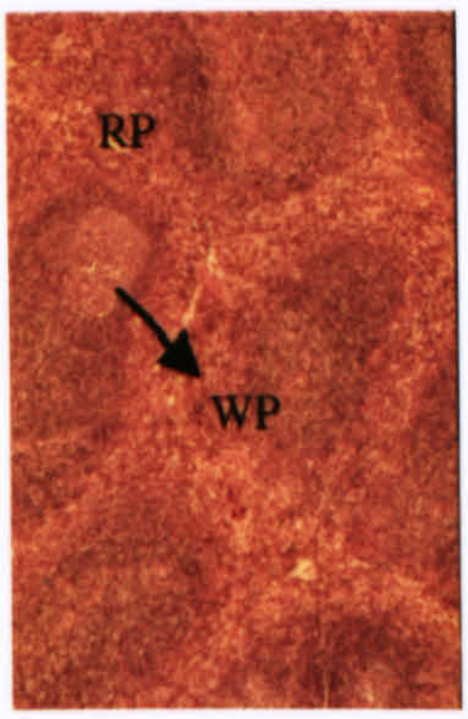


Figure 6.2A Hematoxylin and eosin staining of WT spleens indicating white and red pulp. Magnification x45

The white pulp (WP) consists of lymphoid aggregations and the red pulp (RP) of highly vascularised tissue.



Figure 6.2B Hematoxylin and eosin staining of KO spleens indicating loss of splenic architecture. Magnification x45

Figure 6.2B illustrates the loss of normal splenic architecture in IL-2 deficient mice. The WP nodules are less distinct when compared to WT littermates (fig. 6.2A).

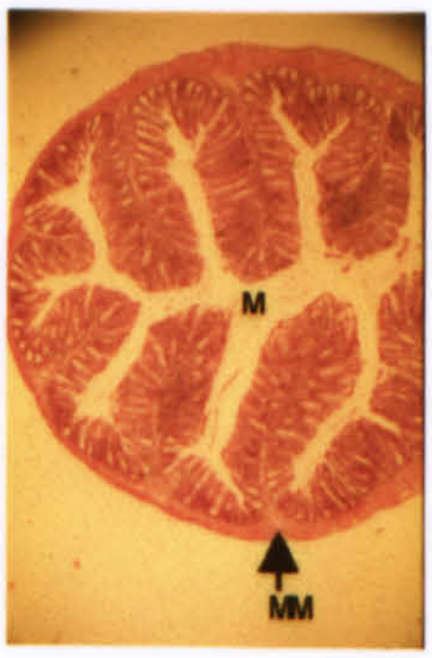


Figure 6.3A Colon H and E staining of WT mice. Magnification x45

The muscularis mucosae MM is a prominent feature of the large intestinal mucosa M. The rhythmic contractions of MM prevent clogging of the glands of the large intestine and enhance the expulsion of mucus to lubricate the colon.

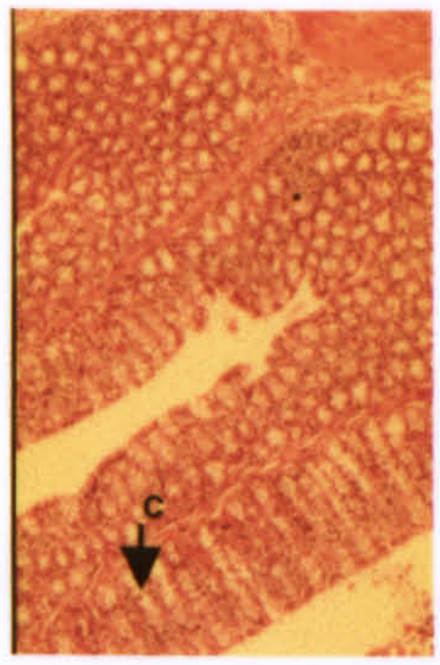


Figure 6.3B Demonstration of WT tubular glands. Magnification x90

Tubular glands mediate water absorption and faecal lubrication, mucus secreting goblet cells and absorptive cells (enterocytes) line the tubular glands. These glands are analogous to the crypts, c, of lieberkuhn of the small intestine and this term is also applicable to the glands of the large intestine.

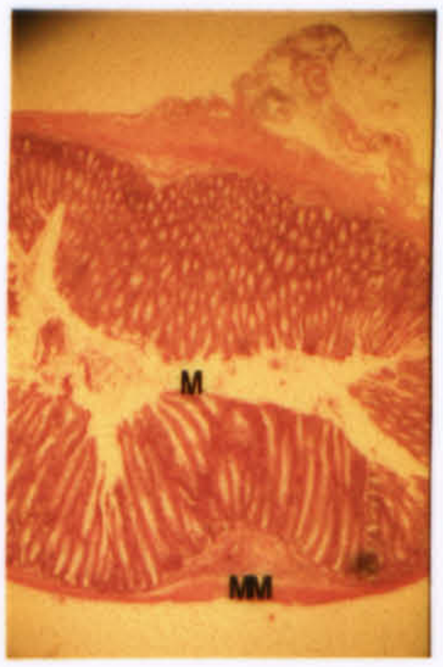


Figure 6.4A Hyperplasia of KO large intestine. Magnification x45

The mucosa consists of two cell types, absorptive cells and mucus secreting goblet cells. These are arranged in closely packed tubular glands and crypts. These crypts appear to be enlarged and deformed in KO large intestines.



Figure 6.4B Illustration of crypt hyperplasia and simplification of mucosal folds in KO large intestines. Magnification x90

As inflammatroy bowel disease progresses in KO mice, the colon changes in architecture. The crypts are enlarged and alignment of tubular glands is disorganised.

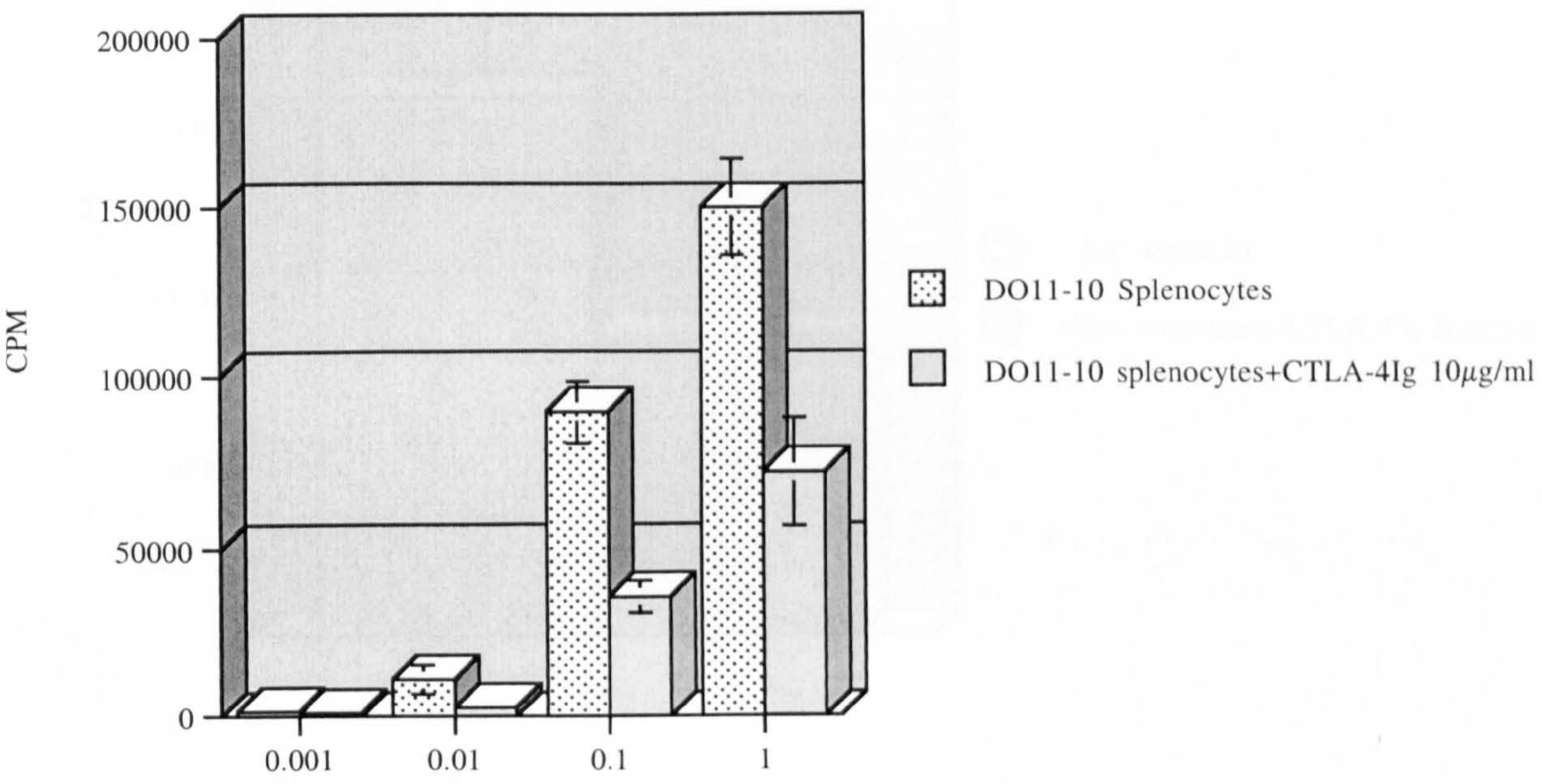
6.4-In vitro controls for CTLA-4Ig function.

hCTL-4Ig binds to B7 ligands and as a result prevents B7 interaction with CD28 and CTLA-4 receptors. In order to test its integrity, hCTLA-4Ig was used to block antigen-specific proliferation and a mixed lymphocyte reaction *in vitro*. The BALB/c OVA-specific H2-A^d-restricted DO11-10 TCR Tg model was used for the proliferation assay. DO11-10 splenocytes were stimulated with the OVA 323-339 peptide (0.001-1μg/ml); as mentioned previously in chapter 4, maximum proliferation occurred at 1μg/ml peptide concentration. When hCTLA-4Ig was added at 10μg/ml to cell cultures, peptide induced proliferative responses were inhibited by approximately 50% (fig. 6.5A). With regard to the mixed lymphocyte reaction, C57BL/6 allo-stimulator irradiated APCs were used at $2.5x10^5$ cells/ml and CBA responder cells were used at $5x10^5$ cells/ml in a 4 day mixed culture. The addition of hCTLA-4Ig at 10μg/ml again led to around a 50% decrease in this allo-response (fig. 6.5B).

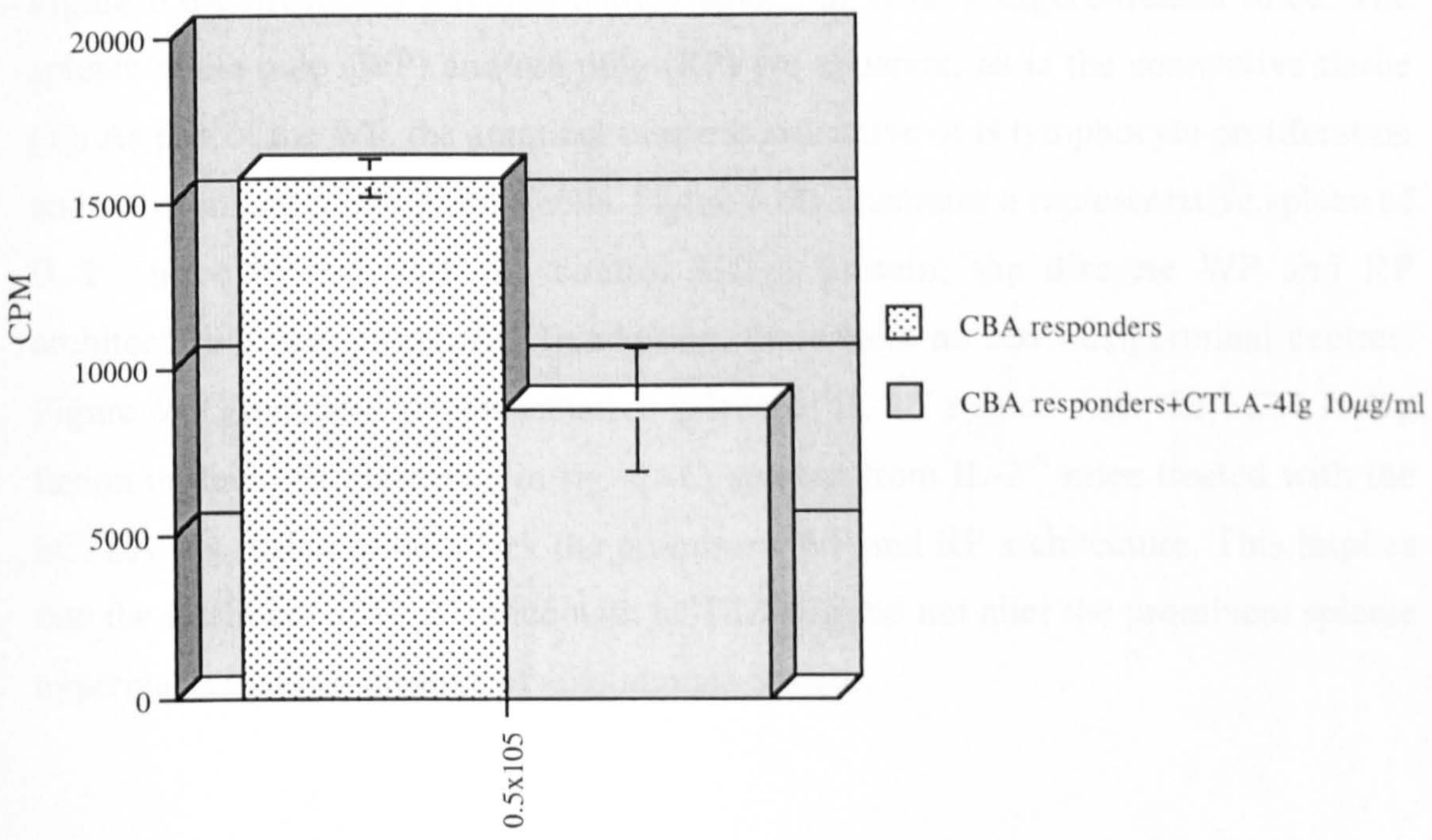
Figure 6.5A illustrates the partial blockade of the B7: CD28 costimulatory pathway using hCTLA-4Ig *in vitro*. Proliferative responses of DO11-10 $CD4^+$ T cells specific for the OVA peptide 323-339 in the context of $H2\text{-}A^d$ were blocked using hCTLA-4Ig at 10μg/ml.

Figure 6.5B demonstrates the partial blockade of an allo-response by hCTLA-4Ig. Irradiated C57BL/6 allo-stimulator APCs and responder CBA cells were co-cultured for 4 days. The addition of 10μg/ml hCTLA-4Ig to the culture resulted in a 56% decrease in proliferation.

Blockade of DO11-10 CD4$^+$ T cells' antigen-specific responses by CTLA-4Ig



Blockade of an allo-response by hCTLA-4Ig



C57BL/6 stimulator APCs

6.5-Hematoxylin and eosin staining of spleen and colon sections from IL-2$^{+/+}$ and IL-2$^{-/-}$ mice following hCTLA-4Ig or hIgG1 treatments.

Hematoxylin and eosin staining is the most commonly used technique in animal histology. Structures such as nuclei, ribosomes and endoplasmic reticulum are stained purple-blue by Hematoxylin because of their high acidic DNA and RNA content. Eosin is an acidic dye which stains basic structures red or pink. Most cytoplasmic proteins are basic; hence the cytoplasm usually stains pinkish-red.

Figure 6.6A illustrates a representative spleen of IL-2$^{+/+}$ hIgG1-treated mice. The splenic white pulp (WP) and red pulp (RP) are apparent, as is the connective tissue (T). As part of the WP, the germinal centre is indicative of B lymphocyte proliferation and transformation into plasma cells. Figure 6.6B illustrates a representative spleen of IL-2$^{-/-}$ mice treated with the control hIGg1 protein; the discrete WP and RP architecture is less prominent. In addition, there were no obvious germinal centres. Figure 6.6C indicates a representative spleen of IL-2$^{-/-}$ treated with the hCTLA-4Ig fusion protein. As illustrated in fig. 6.6C, spleens from IL-2$^{-/-}$ mice treated with the hCTLA-4Ig fusion protein lack the prominent WP and RP architecture. This implies that the treatment of IL-2$^{-/-}$ mice with hCTLA-4Ig did not alter the prominent splenic hyperplasia that is indicative of autoimmune disease.

Figure 6.6A is representative of a spleen from the hIgG1-treated IL-$2^{+/+}$ group. This protein was used as a control for hCTLA-4Ig specificity. The splenic WP and RP are apparent, as is the connective tissue trabeculae (T). As part of the WP, the germinal centre is indicative of B lymphocyte proliferation and transformation into plasma cells. All animals were analysed at 7 week of age.

Figure 6.6B illustrates a representative spleen of IL-$2^{-/-}$ mice treated with the control hIGg1 protein. The discrete WP and RP architecture is less prominent in IL-$2^{-/-}$ spleens. In addition, there were no obvious germinal centres.

Figure 6.6C indicates a representative spleen of IL-$2^{-/-}$ treated with the hCTLA-4Ig fusion protein. Like the control IL-$2^{-/-}$ tissue, spleens from IL-$2^{-/-}$ mice treated with the hCTLA-4Ig fusion protein lack the prominent WP and RP architecture.

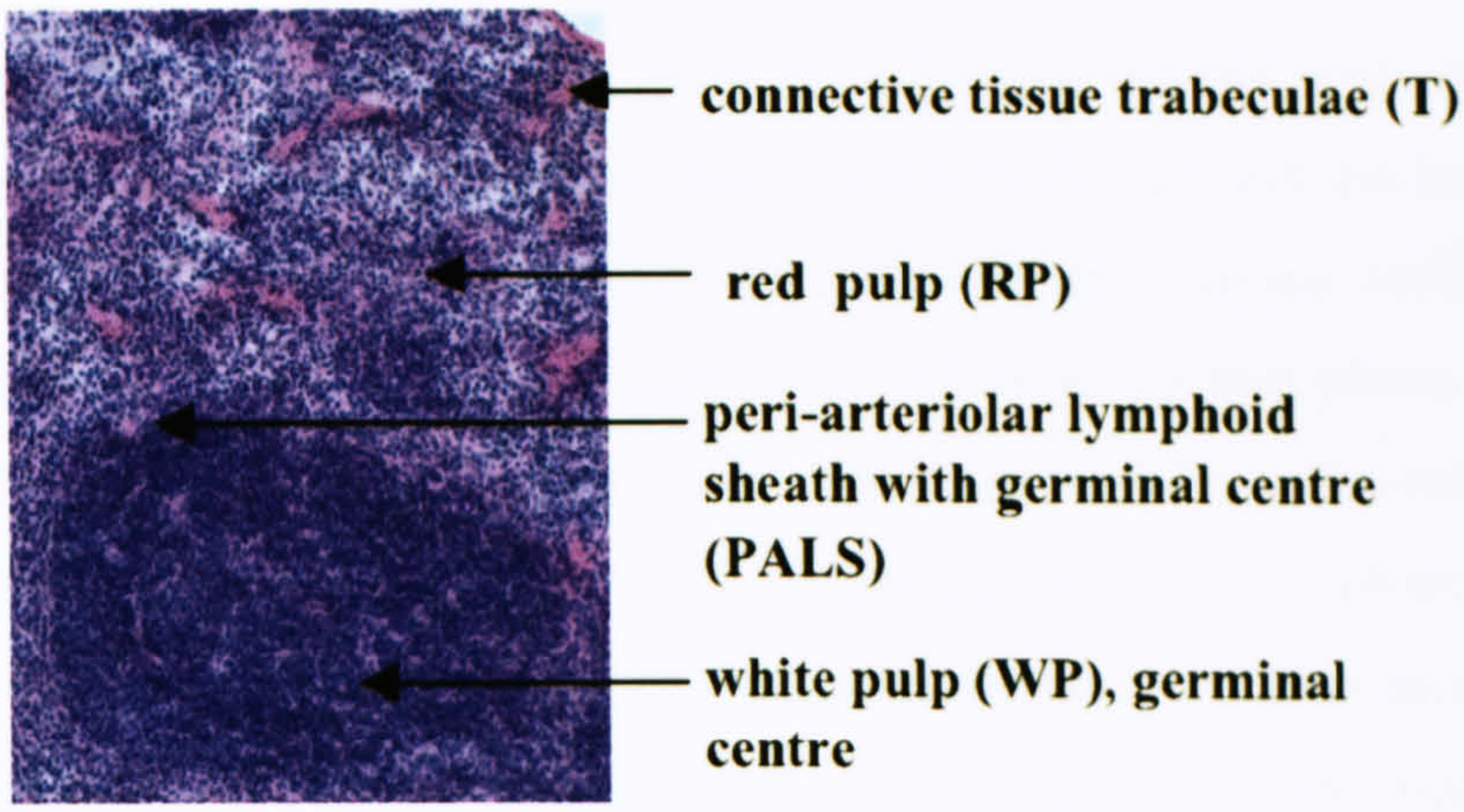


Figure 6.6 A (x10)

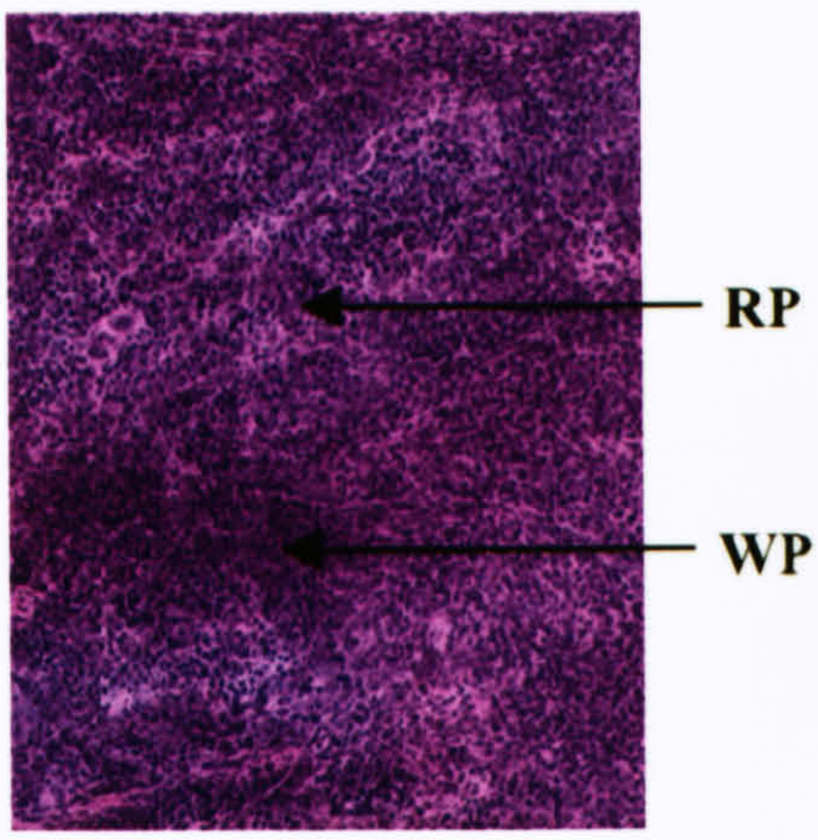


Figure 6.6 B (x10)

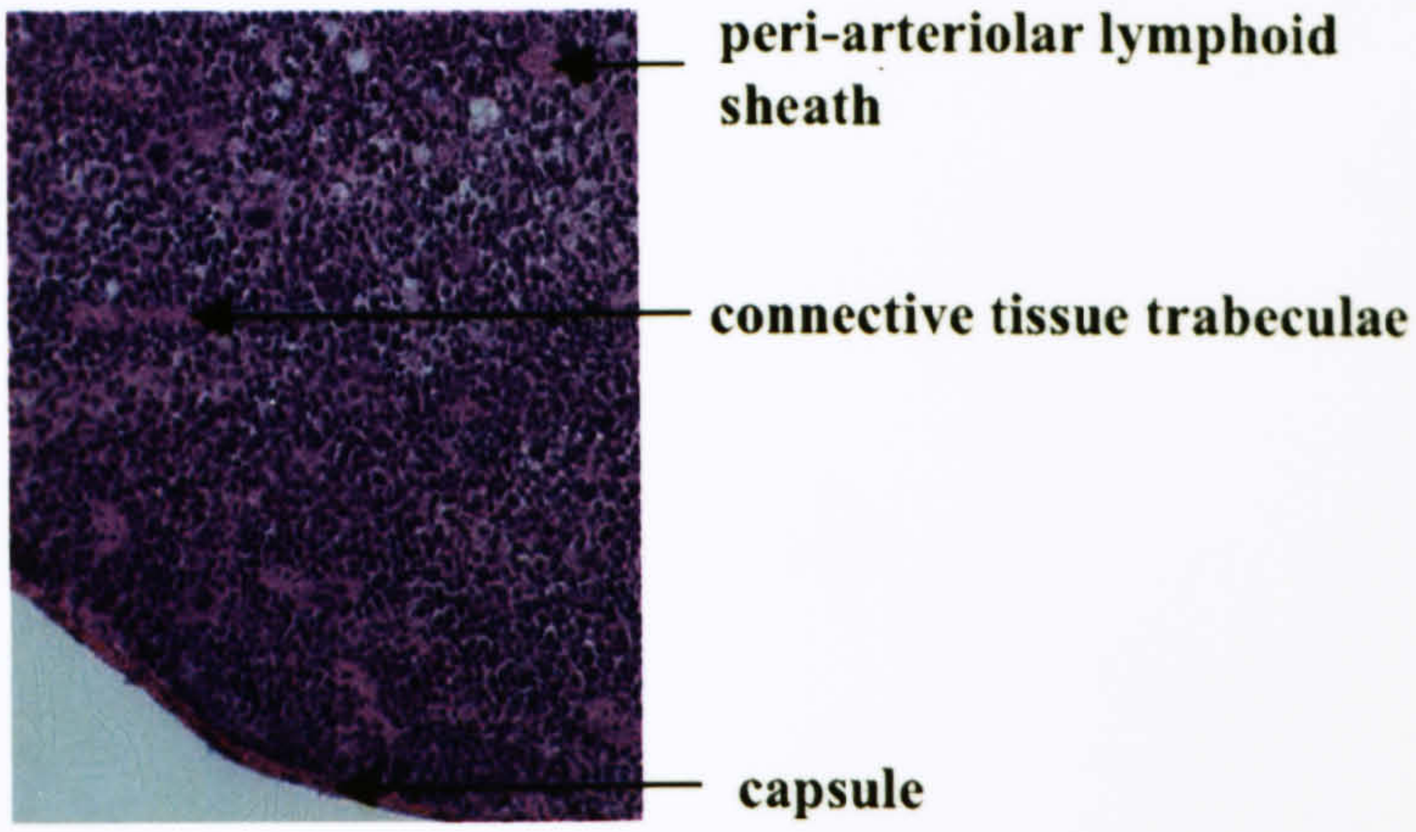


Figure 6.6 C (x10)

Figure 6.6D is a representative section from IL-$2^{+/+}$ mice treated with the control hIgG1; the colonic architecture is typical and illustrates the closely packed tubular glands and goblet cells. The tubular glands extend from the luminal surface to the muscularis mucosae (MM) and are separated from each other by thin plates of lamina propria. Figure 6.6E is representative of IL-$2^{-/-}$ colon from hCTLA-4Ig-treated mice (for control-treated, refer to fig. 6.6I); there is depletion of mucin goblet cells and hyperplasia of the crypts. This is typical of UC. It is noteworthy that in this example, there is no significant infiltration of lymphocytes and no visible crypt destruction.

Figure 6.6D is representative of a large intestine section from IL-$2^{+/+}$ mice treated with the control hIgG1. The typical colonic architecture includes the closely packed tubular glands and goblet cells. The tubular glands extend from the luminal surface to the muscularis mucosae (MM) and are separated from each other by thin plates of lamina propria.

Figure 6.6E is representative of IL-$2^{-/-}$ large intestine from hCTLA-4Ig-treated mice. There is depletion of mucin goblet cells and hyperplasia of the crypts.

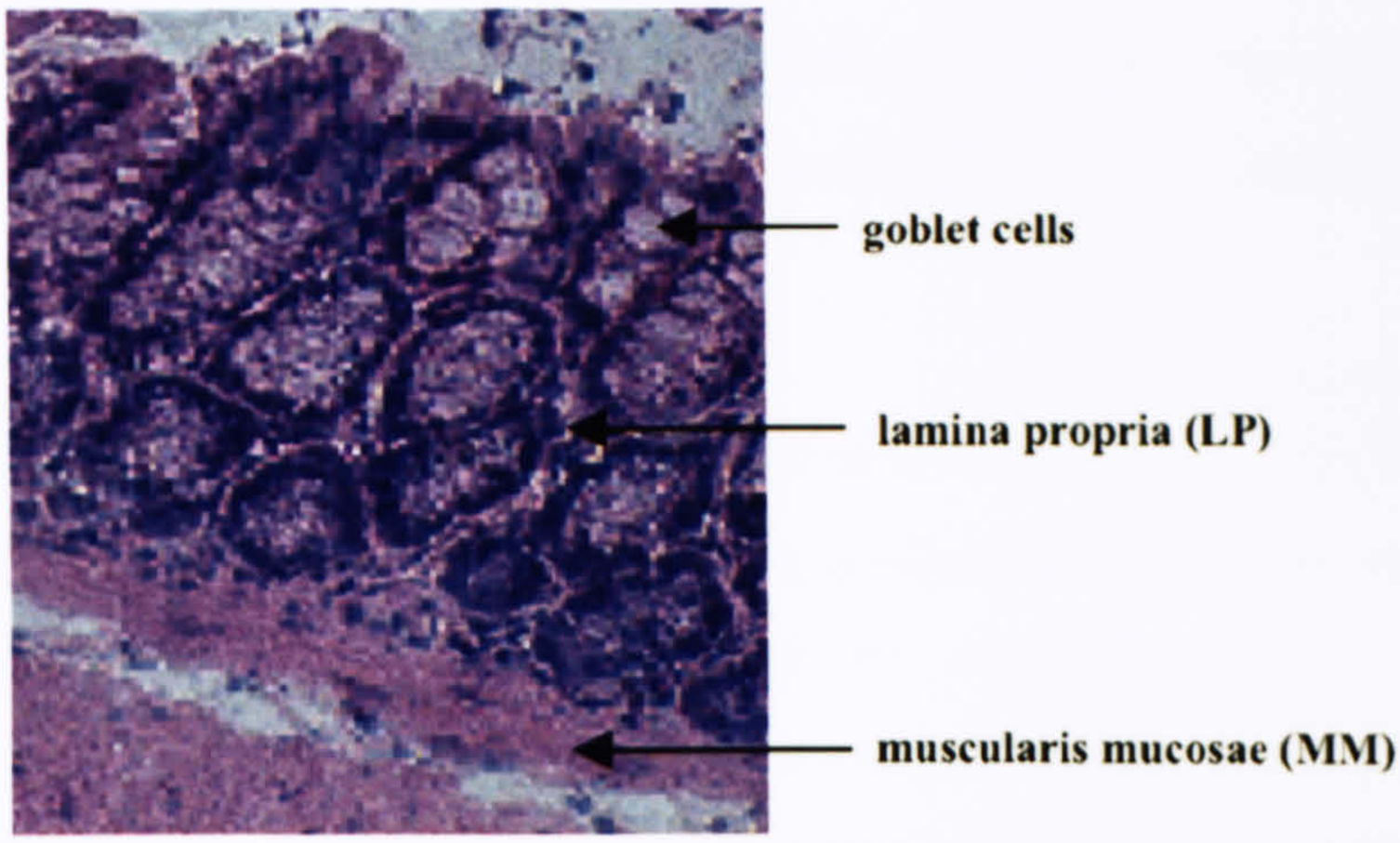


Figure 6.6 D (x10)

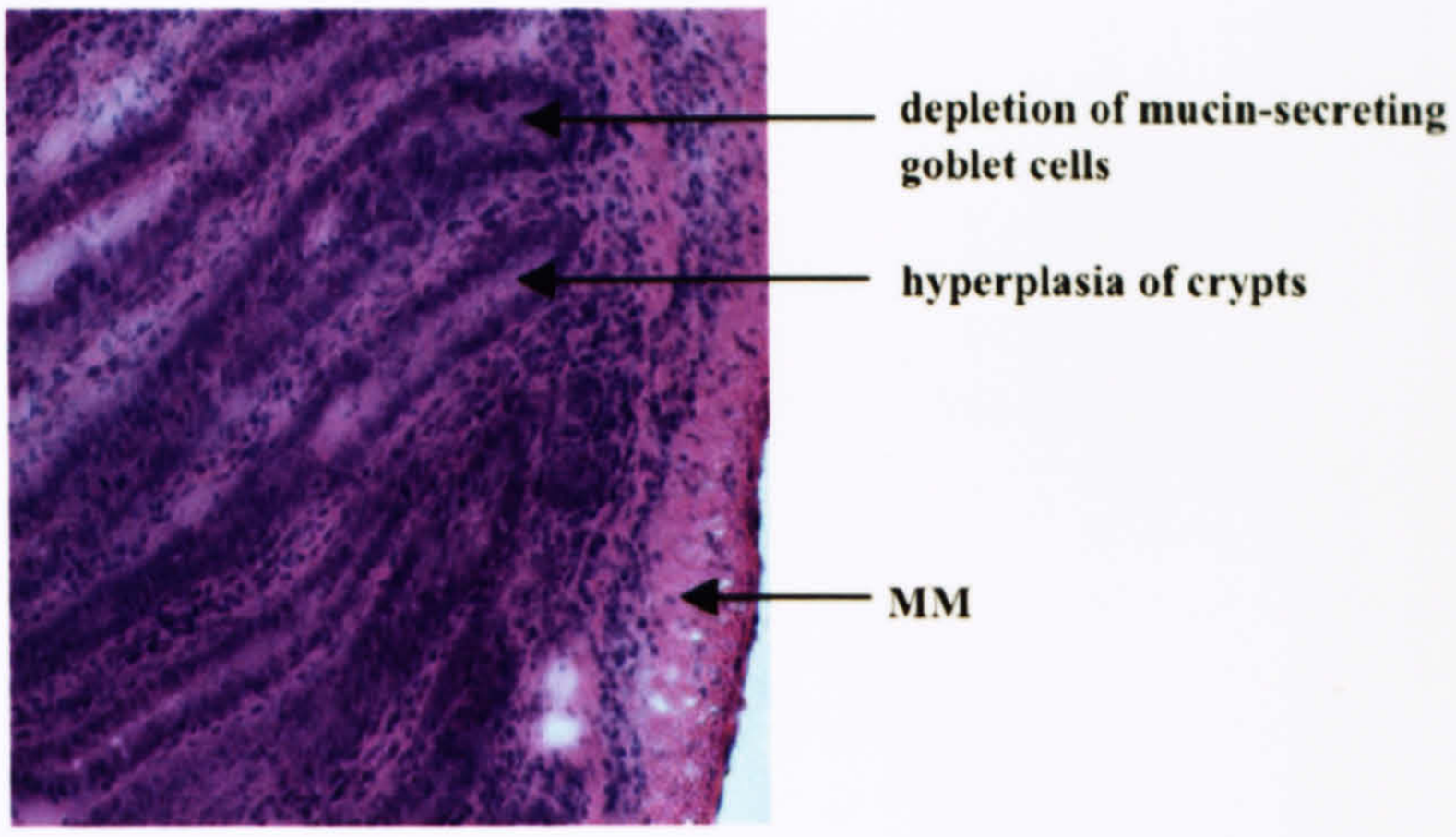


Figure 6.6 E (x10)

Figure 6.6F is a colon section from control hIgG1-treated IL-$2^{+/+}$ mice. The organised tubular gland structure is more apparent in this section. Tubular glands mediate water absorption and faecal lubrication as mucus-secreting goblet cells and absorptive cells (enterocytes) line the glands.

Figures 6.6G and 6.6H indicate colonic sections from hCTLA-4Ig-treated IL-$2^{-/-}$ mice. Colonic invasion by immune lymphocytes is most obvious in these sections. Likewise, the inflammation and destruction of crypts are significant. These features are characteristic of UC. Figure 6.6I represents a colonic section from control hIgG1-treated IL-$2^{-/-}$ mice; as with the hCTLA-4Ig-treated mice, there is immune invasion and destruction of the gut.

Figure 6.6F demonstrates a colon section from hIgG1 control treated IL-$2^{+/+}$ mice. The organised tubular gland structure is more apparent in this section.

Figures 6.6G and 6.6H indicate colonic sections from hCTLA-4Ig-treated IL-$2^{-/-}$ mice. Mucosal inflammation is evident in these sections with the destruction of crypts.

Figure 6.6I represents a colonic section from control hIgG1-treated IL-$2^{-/-}$ mice.

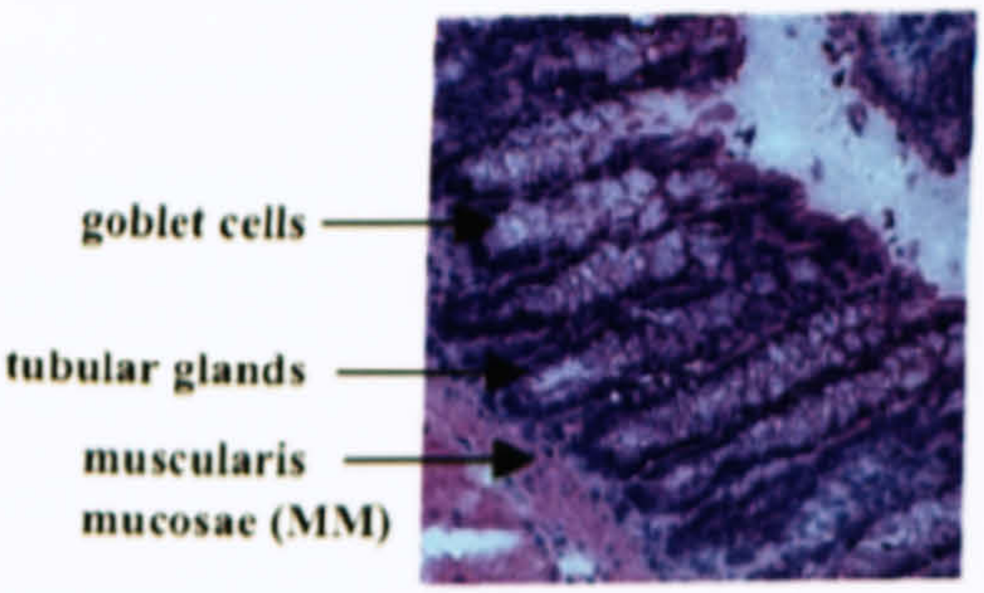


Figure 6.6 F (x10)

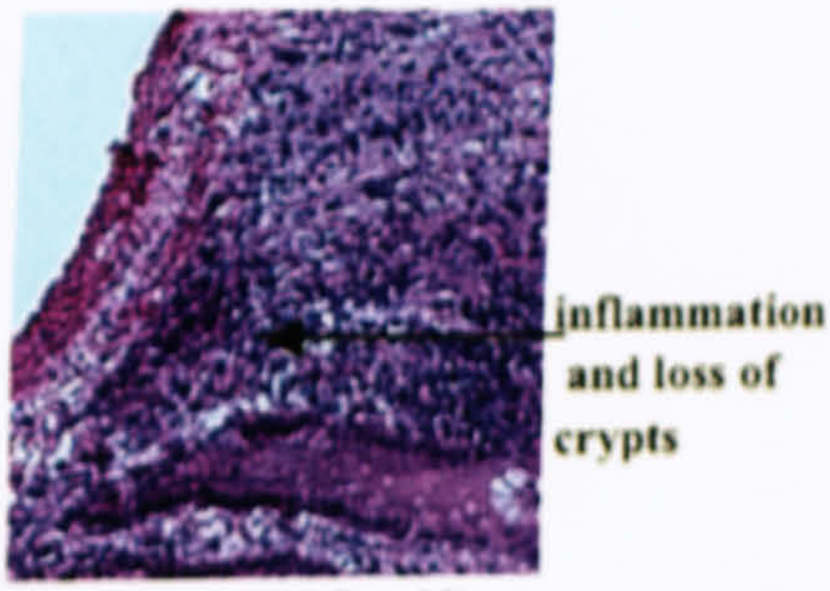


Figure 6.6 G (x10)

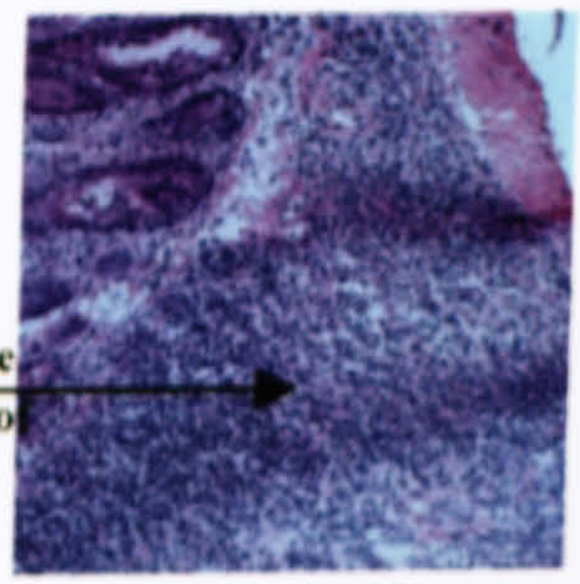


Figure 6.6 H (x10)

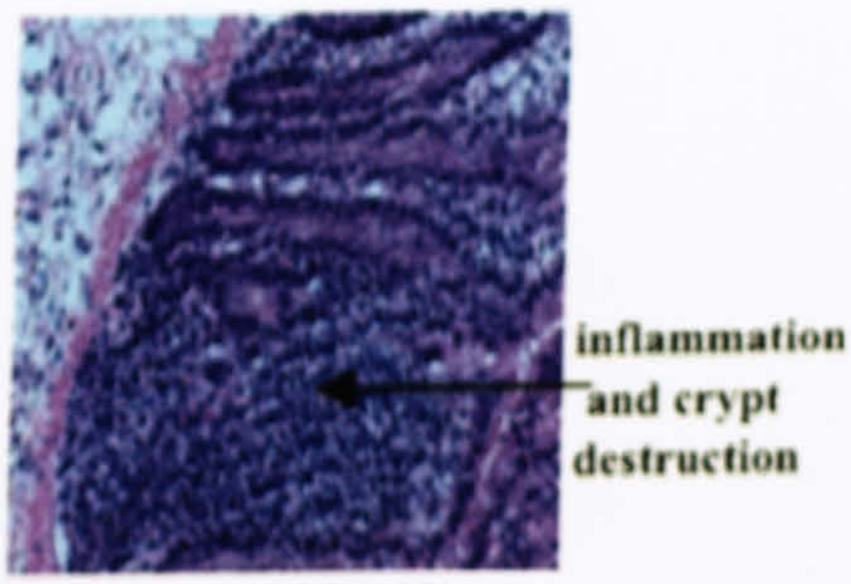


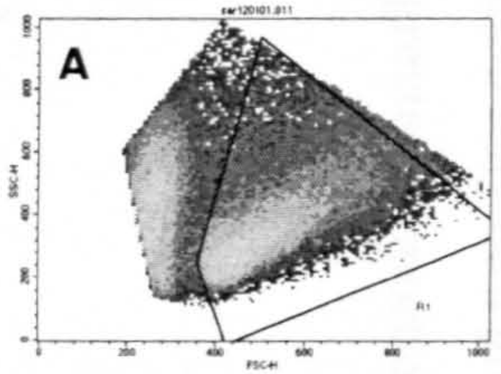
Figure 6.6 I (x10)

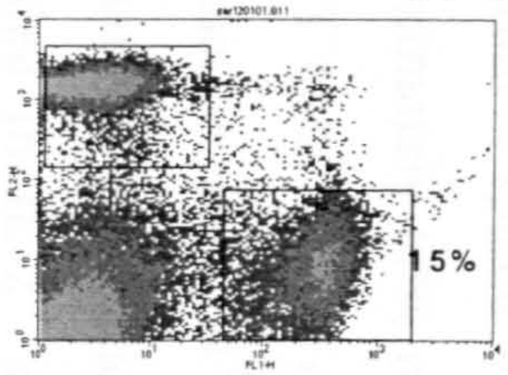
6.6-FACS analysis of spleen cells from IL-2$^{+/+}$ and IL-2$^{-/-}$ mice following hCTLA-4Ig or hIgG1 treatments.

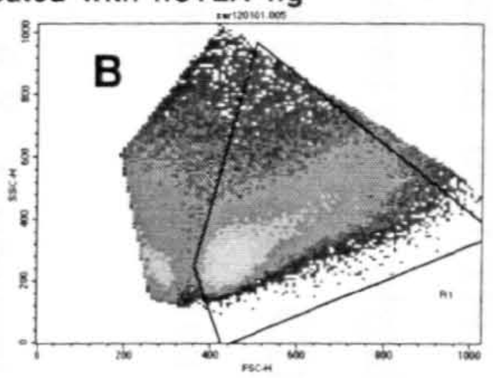
FACS analysis of B220^{+} lymphocytes (B cells) and CD4^{+} CD38^{+} T cells was employed to assess the effects of treatment of IL-2$^{-/-}$ mice with hCTLA-4Ig. As demonstrated in figures 3.3.3 and 3.3.3.1, concomitant with IL-2$^{-/-}$ splenic hyperplasia is a substantial decrease in the percentage of B cells. Figure 6.7 illustrates the percentage of B220^{+} cells in the spleens of IL-2$^{-/-}$ mice treated with hCTLA-4Ig (fig. 6.7A-B), hIgG1 (fig. 6.7 C-E) and spleens of IL-2$^{+/+}$ mice treated with hIgG1 (fig. F-H) after 3 weeks of injections. Each group consists of three mice, with the exception of the IL-2$^{-/-}$-CTLA-4Ig treated group where one mouse died earlier than expected. Despite small group sizes, the percentages of B cells in hCTLA-4Ig and hIgG1-treated IL-2$^{-/-}$ spleens appear comparable, ranging from 3-15% when compared to IL-2$^{+/+}$ B cell percentages which are around 50% of the total cellular compartment. This implies that treatment of IL-2$^{-/-}$ mice with hCTLA-4Ig did not halt splenic enlargement, characteristic of autoimmune disease.

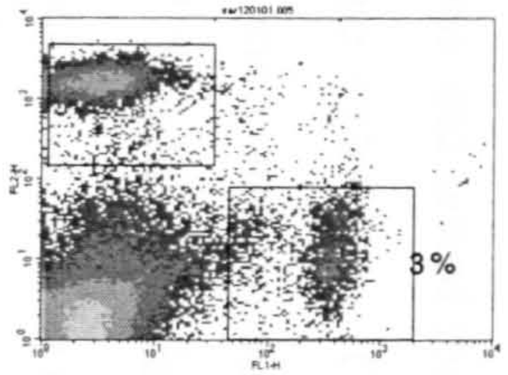
Figure 6.7 shows splenic FACS analysis from IL-$2^{+/+}$ mice treated with hIgG1 and IL-$2^{-/-}$ mice treated with hCTLA-4Ig or hIgG1 at 7 week of age. The characteristic reduction in the percentage of $B220^{+}$ splenic lymphocytes, described in chapter 3, was seen in IL-$2^{-/-}$ mice treated with hCTLA-4Ig (figures 6.7A-B) and IL-$2^{-/-}$ mice treated with hIgG1 (figures 6.7C-E). Normal levels of $B220^{+}$ lymphocytes were apparent in the spleens of IL-$2^{+/+}$ mice treated with hIgG1 (figures 6.7F-H).

IL-2$^{-/-}$ mice treated with hCTLA-4Ig



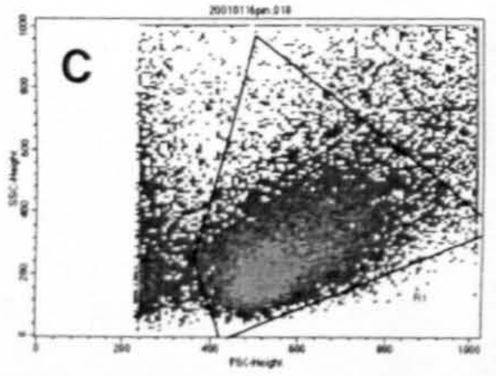


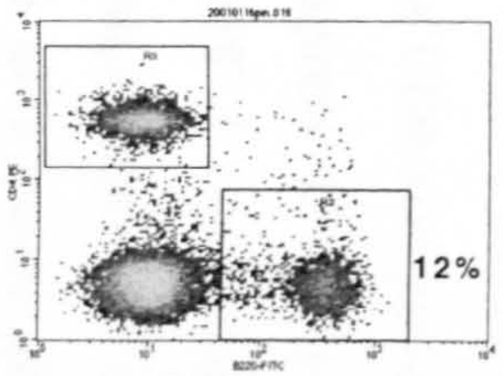


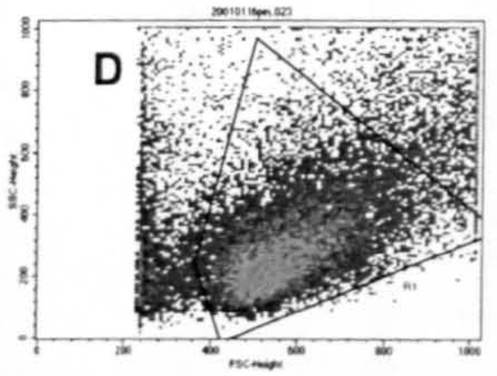


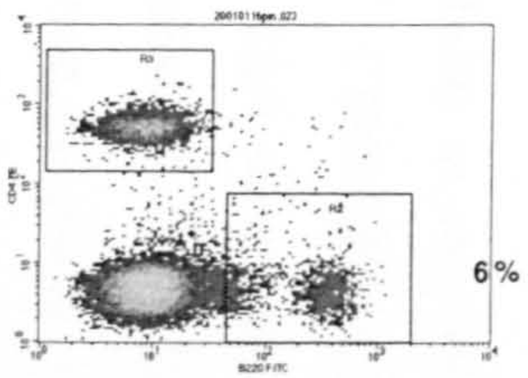


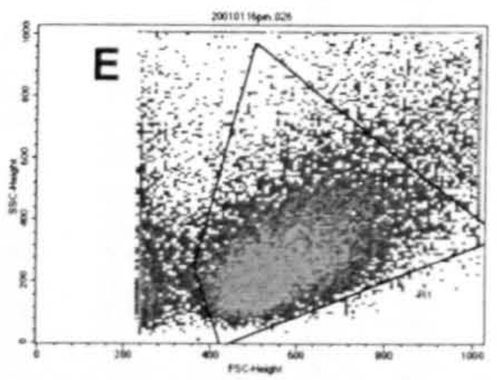
IL-2$^{-/-}$ mice treated with hIgG1

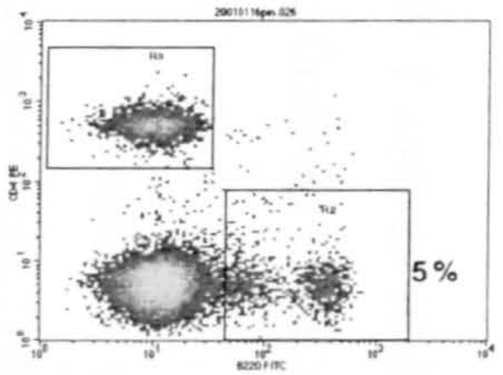




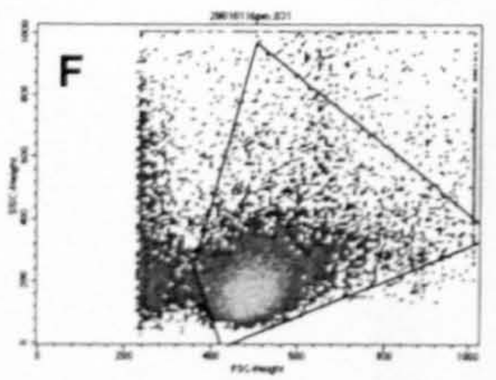


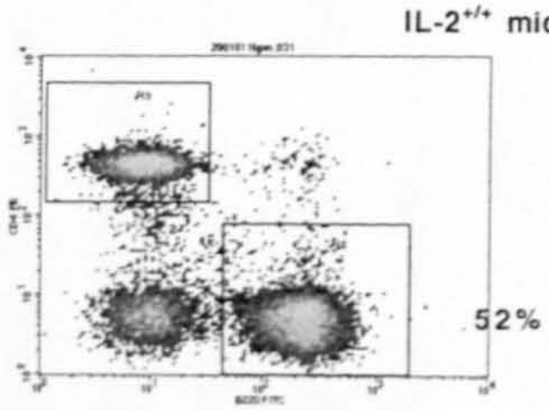


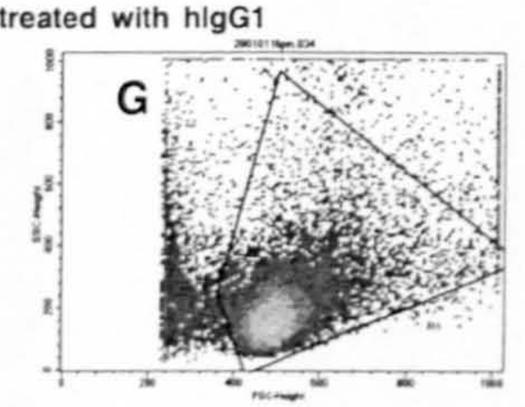


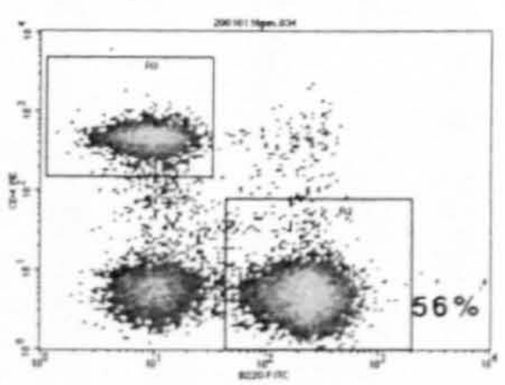


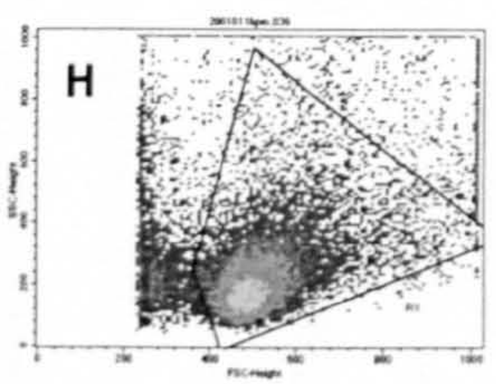
IL-2$^{+/+}$ mice treated with hIgG1

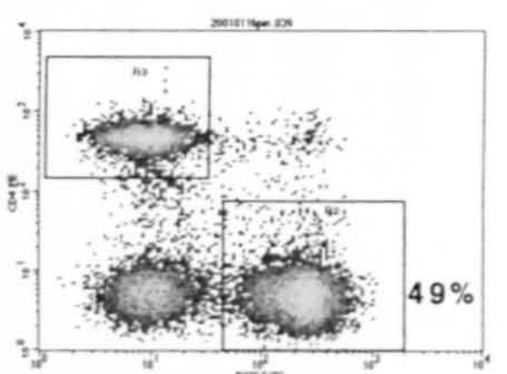












6.7-CD45RB^{lo} CD4^{+} CD38^{+} T cells in IL-2$^{-/-}$ spleens.

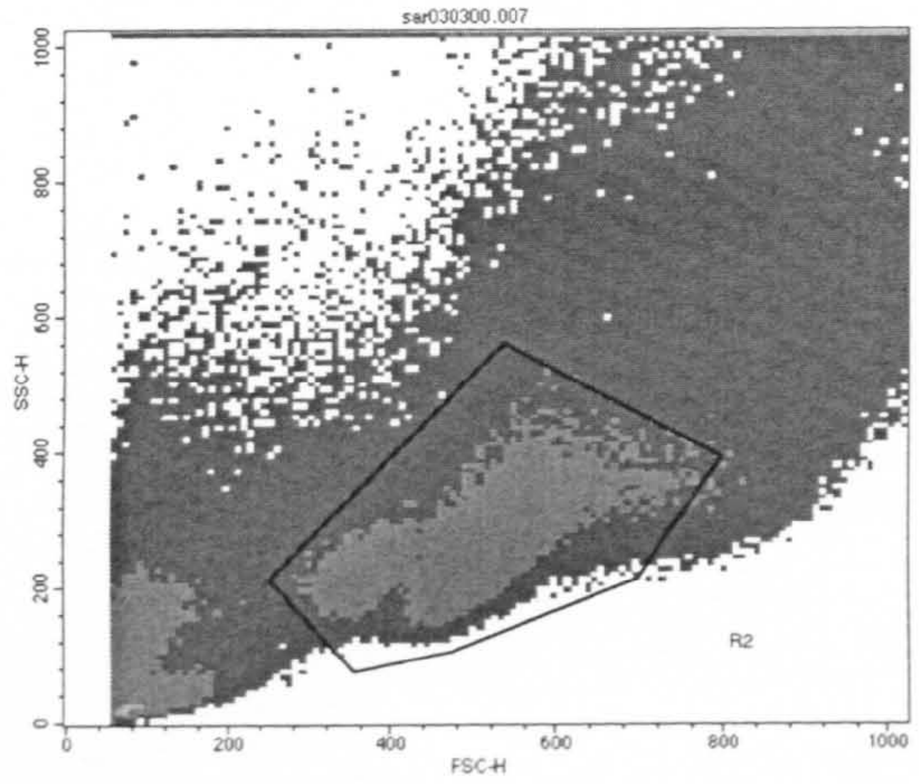
One of the most interesting findings is the expansion of the CD4^{+} CD38^{+} T cells in IL-2$^{-/-}$ spleens, demonstrated in fig. 6.8. CD38 is a 42-46 kDa transmembrane glycoprotein expressed on a variety of murine hematopoietic cells such as macrophages and B cells. Interestingly, it is a B cell marker that is normally present on only a small subpopulation of splenic CD4^{+} T cells (318). The exact function of this ectoenzyme is yet to be deciphered. However, CD38 was found to perform a variety of functions, including the induction of T and B cell proliferation, inhibition of B cell lymphopoiesis and protection of B cells from apoptosis. Importantly, this enzyme was recently revealed to play a role in immune regulation (157). In this study, Read et al. subdivided the regulatory CD4^{+}CD45RB^{lo} T cell subset into CD38^{+} and CD38^{-} fractions and tested their ability to respond to recall antigens, proliferate *in vitro* and produce cytokines. CD4^{+} CD45RB^{lo} CD38^{-} T cells responded to polyclonal stimulation and produced high levels of cytokines, whereas the CD4^{+} CD45RB^{lo} CD38^{+} T cells were incapable of responding to anti-CD3 and producing detectable levels of cytokines. In addition, co-culture of this subpopulation with CD4^{+} CD45RB^{lo} CD38^{-} T cells resulted in inhibition of anti-CD3-induced proliferation and cytokine production by CD4^{+} CD45RB^{lo} CD38^{-} T cells. This immune suppression was dependent on the physical interaction between the regulatory CD4^{+} CD45RB^{lo} CD38^{+} and the responding CD4^{+} CD45RB^{lo} CD38^{-} T cells and independent of the production of immunosuppressive cytokines such as IL-10 and TGF-β.

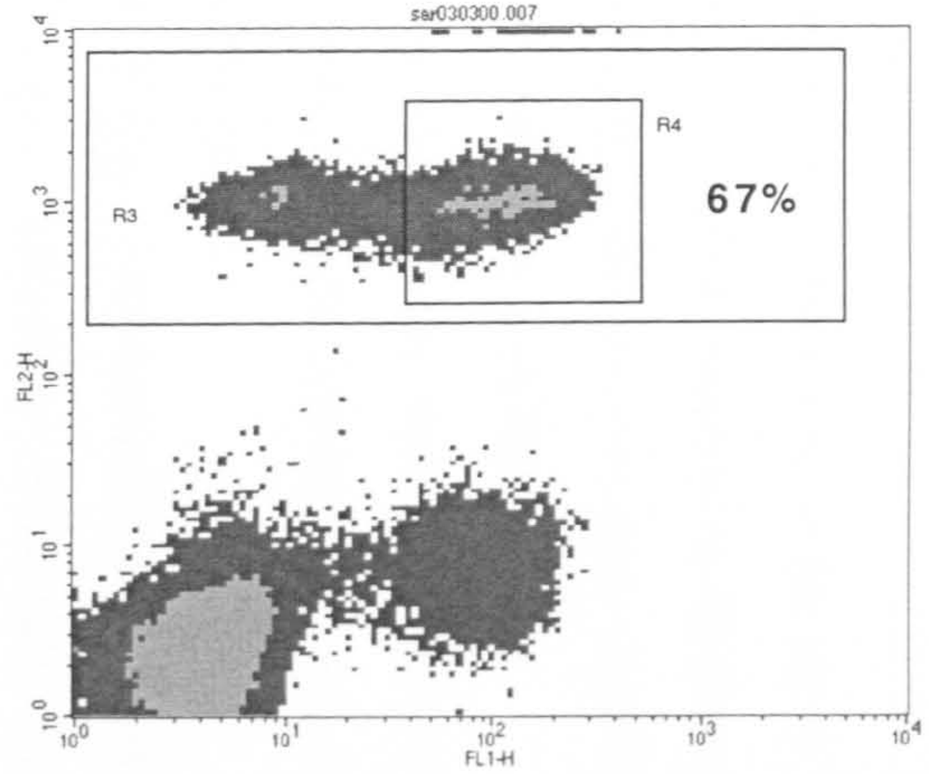
Figure 6.8, representative of four separate experiments, is an example of the population of splenic IL-2$^{-/-}$ CD4^{+} T cells from untreated mice which co-express CD38 (approximately 60% of the CD4 compartment), whereas, the percentage of IL-2$^{+/+}$ CD4^{+} T cells which co-express CD38 is 4%. The implication of this significant difference in the CD4^{+} CD38^{+} T compartment is yet to be clarified. One hypothesis was that this population might represent an immunoregulatory population that has expanded in IL-2$^{-/-}$ mice as a result of chronic inflammation. Therefore, this population may be a good indicator of chronic inflammation. In fact, Lund et al. stated that a greater proportion of murine peripheral T cells express CD38 under conditions

of increased *in vivo* immune activation and inflammation (reviewed in (319)). Therefore, CD38 may be used as an indication of *in vivo* immune activation.

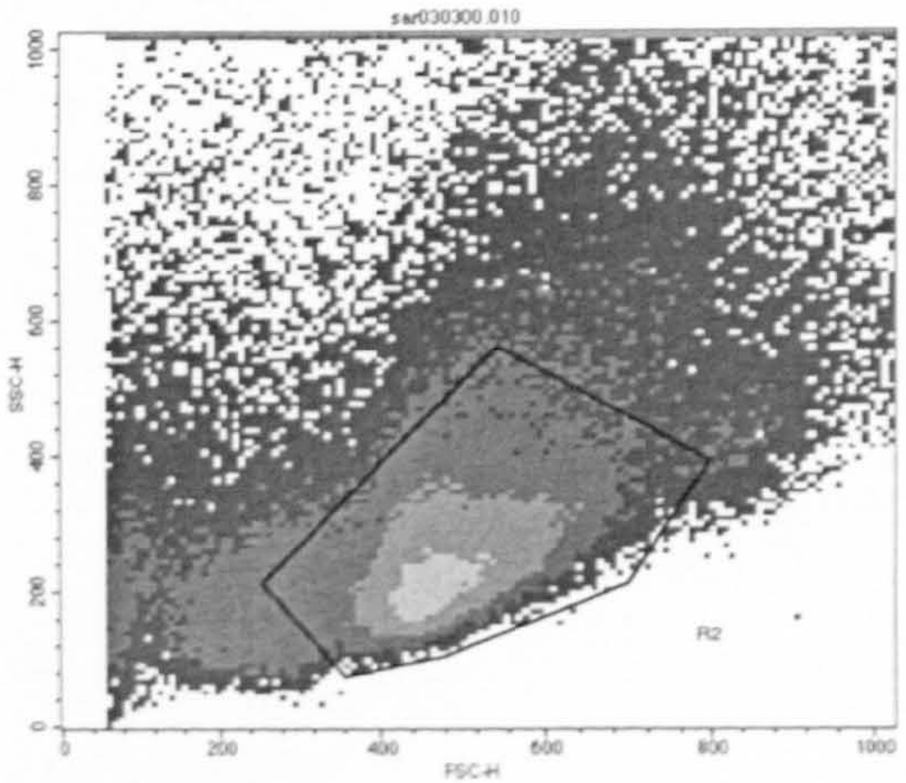
Figure 6.8 demonstrates the expansion of $CD4^+$ $CD38^+$ T cells in $IL\text{-}2^{-/-}$ spleens. **67%** of the $IL\text{-}2^{-/-}$ $CD4^+$ T cell population co-express CD38, whereas **6% of the** $IL\text{-}2^{+/+}$ $CD4^+$ T cell population co-express CD38.

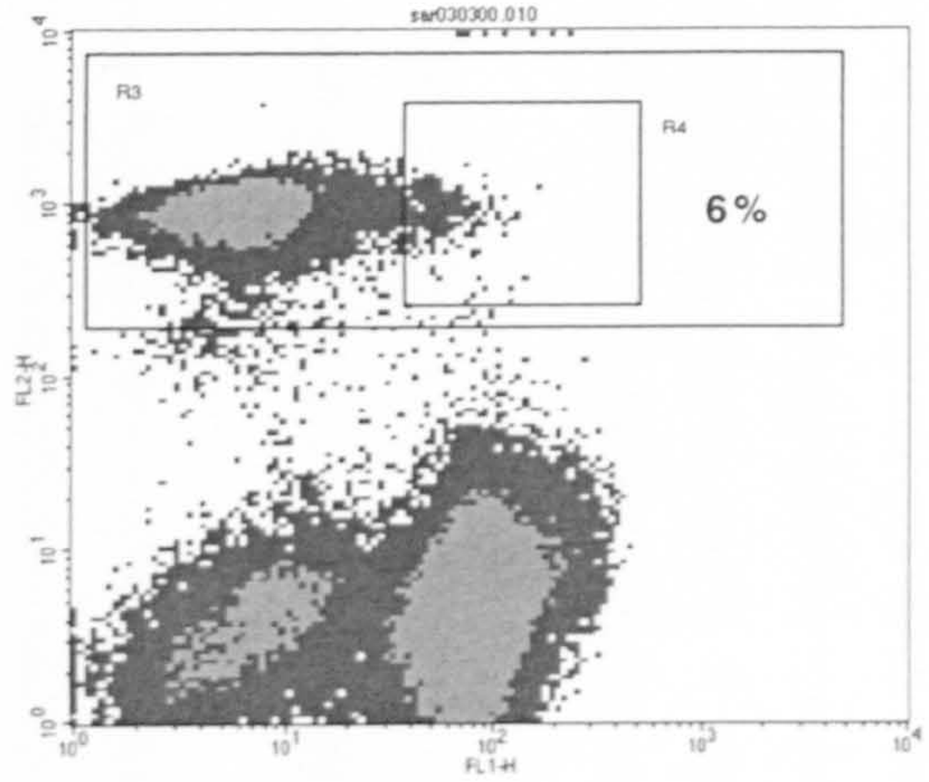
IL-2$^{-/-}$





IL-2$^{+/+}$





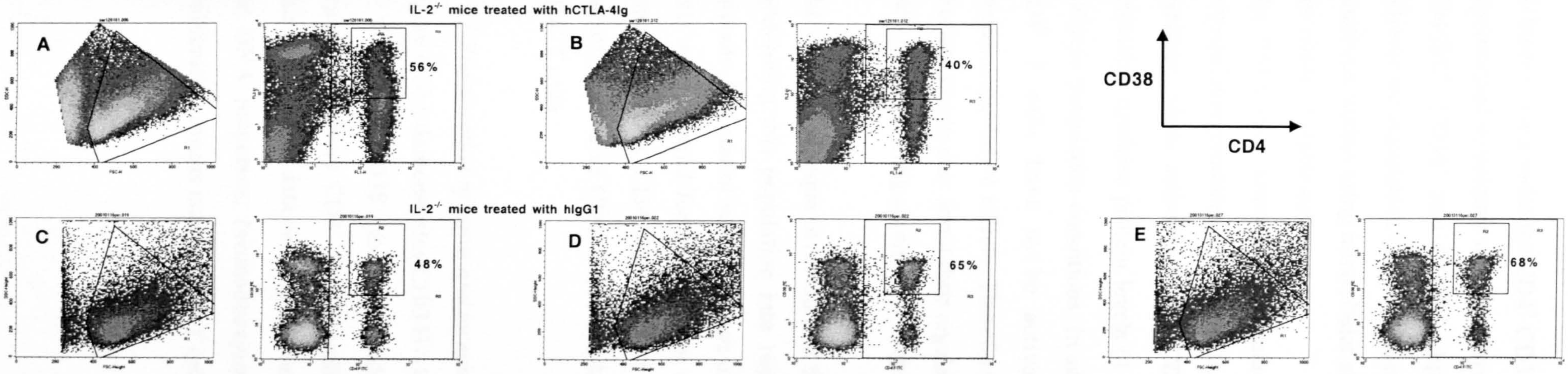
CD4

CD38

Figure 6.9 exhibits the population of $CD4^+$ T cells which co-expresses CD38 in IL-$2^{-/-}$ hCTLA-4Ig-treated mice; ranging from 40-56% (fig. 6.9A-B), in IL-$2^{-/-}$ hIgG1-treated mice; ranging from 50-68% (fig. 6.9 C-E) and IL-$2^{+/+}$ hIgG1-treated mice; ranging from 8-11% (fig. 6.9 F-H). This implies that treatment of IL-$2^{-/-}$ mice with hCTLA-4Ig did not affect the inflammatory milieu, hence the high proportion of T cells that co-express CD4 and CD38. Despite the low numbers of mice per groups, several observations point to an ineffectual role for hCTLA-4Ig in preventing immune activation in IL-$2^{-/-}$ mice. These observations are as follows:

- Treatment of IL-$2^{-/-}$ mice with hCTLA-4Ig appeared to accelerate IBD disease symptoms. In fact, one of the three mice died at week 7 of age and the remaining two were culled because of disease symptoms such as diarrhoea. The IL-$2^{-/-}$ hIgG-1-treated group did not then exhibit overt clinical IBD symptoms, as seen in the IL-$2^{-/-}$ hCTLA-4-treated group.
- Colonic histology sections from the IL-$2^{-/-}$ hCTLA-4Ig-treated group revealed comparable gut destruction, cell infiltrate and pathology to the IL-$2^{-/-}$ hIgG1-treated group.
- FACS data demonstrated the typical decrease in B cell proportion, indicative of the increased cellularity of the non-B cell IL-$2^{-/-}$ splenic compartments, resulting in splenomegaly.

Figure 6.9 shows splenic FACS analysis from IL-$2^{+/+}$ mice treated with hIgG1 and IL-$2^{-/-}$ mice treated with hCTLA-4Ig or hIgG1. The expansion of the $CD38^{+}$ $CD4^{+}$ T cell population was not prominent in the spleens of IL-$2^{+/+}$ mice treated with hIgG1, ranging from 8-11% (figures 6.9F-H).


IL-2-/- mice treated with hCTLA-4Ig
A
56%
B
40%
CD38
CD4
IL-2-/- mice treated with hIgG1
C
48%
D
65%
E
68%
IL-2+/+ mice treated with hIgG1
F
8%
G
10%
H
11%

To further characterise the $CD4^+$ $CD38^+$ population from IL-$2^{+/+}$ and IL-$2^{-/-}$ mice, two experimental techniques were employed. The first entailed FACS sorting of the $CD4^+$ $CD45RB^{lo}$ $CD38^+$ and $CD38^-$ subpopulations from IL-$2^{+/+}$ and IL-$2^{-/-}$ spleens, followed by stimulation of sorted fractions for 18 hrs using PMA plus ionomycin and functional studies using intracellular staining techniques. This method resulted in very low yields of viable cells.

The PMA plus ionomycin-stimulated IL-$2^{-/-}$ $CD38^+$ $CD45RB^{lo}$ $CD4^+$ T cell subpopulation produced IFN- γ and IL-4 (data not shown). This questioned data in the literature, where activated IL-$2^{+/+}$ $CD38^+$ $CD45RB^{lo}$ $CD4^+$ T cells did not produce detectable cytokine protein levels (157). This discrepancy is possibly the result of different stimulation conditions. In addition, Read et al. state that $CD38^+$ $CD45RB^{lo}$ $CD4^+$ T cells have to be activated through their TCR to mediate their immunosuppressive effects. Therefore, stimulation of this subpopulation using PMA plus ionomycin may annul their regulatory function. However, cytokine analysis using intracellular staining requires a strong activation system such as PMA plus ionomycin.

The second technique involved FACS sorting of $CD4^+$ $CD45RB^{lo}$ and subsequently subdividing this population into two fractions, $CD38^+$ and $CD38^-$ using MACS columns. Because of small numbers of $CD38^+$ $CD45RB^{lo}$ $CD4^+$ cells, only the $CD38^-$ cells were assayed for proliferation to CHO B7-1 and anti-CD3 (fig. 6.10A). The remaining $CD38^-$ $CD45RB^{lo}$ $CD4^+$ T cell populations were used to compare IL-$2^{-/-}$ memory and naïve $CD4^+$ T cell cytokine production with IL-$2^{+/+}$ memory and naïve $CD4^+$ T cells.

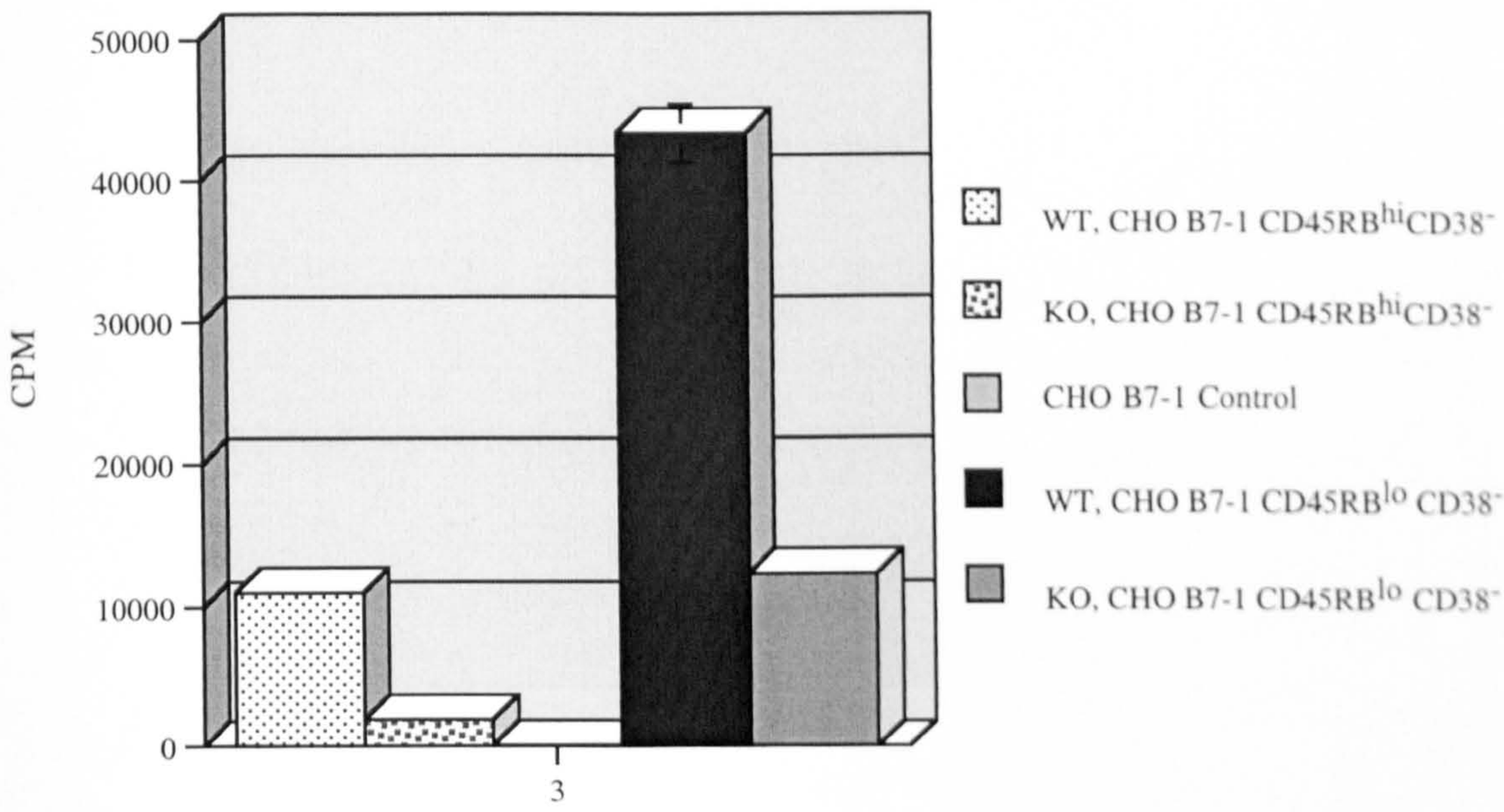
6.7.1-Proliferation of naïve and memory T cells:

Figure 6.10A demonstrates CHO B7-1/anti-CD3-induced activation of IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD45RB^{lo}$ $CD38^-$ (memory) and $CD45RB^{hi}$ $CD38^-$ (naïve) $CD4^+$ T cells. As expected, memory $CD4^+$ T cells proliferated more readily than naïve $CD4^+$ T cells. This concurs with data defining the turnover of T cell subsets in mice by administering the DNA precursor, bromodeoxyuridine (BrdU) in drinking water (320). The proliferation results confirm that T cells with a memory phenotype ($CD4^+$ $CD45RB^{lo}$

T cells) proliferate more than naïve T cells ($CD4^+$ $CD45RB^{hi}$), as manifested in the high incorporation of [^{3}H] thymidine during DNA replication, using CHO B7-1/anti-CD3 stimulation (fig. 6.10A). IL-$2^{-/-}$ memory cells were, however, hyporesponsive when compared to IL-$2^{+/+}$ memory $CD4^+$ T cells.

Figure 6.10A represents the proliferative response of IL-$2^{+/+}$ and IL-$2^{-/-}$ naïve versus memory $CD4^+$ T lymphocytes using CHO B7-1/anti-CD3 activation.

IL-$2^{-/-}$ and IL-$2^{+/+}$ naive and memory CD4 $^{+}$ T cell responses



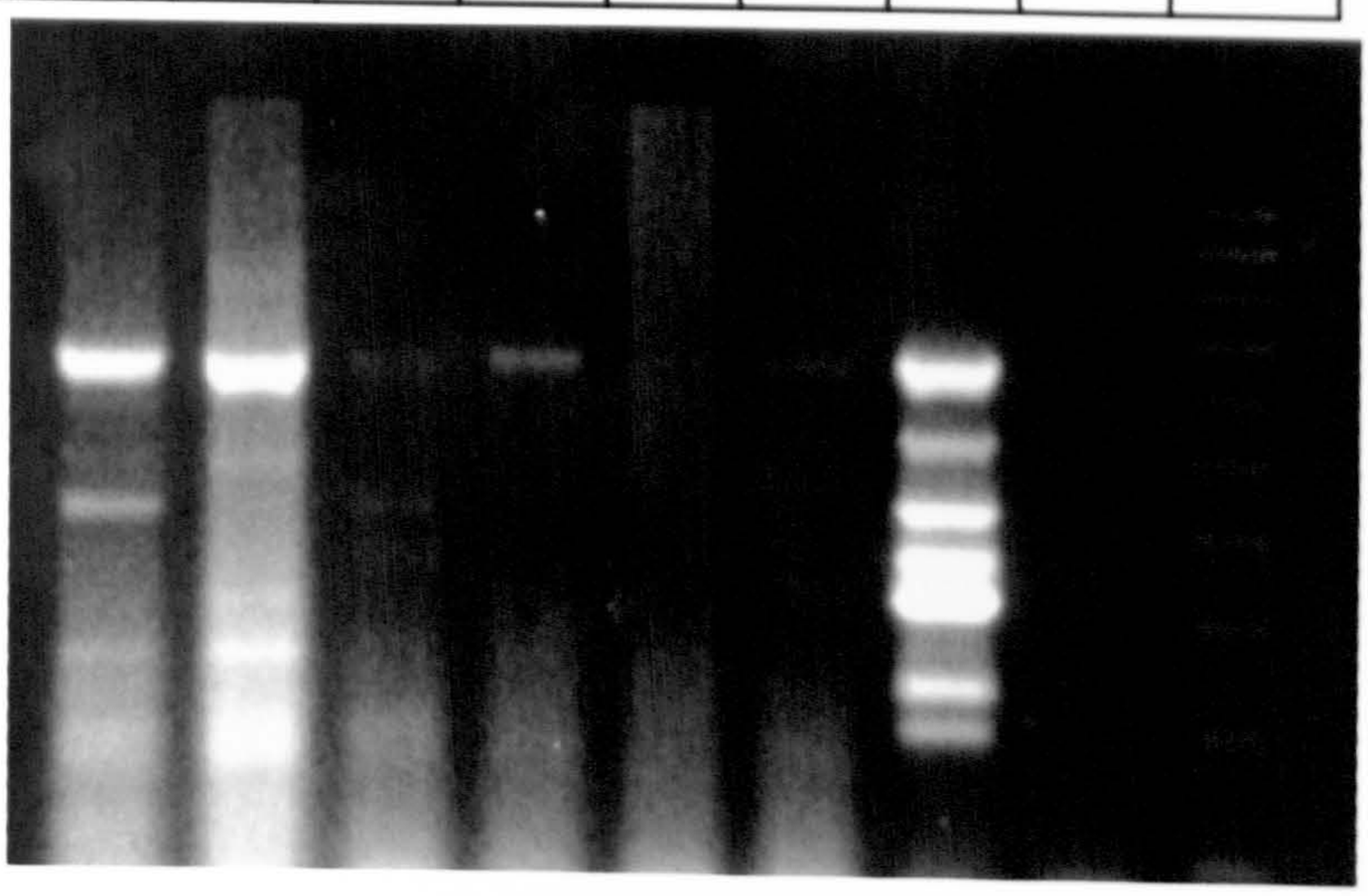
6.7.2-Cytokine mRNA detection of CHO B7-1/antiCD3-stimulated CD45RB^{lo} subpopulations using RT-PCR:

RT-PCR at 48 hrs of culture was used to analyse the cytokine profile of IL-2$^{+/+}$ and IL-2$^{-/-}$ CD38^{+} CD45RB^{lo} CD4^{+} T cells. Cytokine data from experimental samples were inconclusive (fig. 6.10B).

Figure 6.10B represents one of five separate experiments investigating the cytokine profile of IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ $CD45RB^{hi}$ or $CD45RB^{lo}$ T cells. $CD45RB^{lo}$ cells were also fractionated into $CD38^+$ or $CD38^-$ T cells and stimulated with CHO B7-1 and anti-CD3 to analyse their cytokine profile.

Figure 6.10B RT-PCR on IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD38^{+}$ $CD45RB^{lo}$ $CD4^{+}$ T cells.

$CD45RB^{hi}$	+	+							
$CD45RB^{lo}$ $CD38^{-ve}$			+	+					
$CD45RB^{lo}$ $CD38^{+ve}$					+	+			
KO		+		+		+			
WT	+		+		+				
-VE								+	
+VE							+		
Marker									+



DISCUSSION

The role of the costimulatory B7: CD28 pathway in inflammatory bowel disease was investigated using hCTLA-4Ig blocking experiments. hCTLA-4Ig was administered twice weekly to IL-$2^{-/-}$ mice in order to block the activation of T lymphocytes, thereby altering the course of this inflammatory bowel disease model.

Before any general discussion of these studies, I must emphasise that these findings are not conclusive. This is mainly because of the small number of animals per group. The problematic breeding of IL-$2^{-/-}$ mice made it impossible to generate sufficient numbers of litters that are of the same age.

Also, weak blockade of *in vitro* antigen-specific responses and mixed lymphocyte reaction (approximately 50%) using hCTLA-4Ig suggested that *in vivo* blockade would be poor. To address this problem, subsequent experiments must include controls for the efficacy of the hCTLA-4Ig treatment, such as assessing whether treating IL-$2^{+/+}$ mice with hCTLA-4Ig would block the production of antibodies to the hIgG1 or the hCTLA-4 extracellular domain.

The preliminary findings of this study point to a minor role of the B7: CD28 pathway in the activation of pathogenic IL-$2^{-/-}$ $CD4^+$ T cells. The failure to alter disease pathogenesis in IL-$2^{-/-}$ mice was apparent in the obvious destruction of gut architecture, a prominent characteristic of IBD (figures 6.4 and 6.6 G-I). In addition, the unchanged profile of the IL-$2^{-/-}$ splenic cellular composition (another feature of autoimmune disease in the IL-$2^{-/-}$ mouse) implies that blocking the B7: CD28/CTLA-4 did not influence the activation of lymphocytes *in vivo*, despite the functional integrity of the hCTLA-4Ig, as tested *in vitro* using proliferation assays, where it partially blocked proliferation of $CD4^+$ T cells.

In fact, it is tempting to suggest that hCTLA-4Ig accelerated disease in IL-$2^{-/-}$ mice. This is because the hCTLA-4Ig-treated group appeared to have exacerbated disease symptoms; one of the mice in the latter group died earlier than expected, and the

remaining two mice had to be culled because of severe disease symptoms. The hIgG1-treated IL-$2^{-/-}$ group appeared to be healthier and did not show clinical signs of disease such as diarrhoea by that stage.

Additionally, in certain autoimmune models, the blockade of B7: CD28 costimulatory pathway was found to accelerate disease symptoms. This finding was first reported in a study of $CD28^{-/-}$/NOD and CTLA-4Ig transgenic NOD (CTLA-4Ig Tg/NOD) mice (306). $CD28^{-/-}$/NODs (mice that are defective in the B7: CD28 pathway) and CTLA-4Ig Tg/NODs (mice that are defective in the B7: CD28/CTLA-4 pathway) had exacerbated autoimmune disease. This indicated that the blockade of the B7: CD28/CTLA-4 pathway promoted the development and progression of spontaneous autoimmune diabetes. Recently, a study on B7-1/B7-$2^{-/-}$/NODs (NOD mice which are B7-1 and B7-2 deficient) reported a dramatic decrease of the immunoregulatory $CD4^+$ $CD25^+$ Tr cell subset which led to accelerated diabetes (307). In the case of IL-$2^{-/-}$ mice, the regulatory $CD4^+$ $CD25^+$ T cell subset is absent (142). Hitherto, there have been no studies on the presence of a functional regulatory T cell subset in IL-$2^{-/-}$ mice. Therefore, it is most interesting that there is increased expansion of the $CD4^+$ $CD38^+$ T cell subset in IL-$2^{-/-}$ mice (fig. 6.8). Whether this cell subset functions as a regulatory T cell subset in IL-$2^{-/-}$ mice remains to be seen. Indeed, functional studies performed in this study were inconclusive due to the difficult and laborious methods used to isolate this cell subset and the failure in characterizing cytokine profiles produced by these cells. It is noteworthy that other reports in the literature have also failed to characterize cytokine profiles produced by these cells. Assuming that the $CD4^+$ $CD38^+$ T cell subset is a regulatory subset in IL-$2^{-/-}$ mice, as it is in IL-$2^{+/+}$ mice, one possible explanation of why disease was exacerbated maybe that CTLA-4 regulates the function of this regulatory subset, as it does the $CD4^+$ $CD25^+$ Tr subset (144). Therefore, blockade of B7: CD28/CTLA-4 pathway will exacerbate IBD in IL-$2^{-/-}$ mice.

As mentioned previously, the effect of blocking the B7/CD28 pathway is dependent on the nature of disease, as B7/CD28 signals regulate TH_1/TH_2 signals. Since B7/CD28 signals are essential for the differentiation of TH_2 cells, the inhibition of TH_2 responses following B7/CD28 blockade may exacerbate TH_1 pathogenic responses in some disease models. Therefore, another possibility may be that blockade of B7: CD28 pathway may have accelerated disease in IL-$2^{-/-}$ mice as a result of TH_2 inhibition, which suggests that there would not be a counter-balance to the pathogenic TH_1 immune response.

Finally, these preliminary results point to a non-essential role for the B7: CD28 in the pathogenesis of IBD in the IL-2 deficient model. Moreover, cDNA gene arrays revealed an up-regulation of costimulatory molecules such as LFA-1 and CD6 but not B7 molecules in activated IL-$2^{-/-}$ $CD4^+$ T cells in comparison to IL-$2^{+/+}$ counterparts. These data emphasize the complexity of the costimulatory signals that regulate T cell functions.

CHAPTER 7

FINAL REMARKS AND CONCLUSION

FINAL REMARKS

The aim of this study was to characterise IL-$2^{-/-}$ $CD4^+$ T lymphocytes and to investigate the role of the B7: CD28 costimulatory pathway in inflammatory bowel disease pathogenesis. Several experimental techniques were used to characterise IL-$2^{-/-}$ $CD4^+$ T lymphocytes.

Firstly, the *in vitro* cytokine profile and cytokine requirement of B7-1/anti-CD3 activated IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells was examined using:

- Addition of recombinant cytokines to short-term cultures.
- Addition of anti-cytokine mAb to short-term cultures.
- ELISA studies on activated IL-$2^{+/+}$ and IL-$2^{-/-}$ CD4+ T lymphocytes.

These studies did not reveal a significantly distinct cytokine production pattern by IL-$2^{-/-}$ $CD4^+$ T cells when compared to IL-$2^{+/+}$ $CD4^+$ T cells. However, ELISA studies on purified $CD4^+$ T lymphocytes demonstrated an increased production of IL-10 by IL-$2^{-/-}$ $CD4^+$ T cells.

Secondly, analysis of IL-$2^{-/-}$ $CD4^+$ T cells cytokine mRNA levels, using RNase protection assays and RT-PCR, did not reveal distinct expression of cytokine mRNA, apart from IL-10 mRNA in IL-$2^{-/-}$ spleen cells at early time points. This suggests that the over-expression of IL-10 mRNA by activated IL-$2^{-/-}$ splenocytes at early time points *ex vivo* is possibly a reflection of the enhanced anti-inflammatory activity *in vivo*.

Thirdly, cDNA gene microarrays showed the most significant differences, in particular with regard to the differential expression of metabolic enzymes and housekeeping genes, depending on the activation system used. However, cDNA gene microarrays did not reveal any differential expression of cytokines by IL-$2^{-/-}$ $CD4^+$ T lymphocytes.

These findings question the hypothesis that IL-$2^{-/-}$ $CD4^+$ T cells are differentially activated and thus produce different cytokines from IL-$2^{+/+}$ $CD4^+$ T cells, just because

they lack IL-2. Essentially, they suggest that IL-$2^{-/-}$ $CD4^+$ T lymphocytes are not intrinsically different from IL-$2^{+/+}$ $CD4^+$ T lymphocytes. Indeed, studies reported in the literature suggest a minor role for IL-2 in thymic development.

Initial studies by Schorle et al. failed to demonstrate a role for IL-2 in thymic development; however, subsequent studies reported a minimal deficit in IL-$2^{-/-}$ thymic negative selection and a propensity toward TH_1 development by IL-$2^{-/-}$ thymocytes (111). Importantly, the TH_1 propensity was only observed in thymocytes from TNP-KLH-immunized IL-$2^{-/-}$ mice, which produce more IFN-γ and less IL-4 than similarly immunized IL-$2^{+/+}$ mice.

The non-critical role of IL-2 in T cell thymic development agrees with the findings in this thesis which suggest that IL-$2^{-/-}$ $CD4^+$ T cells simply lack the prominent IL-2 signaling pathway and therefore use a residual secondary pathways for proliferation *in vitro*. In fact, the proliferative responses of IL-$2^{+/+}$ $CD4^+$ T cells, when co-cultured with anti-IL-2mAb, are similar to those of IL-$2^{-/-}$ $CD4^+$ T cells. Similarly, the proliferative responses of IL-$2^{-/-}$ $CD4^+$ T cells, when co-cultured with IL-2, are restored to IL-$2^{+/+}$ levels.

Other data which point to insignificant characteristics between IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells are the correspondence of the IL-$2^{+/+}$ and IL-$2^{-/-}$ Vβ repertoire despite disease in IL-$2^{-/-}$ mice. Collectively, these data suggest that autoimmunity is IL-$2^{-/-}$ mice is not the result of an intrinsically different activation pathway and growth factor production by IL-$2^{-/-}$ $CD4^+$ T cells. Rather, it is a failure in peripheral deletion and the clearance of activated T cells.

With regard to the role of B7: CD28 in IBD pathogenesis, preliminary CTLA-4Ig studies did not reveal a protective role for the blockade of the costimulatory B7: CD28/CTLA-4 pathway in IBD. In fact, these preliminary studies suggested an acceleration of disease as a result of blockade. If this proves to be true, it will have significant therapeutic implications for IBD. Interestingly, cDNA gene arrays revealed the up-regulation of costimulatory molecules such as LFA-1 and CD6 but not the B7 molecules.

Another intriguing finding is the accumulation of $CD38^+$ $CD4^+$ T lymphocytes in IL-$2^{-/-}$ spleens. Thus far, $CD38^+$ $CD4^+$T lymphocytes have been reported to be key players in the regulation of immune responses. In addition, as $CD4^+$ $CD25^+$ Tr cells are absent in IL-$2^{-/-}$ mice, the expansion of the $CD38^+$ $CD4^+$ Tr population in IL-$2^{-/-}$ spleens is of particular interest. In effect, it is conceivable that the $CD38^+$ $CD4^+$ Tr population is responsible for the enhanced production of IL-10 in IL-$2^{-/-}$ $CD4^+$ T cells proliferation assays.

Future studies should include:

- Functional analysis of the $CD38^+$ $CD4^+$ T cell subset. This may give insight into the secondary regulatory pathway(s) operating in the absence of IL-2. An example is the isolation of $CD45RB^{lo}$ $CD38^+$ T cells from IL-$2^{+/+}$ and IL-$2^{-/-}$ mice and analysis of gene expression profiles using the Clontech Atlas gene arrays to assess possible differences between WT and KO mice.
- Transfer allotypically marked and CFSE-labeled IL-$2^{+/+}$ $CD4^+$ T cells in a dose-dependent manner into IL-$2^{-/-}$ mice to assess disease development in the presence of WT cells.
- Transfer of allotypically marked and CFSE-labeled IL-$2^{-/-}$ and dose-graded IL-$2^{+/+}$ into a lethally irradiated IL-$2^{-/-}$ host to assess the effect of IL-2 in replenishing the T cell repertoire.
- Transfer of allotypically marked IL-$2^{+/+}$ $CD25^+$ regulatory T cells into IL-$2^{-/-}$ mice to test whether the introduction of this Tr subset will influence disease progression.
- Isolation of IL-$2^{-/-}$ colonic T cells and comparison with IL-$2^{+/+}$ colonic T cells, using RT-PCR and Clontech gene expression assays.
- Injecting recombinant IL-2 at different stages of disease development and assessing whether it will halt disease progression, in particular at late stages of disease (mice aged 14-16 weeks). This will give insight into the protective role of IL-2 and whether there is a specific window period after which IL-2 is unable to promote protection.
- Treatment of IL-$2^{-/-}$ mice with anti-LFA-1 blocking antibodies to assess the role of LFA-1 up-regulation in IL-$2^{-/-}$ $CD4^+$ T cells and disease pathogenesis.

Further time-course studies on activated IL-$2^{+/+}$ and IL-$2^{-/-}$ $CD4^+$ T cells, using Clontech gene arrays, will give insight into the kinetics of cytokine production. Similarly, studies on the blockade of B7: CD28/CTLA-4 costimulation in IL-$2^{-/-}$ mice will strengthen our understanding of its complexities with regard to the complex manipulation of the B7: CD28/CTLA-4 pathway.

Finally, these studies highlight the critical role for IL-2 in peripheral tolerance but not development. They suggest that disease in IL-2 null mice is due to an abnormal mucosal balance, which is in turn a product of the uncleared activated $CD4^+$ T cells. The findings in this thesis do not present evidence in support of a specific cytokine that is substituting for IL-2 in the proliferative responses of IL-$2^{-/-}$ $CD4^+$ T cells *in vitro*.

CHAPTER 8

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