CYTOKINE PRODUCTION BY VACCINIA VIRUS IMMUNE CELLS

by

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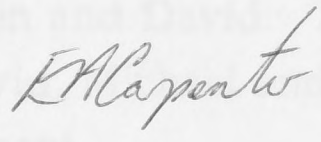
STATEMENT

With the exception of the following, the experiments described in this thesis represent my own work. The antibody neutralization data presented in Table 5.2 was provided by G. Bartell, Mrs. S. Fordham and Dr. K. Rockett.



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PUBLICATION ARISING FROM THIS THESIS

E. A. Carpenter, J. Ruby, and I. A. Ramshaw. Interferon-γ, Tumour Necrosis Factor and Interleukin-6 production by vaccinia virus immune spleen cells: an *in vitro* study. *J. Immunol,* accepted for publication, October, 1993.

ABSTRACT.

T lymphocytes play an important role in recovery from virus infection. These cells can be both directly cytolytic and release cytokines. The cytokines support proliferation of the cells responding to infection, and also function to recruit other cell populations, which themselves secrete cytokines. Several of these cytokines have demonstrated antiviral activity. The experiments described in this thesis were designed to examine the production of the antiviral cytokines TNF, IFN-γ and IL-6 in an immune response to virus infection.

An *in vitro* model was established to investigate the kinetics of cytokine production by virus immune spleen cells. Vaccinia virus primed spleen cells from CBA/H mice were stimulated *in vitro* with virus infected UV-irradiated syngeneic cells. Both $CD4^+$ and $CD8^+$ T cells proliferated following restimulation. TNF, IL-6 and IFN-γ were detected within 12 h of restimulation, with maximal levels reached by 24 h. The virus immune responder cells co-operated to provide costimulatory signals which were not presented by the UV-irradiated stimulator cells.

Depletion of subpopulations of cells provided information on the cells producing the cytokines. The virus immune adherent cells were the major producers of TNF and IL-6. The production of these cytokines did not require T cells and was not antigen specific. In contrast, IFN-γ production was T cell dependent, particularly $CD4^+$ T cell dependent, and production was antigen specific.

Cytokine interaction was examined by the addition of specific antibodies and exogenous cytokine. The results suggested that the production of IFN-γ and TNF was influenced by the presence of other cytokines, whereas IL-6 was not. Neutralization of TNF in cultures reduced IFN-γ production, indicating that at least a portion of IFN-γ production was dependent on TNF. IFN-γ production was also shown to be dependent on IL-2, as neutralization of IL-2 led to decreased IFN-γ levels, whereas levels increased upon addition of recombinant IL-2. The IL-2-induced IFN-γ production was dependent on the presence of T cells. Neither IL-2 nor IL-4 were detectable in the culture supernatant, although neutralization of IL-4 led to a decrease in both TNF and IFN-γ

levels. The antibody to IL-10 had no effect on cytokine production yet IL-10 was detected after 24 h incubation.

Virus immune cells were restimulated with UV-irradiated cells infected with recombinant vaccinia viruses which encode cytokine genes. The virus constructs continued to produce the encoded cytokine after UV-irradiation. TNF production by the responder cells was enhanced when restimulated with the TNF-encoding virus. Similarly, enhanced production of IFN-γ was observed following restimulation with the IFN-γ encoding virus. In addition, increased IFN-γ levels were produced following restimulation with the IL-2-, TNF-, IL-4- and IL-10 encoding viruses.

ABBREVIATIONS.

APC	Antigen presenting cell
BSA	Bovine serum albumin
C	Complement
CMI	Cell-mediated immunity
Con A	Concanavalin A
CTL	Cytotoxic T Lymphocyte
DC	Dendritic cell
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	relative centrifugal force
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	hours
HSV	Herpes simplex virus
i.v.	intravenous
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LT	Lymphotoxin
mAb	Monoclonal antibody
MHC	Major histocompatability complex
min	minutes
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
mM	millimolar
ND	Not determined
ng	nanogram
NK	Natural Killer cell
nm	nanometre
OD	Optical density
OVA	Ovalbumin
p	probability
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline

PE	Phycoerythrin
pfu	plaque-forming units
pg	picogram
r	Recombinant
R	Receptor
scid	Severe combined immunodeficient
SD	Standard deviation
SN	Supernatant
t1/2	half-life
TCR	T cell receptor
TdR	Thymidine deoxyribose
Th	Helper T
TNF	Tumour Necrosis Factor
U	Unit
UV	Ultraviolet
VV	Vaccinia virus
VV-WR	Wild type vaccinia virus

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CHAPTER 1

Introduction and Literature Review

1 Introduction

The host defence to a primary virus infection occurs in three phases (Janeway, 1989). Initially, there is an innate non-specific resistance that is non-inducible. This natural resistance to infection consists of those mechanisms which are triggered immediately upon entry of a pathogen. This is followed by an early, inducible phase that is largely antigen non-specific. Finally, there is a late, T-cell dependent phase that is inducible, highly antigen-specific and generates immunological memory.

The T cell-specific immune response takes several days to become effective, whereas the innate resistance mechanisms are immediately effective upon contact with the invading pathogen. Once activated, this initial defence system sets out to confine the infection, eradicate the pathogen and limit host tissue damage (Müller-Eberhard, 1989). The components of this system include monocytes/macrophages, NK cells, interferons and secretory products of macrophages.

It is the later T cell-specific immune response that is responsible for protective immunity following infection. This involves the induction and interaction of specific antibody and cell-mediated responses through the activation of clonally specific T and B cells. Cytotoxic T lymphocytes (CTL) are believed to be important in the control of and recovery from most primary viral infections (Blanden, 1971), although an antibody response may be a contributing factor. An immune response must be tightly regulated to ensure the response is of the right magnitude and quality. The mechanism of regulation is mediated through the profile of cytokines secreted by various cell populations (Coffman *et al.*, 1988).

2 Innate immunity

The innate non-specific host defences to an infection are an immediate response, triggered by entry of the pathogen. The responses are important in determining the outcome of a viral infection as they interfere with the early stages of viral invasion, subsequent multiplication and the spread of virus to susceptible organs. Macrophages and natural killer cells are active participants in the innate and early inducible phases of the response (Mims, 1964). An inflammatory focus develops around the site of invasion, with the accumulation of phagocytes. Macrophages recognise the pathogen through interaction between pathogen-surface molecules and receptors on the macrophage, and once stimulated begin the process of endocytosis and release TNF, IL-1 and interferons (Müller-Eberhard, 1989). NK cells may become stimulated to lyse infected cells and produce IFN-γ (Trinchieri, 1989).

2.1 Macrophages

Macrophages are placed strategically throughout the body to meet foreign particles and are widely distributed in major organs of the body. They respond to stimulation with secretion of some 100 substances, which range in biologic activity from induction of cell growth to cell death (Nathan, 1987). Therefore, macrophages may be decisive in determining the susceptibility or resistance of an animal to a virus infection (Mims, 1964). If a virus is taken up by a macrophage which does not support virus replication, the outcome is that the virus infection is delayed or prevented from spreading to susceptible cells. However, due to the mobile nature of macrophages, replication of the virus in these cells will result in dissemination of the virus throughout the body (Blanden, 1982). Stimulated macrophages have been demonstrated to inactivate viruses extracellularly in a non-antigen specific manner (Morahan *et al.*, 1977). Growth of HSV, MHV and VSV have all been inhibited by macrophages. The mechanism responsible may differ depending upon the particular macrophage and the particular virus. Reported mechanisms include inhibition of virus adsorption and penetration into permissive cells, and direct inactivation by macrophage secreted products such as interferons and TNF (reviewed by Morahan *et al.* , 1985).

2.2 NK Cells

NK cells are $CD3^-$ T-cell receptor $(TCR)^-$, large granular lymphocytes (Hercend and Schmidt, 1988) which are cytotoxic to a variety of target cells in a MHC-non-restricted fashion (Trinchieri, 1989). There is experimental evidence to suggest a role for NK cells in defense against many virus infections (Welsh *et al.*, 1991). NK cells are activated and numbers increase at the site of virus infection (Biron *et al.*, 1983), and treatment of mice with anti-asialo GM_1 antibody to deplete NK cells results in increased titres of several viruses, compared to control mice (Bukowski *et al.*, 1983). NK cells are present in the peripheral blood and spleen in a resting state, and can be triggered to lyse a target cell within minutes of binding (Trinchieri, 1989). NK cells have also been shown to produce IFN-γ, TNF, GM-CSF, colony-stimulating factor (CSF) and IL-3 when appropriately stimulated (Cuturi *et al.*, 1989). Both IFN-γ and TNF have been shown to have direct antiviral activity (refer to section 2.3), while another important function of these cytokines is to activate phagocytes.

Information about the receptor used by NK cells to distinguish between normal cells, tumour cells and virus infected cells has not been available until very recently. It has been shown that sensitivity to NK cells is inversely correlated with class I MHC expression on target cells (Kärre *et al.*, 1986). A recent proposal suggests that NK cell triggering is inhibited when target cells encode a specific natural ligand bound in the class I MHC peptide groove. Loss of either the class I MHC molecule or the ligand from the target cell surface would render the cell NK sensitive (Raulet, 1992). Either of these events could take place in a virus infected cell; viruses are known to switch off cellular protein synthesis or a viral peptide may displace the NK ligand from the MHC peptide groove.

The ability of the NK cell population to respond without preactivation allows them to participate in the first line of defence against infection by viruses, before the humoral and cellular effects of the adaptive immune response are activated. The rapid release of cytokines by NK cells also enhances their role in the non-adaptive resistance phase by enabling the recruitment of other relevant effector cells.

2.3 Antiviral cytokines

2.3.1 Interferons

Interferon was first identified in 1957 as a secreted factor able to transfer a virus-resistant state (Isaacs and Lindenmann, 1957). This interferon is a family of related proteins which have been classified into two groups, Type I and Type II interferons. Type I interferons, IFNα and IFNβ, are induced by virus infection and are produced by leukocytes and fibroblasts, respectively. Type II IFN, or IFN-γ, also known as immune IFN, is induced by mitogenic or specific antigenic stimulation of T lymphocytes and NK cells (Pestka and Baron, 1981). Both Type I and Type II IFNs have antiviral activity *in vivo*, demonstrated by increased virus growth in animals treated with antibodies to IFN (Gresser *et al.*, 1976; Klavinskis *et al.*, 1989; Leist *et al.*, 1989). There are differences in the sensitivity of various viruses to the effect of IFNs (Rubin and Gupta, 1980; Stewart, 1979) and there may be synergistic amplification of IFN-mediated protection against virus infection when there is a combination of IFN types present (Fleischmann *et al.*, 1979).

All three IFNs, α, β, and γ, up-regulate the expression of class I MHC, while only IFN-γ increases the rate of expression of class II MHC (Wong *et al.*, 1983). The IFN-enhanced expression of MHC may contribute to the antiviral actions of IFNs by making the cells more susceptible to lysis by cytotoxic T cells in the later T cell-dependent phase of response.

2.3.2 Tumour Necrosis Factor

Tumour Necrosis Factor (TNF) was first described as a tumoricidal protein found in the serum of bacillus Calmette-Guérin (BCG)-infected mice following endotoxin treatment (Carswell *et al.*, 1975). It has been demonstrated that TNF-α and lymphotoxin (TNF-β) have antiviral activity against various RNA and DNA viruses (Mestan *et al.*, 1986; Wong and Goeddel, 1986). These cytokines induce resistance in uninfected cells and are also able to selectively kill virus-infected cells, thus preventing further viral spread. Virus-infected cells are more susceptible to the cytolytic action of TNF than uninfected cells (Wong and Goeddel, 1986). Virus infection has been shown to induce the

production of TNF by monocytes (Aderka *et al.*, 1986), indicating the importance of TNF in a physiologic antiviral response.

Interestingly, the antiviral activity of TNF and IFN was found to be enhanced if used in combination (Feduchi *et al.*, 1989; Wong and Goeddel, 1986).

3 Immune response

The third phase in the host defense against virus infection is a late, antigen-specific, T cell-dependent phase leading to immunological memory. The host T cells recognise viral components as foreign in the context of major histocompatability complex (MHC) antigens on the surface of antigen presenting cells. Following recognition, a cascade of cellular and humoral responses to the virus is set in motion. Virtually all adaptive immune responses involve clonal expansion of naive $CD4^+$ T cells. These cells and the cytokines they produce play an important regulatory role in the responses of B cells, other T cells and NK cells (Coffman *et al.*, 1988).

Swain *et al.* (1990a) have identified three developmentally distinct subsets of $CD4^+$ T cells: precursors, effectors and memory cells. Precursor and memory cells are resting $CD4^+$ T cells, while the effector cells are large, activated $CD4^+$ T cells. The development of effector cells from precursors requires an activation signal, cytokines and a source of APC. The activation signal may be a mitogen or, as in a natural infection, antigen in the presence of MHC molecules expressed on the cell surface of a specialized antigen-presenting cell.

3.1 MHC

MHC class I molecules are present on nearly all cells. MHC class II molecules are restricted to a specialized set of antigen presenting cells, ie. macrophages, dendritic cells and B cells (Harding, 1991). T cells recognise proteolytic fragments of antigen that are presented at the cell surface in association with either class I or class II MHC molecules. This was first demonstrated by Zinkernagel and Doherty (1974) using virus-specific CTLs which recognised viral antigens in association with MHC molecules. Later, it was shown the CTL recognised viral antigens that are not normally present on the cell surface, such as nucleoprotein,

indicating the CTL may recognise degraded forms of the antigen bound to an MHC class I molecule (Townsend *et al.*, 1985).

The immune system must be able to present both intracellular and extracellular antigens and elicit the correct immune response. It would appear this has led to the evolution of the two classes of MHC molecule. Peptides derived from intracellular antigens are generally presented by MHC class I molecules, whereas extracellular antigen-derived peptides are presented by MHC class II molecules (Braciale *et al.*, 1987). There has been intense research in the area of processing pathways for antigen presentation by MHC molecules recently (reviewed by Monaco, 1992; Neefjes and Ploegh, 1992). The current model of MHC class-I-restricted antigen processing is that intracellular proteins are degraded in the cytoplasm to, preferentially, nine amino acid peptides. The peptides are delivered to the endoplasmic reticulum (ER) lumen and bind to newly synthesized MHC class I molecules, inducing a conformational change that may facilitate their export to the cell surface (Monaco, 1992). Thus, any protein synthesized within the cytoplasm, including viral protein, is able to be processed and presented by class I molecules.

The processing and presentation of extracellular antigens is more complex. MHC class II molecules are assembled in the ER. The functional unit is a heterodimer, $\alpha\beta$. A third chain, γ, is associated with the $\alpha\beta$ dimer to prevent peptides binding to the dimers in the ER, and to help in the transport process from the ER through the Golgi. Antigen enters the cell, through endocytosis, and is degraded into peptides. Peptide fragments associate with class II molecules after the release of the γ chain. The class II-peptide complex is then transported to the cell surface (Neefjes and Ploegh, 1992).

There are examples, however, where endogenous antigens, which according to the above model should be presented by class I MHC, are presented by class II MHC molecules (Sekaly *et al.*, 1988). This suggests that the models for endogenous and exogenous antigens being presented by class I and class II MHC molecules, respectively, may not hold for all antigens (Nuchtern *et al.*, 1990).

3.2 Antigen presenting cells and immune induction

The three situations in which antigen is presented to T cells are the stimulation of resting T cells in the primary response, the expansion of memory cells on secondary stimulation, and the interaction between cytotoxic effector cells and their targets.

In primary T cell responses, naive T cells need to be selected from the T cell pool and stimulated. Naive, or resting, T cells distribute preferentially from blood to the lymphoid tissues. This means that on primary exposure, antigen must be transported to lymphoid tissue for presentation to the local T cell population. Bone marrow derived dendritic cells (DC) are believed to be the specialized cells that acquire antigen and transport it to lymph nodes (Knight and Stagg, 1993). An increase of DC movement into lymph nodes following peripheral exposure to antigen has been observed (Hill *et al.*, 1990), and dendritic cells are capable of processing and presenting even large antigen (De Bruijn *et al.*, 1992a), despite being nonphagocytic. Once antigen has been carried to the lymph nodes the DCs mature, up-regulate MHC class II expression and become specialized at clustering and activating T cells. These clusters are not seen when antigens are presented on macrophages or lymphocytes (Inaba *et al.*, 1987). Clustering could provide the time required for products on two opposing cell surfaces, such as MHC molecules and receptors, to align and interact. DCs as antigen-presenting cells are able to induce primary anti-viral CTL responses, whereas spleen cells and LPS-induced B cell blasts are not (De Bruijn *et al.*, 1992b). Macrophages are also important APC for CTL induction as demonstrated by the inability of mice to mount CTL following depletion of phagocytes, which was dramatically reversed by infusion of a macrophage hybridoma (Debrick *et al.*, 1991).

T cells, particularly $CD4^+$ T cells, play an important regulatory role within the immune system. Expansion of these cells is critical for development of cytotoxic responses, antibody responses and the generation of delayed-type-hypersensitivity (DTH) responses. Therefore, the proliferation of these cells must itself be tightly regulated to protect against the development of an inappropriate immune response. In 1970, Bretscher and Cohn proposed a two-signal model of lymphocyte activation for B cells to solve the problem of self-nonself

discrimination. It was postulated that signal one was the occupancy of the antigen-specific receptor. Signal one alone induced tolerance. Upon receiving the second signal, however, the cell became activated. Lafferty *et al.* (1977) extended this model to T cell activation, with binding of antigen to the T cell receptor providing signal 1, and the second signal, a nonspecific inductive stimulus, provided by the stimulator cell.

The nature of this costimulatory activity required for the delivery of signal 2 is yet to be fully determined. A review by Mueller *et al.* (1989) outlined a number of experiments which defined signal 2 as a cell-bound or short-range acting costimulatory factor which was rapidly induced on the APC surface. Most candidates for molecules mediating the costimulatory pathways are lymphoid adhesion molecules (van Seventer *et al.*, 1991). Using monoclonal antibodies it has been demonstrated that CD28 is a costimulatory molecule (June *et al.*, 1990) and thus it may provide the second signal. CD28 is a cell surface glycoprotein, expressed on all murine $CD4^+$ and $CD8^+$ T cells (Gross *et al.*, 1992). The ligand for CD28 has been identified as B7 (Linsley *et al.*, 1991a), which is expressed constitutively on splenic and blood dendritic cells, is induced on B cells and monocytes, and is not expressed on resting T cells (reviewed by Jenkins and Johnson, 1993). The physiologic signal for inducing B7 expression on B cells is thought to be contact between the TCR and the MHC/antigen complex on the B cell (Nabavi *et al.*, 1992). Engagement of the CD28 surface receptor results in stabilization of T cell cytokine mRNA and an increased rate of cytokine gene transcription (reviewed by Schwartz, 1992). A second receptor for B7 also exists, CTLA-4, an activation molecule on both $CD4^+$ and $CD8^+$ T cells (Linsley *et al.*, 1991b). It is unclear why there are two receptors for B7, although they may act co-operatively to give maximum $CD4^+$ T cell proliferation (Schwartz, 1992). Delivery of both TCR ligand and the B7 molecule on one cell is at least 30 times more efficient at inducing primary T cell proliferation than a mixture of cells expressing either one of these two molecules alone (Liu and Janeway, 1992). In contrast, cloned or activated T cells are capable of receiving costimulatory signals from bystander cells such as B cells and macrophages (Jenkins *et al.*, 1988).

A number of other molecules on the APC augment the proliferative effects of TCR stimulation by interacting with a specific counter-

receptor on the T cell (reviewed by Liu & Linsley, 1992, see Figure 1.1). For example, lymphocyte function associated antigen (LFA)-3 which binds to the CD2 receptor on T cells plays an important role in signalling. Intercellular adhesion molecule (ICAM)-1 and ICAM-2 are counter receptors for the integrin LFA-1. Their binding enhances T cell activation. Heat stable antigen (HSA), expressed on different APC, has costimulatory activity for murine $CD4^+$ T cells, although the T cell receptor has not been identified. These receptor-ligand pairs may act by promoting T cell-APC adhesion, allowing other signalling molecules, such as the TCR and CD28, to interact more efficiently with their ligands.

On secondary exposure to antigen, memory cells and antibodies are present in the periphery. The memory T cells enter tissues and it is possible to have activation at peripheral sites. Primed T cells can interact with many types of presenting cells (Inaba and Steinman, 1986), including macrophages, B lymphocytes and Langerhans cells, although the antigen presenting function of dendritic cells is more efficient (Nonacs *et al.*, 1992). Activated T cells can be triggered by peptide-MHC on planar membranes (Watts *et al.*, 1984), and thus do not have to interact with viable APCs.

Any cell that is infected with virus is potentially able to present antigens to MHC class I-restricted $CD8^+$ T cells, as all cells have MHC class I molecules. In contrast, stimulation of $CD4^+$ T cells requires viral entry into cells that constitutively express class II MHC molecules.

4 Effector response

A cascade of cellular and humoral responses is initiated following activation of T cells in a virus infection. These responses include generation of cytotoxic T lymphocytes (CTL), activation of macrophages to produce reactive nitrogen intermediates and other secreted factors, and at a later stage, neutralization of pathogen with antibodies. T cell-mediated immunity is usually measurable systemically within 4 - 6 days after general spread of the virus, at a time when the viral titres are beginning to decline. In contrast, neutralizing antibodies can be detected much later, often measurable days or weeks after the virus has disappeared (Zinkernagel and Hengartner, 1985).

4.1 Regulation of response by T cell cytokines

There are a wide variety of different effector mechanisms possible within an immune response, each of which is particularly effective against a certain set of pathogens. For example, a cytotoxic response will be effective in a virus infection, whereas antibodies are required to neutralize soluble toxins. Thus, the regulation of the type of immune response is crucial to the well-being of the host. During the last five years it has become apparent that subsets of T cells secreting distinct patterns of cytokines have a major role in this regulation. Two major patterns of cytokine secretion by T cells have been identified among murine T cell clones (Mosmann *et al.*, 1986), and these patterns appear to be associated with the induction of either CMI or help for antibody production, thus providing a possible explanation for the separate and often reciprocal regulation of these two responses (Parish, 1972).

4.2 Cytokine secretion phenotypes of T cells

Mosmann *et al.* (1986) defined two types of $CD4^+$ T cell, based on studies of $CD4^+$ T cell clones. $CD4^+$ T cells are commonly referred to as helper T cells, hence the two groups were named T helper 1 (Th1) and T helper 2 (Th2). It must be stressed, however, that $CD4^+$ T cells have a variety of functions, only one of which is to activate antigen-specific B cells, the original definition of T cell help (Bottomly, 1988). Upon activation, Th1 clones synthesize IL-2, IFN-γ and lymphotoxin (LT) while Th2 clones synthesize IL-4, IL-5, IL-6 and IL-10. Both types of clone synthesize IL-3 and GM-CSF (reviewed by Mosmann and Coffman, 1989b). $CD8^+$ T cell clones express a pattern of cytokines that corresponds to the Th1 pattern (Fong and Mosmann, 1990).

Th2 clones cultured with antigen-specific B cells enhance the growth and differentiation of the B cells and induce significant IgE responses. In contrast, Th1 clones may suppress B cell responses, although they are able to induce IgG2a production (Stevens *et al.*, 1988). The production of IgG2a antibody, the major opsonising isotype, would be appropriate when CMI is induced. Only Th1 clones are able to elicit DTH, which may be mediated through the production of IFN-γ (Mosmann and Coffman, 1989a).

The Th1 and Th2 cytokine synthesis patterns have been defined using long-term mouse T cell clones. Cytokine secretion by T cell clones at short times after establishment do not follow a pure Th1 or Th2 pattern, as a broad spectrum of cytokines are produced (Firestein *et al.*, 1989; Mosmann and Coffman, 1989a), although Th1 or Th2 clones can develop with prolonged culture. Experiments by Kelso and Gough (1988) have also demonstrated that the co-expression of GM-CSF, IL-3, IFN-γ and IL-4 was random in both $CD4^+$ and $CD8^+$ T cells in short term clones. Thus, it is uncertain whether normal activated T cells display the Th1 or Th2 phenotype. Some of the strongest evidence for the existence of Th1 and Th2 cells *in vivo* comes from studies with several mouse models of parasite infections. Host-parasite models have been used extensively to study the effects of cytokine patterns *in vivo* (reviewed by Sher *et al.*, 1992) because T cell subset and cytokine responses tend to polarize in many experimental parasitic infections.

4.2.1 The murine model of *Leishmania major* infection

The murine response to infection with the protozoan parasite *Leishmania major* has been well characterized. In most inbred strains of mice, subcutaneous infection with *L. major* is resolved due to the induction of a strong cell-mediated immune response. In contrast, BALB/c mice develop a progressive and ultimately fatal disease (reviewed by Müller *et al.*, 1989). An important difference between resistant and susceptible mice is the nature of the $CD4^+$ T cell response. Healing of infection in resistant mice is associated with stimulation of Th1 type cells (Heinzel *et al.*, 1989) and expression of IFN-γ and IL-2 by these cells (Heinzel *et al.*, 1991), whereas susceptiblity is associated with preferential stimulation of the Th2 subset and expression of IL-4 and IL-10. Furthermore, adoptive transfer of Th1 and Th2 cell lines into nonhealer BALB/c mice provided direct evidence that the Th1 cells mediate resistance (Scott *et al.*, 1988).

4.2.2 Regulation of Th1 or Th2 phenotype generation

A question that is still not resolved is what mechanisms operate to regulate the generation of either Th1 or Th2 cell phenotypes from a naive T cell population. There is recent evidence to suggest that the cytokines present during primary stimulation are the most important influence on naive Th cell differentiation (Scott, 1993). However, there

are studies which have indicated that the antigen presenting cell and the antigen dose are also important factors. Many of the studies have used the *L. major* model, and are discussed below.

Both *in vivo* and *in vitro* studies of the response of mice to *L. major* have given clear evidence of the importance of certain cytokines in the regulation of differentiation of naive T cells into Th1 or Th2 cells. Treatment of resistant mice with anti-IFN-γ mAb led to increased susceptibility, with enhanced production of IL-4 and IL-5 (Scott, 1991), whereas treatment of BALB/c mice with anti-IL-4 mAb during the first two weeks of infection allowed these previously susceptible mice to develop healing Th1-like responses (Chatelain *et al.*, 1992). The development of a Th1 or Th2 response is induced soon after infection as lymph node T cells taken from infected mice could be restimulated *in vitro* with *L. major* antigens as early as 3 days after initial infection. Cells from BALB/c mice produced substantial IL-4 and IL-5, while resistant mice secreted IFN-γ (Scott, 1991). The early timing of this response indicates the production of these cytokines is part of the innate response, ie. the cytokines are produced by cells which can respond immediately to antigen, without clonal proliferation, and are present in relatively large numbers.

NK cells appear to be a major source of IFN-γ as shown in a study using *scid* mice, where a host source of IFN-γ appeared to drive transferred naive T cells towards a Th1 phenotype in *L. major* infected mice (Varkila *et al.*, 1993). This would suggest that antigens which activate NK cells may preferentially induce a Th1-like response. Alternatively, antigens which activate macrophages to produce cytokines which then stimulate NK cell production of IFN-γ may also lead to a Th1-like response. NK cells are stimulated by IFN-α (Kasaian and Biron, 1990) and IL-12 (Kobayashi *et al.*, 1989), cytokines which are produced predominantly by macrophages (D'Andrea *et al.*, 1992). It has been proposed that viruses induce specific immune responses of the Th1 type because they either directly stimulate NK cells to produce IFN-γ, or because they induce macrophage production of IL-12 and IFN-α, which in turn stimulate NK cell growth and IFN-γ production (Romagnani, 1992).

The presence of IL-4 at the early stages of infection appear to be critical in the development of a Th2-type response. The cellular source of IL-4

during the innate stage of infection has not been identified. As T cells are not able to differentiate into IL-4 producing cells in the absence of IL-4 (Le Gros *et al.*, 1990), IL-4 must be produced by another cell. Non-T cells from mouse spleen, probably belonging to the mast cell/basophil lineage, can secrete significant levels of IL-4 (Ben-Sasson *et al.*, 1990), thus antigens which are able to activate mast cells may induce the production of a Th2-type response. Recent studies by Morris *et al.* (1992) demonstrate that both resistant and susceptible mice synthesize IL-4 and IFN-γ early in the response to L. major infection. However, the outcome of infection is determined by the number of *L. major*-specific $CD4^+$ cells secreting IL-4 (Morris *et al.*, 1993). Removal of $CD4^+$ cells or treatment with anti-IL4 led to a decreased precursor frequency of cells secreting IL-4 and reduction in parasite number, with no change in IFN-γ secreting precursors. This indicates that IL-4 or associated cytokines down-regulate the effectiveness of IFN-γ, and removal of IL-4 secreting cells allows IFN-γ to exert its protective effects.

The dose of antigen has also been shown to be crucial in determining whether a Th1 or Th2 type of response is induced. A concentration of antigen that is subimmunogenic for the induction of antibody (Th2 type response) can induce DTH (Th1 type response) (Parish, 1972). Recent studies by Bretscher *et al.* (1992) have demonstrated that "susceptible" BALB/c mice mount a cell-mediated Th1 type response when injected with a small number of *L. major* parasites, and aquire systemic resistance. This may have important implications in the design of vaccines for use in a genetically diverse human population.

Another mechanism of differential activation of Th1 or Th2 cells may be at the level of the APC. Using OVA-specific Th1 and Th2 clones derived from the same lymph node cell preparation, Gajewski *et al.* (1991) have demonstrated the two phenotypes proliferate optimally in response to distinct antigen-presenting cell populations. Purified B cells stimulated optimal proliferation of Th2 clones, whereas adherent cells (macrophages/ dendritic cells) stimulated optimal proliferation of Th1 clones. This suggests that a cofactor is necessary for optimal proliferation of each Th subset, and that these cofactors are preferentially expressed by distinct APC populations.

As described above, the Th1 and Th2 phenotypes do exist *in vivo* following priming. However, short term clones exhibit a number of

other cytokine secretion phenotypes. Firestein *et al.* (1989) described a third phenotype, Th0, with cells in this subset having an unrestricted pattern of cytokine production. It has been proposed that the Th1 and Th2 cells represent phenotypes that have differentiated in response to strong and repeated stimulation during an immune response, while the Th0 phenotype may represent an intermediate stage of differentiation (Mosmann and Coffman, 1989a), although this still does not account for the range of cytokine secretion phenotypes which have been described (Kelso *et al.*, 1991).

4.2.3 Cross-regulation of immune response

There is an inverse relationship between the induction of antibody and DTH responses in an immune response (Katsura, 1977; Parish, 1972). As these two responses are associated with preferential expansion of Th2 and Th1 cells, respectively, this reciprocal behaviour could be explained by cross regulatory effects between Th1 and Th2 cells. IFN-γ, a product of Th1 cells, inhibits the proliferation of Th2 but not Th1 cells (Gajewski and Fitch, 1988). An activity produced by a Th2 clone, D10, inhibited Th1 proliferation (Horowitz *et al.*, 1986). Fiorentino *et al.* (1989) characterized a cytokine, IL-10, which is produced by Th2 cells and inhibits synthesis of several cytokines by Th1 clones. IL-10 has been shown to inhibit the APC function of purified macrophages, but not B cells (Fiorentino *et al.*, 1991a). IL-10 also inhibits the production of IFN-γ by NK cells (Tripp *et al.*, 1993). These results indicate that subsets of Th cells can cross-regulate the growth and activities of one another.

4.3 Cytotoxic T Lymphocytes

Adoptive transfer of immune lymphocytes into mice with varying virus infections, including influenza, HSV-1 and LCMV, promotes their recovery and reduces virus titres. The cells with this effector activity are Thy.1$^+$ and Lyt.2$^+$, require class I homology between donor and recipient, and exhibit virus-specific cytotoxicity *in vitro* (Byrne and Oldstone, 1984; Sethi *et al.*, 1983; Yap and Ada, 1978). Two possible mechanisms by which these virus-specific T cells mediate their antiviral activity have been suggested. Firstly, the cytotoxic T cells lyse virus-infected cells before infectious progeny is assembled and released. This suggestion has been supported by the work of Zinkernagel and

Althage (1977), where 90% of vaccinia infected target cells were lysed by immune cells before progeny virus could be assembled. The second mechanism suggested is that the T cells release soluble mediators which then indirectly prevent virus spread, eg. the release of IFN-γ or other lymphokines that activate macrophages to increased virucidal capacity (Blanden, 1982). This is also supported by the demonstration that the antiviral activity of vaccinia virus immune $CD8^+$ T cells is dependent on IFN-γ (Ruby and Ramshaw, 1991).

The process of CTL activation occurs in 2 - 7 days after mitogen or antigen stimulation and involves a differentiation event and, usually, proliferation. This multistep process requires transmembrane signals delivered by cytokines, in addition to signals delivered by the antigen specific T cell receptors (Plate, 1976). Raulet and Bevan (1982) identified a factor, called CDF (CTL differentiation factor), which is required for CTL differentiation. CDF was later identified as IL-6 (Takai *et al.*, 1988). The *in vitro* cytokine requirements for CTL induction differ for thymocytes and spleen cell responders (Takai *et al.*, 1986), as thymocytes required IL-2, IFN-γ and IL-6 for optimal induction, while spleen cell responders only required IL-2. The importance for IL-6 in the generation of CTL *in vivo* has been demonstrated using IL-6 knockout mice (IL-6 -/-). The generation of CTL against vaccinia virus (WR strain) was compromised in IL-6 -/- mice, with increased virus titres in ovaries and lungs (A. Ramsay, personal communication). IL-4 is another cytokine required for CTL induction. IL-4 added late to a culture augments the cytotoxicity, providing a late signal in CTL generation. Conversely, exposure of cells to IL-4 before antigen results in suppression of the CTL response (Horohov *et al.*, 1988). A recent development has been the demonstration that IL-6, which is required for CTL differentiation, acts by inducing Th1 cells to produce IL-2 and IL-4, the mediators for the final differentiation of $CD8^+$ T cells to active CTL (Quentmeier *et al.*, 1992). These studies indicate that the generation of CTL is dependent not only on presentation of antigen in the context of class I MHC, but also the presence of $CD4^+$ T cell-derived cytokines at the site of stimulation.

There has been a great deal of research in the field of cell-mediated cytotoxicity, yet there is still no consensus about the way in which CTL induce target cell death. It would seem that there are multiple mechanisms and mediators. Nevertheless, these mechanisms show

exquisite specificity, as demonstrated by Lukacher *et al.* (1984). When mice were simultaneously infected with two different subtypes of influenza the adoptive transfer of a CTL specific for only one subtype did not promote recovery of the mice. The subtype specific clone was only able to reduce the virus titre of the strain which it recognised.

Two general models have been developed to explain the mechanisms by which cytolytic T cells kill target cells (Berke, 1991). The first model, the membrane attack model, can be resolved into three steps. First, CTLs recognise an appropriate target cell and bind. The second step is the delivery of a "lethal hit", through granule exocytosis by the effector cell. The final step is disintegration and lysis of the cell. Purification of proteins stored in the granules led to the discovery of a membranolytic protein named perforin and a series of serine esterases (reviewed by Lowin *et al* , 1992). There is a great deal of *in vitro* evidence to support this model, although criticism against it is a question of how the killer cell is able to protect itself against its own lethal machinery (Tschopp and Nabholz, 1990). In the second model, the suicide model, the cytotoxic cell is assumed to trigger a self-destructive process in the target cell, with DNA fragmentation occurring within minutes of target cell contact with an effector cell (Golstein *et al.*, 1991).

It appears that a mixture of cytotoxic mechanisms is used by different killer cell types and that any given target cell may be susceptible to multiple effector mechanisms (Smyth *et al.*, 1992). The mechanism may depend on the nature of the target cell, the presence of accessory cells or the lymphokines at the site. Elucidation of the importance of these various mechanisms may depend on strategies such as those recently employed by Lowin *et al.* (1992). His group is generating transgenic mice with a homozygous non-functional perforin gene to determine whether there is an absolute requirement for perforin in cell lysis. The work from these mice has not yet been published. However, two recent studies using sensitive PCR or molecular probes have demonstrated that CTL hybridomas are able to kill targets effectively in the complete absence of perforin transcripts (Berke *et al.*, 1993; Helgason *et al.*, 1992). These results support the argument that populations of lymphocytes do exist which have a perforin-independent CTL lytic mechanism.

4.4 Interactions between CD4+ T cells and B cells

Resting B cells require contact-dependent, antigen-specific MHC class II-restricted interactions with Th cells for the induction of their growth and differentiation. CD4+ T cells provide "help" to B cells through physical contact and through secretion of cytokines. To initiate T-B cell interaction, the B cell must take up and re-express antigen in association with MHC molecules. Antigen binds to membrane IgM and IgD on B cells and is endocytosed. Following processing the antigen is re-expressed on the cell surface in a complex with class II MHC molecules, for CD4+ T cell recognition (Noelle and Snow, 1990). Once recognised, a T-B cell conjugate forms. TCR cross-linking results in a rapid and transient increase in affinity of adhesion molecules, such as LFA-1, for their ligands on the B cell (Dustin and Springer, 1989) which increases the stability of the conjugate. The interaction of CD4/TCR with the class II-Ag complex activates the T cell, resulting in T cell expression of a novel protein, CD40 ligand, on the cell surface (Noelle *et al.*, 1992). Once a T cell is activated, the effector mechanisms mediating the activation of the B cells are genetically unrestricted, non-antigen specific, requiring only contact between the Th cell and B cell (Noelle *et al.*, 1992). Binding of the ligand to the receptor, CD40, on the B cell activates the cognate B cell to proliferate (Banchereau *et al.*, 1991) and induces the cell to become responsive to the cytokines released by the T cell. IL-4, a product of Th2 cells is necessary for Th cell dependent B-cell growth (Hodgkin *et al.*, 1990; Noelle *et al.*, 1991). Differentiation of the B cell to immunoglobulin synthesis requires both IL-4 and IL-5. IL-4 enhances secretion of IgG_1 and IgE, while IL-5 enhances IgM and IgA secretion (Snapper *et al.*, 1988; Takatsu *et al.*, 1988). The isotype pattern induced by Th2 cells is modified by the intervention of Th1 cells, as IFN-γ is a switch factor for production of IgG_{2a}, resulting in secretion of larger amounts of IgG_{2a} and reduced secretion of IgG_1 and IgE (Snapper and Paul, 1987).

4.5 Neutralization by antibody

Neutralization is the loss of virus infectivity caused by antibody. It is of major importance in limiting viremia to prevent the spread of virus to susceptible organs and in controlling reinfection (Daniels, 1975). The mechanism of neutralization is complex as several components are involved. IgG can cause virus neutralization by inhibiting fusion of enveloped viruses with cell membranes, while other antibodies can neutralize virus after it has attached to a cell, although a limiting factor in this situation is the rate at which the attached virus is neutralized. It appears that each virus may be neutralized by a mechanism which is determined by some of the following factors: the virus, the neutralization antigen, the Ig isotype and the cell receptor (Dimmock, 1993).

4.6 Activation of Macrophages

IFN-γ has been described as a potent macrophage activating factor (Nathan *et al.*, 1983). This description has been modified to suggest that IFN-γ only primes the macrophage to respond to a second stimulus, such as LPS, and then will become activated (Pace *et al.*, 1983). Primed macrophages are not cytolytic; a second signal is required (Pace *et al.*, 1983). IFN-γ induces the presentation of TNF-α receptors (Ruggiero *et al.*, 1986; Tsujimoto *et al.*, 1986), so that TNF-α produced by the macrophage can then act in an autocrine manner (Philip and Epstein, 1986), amplifying cytokine secretion and begin the signal cascade leading to antimicrobial effector functions. The majority of studies use LPS as a stimulating agent. These do not reflect the mechanism of macrophage activation in non-septic inflammatory responses, where LPS is not present. A series of studies, reviewed by Stout (1993), have shown there are various mechanisms by which T cells could mediate macrophage activation through production of TNF-α and IFN-γ, in the absence of LPS. Both contact-dependent and independent mechanisms have been demonstrated. The supernatant from cultured Th1 cells containing soluble TNF-α and IFN-γ can activate macrophages, by non-cognate signalling. Macrophages, primed by IFN-γ, are also able to receive a second signal through contact with either Th1 or activated Th2 cells. The Th2 mediated signalling of macrophage activation closely resembles that of T cell mediated B cell activation, where

activated T cells deliver non-specific cognate signals to the B cell (see section 4.4) (Stout, 1993). Despite the ability of Th2 cells to activate primed macrophages *in vitro*, it is probably the Th1 cells that are involved in macrophage activtion *in situ*. Th2 cells do not produce macrophage activating cytokines; rather, they produce cytokines, such as IL-4 and IL-10, which will inhibit the production of the macrophage-priming cytokine IFN-γ and prevent activation of macrophages (Sher *et al.*, 1992).

Activated macrophages secrete a number of cytokines, such as IL-1 and TNF, that are thought to play an important role in inflammation (Beutler and Cerami, 1986; Oppenheim and Gery, 1982). IFN-γ released by antigen-stimulated T cells enhances the transcription of these genes in murine macrophages (Collart *et al.*, 1986). IFN-γ has also been shown to prime macrophages for an enhanced oxidative burst (Nathan *et al.*, 1983), tumour cytolysis (Pace *et al.*, 1983), as well as activate macrophages for enhanced microbial killing (Nathan *et al.*, 1983).

There has been a recent discovery of a new antimicrobial mechanism of activated macrophages - the production of reactive nitrogen intermediates (RNI). Studies from 1985 - 1987 demonstrated that macrophages could generate broadly cytotoxic molecules from the guanidino moiety of L-arginine, and form nitrate and nitrite (reviewed by Nathan, 1991). Further studies led to evidence that RNIs contribute to the ability of macrophages to inhibit a wide variety of intracellular and extracellular pathogens. Importantly, IFN-γ can induce nitric oxide synthase (NOS), the enzyme responsible for generation of RNIs, and there is dramatic synergy between IFN-γ and TNF-α or LT in this production (Ding *et al.*, 1988). This suggests that RNI synthesis occurs during any immune response involving T cell-mediated macrophage activation. Two recent reports have demonstrated that nitric oxide (NO) has antiviral activity. Croen (1993) reported that NO inhibited the replication of HSV-1 virus *in vitro*, and Karupiah *et al.* (1993) demonstrated that the IFN-γ induced antiviral activity of mouse macrophages correlated with the cells' production of NO. This group also restricted viral replication in cells with no detectable NO synthesis by transfecting the cells with a complementary DNA encoding inducible NO synthase. NO has been described as being able to inhibit several cellular ribonucleotide reductases (Lepoivre *et al.*, 1991), thus

inhibition of cellular and viral ribonucleotide reductases may explain the sensitivity of viruses to NO.

4.7 Immunological Memory

A feature of the late T cell-dependent response to infection is the generation of memory. Immunological memory is the term used to describe the rapid and more vigorous response to pathogens that have been encountered previously. Memory T cells can now be distinguished from naive cells by their expression of certain cell surface molecules. In humans, antibodies against different isoforms of the leukocyte common antigen (CD45) distinguish between naive and memory cells (Sanders *et al.*, 1988). $CD45RO^+$ T cells respond to a recall antigen *in vitro,* and are thus memory cells, whereas $CD45RA^+$ T cells are naive. In the mouse, anti-CD44 (Pgp-1) antibodies are used to distinguish memory cells (Cerottini and MacDonald, 1989). In both humans and mice, naive cells synthesize greater levels of IL-2 than memory cells, whereas IFN-γ, IL-3 and IL-6 are preferentially synthesized by memory cells (Akbar *et al.*, 1991).

Naive and memory T cells migrate along different pathways through the circulation (reviewed by Mackay, 1991). Memory T cells localize to nonlymphoid tissues, such as the skin and gut, whereas naive T cells generally migrate to lymph nodes. The frequency of naive T cells reactive to a given antigen is very low, and their presence in the lymph node increases the likelihood of a cell encountering its cognate antigen in the presence of appropriate stimulatory signals. Pre-activated (memory) cells, on the other hand, serve their best function when situated in areas of likely antigen encounter, such as epithelial sites. This will ensure that an immediate response is possible when a recall antigen enters the host.

Several virus infections and vaccinations result in lifelong immunity. Whether such immunity is due to persistence of the initially primed T cells and their progeny, or due to subsequent re-exposure to virus and priming of new T cells, has been the focus of a recent study. Jamieson and Ahmed (1989) report that virus-specific CTL can persist indefinitely *in vivo.* Transferred $CD8^+$ T cells eliminated virus and persisted in small numbers in the recipient. Upon re-exposure to antigen, these resting $CD8^+$ T cells proliferated, independently of $CD4^+$ T cell help. The transferred cells were recovered a year later and still retained

antigen specificity and biological function. These results suggest that virus-specific CTL (memory cells) are present in small numbers in a resting stage, throughout the life of the host, and are capable of expansion after re-exposure to antigen. The possibility cannot be ruled out, however, that the virus-specific CTL were maintained through antigenic stimulation by minute amounts of antigen not detectable by conventional assays. A study into the longevity of memory T cell help by Roost *et al.* (1990) has demonstrated that memory T cell help in the generation of antibody is short-lived, ie. 14 days. These results support the idea that T cell memory is dependent on the presence of antigen. The results of the two studies discussed may indicate that the longevity of T cell memory is different according to the type of T cell.

5 Cytokines

5.1 Common characteristics

Cytokines are a group of proteins which are extremely heterogeneous, with a diverse array of functions, yet have a number of common characteristics. These include low molecular weight, transient and local production, potent activity, high affinity binding to receptors, and overlapping cell regulatory actions (Balkwill and Burke, 1989). Most cytokines are glycoproteins with a monomeric molecular mass of 15-25 kDa (Clark and Kamen, 1987). Each cytokine interacts with high affinity cell surface receptors specific for each cytokine or cytokine group (Balkwill and Burke, 1989). The number of receptors on normal cells is low (10 - 10000 per cell), although only 10 - 100 receptors need to be occupied by the cytokine to induce a biological response (Mita *et al.*, 1988; Tsujimoto *et al.*, 1985). The extracellular event of the cytokine interacting with the receptor is transduced into internal signals, often involving a cascade of kinases (Aderem, 1993). These signals then alter the behaviour of the effector cell. Another feature of these proteins is that each cytokine has multiple functions (pleiotropic), and that more than one cytokine can mediate the same function (redundancy; Paul, 1989). Finally, cytokines do not appear to work alone, they interact in a network by: inducing each other, transmodulating cytokine cell surface receptors and by synergistic, additive or antagonistic interactions on cell function (Balkwill and Burke, 1989).

Cytokines produced by T cells are not antigen-specific, although their production is both antigen specific and MHC restricted (Conta *et al.*, 1985). They appear to function as paracrine hormones as they are both produced and consumed at the site of immune reaction (Kelso, 1989). In contrast, cytokine production by non-lymphoid cells can be triggered without antigen specificity, for example by other cytokines or bacterial products. These cytokines are able to travel via the circulation to exert their effects at distant sites in the body.

As mentioned earlier in this review, cytokines play a major role in mediating the immunoregulatory role of T cells in an immune response. Cytokines have a wide range of effects, both beneficial and inhibitory. Therefore, mechanisms must exist to ensure that the cytokines produced stimulate a beneficial host immune response at the site of infection, and once this has been established, the cytokines must be removed or down-regulated to prevent inappropriate responses. Several mechanisms for such regulation have been identified in *in vitro* studies (reviewed by Kelso, 1989). Firstly, cytokine synthesis by activated T cells is short-lived and tightly regulated by antigen concentration. For example, the IL-2 mRNA induced in primary splenic lymphocytes by Con A has a $t_{1/2}$ of only about 30 min and the expression of IL-2 and IL-4 mRNA rapidly disappears following removal of the stimulating signal (Swoboda *et al.*, 1991). Many cytokines have AU-rich sequences in the 3'-untranslated regions of their mRNAs (Shaw and Kamen, 1986) and it has been shown that mRNA instability is correlated with the abundance of 3' AU sequences (Wreschner and Rechavi, 1988). A second mechanism to regulate cytokine production has been demonstrated using cells cultured with a variety of mitogens (Carding *et al.*, 1989). The different stimuli induced synthesis of different combinations of cytokines. Finally, cytokine secretion can be localized and directional (Kupfer *et al.*, 1991; Poo *et al.*, 1988). This would allow concentration of limiting amounts of cytokine in the vicinity of the interacting cells, and prevent stimulation of non-target cells.

A large number of cytokines are involved in a host response to virus infection. In the following sections of this review some of the major characteristics and functions of several of these cytokines will be outlined. The cytokines discussed bear particular relevance to the work

described in this thesis. As cytokines rarely function alone this discussion will also include information on cytokine interaction and the cytokine network (Balkwill and Burke, 1989).

5.2 Macrophage-derived cytokines

The two cytokines discussed in this section are synthesized by a large number of cell types, however, they were originally thought to be macrophage-derived. In the early innate stage of a host response, cytokines produced by NK cells and macrophages signal the adaptive immune response how to react appropriately (Romagnani, 1992). They may also have direct activity on the pathogen itself. TNF and IL-6 secretion are induced upon infection with virus, indicating they play an important role in the host response to this type of invading pathogen.

5.2.1 Tumour Necrosis Factor

In 1975, Carswell *et al.* reported the existence of a serum factor derived from BCG-primed, endotoxin-treated animals which was able to elicit haemorrhagic necrosis of a transplantable tumour in another animal (Carswell *et al.*, 1975). The molecule responsible for this was called Tumour Necrosis Factor (TNF).

TNF is a 17 kDa polypeptide when released by the cell and interacts with its receptor as a trimer (Jones *et al.*, 1989). A 26 kDa membrane form of TNF also exists on activated macrophages (Kriegler *et al.*, 1988), but this represents only a small fraction of all TNF contained by stimulated cells (Lonnemann *et al.*, 1989). Virtually all types of nucleated cells can respond to TNF as they all have receptors. Two types of TNF receptor have been identified, a low and a higher molecular weight form, called p55 and p75, respectively. Some cells have both receptors, eg NK cells, while others have only one or the other (Vassalli, 1992). The interaction of TNF with its receptor puts in motion a cascade of biological responses, which can continue for up to 36 hours (Cerami, 1992). The half-life of TNF in the circulation is short, estimated to be 6 min (Beutler *et al.*, 1985) which prevents active diffusion and widescale cytotoxicity throughout the body.

TNF is made primarily by activated macrophages (Beutler and Cerami, 1989), although T lymphocytes and NK cells represent a large potential source of TNF. Other cell types, including mast cells, keratinocytes, astrocytes and smooth muscle cells, can also be stimulated to produce TNF (Vassalli, 1992).

Although monocytes and macrophages are a major source of TNF, they are also one of its main targets. TNF is important in the activation of macrophages, in synergy with IFN-γ as outlined in section 4.6, potentially resulting in further TNF release. In addition, TNF is chemotactic for monocytes and may serve to recruit monocytes from the blood compartment to amplify resistance against infectious agents (Ming *et al.*, 1987). Resting T cells do not have TNF receptors, and therefore will not respond to TNF. Receptors are induced upon primary activation, and on exposure to TNF these activated T cells increase their expression of IL-2 receptor. As a consequence, the cells show an enhanced proliferative response to IL-2 (Scheurich *et al.*, 1987). Costimulation of T cells with TNF and IL-2 leads to increased IFN-γ production by these cells. Similarly, in the presence of IL-2, TNF increases IL-2 receptor expression on NK cells and augments their cytolytic activity (Østensen *et al.*, 1987). Thus, TNF enhances the ability of T and NK cells to respond to IL-2. The endothelial cells are another major target of TNF, as shown by the multiple responses of these cells to TNF *in vitro*. These responses have been reviewed by Vassalli (1992), and include the release of growth factors and cytokines, enhanced blood clotting, increased cell adhesion molecules and class I MHC antigens.

TNF is not cytotoxic to most normal cells *in vitro*. In contrast, tumour cell lines can be either a) insensitive to TNF, b) rapidly killed by TNF, or c) prevented from growing by TNF. TNF is able to kill susceptible cells simply by binding to receptors. The probable mechanism is that this induces superoxides and exposes the cells to an oxidative stress. Cell survival depends on the efficiency of its protective mechanisms. Those tumour lines most sensitive to TNF are those with the lowest radical scavenging capacity (Zimmerman *et al.*, 1989).

TNF release can be induced by several cytokines, including TNF itself (Philip and Epstein, 1986). IL-2, GM-CSF and CSF-1 have been reported

to induce TNF release (Vassalli, 1992), while IFN-γ has a strong potentiating activity in many conditions involving TNF.

TNF mediates a large array of activities which are beneficial during a response to an infection. These include direct antiviral activity, and enhancement of responses by T and NK cells, and also macrophages. However, the production of TNF must be tightly regulated as high levels of TNF will induce wasting, fever and even death (Beutler and Cerami, 1986).

5.2.2 Interleukin 6

IL-6 is a pleiotropic cytokine that acts on a variety of cells. This is apparent when the history of IL-6 is considered. During the 1980's several research groups worked on cloning what seemed to be a series of unique molecules. Following the publication of the sequence data of these molecules, however, it was discovered that IFN-β_2 (an antiviral factor), BSF-2 (B-cell stimulatory factor 2), HPGF (hybridoma/ plasmacytoma growth factor), HSF (hepatocyte-stimulating factor) and CDF (cytotoxic T-cell differentiation factor) were all the same molecule (Van Snick, 1990), and it was designated IL-6.

IL-6 is produced by a large number of cells, including fibroblasts, endothelial cells, monocytes/macrophages, keratinocytes, T cell lines and mast cells (Van Snick, 1990). The major source of IL-6 in fresh cells is the monocyte population (Aarden *et al.*, 1987), while T and B cells produce IL-6 to a lesser degree (Horii *et al.*, 1988). IL-6 is not produced constitutively by normal cells, but its expression is readily induced by a number of factors, including viral infections (Sehgal *et al.*, 1988), LPS (Ulich *et al.*, 1991) and other cytokines such as TNF or TNF in combination with IFN-γ (Van Snick, 1990).

Macrophages, T cells and B cells have been found to express IL-6 receptors. IL-6 receptors are constitutively expressed on resting T cells and are down-regulated upon activation. In contrast, normal B cells only acquire IL-6 receptors upon activation (Taga *et al.*, 1987). It is not necessary for normal B cells to constitutively express IL-6 receptors as IL-6 acts at a late stage of B cell maturation. It has been shown that IL-6 induces the terminal differentiation of B cells into plasma cells. IL-6

acts late (4 days) in the response (Muraguchi *et al.*, 1988) to induce Ig production without further proliferation (Beagley *et al.*, 1989). IL-6 knockout mice (IL-6 -/-) have similar total serum immunoglobulin levels as their normal littermates. However, the mutants have impaired serum IgG and mucosal IgA antibody responses to virus infection (M. Kopf, A. Ramsay, personal communication). Antibody secretion by naive B cells is dependent on IL-6, whereas antigen-primed B cell responsiveness is essentially IL-6 independent (Hilbert *et al.*, 1989). Thus, T cell-derived IL-6 plays a critical role in regulation of B cell responses; it is essential for primary B cell differentiation into antibody-secreting cells and it enhances secretion by any antibody secreting cell.

Normal T cells constitutively express IL-6 receptors. The activation of these cells is controlled by IL-6, in synergy with IL-1 (Van Snick, 1990). IL-6 acts early, predominantly by enhancing IL-2 responsiveness, whereas IL-1 acts at the later stage of IL-2 production. The synergistic action between IL-1 and IL-6 is also seen in the induction of CTL responses in allogeneic mixed lymphocyte reactions (Renauld *et al.*, 1989). These cytokines act on the $CD8^+$ T cells capable of secreting IL-2. IL-6 appears to be an essential early accessory signal in the activation of fresh T-cells, and thus is an important factor in the regulation of an immune response.

IL-6 has been reported to down-regulate LPS-induced expression of TNF and IL-1, suggesting it may be part of a negative feedback mechanism in the cytokine cascade (Aderka, 1989). This could be described as an anti-inflammatory activity. IL-6 has a range of other functions, including induction of the acute phase response and inducing fever (Van Snick, 1990), which are beyond the scope of this review.

5.3 T cell-derived cytokines

Unlike the secretion of cytokines by macrophages, cytokine synthesis by T cells requires antigen specific and MHC-restricted stimulation of the T cell. The production of cytokines is tightly regulated, and upon removal of the stimulating antigen, cytokine synthesis ceases. T cell-derived cytokines have multiple biological activities on different target cells. The discussion below has been limited to those cytokines which have direct relevance to the work discussed in this thesis.

5.3.1 Interleukin 2

Interleukin 2 was first identified as a soluble mitogenic factor released by T lymphocytes with activity on T cells, and was called T cell growth factor (TCGF) (Gillis *et al.*, 1978). At the Second International Lymphokine Workshop, in 1979, TCGF was designated IL-2 (Smith, 1988). This was to clarify the terminology, and to distinguish it from a macrophage-derived factor also with mitogenic activity, which had been known as lymphocyte activating factor (LAF) (Gery *et al.*, 1972), and was designated IL-1.

T cells are the principal producers of IL-2 (Smith, 1988). T cells also respond to IL-2 and, during the response, IL-2 is consumed by the T cell. T cells express IL-2 receptors which remove or "consume" IL-2 by internalization of the receptor/IL-2 complex. High-affinity IL-2 receptors consist of two polypeptide chains, the 75 kDa chain (α) and the 55 kDa chain (β), each of which contains a distinct binding site. When expressed individually the chains react with IL-2 quite differently, and bind with distinct affinities. Co-operation of the two chains leads to formation of a heterodimeric receptor with an increased affinity for IL-2.

IL-2 activates a wide range of cell types, including monocytes (Holter *et al.*, 1987), B cells (Mingari *et al.*, 1985), NK cells (Handa *et al.*, 1983), as well as T cells (Morgan *et al.*, 1976). IL-2 also has a stimulatory action on IFN-γ secretion by NK cells (Handa*et al.*, 1983) and CTL (Farrar *et al.*, 1981).

It is the action of IL-2 on T cells which is pivotal for the induction of an immune response (Smith, 1988). Resting T cells do not produce IL-2,

nor do they respond to IL-2. Upon receiving the signal from T cell antigen-receptor complex, these cells begin transcription of both the IL-2 gene and the IL-2 receptor genes. For 3 days the levels of IL-2 receptor on T cells increase, and then decline progressively, proliferation ceases and by 12 - 14 days the cells are back in the resting phase (Smith, 1988). IL-2 receptor expression is dependent on the presence of antigen (Cantrell and Smith, 1983), and thus, as antigen is cleared *in vivo* the receptor expression is reduced, the T cells are no longer able to respond to IL-2, and the T cell dependent immune response subsides.

5.3.2 Interferon-γ

The production of interferon-γ (immune interferon) is a function of activated $CD8^+$ T cells, Th1 type $CD4^+$ T cells and NK cells. Human IFN-γ is 17 kDa and the usual biological form is as a dimer (Ealick *et al.*, 1991). IFN receptors (10^3 to 10^4 per cell) exist on most cells and, following binding of the IFN-γ dimer, a series of signals leads to the synthesis of many new mRNAs and up-regulation of others (Borden, 1992).

IFN-γ was initially characterized by its ability to render homologous cells resistant to virus infection, a property it shares with IFN-α and IFN-β (Isaacs and Lindenmann, 1957). IFN-γ also exerts a number of other effects, including various immunomodulatory activities. For example, IFN-γ is a powerful inducer of MHC antigens, is essential in the activation of macrophages and NK cells, and has an important role in antibody production (Dijkmans and Billiau, 1988).

IFN-γ enhances the expression of both class I and class II MHC antigens in a large number of cell types and is thus of prime importance in adaptive cellular immune responses. Increased class I expression could improve recognition of targets by CTL, while up-regulation of class II MHC expression by IFN-γ may enhance the antigen-presenting function of macrophages. IFN-γ activates a number of other macrophage functions, including cytotoxicity against tumours, induction of reactive oxygen intermediates and RNIs, and production of TNF and IL-1. Macrophages are also stimulated to mediate antibody-dependent cell mediated cytotoxicity (ADCC) by enhancing the expression of IgG-Fc receptors (Perussia *et al.*, 1983). NK cell killing

activity is enhanced by IFN-γ (Weigent *et al.*, 1983). Exposure of activated B cells to IFN-γ stimulates the secretion of IgG2a (Snapper and Paul, 1987) and a decrease in secretion of other isotypes. IgG2a is the predominant antibody response to viral infections (Coutelier *et al.*, 1987), is the major opsonising isotype in the mouse, and is the preferred ligand for the macrophage IgG-Fc receptor (Coffman *et al.*, 1988).

IFN-γ has antiviral activity *in vitro* on a large number of cell types including fibroblasts, macrophages and some lymphoid cells (Stewart, 1979). The *in vivo* antiviral role of IFN-γ has been demonstrated by blocking IFN-γ activity with administration of mAb (Leist *et al.*, 1989). This method has been improved upon in the last year with the generation of IFN-γ knockout mice (Barinaga, 1993). Studies have revealed that although these mice made a normal cytotoxic T cell response, IFN-γ was essential for clearance of vaccinia virus. IFN-γ was also shown to be needed for production of nitric oxide by macrophages. These mice will help provide answers to many basic questions about the role of IFN-γ in the immune system.

IFN-γ is able to modulate expression of other cytokines. It increases TNF-α production by activating macrophages and enhances their TNF receptor expression (Ruggiero *et al.*, 1986; Tsujimoto *et al.*, 1986). IFN-γ is a Th1-type cytokine and inhibits the proliferation of Th2 clones (Gajewski and Fitch, 1988). This leads to a down-regulation of Th2-type cytokines, eg. IL-4, IL-5, and IL-10. Finally, IFN-γ up-regulates its own gene expression (Hardy and Sawada, 1989), which may serve to amplify the amount of IFN-γ expressed at the site of inflammation. At this stage it is not known whether this represents autocrine superinduction of IFN-γ in T cells, or whether an initial source of IFN-γ induces greater expression by NK cells (Cockfield *et al.*, 1993).

The main function of IFN-γ was thought to be its antiviral activity. However, as described above, IFN-γ is an important immune modulator, acting on T and B cells, NK cells, and macrophages.

5.3.3 Lymphotoxin

Lymphotoxin (LT), also known as TNF-β, belongs to the same gene family as TNF-α, binds to the same cell surface receptors, yet has only limited sequence homology (34%) (Ruddle, 1992). LT has generally been thought to be produced by T cells while TNF-α was a macrophage product. Recent studies, however, have shown both TNF-α and LT can be produced by T cell populations (English *et al.*, 1991). They appear to be under different regulation, as shown by different kinetics in mRNA accumulation. TNF-α has a higher baseline transcription rate, and short (30 min) mRNA half life, whereas LT has a lower baseline rate and a mRNA half life of 5.5 hours. TNF-α and LT have structural similarities, as both form trimer molecules (Schoenfeld *et al.*, 1991) which are able to bind to both TNF receptors. As LT binds to the same receptors, it is believed to induce similar responses as TNF-α. There is strong evidence that LT is a mediator of CTL killing and it may play a role in defence against virus infection. LT production is induced by virus infection and LT kills virus infected cells more efficiently than normal cells (Paul and Ruddle, 1988).

5.3.4 Interleukin-10

As outlined in an earlier section, $CD4^+$ T cells can be divided into different subsets that are distinguished by their cytokine production profiles. Th1 clones produce IL-2 and IFN-γ, whereas Th2 clones produce IL-4 and IL-5 (Mosmann *et al.*, 1986). Th1 cells are effective in the generation of a DTH response, and Th2 cells are more efficient in mediating help for antibody synthesis. These two responses are often mutually exclusive (Parish, 1972), suggesting there is a cross-regulatory mechanism involved. This was supported by evidence showing that IFN-γ inhibits proliferation of Th2 cells (Gajewski and Fitch, 1988). With the expectation of there being a similar Th2 product which would inhibit proliferation and effector function of Th1 cells, the cytokine synthesis inhibitory factor (CSIF) was discovered (Fiorentino *et al.*, 1989). CSIF inhibited synthesis of IFN-γ and other cytokines produced by Th1 clones. This was then cloned and, as it was thought to be pleiotropic, was designated IL-10 (Moore *et al.*, 1990). Both mouse (m)-IL-10 and human (h)-IL-10 have been cloned and their primary structures determined (Moore *et al.*, 1993). They show a high degree of

homology and, interestingly, have strong homology with an open reading frame in the Epstein-Barr (EBV) genome, BCRFI (Moore *et al.*, 1990). It has been proposed that BCRFI may represent a cellular cytokine gene captured by the EBV during evolution. BCRFI displays some of the biological activities of IL-10 and has been designated viral IL-10 (v-IL-10) (Moore *et al.*, 1993).

IL-10 is not strictly a Th2-specific cytokine. Mouse IL-10 is produced by Th2 cells (Fiorentino *et al.*, 1989), macrophages (Fiorentino *et al.*, 1991b), Ly.1 B cells and keratinocytes (Moore *et al.*, 1993). In comparison to other T cell derived cytokines, IL-10 is produced relatively late following activation, which may be an important aspect of IL-10's ability to inhibit macrophage and T cell activation. The maximal production of IL-10 protein by $CD4^+$ T cell clones was observed 24 hours after activation (Yssel *et al.*, 1992).

The inhibitory effect of m-IL-10, produced by Th2 cells, on Th1 cell cytokine synthesis is indirect. Antigen-specific Th1 cytokine synthesis was inhibited when macrophages, but not B cells, were used as APC (Fiorentino *et al.*, 1991a). The inhibitory effects were most pronounced for IFN-γ and IL-3, which are produced relatively late after activation. Levels of IL-2, GM-CSF and LT, which are produced at an early stage, are only slightly affected (Moore *et al.*, 1993). IL-10 also inhibits proliferation of T cells in the presence of monocyte/macrophage APC. This indicates that IL-10 is exerting its effect at the T cell activation stage. Although the mechanism has not been determined, the current view is that IL-10 inhibits the production or function of a membrane-bound costimulator required for T and NK cell activation. Studies are now addressing the possibility that IL-10 interferes with B7 function or expression on macrophages (Moore *et al.*, 1993).

Production of monokines is strongly inhibited by IL-10. Mouse IL-10 inhibits production of IL-6, IL-1 and TNF-α by LPS or IFN-γ stimulated macrophages (Fiorentino *et al.*, 1991b). Blocking studies with antibodies revealed that endogenous IL-10 production by macrophages suppresses IL-6 production. Mouse IL-10 also inhibited production of reactive nitrogen intermediates (NO) by macrophages following activation by IFN-γ (Gazzinelli *et al.*, 1992).

In contrast to these immunosuppressive activities, IL-10 has been shown to costimulate a strong proliferative response of most thymocyte subsets in the presence of IL-2/IL-4 (Moore *et al.*, 1993). In addition to promoting proliferation, IL-10 augmented the differentiation of cytotoxic T-cell precursors into antigen-specific $CD8^+$ cytotoxic T cells (Chen and Zlotnik, 1991).

IL-10 plays an important role in the regulation of the immune response. It is a powerful immunosuppressive factor, able to inhibit antigen presentation by macrophages and thus prevent antigen-specific T cell proliferation and cytokine production. On the other hand, it has an enhancing effect on T cell proliferation, and acts as a cytotoxic differentiation factor. It also acts directly on the macrophage to inhibit monokine and NO release, thus it has strong anti-inflammatory activities.

5.3.5 Interleukin-4

IL-4 was originally described by its ability to enhance DNA synthesis by purified resting mouse B cells stimulated with anti-IgM antibodies (Howard *et al.*, 1982). For this reason it was designated B-cell growth factor (BCGF). It was later designated IL-4 when it was realised that the molecule was pleiotropic in its action, with non-B cells being important targets (Noma *et al.*, 1986).

Murine IL-4 is a 19 kDa glycoprotein produced by Th2 cell clones (Mosmann *et al.*, 1986), Th0 cell clones (Firestein *et al.*, 1989) and mast cells (Plaut *et al.*, 1989). Resting T cells isolated from lymph nodes of normal mice produce very little IL-4 in response to stimulation with mitogen (Swain *et al.*, 1988), but the IL-4 producing capacity can be markedly enhanced by *in vitro* culture of these cells in the presence of IL-2 and IL-4 (Le Gros *et al.*, 1990). Both $CD4^+$ and $CD8^+$ T cells produce IL-4 following this priming (Seder *et al.*, 1992). These studies indicate that IL-4 producing capacity is mainly found in the activated T cell population.

Receptors for IL-4 have been reported to exist on freshly prepared B and T lymphocytes, macrophages and a variety of nonhaematopoietic cells (Lowenthal *et al.*, 1988; Ohara and Paul, 1987), and are increased upon activation of the cell (Ohara and Paul, 1988). IL-4 itself is able to

increase IL-4 receptor expression on resting T and B cells (Renz *et al.*, 1991). The ability of IL-4 to regulate receptor expression provides an important mechanism for amplification of IL-4-dependent activation pathways.

IL-4 was first recognized for its ability to enhance B cell growth in response to anti-IgM antibody (Howard *et al.*, 1982). It also has a number of effects on resting B cells, including induction of an increase in MHC class II molecule expression (Noelle *et al.*, 1984) which will improve their antigen presenting ability. IL-4 plays an important role in immunoglobulin isotype regulation, as it stimulates secretion of IgG1 and IgE and suppresses secretion of other Ig isotypes (Snapper *et al.*, 1988). A recent study has demonstrated that IL-4 induces motile morphology and migration of murine B cells, indicating that IL-4 may play a role as a chemoattractant factor that enhances cell contact between $CD4^+$ T cells and B cells (Clinchy *et al.*, 1991).

IL-4 acts as an autocrine growth factor for long-term Th2 cell clones (Kurt-Jones *et al.*, 1987). Th2 cells proliferate in response to IL-2 and IL-4, but this proliferation is dependent on IL-1 as an accessory signal. Normal resting T cells proliferate in response to PMA plus IL-4, but not IL-4 alone (Hu-Li *et al.*, 1987). The IL-4 must be present early in the T cell response, for the resting cells to become sensitive to the growth promoting effects of IL-4, but also is needed late in culture to cause the cells to enter S-phase (Paul and Ohara, 1987). IL-4 is able to suppress T cell subsets involved in a cell-mediated (Th1-type) response. A recent study has demonstrated this regulation may be through down-regulation of IL-2 production, necessary for Th1 proliferation, by inhibiting factors required for transcription of the IL-2 gene (Schwarz *et al.*, 1993).

IL-4 is a potent murine macrophage activation factor. Macrophages treated with IL-4 increased expression of class-II MHC molecules and exhibited increased cytotoxicity against tumour cells (Crawford *et al.*, 1987). IL-4 is also chemotactic for mouse macrophages, whereas IL-2 is not (Hiester *et al.*, 1992). Conversely, human IL-4 has also been shown to modulate macrophage behaviour by down-regulating expression of IL-1 and TNF genes (Essner *et al.*, 1989; Hart *et al.*, 1989). This indicates that IL-4 not only has stimulatory activity on monocytes/ macrophages,

it may also be an anti-inflammatory agent. Thus, IL-4 is yet another cytokine with demonstrated stimulatory and suppressive activity.

6 Th1/Th2 responses in infectious diseases.

There is an increasing volume of evidence suggesting that the failure to control or resolve infectious diseases often results from inappropriate rather than insufficient immune responses (Powrie and Coffman, 1993). There are a number of host-parasite models where T cell derived cytokines may influence the course of infection by down-regulating the host cell-mediated immune response. These cytokines, IL-4 and IL-10, are produced by $CD4^+$ T cells belonging to the Th2 subset. IL-4 inhibits the development of Th1 $CD4^+$ T cells (Swain *et al.*, 1990b), whereas IL-10 suppresses the expression of IL-2 and IFN-γ (Fiorentino *et al.*, 1989).

The protozoa *Trypanosoma cruzi* and *Toxoplasma gondii* invade a large number of different cell types. Cell-mediated immunity plays a major role in resistance to infection, with IFN-γ implicated as a protective cytokine against both pathogens (Silva *et al.*, 1991; Suzuki *et al.*, 1988). The control of these protozoa by activated macrophages can, however, be down-regulated by the cytokines IL-4 and IL-10 (Sher *et al.*, 1992). Helminth infections induce elevated serum IgE and Th2 cytokine levels (Bazin, 1974; Scott *et al.*, 1989). This increase is usually coincident with a down-regulation in Th1 responses (Sher *et al.*, 1992). IL-10 plays a role in the suppression of Th1-type cytokine synthesis, as the addition of neutralizing mAb against IL-10 to spleen cultures up-regulates the suppressed IFN-γ responses to parasite antigen in infected mice (Sher *et al.*, 1991). Activated macrophages are thought to be important effector cells of protective immunity against schistosomes in mice (Sher *et al.*, 1992). Thus IL-10, which is produced during schistosome infection, and is able to suppress many macrophage functions (Fiorentino *et al.*, 1991b), will have a profound effect on the immune response to this infection.

The pattern of mutually exclusive Th1 and Th2 clones, which could be isolated from mice, was not apparent among human T-cell clones for several years. However, as first described by Romagnani (1991), T-lymphocyte clones can be isolated from patients with infections, or

from immunized individuals which display the Th1 or Th2 cytokine profiles. The importance of generation of an appropriate response has been demonstrated in the response to *Mycobacterium leprae.* This disease presents either as tuberculoid (resistant) or lepromatous (susceptible) lesions. Yamamura *et al.* (1991) have shown that mRNA for IL-2 and IFN-γ, ie. Th-1 type cytokines, were expressed in tuberculoid lesions, whereas mRNA for IL-4, IL-5 and IL-10 were characteristic of lepromatous lesions.

The production of an appropriate response to infection with the human immunodeficiency virus (HIV) may also be regulated by cytokines. Individuals infected with HIV have a severe depletion of $CD4^+$ T cells as the infection progresses towards acquired immunodeficiency syndrome (AIDS). Loss of T helper function has been observed prior to the decrease of $CD4^+$ T cell numbers (Clerici *et al.*, 1989). The independence between loss of Th function and $CD4^+$ T cell count indicates that factors other than $CD4^+$ depletion contribute to T cell dysfunction. These factors might include cytokines, and it has been suggested that the Th1- and Th2-type cytokines play a role in the progression of HIV infection to AIDS (Sher *et al.*, 1992). The model proposed is that HIV-specific T cell responses that result in the production of Th1-type cytokines prevents infection, and/or keeps the infection under control. In contrast, HIV seroconversion could be correlated with a transition to a Th2-type response, with increased HIV-specific antibodies. This latter state would be more susceptible to the progression to AIDS (Clerici and Shearer, 1993). This model is consistent with the hypothesis where any immunological stimulus that induced a strong Th2 response would render the individual more susceptible to HIV infection and/or progression to AIDS. This is of particular relevance to the AIDS epidemic in developing regions of the world where helminth infections, which are known to generate Th2 responses, are common (Actor *et al.*, 1993).

7 Scope of this thesis

As has been discussed, an immune response to an infection involves the participation of a large number of distinct cell types. The functions of these cells must be co-ordinated to ensure that the response is not only appropriate, but also of the right magnitude, to the eliciting antigen. The co-ordination is regulated by cytokines secreted by T lymphocytes.

The host response to a virus infection involves the co-ordinated response of macrophages, B cells, NK cells and T cells. Cytokines have been shown to be important in this response, not only as regulatory molecules, but also as direct antiviral effector molecules. The use of recombinant vaccinia virus encoding cytokine genes has revealed that cytokines can indeed influence the outcome of a virus infection. In order to have some further understanding of the role of cytokines in the control of a virus infection, an *in vitro* model was established to analyse cytokine production during the response to restimulation with vaccinia virus, with a focus on the antiviral cytokines.

Chapter 3 establishes the conditions for restimulation of vaccinia virus immune cells and their production of antiviral cytokines. Primed spleen cells could be restimulated with virus infected syngeneic cells to synthesize TNF, IFN-γ and IL-6. Production of TNF and IL-6 was very rapid, occurring within several hours of restimulation, whereas IFN-γ production was delayed by 4 h.

Chapter 4 examines the cells responsible for the cytokine production, using cultures depleted of specific cell phenotypes. The majority of the TNF and IL-6 was produced by the adherent cell population, probably the macrophages. IFN-γ production was dependent on the $CD4^+$ T cells. An interesting finding was the $CD4^+$ T cells had a negative influence on TNF production. TNF and IL-6 production was found to be non-antigen specific, whereas IFN-γ production required restimulation with the immunizing antigen.

Chapter 5 examines the interaction of endogenous cytokines in the production of antiviral cytokines by the immune cells. Antibodies, or an exogenous source of cytokine, were added to the immune cells 2 h prior to restimulation or at the onset of culture. TNF, IL-2 and IL-4

have a role in IFN-γ production, whereas the production of TNF and IL-6 is not significantly influenced by other cytokines.

The effect of stimulation with recombinant vaccinia virus encoding cytokine genes is examined in Chapter 6. A virus encoding IFN-γ stimulated enhanced production of IFN-γ by the responder cells, while stimulation with a TNF-encoding virus resulted in increased levels of both TNF and IFN-γ. Viruses encoding IL-2, IL-4 or IL-10 had no effect on TNF or IL-6 levels, however, these viruses stimulated enhanced IFN-γ production.

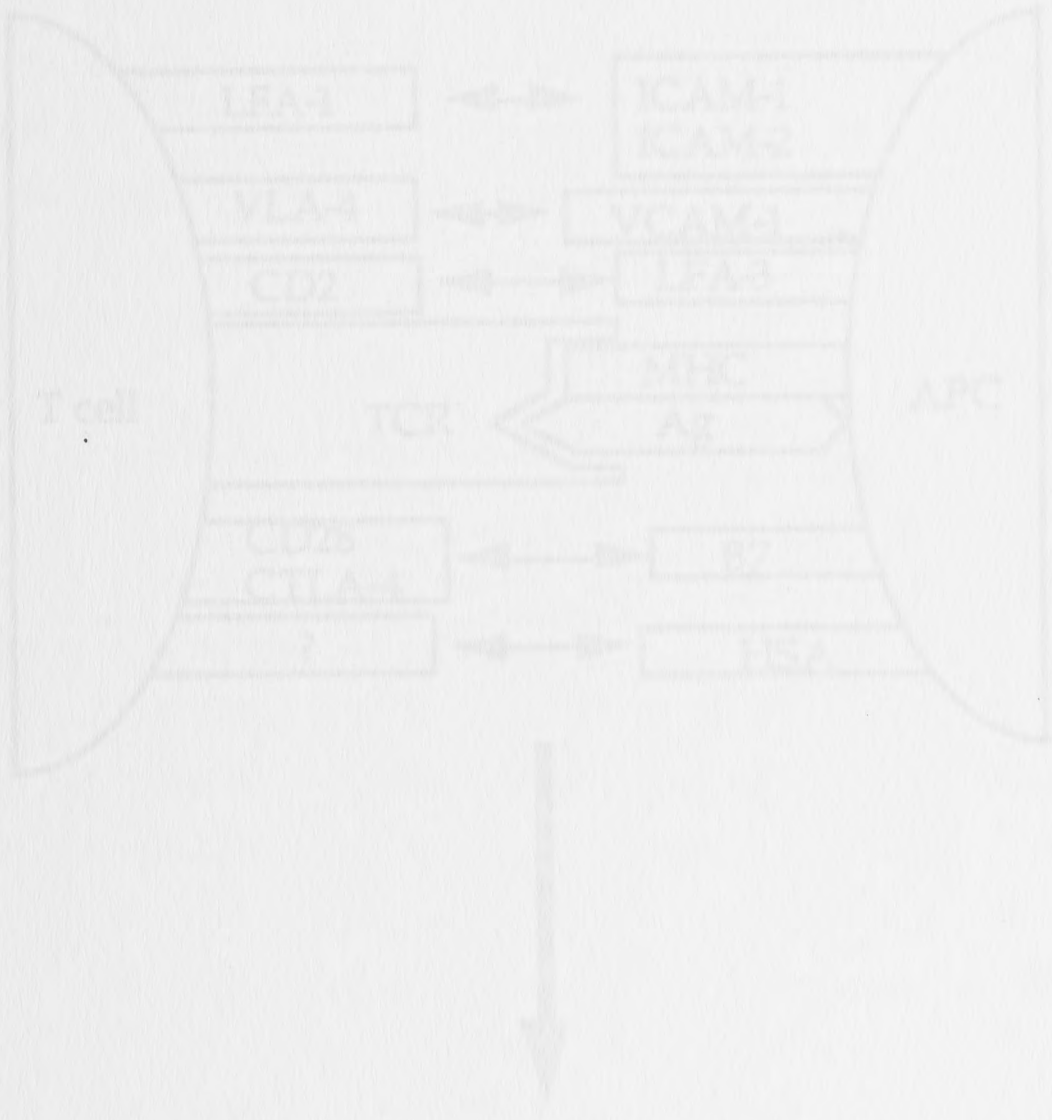


Figure 1.1. Costimulatory signals determine the outcome of T-cell-receptor (TCR) occupancy

From a review by Liu & Linsley (1992)

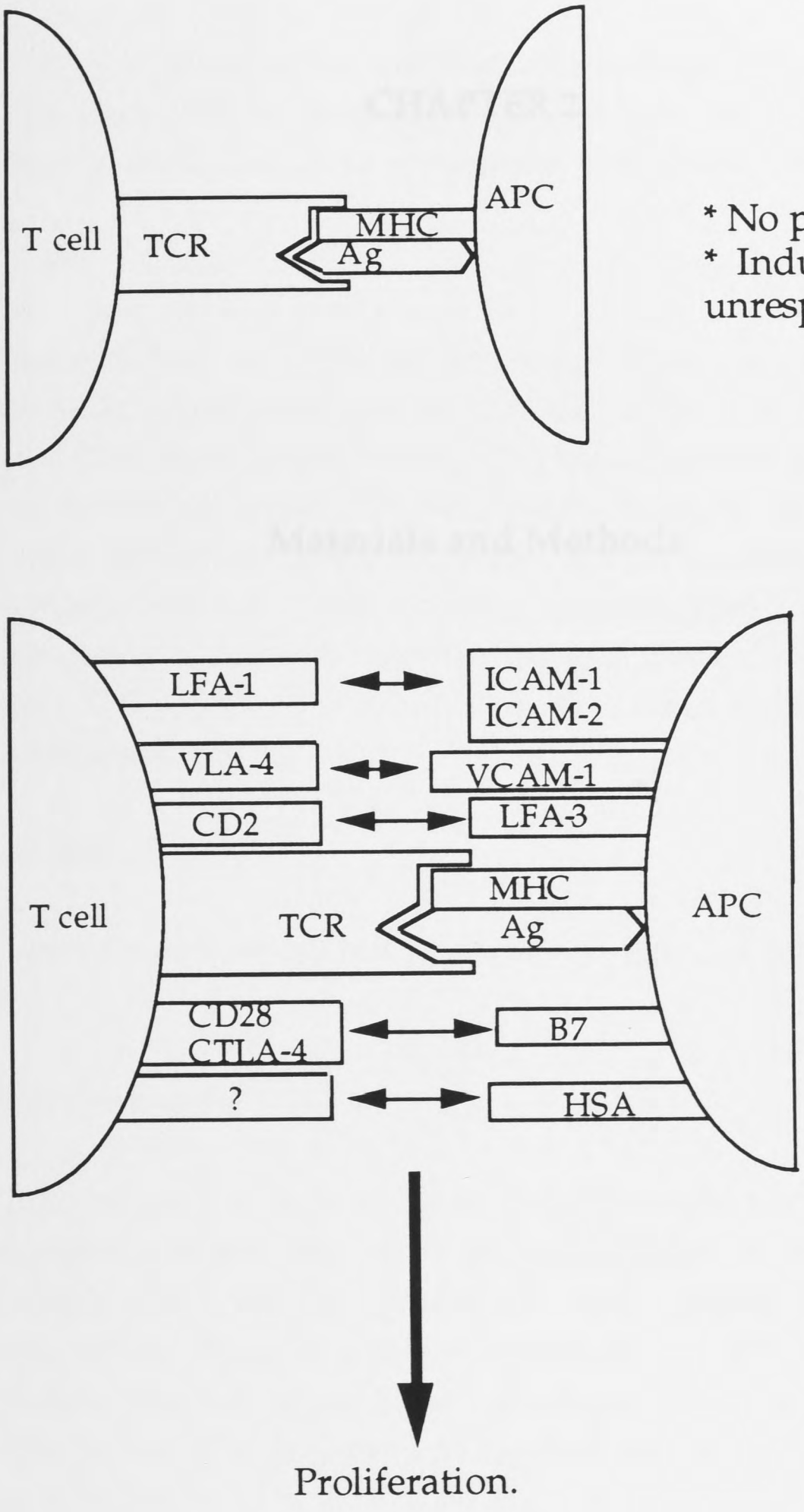


Figure 1.1 *Costimulatory signals determine the outcome of T-cell-receptor (TCR) occupancy*

From a review by Liu & Linsley (1992)

CHAPTER 2

Materials and Methods

2.1 Mice

CBA/H (H-2^k) mice raised under specific pathogen free conditions at the John Curtin School of Medical Research were used at 6 to 7 weeks. Female mice were used in all experiments unless otherwise stated.

2.2 Virus

The neurovirulent, L929 cell-adapted strain of vaccinia virus, VV-WR, was used in all experiments (Wokatsch, 1972). Virus stocks were prepared from infected CV-1 cells (0.01 pfu/cell) grown in 2 litre Schott acid-washed roller bottles. The stocks were stored in 100 µl aliquots at -70^{o}C and titrated on 143B cells. Before use for injection, virus stocks were diluted in 1 ml of gelatin saline and sonicated for 5 seconds at 50 watts using a Branson Sonifier (Branson Sonic Power Company, Danbury, CT. USA). The virus was then made up to the desired concentration in gelatin saline.

2.3 Infection of mice

Mice were infected with 2.5 x 10^6pfu of VV-WR in a volume of 0.2 ml i.v.

2.4 Culture medium

The predominant culture medium used was Dulbecco's Modified Eagle's with supplements. H16 refers to DMEM (GIBCO BRL, Life Technologies, MD, USA) supplemented with L-glutamine (216 mg/l), 10 mM HEPES buffer, 2-mercaptoethanol ($5x10^{-5}$ M), 10% heat-inactivated foetal calf serum (Flow Laboratories North Ryde, Australia), penicillin (30 mg/l), streptomycin sulphate (50 mg/l), and neomycin sulphate (50 mg/l).

The enriched medium, referred to as Mixed Lymphocyte Medium (MLC), is H16 supplemented with glucose (4 g/l), folic acid (6 mg/ml), L-asparagine (36 mg/l), L-arginine (116 mg/l) and 1 mM sodium pyruvate.

F15 consists of Eagles Minimum Essential Medium (Cytosystems, Castle Hill, NSW, Australia) supplemented with sodium bicarbonate (2.2 g/l),

L-glutamine (216 mg/l) and 5% heat-inactivated foetal calf serum, penicillin (30 mg/l), streptomycin sulphate (50 mg/l), and neomycin sulphate (50 mg/l) and 2-mercaptoethanol ($5x10^{-5}$ M).

2.5 Virus titration

100 µl of the sample to be titrated, in gelatin saline, was incubated with an equal volume of trypsin (1 mg/ml) for 30 min at 37°C. The trypsin was neutralized by adding 800 µl F15 medium containing 10% FCS. Serial ten-fold dilutions were then made in gelatin-saline-HEPES (0.02M HEPES) and the virus was titrated as plaques on 143B cell monolayers in 6 well Linbro plates (Flow Laboratories, Virg. USA). The virus was adsorbed for one hour before the cell monolayers were overlaid with F15 medium. Following incubation for 2 days at 37°C in a humidified atmosphere of 5% CO_2, the monolayers were fixed and stained with 0.1% crystal violet in 20% ethanol for 5 min, dried and the plaques counted.

2.6 Cell lines

CV-1, a cell line derived from African green monkey kidney (Jensen *et al.*, 1964), 143B, a human osteosarcoma cell line (Rhim *et al.*, 1985) and L929, a continuous fibroblast line from C3H mouse (Sanford *et al.*, 1948) were maintained in F15 and incubated at 37°C in a humidified atmosphere of 5% CO_2.

The IFN-γ sensitive cell line WEHI 279, a B cell lymphoma (Reynolds *et al.*, 1987) and the IL-6 dependent cell line, B9, a B-cell hybridoma cell line, (Aarden *et al.*, 1987), obtained from L.A. Aarden (University of Amsterdam, Amsterdam, The Netherlands) were maintained in MLC. The B9 cell medium was supplemented with 10% of a Con A supernatant source of IL-6. The TNF sensitive cell line, WEHI 164, a murine fibrosarcoma line (Espevik and Nissen-Meyer, 1986), was maintained in H16 medium.

The Con A supernatant source of IL-6 was generated by stimulating mouse spleen cells with Con A. Single cell suspensions were made from spleens aseptically removed from 15 CBA/H mice. After washing, these cells were resuspended in 200 ml complete H16 supplemented with 1% FCS and 5 µg/ml Con A (Pharmacia, Uppsala,

Sweden). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 3 days. The supernatant was removed and mixed with α-methyl-D-mannoside (2 g/100 ml; Sigma, St Louis, MO, USA) to bind to excess Con A. This was collected by centrifugation at 11,000 g for 10 min at 4°C. The supernatant was sterilized by filtration through a 0.22 μm membrane and frozen at -70°C.

2.7 Preparation of responder cells

Spleens were removed aseptically from mice infected with VV-WR 6 days earlier and pooled. Single cell suspensions were obtained by pressing through stainless-steel mesh into MLC medium. The resultant cell suspension was transferred to a sterile tube and allowed to settle for 5 min to remove clumps. The supernatant was transferred to a fresh sterile centrifuge tube, centrifuged at 300 g for 5 min and the medium decanted. Erythrocytes were lysed by hypotonic shock, in which 4.5 ml sterile water was added to the tube and mixed, quickly followed by 0.5 ml 10 x Hanks buffer and 5 ml medium. The remaining cells were washed and made up to a concentration of 2×10^7/ml in MLC medium.

2.8 Preparation of stimulator cells

Single cell suspensions were made from spleens removed from naive syngeneic mice. The erythrocytes were removed from suspension by hypotonic shock and washed. The cells were infected with VV-WR at 10 pfu/cell at a concentration of 5×10^7 cells/ml, at 37°C for 1 h. The cells were washed three times in culture medium, made up to 10 ml and placed in a 65 cm^2 plastic Petri dish and subjected to 5 min UV irradiation at an intensity of 600 μW/cm^2 in the 230 - 270 nm range. Virus titrations of a sample before and after UV-irradiation are shown in Table 2.1. This level of UV-irradiation completely destroyed virus activity. The cells were then made up to a concentration of 4×10^6 cells/ml in MLC medium.

Table 2.1 *Effect of UV-irradiation onvirus activity*

UV-irradiation	VV-WR titration (pfu/ml)
None	2.2×10^6
5 min	< 100

2.9 Cell culture

Cultures were set up in 1 ml volumes in 24-well plates (Linbro, Flow Laboratories, Virg, USA). 10^7 immune responder cells were mixed with 2×10^6 stimulator cells and incubated at 37°C in a humidified atmosphere of 5% CO_2. Sufficient wells of each culture condition were set up to allow supernatant from one well to be removed at each of the eight time points over the next 48 hours. These were frozen in aliquots at -70°C until assayed for cytokine levels. The set up has been presented schematically in Figure 2.1. As a positive control, 5 µg/ml Con A was added to wells containing 10^7 immune responder cells in the absence of stimulator cells.

2.10 Recombinant cytokine standards

Recombinant standard murine TNF-α and IFN-γ were kindly provided by Boehringer Ingelheim, Vienna, Austria; TNF specific activity was 1.2×10^7 U/mg, IFN-γ specific activity was 1×10^7 U/mg. Recombinant standard murine IL-6, prepared by F. Lee (DNAX) was provided by Dr. P. Hodgkin (John Curtin School of Medical Research). The r-Mu-IL-6 preparation was calibrated against the International Standard reference preparation of recombinant human interleukin-6 (88/514), (National Institute of Biological Standards and Control, Herts, U.K.) and therefore all titres have been given in arbitrary units (AU).

2.11 Antibodies

Polyclonal antisera to recombinant murine TNF-α, or recombinant murine IFN-γ, were produced for use in cytokine ELISAs. NZ white rabbits were immunized with 40 µg r-Mu-TNF–α or r-Mu-IFN-γ emulsified in Complete Freunds Adjuvant (CFA; DIFCO, Mich. USA) supplemented with 4 mg/ml of diced *Mycobacterium tuberculosis*, (H37RA; DIFCO) which was freshly prepared. The emulsion was injected subcutaneously over 3 sites on the back of each rabbit and they were boosted 5-12 weeks later with a subcutaneous injection of recombinant TNF-α or IFN-γ in Incomplete-FA (DIFCO). The sera was collected between 6 to 8 days after the booster injection and tested by ELISA for levels of antibody. The antisera were then IgG precipitated by adding 2 volumes of 27% sodium sulphate while stirring vigorously, followed by an incubation for 2 - 4 h at 37°C. The precipitated antibodies were collected by centrifugation at 10,000 g, and resuspended in distilled water. The antibodies were extensively dialysed against PBS, titrated for use in ELISAs and stored at -20°C.

The hamster anti-mouse TNF mAb, TN3-19.12 (Sheehan *et al.*, 1989) was provided by Dr R. Schreiber (Celltech, UK) and the rat anti-mouse IFN-γ, R4.6A2 hybridoma cell ascites (Spitalny and Havell, 1984; American Type Culture Collection, Rockville, MD), protein A purified, was provided by Dr J. Ruby (John Curtin School of Medical Research).

2.12 Cytokine Assays

2.12.1 TNF Bioassay

TNF was assayed for its cytotoxic effect on WEHI 164 cells (Espevik and Nissen-Meyer, 1986). Assays were prepared in flat bottomed 96 well trays (Nunc, Roskilde, Denmark). 50 µl samples of culture supernatant were serially diluted in complete H16 medium. All titrations were carried out in duplicate. Each well received 50 µl of WEHI 164 suspension, at a concentration of 4×10^5/ml in H16 medium containing 1 µg/ml actinomycin D (Sigma). Control cultures consisted of wells containing medium and WEHI 164 cells alone. The cultures were incubated at 37°C in a humidified atmosphere for 18 to 20 h. 10 µl of 5 mg/ml MTT in PBS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-

tetrazolium bromide; Sigma) was added to each well and plates were incubated for a further 6 h. The crystals were dissolved by adding 150 µl 10% SDS in 0.01 M HCl and incubating overnight. Optical density was measured using a Dynatech Microplate reader at 570 nm with a reference wavelength of 630 nm. TNF titres were determined by comparison to r-MuTNF. Figure 2.2 shows a typical dose response curve obtained from plotting OD against $\log_{10}$ dilution of a sample containing TNF. The resultant curve is sigmoid in shape but approximates a straight line over its central region. The titre of the sample was defined as the reciprocal of the dilution resulting in 50% survival of the TNF sensitive cells. This was converted to pg/ml by comparison with a 50% survival titre of a standard TNF sample of known concentration.

2.12.2 IL-6 Bioassay

IL-6 was measured using the IL-6-dependent cell line, B9 (Aarden *et al.*, 1987). A 50 µl sample of culture supernatant was serially diluted and cultured for 44 h with 10^4 washed B9 cells in a total volume of 100 µl in MLC medium. Control cultures consisted of wells containing medium and B9 cells alone. Proliferation was quantitated using [^{3}H]-thymidine incorporation (78 Ci/mmole; ICN Radiochemicals, CA, USA) which was estimated with the use of a 96 well automatic harvester and a betaplate liquid scintillation counter (Pharmacia). IL-6 titres were determined by comparison of the dilution giving half maximal proliferation of the B9 cells with that of a concurrently run recombinant standard murine IL-6. The method of analysis is similar to that described for TNF.

2.12.3 IFN-γ Bioassay

IFN-γ was measured using the IFN-γ-sensitive cell line, WEHI 279 (Reynolds *et al.*, 1987). A 50 µl sample of culture supernatant was serially diluted in MLC medium and cultured for 3 days with 10^4 washed WEHI 279 cells in a total volume of 100 µl. Control cultures consisted of wells containing medium and WEHI 279 cells alone. 10 µl of 5 mg/ml MTT was added to each well and cultures were incubated for a further 6 h. The crystals were dissolved by adding 150 µl 10% SDS in 0.01 M HCl and incubating overnight. Optical density was measured using a Dynatech Microplate reader at 570 nm with a reference

wavelength of 630 nm. IFN-γ titres were determined by comparison to r-MuIFN-γ as described for the TNF bioassay.

2.12.4 TNF-α and IFN-γ ELISA

TNF-α and IFN-γ in culture supernatants were measured by indirect ELISA methods (Sheehan *et al.*, 1989). Briefly, 50 µl of 10 µg/ml hamster anti-mouse TNF mAb, TN3-19.12 (Sheehan *et al.*, 1989) or 50 µl of 3 µg/ml R4.6A2 hybridoma cell ascites (ATCC), protein A purified, diluted in carbonate-bicarbonate coating buffer (pH 9.6) was incubated at 4°C overnight in 96 well, flat bottomed ELISA plates (Titertek, Flow Laboratories, The Netherlands). The plates were washed 6 times in phosphate-buffered saline (PBS)-Tween (0.01 M PBS, 0.1% Tween (Sigma)). The wells were blocked with 100 µl 3% BSA (Sigma) in PBS-Tween for 2 h at room temperature. After washing with PBS-Tween 6 times, 50 µl of sample was added to each well. Dilutions of r-MuTNF or r-MuIFN-γ were used to generate a standard curve. The plates were then incubated overnight at 4°C. The plates were washed 6 times and 50 µl of polyclonal rabbit anti-mouse TNF-α antibody diluted 1/500, or 50 µl of polyclonal rabbit anti-mouse IFN-γ antibody diluted 1/250 in 1% BSA, was added and incubated for 2 h for TNF-α and 1 h for IFN-γ. After washing, 50 µl of sheep anti-rabbit IgG alkaline phosphatase conjugate (Silenus, Hawthorn, Australia) at 1/1000 dilution was added and incubated for 1 h. The plates were washed and then 50 µl of the substrate disodium-p nitrophenyl phosphate at 1 mg/ml (Sigma) was added to each well. Optical density was measured 30 min later using a microplate reader at 405 nm with reference 630 nm. A standard curve was generated for each ELISA and a curve of best fit applied. The equation was used to determine the cytokine levels in supernatant samples. The lower limit of the assays was defined as background $\pm$ 2 SD. This was routinely found to be 80 pg/ml TNF and 0.3 ng/ml IFN-γ.

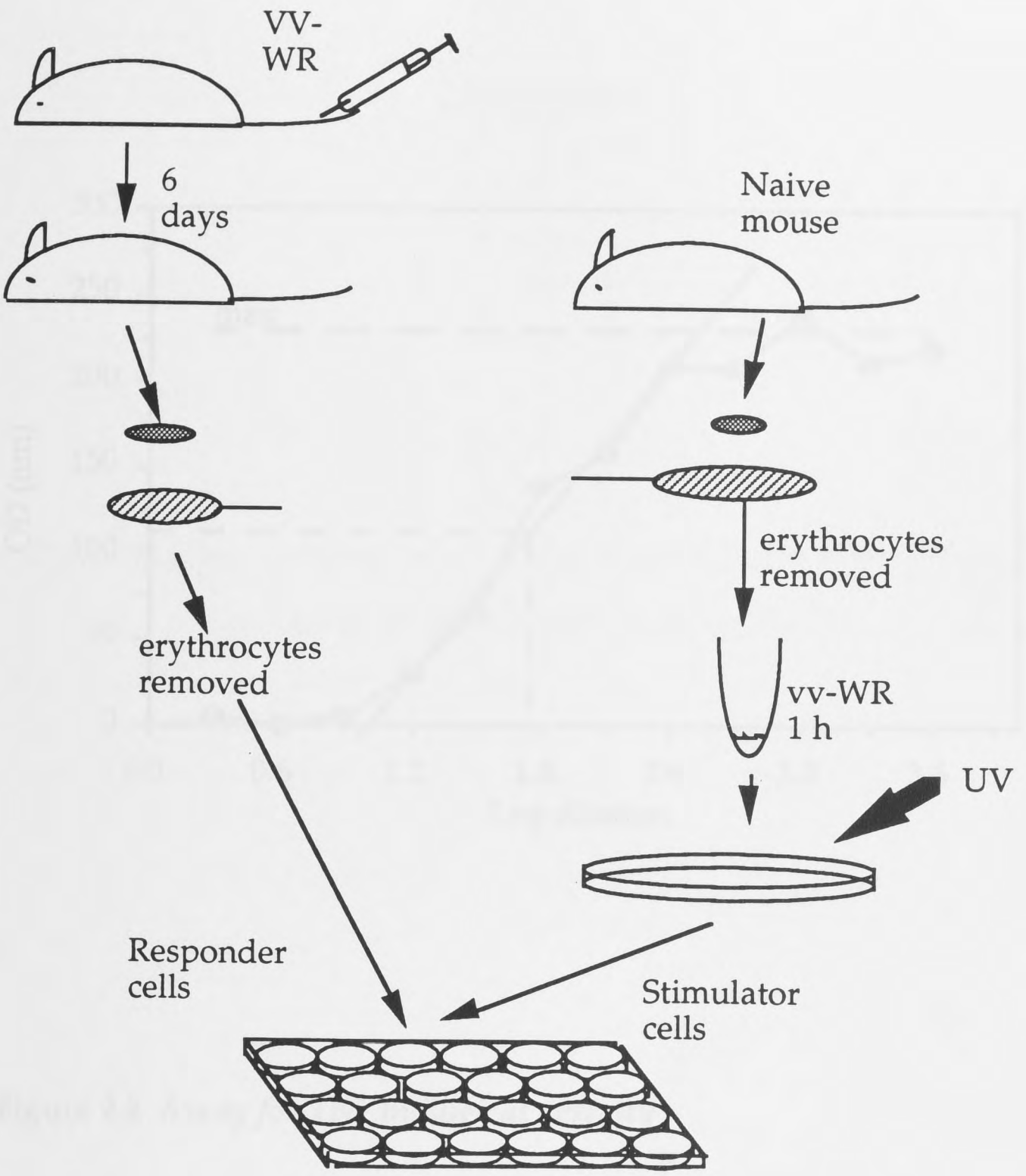


Figure 2.1 *Preparation of cell culture*

6 week CBA/H mice were injected with 2.5 x 10^6 pfu VV-WR i.v. The spleens were removed 6 days later, dispersed through a grid and erythrocytes removed. These cells are referred to as responder cells. For the preparation of stimulator cells, spleens were removed from naive mice and pressed through a grid. Erythrocytes were removed and the cells were infected with VV-WR for 1 h. The stimulator cells were UV-irradiated and 2 x 10^6 cells were put in culture with 10^7 responder cells.

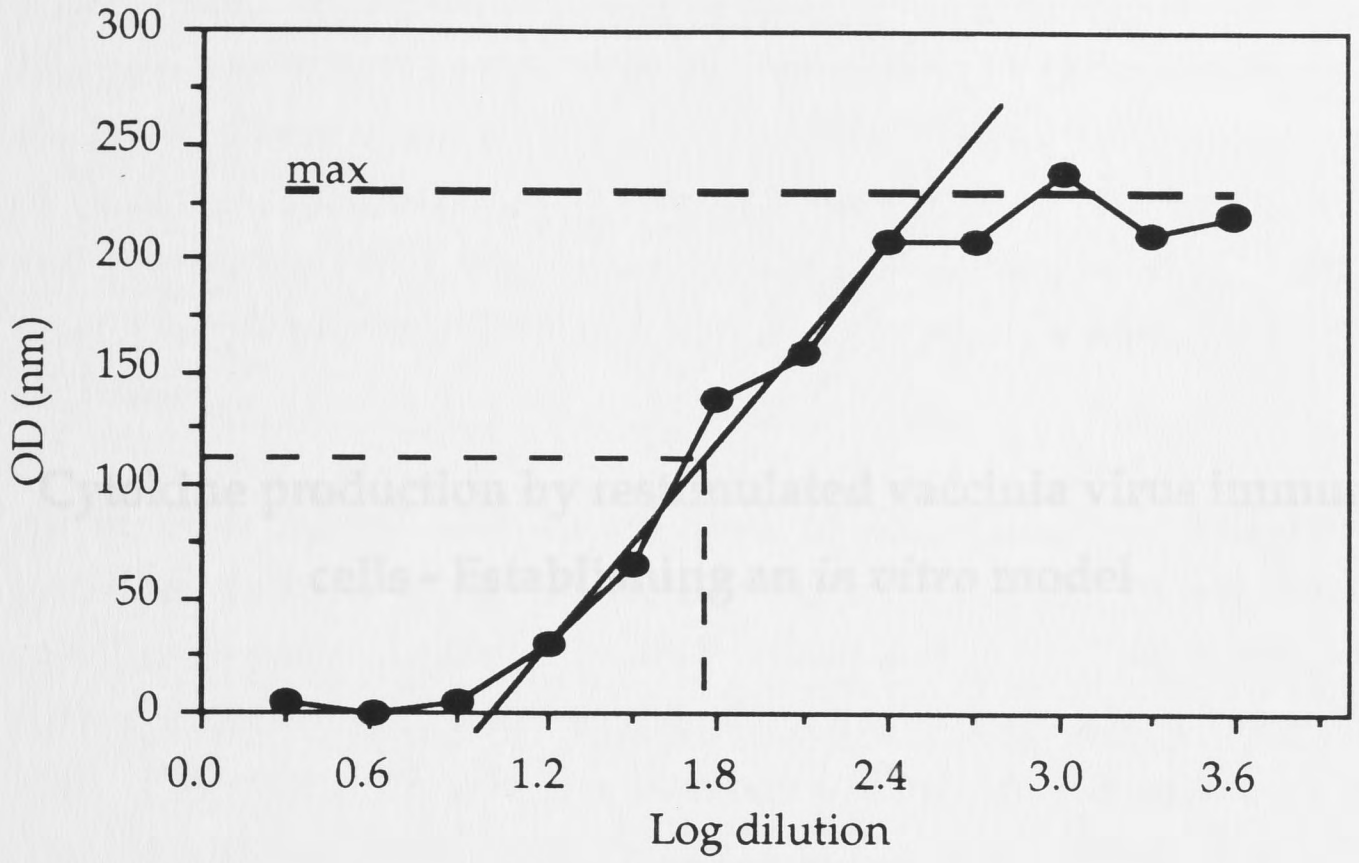


Figure 2.2 *Assay for TNF biological activity*

Culture supernatants taken at various times throughout the culture period were titrated in the TNF bioassay. This graph represents the OD readings for the titration of one sample, having subtracted the background OD reading. The thick dashed line represents the mean maximum OD reading determined in the absence of TNF. A straight line was drawn over the centre part of the graph and the half maximum titration was calculated as shown with the dotted line. This was then compared with a similar graph drawn for the standard to determine TNF levels in the sample.

CHAPTER 3

Cytokine production by restimulated vaccinia virus immune cells - Establishing an *in vitro* model

3.1 INTRODUCTION

T lymphocytes attracted to the site of virus replication play an essential role in recovery from the infection. They can be directly cytolytic and release cytokines that are involved in the recruitment of other cell populations. Inflammatory cells at sites of vaccinia virus infection are composed of 77% Thy1.2$^+$ cells with over 50% Lyt.2$^+$ cells (Hurwitz *et al.*, 1983). Many of the recruited cells are capable of producing a variety of cytokines, for instance Lyt.2$^+$ cells secrete IL-2, IFN-γ and IL-3 (Fong and Mosmann, 1990), NK cells produce IFN-γ (Anegon *et al.*, 1988) and macrophages produce TNF and IL-6 (Beutler and Cerami, 1989; Horii *et al.*, 1988).

An *in vitro* model was established to examine the production of cytokines thought to be involved in the recovery from virus infection, specifically the production of TNF, IFN-γ and IL-6. TNF is antiviral *in vitro* against several RNA and DNA viruses and acts synergistically with IFN-γ, both in inducing resistance in uninfected cells and in the lysis of virus infected cells (Wong and Goeddel, 1986). IFN-γ is also essential for virus clearance *in vivo*, as inhibition with specific mAb prevents mice from clearing the virus (Karupiah *et al.*, 1993; Klavinskis *et al.*, 1989; Ruby and Ramshaw, 1991). Although IL-6, originally described as IFN-β_2 (Weissenbach *et al.*, 1980), has limited antiviral activity, it has been included in this study as high levels are induced in virus infections (Cayphas *et al.*, 1987). As well as having antiviral properties these factors also regulate the type of immune response depending on the pattern of cytokines expressed (Balkwill and Burke, 1989). A Th1 cytokine profile, where IL-2 and IFN-γ are produced, preferentially induces cell-mediated immunity whilst Th2 cytokines, such as IL-4, IL-5 and IL-10, selectively stimulate humoral responses.

This chapter describes the establishment of an *in vitro* model to examine the production of the antiviral cytokines TNF, IFN-γ and IL-6. Splenocytes from mice infected with vaccinia virus were restimulated *in vitro* with virus-infected UV-irradiated spleen cells from naive syngeneic animals. The levels of cytokines produced by the restimulated responder cells were then assessed at various time intervals over the next 48 h.

3.2 MATERIALS AND METHODS

3.2.1

Mice — As described in Chapter 2.1

Virus and infection of mice — As described in Chapter 2.2

Cell lines — As described in Chapter 2.6

Culture medium — As described in Chapter 2.4

Preparation of responder and stimulator cells for 1 ml cultures — As described in Chapter 2.7 and 2.8

Culture conditions (1 ml) — As described in Chapter 2.9

Antibodies — As described in Chapter 2.11

3.2.2 *In vitro* generation of CTL

Responder and stimulator cells were mixed at a ratio of 5:1 with 10^7 6 day vaccinia immune responder cells in a 25 cm^2 flask (Nunc) in H16 medium (Andrew *et al.*, 1986). The flasks were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cytotoxic activity of the cells was determined on the fifth day of incubation.

3.2.3 Cytotoxicity assays

2 x 10^6 L929 target cells were labelled with 50 µCi ^{51}Cr for 1 h at 37°C in a total volume of 0.5 ml H16 medium, washed three times in medium and resuspended at a concentration of 2 x 10^5 cells/ml. For virus-infected targets, L929 cells were infected with VV-WR at 10 pfu/cell during the 1 h labelling period. The standard chromium release assay was carried out in triplicate, using a range of effector to target ratios (E:T). 2 x 10^4 target cells, in 100 µl volumes, were dispensed into wells of 96-well, round bottomed microtitre plates (Linbro). Total releasable ^{51}Cr (maximal release) was determined by adding 100 µl 2% Triton X-100 to wells containing only target cells and spontaneous release

determined by adding 100 µl medium to targets. The plates were incubated for 6 h at 37°C in a humidified atmosphere of 5% CO_2. Radioactivity in 100 µl supernatant from each well was measured in a Packard Auto-Gamma counter and percent specific lysis calculated using the formula

$$\% \text{ specific lysis} = \frac{\text{experimental release - spontaneous release}}{\text{maximal release - spontaneous release}} \times 100$$

3.2.4 Assay of TNF-dependent cytotoxic activity against WEHI 164

The level of TNF in cell lysates was determined using the TNF bioassay, which has been described in Chapter 2.12.1. The bioassay uses WEHI 164 cells, a mouse fibrosarcoma line, which is sensitive to the action of TNF (Espevik and Nissen-Meyer, 1986). To ensure the cytotoxic activity against the WEHI 164 cells was due to TNF, rather than a non-specific factor within the lysate, antibodies to TNF were added and the cytotoxic activity assessed. 50 µl cell lysate was incubated with 2 x 10^4 WEHI 164 cells in H16 medium, in the presence or absence of antibodies to TNF, in a total volume of 100 µl. A control irrelevant antibody was also included. The antibodies used are described in Chapter 2.11. TN3.19.12, a hamster anti-TNF antibody was used at 0.5 and 0.1 mg/ml. R4, a rat anti-IFN-γ antibody was used at 0.1 mg/ml. The TNF bioassay was then carried out as described in Chapter 2.12.1. MTT was added at the appropriate time and the percentage of dead target cells was determined using the formula

$$\% \text{ dead cells} = 100 - \frac{\text{optical density in wells with TNF}}{\text{optical density in control wells}} \times 100$$

3.2.5 Preparation of plasma membrane

Membrane preparations of lymphocytes recovered from the interface of a Ficoll density gradient were prepared, based on a method by Whisnat *et al.* (1978). 5 x 10^7 cells were washed twice in medium, resuspended in 1 ml cold TE buffer for 10 min on ice (10 mM Tris, 1 mM EDTA, pH 7.4) and then disrupted with a glass homogeniser. The cells were transferred to an Eppendorf tube and centrifuged at 500 g for 5 min at 4°C, after which the supernatant was decanted into another tube and centrifuged at 9000 g for 60 min at 4°C. The pellet was gently resuspended in 0.2 ml cold PBS, made up to 1 ml and centrifuged for 5 min at 500 g. The supernatant was decanted into another tube and

centrifuged for a further 60 min at 9000 g. The pellet, containing the crude plasma membrane, was resuspended in 0.5 ml PBS and stored at -70°C.

3.2.6 Protein assay

The protein concentration in the plasma membrane preparations was assayed using the Pierce Micro BCA protein assay reagent (Pierce, Rockford, IL, USA). Protein standards were prepared using BSA in PBS, with concentrations ranging from 20 µg/ml to 1 µg/ml. 1 ml of standard preparation or 1 ml of unknown protein sample was mixed with 1 ml Pierce Micro BCA Working Reagent. Following incubation at 60°C for 1 h, the samples were cooled to room temperature and the absorbance read at 562 nm. A standard curve was drawn up and used to determine the protein concentration of the unknown samples.

3.2.7 Dose response analysis and effect of well type

Serial two-fold dilutions of immune responder cells were made in 100 µl volumes in MLC in 96 well flat-bottomed plates (Nunc), with the first well containing 50 x 10^4 cells. The responder cells were incubated with a series of dilutions of stimulator cells added in 100 µl volumes. To determine whether the two populations required mixing for optimal response, the cells were mixed by pipetting up and down several times. Cells were also added to round-bottomed plates in order to determine whether these plates were better suited for culturing the restimulated immune cells. Cultures were incubated at 37°C and supernatant samples harvested at the determined time of peak response for each cytokine, ie. 8 h for TNF and 24 h for IFN-γ.

3.2.8 Antibodies

Hybridoma supernatants were used as antibody sources for cell depletions. The following rat anti-mouse hybridoma lines were grown in MLC and the supernatant collected for use in cell depletion protocols: J11d secreted mAb (IgM) which reacted with the heat stable antigen on B cells, immature T cells, erythrocytes and neutrophils (Bruce *et al.*, 1981). Clone 31M secreted mAb (IgM) which reacted with Lyt.2 (anti-$CD8^+$ T cells; Sarmiento *et al.*, 1980), and clone RL172.4

secreted an antibody that reacted with L3T4 (anti-CD4$^+$ T cells; Ceredig *et al.*, 1985).

3.2.9 Depletion of cell sub-populations

Immune responder cells were washed and depleted of B cells, and if appropriate CD4$^+$ and/or CD8$^+$ T cells, by resuspending in MLC (1% FCS) at 1 x 10^7 cells/ml. The cells were incubated with J11d (1/5 dilution), to remove B cells, and anti-CD8$^+$ (31M; 1/10 dilution) or anti-CD4$^+$ (RL172.4; 1/10 dilution) for T cell depleted cultures, for 10 min at 37^{o}C. This was followed by incubation with guinea pig serum as a source of C, at a 1/10 dilution, for a further 45 min. Viable cells were recovered on a Histopaque density gradient (Sigma). The antibody depletion step was repeated and the cells washed twice in MLC medium. The J11d treated cells were counted and the volume adjusted to give the desired cell concentration. Samples further depleted of CD4$^+$ or CD8$^+$ T cells were made up to the same volume as cells treated with J11d alone, so that the difference in cell number reflected the loss of cells specific for the anti-CD4$^+$ or anti-CD8$^+$ antibody treatment.

3.2.10 Inactivation of stimulator cells

In this *in vitro* model, vaccinia virus-infected stimlator cells were UV-irradiated, as vaccinia virus is a cytopathic virus. To consider the effect of altering the metabolic state of the stimulator cells on antigen presentation, they were subjected to various treatments before incubation with responder cells. 2 x 10^7 B cell depleted spleen cells were put into each of four 10 ml plastic tubes. The cells were infected with VV-WR at 10 pfu/cell at 37^{o}C for 1 h in a volume of 200 µl. The cells in each tube were washed twice and then treated as follows. One sample of cells was UV-irradiated in a petri dish for 5 min, as described in Chapter 2.8. A second sample was made up to 10 ml with medium and subjected to 2000 rads γ-irradiation. The third sample was treated with 50 µg mitomycin C (Sigma) for 30 min at 37^{o}C and then washed three times in PBS. The fourth sample was left untreated. For initial proliferation studies (see 3.3.9.1), stimulator cells were used at a concentration of 5 x 10^4 cells/well and incubated with 5 x 10^5 immune responder cells. In cultures used for dose-response analysis (see 3.3.9.2), the stimulators were used at 5 x 10^4 cells/well, with serial two-fold

dilutions of responder cells. Proliferation was quantitated by adding [^{3}H]-thymidine for the last 4 h of a 24 h culture.

3.2.11 Proliferation assay

Cultures were pulsed with [^{3}H]-thymidine to assess proliferation. Wells were pulsed with 1 µCi [^{3}H]-thymidine (78 Ci/mmole, ICN Radiochemicals, CA, USA) during the last 4 h of a 24 or 48 h culture at 37^{o}C. The plates were harvested onto filter mats using a Skatron 96 well cell harvester (Pharmacia). Scintillation fluid (LKB Scintillation Products, Leics, UK) was added and the [^{3}H]-thymidine uptake was measured in a Pharmacia liquid scintillation counter.

3.3 RESULTS

3.3.1 An *in vitro* model of antigen-specific stimulation of virus immune spleen cells

To establish the conditions for an *in vitro* model of antigen-specific stimulation of virus immune spleen cells, cultures were set up based on a protocol designed to generate CTL (Andrew *et al.*, 1986). Spleens were taken from mice which had been infected with 2.5 x 10^6 pfu VV-WR i.v. 6 days earlier. These immune responder cells were cultured in the presence of syngeneic VV-infected, UV-irradiated stimulator cells at a ratio of 5:1, in a total volume of 1 ml. Culture wells were also set up containing immune responder cells alone, immune responder cells stimulated with 5 µg/ml Con A, or naive responder cells with VV-infected stimulator cells.

After 3 days of incubation, the wells containing immune responders stimulated by VV-infected stimulators and those stimulated by Con A were densely filled with cells, many of which were clumped. In contrast, wells of immune responders without stimulation appeared to contain fewer cells and these were not clumped.

3.3.2 Antigen specificity of CTL response

To confirm that virus-specific CTL were generated in this system, 6 day vaccinia immune responder cells were stimulated with syngeneic VV-infected, UV-irradiated cells and incubated for 5 days. Figure 3.1 shows that cells taken from these cultures only lysed VV-infected target cells, and not uninfected targets.

3.3.3 Cytokine production by restimulated immune responder cells

3.3.3.1 TNF was produced following restimulation of responder cells

To determine whether 6 day vaccinia virus immune splenocytes would produce cytokines when cultured *in vitro*, these cells were cultured with UV-irradiated virus-infected syngeneic cells or were stimulated with Con A. Immune responder cells stimulated with Con A rapidly began TNF secretion with levels detectable in the supernatant within 2 h of culture (Figure 3.2). Maximal levels were reached by 8 h. Immune responder cells also secreted TNF when stimulated in an antigen-specific manner with virus-infected stimulator cells. This was detectable after 4 h and reached peak levels at 12 h of culture. The non-specific Con A stimulation induced secretion of 3 times the level of TNF produced by the virus stimulated immune cells. TNF was not detected in supernatant taken from cultures of immune responder cells lacking stimulation, nor in naive responder cell cultures stimulated with virus-infected cells.

3.3.3.2 IFN-γ was also secreted by immune responder cells

Con A stimulation of immune responder cells induced rapid production of IFN-γ with detectable levels within 2 h of culture. The levels rose logarithmically to peak at 20 h (Figure 3.3). Stimulation of immune responder cells with virus-infected cells induced IFN-γ with a 2 - 4 h delay compared to the Con A stimulated cultures. Once production began the levels increased logarithmically for 12 h and continued to rise for the rest of the culture period. As seen with the TNF results, cultures of immune cells without stimulation, or stimulation of naive cells did not induce significant levels of IFN-γ production. After 48 h the level of IFN-γ detected in either of these culture conditions was only 1% of that seen in the wells containing stimulated immune responder cells.

3.3.3.3 Restimulated responder cells produced high levels of IL-6

Stimulation of immune responder cells with Con A or with virus-infected cells resulted in similar kinetics and levels of IL-6 production (Figure 3.4). IL-6 was present in the supernatant within 2 h of culture and increased rapidly to peak at 12 - 16 h. Low levels of IL-6 were

detectable in cultures without stimulation and in cultures of stimulated naive responders. However, these were less than 5% of the levels in the corresponding cultures of stimulated immune cells.

3.3.4 TNF was present in cell lysate

TNF exists in secreted or membrane-bound forms (Kriegler *et al.*, 1988). To determine whether cell-associated TNF was a significant component of the TNF response to stimulus it was necessary to release the TNF from the cells. This was done by collecting the cells after an incubation period and subjecting them to a series of freeze/thaw cycles (Lonnemann *et al.*, 1989). Biologically active cell-associated TNF could be detected following this treatment, although the amount released following one, two or three freeze/thaw cycles was not significantly different (Table 3.1).

Table 3.1 ***TNF released from cell lysate by freeze/thaw cycles***

	Biologically active TNF (pg)		
	One cycle	Two cycles	Three cycles
10^6 Con A stimulated cells	18 ± 2	20 ± 7	17 ± 3

10^7 immune responder cells were stimulated with Con A for 12 h. Supernatant was removed, the cells resuspended in 500 µl fresh medium and placed in three tubes which were subjected to 1, 2, or 3 freeze/thaw cycles. Following exposure to these cycles, the lysed cells were assayed for cell-associated TNF activity in the WEHI 164 biological assay. The TNF levels were calculated for 10^6 cells added to the bioassay wells and are expressed as the mean ± SD of triplicate wells.

To ensure that the cytotoxic effect observed was due to TNF, and not to another non-specific factor within the cell lysate, antibodies to TNF were added to the bioassay. Preincubation of the cell lysate with antibody against TNF reduced the cytotoxic activity of the lysate and TNF was no longer detectable above background levels (Table 3.2). Addition of a control irrelevant antibody had no effect on the TNF activity.

Table 3.2 *Cytotoxicity in TNF bioassay was due to TNF in cell lysate*

Time	Antibody added to bioassay			
	None	α-IFN-γ	α-TNF	α-TNF
		0.1 mg/ml	0.5 mg/ml	0.1 mg/ml
2h	65 %	59 %	23 %	15 %
12 h	82 %	82 %	39 %	43 %
48 h	84 %	82 %	40 %	31 %
TNF std	90 %			

Percentage of dead target cells in TNF biological assay. 10^7 immune responder cells were stimulated with Con A for the time indicated. The supernatant was removed for cytokine analysis and cells were resuspended in fresh medium. Following exposure to two freeze/thaw cycles, cell lysates were assayed for cytotoxic activity on the TNF sensitive cell line WEHI 164. Cell lysates were incubated with 10 µl of antibodies at dilutions indicated, for 90 min prior to addition of TNF sensitive cells. The antibodies used were a hamster anti-TNF antibody (TN3-19.12) and a rat anti-IFN-γ antibody (R4.6A2).

Cell lysate preparations were prepared from cultures of immune responder cells incubated with Con A or virus-infected stimulator cells. These were assayed for biologically active TNF. As shown in Figure 3.5.1, the amount of cell-associated TNF in cells restimulated with Con A was similar to that secreted. Following antigenic stimulation of the responder cells, the level of secreted TNF was greater than that which remained cell-associated, although this was not statistically significant (Figure 3.5.2). The kinetics of production were similar in both stimulation conditions, with a rapid increase in TNF levels in the first 8 h, followed by a plateau. TNF levels decreased in the last 4 h of culture.

It was uncertain whether the cell-associated TNF detected in the bioassay was cytosolic or membrane bound. In order to determine the relative contribution of each to the cell-associated TNF pool, crude plasma membrane preparations were made from Con A stimulated lymphocytes, as described in section 3.2.5. These preparations had cytotoxic activity against WEHI 164 cells which was inhibited by preincubation with antibodies to TNF (Table 3.3). The level of TNF detected in these preparations from 10^7 cells was less than 5% of that

found in the culture supernatant or in cell-associated preparations (Table 3.4).

Table 3.3 ***Plasma membrane preparations of Con A stimulated lymphocytes contained TNF biological activity***

	protein (ng)	anti-TNF dil.	% cell death
Assay 1	625	-	68 %
	312	-	44 %
	150	-	28 %
	75	-	17 %
Assay 2	375	-	43 %
	375	1/50	3 %
	375	1/200	7 %

5×10^7 splenocytes were collected from the interface of a Histopaque gradient. The cells were stimulated with 5 µg/ml Con A for 17 h and cell membranes prepared as outlined in section 3.2.5. Protein concentration was determined using the Pierce Micro BCA protein assay kit. 10 µl of TNF antibody (5 mg/ml) was added to appropriate wells, at the dilutions indicated, 90 min before addition of the TNF sensitive cells.

Table 3.4 ***Membrane bound TNF was a minor component of TNF in these cells***

	secreted TNF	cell-associated TNF	membrane bound TNF
10^7 Con A stimulated cells	175 pg	175pg	5 pg

A comparison of the TNF detected in the extracellular, intracellular and membrane fractions of a Con A stimulated culture. TNF was assayed using the TNF bioassay.

3.3.5 Comparison of biologically active and total levels of IFN-γ and TNF

Bioassays measure biologically active cytokine only, whereas an ELISA may also recognise cytokine molecules which have lost biological activity. Samples from immune responder cells cultured with virus infected cells were assayed for TNF and IFN-γ using these two assay methods to determine which would be suitable for future work. The results for the IFN-γ assays are shown in Figure 3.6.1. The ELISA results were less variable and detected at least twice the level of IFN-γ as the bioassay. The cell-associated IFN-γ was assayed using the ELISA system, and at 26 h was less than 10 % of the levels detected in the supernatant. Thus, the major source of IFN-γ for detecting production by these cells was the supernatant. For future IFN-γ assays supernatant samples were assayed by ELISA. The two TNF assays detected similar levels of TNF in the cell-associated samples, whereas the ELISA results for the 7 h supernatant samples showed greater than 4 times the level of biologically active TNF detected in the bioassay (Figure 3.6.2). By 26 h incubation, the ELISA showed greater than 20 times the level of biologically active TNF, indicating that secreted TNF lost biological activity over time. The ELISA assay was therefore chosen for future TNF assays and thus, TNF levels represent total TNF released by cultured cells.

3.3.6 Modifying the culture conditions to use 96 well plates

3.3.6.1 Greater cytokine production in flat-bottomed culture wells

The results from the section 3.3.3 indicated it was possible to take spleen cells from mice infected with VV-WR 6 days earlier and restimulate them to produce cytokines, in an antigen specific manner. These cultures consisted of 1×10^7 immune responder cells stimulated with 2×10^6 VV-WR infected syngeneic cells in a total volume of 1 ml. For greater efficiency, the culture conditions were modified to use 96 well plates and smaller cell numbers. These culture conditions would allow for greater numbers of individual cultures and enable the estimation of cytokine production from multiple wells. The use of both round-bottomed and flat-bottomed 96 well plates was compared, as well as the necessity to mix the responder and stimulator cell populations.

Table 3.5 shows the results of incubation of two dilutions of responder and stimulator cells. The cells were incubated in either flat or round bottomed wells. Half the cultures in the flat bottomed wells had a mixing step at the beginning of culture.

Table 3.5 ***Effect of well type and mixing on cytokine production***

Cell number (x 10^4)	Well type	Cells mixed	TNF (pg/ml)	IFN-γ (ng/ml)
50 resp/ 25 stim	Flat	no	540 ± 30	630 ± 120
"	"	yes	550 ± 30	420 ± 250
"	Round	no	520 ± 20	140 ± 50
25 resp/25 stim	Flat	no	410 ± 50	90 ± 15
"	"	yes	490 ± 50	62 ± 10
"	Round	no	440 ± 50	50 ± 5

Spleen cells from 6 day VV-WR infected mice were depleted of B cells and incubated in a volume of 200 µl with syngeneic VV-WR infected, UV-irradiated spleen cells. The cytokine levels are expressed as mean ± SD of quadruplicate wells.

Table 3.5 shows that the levels of TNF in the culture supernatants of cells incubated in different well types were not sigificantly different. Mixing of the cells also had no effect on the levels of TNF produced. The production of IFN-γ, however, was influenced by the well type, with significantly increased levels in the flat bottomed wells ($p < 0.05$). Mixing of the cells led to decreased IFN-γ production, although this was statistically significant only in cultures with lower numbers of responder cells. From these results it was decided to use 96 well flat bottomed plates for further experiments, without mixing the responder and stimulator populations.

3.3.6.2 Dose response curves

Experiments are described later in this thesis which involve depletion of individual subsets of cells to assess their contribution to cytokine production (Chapter 4). Therefore, it was important for these experiments that the production of cytokines by the responder cells was on the linear portion of the response curve. To ensure that the level of cytokine was proportional to the cell number, a dose response analysis

was carried out. Cultures with varying numbers of both responder and stimulator cells were established and supernatant samples taken at the time of peak response, ie. 8 h for TNF and 26 h for IFN-γ. The results from one set of cultures (representative of three) are shown in Figure 3.7. A linear IFN-γ response was obtained by varying the number of responder cells and keeping the number of stimulators constant (Figure 3.7.1). Cultures produced significant levels of IFN-γ when they contained 50 x 10^4 responder cells. Over the dilutions tested, the number of stimulator cells in the wells did not appear to be a limiting factor in the IFN-γ response. The TNF response remained linear until the number of stimulator cells fell below 2.5 x 10^4 cells or less (Figure 3.7.2). From these results, and data not shown, it was decided for further experiments to use 50 x 10^4 responder cells and 5 x 10^4 UV-irradiated stimulator cells in a total volume of 200 µl. At each time point, 180 µl supernatant was removed from 4 culture wells. The 180 µl was divided into three aliquots and frozen in 96 well round bottomed plates, until assayed in three different cytokine assays.

3.3.7 B cell depletion did not effect cytokine production

As it was anticipated that futher studies would entail FACS analysis of cultures using antibodies which may bind to B lymphocytes non-specifically, the effect of B cell depletion on cytokine production was examined.

The B cells were removed from both the responder and stimulator populations by incubating the cells with J11d hybridoma supernatant followed by C, as described in section 3.2.9. Viable cells were recovered on a Histopaque gradient. The response of immune cells depleted of B cells was compared to that of immune cells which had erythrocytes removed by hypotonic shock. As shown in Table 3.6, depletion of B cells did not affect the level of cytokines produced by the responder cell population. The increased cytokine levels obtained in B cell depleted cultures would be accounted for by an enrichment of cytokine producing cells.

Table 3.6 *Effect of B cell depletion on cytokine production*

Time (h)	IFN-γ (ng/ml)		TNF (pg/ml)		IL-6 (AU/ml)	
	B cell depleted	Erythrocytes removed	B cell depleted	Erythrocytes removed	B cell depleted	Erythrocytes removed
8	5.4 ± 0.6	2.1 ± 0.8	100 ± 40	60 ± 10	2.7 ± 0.2	1.2 ± 0
12	15.7 ± 2.4	7.7 ± 1.0	110 ± 30	75 ± 10	5.4 ± 1.3	3.8 ± 2.1
18	> 20 [a]	16.7 ± 1.8	240 ± 60	140 ± 40	6.5 ± 0.3	4.8 ± 2
24	> 20	> 20	340 ± 50	150 ± 20	8.3 ± 1.8	5.5 ± 1.1

Spleen cells from 6 day VV-WR infected mice were either depleted of B cells by incubation with J11d antibody followed by complement lysis, or depleted of erythrocytes by hypotonic shock. 5×10^5 responder cells were then incubated with 5×10^4 B cell-depleted syngeneic UV-irradiated cells in a 200 µl volume, and supernatant was harvested at times indicated. Cytokine levels are expressed as mean ± SD of quadruplicate wells.

[a] IFN-γ titres in these samples were greater than the standard and cannot be presented accurately. The levels exceed 20 ng/ml.

3.3.8 Both CD4+ and CD8+ T cells proliferated upon antigenic stimulation

To determine the nature of the cells proliferating in response to antigenic stimulation, the CD4+ and/or CD8+ T cells were depleted from the responder population. In the last 4 h of culture with stimulator cells [^{3}H]-thymidine was added to each well to assess proliferation. The immune responder cells proliferated in response to stimulation by VV-WR infected, UV-irradiated syngeneic spleen cells (Figure 3.8). Removal of the CD8+ T cells from the responding population led to a 40% decrease in cell proliferation, while CD4+ T cell depletion reduced cell proliferation by 90%. Depletion of both CD4+ and CD8+ T cells reduced proliferation to levels observed in the absence of stimulation. Thus, both the CD8+ and CD4+ T cell populations responded to the stimulus provided by the UV-irradiated stimulator cells. The observation that wells containing CD4-/CD8+ T cells had only 10% of the level of proliferation in control wells suggest that CD8+ T cells may require the presence of CD4+ T cells for proliferation.

3.3.9 Effect of subjecting stimulator cell populations to various inactivating treatments

3.3.9.1 Inactivated stimulator cells induced proliferative responses from responder populations

The cultures had been established by basing the conditions on a CTL preparation where immune responder cells were stimulated with UV-irradiated VV-WR infected syngeneic spleen cells. UV-irradiation prevented viral replication, as shown in Chapter 2 (Table 2.1), however the treatment may have impaired the ability of stimulator cells to present antigen (Pamphilon *et al.*, 1991). The stimulatory ability of UV-irradiated cells was compared to that of cells treated with mitomycin C or γ-irradiation. These latter treatments block cell division but do not affect RNA or protein synthesis (Wagner, 1973).

All three treatments of stimulator cells led to a significant decrease in proliferation, when compared to cultures stimulated with virus-infected untreated syngeneic cells (Figure 3.9). The viable stimulator cells did not contribute to the greater proliferation seen in these control

cultures as proliferation of stimulator cells alone was less than 2% of that seen in cultures containing responder cells. In all stimulation conditions both $CD4^+$ and $CD8^+$ T cells proliferated. Among the three inactivating treatments there was little difference in the proliferation induced.

3.3.9.2 Dose response analysis of responder cells

Two-fold dilutions of responder cells were incubated with a constant number of stimulator cells which had been subjected to various treatments. The proliferative response of the responder cells was dependent on the number of reactive cells which were activated while in culture, as a result of cellular interaction. Plotting the logarithm of the number of cultured responder cells against the logarithm of the proliferative response yields a straight line, the slope of which can give information about the requirement for interaction among the responder cells (Tse *et al.*, 1980). A slope of 1 is consistent with there being no effective interaction among the titrated cells in the generation of the response, assuming there are no limiting conditions, such as the presence of inhibitors. This can be avoided by analysing the response early in the culture period. The responding cells may require interaction with the cells which are held constant.

The logarithm of the proliferative responses of B cell depleted 6 day VV-WR immune cells stimulated with UV-irradiated virus infected cells were plotted against the logarithm of the cell number and are shown in Figure 3.10. The results show a linear region in the response of the B cell depleted cells, with a slope much greater than 1. This is indicative of responder cell co-operation in the generation of the proliferative response. A slope much greater than 1 is also seen in cultures depleted of either T cell subsets, which suggests that both the $CD4^+$ and $CD8^+$ T cells required responder cell interaction to receive the appropriate signals to proliferate.

To investigate the effect of the metabolic state of the stimulator cells on the responder cell population, varying numbers of immune cells were cultured with virus-infected stimulators treated with UV or γ-irradiation, mitomycin C or left untreated. These cultures yielded markedly different cell number-response lines (Figure 3.11). Cultures containing untreated stimulator cells, infected with VV-WR, had a

slope of cell number-response close to 1, indicating these stimulator cells were able to provide the necessary signals to induce responder cell proliferation. Treatment of the stimulator cells with γ-irradiation or mitomycin C resulted in steeper slopes, between 2 and 3. The slope was further increased in those cultures stimulated with UV-irradiated stimulator cells.

No change in slope was observed when T cell subsets were depleted from immune responder cells and the remaining cells were incubated with untreated, viable VV-WR-infected stimulator cells (Figure 3.12.1). The slope was consistently close to 1. Immune cells cultured with γ-irradiated stimulator cells had a cell number-response line with a slope greater than 2, which is greater than the slope in cultures with untreated stimulator cells (Figure 3.12.2).

3.3.9.3 IFN-γ production was proportional to number of stimulator cells

The result of measuring the levels of IFN-γ produced in cultures where the number of responder cells was held constant, and the number of stimulator cells was varied in two-fold steps, is shown in Figure 3.13. The relationship between the logarithm of IFN-γ titre and the logarithm of stimulator cell number was linear, with a slope of 1. There was a plateau in cytokine production when the number of stimulator cells exceeded 5×10^4 cells.

3.3.9.4 Responder cell interactions in the production of IFN-γ

The amount of IFN-γ produced by different numbers of immune responder cells when the stimulator cell number was kept constant is shown in Figure 3.14. The slope of IFN-γ titre versus responder cell number was approximately 2, which indicated that there was a requirement for interaction among the responding population in the production of IFN-γ.

3.4. DISCUSSION

The pattern of cytokines produced during an immune response to virus infection plays a vital role in regulating the outcome of the response. Not only do the cytokines influence the cells drawn into the focus of infection, they may up-regulate MHC expression leading to a more efficient CTL response. These cytokines may also have antiviral activity. In order to study the production of these antiviral cytokines in the response to vaccinia infection, an *in vitro* model was established. Spleen cells were removed from mice at the time of peak CTL response to infection with vaccinia virus. These cells were restimulated in culture by incubation with virus infected syngeneic cells. Cytokine secretion was assessed by measuring cytokine levels in culture supernatant. The restimulated immune cells produced substantial levels of the antiviral cytokines TNF, IFN-γ and IL-6.

The production of cytokines occurred in wells containing restimulated immune cells but could not be detected in cultures lacking stimulator cells, indicating the need for restimulation, as was observed by Kelso *et al.* (1982). Cytokines have such a diverse range of effects that their production needs to be tightly regulated (Kelso, 1989). One mechanism of regulation is the requirement for the presence of antigen (Paul, 1989), with production reverting to low levels following the removal of the antigen. Cytokines synthesis is also short-lived, as many cytokines have AU-rich sequences in the 3'-untranslated regions of their mRNAs (Shaw and Kamen, 1986), which has been shown to correlate to mRNA instability (Wreschner and Rechavi, 1988). These mechanisms to regulate cytokine synthesis will protect against systemic spread of the cytokine, which could result in pathological effects.

A study of the kinetics of cytokine production after restimulation *in vitro* showed that TNF appeared in the culture supernatant within the first 2 h. Peak levels were reached by 8 h in cultures stimulated with Con A, whereas responder cells stimulated with virus infected cells produced peak levels at 12 h. Under both stimulation conditions, TNF levels had decreased by 24 h. These kinetics are similar to those observed by Lonneman *et al.* (1989) where human mononuclear cells stimulated with LPS *in vitro* produced significant levels of TNF, with maximal levels at 12 h of incubation, and levels decreasing from 24 to 48 h. The immune responder cells required a stimulatory signal to

undergo TNF production, as cells lacking stimulation did not secrete TNF. This result also demonstrated that contamination of the cultures by endotoxin, which may be present in the medium, was not responsible for the production of TNF.

Restimulation of immune cells with Con A induced low levels of IFN-γ within 2 h. The IFN-γ titre increased logarithmically to peak at 20 to 24 h. A similar profile of IFN-γ production by Con A stimulated mouse spleen cells has been described previously (Ito *et al.*, 1984; Klein *et al.*, 1985). IFN-γ production by responder cells stimulated with virus-infected cells was delayed compared to Con A stimulation, with maximal levels reached 4 h later. In the 24 h following maximal production the IFN-γ titres did not change. These results were similar to those of McKimm-Breschkin *et al.* (1982), who demonstrated that antigen specific IFN-γ production by T cell lines was first detectable after 6 h incubation, and reached maximum levels by 30 h. The IFN-γ levels then remained constant for 3 days, indicating the IFN-γ was stable throughout this period. Morris *et al.* (1982) demonstrated that IFN-γ production by antigen-specific CTL required that the T cells "see" the viral antigen in the context of cellular antigens. This may account for the delay in production by the immune responder cells in the cultures described in this chapter, as the cells must first recognise the antigen in the context of MHC molecules on the surface of the APC, before IFN-γ production begins.

IL-6 was detected in the culture supernatants within 2 h and levels increased rapidly to maximal values after 12 h incubation with virus infected stimulator cells. The levels of IL-6 induced by the virus-infected stimulators were of the same magnitude as those produced by Con A stimulated immune cells. Virus infection was a potent stimulus for IL-6 production, as demonstrated by Sehgal *et al.* (1988). This group has shown that infection of fibroblasts with a variety of viruses strongly enhanced secretion of IL-6, and that the addition of bacterial LPS did not enhance IL-6 secretion further. These findings indicated that virus stimuli were the dominant determinant in IL-6 secretion. An alternative, but not exclusive suggestion, is that TNF may have been an intermediate in the induction of IL-6 production by the responder cells. Levels of mRNA for IL-6 in human fibroblast cultures were enhanced by TNF (van Damme *et al.*, 1987; Walther *et al.*, 1988) and several *in vivo* studies have implicated TNF as an

intermediate in the production of TNF (Engelberts *et al.*, 1991; Fong *et al.*, 1989). The kinetics of cytokine production in this study, where peak TNF levels occur 4 to 6 h before maximal IL-6 levels, suggest TNF may play a role in IL-6 production in this system. This possibility is addressed in Chapter 5.

TNF has been reported to exist as both a secreted and a membrane bound form (Kriegler *et al.*, 1988). Similar levels of biologically active TNF were found to be cell-associated and secreted in the culture conditions described here. This is in contrast to the results of Lonnemann *et al.* (1989) who found less than 10% of the TNF produced in response to LPS stimulation was cell-associated throughout a 48 h culture. Of the cell-associated TNF produced by the immune responder cells, less than 5% was in the membrane fraction, indicating it was predominantly cytosolic.

A comparison of assay methods for IFN-γ and TNF indicated that the ELISA method detected higher levels of the cytokines in the supernatant than the bioassays. This suggested the cytokines may have lost some of their biological activity when released into the supernatant and/or bound to soluble cytokine receptors released into the supernatant by the responder cells (Seckinger *et al.*, 1988). However, the ELISA method was chosen for further studies on TNF and IFN-γ production because of its higher degree of precision, shorter operation period and insensitivity to modulators of cytotoxicity (Meager *et al.*, 1989).

Removal of the B cells from the immune responder population recovered from mice at the peak of the CTL response did not negatively influence cytokine production. B cells produced IL-6 when activated with anti-Ig *in vitro* (Freeman *et al.*, 1989) but did not produce IFN-γ or TNF. However, culturing B cell depleted immune responder cells resulted in increased cytokine levels within a shorter period of culture, including increased IL-6. Thus, the removal of B cells from the responder population effectively enriched for the cytokine producing cells. These results also indicated that the B cells in the immune spleen were not producing significant levels of IL-6 when in culture with UV-irradiated virus infected cells.

Vaccinia virus immune responder cells proliferated when cultured with UV-irradiated, VV-WR-infected stimulator cells. Depletion of $CD4^+$ or $CD8^+$ T cells from the responder cell population led to decreased levels of proliferation, indicating that both T cell subsets proliferated in this culture system. As $CD4^+$ T cells recognise viral antigen in association with class II MHC and $CD8^+$ T cells recognise antigen in the context of class I MHC, this indicates that viral antigen was presented in association with both class I and II MHC, on APCs. The $CD8^+$ T cells accounted for 40% of responder cell proliferation, as demonstrated by a 40% decrease in proliferation following the removal of these cells. However, cultures containing $CD8^+/CD4^-$ cells had a level of proliferation that was only 10% of the control wells. These results indicate that $CD8^+$ T cells may require the presence of $CD4^+$ T cells, or perhaps a secreted factor from these cells, for proliferation. The possibility that the $CD4^+$ T cells provided a cytokine, such as IL-2 or IFN-γ, has been addressed in Chapter 6.

In studies using *in vitro* stimulation of lymphocyte cells with another cell population, it is general practice to inactivate the stimulator cells. This inactivation may take the form of gamma irradiation, mitomycin C treatment, or UV-irradiation (Adler *et al.*, 1970; Lafferty *et al.*, 1974), and ensures that any proliferative response observed is essentially that of the responding population. UV-irradiation was used routinely to inactivate stimulator cells in the work described here. The UV-irradiation step also prevents replication of the vaccinia virus, as demonstrated in Table 2.1. The UV-irradiated cells provided a signal to the immune cells which induced a proliferative response.

To investigate whether the metabolic state of the stimulatory population influenced the response of the immune cells, the stimulators were treated to alter this activity. Viable stimulator cells infected with VV-WR induced a greater proliferative response from vaccinia immune spleen cells than cells which had undergone a form of inactivation treatment; a similar response to that seen in the generation of CTL during a secondary MLC (Röllinghoff and Wagner, 1975). The proliferation of these viable stimulator cells was not sufficient to account for the greater proliferation in these cultures. Treatment of cells with mitomycin C or γ-irradiation blocked cell division but had no effect on RNA or protein synthesis (Wagner, 1973), and UV-irradiated cells have impaired APC ability (Pamphilon *et al.*,

1991). From the results shown in Figure 3.3.9, it appeared that stimulator cells, inactivated by these three treatments, induced a proliferative response of similar magnitude.

The requirement for cellular interaction in the proliferation response was analysed using dose response plots. The slope of log-cell number-log-response plots can provide information about the need for interaction of cells responding to a given stimulus. The slope analysis technique first used by Coppleson and Michie (1966) was further developed by Tse *et al.* (1980). They have shown that plotting the logarithm of [^{3}H]-thymidine incorporation against the logarithm of primed cell number generated a straight line, the slope of which represented the number of interacting populations present in limiting concentrations. The cell number-response plot of immune spleen cells stimulated with UV-irradiated cells yielded a slope with a linear region, as shown in Figure 3.10. The slope was much greater than one indicating there was a degree of co-operation among the responder cells to generate the proliferative response. This was true for both the $CD4^+$ and $CD8^+$ T cells. The form of interaction among the responders was not determined but could include antigen presentation, cytokine production or possibly the provision of costimulatory signals, such as that provided by the B7 molecule.

An interesting result was that the slope of cell number-response plots varied markedly with APC treatment (Figure 3.11). The slope was close to 1 for cells incubated with viable VV-WR infected cells. The slope increased for cells responding to γ-irradiated or mitomycin C treated stimulators. The steepest slope, indicating the greatest interaction, was generated for immune cells responding to UV-irradiated cells. These results are consistent with the view that the viable stimulators were able to replace the need for responder cell interaction, perhaps by providing cytokine, cell contact and correct antigen presentation. The slope for both the $CD4^+/CD8^-$ and $CD8^+/CD4^-$ T cell cultures was also close to 1, when cultured with viable stimulator cells. This indicates that the coactivation signals required by these cells, which could not be provided by the UV-irradiated stimulator cells, were provided by the viable stimulator cells (Figure 3.12.1). The γ-irradiated and mitomycin C treated stimulators were able to replace some need for responder cell interaction compared to cultures with UV-irradiated cells, as shown by the decreased slope. Gamma-irradiated or mitomycin C treated cells are

metabolically active and, therefore, may produce a product, such as a cytokine, that was unable to be provided by UV-irradiated cells. An alternative explanation is that the virus was not completely inactivated in mitomycin C or γ-irradiation treated cells, and was able to infect some of the responder cell population.

These results indicate that UV-irradiation of the stimulator population allowed antigen presentation, but removed the cell's ability to provide costimulatory signals. This phenomenom has been observed in other systems. Hapten-coupled IA^+ low density cells were able to activate hapten-specific CTL. When these APC were exposed to UV irradiation their ability to induce CTL was impaired, although the defect could be by-passed by the addition of IL-1 or IL-2 (Tominaga *et al.*, 1983). In a study by Roska *et al.* (1985) it was shown that freshly primed T cells required additional interactions with APC for effective triggering beyond that mediated by the T cell antigen receptor. The interaction required a metabolically intact accessory cell, which could be accomplished by IA^- cells or by third-party cells, which themselves were not bearing antigen.

The nature of the defect in the UV-irradiated cells is unknown, although UV-B irradiation (290 to 320 nm) has been shown to produce a dose-dependent decrease in membrane expression of ICAM-1 in human monocytes (Krutmann *et al.*, 1990) and of the costimulatory ligands B7/BB1 and ICAM-1/CD54 on human dendritic cells (Young *et al.*, 1993). The UV-B irradiation of dendritic cells reduced their ability to stimulate T cell proliferation and IL-2 production in an MLR, presumably due to the low B7/BB1 expression. Addition of low dose r-human IL-2 or MLR supernatant reconstituted the proliferative response of the T cells (Young *et al.*, 1993). These studies would support the concept that responder cell interaction was required for proliferation when stimulated with UV-irradiated cells. Viable stimulator cells were able to provide the necessary signals for both $CD4^+$ and $CD8^+$ T cell proliferation.

The plot of the IFN-γ response against cell number revealed further information about requirements for IFN-γ production. The slope of 1 for the relationship between stimulator cell and IFN-γ titre (Figure 3.13) in the region of responder cell excess, indicated that one antigenic cell was sufficient to trigger IFN-γ release from one responder cell. Thus,

IFN-γ titre was directly proportional to the number of UV-irradiated stimulators added to the cultures. This result is similar to the cytokine response by murine cytomegalovirus (MCMV)-immune lymph node cells upon restimulation with MCMV (Sinickas *et al.*, 1985), where the slope between stimulator cell number and IL-3 production was also 1. The plateau seen with higher numbers of stimulators was in the region of target cell excess, where the number of stimulator cells and responder cells were comparable (McKinnon and Hodgkin, 1987).

The slope of the relationship between responder cell and IFN-γ titre was 2 (Figure 3.14). This indicated that there was some form of interaction among the responder cells in the production of IFN-γ. This may be due to a direct cellular interaction or the release of a soluble factor or cytokine, for example IL-2 which is known to enhance the production of IFN-γ (Yamamoto *et al.*, 1982). This possibility has been addressed in Chapter 5.

3.5 Summary

Spleen cells taken from mice at the peak of the CTL response to vaccinia virus were restimulated *in vitro* to produce cytokines. Stimulation with Con A or with UV-irradiated vaccinia virus infected cells induced the secretion of TNF, IL-6 and IFN-γ. TNF was first detected in the culture supernatant within 2 h, and peaked at 8 to 12 h. IL-6 production was also rapid with maximum titres reached 12 h after initiation of culture with virus infected stimulators. In comparison, IFN-γ production was delayed by 4 to 8 h, and peak levels were reached by 24 h. The majority of TNF and IFN-γ was in the secreted form, rather than cell-associated. Removal of the B cells from the responder cell population did not reduce cytokine production, rather it resulted in an enriched cytokine-producing population. The metabolic state of the stimulator cells influenced the proliferative response of the responder cell population. Stimulator cells exposed to UV- or γ-irradiation or treated with mitomycin C had reduced ability to provide the necessary signals to induce a proliferative response from the immune cells. However, the responder cells were able to compensate for these missing signals by cellular co-operation. IFN-γ production was proportional to the number of stimulator cells, and required interaction among the responder population.

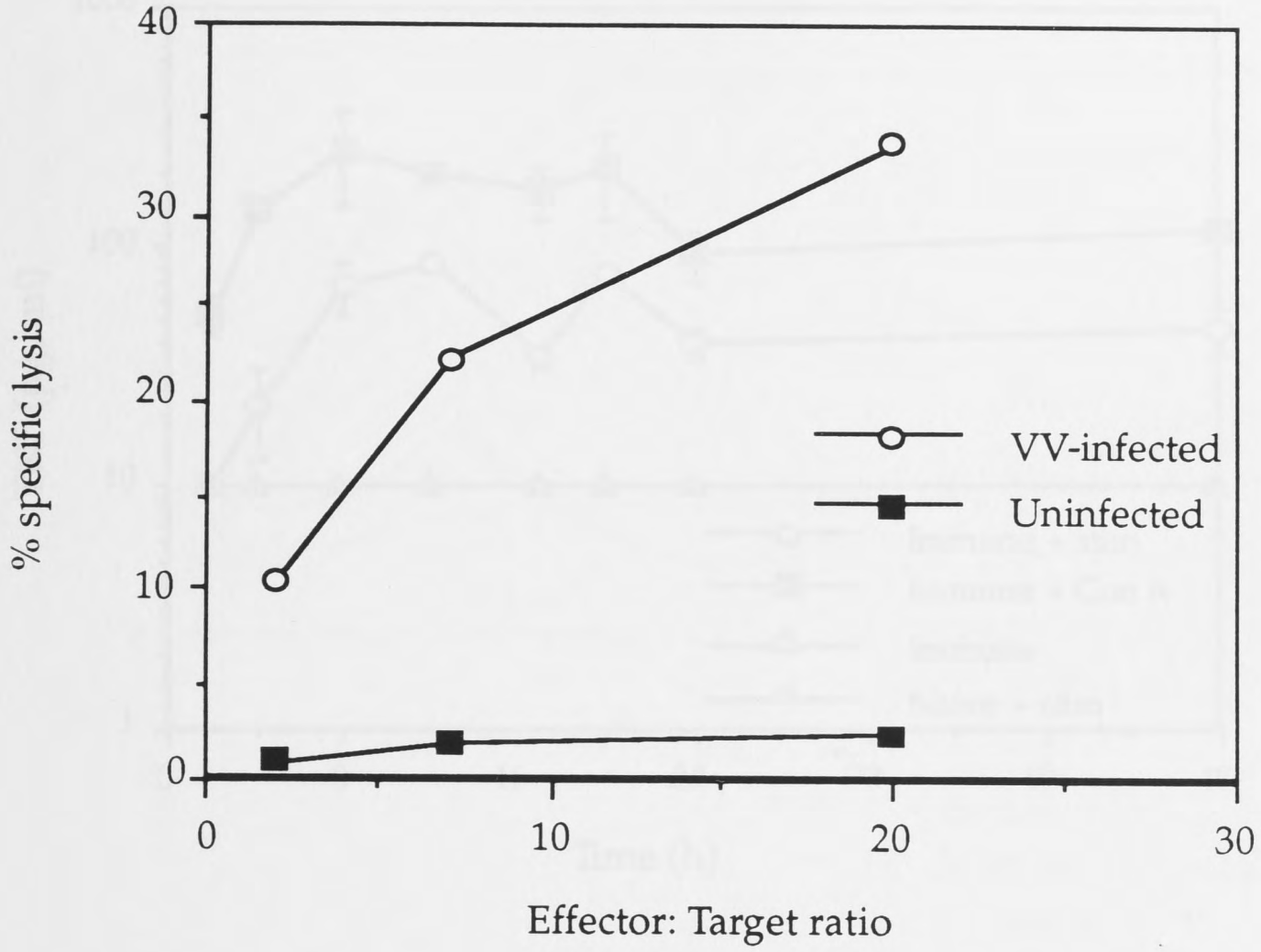


Figure 3.1 ***CTL activity of cultured responder cells***

Mice were injected i.v. with 2.5 x 10^6 pfu of VV-WR and 6 days later the spleens were removed. 10^7 water-lysed spleen cells were cultured with 2 x 10^6 UV-irradiated VV-WR infected syngeneic cells in a final volume of 15 ml. The cells were incubated for 5 days and then tested for their ability to lyse virus infected target cells. Results are shown as means of triplicates.

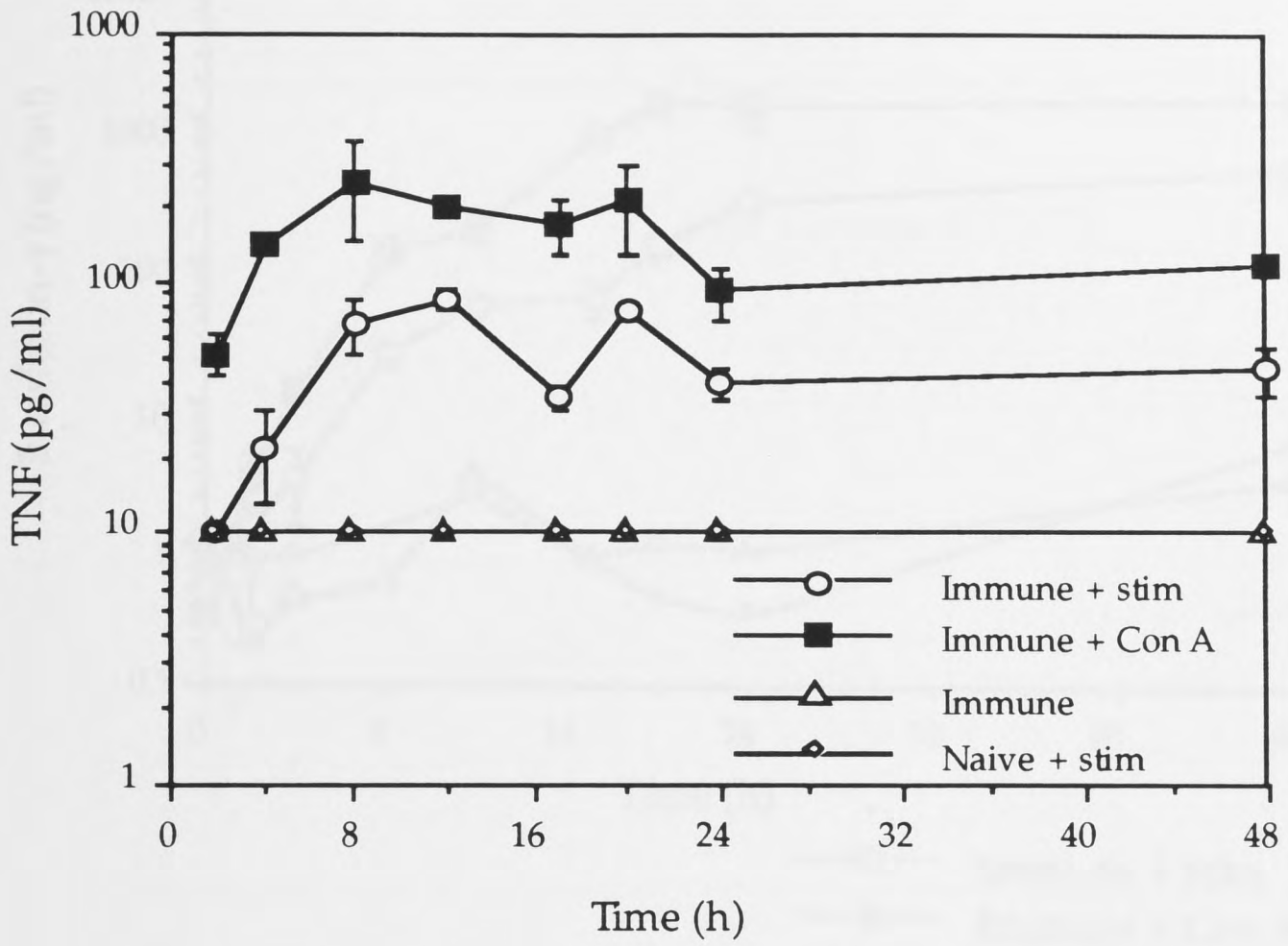


Figure 3.2 ***Time course of TNF production by 6 day VV-WR immune spleen cells***

Spleens were removed from mice injected with VV-WR 6 days earlier, the cells dispersed and erythrocytes removed. 10^7 immune responder cells were incubated with 2 x 10^6 UV-irradiated vaccinia virus infected spleen cells in a total volume of 1 ml. As a positive control 10^7 immune responder cells were stimulated with 5 µg/ml Con A. Cultures of immune responder cells lacking stimulators and cultures of naive spleen cells incubated wth UV-irradiated, VV-WR infected cells were also set up. TNF levels in supernatant were measured by bioassay. Duplicate samples were taken from single culture wells. The results are shown as mean $\pm$ SD.

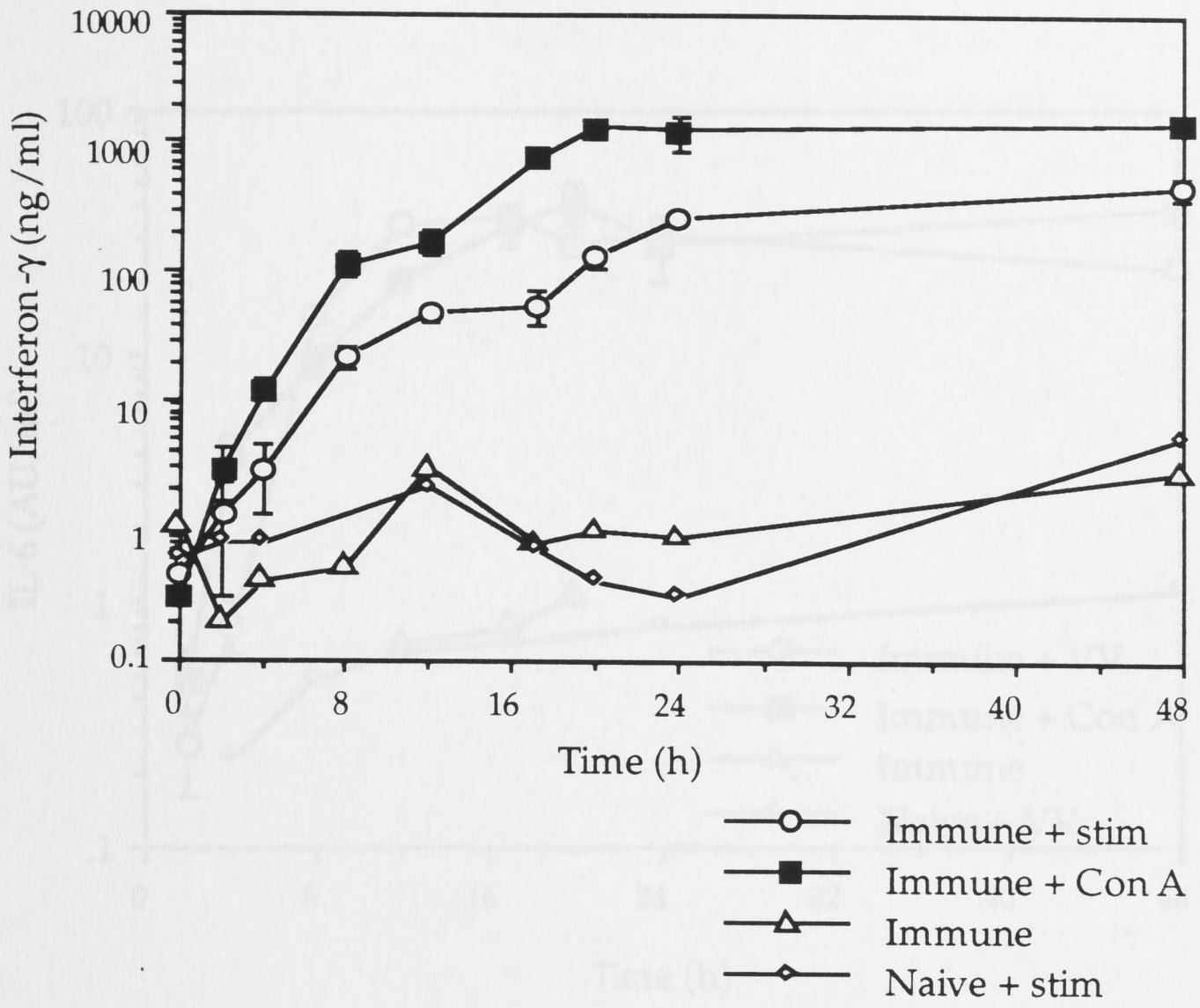


Figure 3.3 ***Time course of IFN-γ production from 6 day VV-WR immune spleen cells***

10^7 immune responder cells were incubated with 2 x 10^6 UV-irradiated vaccinia virus infected spleen cells in a total volume of 1 ml. Other culture conditions are described in legend 3.2. IFN-γ levels were measured by ELISA. Duplicate samples were taken from single culture wells. Results are shown as the mean ± SD.

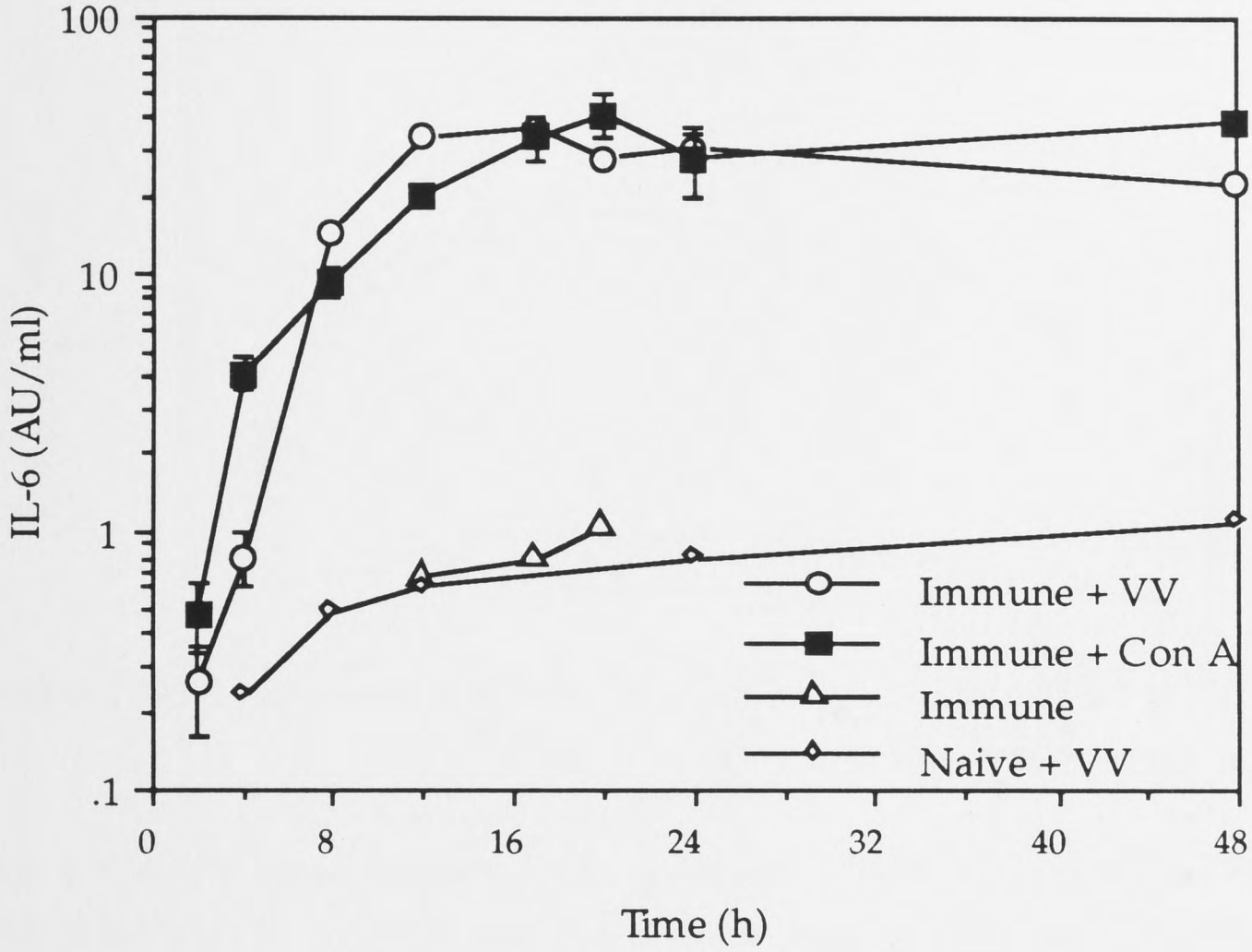
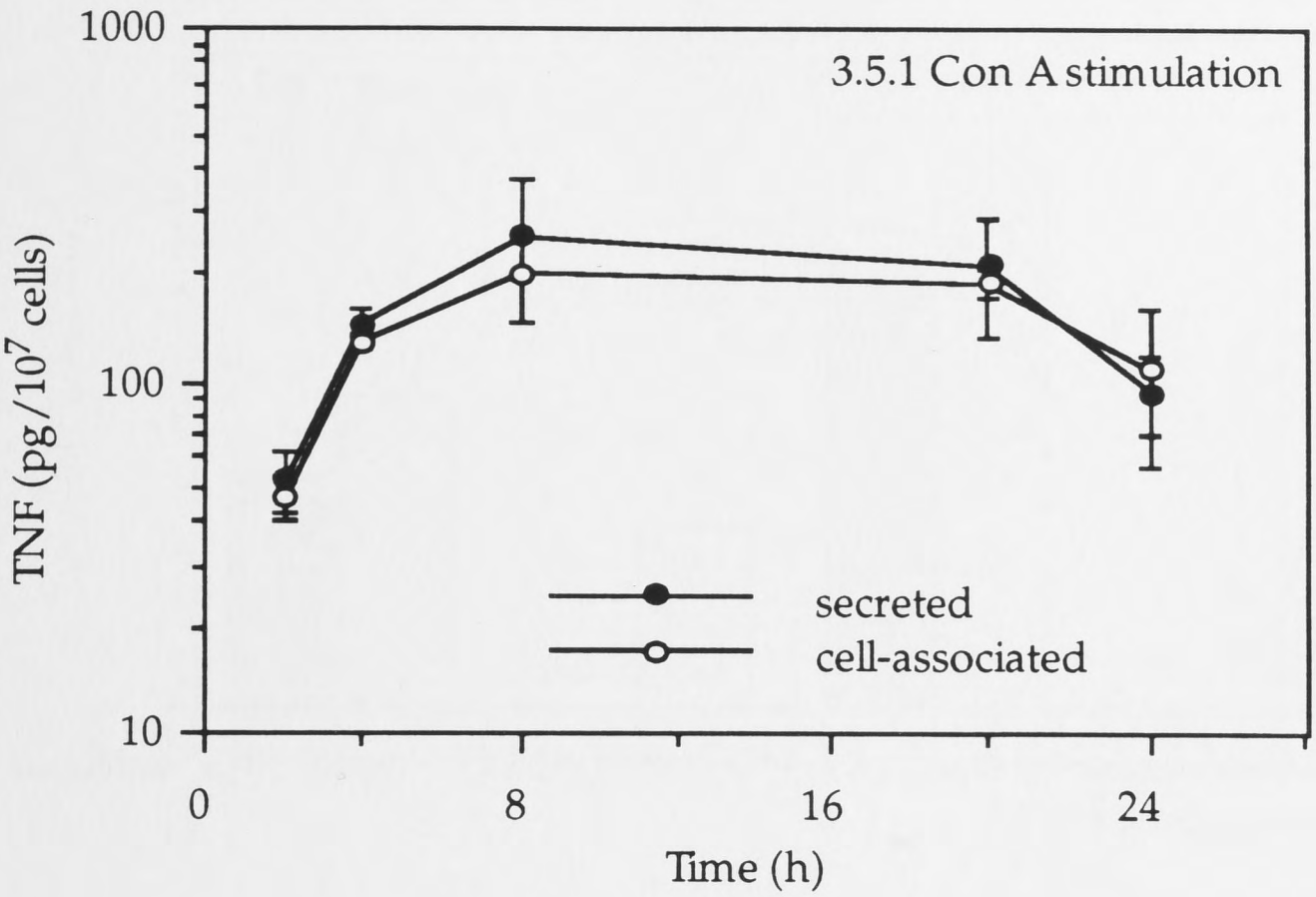


Figure 3.4 ***Time course of IL-6 production by 6 day VV-WR immune spleen cells***

10^7 immune responder cells were incubated with 2 x 10^6 UV-irradiated vaccinia infected spleen cells in a total volume of 1 ml. Other culture conditions are described in legend 3.2. IL-6 levels were measured by bioassay. Duplicate samples were taken from single culture wells. Results are shown as the mean $\pm$ SD.

Figure 3.5 ***Comparison of secreted and cell-associated TNF produced by restimulated immune responder cells***

10^7 water-lysed spleen cells from mice injected with VV-WR 6 days earlier were stimulated with 5 μg/ml Con A or UV-irradiated vaccinia infected syngeneic cells, in a final volume of 1 ml. Supernatant and cells were removed at the times indicated and frozen until assayed for TNF levels. Cell-associated TNF was released by two freeze-thaw cycles. TNF levels were measured by bioassay. Duplicate samples were taken from single culture wells. The results are shown as the mean ± SD.


1000
100
10
TNF (pg/10^7 cells)
3.5.1 Con A stimulation
secreted
cell-associated
0
8
16
24
Time (h)

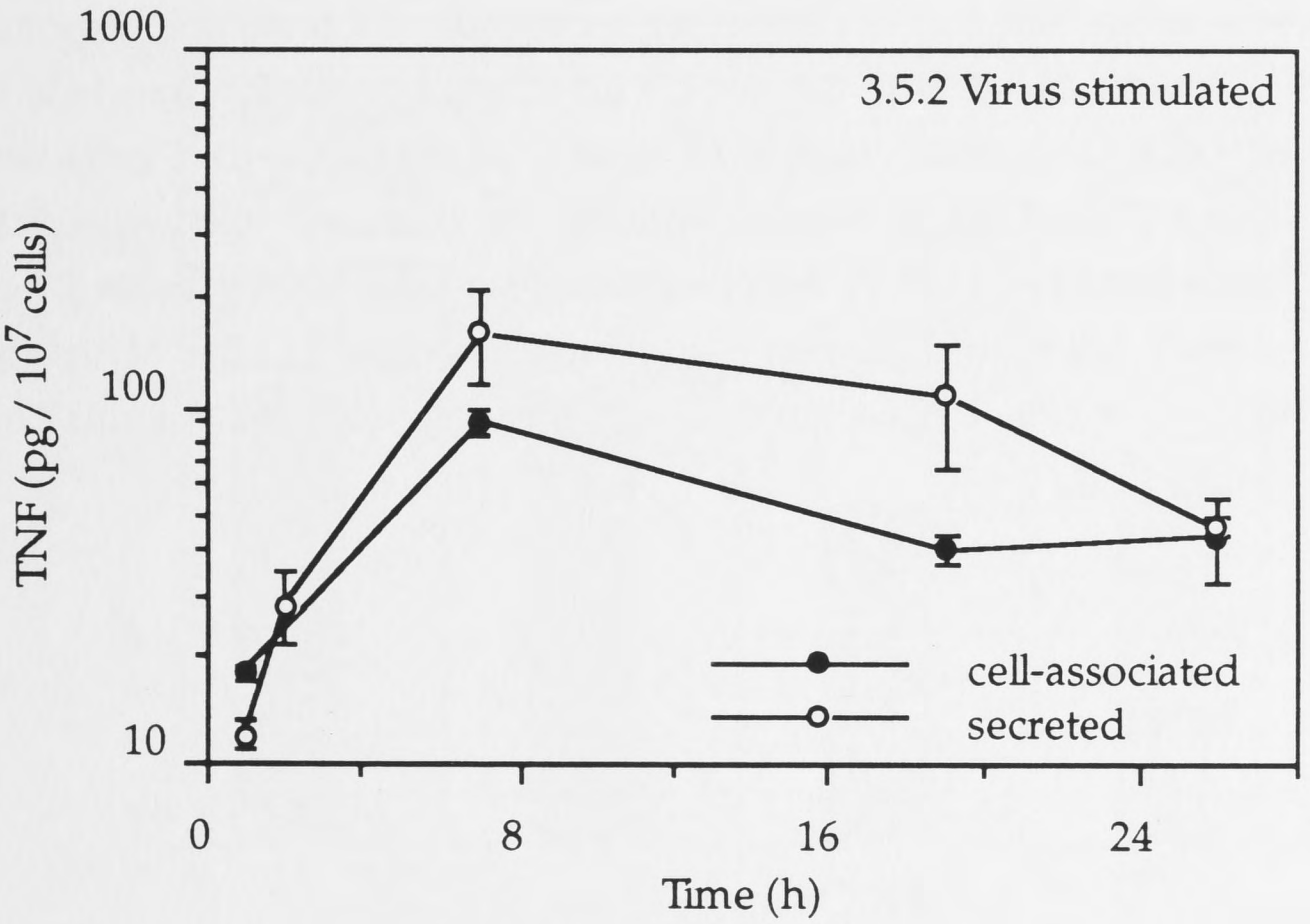
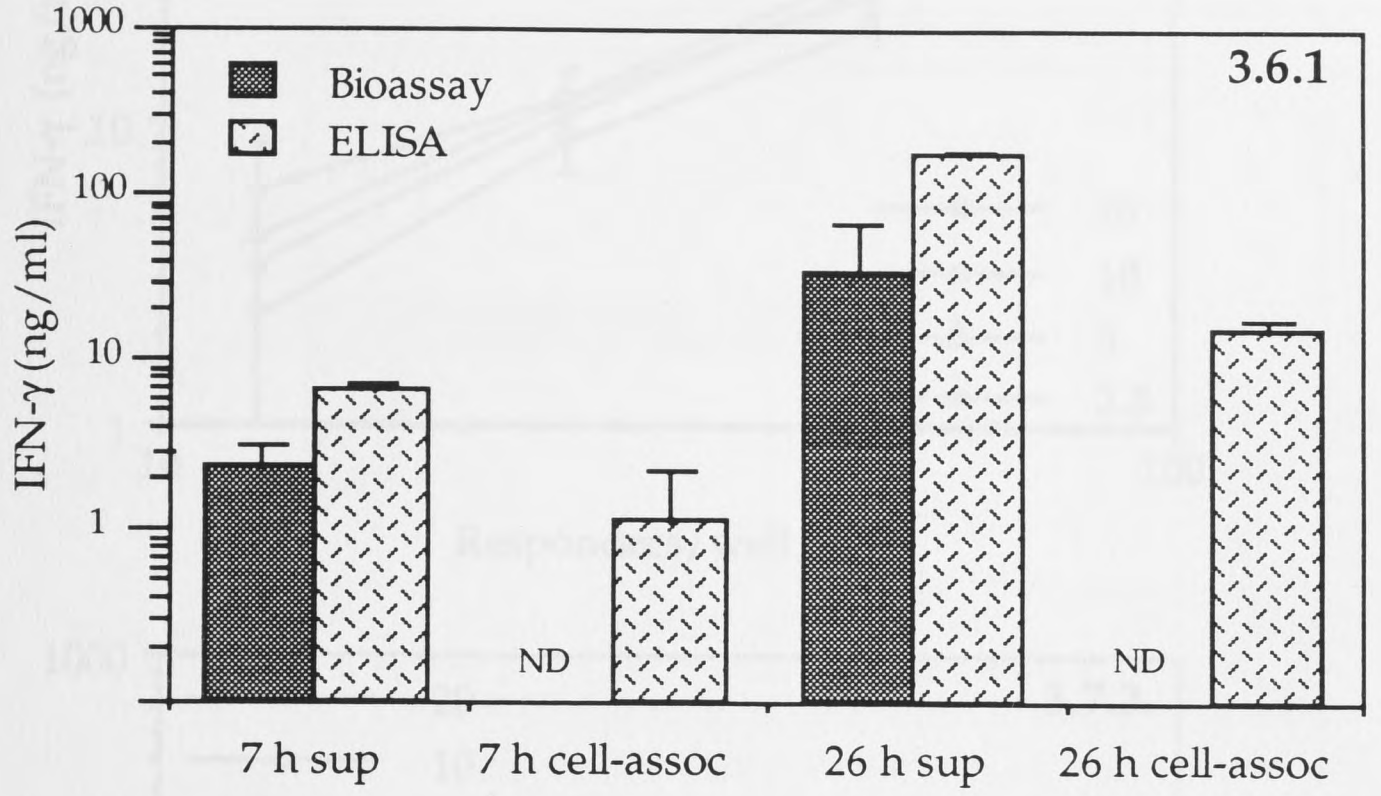
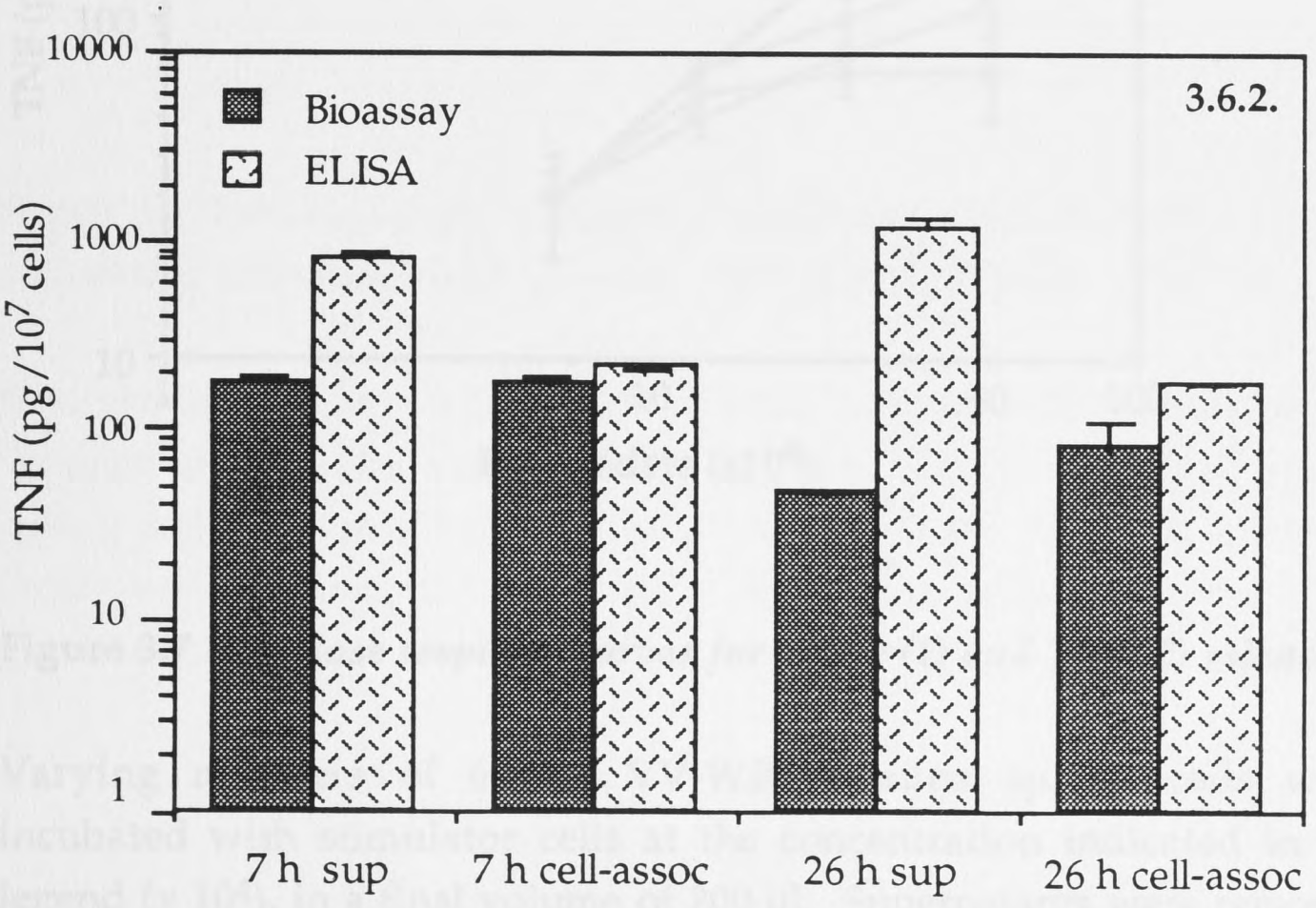
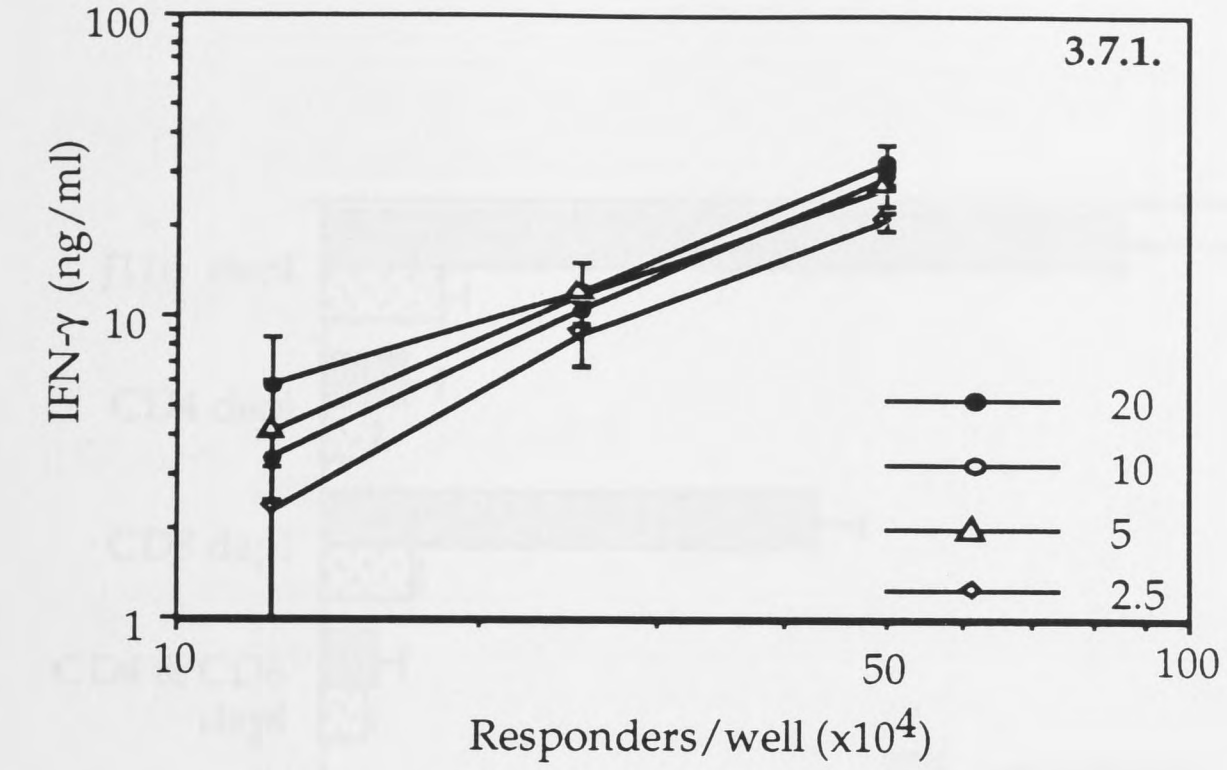

1000
100
10
TNF (pg/ 10^7 cells)
3.5.2 Virus stimulated
cell-associated
secreted
0
8
16
24
Time (h)

Figure 3.6 ***Comparison of the levels of IFN-γ and TNF detected by bioassay and ELISA***

Spleens were removed from mice injected with VV-WR 6 days earlier, the cells dispersed and erythrocytes removed. 10^7 immune responder cells were incubated with 2 x 10^6 UV-irradiated vaccinia virus infected spleen cells in a total volume of 1 ml. Supernatant and cells were harvested 7 and 26 h after initiation of culture. Cell-associated cytokines were released by two freeze-thaw cycles. IFN-γ levels (1) and TNF levels (2) were assayed using bioassay and ELISA. Duplicate samples were taken from single culture wells. Results are shown as the mean $\pm$ SD.


3.6.1
Bioassay
ELISA
IFN-γ (ng/ml)
1000
100
10
1
ND
ND
7 h sup
7 h cell-assoc
26 h sup
26 h cell-assoc


3.6.2.
Bioassay
ELISA
TNF (pg/10^7 cells)
10000
1000
100
10
1
7 h sup
7 h cell-assoc
26 h sup
26 h cell-assoc



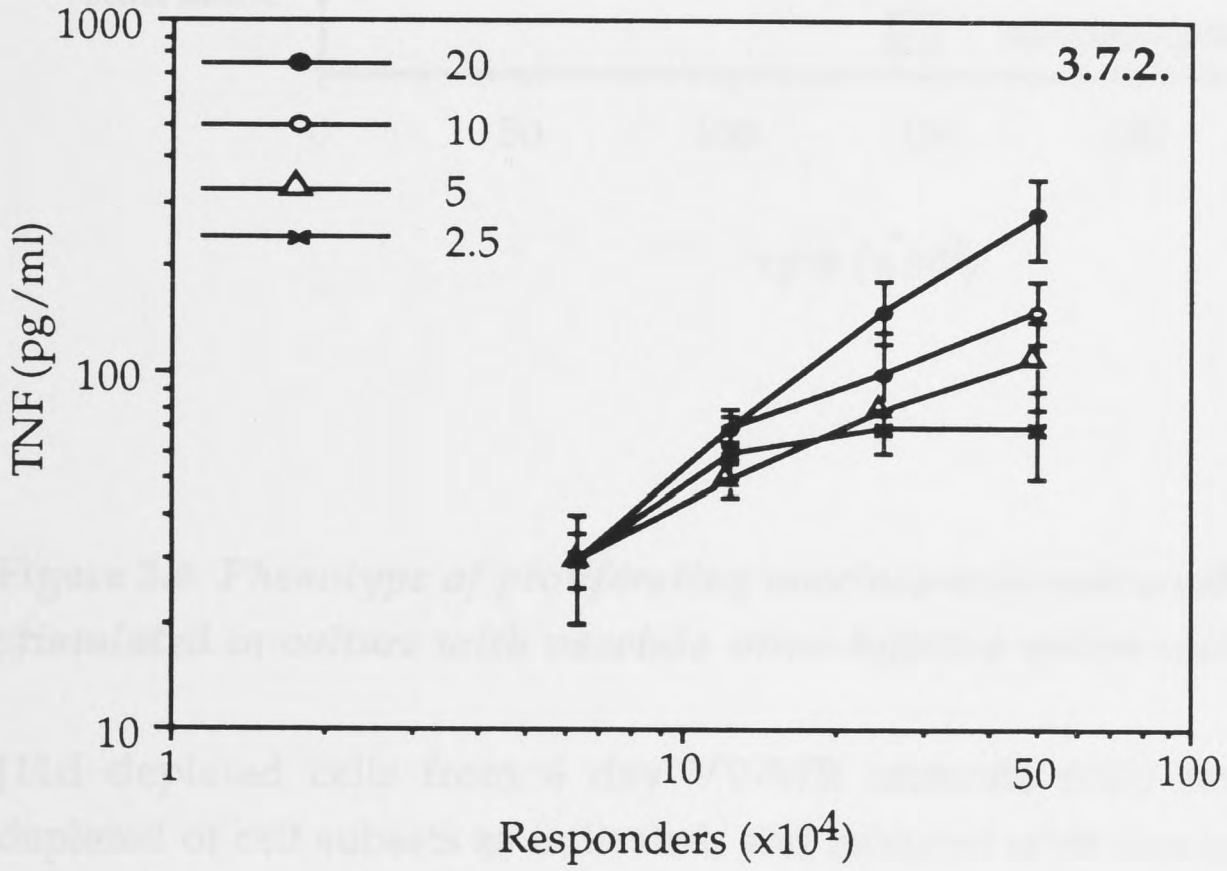


Figure 3.7 ***The dose response curves for IFN-γ (1) and TNF (2) release***

Varying numbers of 6 day VV-WR immune spleen cells were incubated with stimulator cells at the concentration indicated in the legend (x 10^4), in a final volume of 200 µl. Supernatants were removed at 8 h and 24 h and assayed for TNF and IFN-γ, respectively. Cytokine levels are expressed as mean ± SD of quadruplicate wells.

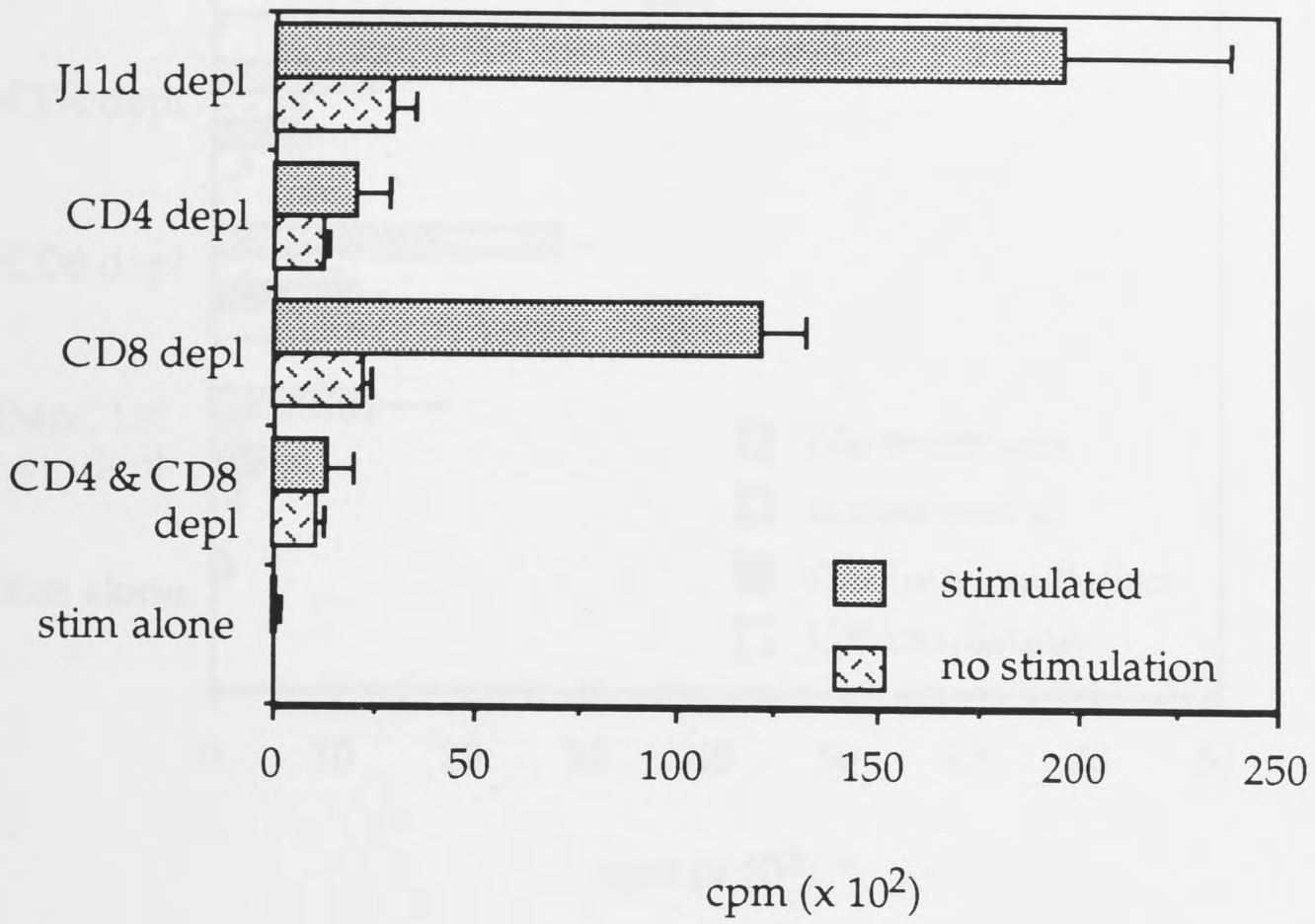


Figure 3.8 ***Phenotype of proliferating vaccinia-responsive cells stimulated in culture with vaccinia virus-infected spleen cells***

J11d depleted cells from 6 day VV-WR immune mice were further depleted of cell subsets as indicated, and cultured with vaccinia-infected spleen cells. Incorporation of [^{3}H]-TdR during the final 4 h of a 48 h incubation was determined. Results are shown as the mean $\pm$ SD of quadruplicate wells.

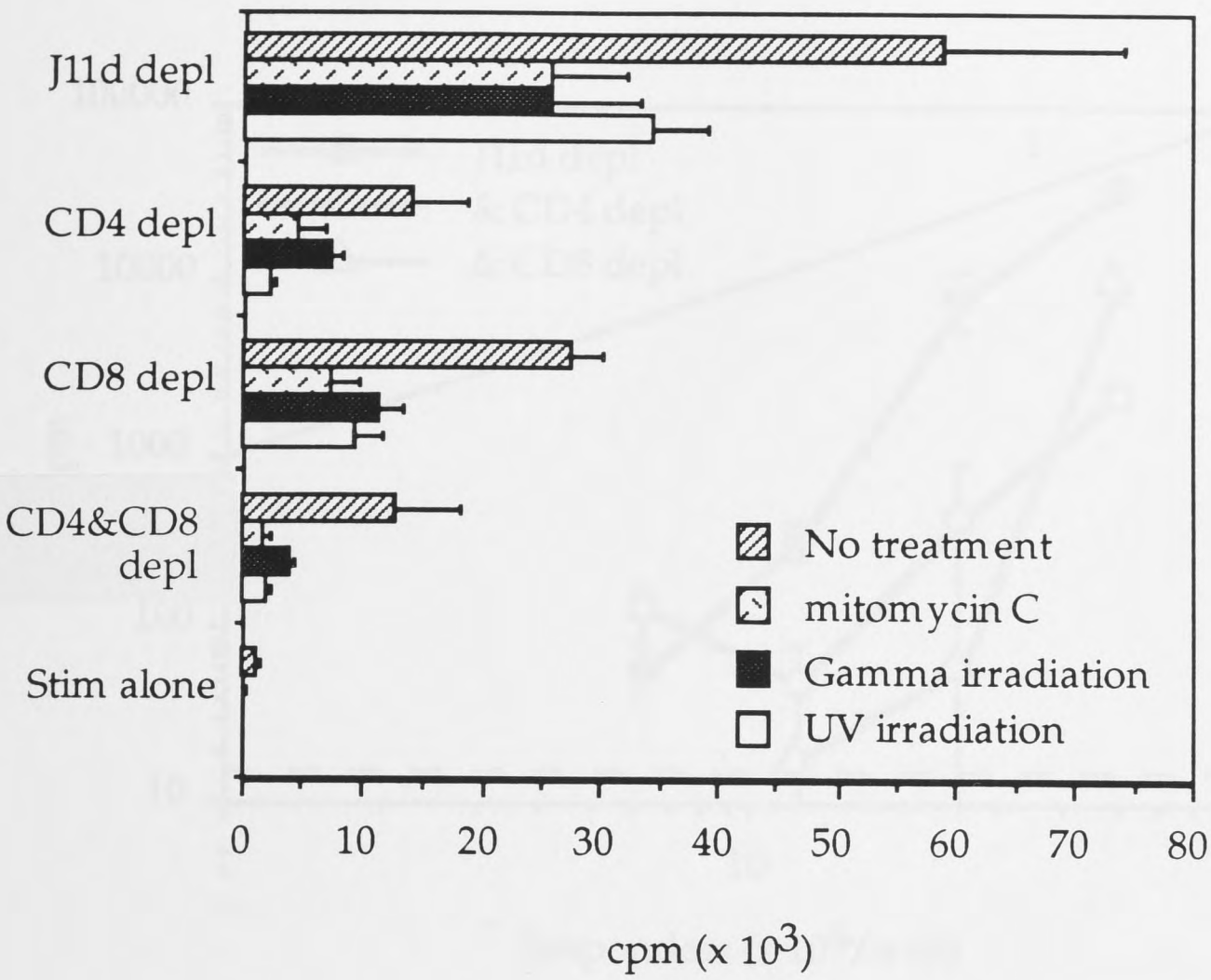


Figure 3.9 ***The proliferative response of 6 day VV-WR immune spleen cells to stimulation with treated VV-WR infected syngeneic cells***

6 day VV-WR immune spleen cells were depleted of specific subpopulations and incubated with VV-WR infected syngeneic cells which had been subjected to various treatments following infection with VV-WR for 1 h at 37°C. Incorporation of [^{3}H]-TdR during the final 4 h of a 48 h incubation was determined. Results are shown as the mean ± SD of quadruplicate wells.

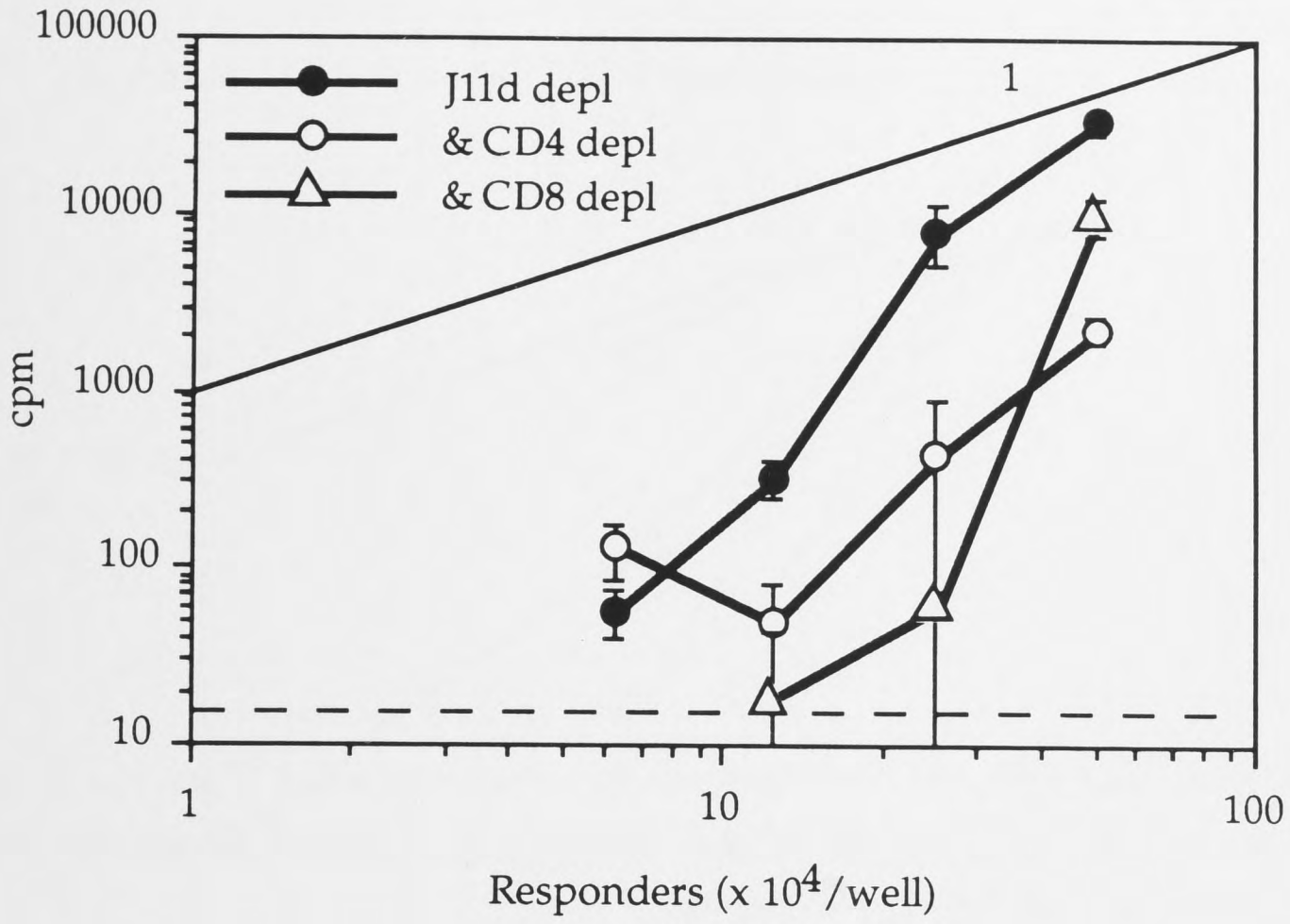


Figure 3.10 ***The cell number-proliferative response curve for 6 day immune spleen cells stimulated with UV-irradiated cells***

Six day VV-WR immune spleen cells were depleted of B cells and $CD4^+$ or $CD8^+$ T cells. Varying numbers of these responder cell preparations were incubated with 5 x 10^4 UV-irradiated virus infected syngeneic cells in a final volume of 200 µl. Incorporation of [^{3}H]-TdR during the final 4 h of a 48 h incubation was determined. Results are shown as the (mean - background) $\pm$ SD of quadruplicate wells. The thin line is included as a reference and represents a slope of 1. The dashed line represents 3 SD of machine background counts.

Figure 3.11 ***The cell number-proliferative response lines for 6 day immune spleen cells incubated with virus infected syngeneic cells following a variety of treatments***

Spleen cells taken from naive animals were infected with VV-WR for 1 h at 37°C. The cells were then either UV- or γ-irradiated, incubated with mitomycin C or left untreated and used as stimulator cells. 5 x 10^4 stimulator cells were incubated with varying numbers of B cell depleted, 6 day vaccinia immune spleen cells in a total volume of 200 µl. Incorporation of [^{3}H]-TdR during the final 4 h of a 48 h incubation was determined. Results are shown as the (mean - background) ± SD of quadruplicate wells. The thin lines are included as a reference and represent slopes of 1 and 2. The dashed line represents 3 SD of β-counter background.

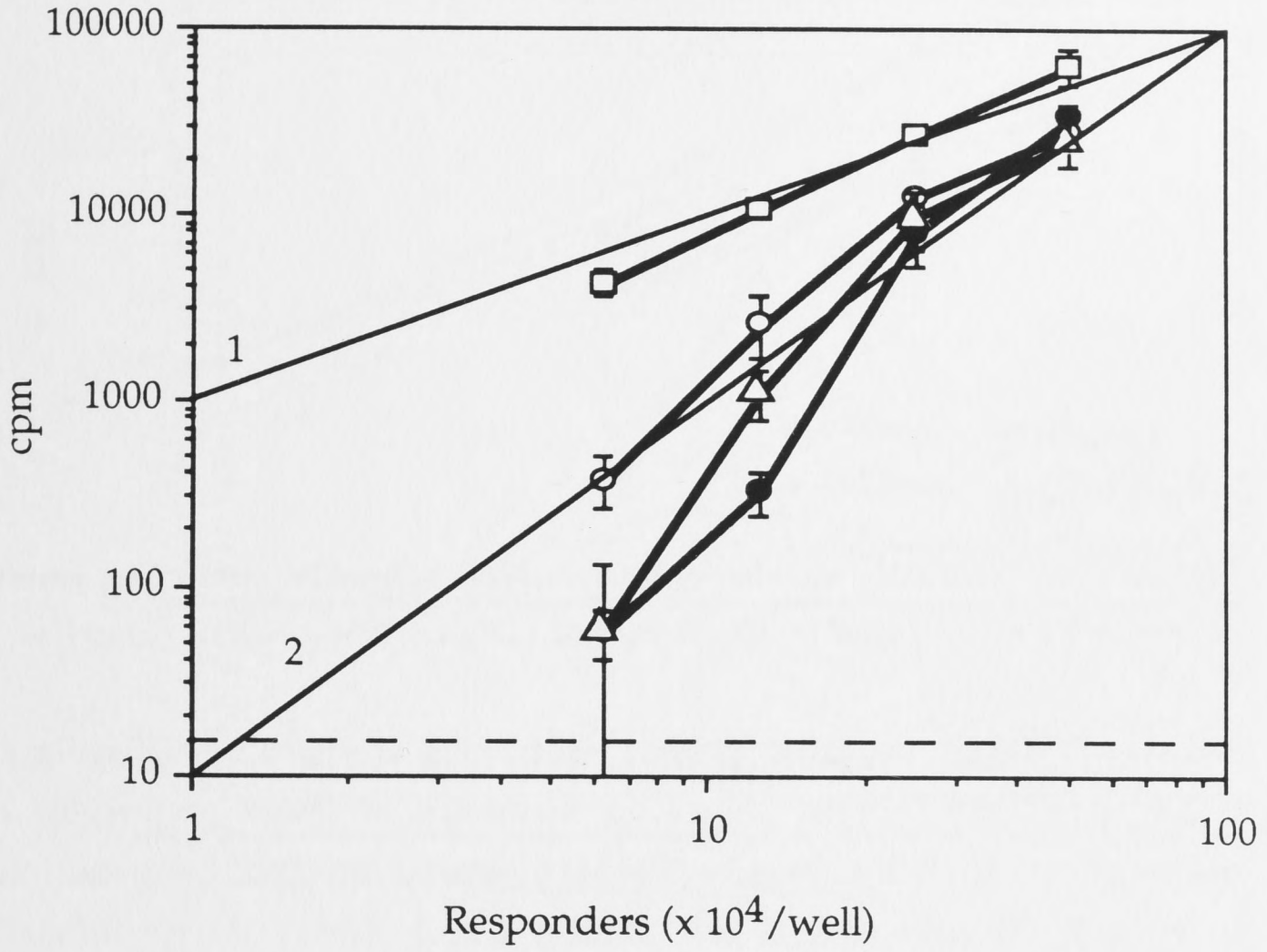
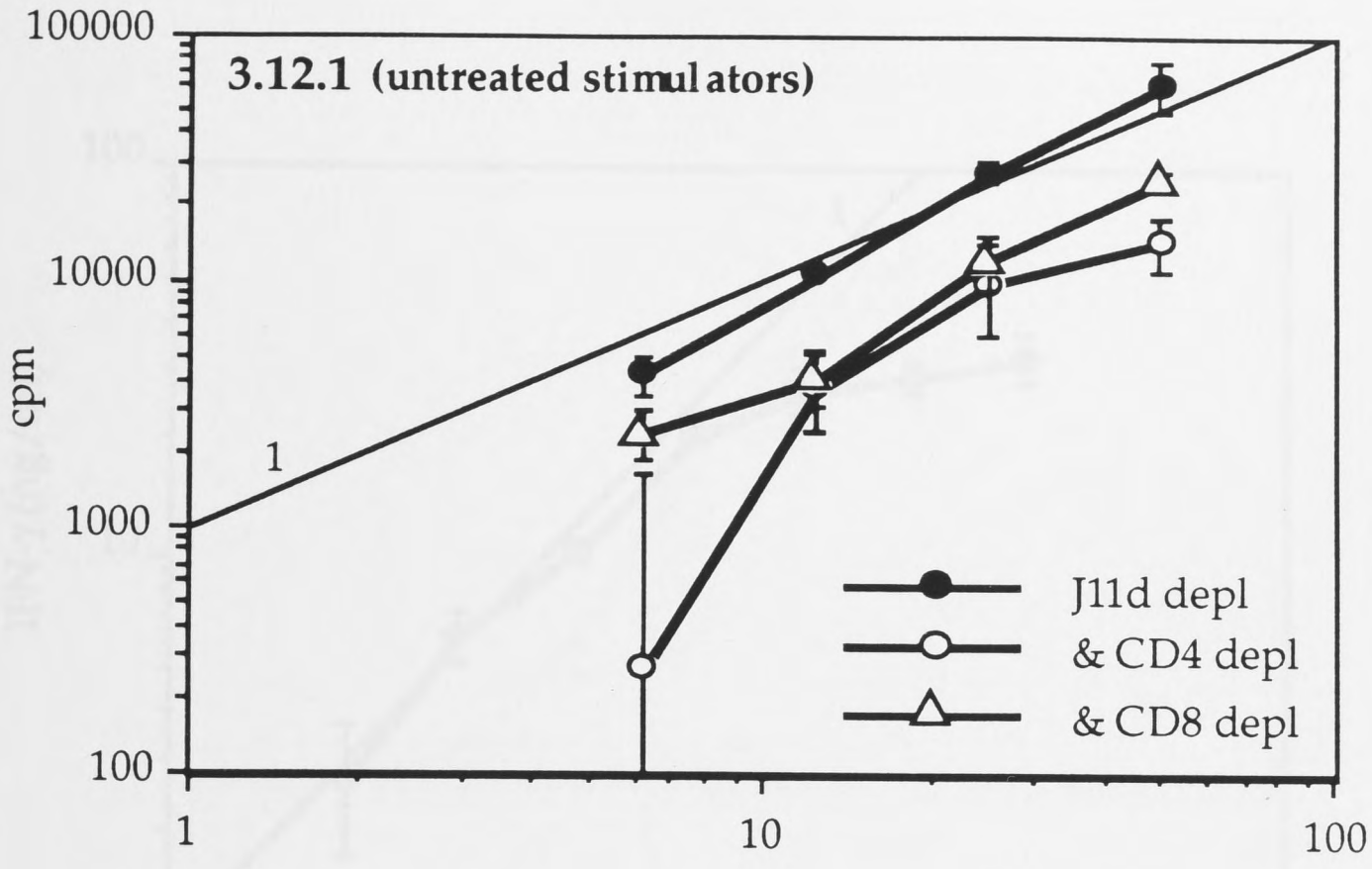
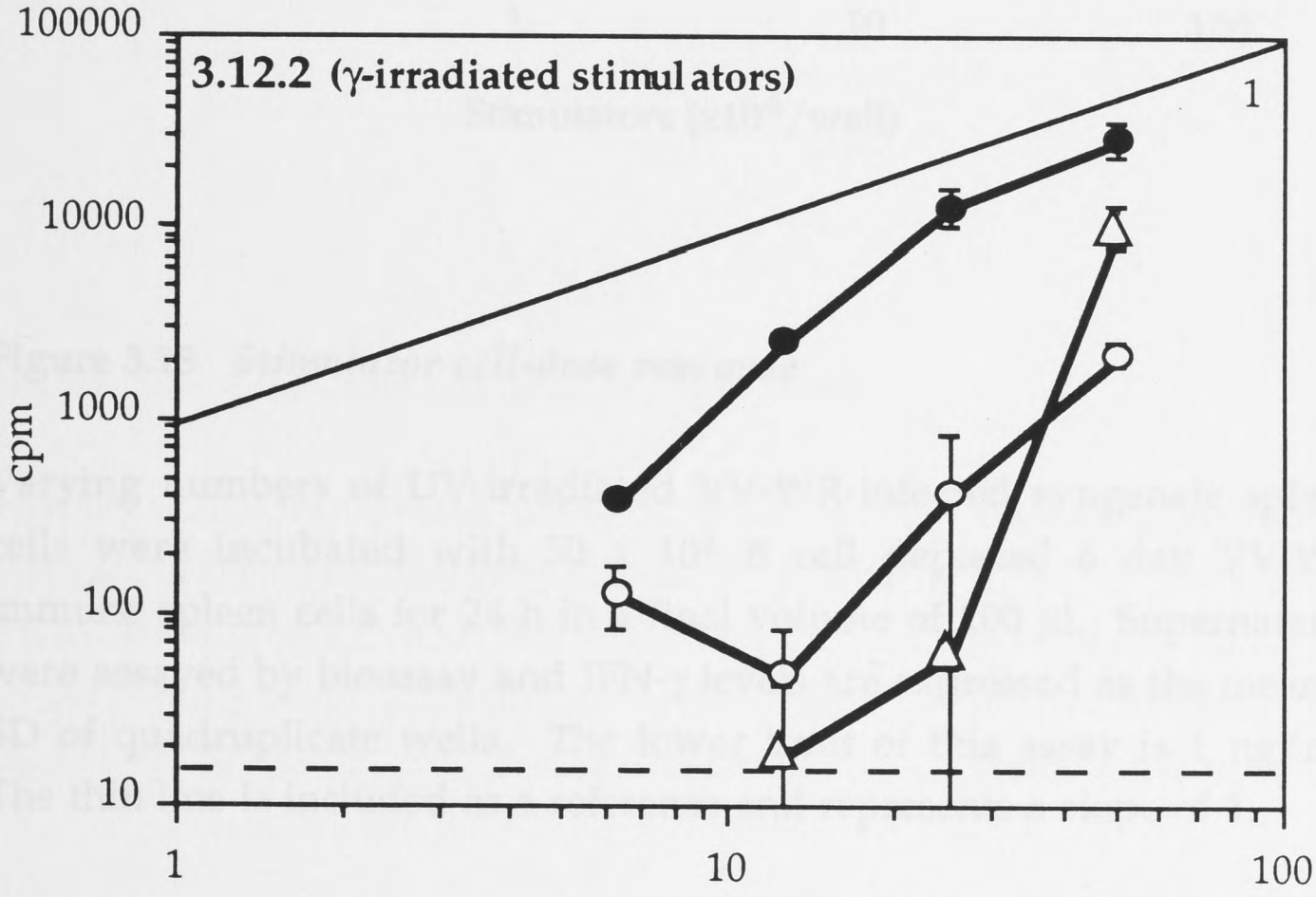

100000
10000
1000
100
10
cpm
1
2
1
10
100
Responders (x 10^4/well)
None
mitomycin C
Gamma irradiation
UV-irradiation

Figure 3.12 ***The cell number-proliferative response curve for immune spleen cells stimulated with untreated (1) and γ-irradiated (2) cells***

Six day VV-WR immune spleen cells were depleted of B cells and $CD4^+$ or $CD8^+$ T cells. Varying numbers of these responder cell preparations were incubated with 5×10^4 virus-infected syngeneic cells in a final volume of 200 µl. Figure 3.12.1 shows the proliferative response induced when the stimulators had not received any further treatment, whereas the stimulators had been γ-irradiated in Figure 3.12.2. Incorporation of $[^3H]$-TdR during the final 4 h of a 48 h incubation was determined. Results are shown as the (mean - machine background) $\pm$ SD of quadruplicate wells. The thin lines included as a reference represent a slope of 1. The dashed line represents 3 SD of the β-counter machine background counts.


3.12.1 (untreated stimulators)
100000
10000
1000
100
cpm
1
1
10
100
J11d depl
& CD4 depl
& CD8 depl


3.12.2 (γ-irradiated stimulators)
100000
10000
1000
100
10
cpm
1
1
10
100
Responders (x10^4/well)

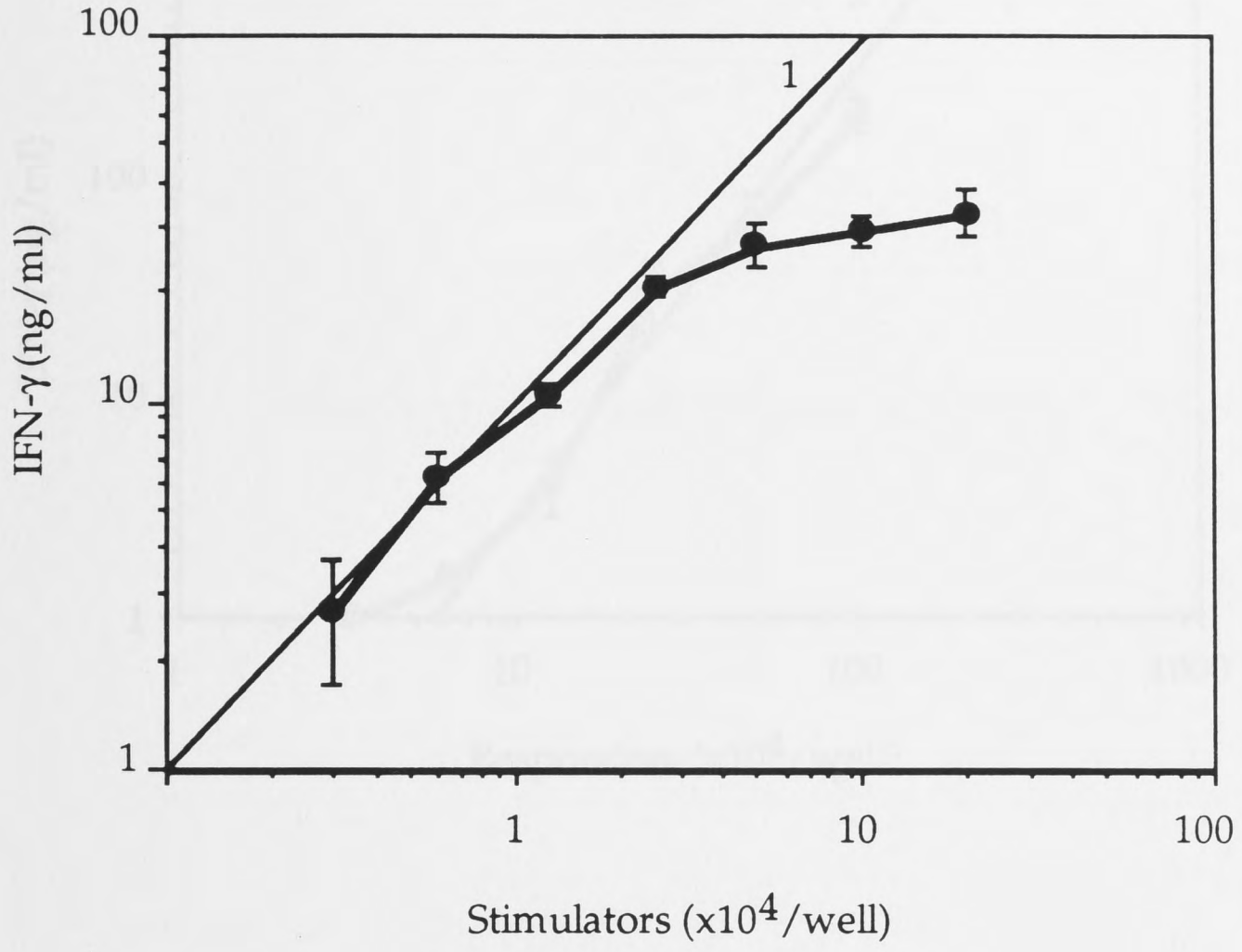


Figure 3.13 ***Stimulator cell-dose response***

Varying numbers of UV-irradiated VV-WR-infected syngeneic spleen cells were incubated with 50 x 10^4 B cell depleted 6 day VV-WR immune spleen cells for 24 h in a final volume of 200 µl. Supernatants were assayed by bioassay and IFN-γ levels are expressed as the mean ± SD of quadruplicate wells. The lower limit of this assay is 1 ng/ml. The thin line is included as a reference and represents a slope of 1.

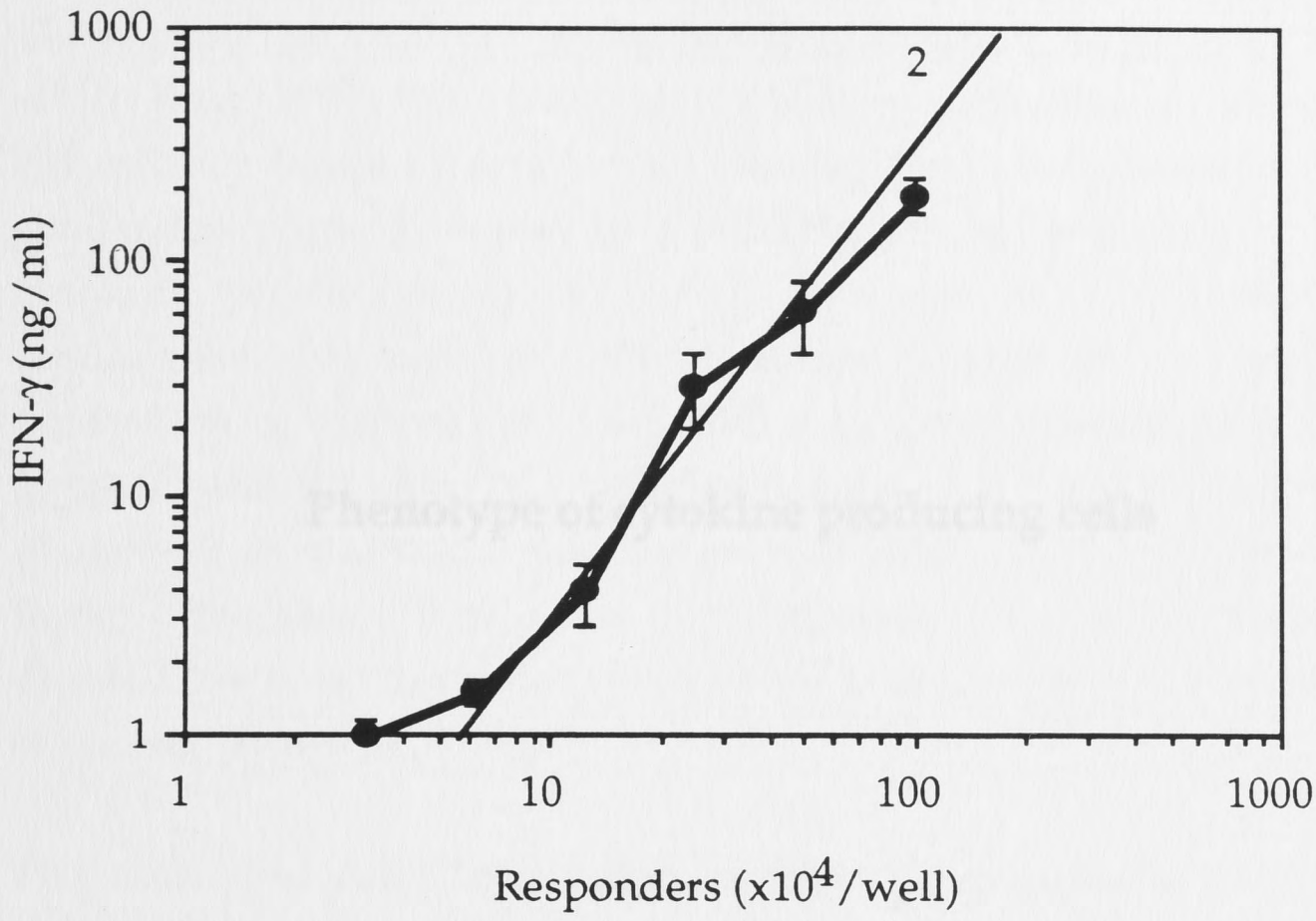


Figure 3.14 ***Responder cell dose response curve***

Varying numbers of B cell-depleted 6 day VV-WR immune spleen cells were incubated with 20 x 10^4 UV-irradiated virus-infected cells for 24 h in a final volume of 200 µl. IFN-γ levels in culture supernatant are expressed as the mean ± SD of quadruplicate wells. The lower limit of this assay is 1 ng/ml. The thin line is included as a reference and represents a slope of 2.

CHAPTER 4

Phenotype of cytokine producing cells

4.1 INTRODUCTION

Cytokines are produced by a large array of cell types, including macrophages, T cells, mast cells and endothelial cells (Kelso, 1989). There is a great deal of overlap in cytokine target range and function, which Paul (1989) has described as pleiotropy (multiple functions) and redundancy (more than one cytokine may mediate the same or a very similar function). This may be a reflection of the levels of control over cytokine production, rather than an overwhelming example of redundancy. For example, different induction signals will result in the production of distinct cytokines with a similar range of actions, while in other cases the effect of a cytokine depends not only on the target cell phenotype but also on the presence of other stimuli (Balkwill and Burke, 1989; Kelso, 1989). The demonstration that cytokine secretion is directed towards bound target cells adds another dimension to the level of control (Poo *et al.*, 1988).

The ability of cells to produce cytokines depends in part on the inducing stimulus. For example, alloantigens or mitogens stimulate both $CD4^+$ and $CD8^+$ T cells to produce IFN-γ (Kelso and MacDonald, 1982), whereas soluble antigens preferentially stimulate $CD4^+$ T cells to secrete IFN-γ (Biondi *et al.*, 1984). T cell clones have been extensively used to study cytokine expression. Two recent papers demonstrated that virus specific cytotoxic T lymphocyte clones release IFN-γ upon contact with target cells presenting viral antigen (Jassoy *et al.*, 1993; Kuwano *et al.*, 1993), while Taylor *et al.* (1989) showed that the protective effects of T cell clones against influenza virus was related to their ability to produce IFN-γ. Other cell types, such as NK cells, also secrete IFN-γ both in *in vitro* and *in vivo* situations in response to a variety of bacterial and viral infections (Guo *et al.*, 1992; Welsh, 1978).

TNF and IL-6 are also produced by a wide variety of cells. Activated macrophages are a major source of TNF. Macrophages can be activated to produce TNF by stimulation with cytokines, cross-linking of FcR and undigested particles (reviewed by Vassalli, 1992). $CD4^+$ T cells, both Th1 and Th2, and $CD8^+$ T cells also produce TNF, as can NK cells, mast cells, keratinocytes and astrocytes (Vassalli, 1992). The major source of IL-6 is the monocyte/macrophage (Aarden *et al.*, 1987), although it is also produced by T cells, B cells, endothelial cells, fibroblasts, keratinocytes and mast cells (Van Snick, 1990).

This chapter sets out to identify the cells in the virus immune responder population which are producing the antiviral cytokines, TNF, IFN-γ and IL-6.

4.2 METHODS

4.2.1

Mice	As described in Chapter 2.1
Vaccinia virus and infection of mice	As described in Chapter 2.2 and 2.3
Cell lines	As described in Chapter 2.6
Culture medium	As described in Chapter 2.4
Depletion of T cell populations	As described in Chapter 3.2.9

4.2.2 Virus

Herpes simplex virus type 1 strain SC16 (HSV-1, SC16) (Field *et al.*, 1979) was provided by Dr. A. Simmons of the Institute of Medical and Veterinary Science, Adelaide, South Australia. Stocks of the virus were propagated in Vero cells infected at 0.1 pfu/cell for 48 h. After this period the infected Vero cells were washed/scraped into the culture media and sonicated for 5 seconds at 50 watts using a Branson sonifier. The stocks were titrated on Vero cells and stored at -70°C. Stocks were provided by Dr. B. Lidbury.

4.2.3 Cell lines

Vero cells, a cell line derived from African green monkey kidney (ATCC, CCL 81), were maintained in F15, while α6310 cells (Karasuyama and Melchers, 1988) were maintained in MLC medium. The components of these media have been described in Chapter 2.4. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

4.2.4 Antibodies

The following antibodies were used for cell surface analysis by cytofluorimetry, to assess the efficacy of cell depletion protocols: anti-CD8, clone 53.6.7 (Ledbetter and Herzenberg, 1979) and anti-CD4, clone GK-1.5 (Dialynas *et al.*, 1983) hybridoma supernatants were used undiluted; biotinylated anti-Thy1.2, clone 30-H12 (Ledbetter and Herzenberg, 1979; Becton Dickinson, Mountain View, CA, USA) was used at 1/25; FITC-conjugated sheep anti-rat IgG (Silenus Laboratories, Hawthorn, Victoria, Australia) was used at 1/30 as a secondary antibody; Streptavidin fluorescein (Amersham Australia, North Ryde, NSW, Australia) was used at 1/40.

4.2.5 Depletion of cell sub-populations

Immune responder cells were washed and depleted of B cells and, if appropriate, $CD4^+$ or $CD8^+$ T cells, as described in Chapter 3.2.9. The efficacy of these T cell depletions was confirmed by flow cytometry, by demonstrating that the treatment with antibody against $CD4^+$ or $CD8^+$ and C resulted in less than 5% of the respective $CD4^+$ or $CD8^+$ T cell subset remaining.

Adherent cells were removed following one B cell depletion step by incubating 5×10^7 cells in a 60 x 15 mm petri dish in 5 ml medium for 1 h at 37°C. The non-adherent cells were washed off with warm medium and were incubated in a second petri dish for a further 30 min at 37°C. Non-adherent cells were collected and resuspended in the same volume as the J11d depleted cells.

4.2.6 Restimulation of immune spleen cells *in vitro*

J11d depleted responder cells were made up to a concentration of 5×10^6 cells/ml. Those responder cells depleted of additional subsets were then made up to this volume. Stimulator cells were prepared as described in Chapter 2.8 and, following UV-irradiation, were made up to 5×10^5 cells/ml. Cultures consisted of 100 µl of the stimulator cell preparation added to 100 µl of a responder cell preparation in a well of a 96 well flat bottomed plastic plate (Nunc). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. At the time of harvest, the plates were centrifuged at 200 g for 3 min and 180 µl of supernatant

was removed from each well. This was divided into three and frozen in wells of a 96 well round bottom plate. Thus, the supernatant from each culture was assayed for the cytokines TNF, IL-6 and IFN-γ.

4.2.7 Immunofluorescent staining

The efficacy of the cell depletion protocols was confirmed by flow cytometry, following staining the cells for the possible contaminating cell type. 1 x 10^6 B cell depleted spleen cells in MLC were pelleted by centrifugation at 200 g for 5 min. Medium was aspirated and the cells resuspended in 100 µl hybridoma supernatant or 50 µl of mAb diluted in MLC and incubated for 20 min on ice. Samples were centrifuged through 0.5 ml heat-inactivated FCS (Flow Laboratories, North Ryde, Australia) and resuspended in 50 µl of the appropriate secondary antibody and incubated for a further 20 min on ice. The cells were centrifuged through FCS and resuspended in 0.5 ml cold PBS for flow cytometry. When flow cytometry was performed the day after staining, the cells were fixed in phosphate-buffered 1% paraformaldehyde, pH 7.4 (10 g/l paraformald-ehyde, 16.83 g/l $NaH_2PO_4.2H_2O$, 3.85 g/l NaOH, 5.4 g/l glucose).

4.2.8 Flow cytometry

Fluorescent labelled samples were analyzed on a FACScan (Becton Dickenson) using 100mW of 488 nm laser light to excite FITC. Fluorescence emissions of FITC were detected through a 530 nm band pass filter. 10,000 events were collected for each sample and gating was performed using forward vs 90° light scatter. Data analysis was performed using a Hewlett Packard workstation with the the Becton Dickinson Lysis II program. The percentage of positively stained cells was calculated relative to control cells stained with the fluorochrome-conjugated reagents.

4.3 RESULTS

4.3.1 Phenotype of the cytokine-producing cells

4.3.1.1 Adherent cells were major IL-6 producers

To identify the cell population responsible for the production of the high levels of IL-6, immune responder cells were depleted of either $CD4^+$ or $CD8^+$ T cells, or adherent cells. IL-6 was detected in the supernatant of the control cell population within 4 h and levels increased steadily throughout the culture period (Figure 4.1.1). Depletion of either $CD4^+$ or $CD8^+$ T cells from the responder cell population had little effect on the level of IL-6 produced. Removal of adherent cells, however, caused a large (70%) reduction of IL-6 produced ($p<0.05$), eg. from 7.5 AU/ml to 2.2 AU/ml at 18 h (Figure 4.1.2). The cellular origin of the remaining IL-6 was not determined. This suggests that the major source of IL-6 production was the adherent cell population and this production was not dependent on either $CD4^+$ or $CD8^+$ T cells.

4.3.1.2 Adherent cells were also the major producers of TNF

With the new culture conditions, (see 4.2.6 ie. reduced number of cells), the profile of TNF production was different to that described in Chapter 3.3.3. TNF levels were still increasing at 24 h of culture, whereas in cultures with a greater concentration of cells a plateau was reached by 12 h. Depletion of $CD4^+$ T cells from the responder population resulted in a significant increase (50 - 100%) in TNF production ($p<0.05$ in 3 out of 4 experiments) within the first 18 h of culture (Figure 4.2.1 and Table 4.1). In contrast, removal of $CD8^+$ cells had no effect on levels of TNF produced. Figure 4.2.2 shows that removal of adherent cells resulted in a dramatic decrease in TNF production, from 400 pg to 150 pg 24 h after initiation of the culture ($p<0.05$). The TNF that was produced by the adherent cell depleted population occurred approximately 6 h later than in non-depleted cultures.

4.3.1.3 IFN-γ production was dependent on $CD4^+$ T cells and adherent cells

Levels of IFN-γ produced by the immune responder cells were not detectable until after 4 h of culture. The delay of several hours, compared to the IFN-γ response in the 1 ml cultures described in Chapter 3, probably results from the culture conditions in which fewer cells were added per well. The production of IFN-γ increased logarithmically and began to plateau at 12 h, in similar kinetics to that seen with the culture conditions described in Chapter 3. Depletion of the $CD4^+$ T cell population resulted both in a delay and decrease in the production of IFN-γ (Figure 4.3.1). Little or no IFN-γ was produced in the first 8 h of culture and by 24 h the level of IFN-γ was still only 14% that of the non-depleted population. Depletion of $CD8^+$ T cells, however, resulted in little change in the IFN-γ profile. Removal of adherent cells also resulted in a significant decrease in IFN-γ production late in the culture period, eg from 36 ng/ml to 22 ng/ml at 24 h ($p<0.005$) (Figure 4.3.2).

4.3.1.4 The adherent population contained a small number of T cells

Adherent cells were isolated from the immune responder cell population by two incubations on plastic with non-adherent cells removed by vigorous washing. To investigate the possibility that activated T cells were present in the adherent population, the cells were scraped off the plastic and incubated with anti-Thy1.2 antibody and analysed by flow cytometry. The adherent cells, which made up only 1% of the original responder cells, contained 11% Thy1.2 positive T cells. Thus the majority of the cells removed by the adherence step were not T cells, and are believed to be macrophages and monocytes.

4.3.2 IL-6 and TNF production were not dependent on T cells

Depletion of both $CD4^+$ and $CD8^+$ T cells did not significantly reduce levels of TNF or IL-6 produced by the immune responder cells. However, the IFN-γ titres reached only 4% that of the non-depleted population at 20 h (Table 4.1). This indicated that IFN-γ production was totally dependent on the presence of T cells, whereas TNF and IL-6 production was T cell independent.

Table 4.1 ***Effect of T cell depletion on cytokine levels***

Antibody treatment of responders	IL-6 (AU/ml)	IFN-γ (ng/ml)	TNF (pg/ml)
J11d + C	7.8 ± 1.1	37.7 ± 3.1	210 ± 80
J11d + anti-CD4 + C	ND	13.9 ± 1.3	450 ± 70
J11d + anti-CD8 + C	9.6 ± 1.7	31.9 ± 2.5	220 ± 60
J11d + anti-CD4 + anti-CD8 +C	12.4 ± 1.7	1.6 ± 0.4	250 ± 40

Spleen cells from mice immunized 6 days earlier were treated as shown and cultured for 20 h with VV-WR infected UV-irradiated syngeneic cells. The IL-6 content of the SN was determined by B9 assay while the TNF and IFN-γ levels were determined by ELISA. The cytokine levels are expressed as the mean ± SD of quadruplicate wells.

4.3.3 IL-6 and TNF production were not antigen specific

The antigen-specificity of an immune response is due to the recognition, by specific T cells, of antigen in association with MHC on antigen presenting cells. The T cell independence of IL-6 and TNF production suggested that the production of these cytokines may not be antigen specific. Cells infected with HSV were used as stimulator cells in culture with vaccinia virus immune responder cells. The VV-immune responder cells produced levels of IL-6 and TNF in response to HSV similar to those produced when the immunizing virus was used (Table 4.2). In contrast, IFN-γ was not detected in cultures stimulated with HSV. With HSV-infected stimulator cells, non-immune cells were able to produce low levels of TNF and IL-6.

Table 4.2 ***Production of cytokines by non-immune or vaccinia virus immune spleen cells stimulated with VV or HSV***

		Cytokine		
Spleen cells	Stim.virus	IFN-γ (ng/ml)	IL-6 (AU/ml)	TNF (pg/ml)
Non-immune	vv-WR	<0.3	0.13 ± 0.01	<50
VV-immune	vv-WR	25 ± 3.5	6.76 ± 0.72	350 ± 80
Non-immune	HSV	<0.3	1.49 ± 0.22	110 ± 10
VV-immune	HSV	<0.3	4.5 ± 0.9	300 ± 20

Spleen cells from non-immune mice or mice immunized 6 days earlier with VV-WR were depleted of B cells and cultured for 24 h with syngeneic spleen cells which had been infected with VV or HSV for 1 h and then UV-irradiated. The cytokine levels of the SN are expressed as the mean ± SD of quadruplicate wells.

4.3.4 Memory cells and IFN-γ production

To determine whether memory cells could be induced to produce cytokines, mice were injected with vaccinia virus i.v. and spleens were removed three weeks later for use in cultures. These 3 week vaccinia immune spleen cells did not produce significant levels of TNF when cultured with vaccinia virus infected UV-irradiated stimulator cells (Table 4.3). The IL-6 titre was also low and by 48 h was less than 5% of that observed in cultures containing responder cells from mice at the peak of the CTL response to VV-WR. However, IFN-γ was produced by immune responder cells, although significant levels did not appear until 48 h of culture.

Table 4.3 ***TNF and IL-6 production was not associated with memory cells***

Time (h)	IFN-γ (ng/ml)		TNF (pg/ml)		IL-6 (AU/ml)	
	6 day [a]	3 week [b]	6 day [a]	3 week [b]	6 day [a]	3 week [b]
12	13.2 ± 1.9	< 0.3	170 ± 35	< 100	5.8 ± 1.2	0.2 ± 0.1
24	28.6 ± 6.8	< 0.3	260 ± 45	110 ± 70	19.3 ± 4.7	0.4 ± 0.1
48	49.3 ± 4.1	2.8 ± 0.7	510 ± 150	< 100	22.0 ± 5.0	0.8 ± 0.1

Spleens were removed from male mice which had been injected with VV-WR i.v. either 6 days or 3 weeks earlier. 50×10^4 B-cell depleted responder cells were incubated with 5×10^4 UV-irradiated, vaccinia virus infected stimulator cells. Supernatant was harvested at the times indicated. Cytokine levels are expressed as mean ± SD of quadruplicate wells.

[a] Mice injected with VV-WR 6 days earlier.

[b] Mice injected with VV-WR 21 days earlier.

4.4 DISCUSSION

The immune response to a virus infection involves a variety of cell types. The peak of the CTL response to vaccinia infection occurs 6 days post infection (Koszinowski and Thomssen, 1975). At this time in the response the relative numbers of $CD4^+$ and $CD8^+$ T cells are equal and account for 70% of the non-B cells in the spleen (Ruby *et al.*, 1993), whereas the NK cytotoxic activity has declined and is barely detectable (Karupiah, 1990). The results in Chapter 3 showed that spleen cells taken from mice at the peak of the CTL response to vaccinia virus secreted TNF, IFN-γ and IL-6 after restimulation *in vitro* .

IL-6 is known to be produced by a variety of cell types, including macrophages /monocytes and T cells (Horii *et al.*, 1988; Tosato *et al.*, 1988). In cultures stimulated with UV-irradiated virus infected cells, removal of either the $CD4^+$ or $CD8^+$ T cells from the responder cell population had little effect on the production of IL-6 (Figure 4.1). Depletion of the adherent cell population decreased the level of IL-6 produced by as much as 70%, indicating that these cells were the major source of IL-6. This result is in agreement with Aarden *et al.* (1987) who have reported that monocytes were the major source of IL-6 in freshly isolated cell preparations.

The adherent cells were also the major producers of TNF in this culture system (Figure 4.2). This has also been seen in a study of PBMC cultured with Sendai virus (Aderka *et al.*, 1986). Removal of the $CD4^+$ T cells significantly enhanced levels of TNF in the culture supernatant. In contrast, $CD8^+$ T cell depletion did not alter TNF levels. Finally, cultures in which both $CD4^+$ and $CD8^+$ T cells were removed had levels of TNF which were similar to control cultures. A possible explanation for these results is that the $CD8^+$ cells are a major source of TNF, but in the presence of $CD4^+$ T cells their production is down-regulated. $CD8^+$ T cells have been shown to produce TNF (Fong and Mosmann, 1990) and there are examples of virus specific CTL clones releasing TNF upon exposure to their target antigen (Jassoy *et al.*, 1993; Kuwano *et al.*, 1993).

It would appear that the IL-6 and TNF production by adherent cells was not T cell dependent in this system, as depletion of both $CD4^+$ and $CD8^+$ T cells had no effect on production of these cytokines (Table 4.1).

It has been shown that some viruses are able to trigger cytokine release from monocytes, *in vitro* (Aderka *et al.*, 1986; Houde and Arora, 1990). Culturing human monocytes with coxsackievirus 3B induced IL-1, IL-6 and TNF expression by the cells, and IL-1 and IL-6 were still produced when infectivity of the virus was destroyed by UV-irradiation (Henke *et al.*, 1992). Also, UV-irradiated human herpes virus was able to stimulate IL-6 and TNF production by PBMC cultures, suggesting that virus particle-cell contact is sufficient to enhance monokine release (Gosselin *et al.*, 1992). A recent study of a murine model of influenza showed an increase in IL-1α, IL-1β, IL-6 and TNF production by lung resident cells, suggesting the alveolar macrophages participate actively in the early response against the virus (Hennet *et al.*, 1992). It is interesting to note that vaccinia immune responder cells cultured with HSV-infected UV-irradiated cells produced three times the amount of TNF and IL-6 as non-immune cells (Table 4.2). The vaccinia virus immune responder cells contain a population of activated macrophages (Blanden, 1982), which may have been primed *in vivo* by T cell-dependent lymphokines such as IFN-γ (Stein and Gordon, 1991), and these appear to be triggered to release higher levels of monokines on contact with viral antigen. The macrophages seem to lose their primed status over time, as cells taken from mice immunized three weeks before restimulation *in vitro* did not produce significant levels of TNF and IL-6. This may reflect a mechanism of regulation over cytokine production. Both TNF and IL-6 have been shown to bring about a cascade of events, eg. TNF activates several different cell types, including macrophages, T cells, NK cells and endothelial cells (Vassalli, 1992), while IL-6 acts on T and B cells and induces acute phase response and fever (Van Snick, 1990). During a period of acute infection, it would be advantageous to the host to have macrophages moving throughout the body able to respond rapidly to virus antigen which may have escaped the T cell response. Thus, a co-ordinated response by T cells at the focus of infection and surveillance by mobile macrophages would ensure the antigen was not able to escape detection and thus, would not persist. However, upon clearance of the antigen, the possible detrimental effects of inappropriate TNF and IL-6 production would necessitate a down-regulation of the cells responsible for their production. This may be achieved through immunosuppressive cytokines, such as IL-10 or TGF-β, which suppress macrophage effector activity and appear late in the immune response (Sher *et al.*, 1992; Tsunawaki *et al.*, 1988).

Several groups have suggested recently that the $CD8^+$ T cells control virus infections by an IFN-γ-dependent mechanism (Klavinskis *et al.*, 1989; Ruby and Ramshaw, 1991). However, in this *in vitro* model the $CD8^+$ T cells were not the major producers of IFN-γ, as their removal had little effect on the level of IFN-γ produced. Removal of $CD4^+$ T cells, on the other hand, delayed production by 8 h and only low levels were detected by 24 h. These low levels are believed to be produced by $CD8^+$ T cells as depletion of both $CD4^+$ and $CD8^+$ T cells resulted in less than 5% the level of IFN-γ detected in complete cultures. Thus, production of the majority of IFN-γ in this system was dependent on the $CD4^+$ T cells. The ability of $CD4^+$ T cells to produce IFN-γ may explain why β2 microglobulin disrupted mice, although lacking $CD8^+$ cells, are able to resolve vaccinia virus infection via a $CD4^+$ mediated mechanism (Spriggs *et al.*, 1992). However, care must be taken in interpreting these *in vitro* results as it has been shown recently, using single cell analysis, that the majority of the cells producing IFN-γ during a vaccinia virus infection were $CD8^+$ T cells (Ruby *et al.*, 1993). In contrast, the *in vitro* restimulation of spleen cells taken from mice during a vaccinia virus infection induced production of IFN-γ which was dependent on $CD4^+$ T cells. Production of IFN-γ by $CD4^+$ T cells in a secondary culture has been reported (Budd *et al.*, 1987; Kelso and Glasebrook, 1984). Spleen cells taken from mice immunized three weeks earlier also produced IFN-γ in an antigen-specific manner suggesting that IFN-γ producing cells are able to persist beyond the period of virus infection.

The results from this *in vitro* study indicate that T cells removed from mice at the peak of the CTL response to vaccinia virus infection can be restimulated to produce high levels of IFN-γ. Using flow cytofluorimetric analysis to simultaneously detect cytokine production and T cell phenotype, Ruby *et al.* (1993) demonstrated that of splenocytes removed from mice at this stage of infection, 25.6% were $CD8^+$/IFN-γ^+ and 13.4% were $CD4^+$ /IFN-γ^+. The increases in IFN-γ production described are beyond what can be expected as a specific response to the virus. For example, the frequency of influenza specific CTL was estimated to be only 1/2500 following virus clearance (Owen *et al.*, 1988). This would indicate that a large proportion of the cells increasing cytokine expression in these restimulated cultures must be responding to non-antigen-specific factors. Similarly, of the

bronchoalveolar lavage cells obtained from mice during a secondary infection to influenza A, at least 20% showed increased expression of IFN-γ mRNA (Carding *et al.*, 1993). Yang *et al.* (1989) observed that spleen cells taken from mice infected with a variety of viruses, including vaccinia virus, generated allospecific CTL coincidental with the generation of virus-specific CTL. This generation of CTL was dependent on $CD4^+$ secretory factors, which suggested that lymphokines produced as a consequence of virus infection may act to stimulate the proliferation and activation of CTL not specific to the challenging virus. These cells may have direct cytotoxic activity *in vivo*, or secrete cytokines which will amplify other clearance mechanisms, and possibly enhance the proliferation of $CD8^+$ T cells (Nahill and Welsh, 1993). Thus, the increase in IFN-γ production by T cells upon incubation with vaccinia virus-infected stimulator cells may be a result of polyclonal stimulation during the normal response to virus infection, as described by Yang *et al.* (1989).

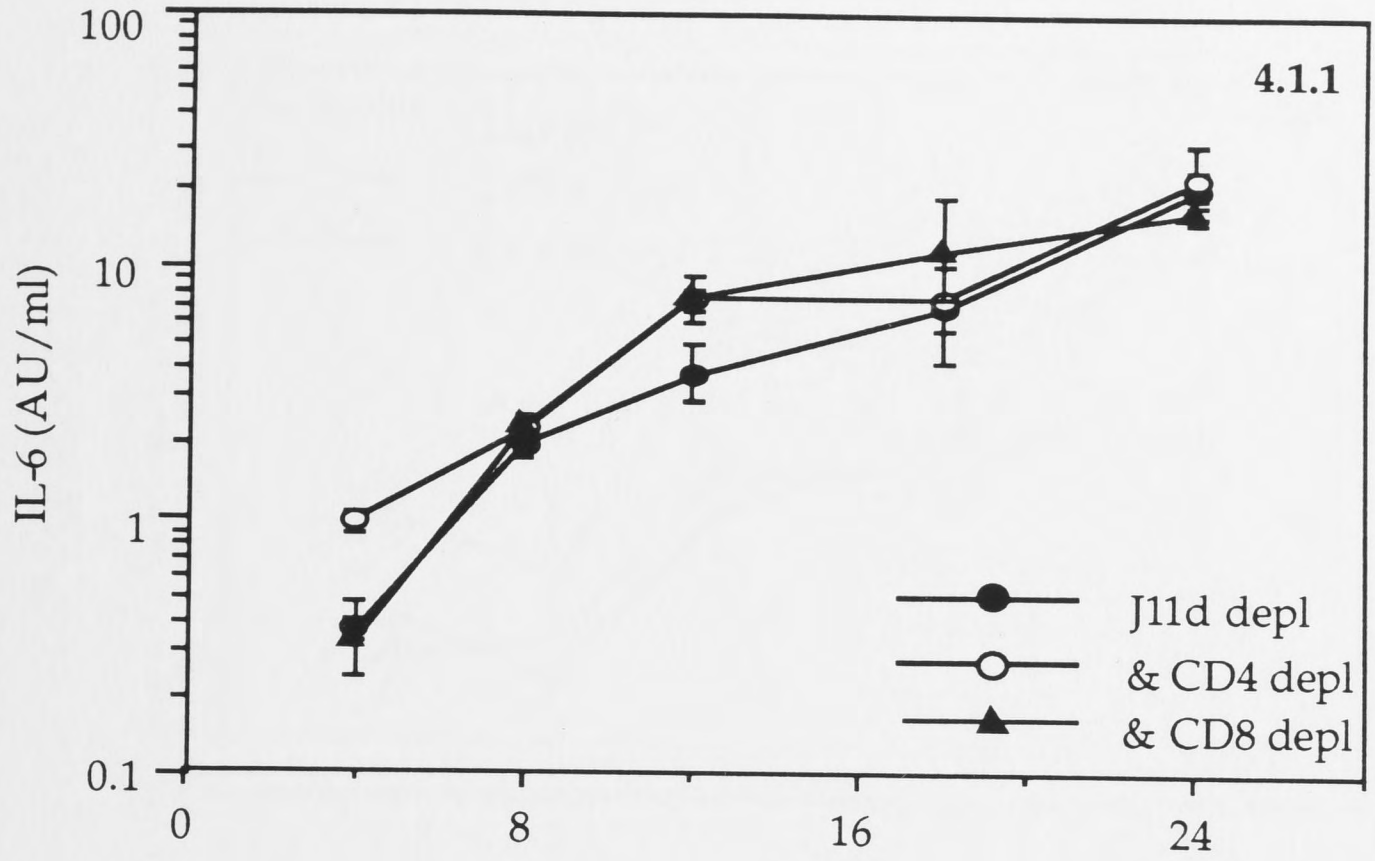
The removal of adherent cells also led to a decrease in IFN-γ production (40%). Two interpretations of this result are that the adherent cells themselves produce IFN-γ after 12 h in culture, or that the adherent cells are required to stimulate another population to produce IFN-γ, perhaps via another cytokine. These results do not distinguish between these two possibilities. Others have reported that depletion of adherent cells from human tonsil cultures resulted in a 50% reduction in IFN-γ-producing cells (Czerkinsky *et al.*, 1991), while lymph node cells produced greater IFN-γ levels when stimulated in the presence of adherent cells (Höiden and Möller, 1991), again suggesting a requirement for an adherent cell population. It is not known whether the adherent cell induction of IFN-γ production is through secretion of soluble molecules, such as IL-6 or IL-1, that affect T cells, or by direct accessory cell-T cell interaction via surface molecules.

4.5 SUMMARY

Depletion of individual cell populations from the vaccinia immune responder cells enabled the identification of the cells producing the antiviral cytokines. The adherent population was the major producer of TNF and IL-6. The secretion of these cytokines was not antigen-specific and did not require the presence of T cells. This may be a demonstration of first order defence mechanisms, which would come into play in the host defence to virus infection, before the antigen-specific T cell response. IFN-γ production was T cell dependent, with the majority dependent on $CD4^+$ T cells. Adherent cells were also shown to play a role in production of maximal levels of this cytokine. IFN-γ production was antigen-specific and the producing cells were able to persist beyond the period of virus infection.

Figure 4.1 ***Effect of cell depletion on the kinetics of IL-6 production***

B cell depleted vaccinia immune spleen cells were stimulated with vaccinia virus-infected cells in a 200 µl volume. The responder cells were also depleted of $CD4^+$ or $CD8^+$ T cells (4.1.1) or the adherent cells were removed (4.1.2). IL-6 levels are expressed as the mean $\pm$ SD of quadruplicate wells.


4.1.1
100
10
1
0.1
IL-6 (AU/ml)
0
8
16
24
J11d depl
& CD4 depl
& CD8 depl

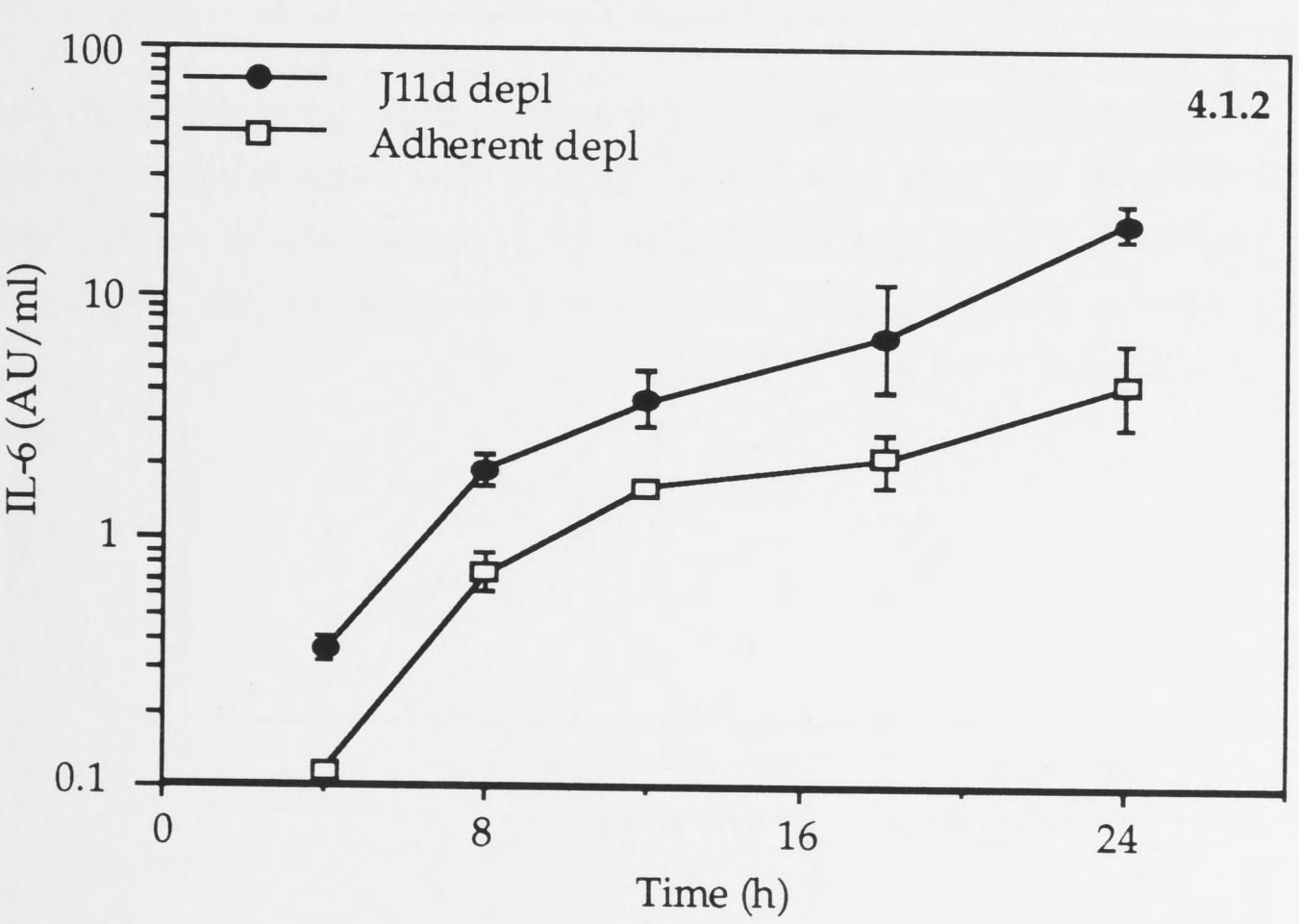
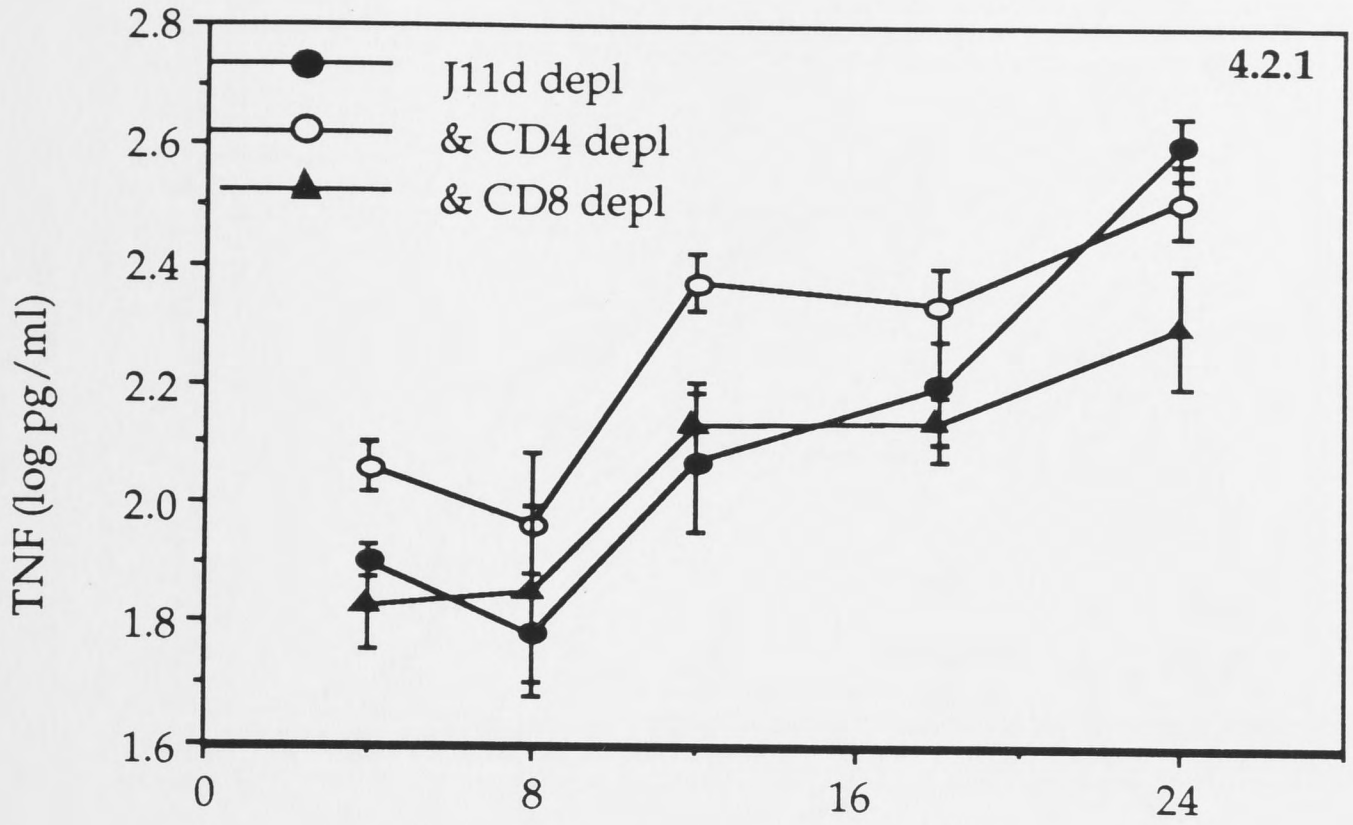

4.1.2
100
10
1
0.1
IL-6 (AU/ml)
0
8
16
24
Time (h)
J11d depl
Adherent depl

Figure 4.2 ***Effect of cell depletion on the kinetics of TNF production***

B cell depleted vaccinia immune spleen cells stimulated with vaccinia virus-infected cells in a 200 µl volume. The responder cells were also depleted of $CD4^+$ or $CD8^+$ T cells (4.2.1) or the adherent cells have been removed (4.2.2). TNF levels are expressed as the mean $\pm$ SD of quadruplicate wells.


4.2.1
J11d depl
& CD4 depl
& CD8 depl
TNF (log pg/ml)
2.8
2.6
2.4
2.2
2.0
1.8
1.6
0
8
16
24

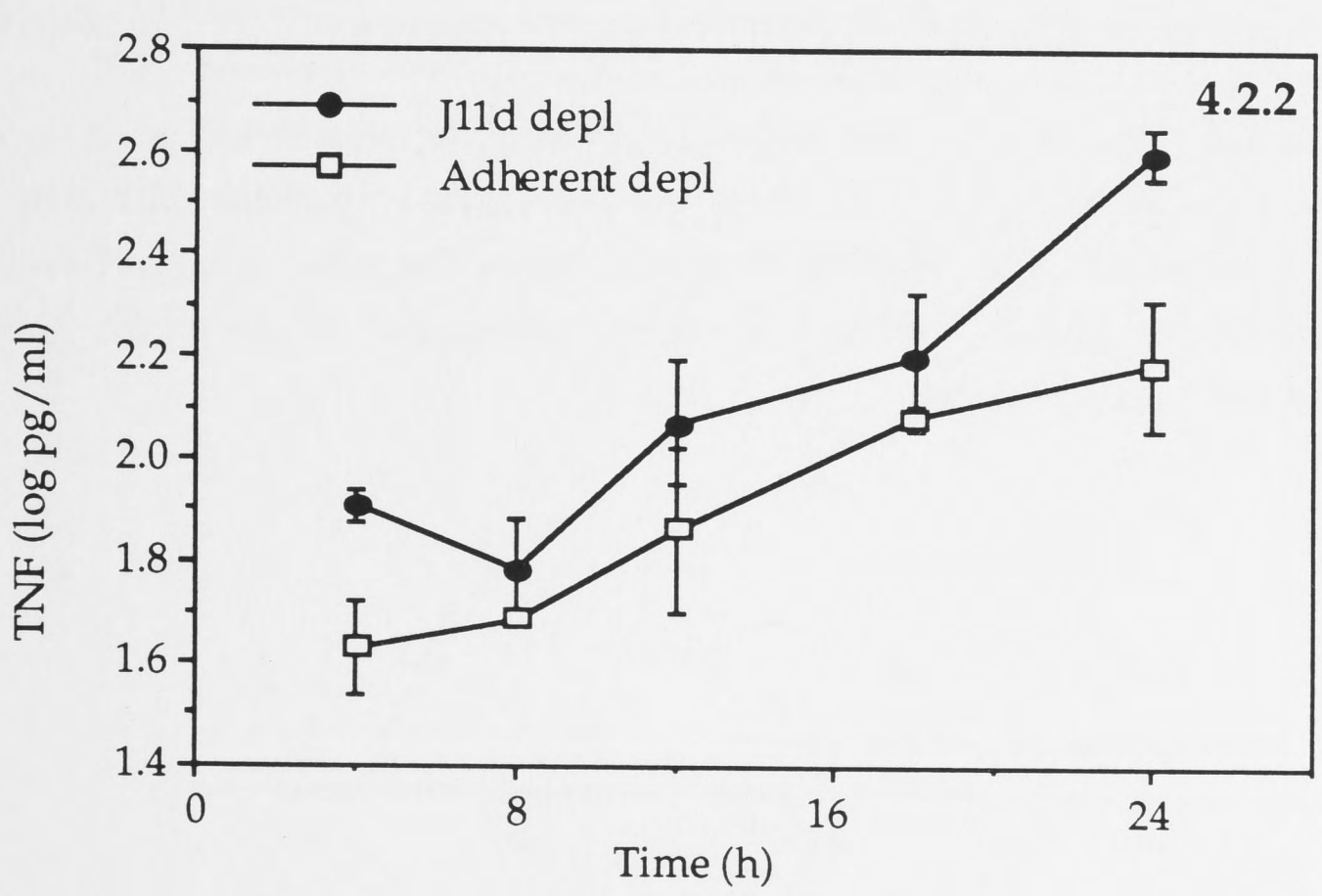
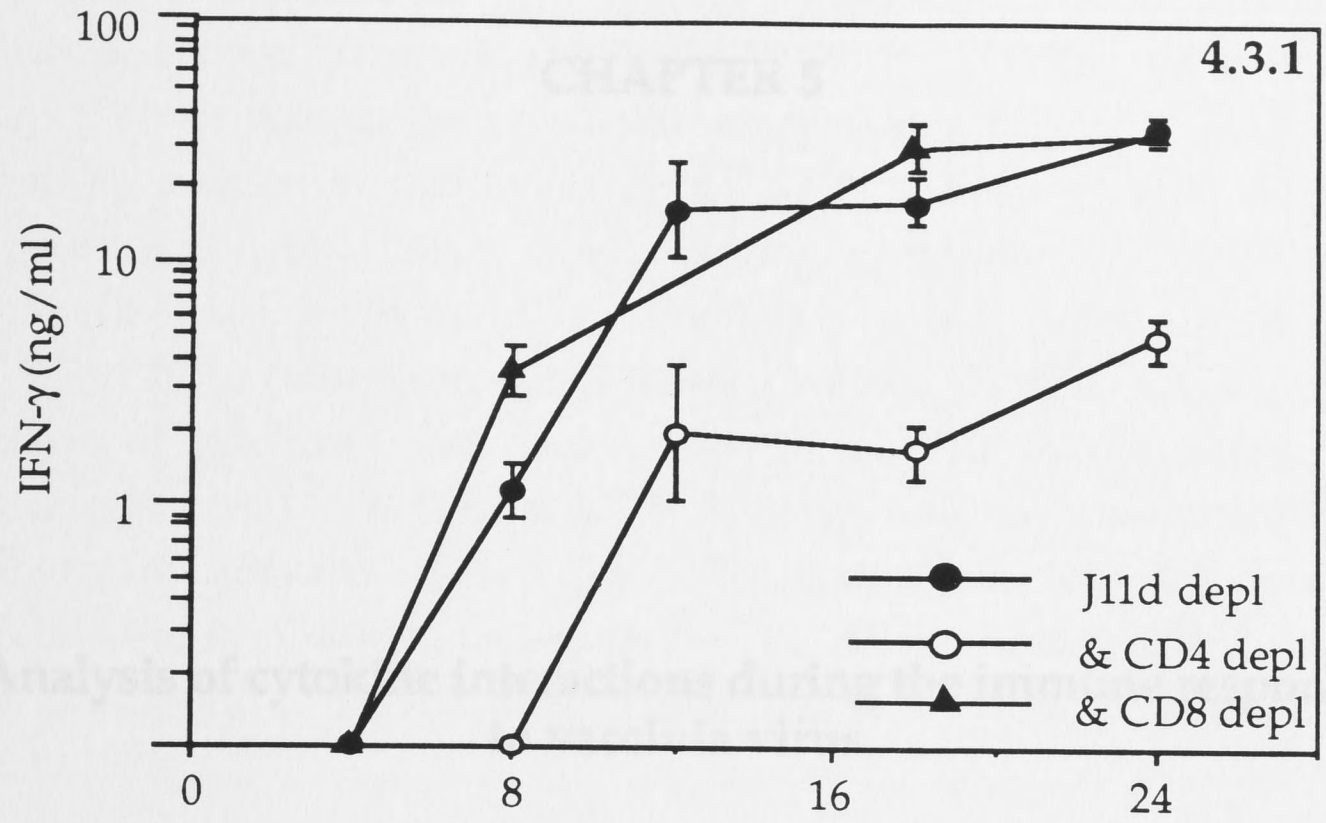
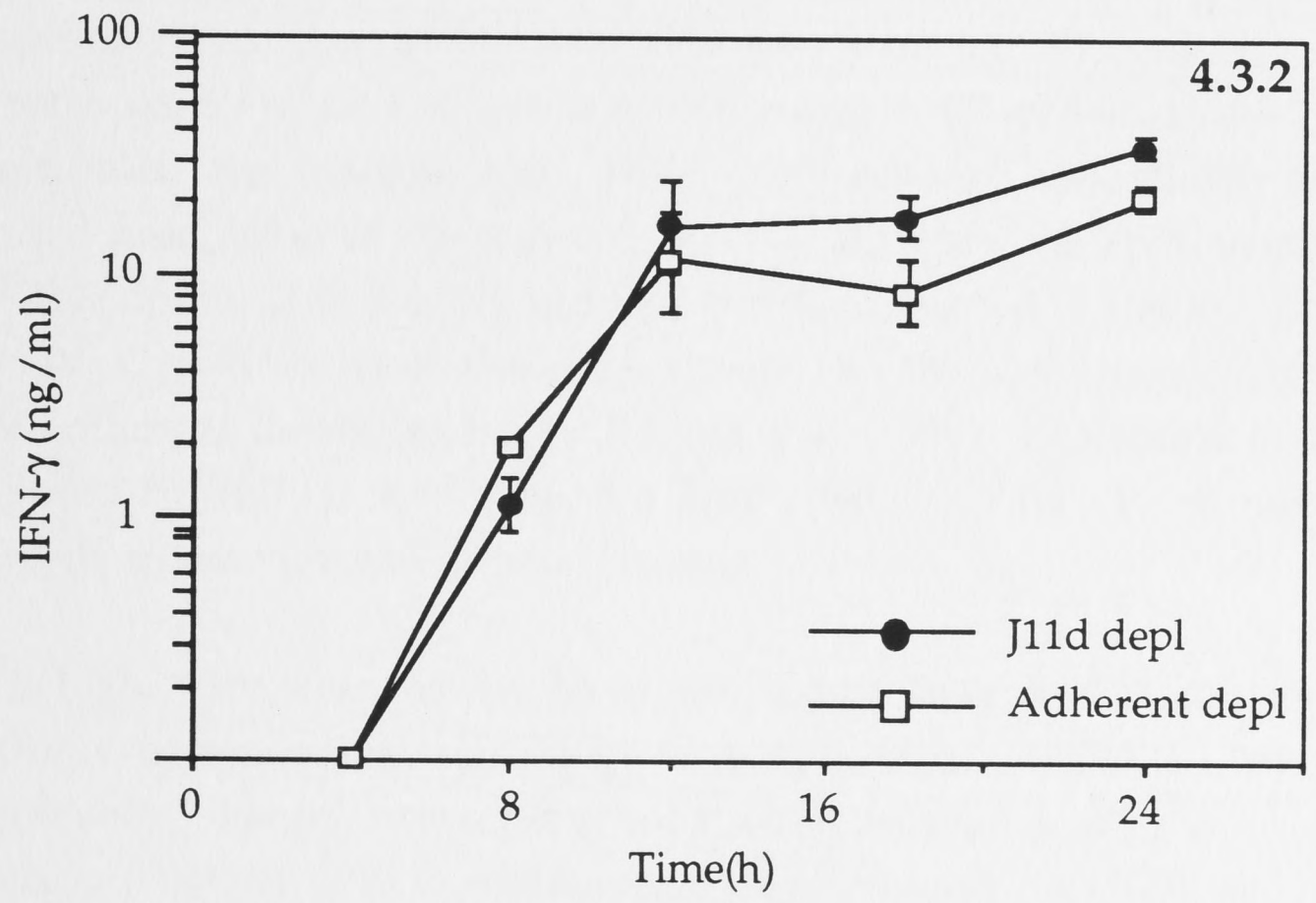

4.2.2
J11d depl
Adherent depl
TNF (log pg/ml)
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
0
8
16
24
Time (h)

Figure 4.3 ***Effect of cell depletion on the kinetics of IFN-γ production***

B cell depleted vaccinia immune spleen cells stimulated with vaccinia virus-infected cells in a 200 µl volume. The responder cells were also depleted of $CD4^+$ or $CD8^+$ T cells (4.3.1) or the adherent cells have been removed (4.3.2). IFN-γ levels are expressed as the mean ± SD of quadruplicate wells.


4.3.1
100
10
1
IFN-γ (ng/ml)
0
8
16
24
J11d depl
& CD4 depl
& CD8 depl


4.3.2
100
10
1
IFN-γ (ng/ml)
0
8
16
24
Time(h)
J11d depl
Adherent depl

CHAPTER 5

Analysis of cytokine interactions during the immune response to vaccinia virus

5.1 INTRODUCTION

A wealth of evidence has revealed that cytokines interact in a complex network *in vivo* (Balkwill and Burke, 1989; Paul, 1992). $CD4^+$ T cells play a major role in the regulation of cytokines produced during an immune response with two types having been clearly defined (Mosmann *et al.*, 1986). Cells belonging to the Th1 phenotype synthesize IL-2, IFN-γ and LT, while Th2-type cells produce IL-4, IL-5, IL-6 and IL-10 (Mosmann and Coffman, 1989b). $CD8^+$ T cells express a pattern of cytokines that corresponds to the Th1 pattern (Fong and Mosmann, 1990). The Th1 and Th2 cells are able to cross-regulate the growth and activities of each other. For example, IL-10, a Th2 product, inhibits the synthesis of cytokines by Th1 cells, whereas IFN-γ inhibits the proliferation of Th2 cells (Fiorentino *et al.*, 1989; Gajewski and Fitch, 1988).

Cytokines often work in an autocrine or paracrine, rather than endocrine, manner (Balkwill and Burke, 1989), being both produced and consumed at the site of immune reaction. Autocrine induction has been reported for a number of cytokines. This has been demonstrated for TNF and IFN-γ *in vitro* by the addition of recombinant cytokine material to cell cultures (Cockfield *et al.*, 1993; Descoteaux and Matlashewski, 1990). TNF has also been shown to act on the production of other cytokines. For example, the administration of TNF to humans rapidly induced production of IL-6 (Jablons *et al.*, 1989). Cytokines have also been shown to inhibit the production of each other, as demonstrated by Aderka *et al.* (1989). Interleukin 6, itself induced by TNF, is able to inhibit TNF production by LPS stimulated human monocytes and in mice *in vivo*.

It is highly probable that in the *in vitro* model described in this thesis, various cytokine interactions govern the overall cytokine response observed. These interactions may have relevance to the *in vivo* situation. Other *in vitro* studies have demonstrated that TNF activates macrophages and is a potent inducer of IL-6 in cultured fibroblasts (Kohase *et al.*, 1986). IL-6 was shown to play a role in the induction of CTL (Renauld *et al.*, 1989), cells which are able to produce IFN-γ upon appropriate stimulation (Klein *et al.*, 1982; Morris *et al.*, 1982). IL-2 also acts on a variety of cells, including monocytes and T cells (Holter *et al.*, 1987; Morgan *et al.*, 1976) and has stimulatory activity on IFN-γ

secretion by NK cells and CTL (Farrar *et al.*, 1981; Handa *et al.*, 1983). IL-4 and IL-10, Th2 cytokines, as well as being able to suppress production of Th1 cytokines, such as IFN-γ (Mosmann *et al.*, 1986) have an inhibitory effect on LPS-induced cytokine production by macrophages, in both human (Hart *et al.*, 1989) and mouse (Fiorentino *et al.*, 1991b).

In order to examine cytokine interactions in more detail, individual cytokines were neutralized using specific antibodies or exogenous cytokines were added to the culture system.

5.2 METHODS

5.2.1

Vaccinia virus	As described in Chapter 2.2
Infection of mice	As described in Chapter 2.3
Culture medium	As described in Chapter 2.4

5.2.2 Mice

Male CBA/H mice were used at 7 weeks of age. A preliminary experiment demonstrated that the proliferative and cytokine response of cells from vaccinia immune male mice was of the same magnitude as cells taken from female mice (data not shown).

5.2.3.1 Antibodies

Table 5.1 describes the anti-cytokine monoclonal antibodies added to the cultures. The neutralizing abilities of the antibodies are described in Table 5.2. The antibodies S4B6, 11B11, 20F3 and 2A5 were provided by Dr P. Hodgkin (John Curtin School of Medical Research). TN3.19.12 antibody has been described in Chapter 2.11. The antibodies were diluted in medium and added in a 20 µl volume either at the initiation of culture, or the immune responder cells were incubated with the antibody for two hours at 37°C before addition of the stimulator cells.

The SXC1, SXC4 and BVD6 antibodies were also provided by Dr. P. Hodgkin. Biotinylated BVD6 and SXC1 antibodies were provided by K. Doherty (John Curtin School of Medical Research).

Rabbit anti-gangliotetraosylceramide (anti-asialo-GM_1) (Wako Pure Chemicals Industries, Osaka, Japan) was used at a final dilution of 1/50.

Table 5.1 *Antibodies used in cell culture*

Antibody		Isotype	Protein Conc.	Preparation	Purification	Reference
S4B6	rat-α–mu-IL2	IgG_{2a}	1 mg/ml	Serum free culture supernatant	IgG precipitated	(Mosmann,1986)
11B11	rat-α–mu-IL4	IgG_1	1 mg/ml	Serum free culture supernatant	Ion exchange column	(Ohara and Paul, 1985)
20F3	rat-α–mu-IL6	IgG_1	10 mg/ml	Ascites	IgG precipitated	(Starnes *et al.*, 1990)
2A5	rat-α-mu-IL-10	IgG_1	12 mg/ml	Ascites	IgG precipitated	(Ishida *et al.*, 1992)
TN3.19.12	hamster-α-mu-TNF	IgG	5 mg/ml	Culture supernatant	Protein A-agarose	(Sheehan et al., 1989)

Table 5.2 *Neutralizing ability of antibodies*

Antibody	Protein conc.[a]	Neutralization activity	Assayed on:
S4B6	20 µg/ml	10 µg/ml : 0.2 U/ml IL-2	HT-2 cell line
11B11	20 µg/ml	3 µg/ml : 70 U/ml IL-4	HT-2 cell line
20F3	200 µg/ml	4.8 ng/ml : 0.03 AU/ml IL-6	B9 cell line
TN3.19.12	100 µg/ml	2.5 µg/ml : 83 pg/ml TNF	WEHI 164 cell line
2A5	250 µg/ml	ND	

[a] Concentration of antibody in first well of subsequent cultures (1/5 dilution).

5.2.3.2 Determination of cytokine contamination in ascites-derived antibodies

Antibody preparations active against several murine cytokines were available. A number of these have been prepared from ascites fluid, which may contain inflammatory cytokines. To determine whether such contaminants were present, the antibodies were added to wells containing naive responder cells and UV-irradiated stimulator cells. This incubation would also allow the detection of endotoxin contaminants, as low levels of endotoxin would stimulate TNF production from the naive cells. In the absence of antibody the cultures did not produce detectable levels of any of the cytokines assayed (Table 5.3, medium alone).

Table 5.3 *Determination of presence of cytokines in antibody preparations*

Antibody [a]	Activity	Naive Responders, stimulated [b]		
		TNF (pg/ml)	IFN-γ (ng/ml)	IL-6 (AU/ml)
medium		< 50	< 0.3	< 0.2
S4B6	IL-2	< 50	< 0.3	ND
11B11	IL-4	< 50	< 0.3	ND
20F3	IL6	< 50	< 0.3	< 0.2
2A5	IL-10	< 50	< 0.3	0.2 ± .01
TN3.19.12	TNF	[c]	< 0.3	0.6 ± 0.2

[a] Antibody was added at the first of the four dilutions used in subsequent cultures.

[b] 50×10^4 B cell depleted naive spleen cells were cultured with 5×10^4 UV-irradiated virus infected cells. Antibodies were added at initiation of culture to give a final volume of 220 µl. Supernatants were harvested after 20 h incubation. Cytokine levels are expressed as mean ± SD of triplicate wells.

[c] Supernatants were not able to be assayed for this cytokine as the antibody interfered with the detection of TNF.

TNF was not detected in any of the cultures, indicating endotoxin contaminants were not present. Low levels of IL-6 were present in the 2A5 and TN3.19.12 preparations.

5.2.3.3 Cytokine contaminants in antibody preparations may stimulate immune cell cytokine production

To determine whether the antibody preparations were able to stimulate the responder cells to produce cytokines, they were added to wells containing immune responder cells, in the absence of stimulator cells. The presence of antibodies did not influence the background proliferation of the responder cells (Table 5.4).

Several antibodies induced some cytokine secretion by these cells. The IL-6 antibody, 20F3, stimulated production of IFN-γ to levels approximately 10% of that produced by restimulated immune cells. The hamster antibody specific for TNF, TN3.19.12, stimulated low levels of IL-6, less than 10% that secreted by the immune cells upon antigenic restimulation.

Table 5.4 ***Determination of the stimulatory effects of antibody preparations on immune responder cells***

Antibody [a]		TNF (pg/ml)	IFN-γ (ng/ml)	IL-6 (AU/ml)	Proliferation (x 10^3)
medium		< 50	< 0.3	< 0.2	22 ± 5
S4B6	IL-2	< 50	< 0.3	ND	18 ± 2
11B11	IL-4	< 50	< 0.3	ND	18 ± 3
20F3	IL6	< 50	2.1 ± 0.8	0.6 ± 0.2	23 ± 3
2A5	IL-10	< 50	< 0.3	1.2 ± 0.1	13 ± 2
TN3.19.12	TNF	[b]	< 0.3	1.8 ± 0.3	15 ± 2
VV-WR stim [c]		290 ± 30	28.2 ± 3.9	24.3 ± 2.4	ND

50×10^4 B cell depleted spleen cells from mice injected with VV-WR 6 days earlier were cultured in wells lacking stimulator cells. Antibodies were added at the initiation of culture to give a final volume of 220 µl. Supernatant was harvested after 20 h incubation. Cytokine levels and proliferation are expressed as mean ± SD of quadruplicate wells.

[a] Antibodies added at first of four dilutions used in subsequent experiments.

[b] Supernatants were not able to be assayed for TNF as the antibody interfered with the assay.

[c] Immune cells restimulated with 5×10^4 UV-irradiated virus infected cells.

5.2.4 Cell lines

HT-2 cells (Watson, 1979) were maintained in MLC medium, described in Chapter 2. α6310 supernatant was added as a source of IL-2 at 50 U/ml. The α6310 cells (Karasuyama and Melchers, 1988) were maintained in MLC medium also. YAC-1 ($H\text{-}2^a$) cells, a cell line derived from Moloney leukemia virus-induced lymphoma of A/Sn mouse (Kiessling *et al.*, 1975), were maintained in F15 medium, described in Chapter 2.4. YAC-1 cells are sensitive to the cytotoxic activity of NK cells and were used as targets in NK cell cytotoxicity assays.

α6310 cell supernatant was used as a source of recombinant IL-2. The cells were incubated in MLC medium for two days at 37°C in a humidified atmosphere of 5% CO_2. The cells were removed by centrifugation and the supernatant sterilized by filtration through a 0.22 μm membrane and frozen at -70°C. The IL-2 content of the supernatant was assayed on the HT-2 cell line (Watson, 1979) and found to be approximately 5000 U/ml.

5.2.5 Cytokines

Recombinant murine TNF was provided by Boehringer Ingelheim, Vienna, Austria, and was diluted in MLC medium for use in cultures.

Recombinant standard murine IL-2, prepared by N. Arai (DNAX) and recombinant murine IL-4, prepared by R. Kastelein (DNAX), were provided by Dr. P. Hodgkin (John Curtin School of Medical Research). Recombinant murine IL-10 was purchased from Genzyme. These standards were used in cytokine assays.

5.2.6 Cultures

Spleens were taken from mice which had been injected with VV-WR i.v. 6 days earlier. Single cell suspensions from pooled spleens were obtained and B cells depleted by incubation with J11d antibody and C, as described in Chapter 3.2.9. For these experiments the complement source was low toxicity rabbit complement (Cedarlane Laboratories, Ltd, Canada). This was used at a final dilution of 1/15. Cultures were set up as described in Chapter 4.2.6, with 50 x 10^4 immune responder cells

incubated with 5 x 10^4 UV-irradiated vaccinia infected cells, in 96 well plates in MLC medium. Immune cells were depleted of various cell sub-populations as described in Chapter 3.2.9.

5.2.7 Cytokine Assays

IL-2 Bioassay

The HT-2 cell line (Watson, 1979) was used to determine the presence or absence of IL-2 in the culture supernatants. The supernatant samples and standard were titrated across a 96 well flat-bottomed plate (Nunc) in a final volume of 50 µl. After washing several times in medium, the HT-2 cells were added to the wells in a 50 µl volume, at a concentration of 2000 cells/well. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. Proliferation was quantitated using [^{3}H]-thymidine incorporation during the last 4 h of culture, as described in section 3.2.11. This cell line proliferates in response to IL-2 and IL-4, therefore antibodies were used to determine the relative concentration of each cytokine. However, the HT-2 cells did not proliferate in the presence of culture supernatant, indicating that neither IL-2 nor IL-4 were present.

IL-4 and IL-10 ELISA

IL-4 and IL-10 in culture supernatants were measured by ELISA. The coating antibody was bound to 96 well, flat bottomed ELISA plates (Dynatech Laboratories, Inc.) by incubation overnight at 4°C, in 50 µl volumes. The antibodies used were the anti-IL 4 antibody, 11B11 (Ohara and Paul, 1985), at a concentration of 5 µg/ml or the anti-IL-10 antibody, SXC4 (Mosmann *et al.*, 1990), at a concentration of 1.25 µg/ml, diluted in carbonate, bicarbonate coating buffer (pH 9.6). The plates were washed three times in phosphate-buffered saline (PBS)-Tween (0.01 M PBS, 0.1% Tween) and then excess protein binding sites were blocked by the addition of 100 µl 3% BSA PBS-Tween for 1 h at 37°C. After washing three times in PBS-Tween, 50 µl of sample was added to each well and the plates were incubated for 2 h at room temperature. Dilutions of recombinant murine IL-4 or IL-10 were used to generate a standard curve. The plates were washed again and 50 µl of biotinylated antibody was added to each well for 1 h at room temperature. Biotinylated BVD6 (DNAX), anti-IL-4, was used at 625 ng/ml and

biotinylated SXC1 (Mosmann *et al.*, 1990), anti-IL-10, was used at 1.25 µg/ml. After washing, 50 µl of streptavidin-alkaline phosphatase conjugate (Amersham Life Science) at 1/1000 dilution was added to each well and incubated for 1 h at room temperature. The plates were washed and then 50 µl of the substrate disodium-p nitrophenyl phosphate at 1 ng/ml (Sigma) was added to each well. Optical density was measured 30 min later using a microplate reader at 405 nm with a reference of 630 nm. A standard curve was generated for each ELISA and a curve of best fit applied. The equation was used to determine the cytokine levels in supernatant samples.

5.2.8 NK cell depletion

Immune responder cells were depleted of B cells as described in section 3.2.9. The cells were then incubated with anti-asialo-GM_1 for 10 min at 37°C, followed by a 45 min incubation with rabbit C at 37°C. Viable cells were recovered on an Histopaque gradient, washed in cold MLC and made up to the same volume as control cells, ie. J11d depleted only, which had a concentration of 5 x 10^6 cells/ml. The efficiency of the depletion was assessed with the NK cytotoxicity assay. The specific lysis decreased from 29% to 5%, at the effector:target ratio of 20:1, following treatment of the immune cells with anti-asialo-GM_1 and C.

5.2.9 NK cytotoxicity assay

The standard chromium release assay was performed, as described in section 3.2.3. 2 x 10^6 YAC-1 cells were labelled with 50 µl ^{51}Cr in a volume of 200 µl for 90 min at 37°C. The cells were washed three times in complete medium and resuspended at a concentration of 2 x 10^5 cells/ml for use as targets. The assay was carried out in triplicate in round-bottom 96 well plates (2 x 10^4 target cells plus the appropriate number of effector cells) for 4 h at 37°C in a humidified atmosphere of 5% CO_2 in air.

$$\% \text{ specific lysis} = \frac{\text{experimental release - spontaneous release}}{\text{maximal release - spontaneous release}} \times 100$$

5.3 RESULTS

5.3.1 Endogenous TNF had a role in IFN-γ production

To determine whether TNF influenced the production of IFN-γ or IL-6, the cytokine was neutralized in the cultures using the hamster TNF antibody. This neutralization led to a significant decrease in IFN-γ production by the restimulated immune responder cells (Table 5.5). The decreased response was more evident at earlier time points. Preincubation of the responder cells with the antibody led to a further reduction in IFN-γ levels, throughout the culture period. Despite this antibody inducing low levels of IL-6 from unstimulated immune cells (Table 5.4), it had no significant effect on the levels of IL-6 produced by restimulated cells, in a 20 h culture (Table 5.5).

To determine whether the addition of TNF to the cultures would have a reverse and stimulatory effect on IFN-γ production, recombinant TNF was added to the cultures. However, the presence of the recombinant TNF had little or no effect on IFN-γ or IL-6 produced by the restimulated immune cells. Four dilutions of TNF were added, with the first dilution containing 200 pg recombinant murine TNF. This amount of TNF was similar to that produced by stimulator cells infected with a vaccinia virus encoding TNF, which is discussed in the next chapter. Following a 24 h incubation, cytokine levels in the supernatant were assayed. The TNF levels in cultures with 200 pg TNF added were less than expected when compared to cultures with medium alone (Table 5.6). The difference between the TNF level expected and TNF detected, approximately 60 pg, may indicate that TNF was bound to receptors on the restimulated responder cells. At the lower TNF concentrations, the TNF detected was similar to control wells.

Table 5.5 *Effect of neutralizing TNF on IFN-γ and IL-6 levels produced by restimulated vaccinia immune cells*

Antibody dilution	IFN-γ (ng/ml) 8 h culture		IFN-γ (ng/ml) 20 h culture		IL-6 (AU/ml) 20 h culture	
	No preincub. [a]	Preincub. [b]	No preincub. [a]	Preincub. [b]	No preincub. [a]	Preincub. [b]
None	5.5 ± 0.6		28.2 ± 3.9		13.6 ± 2.3	13.6 ± 2.3
1/5	3.8 ± 0.2 **	2.1 ± 0.7 **	22.6 ± 3.1	20.2 ± 2.2 **	10.0 ± 1.8	12.3 ± 0.6
1/10	4.3 ± 0.4 *	2.7 ± 0.2 **	24.8 ± 2.5	17.6 ± 1.8 **	13.0 ± 7.0	15.2 ± 3.1
1/20	4.5 ± 0.8	2.2 ± 0.7 **	26.2 ± 1.3	22.3 ± 1.8 **	8.7 ± 2.8	13 ± 2

6 day vaccinia immune spleen cells were depleted of B cells. 50×10^4 of these responder cells were incubated with 5×10^4 UV-irradiated virus infected cells in a total volume of 200 µl. The TNF antibody, was added in a 20 µl volume and supernatant was harvested at times indicated. Cytokine levels are expressed as the mean ± SD of quadruplicate wells.

[a] Antibody added to responder cells in culture wells two hours before addition of stimulator cells.

[b] Antibody added to responder cells in culture wells at time of addition of stimulator cells.

* significant < 0.05, Students t-test.

** significant < 0.005, Students t-test.

Table 5.6 ***Exogenous TNF did not alter IFN-γ or IL-6 levels produced by immune responder cells***

r-TNF added (pg) [a]	TNF (pg/well)	IFN-γ (ng/ml)	IL-6 (AU/ml)
Medium	150 ± 10	35.8 ± 4.7	13.6 ± 2.3
200	290 ± 20	31.5 ± 3.7	16.6 ± 1.7
100	240 ± 20	34.7 ± 3.0	22.0 ± 3.7
50	210 ± 20	41.0 ± 3.1	18.2 ± 4.9
25	200 ± 10	39.7 ± 3.4	18.4 ± 4.4

Spleen cells were removed from mice infected with VV-WR 6 days earlier. The immune cells were depleted of B cells and made up to a concentration of 50×10^5/ml in medium. 5×10^4 UV-irradiated stimulator cells were added to 100 µl immune cells, with a final volume of 220 µl. Supernatant was harvested 24 h after initiation of culture. Cytokine levels are expressed as mean ± SD of quadruplicate wells.

[a] Recombinant murine TNF-α was diluted in medium and added to the culture wells in a 20 µl volume.

5.3.2 IL-6 antibody enhanced IFN-γ levels

Preincubation of the immune responder cells with the IL-6 antibody preparation led to a significant increase in IFN-γ production in the first 12 h of restimulation (Table 5.7). This level of IFN-γ was twice that seen in cultures lacking antibody. Although the antibody preparation was able to directly induce IFN-γ production from immune responder cells (2.1 ng/ml, Table 5.4), the increase shown here in restimulated wells was 8 times the level of IFN-γ produced in the unstimulated wells. A decrease in TNF levels was observed in the cultures incubated with a 1/5 dilution of the IL-6 antibody preparation after 12 h of culture, however, by 24 h there was no difference in TNF levels (Table 5.7).

Table 5.7 *Effect of neutralizing IL6 on IFN-γ and TNF levels*

	12 h		24 h
Antibody dil.	IFN-γ (ng/ml)	TNF (pg/ml)	TNF (pg/ml)
Medium	19.3 ± 1.4	160 ± 30	220 ± 30
1/5	37.0 ± 3.8**	90 ± 10 **	180 ± 30
1/10	28.6 ± 6.9*	130 ± 20	250 ± 60
1/20	24.2 ± 9.4	130 ± 50	430 ± 10 **
1/40	22.1 ± 1.8	150 ± 50	290 ± 50

Antibody to IL-6 was added in 20 µl volumes to wells containing 50 x 10^4 immune responder cells. Two hours later 5 x 10^4 stimulator cells were added to each well, with a final volume of 220 µl. Cytokine levels are expressed as mean ± SD of quadruplicate wells. No IL-6 was detected in the supernatants of cultures treated with the IL-6 antibody, using the B9 assay.

Cultures incubated with the IL-10 antibody, 2A5, had no significant difference in IFN-γ of TNF levels at 12 h incubation.

* significant < 0.05, Students t-test.

** significant < 0.005, Students t-test.

5.3.3 IFN-γ production was dependent on IL-2

The presence of an antibody against IL-2 in cultures of restimulated immune cells led to a significant reduction in the production of IFN-γ (Table 5.8), with levels less than 40% of those seen in control wells. After 12 h of culture, TNF levels were increased in the presence of anti-IL-2, however, by 24 h the TNF levels were similar to wells lacking antibody.

Table 5.8 *Effect of neutralizing IL2 on IFN-γ and TNF levels*

	12 h		24 h
Antibody dil.	IFN-γ (ng/ml)	TNF (pg/ml)	TNF (pg/ml)
Medium	19.3 ± 2.2	160 ± 30	220 ± 30
1/5	6.7 ± 1.3 **	250 ± 40 **	300 ± 60
1/10	8.4 ± 2.7 **	220 ± 30 **	210 ± 70
1/20	10.0 ± 1.1 **	250 ± 50 *	160 ± 40
1/40	8.0 ± 1.4 **	220 ± 60 *	200 ± 75

Antibody to IL-2 was added in 20 µl volumes to wells containing 50×10^4 immune responder cells. Two hours later 5×10^4 stimulator cells were added to each well, with a final volume of 220 µl. Cytokine levels are expressed as mean ± SD of quadruplicate wells.

* significant < 0.05, Students t-test.

** significant < 0.005, Students t-test.

The supernatant of the α6310 cell line contains IL-2, and was used as a source of exogenous IL-2 for these cultures. IFN-γ was not detected in the α6310 supernatant when assayed by ELISA, nor in supernatant harvested from wells containing stimulated naive responder cells cultured in the presence of the α6310 supernatant. Responder cells lacking restimulation did not produce IFN-γ (Table 5.4), however, the addition of 100 U/ml IL-2 induced the production of significant levels of IFN-γ (Table 5.9). These results indicate that the IL-2 source was not contaminated with IFN-γ, and that only activated cells responded with IFN-γ production.

The IFN-γ titre in cultures of non-stimulated responder cells supplemented with the IL-2-containing supernatant was 10% of that produced by restimulated immune cells. However, it was only 5% of that produced by restimulated cells in the presence of an exogenous source of IL-2 (Table 5.9). Depletion of both the $CD4^+$ and $CD8^+$ T cells abrogated the IFN-γ response, indicating the IFN-γ production was dependent on the presence of T cells. The addition of 100 U/ml IL-2 to the cultures led to a significant increase in the production of IFN-γ by restimulated immune cells, with increased IFN-γ titres of about

30 ng/ml. Cultures containing $CD4^-/CD8^+$ T cells also had marginally increased levels of IFN-γ when incubated with the IL-2 source (5 ng/ml). The $CD8^-/CD4^+$ T cell cultures, however, did not respond by increased IFN-γ production to the IL-2 added. These results indicate that both $CD4^+$ and $CD8^+$ T cells were required for the optimal inducing effect of the exogenous IL-2.

Table 5.9 ***Effect of an exogenous source of IL-2 on IFN-γ production by immune cells***

Responder cells	No stim. [a] + α6310 sup [b]	With stimulators	With stimulators + α6310 sup [b]
B cell depl.	2.5 ± 0.7	27.3 ± 5.6	61.6 ± 12.6 **
& CD4 depl.	1.3 ± 0.6	4.4 ± 1.1	9.3 ± 2.2 *
& CD8 depl.	0.4 ± 0.1	16.2 ± 1.5	14.0 ± 3.6
& CD4/8 depl.	0.4 ± 0.2	1.8 ± 0.6	5.9 ± 1.8 *
Naive [c]	< 0.3	< 0.3	< 0.3

Spleens were removed from 6 day vaccinia immune mice and depleted of B cells and T cell subsets. The B cell depleted cells were made up to a concentration of 5×10^6 cells/ml and the other cells were made up to the same volume. 100 µl of responder cells was cultured with 5×10^4 UV-irradiated stimulator cells in a total volume of 200 µl. Supernatant was harvested after 24 h culture. Levels of IFN-γ are expressed in ng/ml as mean ± SD of triplicate wells.

[a] Cultures received 100 µl medium instead of stimulator cells.

[b] 20 µl of α6310 supernatant, with a concentration of approximately 1000 U/ml IL-2, was added to each well at the initiation of culture.

[c] Responder cells were B cell depleted spleen cells taken from uninfected mice.

* significant, $p < 0.05$, Students t-test.

** significant < 0.005, Students t-test.

Although 100 U/ml IL-2 resulted in a marginal increase in IFN-γ levels in $CD4^-/CD8^+$ cultures, the addition of 500 U/ml increased the response significantly such that levels were restored to non-depleted levels of IFN-γ (Table 5.10). The increased TNF levels following removal of the $CD4^+$ T cells remained unchanged. Depletion of asialo-GM^+ cells from the immune responder cells led to a significant decrease in IFN-γ levels. Supplementing the cultures with 500 U/ml IL-2 restored the IFN-γ production, indicating the IFN-γ produced in response to the IL-2 was not derived from the asialo-GM_1^+ cells

Table 5.10 ***High levels of endogenous IL-2 restored IFN-γ production by $CD4^-/CD8^+$ immune T cells***

Responder cells	α 6310 sup [a]	IFN-γ (ng/ml)	TNF (pg/ml)
B-cell depleted	absent	37.7 ± 3.1	170 ± 3
& CD4 depleted	absent	13.9 ± 1.3	450 ± 70
& CD4 depleted	present	34.9 ± 1.2	430 ± 60
& asialo-GM_1 depleted	absent	27.3 ± 5.0	ND
& asialo-GM_1 depleted	present	50.6 ± 7.9	ND

Spleen cells were removed from mice infected with VV-WR 6 days earlier, depleted of B cells and made up to a concentration of 5×10^6/ml in medium. $CD4^+$ T cells were also depleted from immune cells and made up to the same volume as the B cell depleted cells. 100 µl of responder cells was cultured with 5×10^4 UV-irradiated stimulator cells in a total volume of 200 µl. Supernatant was harvested 20 h after initiation of culture. Cytokine levels are expressed as mean ± SD of quadruplicate wells.

[a] 20 µl of α6310 supernatant, at a concentration of approximately 5000 U/ml was added to each well at initiation of culture.

Interestingly, it was not possible to detect significant levels of IL-2 in the supernatant of restimulated responder cells during the first 12 h of culture, using the HT-2 cell line to assay IL-2 (Table 5.13).

5.3.4 Endogenous IL-4 influenced IFN-γ and TNF production

IL-4 was not able to be detected in the supernatant harvested from restimulated vaccinia immune cultures, over a 3 day period (Table 5.13). Despite this, the addition of antibodies to IL-4 led to a decrease in TNF production by the restimulated immune cells (Table 5.11). This was observed whether the responder cells were preincubated with the antibody, or not. In contrast, the presence of an antibody specific for IL-10 had no effect on TNF levels. Neutralization of IL-4 also led to decreased levels of IFN-γ, although this effect was seen later in the culture period, after 20 h incubation of the restimulated responder cells (Table 5.12).

Table 5.11 ***Effect of neutralizing IL-4 or IL-10 on TNF levels***

Antibody dil.	Experiment 1 (20 h)[a]		Exp. 2 (24 h)[b]
	anti-IL-10	anti-IL-4	anti-IL-4
Medium	300 ± 40	300 ± 40	220 ± 25
1/5	340 ± 30	160 ± 20 *	150 ± 40
1/10	360 ± 50	230 ± 20	140 ± 20 *
1/20	370 ± 60	210 ± 40	150 ± 10 *

6 day vaccinia immune responder cells were incubated with UV-irradiated virus infected cells. Antibody to IL-4 or IL-10 was added in 20 µl volume to give a final volume of 220 µl. Supernatant was harvested at the times indicated. TNF levels are expressed as mean ± SD of triplicate wells.

[a] Antibody added to the wells at the same time as stimulator cells.

[b] Antibody added to responder cells two hours prior to addition of stimulator cells.

* significant < 0.05, Students t-test.

Table 5.12 ***Effect of neutralizing IL-4 on IFN-γ levels produced by restimulated vaccinia virus immune cells***

Antibody dil.	Experiment 1 [a] (12 h)	Experiment 2 (20 h) No preincub. [b]	Preincub. [a]
Medium	16.9 ± 1.5	28.2 ± 3.9	
1/5	16.1 ± 0.6	20.6 ± 1.4 *	10.7 ± 1.4 *
1/10	18.2 ± 1.5	22.6 ± 2.5	25.2 ± 1.7
1/20	16.7 ± 2.8	23.8 ± 2.6	24.7 ± 3.1

6 day vaccinia immune spleen cells were depleted of B cells. 50×10^4 of these responder cells were incubated with 5×10^4 UV-irradiated virus infected cells in a total volume of 200 µl. Antibody was added in a 20 µl volume and supernatant was harvested at times indicated. IFN-γ levels are expressed as ng/ml and are mean ± SD of quadruplicate wells.

The presence of antibody to IL-10, 2A5, in the cultures had no significant effect on IFN-γ levels.

[a] Antibody added to responder cells in culture wells two hours before addition of stimulator cells.

[b] Antibody added to responder cells in culture wells at time of addition of stimulator cells.

* significant < 0.005, Students t-test.

5.3.5 IL-10 was produced later in culture period

IL-10 was detected in the culture supernatant of the restimulated immune cells, but not until after 24 h of culture (Table 5.13). The addition of antibodies to this cytokine had no effect on TNF levels, within the first 20 h of culture, by which time the TNF levels had begun to plateau (Table 5.10).

Table 5.13 ***Culture supernatant did not contain significant levels of IL-2 or IL-4***

Time (h)	IL-2 (U/ml)	IL-4 (U/ml)	IL-10 (pg/ml)
4	< 10	< 0.2	ND
12	< 10	< 0.2	1.5 ± 0.6
24	ND	< 0.2	11 ± 1
48	ND	< 0.2	30 ± 3
72	ND	< 0.2	21 ± 5

50×10^4 B cell depleted vaccinia immune responder cells were incubated with 5×10^4 UV-irradiated virus infected spleen cells in a total volume of 200 µl. Supernatants were harvested at the times indicated and frozen until assayed for cytokine levels. IL-2 was assayed on the HT-2 cell line, whereas IL-4 and IL-10 were assayed using ELISAs. IL-2 was not detected above the limit of the assay (10 U/ml), nor was IL-4 (0.2 U/ml). IL-10 levels are given as pg/ml and are the mean ± SD of quadruplicate wells.

A summary table of the results presented in this chapter can be found in Chapter 7, the general discussion (Table 7.2).

5.4 DISCUSSION

Vaccinia immune spleen cells produced the cytokines TNF, IL-6 and IFN-γ upon restimulation with virus infected cells. There have been numerous reports demonstrating that the production of these three cytokines is closely interrelated. TNF induced the production of IL-6 in cell culture (Kohase *et al.*, 1986) and *in vivo* (Fong *et al.*, 1989; Jablons *et al.*, 1989), whereas IL-6 may have served to down-regulate TNF production (Aderka *et al.*, 1989). TNF has been shown to be necessary for production of IFN-γ from NK cells (Wherry *et al.*, 1991) while IFN-γ may be involved in the negative feedback control of TNF (Doherty *et al.*, 1992). On the other hand, there are also examples where the induction of cytokine production occurs through independent pathways (Havell and Sehgal, 1991). In order to investigate whether the production of the cytokines IL-6, TNF and IFN-γ by vaccinia immune cells involved cytokine interaction, antibodies specific for several cytokines were added to the cultures of restimulated cells.

Treatment of the vaccinia immune cells with the TNF antibody did not significantly affect IL-6 levels produced by the cells upon restimulation (Table 5.5), indicating the production of IL-6 occurred through a TNF-independent mechanism. This is in agreement with the work presented in Chapter 3 which indicated that the responder cells contained a population of activated macrophages which could be triggered to produce IL-6 directly by the UV-irradiated virus-infected stimulator cells, as was shown by Gosselin *et al.* (1992) in a study using EBV and HSV. In contrast to these results, there have been several reports of TNF acting as an intermediate in IL-6 production. Pretreatment with anti-TNF antibodies significantly lowered IL-6 serum levels after *E. coli* challenge in baboons and mice (Fong *et al.*, 1989; Starnes *et al.*, 1990), whereas treatment of fibroblasts with recombinant TNF induced IL-6 mRNA (Kohase *et al.*, 1986). The TN3.19.12 TNF antibody preparation was used by Engelberts *et al.* (1991) and was shown to be more effective at reducing IL-6 levels *in vivo* than in cultures of LPS stimulated macrophages.

Treatment of vaccinia immune responder cells with anti-TNF did, however, affect their ability to produce IFN-γ, with a 30% decrease observed in IFN-γ titre (Table 5.5). This result suggests that at least a

portion of IFN-γ production was dependent on TNF. A similar result was seen *in vivo*, with mice treated with LPS and anti-TNF having lower IFN-γ levels in their serum (Doherty *et al.*, 1992). In addition, the production of IFN-γ by NK cells isolated from *scid* mice, cultured with macrophage conditioned medium was reduced in the presence of antibodies to TNF (Wherry *et al.*, 1991). The responder cells in the studies described here were taken from mice injected with VV-WR 6 days earlier. It has been shown that the NK cell activity (lysis of YAC-1 cells) increased following VV-WR infection of normal mice, with peak activity at 3 days. By 6 days, however, the NK cytolytic activity had decreased to below the levels of unimmunized controls (Karupiah *et al.*, 1990a). Thus, NK cells are not believed to be major contributors to IFN-γ production in the system described here. The observation that there was no more than a 30% decrease in IFN-γ titres may suggest the antibody was acting on one IFN-γ producing cell population, with another population producing IFN-γ in a TNF-independent manner. This suggestion is supported by the kinetics of IFN-γ production following depletion of adherent cells, as described in Chapter 4. The decrease in IFN-γ titres following removal of adherent cells was not evident until after 12 h incubation, indicating the initial IFN-γ production was independent of the adherent cells.

Preincubation of the responder cells with TNF antibodies augmented the suppressive effect on IFN-γ production by the cells. The preincubation may have allowed for binding of antibody to the immune responder cells expressing membrane bound TNF before secretion of soluble TNF (Sheehan *et al.*, 1989), and thus interfered with subsequent TNF release or interaction with a target cell.

The addition of recombinant TNF to cultures of restimulated vaccinia immune cells led to only a marginal increase in the levels of IL-6 (Table 5.6). Similarly, IFN-γ levels were not influenced by this level of exogenous TNF. This lack of response suggests several possibilities. Firstly, the TNF may not be active in this form; membrane bound TNF may be more active. Secondly, TNF may not be involved in the generation of IL-6 and IFN-γ and, finally, the cells producing these cytokines were optimally stimulated and could not respond further. In contrast, treatment of the responder cells with antibody to TNF indicated that TNF was involved in production of IFN-γ by a subset of the immune responder cells, but that IL-6 production was not

influenced by TNF. These results indicate that IL-6 production was TNF independent, and that the level of exogenous TNF added was not able to stimulate increased IFN-γ production.

Responder cells produced greater levels of IFN-γ when antibody to IL-6 was present in cultures, compared to cells cultured without antibody (Table 5.7). No IL-6 was demonstrable in the supernatants from the IL-6 antibody treated cultures, suggesting that IL-6 produced by the responder cells following restimulation was neutralized by the antibody. This may indicate that IL-6 was able to down-regulate IFN-γ production. Another possibility is that the antibody interfered with binding of IL-6 to the B9 IL-6 receptor, while the IL-6 retained activity on IFN-γ producing cells. As shown in the previous chapter, removal of the adherent cells, the major IL-6 producers, resulted in a decrease in IFN-γ levels. This argues against the possibility that IL-6 is able to down-regulate IFN-γ production.

Decreased levels of TNF were observed in cultures of responder cells restimulated in the presence of the IL-6 antibody. However, this effect on TNF was only seen at high concentrations of the antibody, despite its potent neutralizing activity against IL-6. Twelve hours later the decreased TNF response was no longer apparent. This is in contrast to the increased levels of TNF observed following anti-IL-6 treatment of *E. coli* induced TNF *in vivo* in mice (Starnes *et al.*, 1990). This *in vivo* result, and the decreased levels of TNF in IL-6 treated cultured cells (Aderka *et al.*, 1989), has led to the conclusion that IL-6 may be part of a feedback loop that blocks TNF production. Such a loop was not consistent with the observations made of the culture conditions described here. A recent study examined the regulation of IFN-γ receptor expression by a monocytic cell line. The results indicated that TNF and IL-6 up-regulated the IFN-γR gene via different mechanisms (Sanceau *et al.*, 1992). Perhaps removal of IL-6 from these cultures led to decreased IFN-γR expression by the macrophages such that they were less able to be activated, and in consequence led to a decrease in TNF and IL-6 production.

The addition of antibodies to IL-2 to cultures of restimulated vaccinia virus immune spleen cells led to a significant decrease in IFN-γ titres (Table 5.8). A study of IFN-γ producing tonsillar cells has similarly shown a decrease in IFN-γ production following treatment with

anti-IL-2 (Quiding *et al.*, 1993). In contrast, TNF levels were enhanced after 12 h culture in the presence of this antibody. Enhanced TNF levels have been described in Chapter 4, in cultures lacking $CD4^+$ T cells. Recombinant IL-2 added to cultures depleted of $CD4^+$ T cells did not alter the enhanced levels of TNF (Table 5.10), indicating that the negative influence $CD4^+$ T cells have over TNF production was not mediated through IL-2.

Addition of α6310 supernatant, as a source of r-IL-2, to cultures of unstimulated vaccinia immune spleen cells led to a production of low, but significant levels of IFN-γ (Table 5.9), as seen by Yamamoto *et al.* (1982). IL-2 is known to be involved in the production of IFN-γ (Farrar *et al.*, 1981) and was required for maximum synthesis of IFN-γ *in vitro* (Reem and Yeh, 1984). In the absence of restimulation, $CD4^-/CD8^+$ responder cells produced 50% of the level of IFN-γ upon incubation with IL-2, as compared to B cell depleted cells. The $CD8^-/CD4^+$ immune cells did not respond to the exogenous IL-2, suggesting the IFN-γ inducing effect of IL-2 is acting on the $CD8^+$ T cell population. This is in agreement with the work of Torres *et al.* (1982) in which $Lyt.2^+$ cells were the IFN-γ producers in cultures of unprimed lymphocytes incubated with IL-2. Vaccinia immune spleen cells produced higher levels of IFN-γ upon restimulation. The addition of 100 U/ml IL-2, in the form of α6310 supernatant, enhanced the secretion two-fold (Table 5.9). This level of IL-2 was in the same order of magnitude as other *in vitro* studies (Reem and Yeh, 1984; Torres *et al.*, 1982). A similar two-fold enhancement was observed in cultures of $CD4^-/CD8^+$ responder cells, although the level of IFN-γ production was still only 15 % of that seen in non-T cell depleted cultures. Those cultures containing $CD8^-/CD4^+$ cells were the only ones not to respond to the IL-2 with increased IFN-γ production. Similarly, Kelso *et al.* (1984) reported that IL-2 was found to enhance the precursor frequency of MAF secreting $Lyt.2^+$ cells while not affecting the $Lyt.2^-$ cells. The addition of 500 U/ml IL-2, in the form of α6310 supernatant, completely restored levels of IFN-γ produced by $CD4^-/CD8^+$ T cell responders to that produced by non-T cell depleted cultures (Table 5.10). The fact that depletion of $CD4^+$ T cells dramatically reduced IFN-γ titres which could only be restored with excessive levels of IL-2 suggested that cells other than $CD4^+$ T cells responded to the high levels of IL-2 with IFN-γ production. These may have been $CD8^+$ T cells or NK cells. NK cells are able to produce IFN-γ (Cuturi *et*

al., 1989) and IL-2 is known to augment NK cell activity, in the absence of IFN-γ (Henney *et al.*, 1981; Sayers *et al.*, 1986). Removal of the NK cells from responder cells with anti-asialo GM_1 and C resulted in decreased IFN-γ production. The addition of 500 U/ml IL-2 to the culture restored IFN-γ levels, indicating that the IFN-γ production in response to IL-2 was not dependent on NK cells, but was dependent on IL-2. The IL-2 was acting on $CD8^+$ T cells to increase IFN-γ expression, however, $CD4^+$ T cells must also be present for this increase to occur. This suggests the $CD4^+$ T cells are providing another signal, perhaps another cytokine, to the $CD8^+$ T cells. This may account for the interaction observed among the responder cells in Chapter 3.

IL-4 was not detected in the supernatant of restimulated vaccinia immune spleen cells at any time throughout the 48 h culture. IL-4 may be secreted by these cells, but if so, the levels were below the detection limit of the assay, or the cytokine was bound to receptors on responder cells. Similarly, IL-2 was not detected in culture supernatant. Cytokines are released in a localized space in the vicinity of interacting cells (Kupfer *et al.*, 1991), and thus, lack of detection in the supernatant of these cells does not necessarily indicate the cytokine was not being produced. IL-4 has been shown to be a macrophage activating factor, increasing their tumoricidal activity and expression of class I and II MHC antigens (Crawford *et al.*, 1987; Stuart *et al.*, 1988). A recent report, however, has demonstrated that IL-4 inhibited the expression of TNF mRNA in murine peritoneal macrophages following stimulation with IFN-γ or IL-2, whereas LPS-induced TNF mRNA remained unaffected (Gautam *et al.*, 1992). Antibodies to IL-4 in the cultures of restimulated vaccinia immune cells led to a decrease in TNF levels (Table 5.11). As TNF appeared to be produced upon contact with virus antigen in these cultures, the role of IL-4 was likely to be minor, although IL-4 may have enhanced macrophage activation.

The IFN-γ levels also decreased upon incubation of the responder cells with the IL-4 antibody (Table 5.12). It was shown in the previous chapter that the majority of IFN-γ produced was dependent on the $CD4^+$ T cells. Although the $CD4^+$ T cells may be regulating IFN-γ production by another cell type through the production of cytokines, a significant level of IFN-γ was produced by these cells, as seen by the lack of IFN-γ response in cultures lacking these cells supplemented with 100 U/ml IL-2. $CD4^+$ T cells which produce IFN-γ belong to the Th1

phenotype (Mosmann *et al.*, 1986). IL-4, a Th2-type cytokine, has been shown to suppress a Th1 response, through down-regulation of IL-2 production (Schwarz *et al.*, 1993). Neutralization of IL-4 in the cultures might be expected to lead to increased IFN-γ levels, if the IL-4 was involved in such an inhibitory activity. The results presented using the IL-4 antibody treatment do not readily fit with the Th1/Th2 model. The decrease in IFN-γ levels following neutralization of IL-4 was observed later in the culture period (20h). A similar, late, decrease in IFN-γ levels was observed following removal of the adherent cells (Chapter 4.3.1.3). Neutralization of IL-4 also resulted in decreased TNF levels, as discussed above. This may suggest that the decreased IFN-γ levels following neutralization of IL-4 were a consequence of the reduced TNF levels. Interestingly, as will be discussed in the following chapter, stimulation of responder cells with a recombinant vaccinia virus encoding IL-4 enhanced IFN-γ levels two-fold. Alternatively, the IL-4 may act on the vaccinia immune $CD8^+$ T cells. IL-4 has been associated with secondary responses, as a high proportion of primed cells were able to produce IL-4 following restimulation whereas it was not readily produced by lymphoid cells freshly isolated from mice (Powers *et al.*, 1988). IL-4 was demonstrated to be able to drive $CD4^-/CD8^+$ CTL-precursors to maturation (Quentmeier *et al.*, 1992), and was involved in the induction of allo-CTL induction in a secondary response. In both these studies IL-4 was provided by primed $CD4^+$ T cells (Suzuki *et al.*, 1992).

The addition of antibodies to IL-10 to the culture system described here had no effect on TNF production by restimulated immune responder cells. The supernatants were harvested after 12 h of culture. Significant levels of IL-10 were not detected in the culture until 24 h of culture. This later stage production has been observed with anti-CD3 stimulated T cell clones and peripheral blood T cells (Yssel *et al.*, 1992). TNF levels had begun to plateau by the time IL-10 was detected in the culture supernatant. The kinetics of production could suggest the IL-10 had a regulatory effect on TNF production. IL-10 was first described as a cytokine produced by Th2 cells which inhibited macrophage APC-dependent cytokine synthesis by Th1 cells (Fiorentino *et al.*, 1989). IL-10 has been found to have a number of effects on other cells, including an inhibitory effect on TNF and IL-6 production by activated macrophages (Fiorentino *et al.*, 1991b). The lack of effect in cultures treated with antibody to IL-10 did not necessarily imply that the cytokine is not

involved. Cytokines are released into a localized space in the vicinity of the interacting cells (Kupfer *et al.*, 1991), possibly beyond reach of antibodies. The lack of effect with antibodies was alluded to in a recent review: "it is our personal experience that neutralizing the effects of an autocrine cytokine is not always easy" (De Maeyer and De Maeyer-Guignard, 1992).

5.5 SUMMARY

It was highly probable that cytokines produced by restimulated vaccinia immune cells interacted in a network, and induced the production of each other. Individual cytokines were neutralized by the addition of specific antibodies in order to examine this more closely. Neutralizing TNF reduced IFN-γ production by 30%, indicating at least a portion of IFN-γ production was dependent on TNF. In contrast, IL-6 production was TNF independent. IFN-γ production was also dependent on IL-2. Neutralization of IL-2 reduced IFN-γ production, while the addition of IL-2 led to an increase in IFN-γ levels. The induction of IFN-γ by IL-2 was dependent on the presence of both $CD4^+$ and $CD8^+$ T cells. Neutralization of IL-4 led to a decrease in levels of TNF and IFN-γ; a result which does not fit with the Th1/Th2 model. IL-4 was not detected in culture supernatant, although antibody to IL-4 reduced both TNF and IFN-γ levels.

CHAPTER 6

Effect of restimulation of vaccinia virus immune cells with recombinant vaccinia virus encoding cytokine genes

6.1 INTRODUCTION

An immune response to virus infection involves a number of distinct cell types, whose functions must be co-ordinated to ensure an appropriate response. The co-ordination of these functions is regulated through the production of cytokines. The previous chapter examined the influence of several cytokines on the generation of antiviral cytokines by vaccinia virus immune spleen cells. The approach taken was to inhibit cytokine activity by the addition of antibody, or to add exogenous cytokine. However, this approach has its limitations. Cytokine release by T cells is polarized towards the interactive cells (Kupfer *et al.*, 1991; Poo *et al.*, 1988) which leads to very high local concentrations of the cytokine. Thus, antibodies against the cytokines may be unable to reach the localized area of cytokine production. Likewise, the addition of exogenous cytokine to cultures is unlikely to mimic the localized concentrated effect of natural cytokine activity on a responsive cell.

An alternative method of presenting cytokines in a virus infection is readily available through the use of recombinant vaccinia viruses which encode cytokine genes (Ramshaw *et al.*, 1987). The foreign gene encoded by the recombinant vaccinia virus is expressed independent of the type of cell infected. Thus, although IL-2 is normally secreted only by T cells, any cell infected with a recombinant IL-2 virus will produce the cytokine (Ramshaw *et al.*, 1992). Therefore, infection of cells with recombinant vaccinia viruses ensures the cytokine of interest is produced at the site of antigen presentation.

A number of recombinant vaccinia viruses which encode cytokine genes have been constructed. These recombinant viruses can be regarded as belonging to two groups. The first group includes those viruses that encode cytokines which act as antiviral effector molecules, ie. the viruses which encode IL-2, IFN-γ and TNF. Several important observations have been made following replication of these viruses *in vivo*. To summarise these observations:

(1) vector-encoded IL-2 enhanced host antiviral responses mediated by NK cells;

(2) IL-2 was critically dependent on host-derived IFN-γ for antiviral activity;

(3) vector-encoded IFN-γ and TNF, unlike IL-2, mediated direct antiviral activity (reviewed by Ramshaw *et al.*, 1992).

The second group of recombinant vaccinia viruses contains those which encode cytokines that act at the level of antigen presentation and regulation of immune response. Vaccinia virus encoding IL-4 was lethal in normal mice, but the toxicity was overcome by coexpression of IFN-γ, indicating the IL-4 abrogated the IFN-γ response to virus infection (Andrew and Coupar, 1992). In contrast, IL-10 encoded by a recombinant vaccinia virus had no significant effect on immune response *in vivo* (Milbourne *et al.*, submitted).

This chapter sets out to examine the effect of virus-encoded cytokine production, at the site of antigenic stimulation, on the production of the antiviral cytokines by vaccinia virus immune cells. Viruses encoding Th-1 type cytokines were found to enhance production of IFN-γ by responder cells. Viruses encoding the Th-2 type cytokines, IL-4 and IL-10 were also used to examine whether the presence of these cytokines at the site of restimulation would skew the response by the immune cells towards a Th-2 cytokine response.

6.2 METHODS

6.2.1

Mice	As described in Chapter 2.1
Culture medium	As described in Chapter 2.4

6.2.2 Viruses

The viruses have been constructed using the neurovirulent, L929 cell-adapted WR strain of vaccinia virus (Wokatsch, 1972), which is designated VV-WR. VV-HA-IL-2 (Ramshaw *et al.*, 1987) and VV-HA-TNF (Sambhi *et al.*, 1991), VV-HA-IL-4 and VV-HA-IL-10 were constructed using the recombinant VV-PR8-HA6 (Andrew *et al.*, 1986), which encodes the haemagglutinin (HA) of influenza A/PR/8/34. VV-IFN-γ was constructed without the HA gene (Kohonen-Corish *et al.*, 1990). Virus stocks were prepared from infected CV-1 cells grown in 2 litre Schott acid-washed roller bottles. VV-HA-TNF, VV-IFN-γ and VV-HA-IL-2 stocks were provided by Mrs Jill Medveczky; VV-HA-IL-4 stocks were provided by Dr D. Sharma; and VV-HA-IL-10 stocks were provided by Dr E. Milbourne (John Curtin School of Medical Research).

6.2.3 Preparation of stimulator cells

To analyse the ability of cytokine-encoding recombinant viruses to synthesize cytokines following UV-irradiation, spleen cells were infected with the VV-HA-TNF or VV-IFN-γ virus at 10 pfu/cell for 1 h at 37°C. After washing twice in medium the cells were made up to a concentration of 10^6 cells/ml and subjected to increasing periods of UV-irradiation. 10^6 cells were placed in a well of a plastic 24-well plate (Linbro) and subjected to 1 min UV-irradiation at an intensity of 600-μW/cm^2 in the 230 - 270 nm range. 10^6 cells were added to a second well and the plate further exposed to 1 min UV-irradiation. This was continued until the first well had been subjected to 5 min irradiation. The 30 second irradiation was performed separately, then a final 1 ml containing 10^6 cells was added to a further well, as a non-UV-irradiated control. The cells were incubated at 37°C in a humidified atmosphere

of 5% CO_2 for 48 h. Supernatant was harvested and frozen until assayed for cytokine levels.

As shown in Table 6.1, subjecting the cells to up to 5 min UV-irradiation reduced the level of cytokine produced by up to 25%. However, levels were still sufficiently high that it was possible to use these recombinant viruses to infect stimulators and set up cultures using the same protocols as described in Chapter 4.

Table 6.1 ***Effect of UV-irradiation on cytokine production by spleen cells infected with recombinant vaccinia viruses encoding cytokine genes***

UV-irradiation (min)	VV-IFN-γ IFN-γ (ng/ml)		VV-HA-TNF TNF (ng/ml)	
	5 h	19 h	5 h	19 h
0	2.6 ± 0.0	2.2 ± 0.0	1.45 ± 0.08	2.35 ± 0.05
0.5	3.0 ± 0.1	2.6 ± 0.1	1.22 ± 0.03	2.34 ± 0.05
1	2.4 ± 0.1	2.1 ± 0.0	1.25 ± 0.1	2.22 ± 0.05
2	2.5 ± 0.2	2.0 ± 0.2	1.31 ± 0.03	2.07 ± 0.1
5	2.3 ± 0.6	1.6 ± 0.1	1.01 ± 0.09	1.91 ± 0.01

6.2.4 Culture conditions

The culture conditions were the same as described in Chapter 4.2.6. Spleens were removed from mice injected with VV-WR 6 days earlier, dispersed through a grid, and depleted of B cells and other cell populations, if appropriate. The B cell depleted cells were made up to a concentration of 5×10^6 cells/ml. Responder cells depleted of other populations were made up to the same volume, as described in Chapter 3.2.9. The immune cells were incubated with 5×10^4 stimulator cells in a 96 well plate, in a total volume of 200 µl. The stimulator cells were prepared as previously described, with the exception that these cells had been infected with either VV-WR or one of the recombinant vaccinia viruses.

6.2.5 Immunofluorescent staining of cells

Following three days incubation of vaccinia immune cells with recombinant virus infected stimulators, the cells were stained for cell surface phenotype for analysis by flow cytometry. The previous protocol for surface staining, described in Chapter 4.2.7, was scaled down, as only 5×10^5 cells were to be stained, rather than the usual 10^6 cells. Cells from the culture wells were transferred to wells of a V-bottomed 96 well (Linbro/ Titertek) plate. The plate was centrifuged at 200 g for 3 min and the supernatant flicked off. The cells were resuspended in 25 µl of the biotinylated anti-Lyt.2 (clone 53-6.7; Becton Dickinson) or the PE-conjugated anti-L3T4 (clone GK1.5; Becton Dickinson) diluted in medium, 1/25. After 20 min incubation at 4°C, 200 µl of cold PBS was added to each well and the plate centrifuged again. The supernatant was flicked off and those cells stained with the biotinylated antibody were incubated for a further 20 min at 4°C with streptavidin-phycoerythrin, at a dilution of 1/30. The cells were washed with PBS, and then resuspended in 200 µl PBS for analysis on the FACScan.

6.2.6 Proliferation assay As described in Chapter 3.2.9

6.3 RESULTS

6.3.1 Virus-encoded cytokine production was rapid

Spleen cells infected with the TNF-encoding or IFN-γ-encoding virus were UV-irradiated and then incubated at 37°C for up to 48 h. Cells infected with VV-WR did not produce TNF or IFN-γ upon incubation, as shown in Chapter 3. High levels of TNF and IFN-γ were detected in the supernatant of cells which had been infected with VV-HA-TNF and VV-IFN-γ, respectively, and incubated at 37°C. The cytokines were detected in the supernatant within 4 h of culture (Figure 6.1), with peak levels reached at 24 h. To put these cytokine levels in the context of cytokine production by immune responder cells, levels of IFN-γ produced by these VV-IFN-γ infected stimulator cells was less than 5% of the IFN-γ produced in restimulated cultures. In contrast, the TNF levels were 50 - 100% of that detected in previous cultures of restimulated responder cells.

6.3.2 Virus encoded TNF dramatically enhanced TNF levels in cultures

Incubation of the responder cells with VV-HA-TNF infected stimulator cells led to a dramatic increase in TNF levels in the culture supernatant (Figure 6.2). The TNF levels were 5 to 8 times the levels seen in cultures with VV-WR infected stimulator cells. The initial increase can be ascribed to TNF production by the TNF-encoding virus. However, the increase during the later period may be from responder cells, as the kinetic study of virus-encoded cytokine production showed that the majority of the virus-encoded TNF was produced within the first 8 h (Figure 6.1).

Significant levels of TNF were secreted into the supernatant by stimulator cells infected with VV-HA-TNF, in the absence of responder cells (Table 6.2). TNF was not detected in supernatant of stimulator cells infected with wild type or the IFN-γ- or IL-2-encoding viruses when cultured alone. The TNF produced by the TNF-encoding virus augmented the TNF produced by the responder cells to levels three times that in cultures stimulated with wild type virus. Stimulation with an IFN-γ- or an IL-2-encoding virus had no effect on the level of TNF produced by the responding cells.

Table 6.2 ***Production of TNF by vaccinia virus primed spleen cells stimulated with recombinant vaccinia virus***

Antibody treatment of responders	STIMULATING VIRUS			
	vv-WR	vv-IFN-γ	vv-HA-TNF	VV-HA-IL-2
No responders	< 100	<100	170 ± 40	< 100
J11d + C	210 ± 80	260 ± 40	630 ± 80 **	180 ± 50
J11d + anti-CD4 + C	450 ± 70	430 ± 130	970 ± 50 **	350 ± 80
J11d + anti-CD8 + C	220 ± 60	170 ± 50	630 ± 130 **	120 ± 50
J11d + anti-CD4 + anti-CD8 + C	250 ± 40	240 ± 60	1030 ± 100 **	310 ± 40
J11d + anti-asialo-GM_1 + C	160 ± 10	330 ± 30 **	740 ± 150 **	190 ± 70

Spleen cells from mice immunized 6 days earlier were treated as shown and cultured for 20 h with syngeneic spleen cells infected with viruses indicated. The TNF level (pg/ml) of the supernatant was determined using ELISA and expressed as the mean ± SD of quadruplicate wells.

* levels of TNF were significantly different to those in similar cultures stimulated by VV-WR, $p < 0.05$, Student's t-test.

** levels of TNF were significantly different to those in similar cultures stimulated by VV-WR, $p < 0.005$, Student's t-test.

To determine the spleen cell subpopulations which responded to the TNF-encoding virus with increased TNF production, responder cells were depleted of cell subsets prior to incubation with stimulator cells. Cultures containing $CD4^-/CD8^+$ T cells had increased TNF levels, as seen following stimulation with wild type virus. This suggests that the $CD4^+$ T cells were negatively influencing the TNF production of another cell type. Cultures containing $CD8^-/CD4^+$ T cells had similar levels of TNF as non-T cell depleted cultures stimulated with this virus. Responder cells lacking both T cell subsets produced similar levels of TNF as seen in cultures lacking $CD4^+$ T cells. These results would suggest a cell type other than $CD4^+$ or $CD8^+$ T cells produced TNF in response to the virus-encoded TNF. Responder cells lacking asialo-GM_1^+ cells also produced increased levels of TNF, indicating the NK cells were not the cells responding to the virus-encoded TNF. TNF levels were also increased following removal of the asialo-GM_1^+ cells in cultures stimulated with VV-IFN-γ ($p < 0.025$).

6.3.3 IFN-γ production by responder cells was enhanced by virus encoded cytokines

Incubation of responder cells with stimulator cells infected with the TNF-, IFN-γ- or IL-2-encoding virus led to enhanced levels of IFN-γ in the culture supernatant. This was observed throughout the period of the 24 h incubation in cultures stimulated with VV-IFN-γ and VV-HA-TNF (Figure 6.3). The IFN-γ levels in the VV-IFN-γ stimulated cultures were almost twice the level detected in cultures stimulated with VV-WR infected cells, eg. 53 ng/ml compared to 30 ng/ml at 24 h culture. The VV-HA-TNF infected cells stimulated three times the levels of IFN-γ, eg. 22 ng/ml compared to 8 ng/ml at 15 h.

Low levels of IFN-γ were secreted by cultured stimulator cells infected with recombinant vaccinia virus encoding this factor, whereas IFN-γ was not detected in supernatant harvested from cultured stimulator cells infected with VV-WR or viruses encoding TNF or IL-2 (Table 6.3). The augmented levels of IFN-γ in cultures of B cell depleted responder cells incubated with VV-IFN-γ–infected stimulators could not be accounted for by the IFN-γ produced by stimulator cells alone.

Table 6.3 ***Production of IFN-γ by vaccinia virus immune spleen cells stimulated with recombinant vaccinia virus***

Antibody treatment of responders	STIMULATING VIRUS			
	VV-WR	VV-IFN-γ	VV-HA-TNF	VV-HA-IL-2
No responders	< 0.3	1.3 ± 0.4	< 0.3	< 0.3
J11d + C	37.7 ± 3.1	52.3 ± 4.5 **	60.7 ± 6.0 **	48.1 ± 2.0 **
J11d + anti-CD4 + C	13.9 ± 1.3	22.2 ± 4.1 *	20.7 ± 1.4 **	17.2 ± 3.7
J11d + anti-CD8 + C	31.9 ± 2.5	36.2 ± 8.1	42.5 ± 7.5 *	42.3 ± 2.8 **
J11d + anti-CD4 + anti-CD8 + C	1.6 ± 0.4	2.8 ± 0.8	2.4 ± 0.5	1.7 ± 0.0
J11d + anti-asialo-GM_1 + C	27.3 ± 5.0	53.5 ± 15.6 *	35.0 ± 0.03 *	24.1 ± 3.6

Spleen cells from mice immunized with VV-WR 6 days earlier were treated as shown and cultured for 20 h with syngeneic spleen cells infected with viruses indicated. The supernatant was harvested and frozen until assayed for levels of IFN-γ. Results shown are expressed as ng/ml and are the mean ± SD of quadruplicate wells.

* levels of IFN-γ were significantly different to those in similar cultures stimulated by VV-WR, $p < 0.05$, Student's t-test.

** levels of IFN-γ were significantly different to those in similar cultures stimulated by VV-WR, $p < 0.005$, Student's t-test.

To determine the cells producing the enhanced levels of IFN-γ in response to the virus encoded cytokines, responder cells were depleted of cell subsets before culture with the virus infected stimulator cells. Removal of the $CD4^+$ T cells from the responder cell population dramatically reduced the level of IFN-γ produced in all cultures (Table 6.3). The levels of IFN-γ in the $CD4^-/CD8^+$ T cell cultures were significantly greater in the cultures stimulated with recombinant viruses encoding TNF or IFN-γ, compared to restimulation with wild type virus. Stimulation of these $CD4^-/CD8^+$ T cell cultures with the IL-2-encoding virus, on the other hand, did not increase levels of IFN-γ compared to cultures stimulated with control virus.

Cultures containing $CD8^-/CD4^+$ T cell responders did not respond with enhanced production of IFN-γ when stimulated with the IFN-γ-encoding virus, when compared to cultures stimulated with VV-WR. In contrast, stimulation of these $CD8^-/CD4^+$ cells with VV-HA-IL-2 or the TNF-encoding virus produced increased levels of IFNγ compared to stimulation with the wild type virus. Cultures lacking both $CD4^+$ and $CD8^+$ T cells produced less than 5% of the IFN-γ found in non-depleted cultures, irrespective of the stimulating virus. These results suggested that the increased levels of IFN-γ observed following stimulation with the IFN-γ-encoding virus was largely derived from the $CD8^+$ T cells, although some may be produced by the $CD4^+$ T cells.

Removal of the asialo-GM_1^+ cells led to decreased IFN-γ titres in cultures stimulated with either wild type virus ($p < 0.05$) or the TNF- or IL-2-encoding viruses ($p < 0.005$) compared to non-depleted cultures. Cultures stimulated with VV-IFN-γ had no change in IFN-γ levels, following removal of the asialo-GM_1^+ cells.

6.3.4 Effect of virus-encoded cytokines on proliferative response of responder cells

The proliferative response of responders to stimulation by cells infected with the recombinant viruses was examined in order to determine whether this could account for the increased IFN-γ production (Figure 6.4). The proliferation of responder cells depleted of B cells was greater in cultures stimulated with recombinant vaccinia viruses than control virus-stimulated cells ($p < 0.05$). In cultures containing $CD4^-/CD8^+$ T cells, the IFN-γ encoding virus stimulated enhanced proliferation at a low, but significant level. Both the TNF and IFN-γ-encoding viruses stimulated significantly greater proliferation of $CD8^-/CD4^+$ responders, compared to stimulation with the wild type virus. The proliferative response of responders depleted of both $CD4^+$ and $CD8^+$ T cells was barely above background levels, irrespective of the stimulating virus. Asialo-GM_1^- responder cells also had increased proliferation following stimulation with recombinant vaccinia viruses, compared to stimulation with wild type virus, indicating the asialo-GM_1^+ cells were not responsible for the increased proliferation in non-depleted cultures.

6.3.5 Prolonged incubation with recombinant stimulators did not alter frequency of $CD4^+$ and $CD8^+$ T cells

The proliferation data presented above suggested the recombinant viruses may induce enhanced proliferation of specific populations of cells. This was further examined by analysing the frequency of $CD4^+$ and $CD8^+$ T cells in cultures after 84 h (3.5 days) incubation with recombinant virus infected stimulator cells. Table 6.4 shows the results of flow cytometric analysis of cells from two experiments. The percentage of $CD4^+$ and $CD8^+$ T cells did not vary beyond 7%.

Table 6.4 ***Effect of virus-encoded cytokines on CD4+ and CD8+ T cell frequency***

Virus	% $CD4^+$ T cells		% $CD8^+$ T cells	
	Exp 1.	Exp. 2	Exp. 1	Exp. 2
VV-WR	30	26	27	22
VV-HA-IL-2	29	29	31	24
VV-HA-TNF	29	22	29	22
VV-IFN-γ	31	28	33	20
(range)	(2%)	(7%)	(6%)	(4%)

50×10^4 B-cell depleted vaccinia immune splenocytes were incubated with 5×10^4 UV-irradiated syngeneic spleen cells infected with VV-WR or a recombinant virus encoding a cytokine in a total volume of 200 µl. After 84 h incubation, cells were transferred to a V-bottomed 96 well plate for antibody staining and were analysed by flow cytometry. The results given are frequencies of $CD4^+$ and $CD8^+$ T cells in all cells in the restimulated cultures.

The frequency of T cells within the cultured $CD4^-/CD8^+$ and $CD8^-/CD4^+$ responder populations was also examined. The responder cells were depleted of $CD4^+$ or $CD8^+$ T cells by incubation with antibody and C. This procedure was not 100% efficient, and contaminating cells of up to 5% were considered acceptable, ie. cultures described as $CD4^+$ depleted ($CD4^-/CD8^+$) may have up to 5% $CD4^+$ T cells present. The cytokines encoded by the recombinant viruses may have stimulated the few contaminating cells ($CD4^+$ T cells if continuing with the above example), in the cultures and induced their proliferation to numbers which may then have been able to influence cytokine titres. Table 6.5 shows the frequency of contaminating $CD8^+$ or $CD4^+$ T cells in cultures containing responder cells which had been depleted of $CD8^+$ or $CD4^+$ T cells, respectively. Following three days incubation with recombinant or wild type virus-infected stimulators, the difference in frequency of contaminating cells was within a 2% range.

Table 6.5 ***Recombinant vaccinia viruses did not influence T cell regeneration***

Virus	% $CD4^+$ cells	% $CD8^+$ cells
	CD4 depl. responders	CD8 depl. responders
before restim.	3.4	0.1
VV-WR	3.8	0.6
VV-HA-IL-2	5.2	1.8
VV-HA-TNF	3.9	2.1
VV-IFN-γ	3.3	1.6
range (restim. cells)	1.9%	1.5%

Vaccinia immune spleen cells were depleted of B cells and $CD4^+$ or $CD8^+$ T cells, as described in 3.2.9. These responder cells were incubated with UV-irradiated, syngeneic spleen cells which had been infected with VV-WR or recombinant vaccinia viruses encoding cytokines for 1 h at 37°C. Following an 84 h incubation at 37°C, the cells were transferred to a 96 well V-bottomed plate for antibody staining, resuspended in 200 µl PBS and analysed by flow cytometry. The results given are the frequency of $CD4^+$ or $CD8^+$ T cells in all cells.

6.3.6 Restimulation with recombinant viruses encoding Th2- type cytokines did not down-regulate IFN-γ production

Vaccinia immune spleen cells were incubated with cells infected with viruses encoding IL-4 or IL-10, to examine whether the production of either of these factors would alter the cytokines produced by the responder cells. IL-4 was not detected by the IL-4 ELISA throughout the incubation period in any of the cultures, including wells with the VV-HA-IL-4 infected stimulators. IL-10 levels in culture supernatant were very low also, with maximal titres of 30 pg/ml in cultures restimulated with VV-WR, and 55 pg/ml following restimulation with the IL-4- or IL-10-encoding virus for 3 days (Figure 6.5). Low levels of these cytokines were detected in supernatant harvested from stimulator cells infected with the IL-4 or IL-10-encoding virus and cultured alone, at 5 x 10^4 cells/ml. After 40 h incubation, supernatant taken from UV-irradiated, VV-HA-IL-4 infected spleen cells contained 2.6 U/ml IL-4. Supernatant harvested from UV-irradiated VV-HA-IL-10-infected spleen cells contained 900 pg/ml IL-10.

Restimulation of the immune cells with the IL-10-encoding virus led to increased production of IFN-γ (Table 6.6). The VV-HA-IL-10 had no effect on TNF levels compared to restimulation with wild type virus. The effect on IL-6 production was inconclusive, and the results are not shown. The proliferative response was enhanced in cultures stimulated with VV-HA-IL-10.

Restimulation of the vaccinia immune responder cells with cells infected with the IL-4-encoding virus resulted in enhanced secretion of IFN-γ (Table 6.6), compared to levels stimulated by the wild type virus. This effect was observed in two similar experiments. Stimulation with VV-HA-IL-4 also led to enhanced proliferation of the responder cells compared to cultures stimulated with the wild type virus (Table 6.6).

Table 6.6 ***Effect of restimulation with recombinant viruses encoding IL-4 or IL-10 on cytokine production by vaccinia virus immune cells***

Virus	Time (h)	TNF (pg/ml)	IFN-γ (ng/ml)	IL-6 (AU/ml)	Proliferation (cpm x 10^3)
VV-WR	12	170 ± 40	13.2 ± 1.9	5.8 ± 1.2	
	24	260 ± 50	28.6 ± 6.8	18.9 ± 4.6	33.9 ± 7.9
	48	510 ± 160	49.3 ± 4.1	21.4 ± 4.9	
VV-HA-IL-10	12	200 ± 10	19.4 ± 2.7 *		
	24	300 ± 40	53 ± 6 **	ND	65.0 ± 11.9 *
	48	550 ± 90	120 ± 15 **		
VV-HA-IL-4	12		19.4 ± 3.2 *		
	24	ND	60 ± 10 **	ND	51.5 ± 6.7 *
	48		80 ± 14 **		

Spleen cells from 6 day vaccinia immune mice were depleted of B cells and cultured with syngeneic spleen cells infected with the VV-WR or the IL-4- or IL-10-encoding viruses. Supernatant was harvested at the times indicated and frozen until assayed for cytokine titres. Results are expressed as mean ± SD of quadruplicate wells.

* significantly different to levels in similar cultures stimulated with VV-WR, $p < 0.05$, Student's t-test.

** significantly different to levels in similar cultures stimulated with VV-WR, $p < 0.005$, Student's t-test.

6.4 DISCUSSION

Recombinant vaccinia viruses which encode cytokine genes were used to infect naive spleen cells which were then used as the stimulatory population in culture with vaccinia virus immune spleen cells. The advantage of using this approach was that the cytokines encoded by the recombinant virus were produced at the site of virus presentation and thus, were in the immediate vicinity of the cells responding to the presentation of antigen.

The cytokine gene in the vaccinia recombinants was under the control of the P7.5 vaccinia promotor, leading to gene expression early and late in the vaccinia virus replicative cycle. Genes under the control of this promotor were able to be expressed in the absence of DNA replication (Coupar *et al.*, 1986). UV-irradiation of cells infected with recombinant vaccinia virus prevented replication of the virus, yet did not inhibit production of the virus-encoded cytokine (Table 6.1). Cytokine production by UV-irradiated cells infected with VV-HA-TNF or VV-IFN-γ was rapid, with levels detected within 4 to 8 h incubation and reaching maximum between 16 and 24 h culture. A similar profile was observed following the infection of human 143B cells with VV-HA-IL-2 virus. Secretion of a significant amount of IL-2 was detected within 4 h, with maximum activity about 12 h after infection (Ramshaw *et al.*, 1987).

Stimulation of vaccinia immune cells with a TNF-encoding virus led to a threefold increase in TNF production by the responder cells, compared to cultures stimulated with either the wild type or IFN-γ-encoding virus (Table 6.2). The increase was 2.5 times that which could be attributed to the TNF produced by the recombinant virus, indicating that the viral-encoded TNF was stimulating the production of enhanced levels of TNF. Autoinduction of cytokine expression has been reported for several monokines and growth factors, including TNF. Recombinant TNF added to cultures of human monocytes increased their cytotoxic activity on the TNF sensitive cell-line WEHI-164 (Philip and Epstein, 1986), while recombinant TNF added to murine bone marrow-derived macrophages induced the production of TNF mRNA (Descoteaux and Matlashewski, 1990).

Removal of the $CD8^+$ T cells did not affect the increased TNF levels stimulated by the TNF-encoding virus, indicating the majority of TNF was not produced by these cells types in agreement with the results in Chapter 4. The asialo-GM_1^+ cells were not responsible for the enhanced TNF production either, as removal of these cells did not affect the TNF levels. A further increase in TNF levels was observed in cultures lacking $CD4^+$ T cells, suggesting that that these cells had a negative regulatory activity on TNF production. However, in cultures stimulated with the TNF-encoding virus, the regulatory activity of the $CD4^+$ T cells was not directed against the $CD8^+$ T cell TNF production, as was observed in Chapter 4. This was demonstrated by the continued high levels of TNF in cultures lacking both $CD4^+$ and $CD8^+$ T cells, which was not seen following restimulation with the wild type vaccinia virus. Thus, the source of enhanced TNF induced by the virus-encoded TNF was neither a $CD4^+$, $CD8^+$ or asialo-GM_1^+ cell. These results and the studies by Philip and Epstein (1986) and Descoteaux and Matlashewski (1990) point to the macrophage/monocyte cells in the responder cell population as the source of the enhanced TNF. However, these experiments do not rule out the possibility that another population of $CD4^-/CD8^-$ T cells, possibly γ/δ T cells, may be induced to produce this factor. In mice infected with influenza virus, cells expressing TCR-γ/δ mRNA were prevalent at the site of inflammation late in the course of infection (Carding *et al.*, 1990). Using *in situ* hybridization, Carding *et al.* (1993) also demonstrated that γ/δ T cells isolated from influenza virus-infected mouse lung expressed mRNA for a number of cytokines. 49% of the γ/δ T cells expressed mRNA for IFN-γ and 27% expressed LT (TNF-β) mRNA. The numbers of γ/δ-TCR^+ T cells were also shown to be elevated in the spleen of mice during infection with Ectromelia virus (Karupiah *et al.*, 1993). There is also another population of $CD3^+$ $CD4^-/CD8^-$ T cells present in the spleens of mice infected with vaccinia virus. In contrast to the influenza system, these cells express message for the α/β TCR chain, with no evidence for the γ/δ TCR chain. These cells have antiviral activity *in vivo*, as the transfer of $CD4^-/CD8^-$ T cells to *scid* mice allows the mice to resolve virus infection (J. Ruby, personal communication). The contribution of these cells to the antiviral response is unclear, however, they are a potential source of cytokines in this *in vitro* model.

Stimulation with VV-HA-TNF also led to increased levels of IFN-γ compared to stimulation with wild type virus (Table 6.3) in all cultures except those lacking T cells. The virus-encoded TNF required the presence of both $CD4^+$ and $CD8^+$ cells to stimulate increased IFN-γ production. Whether the $CD4^+$ or $CD8^+$ T cells were producing the IFN-γ, or were required for another population to produce the cytokine, is unclear. Treatment of the responder population with asialo-GM_1 antibody substantially reduced the IFN-γ response, indicating that these cells were a major source of the IFN-γ induced by the TNF virus. Bancroft *et al.* (1989) demonstrated that the production of IFN-γ by NK cells isolated from *scid* mice was dependent on TNF. However, recombinant TNF alone was not sufficient to stimulate NK cell production of IFN-γ (Wherry *et al.*, 1991) and IL-2 induced maximum production of IFN-γ in response to TNF or IL-12 (Tripp *et al.*, 1993). This would explain the lack of IFN-γ in the T cell depleted cultures, where NK cells may require a T-cell derived stimulus as well as TNF to produce IFN-γ.

Stimulation of the immune responder cells with the IFN-γ- or IL-2-encoding virus did not significantly alter TNF levels compared to those seen in cultures stimulated with wild type virus, with one exception. Responders depleted of the asialo-GM_1^+ cells cultured with VV-IFN-γ secreted increased levels of TNF. Anti-asialo-GM_1 was used to deplete NK cells from the vaccinia immune spleen cells (Kasai *et al.*, 1980), although this antibody may have activity against other cell types, including T cells (Stein *et al.*, 1978). The increase in TNF levels observed following the removal of these cells may indicate that IFN-γ may negatively regulate TNF production by NK cells. IFN-γ has been implicated in the negative feedback control of TNF in an *in vivo* study which demonstrated that serum TNF levels in LPS treated mice were higher in animals pretreated with antibody to IFN-γ (Doherty *et al.*, 1992).

IFN-γ did not appear to be the mediator of the negative regulatory effect of the $CD4^+$ T cells on TNF production. Cultures lacking $CD4^+$ T cells and stimulated with wild type virus-infected cells had increased levels of TNF The increase in TNF was also observed in $CD4^-$ T cell cultures stimulated with the IL-2- or IFN-γ-encoding virus (Table 6.2). In addition, in results presented in Chapter 5, high levels of IL-2 added to $CD4^-$ T cell cultures restored the production of IFN-γ. However, levels

of TNF remained unchanged (Table 5.10). The results from the recombinant virus studies and work presented in Chapter 5 indicate that neither IL-2 nor IFN-γ mediated the $CD4^+$ T cell negative regulation of TNF production.

The production of low levels of IFN-γ by the stimulator cells infected with the IFN-γ encoding virus augmented the production of IFN-γ by the responder cells (Figure 6.3 and Table 6.3). This finding is in agreement with studies where the addition of recombinant IFN-γ to murine splenocyte cultures led to increased expression of IFN-γ mRNA within 2 h, with maximal induction requiring 8 to 12 h culture (Cockfield *et al.*, 1993). A similar result was observed with human peripheral blood lymphocytes (Hardy and Sawada, 1989). Cultures lacking $CD8^+$ T cells did not respond to the VV-IFN-γ stimulation with enhanced IFN-γ production (Table 6.3), thus the enhanced levels of IFN-γ following stimulation with the virus encoding this factor, were largely derived from $CD8^+$ T cells. The increased IFN-γ in cultures stimulated with the IFN-γ encoding viruses did not appear to be derived from NK cells as the removal of asialo-GM_1^+ cells did not reduce IFN-γ titres.

The use of VV-HA-IL-2 as a stimulating virus also significantly enhanced IFN-γ production from the vaccinia immune responder cells. IFN-γ secretion by these cells has been shown to be enhanced upon the addition of an exogenous source of IL-2, as described in the previous chapter. IL-2 has an important role in augmenting IFN-γ synthesis by activated T cells (Reem and Yeh, 1984). IL-2 is also a potent stimulator of murine NK cells (Henney *et al.*, 1981) and enhances NK cell production of IFN-γ (Tripp *et al.*, 1993). Studies examining the growth of the recombinant vaccinia virus encoding IL-2 have shown the IL-2 acts on NK cells to enhance their antiviral response (Karupiah *et al.*, 1991). This is mediated through host-derived IFN-γ, as demonstrated by administration of mAb against this cytokine (Karupiah *et al.*, 1990b). The work presented in the *in vitro* study described here would suggest the virus-encoded IL-2 enhanced secretion of IFN-γ from both the $CD4^+$ T cells and the asialo-GM_1^+ cells (NK cells), as enhanced production of IFN-γ was not seen in cultures depleted of these cells. However, the previous chapter demonstrated that high levels of exogenous IL-2 could restore the IFN-γ producing ability of responder cells depleted of $CD4^+$ or asialo-GM_1^+ cells. This may indicate that

virus-encoded IL-2 produced at the site of antigenic stimulation acted upon a different population(s) of cells compared to the exogenous IL-2 source.

It may be argued that the augmented cytokine production in cultures stimulated with recombinant viruses is due to an increased level of cell proliferation caused by the virus-encoded cytokine. Indeed, proliferation was significantly increased in cultures of responder cells stimulated with most recombinant viruses (Figure 6.4 and Table 6.6). However, there was not always a direct correlation between increased proliferation and increased cytokine levels. For example, $CD8^+$ T cell depleted responders had a higher level of proliferation when stimulated with VV-IFN-γ, compared to the same cells stimulated with the wild type virus (Figure 6.4). The IFN-γ levels following stimulation with these viruses, however, were not significantly different (36 ng/ml vs 32 ng/ml, Table 6.3). In another example, these same cells stimulated with VV-HA-IL-2 did not have increased proliferation but did secrete significantly enhanced levels of IFN-γ. Therefore, increased proliferation may account in part for the augmented cytokine levels, however, this was not the only factor involved. Furthermore, increased cytokine levels were observed in some recombinant stimulated cultures within 8 h of culture, eg. IFN-γ levels following restimulation with VV-HA-TNF (Figure 6.3). This increase occurred before substantial cell division would have taken place. Removal of the $CD8^+$ T cells led to a decrease in proliferation in cultures stimulated with all viruses, while a more dramatic decrease in proliferation was observed in cultures lacking $CD4^+$ T cells. This indicates that both these cell types proliferated in response to restimulation with virus infected cells. However, the poor proliferative response of $CD8^+$ T cells in the absence of $CD4^+$ T cells suggests that some interaction is occuring between these cell populations. The only significant increase in proliferation among the $CD8^+/CD4^-$ cells was among those cells stimulated with the IFN-γ-encoding virus. Novelli *et al.* (1991) demonstrated that the activation of human T cells was inhibited by antibodies to IFN-γ, indicating that IFN-γ is a critical signal in the early phases of T cell proliferative responses. Thus, a possible candidate for the molecule required by the $CD8^+$ cells is IFN-γ, which was also decreased in those cultures lacking $CD4^+$ T cells. The increased proliferation in VV-IFN-γ stimulated cultures was low, which may reflect the low level of IFN-γ produced by the VV-IFN-γ infected

stimulators, ie. less than 5% of the IFN-γ levels in cultures of non-depleted responder cells stimulated with wild type virus. The expression of different cytokines did not selectively stimulate the proliferation of sub-types of T cells as the ratio of $CD4^+$ to $CD8^+$ T cells within the cells in culture did not change after 3 days incubation (Table 6.4).

The analysis of $CD4^+$ and $CD8^+$ frequencies among the responder population accounted for only 60% of the cells (Table 6.4). The responder cells had been depleted of B cells by incubation with J11d hybridoma supernatant and C. The phenotype of the remaining 40% of cells, the non-$CD4^+/CD8^+$ cells, was not determined. However, these cells made a significant contribution to cytokine levels. IL-6 and TNF were produced by non T cells which were plastic adherent. These cells are believed to be macrophages and monocytes. NK cells and $CD4^-/CD8^-$ T cells (α/β TCR^+, as discussed earlier) would make up a proportion of these non-defined cells. The TNF production of these cells was potently stimulated by the TNF-encoding virus.

It has been suggested that the cytokines present at the initiation of an immune response will determine the subsequent production of either a Th1- or Th2-type of response (Romagnani, 1992). There is clear evidence of the importance of certain cytokines in the regulation of the response to infection with *L. major* in mice. Treatment of resistant mice with an anti-IFN-γ antibody led to increased susceptibility (Scott, 1991), whereas treatment of BALB/c susceptible mice with anti-IL-4 antibody allowed them to develop a healing Th1-type response (Chatelain *et al.*, 1992). To examine whether the presence of Th2 type cytokines at the initiation of the response would alter the cytokine production of the restimulated vaccinia immune cells towards a Th2 profile, the responders were cultured with cells infected with either the IL-4- or IL-10-encoding virus. Supernatant harvested from cultures of UV-irradiated VV-HA-IL-4 or VV-HA-IL-10 infected stimulator cells contained barely detectable levels of the encoded factors. This contrasts with high levels of cytokine produced by 143B cells infected with these viruses (D. Sharma, personal communication). The reason for this is unknown, but may be a reflection of utilization of the cytokine by the responder cells, or the effect of UV-irradiation treatment of the virus infected cells. However, these recombinant viruses did have an effect when used as the stimulatory virus in culture with immune responder

cells. The results suggest that stimulation with the recombinant viruses encoding these Th2-type cytokines was not able to redirect the response of the immune cells (Table 6.6). In fact, restimulation of the immune cells with these recombinant viruses induced the production of 2 to 3 times the level of IFN-γ, compared to levels stimulated by VV-WR infected cells. The effect of these Th2 virus-encoded cytokines on the production of TNF and IL-6 was not so clear. Proliferation of the immune cells was also increased when cultured with the IL-4- or IL-10-encoding viruses.

IL-4 and IL-10 are produced by Th2-type cells which are able to down-regulate production of Th1-type cytokines (Fiorentino *et al.*, 1989; Mosmann *et al.*, 1986). However, stimulation of the vaccinia immune spleen cells with these viruses actually led to an increase in IFN-γ titres. IL-4 was not able to be detected in the culture supernatant throughout the incubation period. IL-10 was detected, but significant levels were not reached until 24 h of culture, by which time IFN-γ levels were close to maximal. The responder cells had been taken from mice primed with vaccinia virus, a type of infection that induces specific immune responses of the Th1-type. This is achieved through direct stimulation of NK cells to produce IFN-γ (Romagnani, 1992), or through induction of macrophage derived-IFN-α and IL-12, which in turn stimulate NK cell IFN-γ production (Tripp *et al.*, 1993). The responder cells also contain a significant number of activated $CD8^+$ T cells which may have been producing IFN-γ, as the spleens were removed at the height of the CTL response to the vaccinia infection (Ruby *et al.*, 1993). This priming towards a Th1 response may have prevented the virus-encoded IL-4 and IL-10 altering the cytokines produced by the vaccinia immune cells. The reason for the increased IFN-γ levels in the presence of the IL-4- or IL-10-encoding virus is unknown, although it has been demonstrated that IL-4 plays a role in the generation of secondary allo-CTL (Suzuki *et al.*, 1992).

6.5 SUMMARY

This chapter examined the effect of virus-encoded cytokine production, at the site of antigenic stimulation, on the production of TNF and IFN-γ. UV-irradiation of the recombinant virus-infected stimulator cells did not inhibit the rapid production and release of virus-encoded cytokines into culture supernatant. Restimulation of vaccinia virus immune cells with the TNF-encoding virus induced enhanced production of TNF and IFN-γ, compared to cells restimulated with the wild type virus. The virus-encoded TNF stimulated increased production of TNF by a non-$CD4^+/CD8^+$ cell, whereas the increased IFN-γ production was T cell dependent. $CD8^+$ T cells among the responder cells were stimulated to produce enhanced levels of IFN-γ when incubated with stimulators infected with the IFN-γ-encoding virus. Virus-encoded IL-2 acted on the $CD4^+$ and asialo-GM_1^+ cells to enhance their production of IFN-γ. Restimulation of vaccinia virus immune cells with recombinant viruses encoding IL-10 or IL-4 did not influence the cytokine response towards production of Th2 cytokines, rather, increased levels of IFN-γ were observed.

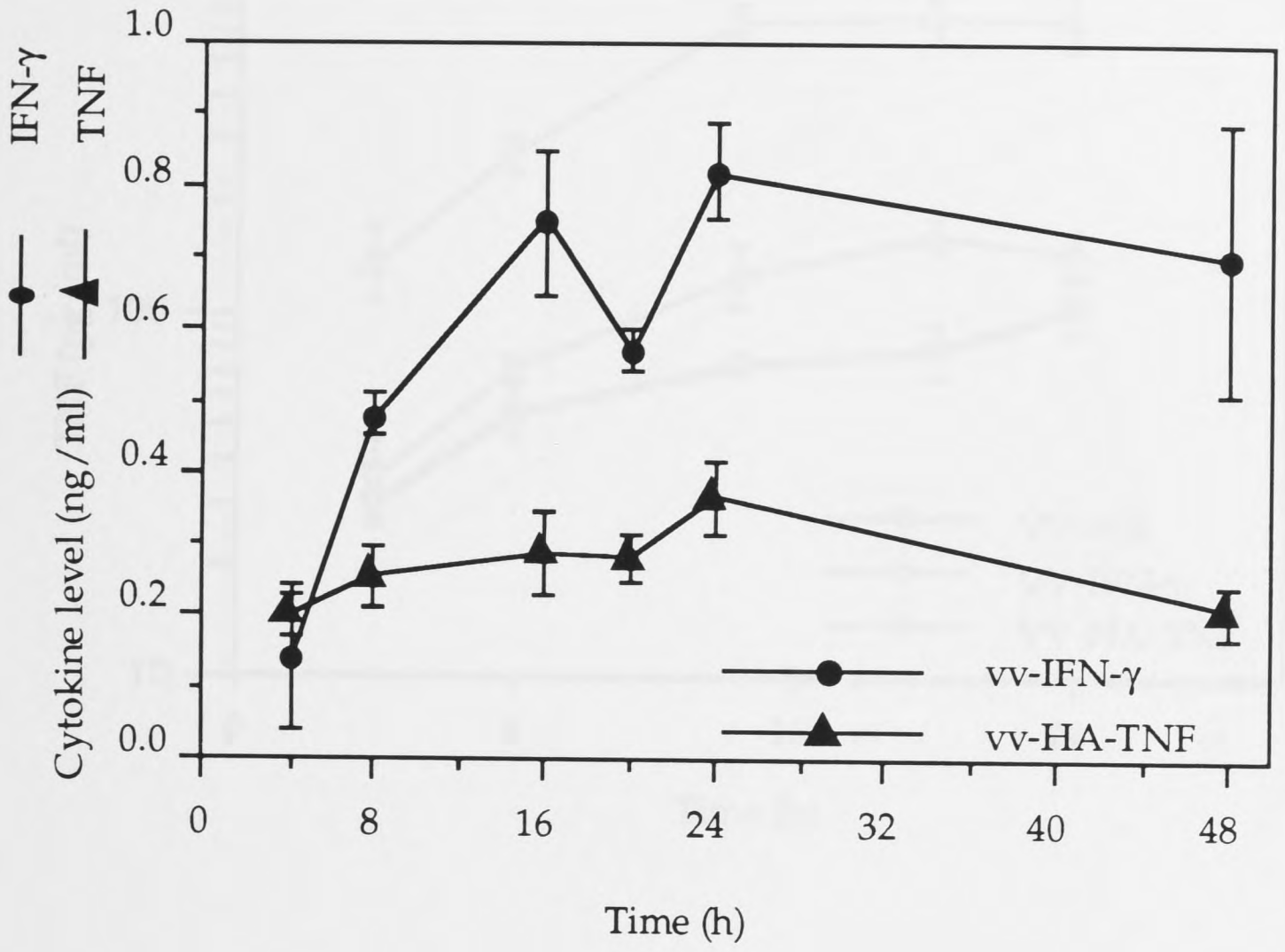


Figure 6.1 ***Time course of cytokine production by stimulator cells infected with vaccinia virus encoding cytokine genes***

B cell depleted spleen cells taken from naive mice were infected with VV-HA-TNF or VV-IFN-γ at 10 pfu/ml for 1 h at 37°C. After two washes and 5 min UV-irradiation, the cells were made up to a concentration of 5×10^5 cells/ml. 200 µl volumes were placed in the wells of 96 well plates and these were incubated at 37°C. Supernatant from four wells was harvested at each time point and frozen until assayed for cytokine levels. The data is presented as mean ± SD of quadruplicate wells.

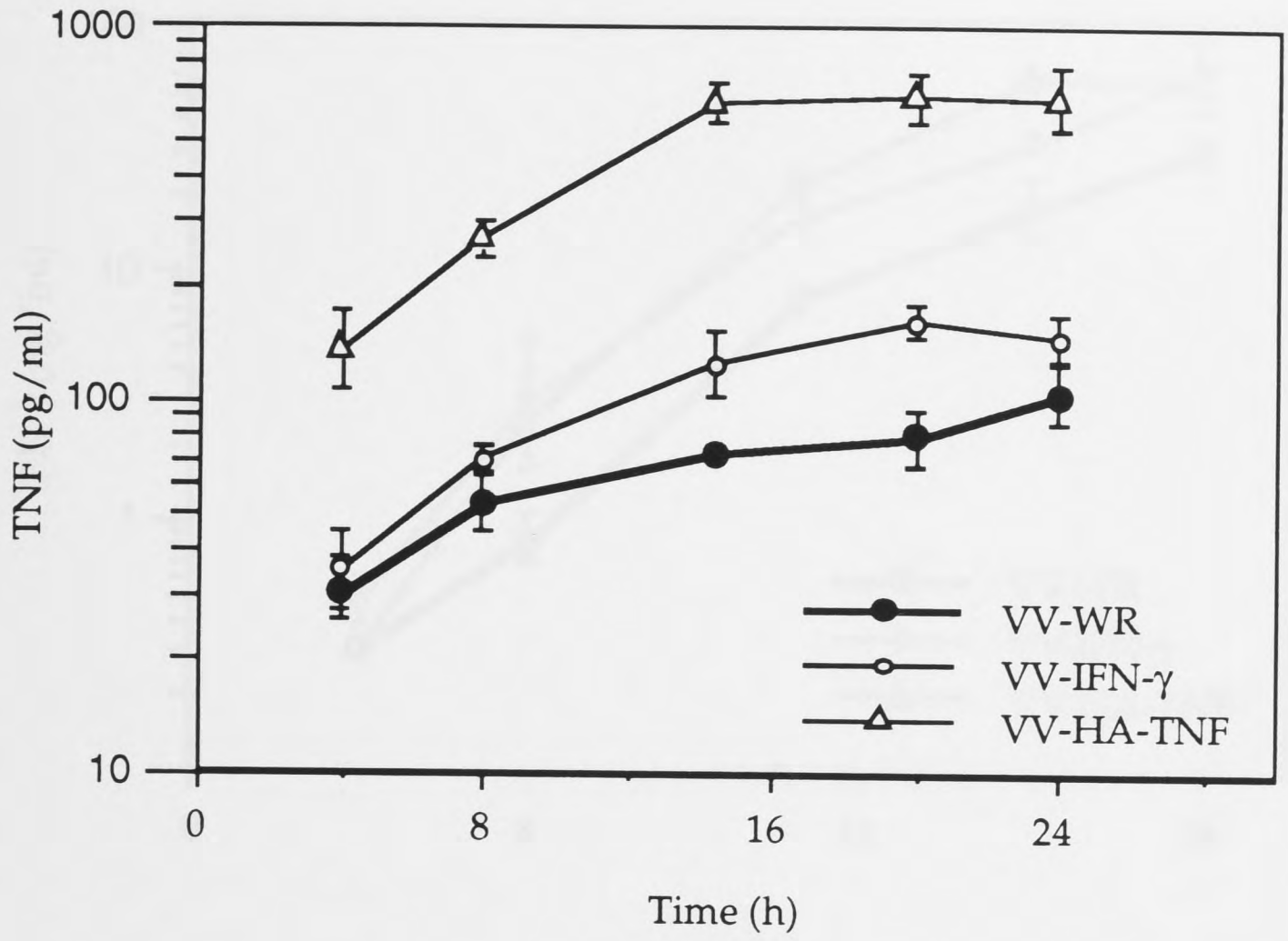


Figure 6.2 ***Time course of TNF production by virus immune spleen cells cultured with recombinant vaccinia virus infected cells.***

Stimulator cells were prepared by infecting naive B-cell depleted spleen cells with either wild type vaccinia virus (VV-WR) or a recombinant vaccinia virus encoding a cytokine gene. Following 1 h incubation at 37°C, these cells were UV-irradiated and then incubated, at 37°C in a humidified atmosphere in 200 µl volumes, with B cell depleted spleen cells from 6 day vaccinia virus immune mice. Supernatant was harvested at the times indicated. The data is presented as pg/ml, on a log scale, and is expressed as mean ± SD of quadruplicate wells.

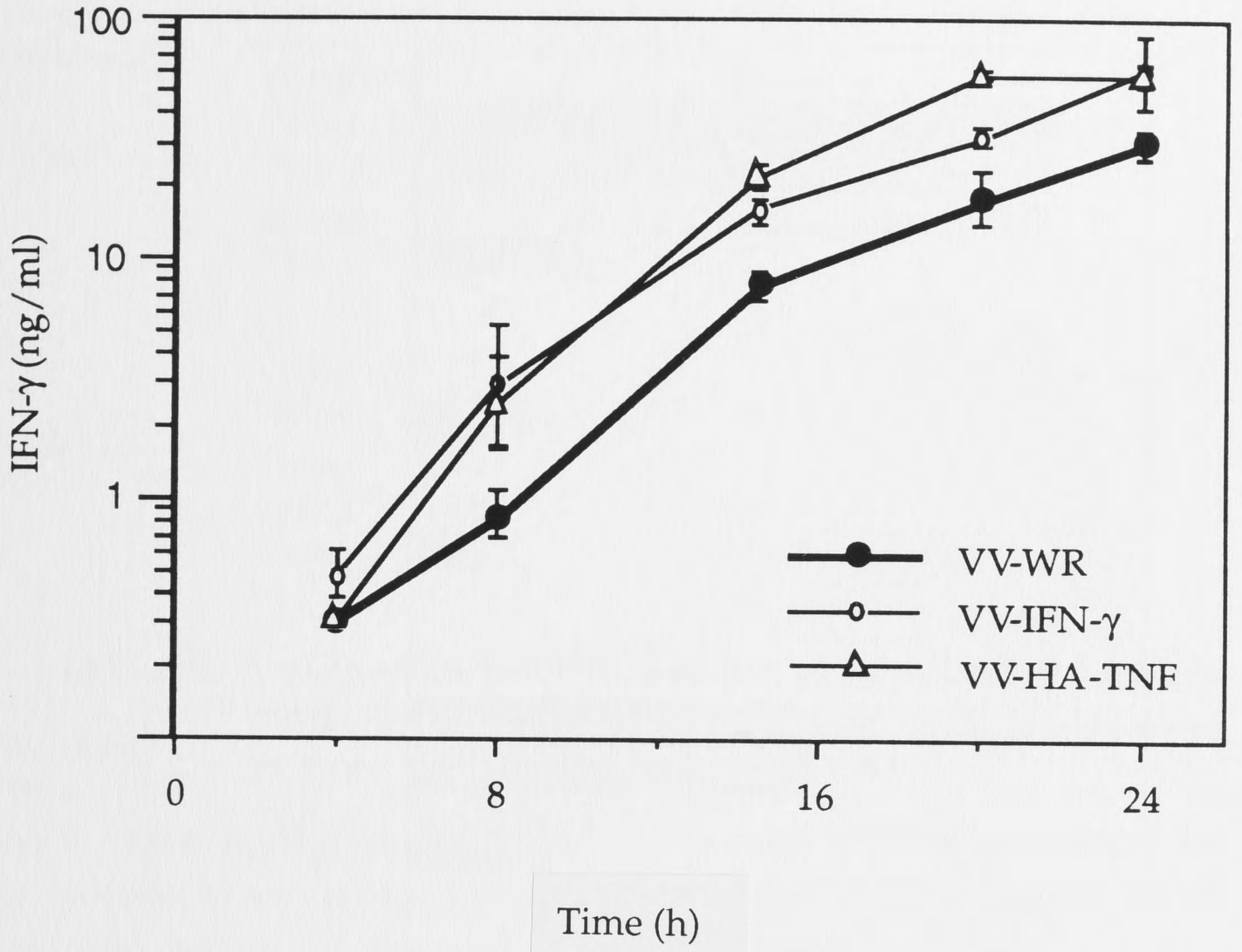


Figure 6.3 ***Time course of IFN-γ production by virus immune spleen cells cultured with recombinant vaccinia virus infected cells.***

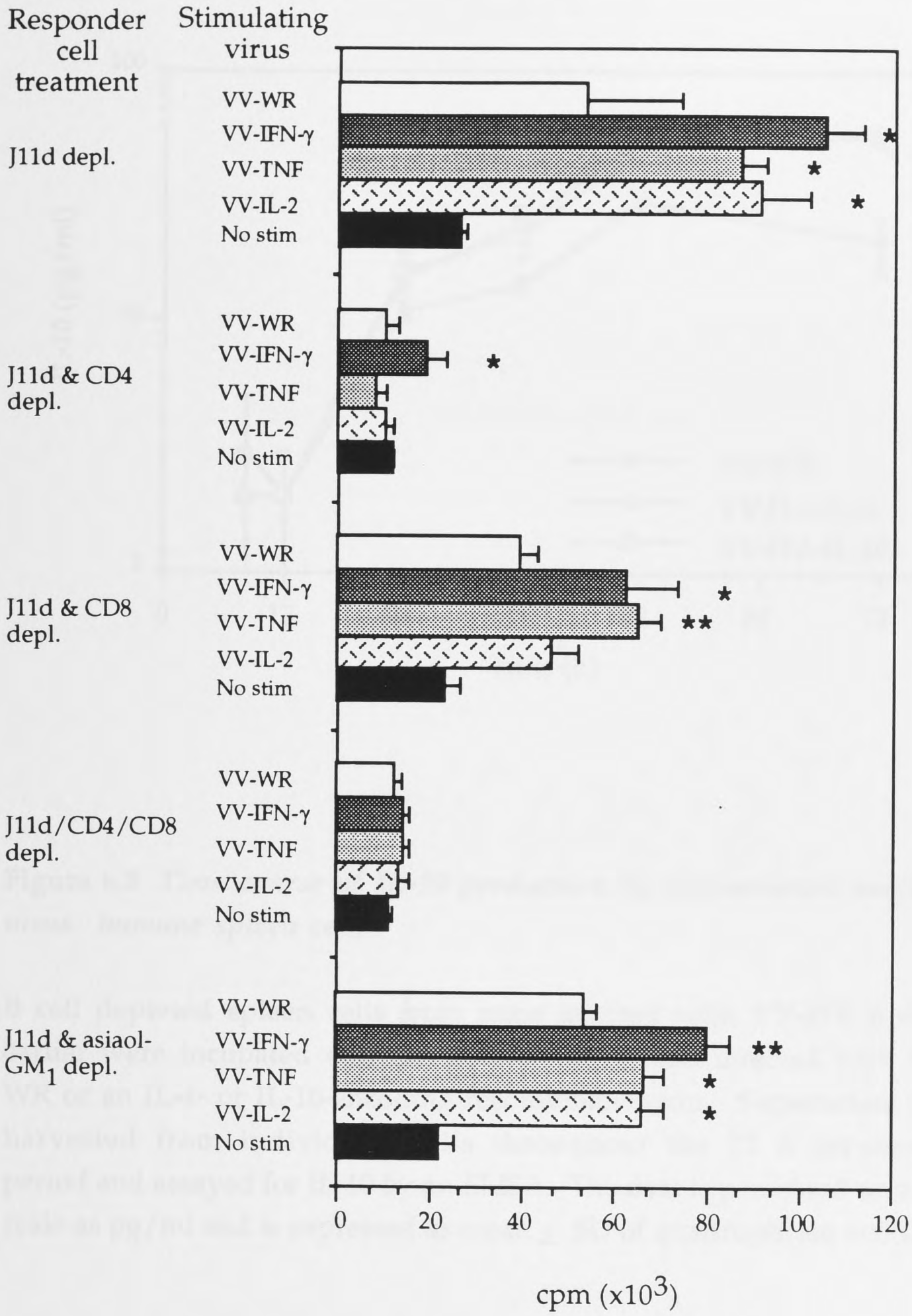
Stimulator cells were prepared by infecting naive B cell depleted spleen cells with either wild type vaccinia virus (VV-WR) or a recombinant vaccinia virus encoding a cytokine gene. Following 1 h incubation at 37°C, these cells were UV-irradiated and then incubated, at 37°C in a humidified atmosphere in 200 µl volumes, with B cell depleted spleen cells from 6 day vaccinia virus immune mice. Supernatant was harvested at the times indicated. The data is presented as ng/ml, on a log scale, and is expressed as mean ± SD of quadruplicate wells.

Figure 6.4 ***Proliferative response of virus immune cells stimulated with recombinant vaccinia virus infected spleen cells***

Responder cells were prepared by taking spleens from 6 day VV-WR immune mice and depleting of specific cell subsets by incubating with antibody and complement. These cells were incubated with virus infected stimulator cells for 48 h at 37°C. The viruses used to infect the stimulators are shown in the figure. Incorporation of [^{3}H]-TdR during the final 4 h of culture was determined. The data is shown as the mean ± SD of quadruplicate wells.

* significant, $p < 0.05$, Student's t-test

** significant, $p < 0.005$, Student's t-test


Responder cell treatment
Stimulating virus
J11d depl.
VV-WR
VV-IFN-γ
VV-TNF
VV-IL-2
No stim
J11d & CD4 depl.
J11d & CD8 depl.
J11d/CD4/CD8 depl.
J11d & asiaol-GM1 depl.
0
20
40
60
80
100
120
cpm (x10^3)

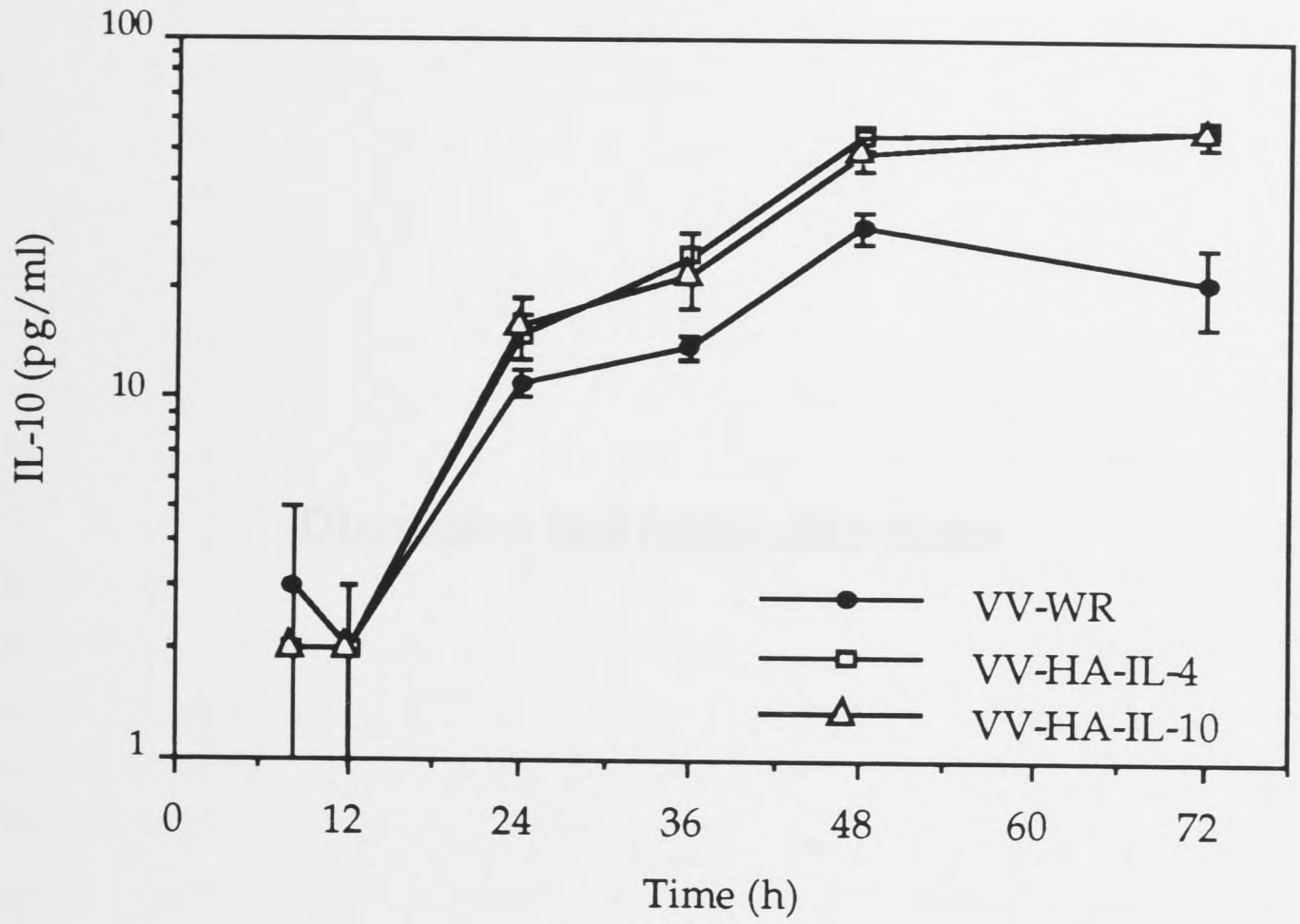


Figure 6.5 ***Time course of IL-10 production by restimulated vaccinia virus immune spleen cells***

B cell depleted spleen cells from mice injected with VV-WR 6 days earlier were incubated with syngeneic spleen cells infected with VV-WR or an IL-4- or IL-10-encoding recombinant virus. Supernatant was harvested from individual wells throughout the 72 h incubation period and assayed for IL-10 by an ELISA. The data is presented on a log scale as pg/ml and is expressed as mean ± SD of quadruplicate wells.

CHAPTER 7

Discussion and future directions

T lymphocytes attracted to the site of virus replication play a crucial role in the control and recovery from the infection (Blanden, 1974). These cells can be directly cytolytic and release cytokines that are involved in the recruitment of other cell populations. Many of the recruited cells in turn are able to secrete cytokines, including IFN-γ and TNF, both of which have antiviral activity. Recovery from a pox virus infection is dependent on the development of virus-specific class-I-restricted CTLs. The mechanism of action of these $CD8^+$ cytotoxic T cells in virus elimination has always been assumed to be direct cytolytic destruction of the virus infected cells, as can be demonstrated in *in vitro* studies. On the other hand, there are reports that demonstrated that soluble factors may be important in antiviral protection (Lehmann-Grube *et al.*, 1988). The antiviral activity of a number of cytokines has been clearly demonstrated in *in vitro* studies. IFN-γ, a product of activated lymphocytes, was first defined by its antiviral effect *in vitro* (Wheelock and Toy, 1973). TNF exhibits potent antiviral activity *in vitro*, which may be mediated in part by IFN-β (Mestan *et al.*, 1986). The antiviral activity of TNF is dramatically enhanced in synergy with both IFN-β and IFN-γ (Wong and Goeddel, 1986). TNF is able to enhance the induction of an antiviral state in uninfected cells and selectively kill virus-infected cells (Wong and Goeddel, 1986). IFN-γ has been demonstrated to be essential for virus clearance *in vivo* as treatment with specific mAb against IFN-γ prevents mice from clearing the virus (Karupiah *et al.*, 1993; Klavinskis *et al.*, 1989; Ruby and Ramshaw, 1991). IFN-γ also activates NK cells and macrophages which are themselves able to produce the antiviral cytokines IFN-γ and TNF. Thus, cytokines may play an important direct role in antiviral immunity as well as having immunoregulatory functions.

Chapter 3 of this thesis described the establishment of an *in vitro* model to examine the production of antiviral cytokines during an immune response. Using this model, the cells in the vaccinia virus immune responder population which produced the antiviral cytokines TNF, IL-6 and IFN-γ were identified. The following table summarises these results.

Table 7.1 *Phenotype of cytokine producing cells*

Cytokine	Cell producing cytokine	Further observations
TNF	Adherent cells	
	Some by $CD8^+$ T cells	Negative regulation by $CD4^+$ T cells
IL-6	Adherent cells	
IFN-γ	T cell	Majority dependent on $CD4^+$ T cells
		Adherent cells may play a role

The adherent cells among the splenocytes isolated from mice at the peak of the CTL response rapidly produced TNF and IL-6 upon contact with virus antigen. This indicates that the splenocytes contained a population of activated macrophages which may have an important function in restricting viral growth *in vivo*. The macrophages went through a process of de-activation prior to three weeks post infection.

Macrophages play a major role in maintaining homeostasis. These cells can recognise, phagocytose and ultimately dispose of dead cells, cellular debris and invading organisms (Fidler and Schroit, 1988). Those functions which macrophages perform continuously, such as removal of dead cells, do not require regulation by other cells. However, functions which occur infrequently, such as participation in host defense against virus infection, require a process of recruitment and activation. Once the viral infection is resolved however, the cells revert to the non-activated state. Viral infections are known to elicit an inflammatory response, including an accumulation of mononuclear phagocytic cells (Allison, 1974). The accumulation of these cells at the site of a virus infection is enhanced by release of both immunologically specific and non-specific chemotactic factors (Allison, 1974). The immunologically specific factors include cytokines released by T cells, which have also been drawn in to the site of infection (Blanden, 1982). Upon interaction with antigen, T cells produce IFN-γ which is an important priming cytokine of macrophages for activation of killing microorganisms (Morris *et al.*, 1982; Russell, 1986). Although IFN-γ alone cannot activate the macrophages, it is likely that the T cells also provide the activating signal, which can be fulfilled by TNF (Stout,

1993). Activated macrophages are able to secrete a number of factors with antiviral activities, including TNF (Wong and Goeddel, 1986) and nitric oxide (Karupiah *et al.*, 1993). An advantage of these molecules as antiviral agents is that their action is independent of immune recognition of the infected cell, as demonstrated by the production of TNF in response to HSV infected cells. Thus, a rapid antiviral response is ensured.

An important finding of the work presented is that $CD4^+$ T cells isolated from vaccinia virus immune spleen cells were the major producers of IFN-γ upon restimulation. That IFN-γ is essential for clearance of some viruses, including vaccinia virus, has recently been demonstrated using mice lacking the IFN-γ receptor gene (Huang *et al.*, 1993). Although these mice appeared to develop a normal immune system with normal cytotoxic and T helper cell responses, they had increased susceptibility to vaccinia virus infection. There are a number of reports demonstrating the control of virus infection by $CD4^+$ T cells, in the absence of $CD8^+$ T cells. For example, the transfer of measles virus-specific $CD4^+$ T cells is sufficient to control an intracerebral measles virus infection (Reich *et al.*, 1992). These cells secreted IFN-γ and IL-2 *in vitro* following antigen restimulation, typical of a Th1 response. Influenza virus is cleared from the lungs of mice depleted of $CD8^+$ T cells, and also from β2 microglobulin (β2-m) gene disrupted mice which do not develop $CD8^+$, αβ T cells (Eichelberger *et al.*, 1991). These β2-m (-/-) mice are also capable of resolving infection with vaccinia virus and Sendai virus (Hou *et al.*, 1992; Spriggs *et al.*, 1992). The mechanism by which the $CD4^+$ cells mediate their antiviral effectivity is unknown, although the ability of the vaccinia virus immune $CD4^+$ T cells to produce IFN-γ clearly points to this being a possible factor. The specific recruitment and activation of macrophages by $CD4^+$ T cells may also be an important factor in the elimination of virus.

In order to study the network of cytokines interacting in the generation of antiviral cytokines, endogenous cytokines were neutralized using specific antibodies. The results are summarised in the following table.

Table 7.2 ***Summary of cytokine interactions using monoclonal antibodies to neutralize specific endogenous cytokines***

Cytokine neutralized	TNF levels	IFN-γ levels	IL-6 levels
IL-2	Increase (40%)	Decrease (60%)	ND
TNF	ND	Decrease (30 - 60%)	no change
IL-6	Decrease (40%)	Increase (100%)	ND
IL-4	Decrease (30%)	Decrease (25%)	ND
IL-10	no change	no change	ND

Neutralization of endogenous IL-2 led to a dramatic decrease in IFN-γ production by the vaccinia virus immune cells, indicating the important role that IL-2 plays in the production of IFN-γ. IL-2 is thought to have a key role in immune responses as it is a major T cell growth factor (Smith, 1988). It was further demonstrated that IL-2 was a limiting factor in IFN-γ production in this model, by the increase in IFN-γ levels following the addition of recombinant IL-2 or stimulation with the IL-2 encoding virus. These results are presented in Table 7.3, which summarises the effects of adding recombinant cytokine to immune cells cultures or stimulating the immune cells with recombinant vaccinia viruses which encode cytokine genes. IL-2 has been demonstrated to induce or enhance IFN-γ production in both primary and secondary cultures (Farrar *et al.*, 1981; Kelso *et al.*, 1984; Yamamoto *et al.*, 1982). The $CD8^+$ T cells among the virus immune cells responded to the recombinant IL-2 with increased IFN-γ production. $CD8^+$ T cells have been shown to be the major producers of IFN-γ in a vaccinia virus infection, *in vivo* (Ruby *et al.*, 1993). However, IL-2 may not be a limiting factor in primary virus infections.

Table 7.3 ***Summary of cytokine interactions using recombinant cytokine material or vaccinia virus encoding cytokine genes to expose virus immune cells to an exogenous source of cytokines***

Cytokine	TNF	Cells affected	IFN-γ	Cells affected
Recombinant TNF	no change		no change	
Virus-encoded TNF	Increase (100%)	$CD4^-/CD8^-$	Increase (60%)	$CD4^+$, $CD8^+$
Recombinant IL-2	no change		Increase (100%)	$CD8^+$ T
Virus-encoded IL-2	no change		Increase (25%)	$CD4^+$, asialo-GM_1^+
Virus-encoded IFN-γ	no change		Increase (40%)	$CD8^+$
Virus-encoded IL-4	ND		Increase (60%)	ND
Virus-encoded IL-10	no change		Increase (100%)	ND

The action of the IL-2 encoding virus can be regarded as an example of paracrine activity, a common phenomena of cytokine interactions (Balkwill and Burke, 1989). The virus-encoded IL-2 acted on $CD4^+$ T cells and asialo-GM_1^+ cells to enhance their production of IFN-γ. T cell cytokines function mainly as paracrine hormones, being both produced and consumed at the site of immune reaction (Kelso, 1989). Such a mechanism ensures the response generated by the cytokine acts in the region where it is required, and minimizes the amount of cytokine needed. Resting T cells neither produce, nor respond to, IL-2. Upon receiving the signal from the T cell antigen-receptor complex, the T cells begin transcription of both the IL-2 and the IL-2R genes. IL-2 produced by the T cell can then bind directly to IL-2R present on the cell. The cell will enter a phase of DNA replication only when a critical

threshold of triggered IL-2 receptors has accumulated (reviewed by Smith, 1988). Therefore, the IL-2 produced by T cells, or the IL-2 encoding virus, acts in an autocrine manner to enhance proliferation and cytokine secretion of T cells, particularly of $CD4^+$ T cells. These cells secrete a panel of cytokines which act to recruit more cells to the site and dictate the type of response elicited eg. a cell-mediated or humoral response. The NK cell production of IFN-γ, which is able to induce an antiviral state in uninfected cells (Staeheli, 1990), is upregulated by the IL-2 secreted by T cells.

The *in vitro* model of immune cell restimulation demonstrated other examples of the paracrine and autocrine action of cytokines. TNF was shown to play a role in the production of IFN-γ as neutralization of TNF led to a decrease in IFN-γ levels, whereas stimulation with a TNF-encoding virus enhanced IFN-γ production. The ability of TNF to induce production of IFN-γ allows for a more aggressive response to virus infection. As shown earlier, TNF was released by activated macrophages upon contact with antigen. The production of both IFN-γ and TNF at one site will also act to further increase the numbers of activated macrophages. IFN-γ acts on macrophages to increase the expression of receptors for TNF (Ruggiero *et al.*, 1986; Tsujimoto *et al.*, 1986). TNF produced by the macrophages in response to viral antigen can then act in an autocrine fashion, amplifying cytokine secretion and beginning the signal cascade leading to antimicrobial effector functions, such as nitric oxide production (Langermans *et al.*, 1992; Stout, 1993). The co-ordinated TNF/IFN-γ production by the immune cells would be particularly effective in a response to virus infection. Not only do these cytokines have antiviral activity individually (Mestan *et al.*, 1986; Wheelock and Toy, 1973), these cytokines also have demonstrated synergistic antiviral activity (Wong and Goeddel, 1986).

The TNF- and the IFN-γ-encoding recombinant vaccinia viruses were able to stimulate enhanced levels of TNF and IFN-γ production, respectively. Auto-induction has been described for a number of cytokines, including IL-6, IL-1, TNF and IFN-γ (Hardy and Sawada, 1989; Miyaura *et al.*, 1989; Philip and Epstein, 1986; Warner *et al.*, 1987). Autoamplification allows a cytokine produced by a particular cell to induce further production of the same cytokine by the original cell or bystander cells. Autoamplificaton of TNF may be an advantage to the host in a virus infection. TNF produced by activated macrophages

upon contact with viral antigen could upregulate its own expression and allow rapid production of high concentrations of TNF. This may then bring about local changes which are a prerequisite for the recruitment and activation of immunocompetent cells. For example, TNF induces the expression of cell adhesion molecules, such as ELAM-1, VCAM-1 and ICAM-1, at the surface of endothelial cells which could then function to arrest circulating lymphocytes at the site of infection (Bevilacqua *et al.*, 1987; Osborn *et al.*, 1989; Pober *et al.*, 1986). TNF could also enhance antiviral mechanisms by up-regulation of class I MHC expression which would result in more efficient action of antigen-specific CTL recruited from the circulation (Bukowski and Welsh, 1985; Lapierre *et al.*, 1988). TNF may also act directly by inducing resistance to viral infection in uninfected cells at the site. The amplification of IFN-γ production by the IFN-γ encoding virus specifically acted on $CD8^+$ T cells. This may be of particular relevance in a response to virus infection, where $CD8^+$ cells are the main IFN-γ producing cells at the height of the CTL response to primary infection (Ruby, 1993). As well as inducing an antiviral state in uninfected cells, the presence of IFN-γ would induce greater expression of MHC and thus allow increased display of antigenic peptides and increase the efficiency of CTL.

Mechanisms exist to amplify cytokine responses, for example auto- and paracrine induction of cytokine expression, as discussed above. However, mechanisms must also exist to down-regulate and limit cytokine expression to maintain a balance within the cytokine network. It is apparent that when cytokine production is sustained and/or systemic, the cytokine contributes to the signs, symptoms and pathology of infectious and autoimmune diseases, as has been described for TNF (Balkwill, 1988; Beutler and Cerami, 1986). $CD4^+$ T cells among the virus immune responder cell population had a negative influence on TNF production, as the removal of these cells led to an increase in TNF levels. Thus, these cells provided some mechanism to limit TNF levels within the cultures. The nature of this regulatory mechanism was not determined, although it was possibly a $CD4^+$ T cell-derived cytokine. Several cytokines produced by $CD4^+$ T cells have immunosuppressive activity. IL-4, IL-10 and TGF-β are able to down-regulate macrophage functions involved in the effector phase of cell-mediated immunity (Sher *et al.*, 1992), and IL-4 and IL-10 have demonstrated ability to down-regulate or inhibit TNF expression by

macrophages (Fiorentino, 1991b). In contrast to these results, neutralization of IL-10 or stimulation of vaccinia virus immune cells with the IL-10 encoding virus had no significant effect on TNF production, while neutralization of IL-4 led to decreased TNF levels. This indicates that IL-4 and IL-10 were not involved in the negative regulation of TNF in these restimulated cultures of vaccinia virus immune cells.

Several of the major findings described above can now be drawn together to provide a simple model describing the cellular interaction in the production of cytokines by restimulated virus immune T cells. $CD4^+$ T cells were the major producer of IFN-γ and exerted a negative influence on TNF production by $CD8^+$ T cells. IFN-γ production by $CD8^+$ T cells was dependent on IL-2 and was enhanced by IFN-γ. As $CD4^+$ T cells produced both IL-2 and IFN-γ upon restimulation, these cells play a central role in regulation of cytokine production.

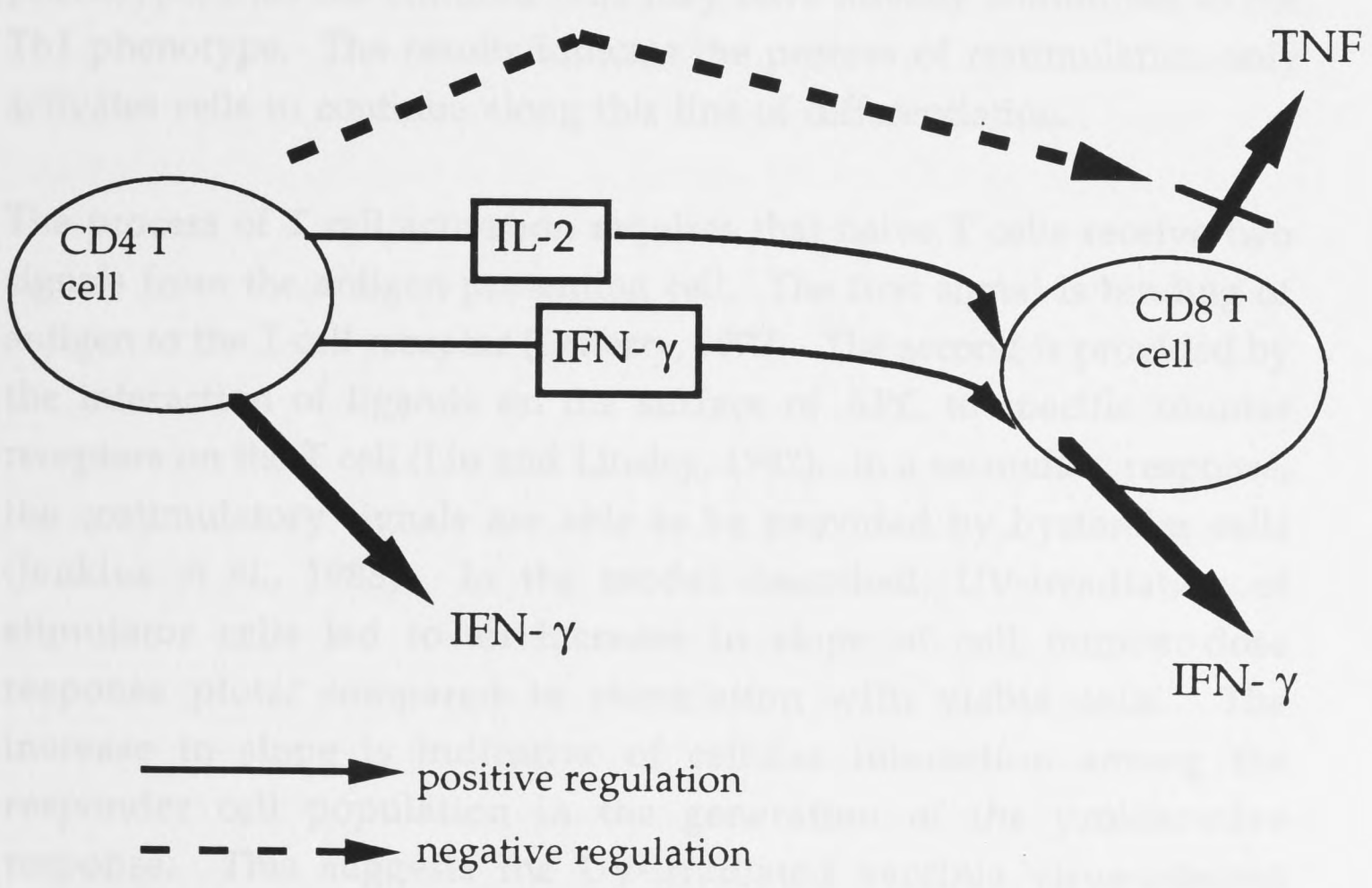


Figure 7.1. ***$CD4^+$ T cell regulation of antiviral cytokine production by $CD8^+$ T cells.***

The Th1 and Th2 pattern of cytokine production was originally described using a panel of long-term mouse T helper cell clones. These two phenotypes have been confirmed in strong mouse immune responses and are mutually inhibitory (Mosmann and Coffman, 1989a). IFN-γ inhibits growth of Th2 cells, and IL-10 inhibits the synthesis of Th1 cytokines. An IL-4 encoding recombinant vaccinia virus is lethal when injected into normal female mice. The mice had a decreased CTL response indicating the IL-4 acted on the immune regulation of CTL activation and expansion (Andrew and Coupar, 1992). Restimulation of the wild type vaccinia immune cells with either the IL-4- or IL-10-encoding virus did not, however, alter the pattern of cytokine production of the virus immune cells. Increased levels of IFN-γ were produced in these cultures compared to cultures restimulated with wild type virus. Thus, the Th2 cytokine produced by the restimulating virus was not able to divert the IFN-γ producing cells. This may reflect the fact that the cells undergoing restimulation were taken from mice at the peak of the CTL response to vaccinia virus infection. A vaccinia virus infection leads to the production of Th1 phenotype, thus the cultured cells may have already committed to the Th1 phenotype. The results indicate the process of restimulation only activates cells to continue along this line of differentiation.

The process of T cell activation requires that naive T cells receive two signals from the antigen presenting cell. The first signal is binding of antigen to the T cell receptor (Lafferty, 1977). The second is provided by the interaction of ligands on the surface of APC to specific counter receptors on the T cell (Liu and Linsley, 1992). In a secondary response, the costimulatory signals are able to be provided by bystander cells (Jenkins *et al.*, 1988). In the model described, UV-irradiation of stimulator cells led to an increase in slope of cell number-dose response plots, compared to stimulation with viable cells. The increase in slope is indicative of cellular interaction among the responder cell population in the generation of the proliferative response. This suggests the UV-irradiated vaccinia virus-infected stimulator cells were not able to provide the costimulatory signals to the responder cells and that, as this was a secondary response, bystander cells among the immune responder cell population could provide the necessary signals. The receptor for vaccinia virus is thought to be the epidermal growth factor receptor (EGF-R) (Eppstein *et al.*, 1985), and thus, vaccinia virus is able to infect a wide variety of

cell types, including fibroblasts, endothelial cells and epithelial cells *in vivo*. However, costimulatory molecules, such as B7, heat stable antigen (HSA) are found on the surface of specialised antigen presenting cells. The results of this study would indicate that in the absence of costimulatory surface molecules on the virus infected cells, bystander cells are able to present the costimulatory signals to the activated T cells. The nature of the signal provided by the bystander responder cells was not investigated.

Future Directions

Using the *in vitro* model described here it would be possible to further investigate the nature of the costimulatory signals required by the immune cells to respond to virus presentation with cytokine secretion. Responder cell interaction was analysed by plotting cell number-response lines, with the slope giving an indication of the requirement for interaction. A slope of 1 indicated all the necessary stimulatory signals were provided by the antigen presenting cells. An increase in slope revealed responder cell interaction to provide signals which were lacking, such as was observed in cultures stimulated with UV-irradiated virus infected cells. A variety of recombinant vaccinia virus constructs are available. These viruses encode cytokines or molecules which may be necessary as costimulatory signals, such as B7 or CD40 ligand. The work presented in Chapter 6 of this thesis indicates that recombinant vaccinia virus continue to produce the recombinant protein following UV-irradiation. Thus, it would be possible to infect naive spleen cells with a vaccinia virus recombinant and UV-irradiate, as described in Chapter 2. Cell number-response analysis of cultures stimulated with these cells would indicate whether the encoded gene replaced a need for responder cell interaction in the generation of a proliferative, or cytokine response. This would be observed as a decrease in cell number-response slope. This is a simple method for investigating the role of particular molecules, both cytokines and cell surface ligands, in the generation of an immune response. The results described in this thesis indicate that IL-2 was a limiting factor in the production of IFN-γ. Therefore, analysis of the response generated using the IL-2 encoding virus as a source of stimulation and provision of the limiting factor would be interesting.

A further area of study is the investigation of the cytokine profile of mice infected with the recombinant IL-4 encoding virus. Virus expression of IL-4 inhibits the development of the CTL response and increases the pathogenicity of the virus in normal mice (Andrew and Coupar, 1992). It would be interesting therefore, to determine whether cells taken from these mice have generated a Th2 type response, as would be predicted when IL-4 is present at the early stage of T cell response (Swain, 1990b). Also, results presented in Chapter 5 suggested that IL-4 may have enhanced TNF secretion by the restimulated vaccinia virus immune cells. IL-4 enhancement of TNF production during an immune response could also be studied in these mice and may provide further insight into the lethality of this recombinant vaccinia virus construct.

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