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COMPARISON OF CYCLOSPORIN A WITH MITOMYCINC AND GAMMA IRRADIATION AS INACTIVATORS OF STIMULATOR CELLS IN THE ONE-WAY MIXED LYMPHOCYTE REACTION

BY

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With greatest love, to my mum and dad, for their unwavering love and support thoughout my life.

For my Eugene, Raistlin Martha and Tommy Tom!

Summary

The one-way mixed lymphocyte culture (MLC) is used to assess histocompatibility between donor and recipient. First introduced in 1966, this method involves the co-culture of lymphocytes from the peripheral blood of the donor and the recipient for a period of 6 to 7 days: antigen disparities, primarily in the HLA-DR region, stimulate proliferation of the responding cells, which is detected by addition of ^{3}H-labelled thymidine and subsequent measurement of radioactivity. The lymphocytes of either the donor, used to predict graft-versus-host disease (GVHD) or recipient, used to predict host-versus-graft disease (HVGD)/graft rejection, are inactivated by exposure to radiation or mitomycin C, so that the observed proliferation is that of the other set of lymphocytes, hence the name "one-way" MLC. The amount of measured radioactivity is directly proportional to the amount of DNA synthesized, which is a reflection of the number of disparities at the major histocompatibility complex (MHC).Previous studies have established that inactivation of the lymphocytes by radiation and mitomycin C, has a negative effect on the structure/expression of HLA-DR molecules on the cell surface, which provides the primary stimulus for the MLC reaction.

The laboratory research presented in this dissertation was designed i) to compare the viabilities and HLA-DR levels on stimulator cells exposed to cyclosporin A, mitomycin C and ionizing irradiation , in order to determine whether cyclosporin A can be used as an alternative to mitomycin C or radiation as inactivator of the stimulator cells in the one-way MLC; ii) to improve sensitivity and accelerate the MLC reaction

by addition of IL-2; iii) establish a flow cytometric mixed lymphocyte assay using the fluorochrome 5,6 carboxyfluorescein diacetate succinimidyl ester (CFSE).

Cyclosporin A showed striking similarities to mitomycin C and ionizing radiation in its effect on viability and reduction/structural changes in HLA-DR molecules of the stimulator cells. Exposure of the stimulator cells to 20µM cyclosporin A, demonstrated a significant loss of both cell viability and HLA-DR molecule cell surface expression. Thus, in evaluating these three methods of inactivation of the stimulator cells in the one-way MLC, it was concluded that a one-way MLC may not in fact be an accurate and qualitative reflection of the histocompatibility between donor and recipient. Instead the two-way MLC , in which neither the donor's nor recipient's cells are inactivated, may be a more reliable alternative. The only limitation associated with a two-way MLC is the inability to distinguish between a host-versus-graft-rejection and a graft-versus-host reaction in the observed allogeneic response

Addition of 5 and 10 IU/ml IL-2 to the MLC showed the opposite effect to that intended, inhibiting proliferation in the MLC. Previous studies have shown that an excess of IL-2 results in the production of suppressor T cells. The amount of IL-2 produced during the MLC depends on the number of disparities in the MLC between donor and recipient, which will be different for each MLC reaction.

Since the number of allogeneic T cells involved in the MLC reaction is not known, the amount of IL-2 produced during the allogeneic immune response in the MLC can not be predicted and addition of exogenous IL-2 may result in production of suppressor T cells and an inhibition of proliferation.

The two-way MLC was modified by staining one of the participating set of lymphocytes (donors or recipients) with CFSE and tracking proliferation in this population, using flow cytometry. The two-way CFSE-based MLC analyzed in this study were counterstained with CD25 (IL-2R). An increase in CD25 expression on the cell surface is an indicator of cell activation and proliferation. Proliferation, as indicated by a progressive loss of CFSE fluorescence correlated well with the corresponding increase in CD25 expression and accumulated daughter cells. In addition, by loading only one of the participating donors in the two-way MLC, the responder/stimulator interaction, observed in the one-way MLC, is re-established. Thus the modified, CFSE-based two-way MLC can be used to predict GVHD. To conclude, the use of CFSE labeling and flow-cytometry to measure proliferation in a two-way MLC, together with CD25 counterstaining provides an alternative, reliable and probably superior method to ^{3}H thymidine uptake.

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LIST OF ABBREVIATIONS

AP-1	Activator protein 1
APCs	Antigen presenting cells
ATP	Adenosine 3', 5'-triphosphate
Brd U	Bromodeoxyuridine
CD	Cluster of differentiation
CFSE	5,6 carboxyfluorescein diacetate succinimidyl ester
CPM	Counts per minute
CsA	Cyclosporin A
CSIF	Cytokine synthesis inhibitory factor
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTL-p	Cytotoxic T lymphocyte precursor
DAG	Diacylglycerol
DNA	Deoxyriboneucleic acid
dNTP	Deoxyribonucleotide triphosphate
EGTA	Ethylene glycol-bis (beta-amino-ethyl-ether)-N, N, N', N'-tetraacetic acid
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FL-AAM	Firefly luciferase ATP assay mix
FS	Forward scatter
G-CSF	Granulocyte-colony stimulating factor

GM-CSF	Granulocyte/macrophage colony stimulating factor
GVHD	Graft-versus-host-disease
HLA	Human leukocyte antigen
HTL-p	Helper T lymphocyte precursor
HVGD	Host-versus-graft-disease
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
IL	Interleukin
IL-2R	Imterleukin 2 receptor
IP_3	Inositol triphosphate
JAK	Janus kinases
LFA-1	Leukocyte functional antigen 1
LPA	Lymphocyte proliferation assay
MAP	Mitogen activated protein
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
MNL	Mononuclear leukocyte
MTX	Methotrexate
$NFAT_c$	Nuclear factor for activated T $cells_{cytoplasm}$
$NFAT_n$	Nuclear factor for activated T $cells_{nucleus}$
NFκB	Nuclear factor kappa B
NH_4CL	Ammonium Chloride

PBS	Phosphate-buffer saline
PCR	Polymerase chain reaction
PCR-SSP	Polymerase chain reaction-sequence specific primers
PLC-γ 1	Phospholipase C-γ 1
PHA	Phytohaemagglutinin
PI	Propidium iodide
PIP_2	Phosphatidyl inositol biphosphate
PPD	Purified protein derivative
PTKs	Protein tyrosine kinases
RNA	Riboneuclotide acid
SS	Side scatter
SSO	Sequence specific oligoneuclotide
STAT	Signal transducers and activators of transcription
TCR	T cell receptor
TNF	Tumor necrosis factor
Th	T helper cell

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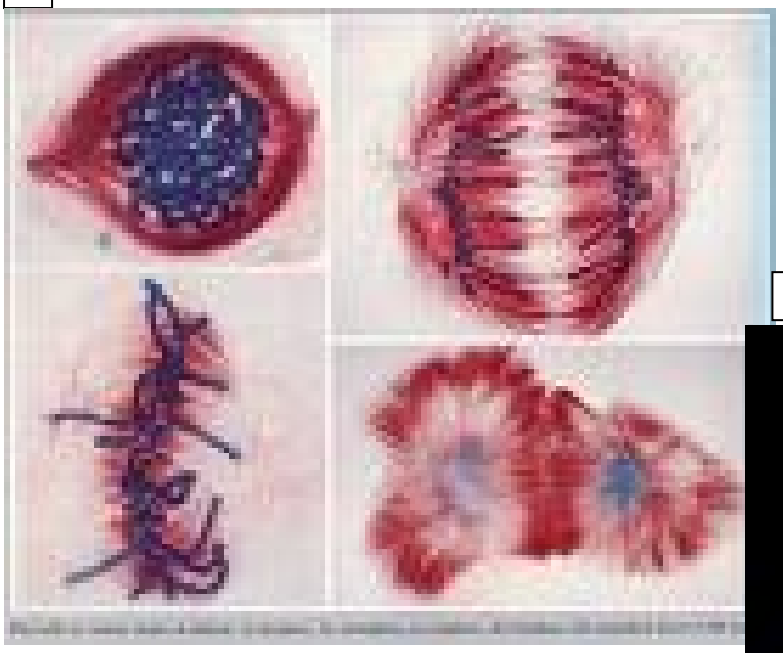

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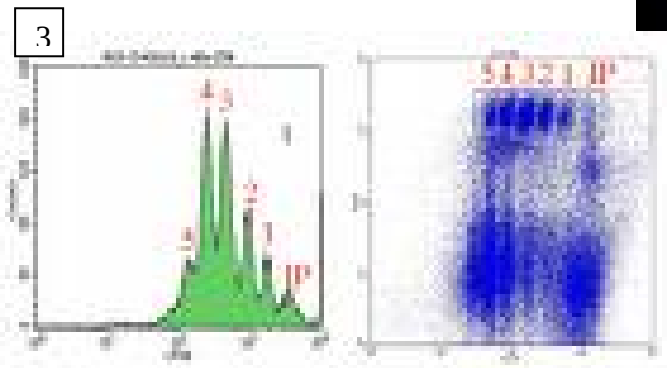

Chapter 1
Introduction

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1. Introduction

Since the introduction of the one-way mixed lymphocyte culture (MLC) in 1966 by Bach and Voynow, which is used to determine histocompatibility between two individuals and to predict graft-versus-host disease (GVHD), very little has changed in the methodology of this assay. This method employs the inactivation of the cells of one of the participating donors, by exposure to radiation or mitomycin C to act as stimulator cells. However, studies undertaken to determine the effects of the exposure of the stimulator cells to radiation or mitomycin C, have demonstrated that these procedures may have a negative affect on the structure/expression of HLA-DR molecules, as well as cell viability (Kasakura and Lowenstein, 1967; Mettler *et al.*, 1985; Malinowski *et al.*, 1992). Since HLA-DR molecules on the surface of the stimulator cells provide the primary activation signal for an MLC reaction, it is clear that any procedure used to inactivate these cells must not negatively affect structure or surface expression of HLA-DR molecules. Despite such observations, the one-way MLC is still performed using radiation or mitomycin C to inactivate the stimulator cells. The main objectives of the research performed in this study were to evaluate existing methodology in the one-way MLC and to investigate alternative procedures to improve the standard one-way MLC. Briefly, this included the use of cyclosporin A as an alternative to ionising irradiation or mitomycin C for inactivation of the stimulator cells, the potential use of IL-2 to improve sensitivity of the MLC and the use of the fluorochrome 5,6 carboxyfluorescein diacetate succinimidyl ester (CFSE), to track proliferation in the MLC flow cytometrically.

1.1 Graft-versus-host-disease

In addition to allograft rejection, graft-versus-host-disease (GVHD) also remains a common, major complication in both organ and bone marrow transplants, even in the post-cyclosporin A era (Parkman, 1991).

Bone marrow transplantation is widely used in the treatment of haematological, immunological, neoplastic and genetic disease (Thomas *et al.*, 1975). However, the use of donors that are not human leukocyte antigens (HLA) matched to the recipient, results in a major complication known as GVHD, both acute and chronic.

Acute GVHD is characterised by the following symptoms:

- onset occurs 7-21 days following bone marrow transplantation
- elevated hepatocellular enzymes
- diarrhoea
- capillary leak syndrome

Chronic GVHD is characterised by the following symptoms:

- onset occurs 30-100 days following bone marrow transplantation

- increased collagen deposition which results in sclerodermous skin changes, pulmonary fibrosis, gastric malabsorption, decreased oesophagal motility
- auto-antibody production to thyroid, muscle, erythrocytes and granulocytes
- immunodeficiency, patients lose the ability to produce protective antibodies to environmental pathogens , especially encapsulated respiratory bacteria

There is a striking clinical similarity between chronic GVHD patients and those with autoimmune and immunodeficiency diseases eg. scleroderma (Parkman, 1991). The pretransplant cytoablation of a recipient's bone marrow eliminates their antigen-specific immunological memory, which must be re-established using reimmunization with standard vaccines. However, the transplanted recipients show abnormalities in both T- and B- lymphocyte function (Lum *et al.*, 1985; Witherspoon *et al.*, 1986), particularly with respect to production of anti-carbohydrate antibodies. This immunodeficiency of the post-transplant patient, together with the pre-transplant cytoablation and subsequent immunosuppressive therapy of transplantation, results in the high morbidity and mortality associated with chronic GVHD (Parkman, 1991).

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Recent developments in [illegible] prophylaxis, have resulted in a decrease in the incidence of acute GVHD, but the incidence of chronic GHVD remains similar to that of the past. (Parkman,1991).

According to Billingham, Brent and Medawar (1959), three prerequisites must be fulfilled for GVHD to develop:

1. Differences at one or more major histocompatibilty complex (MHC) loci (Beatty *et al.*, 1985). The greater the disparities, the greater the grade (II-IV) and incidence of significant GVHD. Thus, in adult patients, 40 % will develop acute GVHD when no MHC difference exists, 70% will develop acute GVHD when a single MHC difference exists and 85% will develop acute GVHD when up to three MHC differences exist (Parkman, 1991).

2. A source of donor immunocompetent cells known as "passenger cells".

2. Failure of the recipient to reject the immunocompetent donor cells. This failure to mount an immune response against the "passenger cells" is due to the pre-transplant cytoablation of the bone marrow and post-transplant immunosuppressive therapy to prevent graft rejection (Parkman, 1991).

The incidence of GVHD, both acute and chronic, is greater in adults than in children (Gale *et al.*, 1987).

The following are currently utilised in the treatment of GVHD:

- Immunosuppressive therapy, which reduces the incidence of acute GVHD, usually involves combined regimens of methotrexate (MTX), cyclosporin A, tacrolimus (FK 506) and/or corticosteroids (Storb *et al.*, 1989). These drugs suppress different stages of donor T cell activation. MTX is an antiproliferative agent, cyclosporin A and tacrolimus block IL-2 synthesis and corticosteroids are lympholytic and prevent IL-1 synthesis by the antigen presenting cells (APC's) (Kahan, 1983; Nash *et al.*, 1996).

- T-lymphocyte depletion. T lymphocytes can be eliminated from a bone marrow graft either by lysis with monoclonal antibodies specific for T lymphocytes plus complement/toxin or physical removal by lectin agglutination with soybean agglutinin (SBA) and E-rosette formation (Reisner *et al.*, 1983; Pavletic *et al.*, 2005). However, although T lymphocyte depletion markedly decreases the incidence of acute and chronic GVHD (Mitsuyasu *et al*, 1986), there is an increase in the incidence of haematopoietic nonengraftment and leukaemic relapse, which again decreases the survival rate of the transplanted patient (Ash *et al.*,1990).

1.2 Immunological strategies to assess donor/recipient compatibility in clinical organ transplantation

Organ transplantation has now become a routine medical therapy. Such a procedure is very costly, which has led to the development of immunological strategies to assess donor/recipient compatibility, thereby improving the chances of a successful transplantation. Such strategies include the following:

1.2.1 HLA-Typing

Recognition of nonself, allogeneic Major Histocompatibility Complex (MHC) molecules on the graft, triggers an immune response that leads to graft rejection. Thus, matching the MHC type of donor and recipient significantly increases the chances of a successful transplantation and ensures that the few healthy organs available are matched to the most suitable recipient. However, HLA-typing in itself, does not prevent rejection for the following two reasons. Firstly, HLA-typing is imprecise due to MHC polymorphism: a donor and recipient, who type HLA-identical, rarely have identical MHC genotypes.

Secondly, in MHC-identical grafts, graft rejection can still occur due to an immune response arising from differences between minor histocompatibilty antigens. HLA-typing is either carried out at the

phenotypic level (the proteins expressed on the cell surface) or the genotypic level (the DNA sequences of the MHC genes).

Phenotypically, HLA-typing is based on the use of serum-derived antibodies and involves a technique known as microcytotoxicity. The wells of microcytotoxicity plates contain antibodies specific for HLA antigens. An isolated lymphocyte suspension from the person to be typed is added to each plate. Following incubation, rabbit complement is added: lysis of the cells indicates recognition of a certain HLA molecule by the antibody. Class I HLA-typing is mostly done using the microcytotoxicity assay (Janeway *et al.*, 2005).

For class II HLA-typing the microcytotoxicity assay is not reliable, since antisera available are not able to distinguish between very similar surface HLA molecules that are actually coded by different alleles at the same locus. Class II HLA-typing is done at the genotypic level, using PCR. The polymerase chain reaction (PCR)-based genomic HLA typing for class II, uses sequence specific primers (PCR-SSP). Briefly, the DNA extraction of the individual to be typed is added to 96-tube PCR trays: each tube contains dNTP, allele- and group-specific class II primers, PCR buffer and *Taq*I polymerase enzyme. Amplification cycles are carried out in a PCR system, a thermocycler, in 3 steps: denaturation of DNA at 94°C for 10s; annealing of primers at 65°C; DNA amplification at 72°C for 30s. The first two steps are repeated between 10 –20 cycles before amplification begins. Absence or presence of DNA product is visualized by

agarose gel electrophoresis (Zetterquist *et al.*, 1997). Detection of specific amplification products can also be identified using sequence specific oligonucleotide (SSO)-based procedures.

1.2.2 Mixed Lymphocyte Reaction (MLR)

The MLR can be used to assess histocompatibility between two individuals. The lymphocytes from two unrelated individuals are isolated from peripheral blood and cultured together. If the two individuals are not MHC-matched, the T cells will proliferate in response to the allogeneic MHC molecules on the cells of the other donor (Janeway *et al.*, 2005). The MLR can be used to predict both allograft rejection and graft-versus-host disease (GVHD). GVHD remains a common, major complication in both organ and bone marrow transplantation (Parkman, 1991).

1.2.3 Limiting-dilution Assay

The limiting-dilution assay precisely counts the frequency of alloreactive T cells in a heterogeneous sample (Janeway *et al.*, 2005). This assay uses the Poisson distribution, a statistical function that describes how objects are distributed at random. Varying dilutions of a heterogeneous sample of T lymphocytes are added to a series of culture wells. Some wells will receive no T cells specific for a given antigen, others will receive one T cell specific for that antigen, others two and so on. The T cells are stimulated with antigen, antigen presenting cells and

appropriate growth factors. After one week, the cells are tested for a response to the antigen, such as by cytokine production or by ability to kill an antigen-specific target cell. From the Poisson distribution it is known that when 37% of the wells are negative for a response each well contained, on average, one antigen-specific T cell at the beginning of culture. Thus, if 37% of the wells are negative when 150 000 T cells were added to each well, then the frequency of the antigen-specific T cells is one in 150 000.

The limiting-dilution technique can be used to measure either the frequency of $CD8^+$ cytotoxic T cells or $CD4^+$helper T cells:

- Cytotoxic T lymphocyte precursor (CTL-p) assay. $CD8^+$T cells are purified using magnetic microbeads coated with anti-CD8 antibody and cultured by limiting dilution. After one week, the cells are incubated with ^{51}Cr-labeled target cells. The amount of radioisotope released is then measured: wells are positive for lytic activity if total counts per minute are >3 x SD over the value of control wells.

- Helper T lymphocyte precursor (HTL-p) assay, which uses the measurement of IL-2 production. (Bouma *et al.*, 1995; Young *et al.*, 1996; Janeway *et al.*, 2005).

1.2.4 Cytokine Analysis

Cytokines play an important role in graft rejection and GVHD. Notwithstanding the most recent idetification of Th3 and Th17 cells, T helper (Th) lymphocytes are divided into two subpopulations, Th1 and Th2, based on cytokines they secrete. Th1 cytokines, such as IL-2 and IFN-γ, are associated with rejection, whilst Th2 cytokines such as IL-4 and IL-10 are associated with tolerance. Measurement of these cytokines in the supernatant of MLC can be used to predict graft rejection and GVHD (Salgame *et al.*, 1991; Dallman, 1993; Joseph *et al.*, 1995; Lambert *et al.*, 1995).

1.3 Detailed review of the MLC

1.3.1. Types of MLC

Two types of MLC exist, the two-way MLC and one-way MLC.

1.3.1.1 The two-way MLC

The two-way MLC was introduced in 1964 by Bain *et al* and Bach and Hirschhorn. Briefly, the mononuclear layer (MNL) from two unrelated individuals are cultured together for 5-7 days. If alloreactive T cells are present, they will be activated to proliferate. Proliferation is measured by incorporation of ^{3}H-thymidine into the DNA of the dividing cells.

1.3.1.2 The one-way MLC

The one-way MLC was introduced in 1966 by Bach and Voynow. Using this procedure the response of only one population of cells is measured. Briefly, this method involves culturing the MNL from a potential recipient, known as the responder cells, with the inactivated MNL from a potential donor, known as the stimulator cells. Thus, in the standard one-way MLC, the stimulator cells represent the graft and as such are considered to be the more passive target of the recipient's immune system (Sato *et al.*, 1999). The reverse strategy, using responder cells from

the donor and stimulator cells from the recipient, may be used to predict GVHD (Kabelitz *et al.*, 1985; Jooss *et al* 1988). The principles of the one-way MLC, using both thymidine uptake and chromium release procedures are shown in Figure 1, page 13.

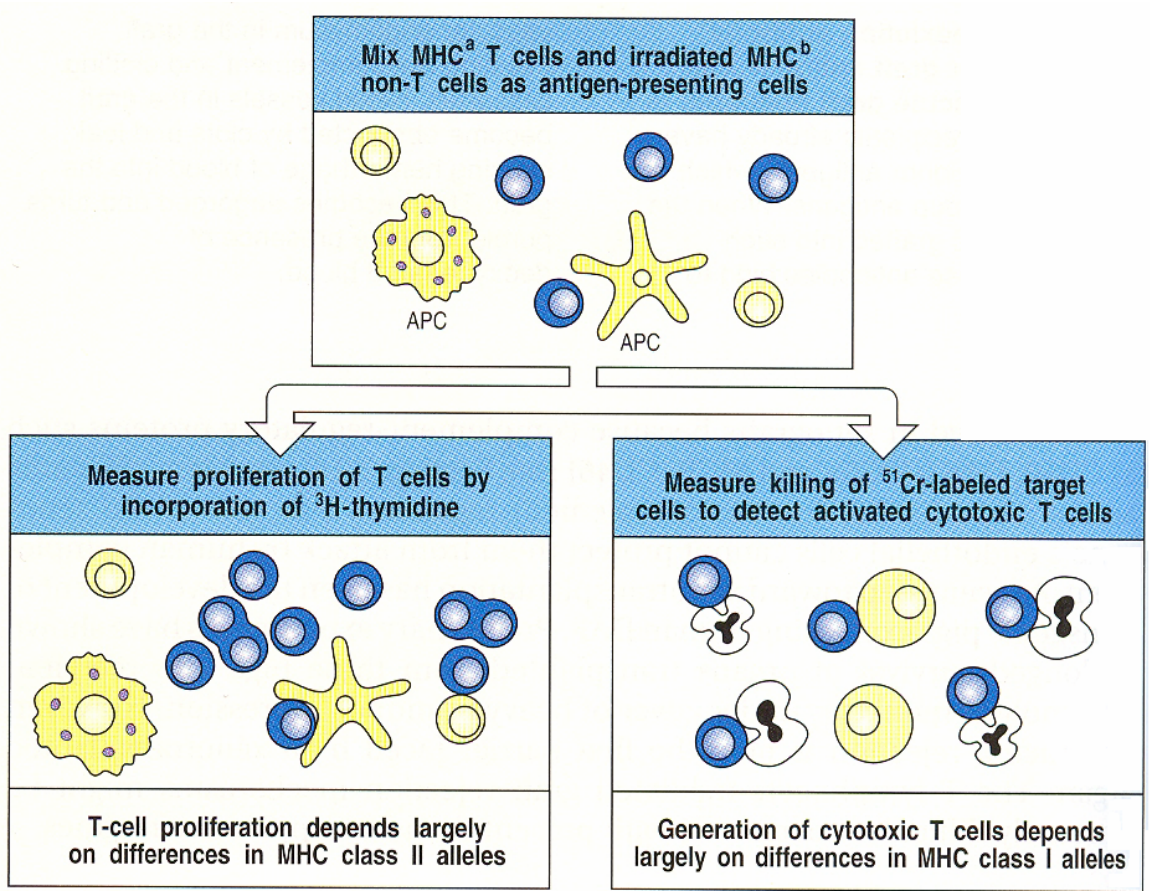

Fig 1. The one-way MLC. MNL from two individuals to be tested for histocompatibility are isolated from peripheral blood. The cells from one individual (yellow), which will also contain antigen-presenting cells, are inactivated by irradiation or exposure to mitomycin C and act as stimulator cells.

The cells from the two individuals are mixed (top panel). If the responder cells (blue) contain alloreactive T cells, these will be activated to proliferate and differentiate into effector cells. Following a 5-7 day culture period, the culture is assessed for T- cell proliferation (bottom left panel), which is the result of CD4 T cell recognition of MHC class II differences. The bottom right panel is to assess generation of functional CD8 T cells (Source: Janeway *et al.*, 2005).

The stimulator cells in the one-way MLC are most commonly inactivated using one of the following two techniques:

1. γ-Irradiation

   Exposure of stimulator cells to γ-irradiation results in breaks in DNA strands, thereby interfering with DNA replication. The treated cells are arrested in the S phase of the cell cycle (Malinowski *et al.*, 1992).

2. Mitomycin C

   Exposure of the stimulator cells to the cytostatic drug, mitomycin C, inhibits DNA synthesis by crosslinking with DNA. RNA and protein synthesis are arrested to a lesser degree. The treated cells cycle more slowly through the S phase and into the G_2 phase (Malinowski *et al.*, 1992).

1.4 Alloantigens

Alloantigens are histocompatibility antigens present in different forms in different individuals of the same species. Thus an alloantigen from one individual will elicit an immune response in a genetically dissimilar individual of the same species.

1.4.1 Structure

Histocompatibility antigens are highly variable glycoproteins expressed on the surface of almost all somatic cells and which are involved in antigen recognition. The particular set of histocompatibilty antigens displayed by an individual is known as that individual's tissue type. The major histocompatibilty antigens are encoded by the major histocompatibility complex (MHC), found on the short arm of chromosome 6 in humans.

HLA molecules are divided into two classes, class I HLA-A, -B and –C, and class II HLA-DR, -DQ and –DP. Class I and class II are both transmembrane glycoprotein heterodimers. Class I consists of an α heavy chain and the $\beta_2$microglobulin (β_2m) light chain, whilst class II dimers consist of an α- and β chain (Peakman and Vergani,1997); the α- and β- chains have a similar structure, consisting of distinct domains. The α-chain of class I molecules comprises 3 domains, and the β_2m-chain comprises one domain. The α- and β- chains of class II molecules consist of two domains each. Thus, each MHC molecule is made up of 4 domains, which can be

divided into two "immunoglobulin-like" domains and two "peptide-binding" domains. For class I molecules, the "immunoglobulin-like" domain consists of the α3 domain and the $\beta_2$microglobulin chain, while the "peptide-binding" domain is comprised of the α1 and α2 domains. For class II molecules, the "immunoglobulin-like" domain consists of the α2 and β2 domains, while the "peptide-binding " domain is comprised of the α1 and β1 domains (ed. Wood, 1995; Peakman and Vergani, 1997). Class I molecules are expressed on the surface of almost all nucleated cells, while class II expression is restricted to the surface of the monocyte/macrophage lineage, antigen presenting cells including dendritic cells and B lymphocytes as well as activated T lymphocytes (Peakman and Vergani, 1997).

1.4.2 The peptide- binding site

As shown in Figure 2, page 17, the α1 and α2 domains of class I, and α1 and β1 domains of class II molecules, form a groove, which has a platform at the bottom built of β-pleated sheets and is flanked by two bent α-helices. This groove represents the peptide-binding site (ed. Wood, 1995).

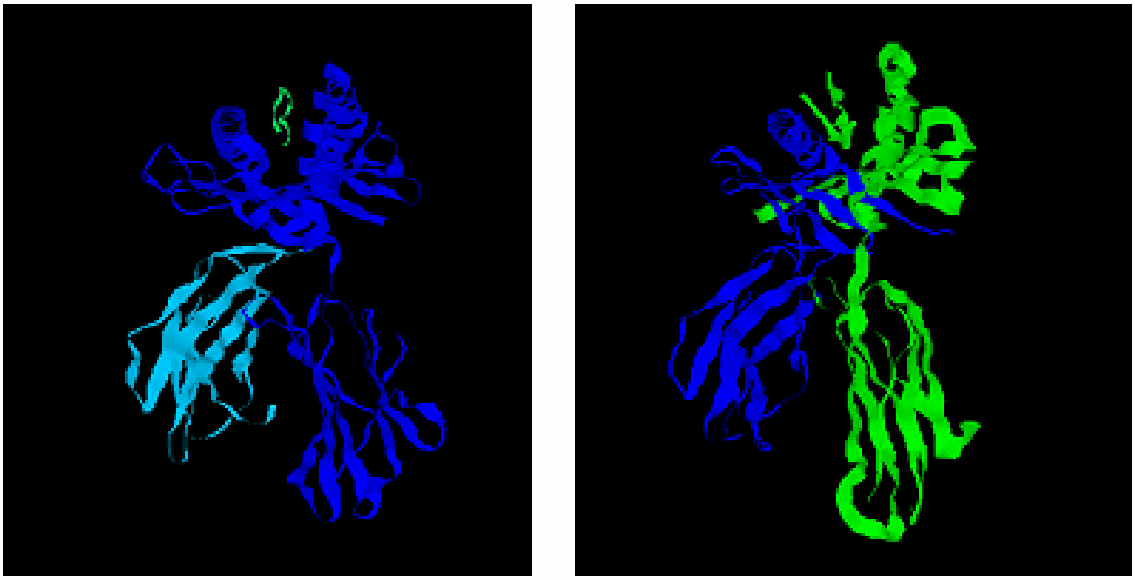

Fig. 2. MHC class I (left panel) and MHC class II (right panel), complexed with influenza hemagglutinin-derived peptide. MHC class I: the dark blue ribbon is the α heavy chain and the light blue ribbon the β_2m light chain. MHC class II: the dark blue ribbon is the α chain and the green ribbon the β chain. The dark green strand in the peptide-binding groove is the infuenza hemagglutinin-derived peptide (Source: Mazza et al., 2005; Scott et al., 1998)

1.4.3 Function of HLA molecules

The main function of HLA molecules is to present peptide fragments of antigens degraded inside cells to the T-cell receptor (TCR). Class I molecules present peptide from endogenous sources such as viral antigens produced during infection in cells, whilst class II molecules present peptide from exogenous sources such as bacterial antigens taken up by B lymphocytes.

The MHC molecules also play an important role in shaping the T cell repertoire. During $CD4^+$ and $CD8^+$ T lymphocyte maturation in the thymus, the T lymphocytes "learn" to recognise foreign antigen in the context of autologous MHC molecules. This occurs in a two-step process of positive and negative selection. First there is positive selection for T cells with receptors which recognise autologous MHC molecules, followed by negative selection to eliminate those T cells with receptors which recognise autologous antigen in the context of autologous MHC molecules. Thus, only T cells recognizing foreign antigen associated with autologous MHC molecules are released into peripheral circulation (Janeway *et al.*, 2005).

1.4.4 HLA Polymorphism

Polymorphism is the phenomenon whereby two or more different forms of a specific gene at a given locus exist in the population. The different forms of the gene are

known as alleles and the locus is polymorphic when several alleles exist in the population (Janeway *et al.*, 2005). For a polymorphic locus, any given individual can be either homozygous, having the same allele on both chromosomes, or heterozygous, with different alleles on each chromosome. The MHC region is polymorphic, with the most extensive polymorphism associated with the peptide-binding site. Thus, in terms of HLA function, polymorphism provides a mechanism for increasing the diversity of peptides that can be displayed to T lymphocytes (ed. Wood, 1995).

1.4.5 MHC molecules as transplantation antigens

MHC molecules are involved in regulating the immune response in an individual-specific manner based on an individual's HLA profile. This results in the development of the T cell repertoire, determining immune responsiveness and self-tolerance (Janeway *et al.*, 2005). Transplantation results in the introduction of foreign HLA molecules which, although similar to the recipients, are different with respect to peptide binding, thereby disrupting the pattern of established immune responsiveness and self-tolerance (ed. Wood, 1995).

The most extensive polymorphic sites on the MHC molecule are amino acid exchanges in the peptide binding site or T cell receptor binding site. Differences at these sites between donor and recipient will result in the following immune interactions, which ultimately lead to graft rejection (ed. Wood, 1995):

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- direct recognition of [illegible] de complex

- indirect recognition of a donor MHC derived peptide presented by either a donor MHC or recipient MHC

- allogeneic donor MHC will present a different set of peptides derived from donor and/or recipient than those presented by recipient MHC molecules

- the same peptide, even a self-peptide, will be presented by donor MHC molecules in different way than when presented by recipient MHC molecules

1.4.6 Minor H Antigens

Minor histocompatibilty antigens (minor H antigens) are endogenous, self proteins that are digested by proteasomes in the cell and the peptides thereof delivered to the endoplasmic reticulum (ER) to be presented in the peptide-binding site of MHC class I molecules on the surface.

Polymorphisms of the genes encoding these proteins means that different peptides will be produced in different members of the same species eliciting an immune response in a donor/recipient pair (Janeway *et al.*, 2005).

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Minor H antigens are not [illegible] throughout the genome and most are ubiquitously expressed. Thus, even if donor and recipient are identical at the MHC, in all probability they will differ with respect to minor H antigens, which will elicit an immune response, and, except for monozygotic twins, result in prolonged graft rejection. Although an immune response directed against a minor H antigen is weak, if many single minor H antigens are involved, their effects are synergistic, resulting in graft rejection as rapid as that against MHC antigens or the development of severe GVHD ([ab]van Els *et al.*, 1990).

1.5 [illegible] MLC

The mechanisms involved during a classical protective immune response, are essentially the same as those that prevail during transplant rejection and in the MLC (Peakman and Vergani, 1997). As in an immune response to pathogen-derived antigen, it is the balance between the different components of the immune system, that determines the magnitude of the rejection process and the intensity of the activation observed in the MLC.

The mixed lymphocyte reaction detects the degree of immune cellular stimulation of recipient T lymphocytes by donor cells.

1.5.1 Allorecognition by T cells

Allorecognition can occur by two possible mechanisms:

1. Direct Allorecognition

Direct allorecognition involves recognition of an intact donor MHC molecule on donor antigen presenting cells by the recipient's T cells. The MHC molecule may be empty or loaded with peptide generated within the donor's cells before tranplantation. The recipient's T cells can recognise amino acid differences in the

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α helices of the donor [illegible], the donor MHC peptide (ed. Wood, 1995).

2. Indirect Allorecognition

Indirect allorecognition involves recognition of donor MHC peptides or non-MHC donor peptides loaded on class II molecules of the recipient's antigen presenting cells (APC's) by the recipient's T cells. The donor cells in the graft, shed antigen, typically MHC class I and II molecules that are endocytosed and processed by the recipient's APC's to be presented to autologous T cells. In addition, the donor peptide alters the recipient's MHC molecule α helices, and T cells may also recognise this difference in autologous MHC stricture (ed. Wood, 1995). These events are summarized in Figure 3, page 24.

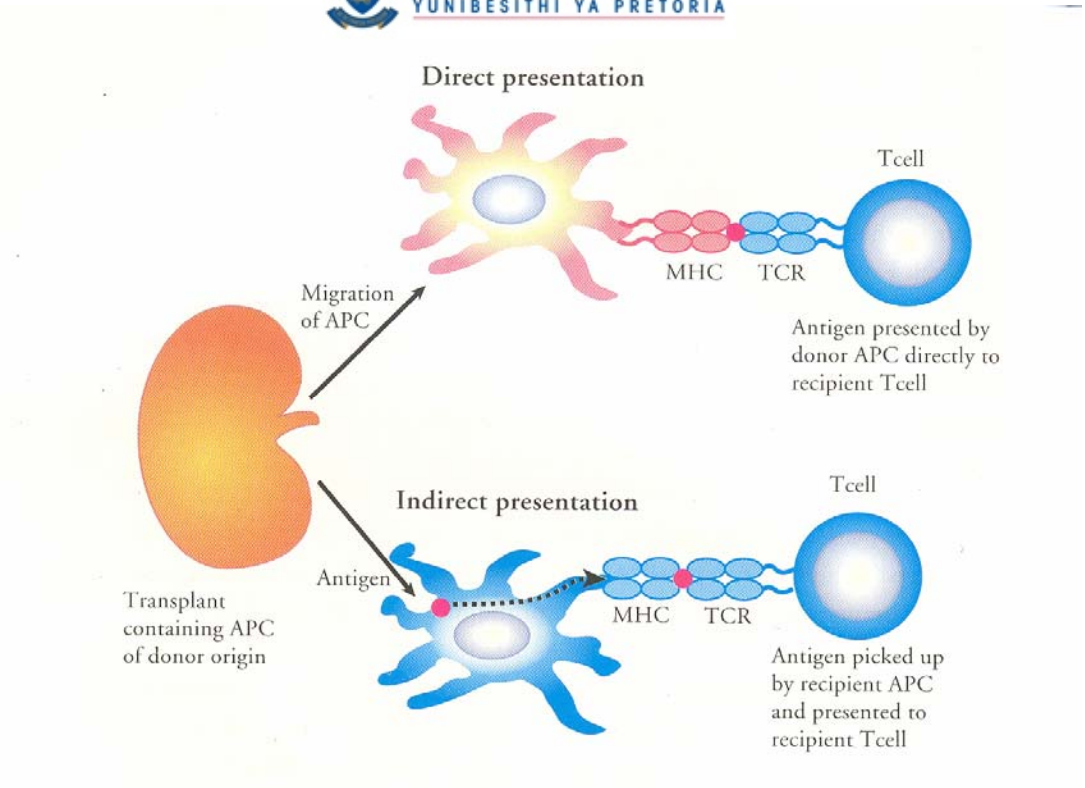


Fig 3. Direct and Indirect Allorecognition. Direct and indirect presentation of alloantigens during an MLC reaction (Source: ed. Wood, 1995).

It has been shown that alloreactive T cells which recognise loaded donor MHC molecules are present at 1000-10 000 times the concentrations of those which recognise loaded recipient MHC molecules. This difference is due to the fact that the peptides carried in the donor MHC molecule were generated during antigenic encounters occurring in the donor at the time of organ/marrow donation. Therefore, allorecognition by the recipient's T cells involves donor MHC molecules and the "carry-over" peptides, thereby providing supplementary allogeneic stimuli. In addition the "carry-over" peptides stabilize the donor's MHC molecule confirmation, providing a strong allogeneic stimulus. An empty MHC molecule provides a poor allogeneic stimulus (Peakman and Vergani, 1997).

1.5.2 T cell activation

Ligation of a T cell's antigen-specific receptor, the T-cell receptor (TCR) provides the primary stimulus for T cell activation. Although the TCR interacts with the antigen with high specificity, the interaction is of low affinity. The T cell is held in contact with the APC by interactions between adhesion molecules such as LFA-1 with ICAM-1, which allows the TCR to survey MHC grooves for foreign peptide. The TCR is composed of two chains, the α and the β chains, which are complexed with the chains of the CD3 complex. Binding of alloantigen by the TCR transduces a signal of activation to the T cell via the CD3 complex.

In addition to the primary stimulus received via the TCR, a naive T cell also requires a secondary signal for activation to be complete. This is provided by co-stimulatory molecules on or from the APC's, which interact with surface receptors on the T cell (ed. Wood, 1995).

Surface molecules involved in co-stimulation:

- The CD4 and CD8 molecules on the T cell, which bind to class II and class I molecules on the APC, respectively. The transmembrane and cytosolic regions of the CD4 and CD8 molecules are associated with intracellular signalling proteins such as $p56^{lck}$, which transduce further signals to the T cell.
- The CTLA-4/CD28 molecule on the T cell, which bind to the B7 (CD80/CD86) molecules on the APC. Interaction between these surface molecules triggers a signalling pathway distinct from the pathway initiated via the TCR.
- The CD40L molecule on the T cell, which binds to CD40 on the APC.
- Interaction between the following surface molecules results in increasing the affinity between the T cell and APC and transduces further signals to the T cell: CD54 to CD11a/CD18, CD2 to CD58 and CD5 to CD72.

These events are summarized in Figure 4, page 27.

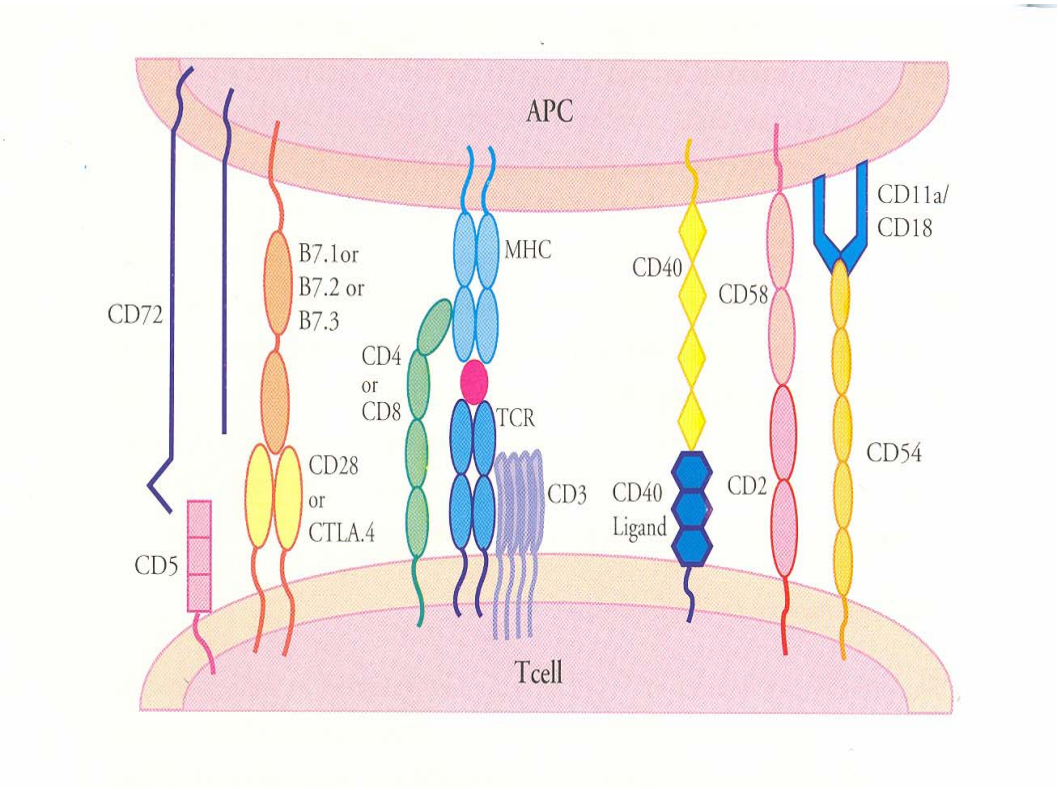


Fig 4. Surface molecules involved in T cell activation. Surface molecules involved in co-stimulation of a T cell by the APC (Source: ed. Wood, 1995).

Membrane-bound forms of cytokines, as well as soluble cytokines, are also involved in co-stimulation. Membrane-bound forms include IL-1 and TNF-α, which interact with surface receptors on the T cell. Soluble cytokines include IL-1, IL-6, IL-12 and TNF-α, which bind to surface receptors on the T cell surface. Cytokines influence both the magnitude and nature of the immune response due to the presence of the two aforementioned subsets of helper T (Th) cells, Th1 and Th2 cells, each with the ability to produce distinct types of cytokines (ed. Wood, 1995). Th1 cells produce cytokines such as IL-2 and IFN-γ, which drive a cell-mediated immune response, whilst Th2 cells produce cytokines such as IL-4 and IL-10, which drive an antibody-mediated response (ed. Wood, 1995).

1.5.3 Intracellular events following TCR ligation

TCR ligation triggers a cascade of kinase activity. Protein tyrosine kinases (PTK's) associated with the TCR are the first to become phosphorylated. These include:

- kinase $p59^{Fyn}$, associated with the TCR/CD3 complex
- kinase $p56^{lck}$, associated with CD4 and CD8
- tyrosine phosphatase associated with CD45 which positively regulates $p56^{lck}$ /$p59^{Fyn}$.

(ed. Wood, 1995; Peakman and Vergani, 1997)

Subsequently, cytosolic tyrosine kinases such as Syk and ZAP-70, are recruited and activated. Phosphorylation of PLC-γ1 and shc link the signal received through the TCR to two key signalling pathways: PLC-γ1 hydrolyzes phosphatidyl inositol biphosphate (PIP_2), a membrane lipid, releasing inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to receptors on the ER, releasing stored calcium from the ER into the cytosol (ed. Wood, 1995). This calcium release, together with membrane events which result in the influx of additional calcium, allows for sustained high calcium concentrations in the cytosol, activating calcium-regulated enzymes. Calcineurin is a calcium-dependent protein phosphatase, which dephosphorylates the transcriptional regulatory protein NFATc (nuclear factor for activated T $cells_{cytoplasm}$), enabling it to translocate from the cytoplasm to the nucleus ($NFAT_n$). $NFAT_n$ moves to its DNA binding site in the regulatory region of the IL-2 gene, resulting in IL-2 gene transcription. $NFAT_n$ also induces transcription of IFN-γ, IL-4, GM-CSF, TNF-α etc, some of which also require the binding of transcription factor activator protein-1 (AP-1). The diacylglycerol released by PLC-γ1 activates protein kinase C, which drives the synthesis of Jun and Fos to form AP-1 (ed. Wood, 1995; Peakman and Vergani, 1995), and is also involved in the chain of cytsolic signalling events leading to the activation of transcription factor nuclear factor kappa B (NFκB).

The result of these signalling events is commitment of the T cell to cytokine expression, activation, blast formation, mitosis and differentiation into functional effector cells.

1.6 Limitations of Existing Procedures

1.6.1 Two-way MLC

The major limitation associated with a two-way MLC is the inability to distinguish between a host-versus-graft-rejection and a graft-versus-host reaction in the observed allogeneic response (Danzer *et al.*, 1996).

1.6.2 One-way MLC

There are 2 major limitations in the one-way MLC:

1. A transplanted organ contains "passenger cells". These "passenger cells" are antigen-presenting cells and immunocompetent cells such as T, B and NK lymphocytes, which leave the graft and persist in host tissues for long periods of time (Schlitt *et al.*, 1993; Richter *et al.*, 1994). These cells are not resting peripheral blood mononuclear cells, but in fact preactivated (HLA-DR$^+$), CD8$^+$ and CD45RO$^+$ T lymphocytes (Schlitt *et al.*, 1993; Richter *et al.*, 1994), which recognise the tissues of the recipient as foreign, resulting in GVHD (Janeway *et al.*, 2005). Therefore the immune reactions following organ transplantation are bilateral and as such cannot be qualitatively represented by the standard one-way MLC (Lawler *et al.*, 1975).

In a study undertaken by Sato *et al*, (1999), which evaluated the interactions of allogeneic cells in the two-way MLC, the following was demonstrated:

- "Domination/Elimination" Process

  Over a period of 3-4 weeks of co-culture, one of the two populations' significantly decreased or disappeared, while the other population survived, proliferating strongly. Reciprocal studies of one-way MLC have demonstrated that the allogeneic responses in the two directions differ in strength (Borel *et al*., 1996). In the same way, the bilateral immune interactions in a two-way MLC could also differ in their relative strengths, resulting in this "domination/elimination" process.

- The role of cytotoxic T cells

  The *in vivo* conditions following transplantation are not adequately reproduced by the 50:50 ratio of allogeneic cells in the two-way MLC set-up. *In vivo*, the recipient cells will far outnumber those of the donor. In *in vitro* assay systems with unequal starting conditions, the larger of the two populations was found to dominate the smaller one. However, this was only found when resting, unseparated cells or $CD2^+$ cells were used. When the smaller of the two populations consisted predominantly of $CD8^+$ T cells or preactivated T cells, the domination of the larger population was reversed. This phenomenon was particularly striking when preactivated T

cells were used: even 5% of preactivated cells in the co-culture could dominate over 95% of resting, allogeneic cells. This demonstrates that small numbers of certain types of immunocompetent cells override the potential dominance of excessive numbers of allogeneic cells.

2. The class II HLA-DR region codes determinants which provide the primary activation signals for an MLC reaction (Mann *et al.*, 1980; Malinowski *et al.* 1992). To achieve unidirectionality in a one-way MLC, the cell cycle of the stimulating cells is arrested, so that although they remain viable, they are unable to divide and proliferate (Dower *et al.*, 1985). The most commonly used techniques to inactivate the stimulator cells in this manner are exposure of the cells to γ-irradiation or to mitomycin C (Kasakura and Lowenstein, 1967). However, these procedures also have the disadvantage of significantly altering cell surface antigen expression and density of individual HLA-DR products and percentages of lymphocyte subsets (Kasakura and Lowenstein, 1967; Mettler *et al.*, 1985). In a study undertaken by Malinowski *et al.* (1992) which evaluated the effects of γ-irradiation and mitomycin C on lymphocyte surface antigen expression, the following changes on HLA-DR expression, after 72 hours culture, were demonstrated:

- HLA-DR expression decreased by 26% and 49% on $CD3^+$ and $CD8^+$ T cells, respectively, following exposure of the cells to γ-irradiation.

- HLA-DR expression decreased by 46% and 30% on $CD3^+$ and $CD8^+$ T cells, respectively, following exposure of the cells to mitomycin C.

These results indicate that not only is the density of HLA-DR expression significantly decreased following exposure of the cells to γ-irradiation and mitomycin C, but that MLC results obtained with γ-irradiated stimulator cells may not be comparable to results obtained with mitomycin C exposed cells. Moreover, the observed decrease in HLA-DR expression may be due to structural changes in the constituent proteins as a result of γ-irradiation or mitomycin C treatment. Structural changes in class II determinants that are common to both stimulator and responder cells may therefore result in a potential amplification of activation signals in the MLC reaction. In addition, γ-irradiation, and to a lesser extent mitomycin C, also result in loss of viability, loss of function, biochemical changes and alterations in biophysical structure and subcellular components of the cells (Kasakura and Lowenstein, 1967: Mettler *et al.*, 1985). Lymphocytes isolated from irradiated leukaemia patients show a decrease in their ability to stimulate normal lymphocytes in the MLC (Stefani and Schrek, 1964; Mickelson *et al.*, 1990). Clearly the procedures used to inactivate the

stimulator cells in the one-way MLC should not affect either the expression or structure of surface molecules recognized by the responder cells.

1.7 CFSE-based MLC assay

5,6 Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a green fluorescent dye with excitation and emission wavelengths of 491 nm and 518 nm respectively (Weston and Parish, 1990). Once taken up by the cells, intracellular esterases hydrolyze CFSE into a fluorescent dye that binds covalently to lysine residues of cytoplasmic proteins (De Clerck *et al.*, 1994). When a cell divides, the CFSE-labeled macromolecules of the cell separate into two daughter cells such that each daughter cell contains half the CFSE content of the parent cell. Therefore the fluorescence intensity in each generation is half of that of the previous generation. See Fig. 5, Page 36. Proliferating cells can then be tracked using flow cytometry. CFSE-labeled cells can also be counterstained with other fluorescent dye-labelled monoclonal antibodies and viability markers to allow for the phenotyping of the proliferating population, and to determine the viability of these cells.

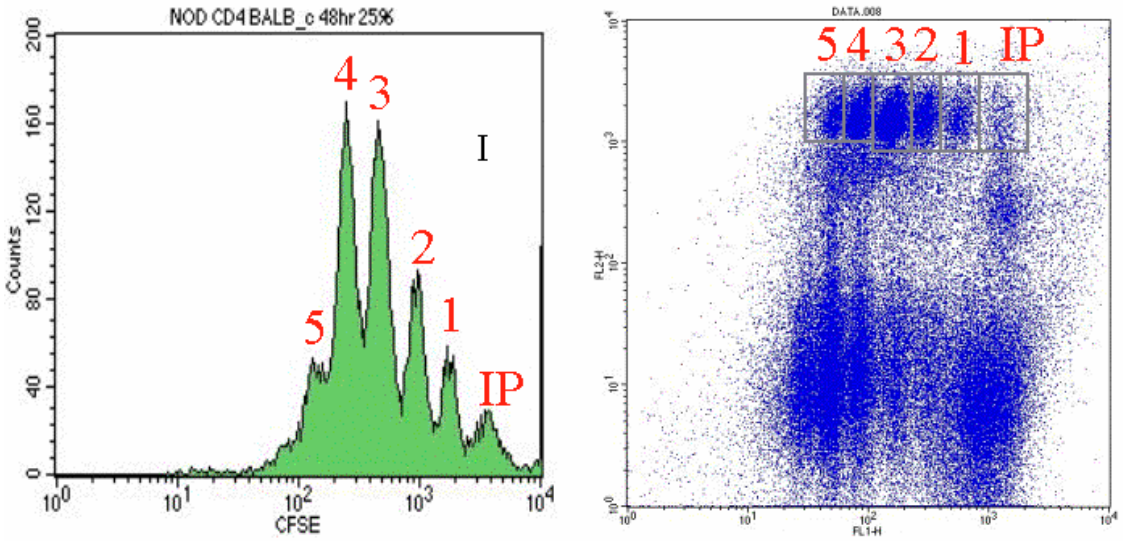


Fig. 5. CFSE staining. Progressive reduction in CFSE fluorescence intensity with each successive generation of mitosis (IP 1-5). (Source: www.med.umich.edu/flowcytometry/training/lessons/lesson7/images/cfse.gif).

1.7.1 Advantages of a CFSE MLC assay over a ^{3}H-Thymidine MLC assay

The following are the advantages of CFSE/flow cytometric based procedures over the ^{3}H-thymidine-based procedures in determining reactivity in the MLC:

- The measuring of T cell DNA synthesis by incorporation of ^{3}H-thymidine indicates a commitment of these cells to proliferation but does not indicate actual cellular division. This may lead to misinterpretation since a late cell cycle arrest results in minimal cellular division. An arrest in cell cycle may be caused by a loss of cell-cell contact,

especially where the stimulator cells have been irradiated or exposed to mitomycin C (Krupnick *et al.*, 2001).

- T cells that show a high level of ^{3}H-thymidine incorporation may be destined to die by apoptosis shortly thereafter. It has been shown that proliferation in response to antigen is followed by a wave of apoptosis (Renno *et al.*, 1999; Mannering *et al.*, 2003). Thus, measuring the magnitude of a T cell response by the ^{3}H-thymidine assay actually measures the peak of DNA-synthesis and not actual proliferation.

- CFSE is able to detect a T cell response to a 10-fold lower antigen concentration than the ^{3}H-thymidine assay, making it particularly useful for an MLC (Mannering *et al.*, 2003).

- Labelling with a CFSE enables detection of the proliferating cells within a heterogenous MLC, since CFSE labelled cells show defined narrow peaks for each generation of dividing cells (Popma *et al.*, 2000).

- CFSE labelling is a simple procedure, which eliminates the use of radioactive materials and their disposal (Popma *et al.*, 2000).

- CFSE produces an extremely bright fluorescence signal, which is easily detected by most tabletop flow cytometers (Popma *et al.*, 2000).

1.8 Cyclosporin A

Cyclosporin A (CsA) is a metabolite, produced during fermentation, of the fungus *Trichoderma polysporum*. It was first isolated by Thiele and Kis in 1970 and its immunosuppressive properties were discovered by Borel in 1972 (ed. Wood, 1995).

1.8.1 Structure

CsA is a cyclic undecapeptide with a molecular weight of 1203 (ed. Wood, 1995).

1.8.2 Mechanism of Action

CsA binds intracellularly to an immunophilin known as cyclophilin, which is a cis-transpeptidyl-propyl isomerase involved in protein folding. The protein folding function of cyclophilin is not associated with the immunosuppressive activity of CsA (ed. Wood, 1995). Once CsA binds to cyclophilin, the latter undergoes a subtle conformational change which allows the CsA-cyclophilin complex to bind to the calcineurin-calmodulin complex with a very high affinity. This interaction between CsA and calcineurin blocks the phosphatase activity of calcineurin. Calcineurin is a fundamental component of the T-cell activation pathway (O'Keefe *et al.*, 1992; Yang, 1992; Ho *et al.*, 1996). Calcineurin is a phosphatase which

dephosphorylates the cytoplasmic form of $NFAT_c$, allowing it to translocate to the nucleus. In the nucleus, together with other transcription factors such as AP-1, $NFAT_n$ acts as a transcription regulator, activating genes required for T-cell activation, including IL-2, IL-3, IL-4, TNF-α, IFN-γ and GM-CSF. The interaction between CsA and cyclophilin is shown in Figure 6.

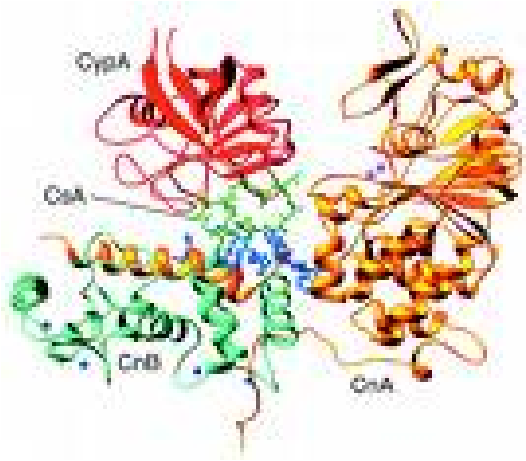


Fig. 6. Structure of CsA. CsA (ball and stick structure) bound to its cell target, cylophilin A, CypA (red ribbon structure) and calcineurin CnA and CnB. (Source: http://www.3dchem.com/molecules.asp?ID=3)

Thus, the result of this block of calcineurin activity by the CsA:cyclophilin complex, is complete inhibition of translocation of NFATc from the cytoplasm to the nucleus and hence a lack of T cell activation and cytokine gene transcription

(Flanagan *et al.*, 1991; Ho *et al.*,1996). CsA suppression of the IL-2 promoter/enhancer function is associated with the two NFAT sites and Oct-2 sites (Ho *et al.*, 1996).

1.9 Cytokines

The process of allorecognition and proliferation which occurs in the MLC results in the secretion of numerous cytokines (Danzer *et al.*, 1996). The profile of cytokines secreted ultimately determines the nature of the ensuing immune response and the outcome of the MLC. Th1 cytokines such as IL-2 and IFN-γ correlate with proliferation in the MLC and as such predict graft rejection (Vandenbroecke *et al.*, 1991; Steinmann *et al.*, 1994; Azuma *et al.*, 1996). Th2 cytokines such as IL-4 and IL-10 correlate with no proliferation in the MLC and as such predict graft tolerance (Dallman *et al.*, 1993; Joseph *et al.*, 1995). The cytokine profile of Th0, Th1 and Th2 cells are summarized in Figure 7, page 43.

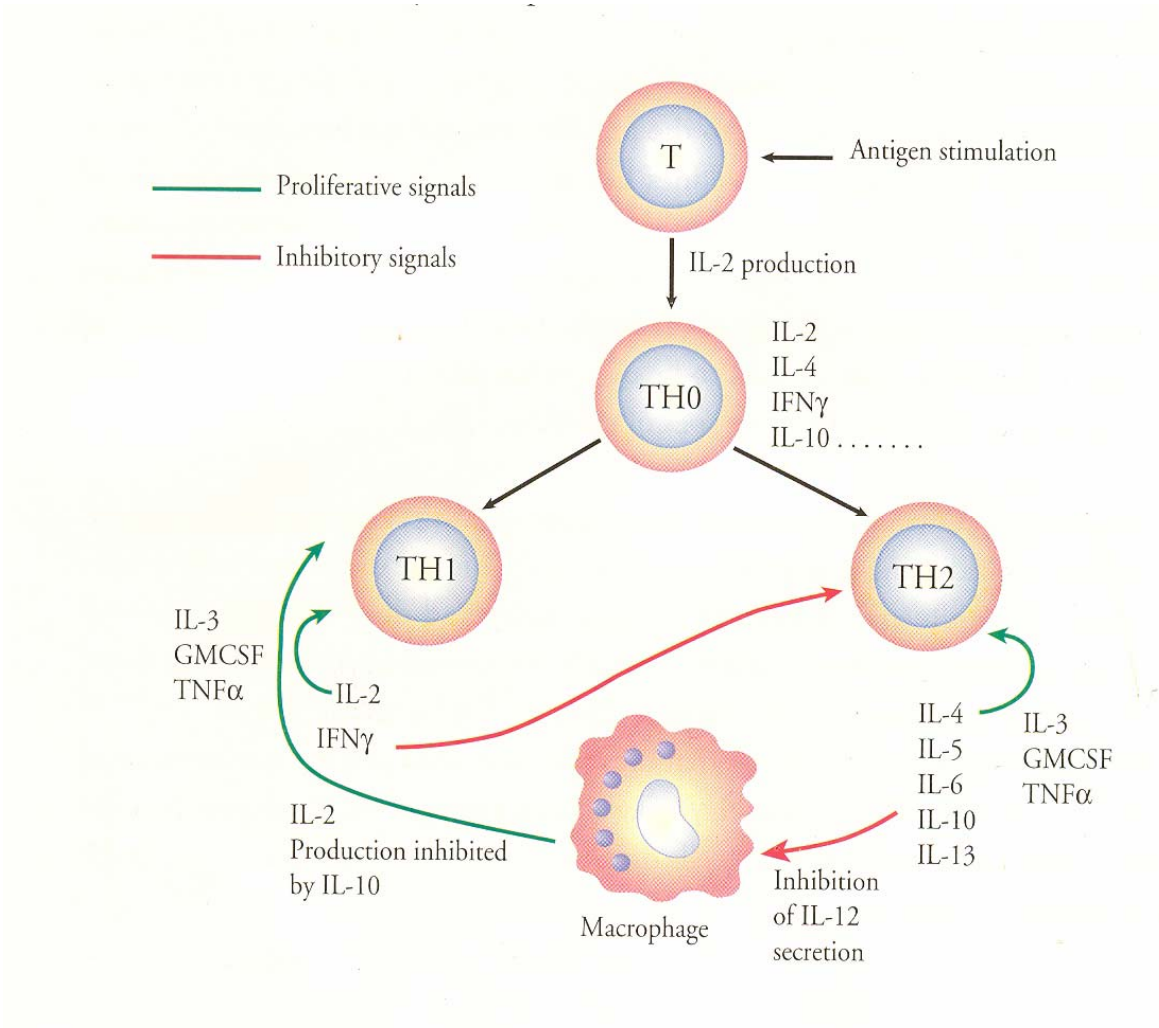


Fig 7. Cytokine profile of Th1 and Th2 lymphocytes. Cytokines released during an immune response determine whether the response is cell-mediated (Th1) or antibody-mediated (Th2) (Source: ed. Wood, 1995).

The following cytokines have been measured in MLC supernatants:

IL-1β

IL-1β is a 17 kDa, pro-inflammatory cytokine secreted by macrophages and epithelial cells, which induces expression of adhesion molecules on endothelium, synthesis of acute phase proteins and IL-6 synthesis and secretion (Peakman and Vergani, 1997).

IL-2

Refer to pages 72-73.

IL-4

IL-4 is a highly pleiotropic cytokine, whose early secretion during the time course of an immune response, leads to the development of a Th2 response (Mosmann *et al.*, 1986). IL-4 is a 20 kDa glycoprotein secreted by mature Th2 cells, mast cells and basophils IL-4 is involved in the activation and differentiation of B cells into Ig-E secreting plasma cells, and as such is a key regulator of the humoral immune response (Brown and Hural, 1997). IL-4 is also viewed as an anti-inflammatory cytokine, blocking the production of IL-1, TNF-α, IL-6, IL-8 and MIP-1α and stimulating the synthesis of IL-1ra, an antagonist of IL-1 (Te Veldte *et al.*, 1990; Brown and Hural, 1997).

IL-5

IL-5 is a 12 kDa cytokine produced by Th2 cells and mast cells. IL-5 is involved in activation and differentiation of IgE producing B cells and mediates activation of eosinophils (Milburn *et al.*, 1993; Janeway *et al.*, 2005).

IL-6

Although IL-6 is commonly seen as a pro-inflammatory cytokine, it does exhibit anti-inflammatory properties as well. IL-6 is secreted by T cells, macrophages and endothelial cells in response to pathogen-associated molecular patterns and any tissue damaging inflammation. IL-6 is a potent inducer of the acute phase protein response (van der Poll *et al.*, 1997; Janeway *et al.*, 2005).

Il-6 also down-regulates synthesis of pro-inflammatory cytokines such as IL-1 and TNF, whilst having no effect on anti-inflammatory cytokines such as IL-10 (Libert *et al.*, 1994; Ruzek *et al.*, 1997).

IL-7

IL-7 is a hematopoietic growth factor secreted by bone marrow stromal cells and the thymus. IL-7 is involved in maturation of pre-B cells, pre-T cells and natural killer

(NK) cells, as well as the survival and function of memory T cells (Janeway *et al.*, 2005).

IL-10

IL-10 is the most potent anti-inflammatory cytokine of the immune response, and as such is also known as the human cytokine synthesis inhibitory factor (CSIF). IL-10 is secreted by $CD4^+$T cells, monocytes and B cells, circulating as a homodimer, each unit consisting of 160 amino acids (Howard and O'Garra, 1992; Opal *et al.*, 1996). IL-10 inhibits synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-3, TNF-α and GM-CSF, promotes degradation of the mRNA of these cytokines, inhibits cell surface expression of MHC class II and the B7 molecules, inhibits translocation of NF-κB and promotes the shedding of TNF receptors (Gerard *et al.*, 1993, Marchant *et al.*, 1994).

IL-12

IL-12 is a heterodimeric cytokine composed of 4 α helices. IL-12 is secreted by macrophages, dendritic cells and B-lymphoblastoid cells in response to foreign antigen (Janeway *et al.*,2005). IL-12 induces the differentiation of naïve T cells into Th1 cells and enhances the cytokine killing activities of $CD8^+$T cells. IL-12 also

stimulates the synthesis and secretion of IFN-γ and TNF-α, whilst suppressing IL-4 mediated suppression of IFN-γ (Janeway *et al.*, 2005)

IL-13

IL-13 is a 10kDa, non-glycosylated cytokine with a molecular weight of 10 kDa. IL-13 is secreted by Th2 cells. Many functions of IL-13 overlap with those of IL-4, however IL-13 is a key mediator of the physiologic changes which occur following allergic inflammation (de Waal., 1993; Mckenzie *et al.*, 1993).

IFN-γ

IFN-γ is a homodimer composed of 25kDa subunits. IFN-γ is secreted by $CD4^+$Th1 lymphocytes, $CD8^+$T lymphocytes and activated NK cells. IFN-γ is a pro-inflammatory cytokine: it is a potent activator of macrophages, increasing their metabolic, phagocytic and killing activity; it increases MHC class II expression on APCs; it induces development of Th1 lymphocytes; activation of $CD8^+$T lymphocytes; it induces a class switch to IgG, and activation of NK cells (Peakman and Vergani, 1997).

TNF-α

TNF-α is a 25 kDa trimeric protein secreted predominantly by macrophages. TNF-α is a pleiotropic, pro-inflammatory cytokine. TNF-α is an acute phase protein whose local release at a site of inflammation initiates a cascade of cytokine secretion (IL-1, IL-6, TNF-α), which leads to the recruitment and activation of macrophages and neutrophils (Peakman and Vergani, 1997; Janeway *et al.*, 2005).

1.10 Hypothesis and Objectives

1.10.1 Hypothesis

The hypotheses to be tested are i) that the use of cyclosporin A in the one-way MLC as the inactivator of the stimulator cells is superior to the standard MLC with mitomycin C or ionizing irradiation, by promoting both sustained viability of the cells and expression of surface HLA molecules; ii) that a CFSE-based, two-way MLC is superior to the conventional one-way MLC.

1.10.2 Objectives

1. To determine whether cyclosporin A can be used as an alternative to mitomycin C, or ionizing irradiation, for the pre-treatment of stimulator cells in the MLC.

2. To assess whether cyclosporin A is superior to mitomycin C or ionizing irradiation as inactivator of the stimulator cells.

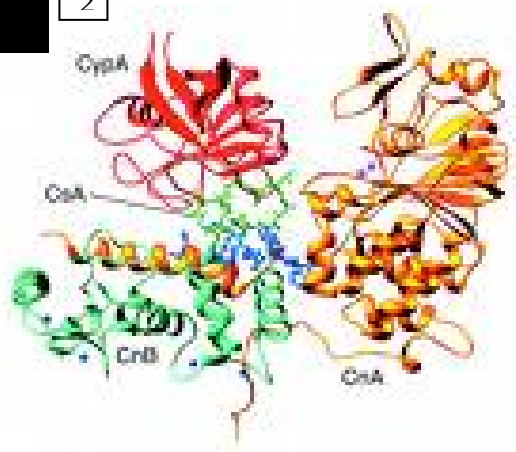
3. To compare the viabilities and levels of expression of HLA class II molecules on stimulator cells inactivated by exposure to cyclosporine A, mitomycin C, or ionizing irradiation throughout the time-course of the one-way MLC.

4. To investigate the potential of IL-2 to improve the sensitivity and accelerate the time-course of the one-way MLC.

5. To establish a CFSE-based flow cytometric mixed lymphocyte assay to the standard MLC, which uses ^{3}H-thymidine uptake to detect proliferation.

1

2



Chapter 2 Evaluation and modification of the one-way MLC

1. Homology Modelling, Cyclophilin, retrieved 21 February 2008, <http--xray_bmc_uu_se-~marian-bioinfo-modeling-cyclophilin_jpg.htm
2. Harrison, K, 1996, 3Dchem, Molecule of the month for March 1996, retrieved 21 February 2008, <http://www.3dchem.com/molecules.asp?ID=32>

Chapter 2: Evaluation and Modification of the one-way MLC

2.1 Introduction

Cyclosporin A is an attractive alternative to mitomycin C or irradiation of stimulator cells in the one-way MLC for several reasons. Firstly, unlike mitomycin C and irradiation, cyclosporin A has a specific cytosolic target (calcineurin/NFATc) and does not, apparently, directly induce DNA damage resulting in apoptosis. Secondly, cyclosporin A is extremely lipophilic, such that pre-treatment of stimulator cells with this agent is unlikely to result in its leakage from the cells with consequent negative effects on the responder cells, as may occur with the water-soluble agent, mitomycin C. Thirdly, abolishment of the requirement for use of an ionising irradiation source has considerable safety implications.

The primary objective of the laboratory research described in this chapter was to investigate the use of cyclosporin A as an inactivator of the stimulator cells in the one-way MLC and to determine whether it is superior to the standard MLC with mitomycin C or ionising radiation, by promoting sustained viability and surface HLA expression of the treated cells

2.2 Materials and Methods

2.2.1 Isolation of mononuclear leukocytes (MNL)

Peripheral blood MNLs were isolated from heparinized (5 units of preservative-free heparin/ml) venous blood of healthy adult humans using standard barrier centrifugation procedures. Peripheral blood was layered on Histopaque®-1077 ficoll cushions and centrifuged at 400g for 25 min at room temperature. Due to differences in the densities of the various cell types, differential sedimentation velocity results in the formation of four cell fractions; plasma containing platelets, mononuclear leukocytes, ficoll and a mixture of granulocytes and erythrocytes. The mononuclear leukocyte (MNL) layer was removed, washed with PBS containing ethylene glycol-bis (beta-amino-ethyl-ether)-N, N, N', N'- tetraacetic acid (EGTA, 1 mM) which prevents aggregation of cells, and centrifuged at 250g for 10 minutes. Contaminating erythrocytes were then removed by selective lysis with 0.84% NH_4Cl for 10 minutes at 4°C, and centrifuged at 250g for 10 minutes. The resultant pellet was then washed with PBS. Enumeration of the MNLs were performed flow cytometrically using Flow-Count™ Fluorospheres (Beckman Coulter, Miami, FL, USA), and the cells resuspended to 1×10^6 cells/ml in RPMI 1640 tissue culture medium and held on ice until use. Purity and viability of isolated lymphocytes was also assessed flow cytometrically using the forward scatter (FS) vs. side scatter (SS) scattergram.

2.2.2 Lymphocyte proliferation assay (LPA)

This assay measures the ability of lymphocytes to undergo polyclonal/clonal proliferation when stimulated *in vitro* by a foreign molecule, antigen or mitogen. With respect to the laboratory research conducted in this chapter, this assay was used to assess the ability of cyclosporin A-treated MNL to respond to the mitogen phytohaemaglutinin (PHA).

Exposure of MNL to Cylosporin A:

Cells resuspended at 1 x 10^6 cells/ml in RPMI 1640 culture medium were incubated in 15ml, pyrogen-free sterile tubes with 2.5-20 µM cyclosporin A (Sigma Diagnostics, St. Louis, Mo, USA) for 24 and 48 hours. Following incubation, the cells were washed with PBS to remove any residual cyclosporin A, centrifuged at 250 g for 10 minutes and resuspended at 1 x 10^6 cells/ml.

Each of triplicate wells in a 96-well micro-titre culture plate received the following: 50 µl of a suspension containing 1 x 10^6 cells/ml control or cylosporin A-treated MNL (5 x 10^4 cells/well), 20 µl fetal calf serum (FCS; 10% final, 20µl/well) and 110µl 1640 RPMI culture medium. Control wells did not receive PHA. The mitogen was added at concentrations of 0.3, 0.6 and 1.25 µg/ml (20 µl/well). The final volume in each well was 200 µl. The plates were agitated gently on a microplate agitator for 5 seconds and then incubated for 48 hrs at 37°C in a humidified CO_2 incubator (5% CO_2). Each well then received 0.2 µCi tritiated thymidine (^{3}H-thymidine; 20µl/ml; Du Pont-NEN, Research Products, Boston,

MA, USA), followed by incubation for a further 18 hours. The extent of MNL activation and proliferation was determined according to the magnitude of uptake of ^{3}H-thymidine into the newly synthesized DNA of the dividing cells as measured by liquid scintillation spectrometry. Briefly, cells were harvested on glass fibre filters using the PHD multi-well cell harvester (Cambridge Technology, USA). The disks were dried using methanol, placed in glass vials, followed by the addition of 4 ml scintillation fluid (Packard Bioscience, USA). The amount of radioactivity incorporated into DNA in each well was measured using a liquid scintillation counter (TRI-CARB – 2100TR, Packard, Canberra Co, USA) and expressed as counts per minute (CPM).

2.2.3 Cell viability and HLA class II molecule expression

The viability of MNL pre-treated with cyclosporin A (20µM), mitomycin C (0.09µM) or exposure to ionzing irradiation, was measured according to intracellular ATP levels and propidium iodide (PI) uptake.

Exposure of MNL to cyclosporin A:

As described above.

Exposure of MNL to mitomycin C:

Mitomycin C (Sigma Diagnostics, St. Louis, Mo, USA) exposure was performed similarly on matched cell suspensions at a final fixed concentration of 0.09µM for 30 minutes.

Exposure to ionising irradiation:

Cells were exposed to 1000 cGys from a Co 60 source for 5 minutes, at a distance of 50 cm from the source.

2.2.3.1 Cellular ATP levels

Measurement of intracellular ATP levels was performed to determine the cytotoxic potential of cyclosporin A, mitomycin C and ionising irradiation for MNL. Control and pre-treated MNL (1×10^6 cells/ml) were cultured for 48 hours at 37°C in a humidified CO_2 incubator (5% CO_2). At incubation time 0, 24 and 48 hours, intracellular ATP levels were measured using a sensitive luciferin/luciferase chemiluminescnce method (Holmes *et al.*, 1972). Briefly 20 µl of MNL suspension was added into pre-prepared chemiluminometer cuvettes containing 100 µl of nucleotide releasing agent (NRS), which causes release of ATP from the cells, and 30 µl of ATP assay mix dilution buffer (FL-AAM). After vortexing, 20 µl of ATP assay mix was added to the mixture, and chemiluminescence measured using the Lumac Biocounter® 2010M and expressed as relative light units (r.l.u.).

2.2.3.2 Propidium iodide uptake

This test depends on the ability of propidium iodide (PI), (Beckman Coulter, Miami, FL, USA) to bind to double stranded nucleic acids, producing red fluorescence proportional to the nucleic acid content. PI is unable to pass through intact cell membranes. Thus, only apoptotic cells will allow for PI uptake. PI also binds to double stranded RNA, which must be removed for accurate DNA measurement.

Control and pre-treated MNL (1×10^6 cells/ml) were cultured for 48 hours at 37°C in a humidified CO_2 incubator (5% CO_2). At incubation time 0, 24 and 48 hours, PI uptake was measured flow cytometrically. Briefly, 500µl of MNL suspension was added to 500µl of PI (DNA Prep™ Stain, Beckman Coulter, Miami, USA) and incubated for 30 minutes in the dark. Samples were analyzed on the Beckman Coulter EPICS XL MCL flow cytometer fitted with a 2-watt argon laser. The lymphocyte population was gated and identified using a FS vs. SS scattergram. The percentage positive cells for PI uptake were derived from the gated lymphocytes.

2.2.3.3 HLA class II molecule expression

Measurement of HLA class II molecule expression was performed to determine reduction and/or structural changes following exposure of MNL to cyclosporin A, mitomycin C and ionising irradiation. Control and pre-treated MNL (1×10^6 cells/ml) were cultured for 48 hours at 37°C in a humidified CO_2 incubator (5% CO_2). At incubation time 0, 24 hours and 48 hours, HLA class II molecule

expression was measured flow cytometrically. Briefly, 500µl each of MNL was added to 10µl of anti-HLA-DR monoclonal antibody (Anti-HLA-DR, BD Biosciences, San Jose, California (USA) and anti-IgG FITC conjugate for detection of nonspecific background staining and incubated for 10 min at room temperature in the dark. Samples were analyzed on the Beckman Coulter EPICS XL MCL flow cytometer fitted with a 2-watt argon laser. The lymphocyte population was gated and identified using a FS vs. SS scattergram. The percentage positive HLA-DR cells were derived from the gated lymphocytes.

2.3 Results

2.3.1 Effects of Cyclosporin A on PHA-activated LPA

To investigate at what concentrations cyclosporin A will inactivate the stimulator cells in the MLC, incorporation of ^{3}H-thymidine into newly synthesized DNA was measured following pre-treatment of the cells with cyclosporin A, followed by PHA activation. Cells were incubated with cyclosporin A (2.5-20µM) for 24 and 48 hours. The results are shown in Figure 2.1 and 2.2, and the most significant observations were as follows:

24 hours incubation with cyclosporin A

♦ At a concentration of 0.3 µg/ml PHA, there was a significant decrease ($P < 0.05$) in mean counts from 18 632.5 ± 1211.2 CPM for untreated cells to 5259.7 ± 209.7, 4556.1 ± 131.6, 2904.5 ± 1043.9 and 406.7 ± 95.7 CPM for cells treated with 2.5, 5, 10 and 20 µM cyclosporin A, respectively. *

♦ At a concentration of 0.6 µg/ml PHA, there was a significant decrease ($P < 0.05$) in mean counts from 18 548.3 ± 1274.3 CPM for untreated cells to 5219.6 ± 108.8, 6005.1 ± 549.5, 4036.7 ± 1049.5 and 613.3 ± 287.3 CPM for cells treated with 2.5, 5, 10 and 20 µM cyclosporin A, respectively. *

♦ At a concentration of 1.25 µg/ml PHA, there was a significant decrease ($P < 0.05$) in mean counts from 19537.9 ± 1427.6 CPM for untreated cells to 6614.82 ± 413.3, 5984.7 ± 718.7, 3703.8 ± 1051.5 and 555.1 ± 203.7 CPM for cells treated with 2.5, 5, 10 and 20 µM cyclosporin A, respectively. *

* *Counts per minute for control systems (untreated cells) were 266 ± 15.2.*

48 hours incubation with cyclosporin A

♦ At a concentration of 0.3 µg/ml PHA, there was a significant decrease ($P < 0.05$) in mean counts from 11 729.1 ± 1719.6 CPM for untreated cells to 2809.1 ± 135.4, 2489.9 ± 192.6, 358.7 ± 61.3 and 289.16 ± 27.8 CPM for cells treated with 2.5, 5, 10 and 20 µM cyclosporin A, respectively. *

♦ At a concentration of 0.6 µg/ml PHA, there was a significant decrease ($P < 0.05$) in mean counts from 12 619.6 ± 1874.4 CPM for untreated cells to 5463.8 ± 231.8, 2260.3 ± 137.5, 458.3 ± 76.6 and 404.3 ± 74.2 CPM for cells treated with 2.5, 5, 10 and µM cyclosporin A, respectively. *

♦ At a concentration of 1.25 µg/ml PHA, there was a significant decrease ($P < 0.05$) in mean counts from 13004.8 ± 1557.7 CPM for untreated cells to 6373.1 ± 200.3, 2487.7 ± 61.4, 445.5 ± 27.9 and 453.1 ± 70.0 CPM for cells treated with 2.5, 5, 10 and µM cyclosporin A, respectively. *

* *Counts per minute for control systems (untreated cells) were 296 ± 15.6*

These results indicate that incubation of MNL with cyclosporin A for 24 and 48 hours, followed by washing the cells, abolishes the ability of the cells to proliferate in response to stimulation by PHA. Inhibition of proliferation was concentration-dependent, showing complete absence of PHA-activated response following pre-treatment with 20 µM cyclosporin A.

2.3.2 Effect of exposure of MNL to ionising radiation, mitomycin C and cyclosporin A on HLA-DR expression of MNL

To investigate the effect of ionising irradiation, mitomycin C and cyclosporin A on expression of HLA-DR molecules on the surface of stimulator cells, HLA-

DR levels were measured by flow cytometry following pre-treatment of the cells with ionising irradiation, mitomycin C and cyclosporin A. Cells were exposed to ionising radiation???, mitomycin C (0.09µM) or cyclosporin A (20 µM) and HLA-DR expression measured at 24 and 48 hours of culture. The results are shown in Figure 2.3, and the most significant observations were as follows:

♦ At 24 hours, there was a significant decrease ($P < 0.05$) in HLA-DR expression from 17.8 ± 2.7 % HLA-DR for untreated cells to 5.1 ± 1.9 % HLA-DR for cyclosporin A-treated cells.

♦ At 48 hours, there was a significant decrease ($P < 0.05$) in HLA-DR expression from 16.9 ± 2.5 % HLA-DR for untreated cells to 3.6 ± 1.2, 2.5 +/- 1.3 and 2.2 ± 1.1 % HLA-DR for irradiated and mitomycin C- and cyclosporin A-treated cells, respectively.

These results indicate that pre-treatment of cells with ionising radiation, mitomycin C or cyclosporin A has a significant effect on levels of HLA-DR expression on the cell surface.

2.3.3 Effect of exposure of MNL to ionising radiation, mitomycin C and cyclosporin A on ATP levels

To investigate the cytotoxic potential of ionising radiation, mitomycin C and cyclosporin A on the stimulator cells, the ATP content of the lymphocytes was

measured using the luciferin-luciferase firefly luminescence method (Jabs *et al.,* 1997), following pre-treatment of the cells with ionising irradiation, mitomycin C and cyclosporin A. Cells were exposed to ionising radiation???, mitomycin C (0.09µM) or cyclosporin A (20 µM) and ATP levels measured at 24 and 48 hours of culture. The results are shown in Figure 2.4, and the most significant observations were as follows:

♦ At 24 hours there was a significant decrease ($P < 0.05$) in ATP levels from 11 037.3 ± 508.2 r.l.u for untreated cells to 584.3 ± 75.8, 1541 ± 174.2 and 2345.6 ± 417.7 r.l.u for irradiated and mitomycin C- and cyclosporin A-treated cells, respectively.

♦ At 48 hours there is a significant decrease ($P < 0.05$) in ATP levels from 10 071 ± 356.2 r.l.u for untreated cells to 425.7 ± 93.6, 684.3 ± 84.5 and 1901.7 ± 221.5 r.l.u for irradiated and mitomycin C- and cyclosporin A-treated cells, respectively.

These results indicate that pre-treatment of cells with ionising radiation, mitomycin C and cyclosporin A has a significant effect on cell viability.

2.3.4 Effect of exposure of MNL to ionising radiation, mitomycin C and cyclosporin A on MNL viability.

To investigate the effects of exposure of cells to ionising radiation, mitomycin C or cyclosporin A on cell viability, uptake of PI into cells pre-treated with mitomycin C(0.09μM), cyclosporin A(20μM), and ionising radiation was measured by flow cytometry at 24 and 48 hours of culture. The results are shown in Figure 2.5, and the most significant observations were as follows:

♦ After 24 hours post-irradiation, there was a significant decrease in cell viability from 99.1 ± 0.1 % for untreated cells to 55.7 ± 3.8 % for irradiated cells. Similarly, albeit to a lesser magnitude, there was a decrease in cell viability for cyclosporin A- and mitomycin C-treated cells are decreased from 99.1 ± 0.1 % for untreated cells to 73.2 ± 9.2 and 68.3 ± 13.9 % for cyclosporin A and mitomycin C treated cells, respectively.

♦ After 48 hours of culture, there was a significant decrease in cell viability from 97 ± 1.2 % for untreated cells to 39.7 ± 2.9, 67.9 ± 10.6 and 57.8 ± 10.5 % for irradiated and mitomycin C- and cyclosporin A-treated cells, respectively.

These results indicate that pre-treatment of cells with ionising irradiation, mitomycin C and cyclosporin A has a significant effect on cell viability, with ionising irradiation demonstrating the most profound effect.

2.4 Discussion

The one-way MLC appears to be a straightforward, simple procedure, based upon inactivation of the stimulator cells, or recipient cells, such that they remain viable but unable to divide. This unidirectionality of the MLC allows for the measurement of proliferation of only one of the populations, that of the donors, that can be used to predict GVHD (Malinowski *et al.*, 1992). To predict HVGD, and ultimately graft rejection, the reverse strategy (responder cells from the recipient/stimulator cells from the donor) may be used.

The primary activation signal for the alloreactive immune response reproduced in the MLC is largely dependent on the cell surface expression of HLA class II molecules, particularly HLA-DR molecules (Levis and Robbins, 1970). The stimulator cells are however inactivated by pre-treatment with ionising irradiation or mitomycin C, which also has a significant effect on HLA-DR molecule expression and structure. HLA-DR molecules are greatly reduced and/or undergo structural changes following exposure to these agents. This may lead to no alloreactive immune response or result in its' amplification, demonstrating a weaker or stronger MLC response, regardless of the degree of compatibility between donor and recipient (Malinowski *et al.*, 1992).

Previous studies have also demonstrated that irradiated cells show loss of viability, biochemical changes and damage to subcellular components (Mettler and

Moseley, 1985). $CD8^+$ T cells are twice as radiosensitive as $CD4^+$ T cells, while radiosensitivity of B cells changes throughout B cell ontogeny, with mature B cells being the most radioresistant (Drewinko *et al.*, 1972).

Preliminary experiments in this chapter demonstrated that cyclosporin A can be used as an alternative to mitomycin C or ionising irradiation, for the inactivation of the stimulator cells in the MLC: incubation of MNL with 20µM cyclosporin A for 48 hours revealed a complete absence of PHA-activated proliferative responses.

However, when comparing the viabilities and levels of HLA-DR expression on cells inactivated by pre-treatment with ionising irradiation, mitomycin C or cyclosporin A, cyclosporin A was shown to have a negative effect on cell viability and HLA-DR levels equivalent to that of mitomycin C and ionising radiation. Cyclosporin A treated cells showed a significant reduction in cell surface HLA-DR expression, ATP levels and loss of viability. In fact, cyclosporin A-treated cells showed the greatest reduction in HLA-DR levels when compared to mitomycin C and ionising irradiation. Previous studies involving renal epithelial cell lines demonstrated a permanent and significant decrease in ATP levels following a 24 hour incubation with cyclosporin A (Massicot *et al.*, 1997). Cyclosporin A has also been shown to inhibit HLA-DR expression on monocytes (Younish-Rouach *et al.*, 1991).

Chapter 2: Evaluation and Modification of the one-way MLC

2.1 Introduction

Cyclosporin A is an attractive alternative to mitomycin C or irradiation of stimulator cells in the one-way MLC for several reasons. Firstly, unlike mitomycin C and irradiation, cyclosporin A has a specific cytosolic target (calcineurin/NFATc) and does not, apparently, directly induce DNA damage resulting in apoptosis. Secondly, cyclosporin A is extremely lipophilic, such that pre-treatment of stimulator cells with this agent is unlikely to result in its leakage from the cells with consequent negative effects on the responder cells, as may occur with the water-soluble agent, mitomycin C. Thirdly, abolishment of the requirement for use of an ionising irradiation source has considerable safety implications.

The primary objective of the laboratory research described in this chapter was to investigate the use of cyclosporin A as an inactivator of the stimulator cells in the one-way MLC and to determine whether it is superior to the standard MLC with mitomycin C or ionising radiation, by promoting sustained viability and surface HLA expression of the treated cells

2.2 Materials and Methods

2.2.1 Isolation of mononuclear leukocytes (MNL)

Peripheral blood MNLs were isolated from heparinized (5 units of preservative-free heparin/ml) venous blood of healthy adult humans using standard barrier centrifugation procedures. Peripheral blood was layered on Histopaque®-1077 ficoll cushions and centrifuged at 400g for 25 min at room temperature. Due to differences in the densities of the various cell types, differential sedimentation velocity results in the formation of four cell fractions; plasma containing platelets, mononuclear leukocytes, ficoll and a mixture of granulocytes and erythrocytes. The mononuclear leukocyte (MNL) layer was removed, washed with PBS containing ethylene glycol-bis (beta-amino-ethyl-ether)-N, N, N', N'- tetraacetic acid (EGTA, 1 mM) which prevents aggregation of cells, and centrifuged at 250g for 10 minutes. Contaminating erythrocytes were then removed by selective lysis with 0.84% NH_4Cl for 10 minutes at 4°C, and centrifuged at 250g for 10 minutes. The resultant pellet was then washed with PBS. Enumeration of the MNLs were performed flow cytometrically using Flow-Count™ Fluorospheres (Beckman Coulter, Miami, FL, USA), and the cells resuspended to 1×10^6 cells/ml in RPMI 1640 tissue culture medium and held on ice until use. Purity and viability of isolated lymphocytes was also assessed flow cytometrically using the forward scatter (FS) vs. side scatter (SS) scattergram.

2.2.2 Lymphocyte proliferation assay (LPA)

This assay measures the ability of lymphocytes to undergo polyclonal/clonal proliferation when stimulated *in vitro* by a foreign molecule, antigen or mitogen. With respect to the laboratory research conducted in this chapter, this assay was used to assess the ability of cyclosporin A-treated MNL to respond to the mitogen phytohaemaglutinin (PHA).

Exposure of MNL to Cylosporin A:

Cells resuspended at 1 x 10^6 cells/ml in RPMI 1640 culture medium were incubated in 15ml, pyrogen-free sterile tubes with 2.5-20 µM cyclosporin A (Sigma Diagnostics, St. Louis, Mo, USA) for 24 and 48 hours. Following incubation, the cells were washed with PBS to remove any residual cyclosporin A, centrifuged at 250 g for 10 minutes and resuspended at 1 x 10^6 cells/ml.

Each of triplicate wells in a 96-well micro-titre culture plate received the following: 50 µl of a suspension containing 1 x 10^6 cells/ml control or cylosporin A-treated MNL (5 x 10^4 cells/well), 20 µl fetal calf serum (FCS; 10% final, 20µl/well) and 110µl 1640 RPMI culture medium. Control wells did not receive PHA. The mitogen was added at concentrations of 0.3, 0.6 and 1.25 µg/ml (20 µl/well). The final volume in each well was 200 µl. The plates were agitated gently on a microplate agitator for 5 seconds and then incubated for 48 hrs at 37°C in a humidified CO_2 incubator (5% CO_2). Each well then received 0.2 µCi tritiated thymidine (^{3}H-thymidine; 20µl/ml; Du Pont-NEN, Research Products, Boston,

MA, USA), followed by incubation for a further 18 hours. The extent of MNL activation and proliferation was determined according to the magnitude of uptake of ^{3}H-thymidine into the newly synthesized DNA of the dividing cells as measured by liquid scintillation spectrometry. Briefly, cells were harvested on glass fibre filters using the PHD multi-well cell harvester (Cambridge Technology, USA). The disks were dried using methanol, placed in glass vials, followed by the addition of 4 ml scintillation fluid (Packard Bioscience, USA). The amount of radioactivity incorporated into DNA in each well was measured using a liquid scintillation counter (TRI-CARB – 2100TR, Packard, Canberra Co, USA) and expressed as counts per minute (CPM).

2.2.3 Cell viability and HLA class II molecule expression

The viability of MNL pre-treated with cyclosporin A (20µM), mitomycin C (0.09µM) or exposure to ionzing irradiation, was measured according to intracellular ATP levels and propidium iodide (PI) uptake.

Exposure of MNL to cyclosporin A:

As described above.

Exposure of MNL to mitomycin C:

Mitomycin C (Sigma Diagnostics, St. Louis, Mo, USA) exposure was performed similarly on matched cell suspensions at a final fixed concentration of 0.09µM for 30 minutes.

Exposure to ionising irradiation:

Cells were exposed to 1000 cGys from a Co 60 source for 5 minutes, at a distance of 50 cm from the source.

2.2.3.1 Cellular ATP levels

Measurement of intracellular ATP levels was performed to determine the cytotoxic potential of cyclosporin A, mitomycin C and ionising irradiation for MNL. Control and pre-treated MNL (1×10^6 cells/ml) were cultured for 48 hours at 37°C in a humidified CO_2 incubator (5% CO_2). At incubation time 0, 24 and 48 hours, intracellular ATP levels were measured using a sensitive luciferin/luciferase chemiluminescnce method (Holmes *et al.*, 1972). Briefly 20 µl of MNL suspension was added into pre-prepared chemiluminometer cuvettes containing 100 µl of nucleotide releasing agent (NRS), which causes release of ATP from the cells, and 30 µl of ATP assay mix dilution buffer (FL-AAM). After vortexing, 20 µl of ATP assay mix was added to the mixture, and chemiluminescence measured using the Lumac Biocounter® 2010M and expressed as relative light units (r.l.u.).

2.2.3.2 Propidium iodide uptake

This test depends on the ability of propidium iodide (PI), (Beckman Coulter, Miami, FL, USA) to bind to double stranded nucleic acids, producing red fluorescence proportional to the nucleic acid content. PI is unable to pass through intact cell membranes. Thus, only apoptotic cells will allow for PI uptake. PI also binds to double stranded RNA, which must be removed for accurate DNA measurement.

Control and pre-treated MNL (1×10^6 cells/ml) were cultured for 48 hours at 37°C in a humidified CO_2 incubator (5% CO_2). At incubation time 0, 24 and 48 hours, PI uptake was measured flow cytometrically. Briefly, 500µl of MNL suspension was added to 500µl of PI (DNA Prep™ Stain, Beckman Coulter, Miami, USA) and incubated for 30 minutes in the dark. Samples were analyzed on the Beckman Coulter EPICS XL MCL flow cytometer fitted with a 2-watt argon laser. The lymphocyte population was gated and identified using a FS vs. SS scattergram. The percentage positive cells for PI uptake were derived from the gated lymphocytes.

2.2.3.3 HLA class II molecule expression

Measurement of HLA class II molecule expression was performed to determine reduction and/or structural changes following exposure of MNL to cyclosporin A, mitomycin C and ionising irradiation. Control and pre-treated MNL (1×10^6 cells/ml) were cultured for 48 hours at 37°C in a humidified CO_2 incubator (5% CO_2). At incubation time 0, 24 hours and 48 hours, HLA class II molecule

expression was measured flow cytometrically. Briefly, 500µl each of MNL was added to 10µl of anti-HLA-DR monoclonal antibody (Anti-HLA-DR, BD Biosciences, San Jose, California (USA) and anti-IgG FITC conjugate for detection of nonspecific background staining and incubated for 10 min at room temperature in the dark. Samples were analyzed on the Beckman Coulter EPICS XL MCL flow cytometer fitted with a 2-watt argon laser. The lymphocyte population was gated and identified using a FS vs. SS scattergram. The percentage positive HLA-DR cells were derived from the gated lymphocytes.

2.3 Results

2.3.1 Effects of Cyclosporin A on PHA-activated LPA

To investigate at what concentrations cyclosporin A will inactivate the stimulator cells in the MLC, incorporation of ^{3}H-thymidine into newly synthesized DNA was measured following pre-treatment of the cells with cyclosporin A, followed by PHA activation. Cells were incubated with cyclosporin A (2.5-20µM) for 24 and 48 hours. The results are shown in Figure 2.1 and 2.2, and the most significant observations were as follows:

24 hours incubation with cyclosporin A

♦ At a concentration of 0.3 µg/ml PHA, there was a significant decrease ($P < 0.05$) in mean counts from 18 632.5 ± 1211.2 CPM for untreated cells to 5259.7 ± 209.7, 4556.1 ± 131.6, 2904.5 ± 1043.9 and 406.7 ± 95.7 CPM for cells treated with 2.5, 5, 10 and 20 µM cyclosporin A, respectively. *

♦ At a concentration of 0.6 µg/ml PHA, there was a significant decrease ($P < 0.05$) in mean counts from 18 548.3 ± 1274.3 CPM for untreated cells to 5219.6 ± 108.8, 6005.1 ± 549.5, 4036.7 ± 1049.5 and 613.3 ± 287.3 CPM for cells treated with 2.5, 5, 10 and 20 µM cyclosporin A, respectively. *

♦ At a concentration of 1.25 µg/ml PHA, there was a significant decrease ($P < 0.05$) in mean counts from 19537.9 ± 1427.6 CPM for untreated cells to 6614.82 ± 413.3, 5984.7 ± 718.7, 3703.8 ± 1051.5 and 555.1 ± 203.7 CPM for cells treated with 2.5, 5, 10 and 20 µM cyclosporin A, respectively. *

* *Counts per minute for control systems (untreated cells) were 266 ± 15.2.*

48 hours incubation with cyclosporin A

♦ At a concentration of 0.3 µg/ml PHA, there was a significant decrease ($P < 0.05$) in mean counts from 11 729.1 ± 1719.6 CPM for untreated cells to 2809.1 ± 135.4, 2489.9 ± 192.6, 358.7 ± 61.3 and 289.16 ± 27.8 CPM for cells treated with 2.5, 5, 10 and 20 µM cyclosporin A, respectively. *

♦ At a concentration of 0.6 µg/ml PHA, there was a significant decrease ($P < 0.05$) in mean counts from 12 619.6 ± 1874.4 CPM for untreated cells to 5463.8 ± 231.8, 2260.3 ± 137.5, 458.3 ± 76.6 and 404.3 ± 74.2 CPM for cells treated with 2.5, 5, 10 and µM cyclosporin A, respectively. *

♦ At a concentration of 1.25 µg/ml PHA, there was a significant decrease ($P < 0.05$) in mean counts from 13004.8 ± 1557.7 CPM for untreated cells to 6373.1 ± 200.3, 2487.7 ± 61.4, 445.5 ± 27.9 and 453.1 ± 70.0 CPM for cells treated with 2.5, 5, 10 and µM cyclosporin A, respectively. *

* *Counts per minute for control systems (untreated cells) were 296 ± 15.6*

These results indicate that incubation of MNL with cyclosporin A for 24 and 48 hours, followed by washing the cells, abolishes the ability of the cells to proliferate in response to stimulation by PHA. Inhibition of proliferation was concentration-dependent, showing complete absence of PHA-activated response following pre-treatment with 20 µM cyclosporin A.

2.3.2 Effect of exposure of MNL to ionising radiation, mitomycin C and cyclosporin A on HLA-DR expression of MNL

To investigate the effect of ionising irradiation, mitomycin C and cyclosporin A on expression of HLA-DR molecules on the surface of stimulator cells, HLA-

DR levels were measured by flow cytometry following pre-treatment of the cells with ionising irradiation, mitomycin C and cyclosporin A. Cells were exposed to ionising radiation???, mitomycin C (0.09µM) or cyclosporin A (20 µM) and HLA-DR expression measured at 24 and 48 hours of culture. The results are shown in Figure 2.3, and the most significant observations were as follows:

♦ At 24 hours, there was a significant decrease ($P < 0.05$) in HLA-DR expression from 17.8 ± 2.7 % HLA-DR for untreated cells to 5.1 ± 1.9 % HLA-DR for cyclosporin A-treated cells.

♦ At 48 hours, there was a significant decrease ($P < 0.05$) in HLA-DR expression from 16.9 ± 2.5 % HLA-DR for untreated cells to 3.6 ± 1.2, 2.5 +/- 1.3 and 2.2 ± 1.1 % HLA-DR for irradiated and mitomycin C- and cyclosporin A-treated cells, respectively.

These results indicate that pre-treatment of cells with ionising radiation, mitomycin C or cyclosporin A has a significant effect on levels of HLA-DR expression on the cell surface.

2.3.3 Effect of exposure of MNL to ionising radiation, mitomycin C and cyclosporin A on ATP levels

To investigate the cytotoxic potential of ionising radiation, mitomycin C and cyclosporin A on the stimulator cells, the ATP content of the lymphocytes was

measured using the luciferin-luciferase firefly luminescence method (Jabs *et al.,* 1997), following pre-treatment of the cells with ionising irradiation, mitomycin C and cyclosporin A. Cells were exposed to ionising radiation???, mitomycin C (0.09µM) or cyclosporin A (20 µM) and ATP levels measured at 24 and 48 hours of culture. The results are shown in Figure 2.4, and the most significant observations were as follows:

♦ At 24 hours there was a significant decrease ($P < 0.05$) in ATP levels from 11 037.3 ± 508.2 r.l.u for untreated cells to 584.3 ± 75.8, 1541 ± 174.2 and 2345.6 ± 417.7 r.l.u for irradiated and mitomycin C- and cyclosporin A-treated cells, respectively.

♦ At 48 hours there is a significant decrease ($P < 0.05$) in ATP levels from 10 071 ± 356.2 r.l.u for untreated cells to 425.7 ± 93.6, 684.3 ± 84.5 and 1901.7 ± 221.5 r.l.u for irradiated and mitomycin C- and cyclosporin A-treated cells, respectively.

These results indicate that pre-treatment of cells with ionising radiation, mitomycin C and cyclosporin A has a significant effect on cell viability.

2.3.4 Effect of exposure of MNL to ionising radiation, mitomycin C and cyclosporin A on MNL viability.

To investigate the effects of exposure of cells to ionising radiation, mitomycin C or cyclosporin A on cell viability, uptake of PI into cells pre-treated with mitomycin C(0.09μM), cyclosporin A(20μM), and ionising radiation was measured by flow cytometry at 24 and 48 hours of culture. The results are shown in Figure 2.5, and the most significant observations were as follows:

♦ After 24 hours post-irradiation, there was a significant decrease in cell viability from 99.1 ± 0.1 % for untreated cells to 55.7 ± 3.8 % for irradiated cells. Similarly, albeit to a lesser magnitude, there was a decrease in cell viability for cyclosporin A- and mitomycin C-treated cells are decreased from 99.1 ± 0.1 % for untreated cells to 73.2 ± 9.2 and 68.3 ± 13.9 % for cyclosporin A and mitomycin C treated cells, respectively.

♦ After 48 hours of culture, there was a significant decrease in cell viability from 97 ± 1.2 % for untreated cells to 39.7 ± 2.9, 67.9 ± 10.6 and 57.8 ± 10.5 % for irradiated and mitomycin C- and cyclosporin A-treated cells, respectively.

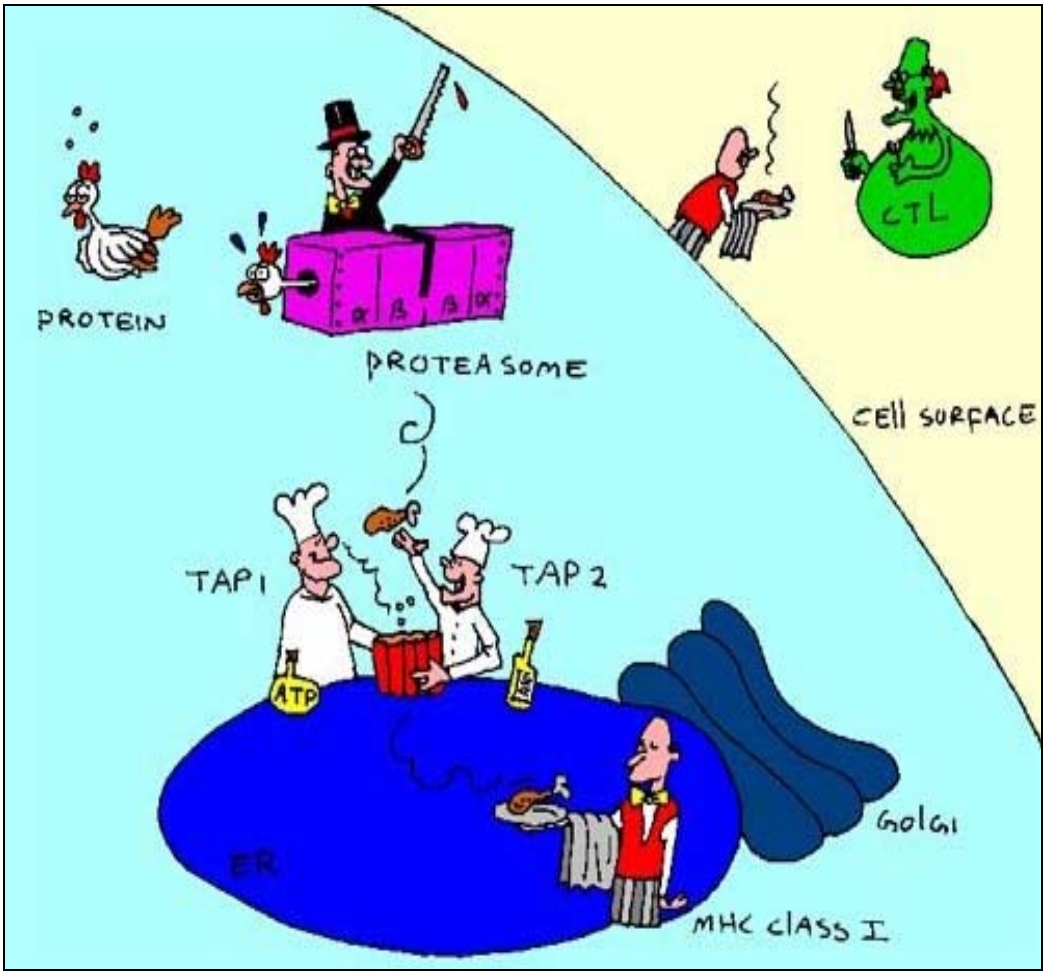
These results indicate that pre-treatment of cells with ionising irradiation, mitomycin C and cyclosporin A has a significant effect on cell viability, with ionising irradiation demonstrating the most profound effect.

2.4 Discussion

The one-way MLC appears to be a straightforward, simple procedure, based upon inactivation of the stimulator cells, or recipient cells, such that they remain viable but unable to divide. This unidirectionality of the MLC allows for the measurement of proliferation of only one of the populations, that of the donors, that can be used to predict GVHD (Malinowski *et al.*, 1992). To predict HVGD, and ultimately graft rejection, the reverse strategy (responder cells from the recipient/stimulator cells from the donor) may be used.

The primary activation signal for the alloreactive immune response reproduced in the MLC is largely dependent on the cell surface expression of HLA class II molecules, particularly HLA-DR molecules (Levis and Robbins, 1970). The stimulator cells are however inactivated by pre-treatment with ionising irradiation or mitomycin C, which also has a significant effect on HLA-DR molecule expression and structure. HLA-DR molecules are greatly reduced and/or undergo structural changes following exposure to these agents. This may lead to no alloreactive immune response or result in its' amplification, demonstrating a weaker or stronger MLC response, regardless of the degree of compatibility between donor and recipient (Malinowski *et al.*, 1992).

Previous studies have also demonstrated that irradiated cells show loss of viability, biochemical changes and damage to subcellular components (Mettler and



Stratikos, Cytotoxic T-lymphocytes "recognize" foreign antigens on target cells and proceed to kill the infected cell, retrieved 21 February 2008, <http--stratikos_googlepages_com-cartoonCell_jpg-cartoonCell-full_jpg.htm>

Chapter 3

Evaluation and modification of the two-way MLC

Chapter 3. Evaluation and modification of the two-way MLC

3.1. Introduction

The complex immune reactions which take place following organ transplantation can not be qualitatively represented by the one-way MLC (Lawler *et al.*, 1975). Due to the presence of "passenger cells" in the graft, the immune reactions taking place will be bilateral and differ in their relative strengths (Borel *et al.*, 1996; Sato *et al.*, 1999). Although "passenger cells" in the transplanted organ will be far outnumbered by host immune cells, the nature of these cells and the cytokines they secrete determines which of the two populations, host or donor, will dominate and therefore drive the immune response. In a study by Sato *et al,* (1999), it was demonstrated that if the smaller of the two populations in an MLC consists of $CD8^+$ T cells, the smaller population will dominate the larger population.

In addition, as shown in earlier studies, and in the previous chapter, exposure of the stimulator cells to cyclosporin A, mitomycin C and ionizing irradiation in the one-way MLC, results in possible structural changes in class II determinants, as well as decreased HLA-DR expression at the cell surface and loss of viability. The structural changes of class II determinants may either lead to no response in the one-way MLC, or an amplification of activation signals in the MLC, making results unreliable.

The laboratory investigations described in this chapter were undertaken with the primary objective of evaluating the two-way MLC and identifying modifications that would lead to its improvement. One such modification is the addition of the T-cell growth factor, interleukin-2 (IL-2), at sub-mitogenic concentrations, to accelerate the reaction and increase sensitivity of the two-way MLC.

3.1.1 IL-2

IL-2 belongs to a family of cytokines, the hematopoietins, which includes other cytokines such as IL-4, IL-7, IL-9 etc (Janeway *et al.*, 2005). The IL-2 molecule is characterised as a variably glycosylated 15 500 Dalton protein which stimulates the proliferation, differentiation and survival of antigen-specific cytotoxic T cells during an immune response (Robb and Smith, 1981). IL-2 is secreted by the activated T cell itself. Binding of the TCR by specific antigen, in the presence of a co-stimulatory signal, drives the T cell to enter into the G1 phase of the cell cycle, while simultaneously inducing synthesis and secretion of IL-2 and the expression of high affinity IL-2 receptors at the cell surface. Binding of IL-2 to its high affinity IL-2 receptor allows the T cell to progress through the rest of the cell cycle and ultimately divide (Janeway *et al.*, 2005).

IL-2 is also important in the immune system's ability to discriminate between "self" and "nonself". IL-2 is involved in the maturation of a subset of T cells called regulatory T cells during T cell ontogeny in the thymus (Sakaguchi *et al.*, 1995; Thornton *et al.*,

2004). The regulatory T cells prevent other T cells from responding to "self-antigens" by preventing their synthesis of IL-2.

IL-2 may also play a role in the regulation of the cytotoxic T cell response. Initial concentrations of IL-2 following T cell activation, induce the differentiation and proliferation of cytotoxic T cells. Higher concentrations of IL-2 produced at a later stage during the immune response, induce the activation and differentiation of suppressor T cells to regulate the cytotoxic T cell response (Ting *et al.*, 1984).
In vitro, IL-2 supports the growth of T cell lines in long-term culture. In a study by Ting *et al*, (1984), concentrations of 0.5 to 1.5 IU/ml IL-2 were able to induce cytotoxic T cells in the MLC, while concentrations of up to 15 IU/ml of IL-2 induced suppressor T cells in the MLC.

3.2 Materials and methods

3.2.1 Lymphocyte proliferation assay (LPA)

This assay measures the ability of lymphocytes to undergo polyclonal/clonal proliferation when stimulated *in vitro* by a foreign molecule, antigen or mitogen. Specific to the laboratory research conducted in this chapter, these experiments were undertaken to determine those concentrations of IL-2, which would enhance T-cell reactivity to specific antigen (PPD), as a preamble to the MLC. Adult human volunteers with known skin test reactivity to PPD were used.

Each 15 ml sterile, non-pyrogenic tube received the following: 1,4 ml of a 1 x 10^6 cells/ml MNL in 1640 RPMI culture medium, 200µl foetal calf serum (10 % final concentration) and 200 µl of the antigen, PPD (Aventis Pasteur, SA) at concentrations of 1, 5 and 10 IU/ml (control tubes did not receive PPD). The tubes were incubated for 24 hours at 37°C in a humidified CO_2 incubator (5% CO_2). Each tube then received IL-2 (Sigma Diagnostics, St Louis, Mo, USA) at concentrations of 10 IU/ml (concentration of PPD determined in preliminary experiments). The final volume of each tube was 2ml. The tubes were then incubated for a further 72 hours at 37°C in a humidified CO_2 incubator (5% CO_2). At the time of addition of ^{3}H-thymidine (0.2 µCi, 20µl/well; Du Pont-NEN, Research Products, Boston, MA, USA), 200 µl from each culture tube was transferred to each of triplicate wells in a 96-well micro-tissue culture plate and incubated for a further 18 hours. The extent of MNL activation and proliferation was determined as previously described, according to the magnitude of uptake of ^{3}H-thymidine into the newly synthesized DNA of the dividing cells as measured by liquid scintillation spectrometry.

To determine the time at which IL-2 should be added, IL-2 was added immediately to the LPAs and 24 hours into the reaction.

3.2.2 Two-way tube MLC

Each 15 ml sterile, non-pyrogenic tube received the following: 0.8 ml of a 1 x 10^6 cells/ml MNL in 1640 RPMI culture medium from each of two donors and 0.2 ml fetal calf serum (10% final). Autologous control systems of each donor received 1.6 ml of a 1 x 10^6 cells/ml MNL in 1640 RPMI culture medium. Control tubes did not receive IL-2. IL-2 was added at concentrations of 5 and 10 IU/ml (0.2 ml/tube), 24 hours into the reaction. The final volume of each tube was 2ml. The tubes were then incubated for a further 72 hours at 37°C in a humidified CO_2 incubator (5% CO_2). At the time of addition of ^{3}H-thymidine (0.2 µCi, 20µl/well), 200 µl from each culture tube was transferred to each of triplicate wells in a 96-well micro-tissue culture plate and incubated for a further 18 hours. The extent of MNL activation and proliferation was determined as previously described, according to the magnitude of uptake of ^{3}H-thymidine into the newly synthesized DNA of the dividing cells as measured by liquid scintillation spectrometry. The data are given as mean counts/min of triplicates ± SEM.

3.2.3 Two-way micro-plate MLC

Each of triplicate cells in a 96-well micro-titre culture plate received the following: 80 µl of a 1 x 10^6 cells/ml MNL in 1640 RPMI culture medium from each donor and 20 µl foetal calf serum (10% final, 20µl/well). Autologous MLC of each donor received 160 µl of a 1 x 10^6 cells/ml MNL in 1640 RPMI culture medium. Control wells did

not receive IL-2. IL-2 was added at concentrations of 5 and 10 IU/ml (20µl/well), 24 hours into the reaction. The final volume of each well was 200µl. The plates were then incubated for a further 72 hrs at 37°C in a humidified CO_2 incubator (5% CO_2). Each well then received 0.2µCi tritiated thymidine (^{3}H-thymidine; 20µl/ml), followed by incubation for a further 18 hours. The extent of MNL activation and proliferation was determined as previously described, according to the magnitude of uptake of ^{3}H-thymidine into the newly synthesized DNA of the dividing cells as measured by liquid scintillation spectrometry. The data are given as mean counts/min of triplicates ± SEM.

3.2.4 Cytokine detection

Experiments were performed to compare the cytokine profiles (both pro-inflammatory and anti-inflammatory) between plate and tube two-way MNLs using suspension bead protein array technology (Bio-Plex™). The Bio-Plex array system utilizes xMAP technology to permit the multiplexing of up to 100 analytes within a single sample. The system uses a liquid suspension of microspopic beads, each internally dyed with different ratios of two fluorophores to assign a unique spectral colour code for each bead, allowing us to discriminate among multiplex assays. Each colour-coded bead is conjugated to a different capture molecule, which is directed to the target analyte. For example, if IL-8 is the target analyte the capture molecule is a monoclonal antibody directed against IL-8. To detect and quantitate the captured analyte, a fluorescently labelled reporter molecule, streptavidin-PE that specifically binds the

analyte is added. In the Bio-Plex array reader, each sandwich immunoassay is excited by two lasers. The red classification laser excites the dyes in the each bead, identifying its spectral address. The green reporter laser excites the reporter molecule associated with each bead, which allows quantitation of each the captured analyte.

Plate and tube two-way MLC were prepared as previously described and cultured for 5 days. Culture supernatants were collected every 24 hours, beginning at time 0 prior to the cytokine assay. Briefly, the assay was performed using 96 well filter plates that were first saturated with 200 µl of assay buffer. The plates were then covered with plastic lids and incubated for 1 hr at room temperature. The assay buffer was removed by using a vacuum manifold apparatus, followed by gentle blotting of the plates on paper. The conjugated beads (50 µl/well) were added, and deposited on the filter by exposing the plate to a vacuum, after which the plate was washed twice with 100 µl of wash buffer. In each designated well, 50 µl of standard, control, or culture supernatant were added in duplicate. The plates were covered with a sealing tape and aluminium foil, agitated for 30 sec on the plate agitator and for 60 min at 300 rpm on the orbital shaker at room temperature to promote bead-cytokine binding. After incubation, the plates were vacuum filtered as before, followed by the addition of the detection biotinylated antibodies (25 µl/well). The plates were again covered with a sealing tape and aluminium foil, agitated for 30 sec and incubated for 30 min at room temperature on the orbital shaker at 300 rpm. The plates were drained and washed three times with 100 µl/well wash buffer. For the detection and

quantification of each captured cytokine, 50 µl of streptavidin-PE (a fluorescently labelled reporter molecule that specifically binds to the analyte), was added to each well. The plates were sealed again, as before, and mixed for 10 min at 300 rpm on the orbital shaker at room temperature, followed by a triplicate wash step, with 100 µl/well wash buffer. After the third wash, assay buffer (125 µl/well) was added into each well, and the plates agitated for 30 sec. The contents of each well were analyzed using Bio-Plex™ plate reader software (version 3.0) and the concentration of each cytokine (pg/ml) calculated from standard curve generated. The following cytokines were assayed: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1, and TNF-α.

3.3 Results

3.3.1 IL-2 and lymphocyte proliferation with PPD as antigen

To investigate the concentrations of IL-2 which *per se* would be non-mitogenic or weakly mitogenic in an antigen-specific cell proliferation, ^{3}H-thymidine uptake into newly synthesized DNA of dividing T cells was measured both in PPD-free systems and following activation of the cells with 10 IU/ml PPD and IL-2 at concentrations of 1, 5 and 10/ml IU. The results are shown in Figure 3.1, and the most significant observations were as follows:

♦ IL-2 at concentrations of 1 and 5 IU/ml did not significantly increase PPD-driven proliferation: the mean count increased from 3077.3 ± 214.6 CPM for 10 IU/ml PPD only, to 3598.0 ± 305.5 CPM and 3660.0 ± 411.2 CPM for 10 IU/ml PPD and 1 and 5 IU IL-2/ml, respectively. Background counts increased from 247.35 ± 31.0 CPM to 1512.15 ± 292.0 CPM and 2286.65 ± 560.0 CPM for 1 and 5 IU IL-2/ml, respectively.

♦ IL-2 at a concentration of 10 IU/ml significantly increased ($P < 0.05$) PPD-driven proliferation: the mean count increased from 3077.3 ± 214.6 CPM to 5448.2 ± 250.3 CPM. However background counts also increased from 247.3 ± 31.0 CPM to 4516.64 ± 996.9 CPM for 10 IU/ml IL-2.

These results indicate that IL-2 *per se* at concentrations of 1 and 5 IU/ml is weakly mitogenic such that the observed increases in ^{3}H-thymidine uptake in the PPD-activated systems are due to this activity and not to increased sensitivity to PPD. At 10 IU/ml, IL-2 is clearly mitogenic.

3.3.2 Timing of addition of IL-2 in the lymphocyte proliferation

To investigate at what point during the time course of a lymphocyte proliferation assay IL-2 should be added in order to ensure proliferation is antigen-driven and not IL-2-driven, 5 and 10 IU IL-2 were added to PPD-activated lymphocyte suspensions at time 0 and 24 hours into the assay.

^{3}H-thymidine uptake into newly synthesized DNA of dividing T cells was measured following activation of the cells by 10 IU/ml PPD and IL-2 at concentrations of 1, 5 and 10 IU/ml. As shown in Figure 3.2, the lymphocyte proliferation systems to which IL-2 was added at time 0 did not differ significantly from the lymphocyte proliferation to which IL-2 was added 24 hours into the assay, for all three concentrations used. However, the responses of the lymphocytes to which IL-2 was added at time 0 were higher than those added 24 hours into the assay: the mean count for PPD-activated cells to which 1 IU/ml IL-2 was added increased from 1382.2 ± 261.5 CPM when added at 24 hours to 1825.7 ± 424.6 CPM when added at time 0; the mean count for PPD-activated cells to which 5 IU/ml IL-2 was added increased from 2252.0 ± 314.9 CPM when added at 24 hours to 3218.8 ± 253.0 CPM when added at time 0; the mean count for PPD-activated cells to which 10 IU/ml IL-2 was added increased from 5695.3 ± 459.6 CPM when added at 24 hours to 6238.1 ± 219.5 CPM when added at time 0.

3.3.3 Effects of addition of IL-2 to the two-way MLC

To investigate the effects of addition of IL-2 to the two-way MLC, incorporation of ^{3}H-thymidine into the newly synthesized DNA of dividing T cells was measured, following addition of IL-2 at concentrations of 5 and 10 IU, 24 hours into the reaction. The results are shown in Figures 3.3, 3.4 and 3.5, and the most significant observations were as follows:

♦ For MLC 1 (Figure 3.3), there was a significant increase ($P < 0.05$) in mean counts from 3044 ± 206.7 CPM for the standard two-way MLC, to 4518 ± 177.5 and 4710.3 ± 3.2 CPM following addition of IL-2 at 5 and 10 IU/ml, respectively. However, comparison of MLC 1 with IL-2 at concentrations of 5 and 10 IU/ml, with the autologous control systems of Donor 1 and Donor 2 with IL-2 at concentrations of 5 and 10 IU/ml, shows a decrease in ^{3}H-thymidine uptake for MLC 1: at 5 IU/ml IL-2 there is a significant decrease ($P < 0.05$) in mean counts from 4654.7 ± 64.8 CPM for Donor 1 and 5319.7 ± 71.9 CPM for Donor 2 in the autologous control systems to 4518.3 ± 177.5 CPM in their corresponding MLC; at 10 IU/ml IL-2 there is a significant decrease ($P < 0.05$) in mean counts from 6147.0 ± 255.7 CPM for Donor 1 to 4710.3 ± 84.0 CPM in MLC 1. The increase in mean count of MLC 1 when compared to the autologous control system for Donor 2 is not considered significant.

♦ For MLC 2 (Figure 3.4), there was a significant increase ($P < 0.05$) in mean counts from 763 ± 3.5 CPM for the standard two-way MLC to 2738.7 ± 101.6 and 3008.0 ± 211.8 CPM following addition of IL-2 at 5 and 10 IU/ml, respectively. However, comparison of MLC 2 with IL-2 at concentrations of 5 and 10 IU/ml, with the autologous control systems of Donor 1 and Donor 3 with IL-2 at concentrations of 5 and 10 IU/ml, shows a decrease in ^{3}H-thymidine uptake for MLC 2: at 5 IU/ml IL-2 there is a significant decrease ($P < 0.05$) in mean counts from 4654.7 ± 64.8 CPM for Donor 1 in the autologous control systems to 2738.7 ± 101.6 CPM in MLC 2; at 10 IU/ml IL-2 there is a significant decrease ($P < 0.05$) in mean counts from

6147.0 ± 85.3 CPM for Donor 1 in the autologous control systems to 3008 ± 211.7 CPM in MLC 2. The increase in mean count of MLC 2 when compared to the autologous control system for Donor 3, at 5 and 10 IU/ml IL-2, is not considered significant.

♦ For MLC 3 (Figure 3.5), there was a significant increase ($P < 0.05$) in mean counts from 2284.0 ± 157.9 CPM for the standard two-way MLC to 3177.0 ± 221.50 CPM following addition of IL-2 at 10 IU/ml. Addition of IL-2 at 5 IU/ml did not significantly increase counts for MLC 3. However, comparison of MLC 3 with IL-2 at concentrations of 5 and 10 IU/ml, with the autologous control systems of Donor 2 and Donor 3 with IL-2 at concentrations of 5 and 10 IU/ml, shows a decrease in ^{3}H-thymidine uptake for MLC 3: at 5 IU/ml IL-2 there is a significant decrease ($P < 0.05$) in mean counts from 5319.7 ± 71.9 CPM for Donor 2 in the autologous control systems to 2619.7 ± 104.4 CPM in MLC 3; at 10 IU/ml IL-2 there is a significant decrease ($P < 0.05$) in mean counts from 4839 ± 84.9 CPM for Donor 2 in the autologous control systems to 3177 ± 221.5 CPM in MLC 3 The increase in mean count of MLC 3 when compared to the autologous control system for Donor 3, at 5 and 10 IU/ml IL-2, is not considered significant.

These results indicate that at concentrations of 5 and 10 IU/ml IL-2, which in the earlier experiments with the PPD-activated LPA's was shown to be weakly mitogenic, immune responsiveness in the two-way MLC is decreased as indicated by a decrease

in proliferation when compared to the autologous control systems of the participating donors.

3.3.4. Plate vs. Tube MLC

To investigate whether the performance of the MLC in microplates or test tubes affects responsiveness, three MLC reactions were performed using both plates and tubes in parallel. In the tube MLC, the cells were transferred to plates in the last 18 hours of culture with the addition of ^{3}H-thymidine. The results are shown in Figure 3.6, and the most significant observations were as follows:

♦ For MLC 1, there was a significant increase ($P < 0.05$) in mean counts from 230.3 ± 23.4 CPM for the plate MLC to 3765.7 ± 123.5 CPM for the tube MLC.

♦ For MLC 2, there was a significant increase ($P < 0.05$) in mean counts from 232.3 ± 19.9 CPM for the plate MLC to 4092.3 ± 183.9 CPM for the tube MLC.

♦ For MLC 3, there was a significant increase ($P < 0.05$) in mean counts from 234.7 ± 4.2 CPM for the plate MLC to 3726 ± 105.4 CPM for the tube MLC.

3.3.5 Cytokine analysis of plate and tube MLC

The cytokine profiles of MLC 1 were analysed and the results are shown in Figures 3.7-3.12, pages 69-74. The most significant observations are indicated in the graphs.

3.4. Discussion

Based on previous studies by Ting *et al*, (1984), IL-2 was added to the two-way MLC at weakly mitogenic concentrations as a growth factor to improve the sensitivity of the two-way MLC and accelerate its time-course, thereby expediting acquisition of results. Preliminary lymphocyte proliferation assays using PPD as the antigen, revealed that at a concentration of 5 IU/ml, IL-2 is weakly mitogenic, increasing background prolferation with no additional, incremental reactivity in the presence of PPD. At a concentration of 10 IU/ml, proliferation becomes IL-2 driven. Addition of IL-2 at a concentration of 1 IU/ml, previously found to be optimal for the induction of cytotoxic T cells in the MLC in the study by Ting *et al*, (1984), was found to be equivalent in effect to 5 IU/ml. Although there was not a significant difference in the lymphocyte proliferation assays pulsed immediately with IL-2 as compared to those pulsed 24 hours into the MLC, the proliferation assays pulsed immediately with IL-2 showed an increased response to those pulsed 24 hours later. Thus, it was decided to add IL-2 to the two-way MLC 24 hours into the response, thereby ensuring that the primary signal for activation and proliferation is delivered through the TCR.

Addition of IL-2 at concentrations of 5 and 10 IU/ml to the two-way MLC demonstrated a significant increase in ^{3}H-thymidine uptake when compared to the corresponding two-way MLC without added exogenous IL-2. However when compared to the corresponding autologous control systems with added exogenous

IL-2, at both 5 and 10 IU/ml, there was a significant decrease in ^{3}H-thymidine uptake. In the study by Ting *et al*, it was demonstrated that at higher concentrations of IL-2 (15 IU/ml), suppressor T cells are induced to differentiate. In the same study it was also demonstrated that the number of stimulatory cells in the MLC has an influence on the immune response: the higher the number of stimulatory cells the greater the levels of IL-2 produced, which ultimately leads to the production of suppressor T cells. MLC 1 and MLC 3 in this series of experiments demonstrated a significant increase in ^{3}H-thymidine uptake, indicating adequate production of IL-2 for the stimulation, proliferation and differentiation of cytotoxic T cells. Addition of exogenous IL-2 to these MLC appears to suppress the immune response via the generation of suppressor T cells.

Since the number of stimulatory T cells in an MLC is not known and therefore the level of IL-2 produced can not be predicted, addition of even sub-mitogenic exogenous IL-2 may not result in an accelerated, more sensitive MLC, but rather to a suppressed response.

An MLC is most commonly performed using 96 flat-bottom well culture plates. MLC 1 and MLC 3 in the series of IL-2 experiments, which demonstrated a significant increase in ^{3}H-thymidine uptake, were cultured in 15 ml, non-pyrogenic, sterile tubes. This difference may be due to the following:

- The total number of cells involved in the immune response is greater in the tube MLC than in the plate MLC, although the cell densities are the same.

• The final volume of the MLC cultured in the tubes is 2ml, while the final volume in the plates is 200 µml. Although the concentration of cells (1 x 10^6 cells/ml) is the same, the larger volume of growth medium in the tube MLC may lead to less toxicity generated through the metabolic activity of the cells, than the plate MLC

• Cytokines. The cytokine profile of one of the two-way MLC was analysed. The final effect of a cytokine depends on the following factors:

- the time it is released during the course of an immune response
- the local environment in which it is released and the presence of competing or synergistic cytokines therein
- cell responsiveness to each cytokine depending on cytokine receptor density

Thus the cytokines released during an immune response cannot be interpreted individually, but as a part of interrelated network (Dinarello *et al.*, 1998).

Although the plate MLC demonstrated higher concentrations of pro-inflammatory cytokines IL-2, IFN-γ and TNF-α than the tube MLC, it also showed higher concentrations of the anti-inflammatory cytokines IL-4, IL-10 and IL-13. In fact, the concentrations of IL-4, IL-10 and IL-13 in the tube MLC remained at background levels. In a study by Danzer *et al*, 1996, addition of exogenous IL-10, at even very low levels prevented secretion of IFN-γ and reduced IL-2 release and surface expression of IL-2R (CD25). The higher levels of IL-2 release in the plate MLC may also lead to the generation of suppressor T lymphocytes with a subsequent suppression of the

immune response (Ting *et al.*, 1984). The timing of IL-6 release demonstrated a different pattern for the plate MLC as compared to the tube MLC: IL-6 levels initially decrease in the plate MLC till day 3 then begin to rise again, whilst in tube MLC the IL-6 levels peak on day 2. However, these results were derived from a single experiment and clearly require confirmation.

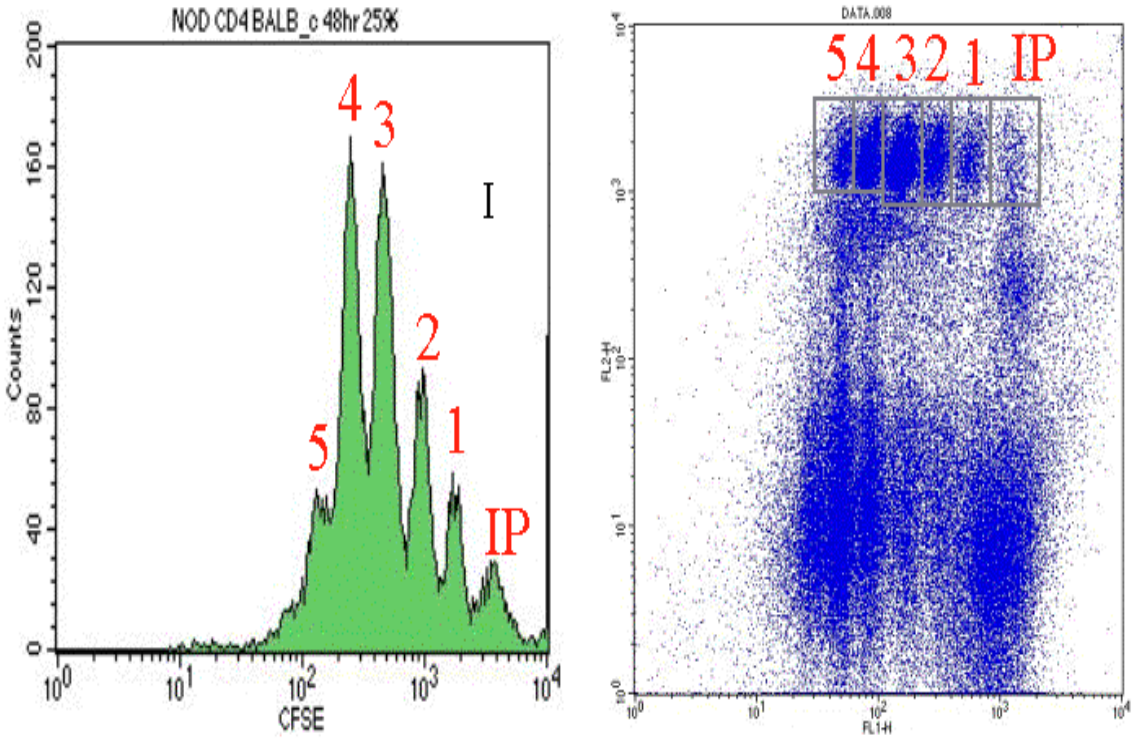
In summary these results demonstrate that: i) IL-2 is of little or no value in enhancing the performance of the two-way MLC, and in fact may have the opposite of the intended effect; and ii) the higher levels of Th2 cytokines produced in the MLC cultured in plates leads to the development of a Th2 response, with little or no cell proliferation taking place, whilst in the tube MLC the absence of these cytokines allows for the development of a Th1 response and subsequent cell proliferation.

Moseley, 1985). $CD8^+$ T cells are twice as radiosensitive as $CD4^+$ T cells, while radiosensitivity of B cells changes throughout B cell ontogeny, with mature B cells being the most radioresistant (Drewinko *et al.*, 1972).

Preliminary experiments in this chapter demonstrated that cyclosporin A can be used as an alternative to mitomycin C or ionising irradiation, for the inactivation of the stimulator cells in the MLC: incubation of MNL with 20µM cyclosporin A for 48 hours revealed a complete absence of PHA-activated proliferative responses.

However, when comparing the viabilities and levels of HLA-DR expression on cells inactivated by pre-treatment with ionising irradiation, mitomycin C or cyclosporin A, cyclosporin A was shown to have a negative effect on cell viability and HLA-DR levels equivalent to that of mitomycin C and ionising radiation. Cyclosporin A treated cells showed a significant reduction in cell surface HLA-DR expression, ATP levels and loss of viability. In fact, cyclosporin A-treated cells showed the greatest reduction in HLA-DR levels when compared to mitomycin C and ionising irradiation. Previous studies involving renal epithelial cell lines demonstrated a permanent and significant decrease in ATP levels following a 24 hour incubation with cyclosporin A (Massicot *et al.*, 1997). Cyclosporin A has also been shown to inhibit HLA-DR expression on monocytes (Younish-Rouach *et al.*, 1991).

In conclusion, the laboratory research presented in this chapter indicates that cyclosporin A is as cytotoxic to the stimulator cells as mitomycin C and ionising radiation, resulting in a similar loss of viability and reduction and/or structural change in HLA-DR molecules. The allogeneic response observed under these conditions may not be a true reflection of the actual compatibility between donor and recipient, making these results unreliable and unpredictable, and clearly demonstrates that none of these treatments represent an ideal strategy for the inactivation of stimulator cells in the one-way MLC.



Adams, D, 2005, CFSE-The Cell Division Marker, retrieved 21 February 2008, <www_med_umich_edu-flowcytometry-training-lessons-lesson7-images-cfse_gif>

Chapter 4
CFSE-based MLC

Chapter 4. CFSE-based MLC

4.1 Introduction

^{3}H-thymidine uptake is the standard method for measuring proliferation in the MLC. However, due to increasing costs involved in the disposal of radioactive isotopes, in addition to the hazards associated in working with radioactivity, alternative non-radioactive methods have been investigated (Chen *et al.*, 2003). These include the use of fluorochromes such as BrdU, for measuring cell cycling, proliferation and tracking migration and position *in vivo* (Weston and Parrish., 1991). Of these, CFSE stands out as the most favourable alternative for many reasons, the most important of which is that it has been shown to correlate well with ^{3}H-thymidine uptake (Fulcher and Wong *et al.*, 1999; Popma *et al.*, 2000).

CFSE-loaded proliferating cells can also be counterstained with other fluorochrome-labelled antibodies to determine viability, the type of cell involved and expression of activation markers. The two-way CFSE MLC analysed in this chapter was counterstained with CD25 (IL-2Rα). CD25 is constitutively expressed at low levels on the surface of resting cells. CD25 expression significantly increases on the surface of activated T and B cells, monocytes and macrophages (Janeway *et al.*, 2005).

4.1.1 IL-2R

IL-2R, is the receptor for the cytokine IL-2. It is composed of three protein chains, α, β and γ. The α and β chains are constitutively expressed on the surface of the cell to form the low affinity IL-2R. Following TCR ligation, low levels of IL-2 are released, which bind to the αβ heterodimer, resulting in a conformational change in the β chain, which allows it to bind the γ chain. Simultaneously, the γ chain is recruited to the cell surface to form high affinity IL-2R (Liparoto and Ciardelli, 1999; Rickert *et al.*, 2004; Janeway *et al.*, 2005). Signal transduction occurs through the β and γ chains; the β chain initiates intracellular signalling through the JAK-STAT pathway ([a b] Morriggl *et al.*, 1999), while the γ chain initiates signalling through the MAP kinase and PI3K pathways (Zmuidzinas *et al.*, 1991). Intracellular signalling *via* these pathways leads to two biochemical events which ultimately lead to progression of the cell through the G1 restriction point of the cell cycle, DNA synthesis and mitosis: activation of the D cyclins (D2 and D3) ([a b] Morriggl *et al.*, 1999) and degradation of the D cyclin inhibitor (Nourse *et al.*, 1994). However, before a cell makes the irrevocable commitment of undergoing mitosis, a critical number of IL-2R's have to be triggered, which through previous studies has been shown to be ~ 30, 000. The mean number of high affinity IL-2R's on activated T cells is ~1000, which means new receptors are synthesized and expressed on the cell surface throughout T cell activation before mitosis occurs (Fazekas *et al.*, 2004). Thus an increase in IL-2R expression can be used as an indicator of cell activation and proliferation.

The primary objective of the research described in this chapter was to investigate an alternative method *viz* the measurement of T cell proliferation during an MLC by labelling the responder cells with CFSE and counterstaining with CD25. CFSE has been adopted for use in both human and non-human one-way MLC (Popma *et al.*, 2000; Chen *et al.*, 2003). To my knowledge, the use of CFSE in a two-way MLC has not previously been reported.

4.2 Materials and methods

4.2.1 CFSE labelling of responder cells

For staining of responder cells with CFSE (Molecular Probes, Eugene, OR,USA), MNL at 1 x 10^6 cells/ml in 1640 RPMI culture medium were incubated with 0.5 µM CFSE (concentration predetermined in preliminary experiments) at 37°C for 7 min. Staining was terminated by washing with cold culture medium containing 20 % FCS. Pellets were resuspended and MNL washed twice more with cold culture medium. CFSE-labelled cells were resuspended and adjusted to a concentration of 1 x 10^6 cells/ml in 1640 RPMI culture medium.

4.2.2 CFSE-based two-way MLC

Each 15 ml sterile, non-pyrogenic tube received the following: 900µl of a 1 x 10^6 cells/ml CFSE-labelled MNL (which would represent the responder cells in the conventional one-way MLC) in 1640 RPMI culture medium, 900µl of a 1 x 10^6 cells/ml unlabeled MNL (which would represent the stimulator cells in the conventional one-way MLC) and 200µl foetal calf serum (10% final). Autologous control systems of each donor received 1.8 ml of a 1 x 10^6 cells/ml CFSE-labelled MNL in 1640 RPMI culture medium. The tubes were then incubated for a further 5 days at 37°C in a humidified CO_2 incubator (5% CO_2).

4.2.3 Flow Cytometric Analysis

MLC were analysed on days 1, 2, 4 and 5 of culture on the Beckman Coulter EPICS XL MCL flow cytometer fitted with a 2-watt argon laser, using a two-colour protocol.

The cells were counterstained with anti-CD25 PE (Beckman Coulter, Miami, FL, USA): 200µl of cells were added to 10µl of monoclonal antibody and incubated for 10 min at room temperature in the dark. For accurate counting, 200 µl of Flow-Count™ Fluorospheres (Beckman Coulter, Miami, FL, USA), were added. Samples were then ready to be analysed:

♦ The lymphocyte and blast populations were identified and gated using a FS vs. SS scattergram (Region A).

♦ Fluorescence data for CFSE and CD25 was collected from the gated region. The collected fluorescence measurements were displayed on a dual parameter plot of CFSE vs. CD25 PE. Cells positive for CD25 and decreasing CFSE fluorescence intensity were identified and gated (Region B).

♦ Region E. Flow-Count™ Fluorospheres.

The percentage positive CD25 cells and counts of accumulated daughter cells were derived from the Region B

4.3 Results

4.3.1 CFSE based two-way MLC

There was a significant increase in proliferating responder T cells ($P < 0.05$) in the two-way CFSE MLC following a 5 day culture period. The results, shown in Figure 4.1, are of 3 experiments (samples were analysed in triplicate for all experiments) are as follows:

1. For MLC 1 (Donor A + Donor B), there was an increase in the mean count of proliferating responder T cells (Donor A) from 4 ± 1.2 cells/µl to 57 ± 4.5 cells/µl.

2. For MLC 2 (Donor A + Donor C), there was an increase in the mean count of proliferating responder T cells (Donor C) from 4 ± 1.5 cells/µl to 30 ± 3.5 cells/µl.
3. For MLC 3 (Donor B + Donor C), there was an increase in the mean count of proliferating responder T cells (Donor B) from 4 ± 1.4 cells/µl to 52 ± 4.3 cells/µl.

4.3.2 CD25 expression

There was a significant increase in CD25 expression on proliferating responder T cells ($P < 0.05$) for the two-way CFSE MLC following a 5 day culture period. The results, shown in Figure 4.2, are of 3 experiments (samples were analysed in triplicate for all experiments) are as follows:

1. For MLC 1 (Donor A + Donor B), there was an increase in the mean percentage of CD25 on proliferating responder T cells (Donor A) from 1.2 ± 0.4% to 21 ± 2.6%.
2. For MLC 2 (Donor A + Donor C), there was an increase in the mean percentage of CD25 on proliferating responder T cells (Donor C) from 0.5 ± 0.2% to 14 ± 3.0%.
3. For MLC 3 (Donor B + Donor C), there was an increase in the mean percentage of CD25 on proliferating responder T cells (Donor B) from 1.0 ± 0.5% to 11 ± 2.7%.

Although monocytes also demonstrated a significant increase in CD25 expression (not shown) and lie in the same gated region as the proliferating T cells, CD25 expression of monocytes can be excluded by gating out the $CFSE^{+++}$(bright) events. Monocytes demonstrate a brighter CFSE fluorescent peak than lymphocytes, which lies to the right of the lymphocyte CFSE peak. (See Figure 4.4)

4.3.3 Reciprocal CFSE labelling in the two-way MLC

In this experiment the CFSE-labelled lymphocytes of Donor A were cultured with the unlabelled lymphocytes of Donor B, allowing us to track the response and proliferation of Donor A's lymphocytes. In the corresponding reciprocal experiment the lymphocytes of Donor B were labelled with CFSE and cultured with the unlabelled lymphocytes of Donor A, allowing us to track the response and proliferation of Donor B's lymphocytes. Thus the relative intensities of the responses of the participating donors in the MLC can be tracked individually and compared.

There are significant differences ($P < 0.05$) in respect of both counts of proliferating responder T cells and level of CD25 expression when each of the participating donors in MLC 1 is tracked separately. Following a 5 day culture period, the mean count of accumulated Donor 1 daughter cells in MLC 1 is 57 ± 4.5 cells/µl; the mean count of accumulated Donor 2 daughter cells is 26.5 ± 1.6 cells/µl. The mean percentage of CD25 expression of

accumulated Donor 1 daughter cells is 21.75 ± 2.6 %; the mean percentage of accumulated Donor 2 daughter cells is 10.76 ± 2.2 %. These results are shown in Figure 4.3.

Figure 4.5. Flow cytometric profile of MLC 1, (Donor A (responder) + Donor B) and autologous control systems for Donor A and B, from day 1 to 5. Histogram 1: light scatter profile gated on lymphocytes and blasts (Region A). Histogram 2A (gated on events in Region A): CFSE vs. CD25. MLC 1 shows a progressive reduction of CFSE fluorescence intensity against increasing levels of CD25 expression (Region B) for proliferating responder T cells. The corresponding autologous control systems for Donor A and Donor B show no proliferation. Region E: Flow Count.

Figure 4.6. Flow cytometric profile of MLC 2, (Donor B (responder) + Donor C) and autologous control systems for Donor B and C, from day 1 to 5. Histogram 1: light scatter profile gated on lymphocytes and blasts (Region A). Histogram 2A (gated on events in Region A): CFSE vs. CD25. MLC 2 shows a progressive reduction of CFSE fluorescence intensity against increasing levels of CD25 expression (Region B) for proliferating responder T cells. The corresponding autologous control systems for Donor B and Donor C show no proliferation.

Figure 4.7. Flow cytometric profile of MLC 3, (Donor A (responder) + Donor C) and autologous control systems for Donor A and C, from day 1 to 5. Histogram 1: light scatter profile gated on lymphocytes and blasts (Region A). Histogram 2A (gated on events in Region A): CFSE vs. CD25. MLC 3 shows a progressive reduction of CFSE fluorescence intensity against increasing levels of CD25 expression (Region B) for proliferating responder T cells. The corresponding autologous control systems for Donor A and Donor C show no proliferation.

Figure 4.8. Comparison of the reciprocal responses in the two-way MLC by CFSE-labelling of cells from either Donor A or Donor B (MLC 1). CFSE staining of either Donor A or donor B in the same MLC reveals differences in the relative strength of the bilateral interactions in the two-way MLC.

4.4 Discussion

^{3}H-thymidine uptake has, in the past, been the standard method for measuring antigen-specific cell proliferation and cell proliferation in the MLC, by measuring incorporation of ^{3}H-thymidine into the DNA of dividing cells. However, the ^{3}H-thymidine assay is limited to this measurement only, providing no further information as to the type of cells proliferating, the nature of the immune response, relative number of reactive T-cell precursors, relative number of mitotic events, etc (Chen *et al.*, 2003). In circumstances where there is substantial non T-cell proliferation, such as is seen in lymph node MLC (Harris *et al.*, 1994), the ^{3}H-thymidine uptake assay will reflect proliferation of both T- and non T-cells. A study by Chen *et al.*, 2003, demonstrated a different pattern of proliferation for non T-cells from T-cells, with proliferation peaking in the first couple of days in the MLC for non T-cells.

The use of CFSE and flow cytometry to track and phenotype proliferation enables us to further understand the cellular interactions during an allogeneic response, as well as accurately quantifying the proliferating cells. The proliferating cells in the CFSE-based MLC described in this chapter were counterstained with CD25, the α chain of the IL-2R, an increase of which is a positive indicator that the cells are committed to mitosis. There was a significant increase in surface expression of CD25 on proliferating CFSE-labelled T cells. The increase in CD25 correlated well with the increase in accumulated daughter cells. The most common method of quantifying cell

proliferation in a CFSE MLC is measurement of the accumulation of daughter cells; accurate counts for accumulated daughter cells were obtained by the addition of FLOW COUNT, making acquisition of this measurement less labour intensive, without the need for complicated calculations. There was a significant increase in accumulation of daughter cells in all CFSE-based MLC experiments described in this chapter. In addition, proliferation as indicated by a progressive loss of CFSE fluorescence, and an increase in CD25 expression can be detected by Day 2 during the time course of the CFSE MLC

Previous studies have shown the following limitations of CFSE analysis:

♦ Cells dying in culture will be excluded during analysis, which may constitute a significant proportion of dividing cells, thereby underestimating the actual degree of proliferation. Dying cells would however, also affect the ^{3}H-thymidine-uptake-based MLC since dying cells will not incorporate DNA.

♦ Cells may proliferate to an extent where the intensity of the CFSE signal is reduced to auto-fluorescent levels. This phenomenon is only a limitation where accurate enumeration of proliferation is important A strong immune response in such a case will still be evident (Angulo and Fulcher, 1998).

In summary the advantages of the modified CFSE-based two-way MLC are summarised as follows:

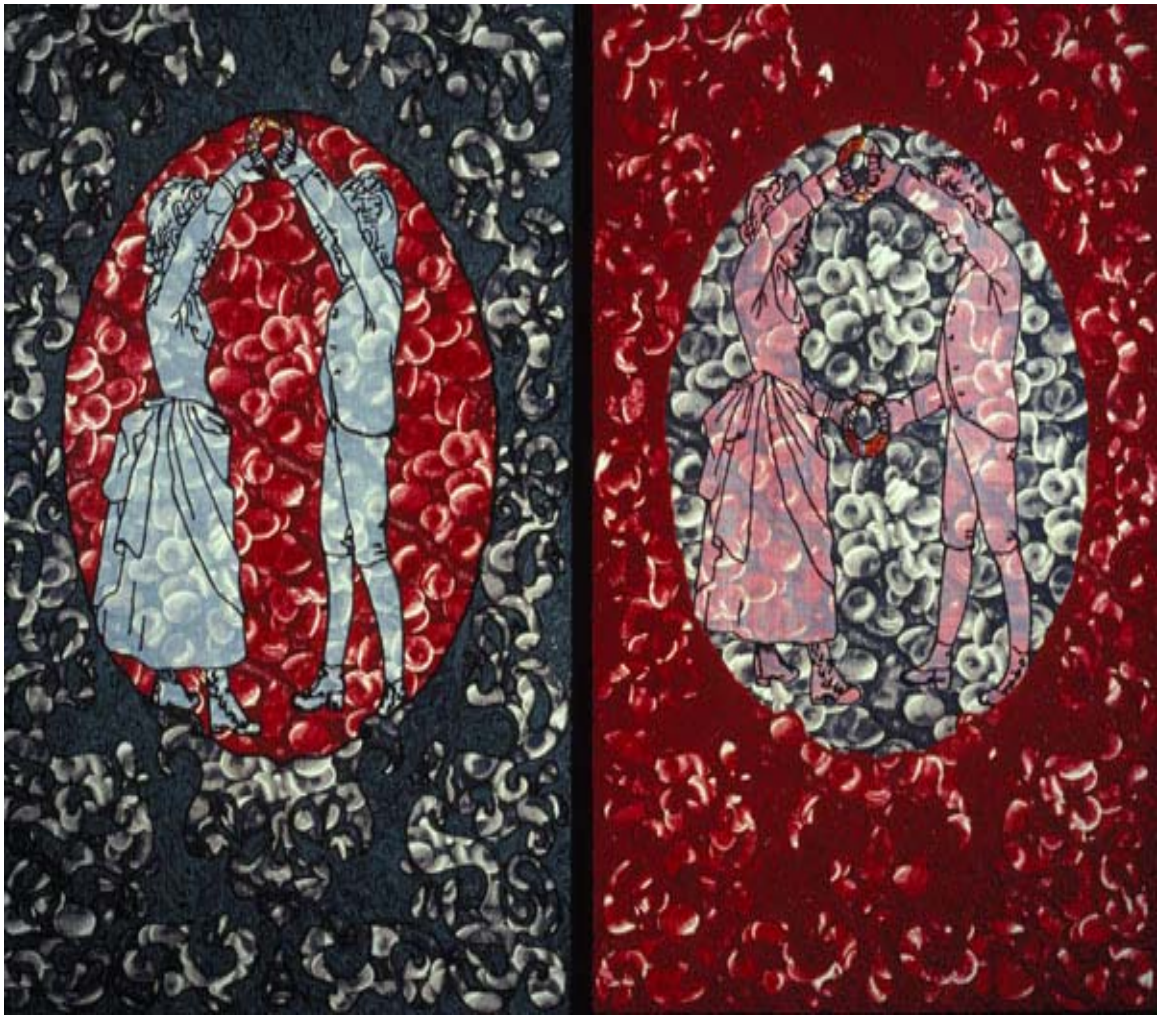
♦ The use of 15ml, non-pyrogenic, sterile tubes for culture of the MLC, which based on the findings described in chapter 3, appears to provide the optimal growth conditions for proliferating cells and release of the necessary cytokines to induce a TH1 response.

♦ Proliferation and an increase in surface expression of CD25 are detected as early as Day 2 of the MLC, thereby expediting results. The ^{3}H-thymidine uptake based MLC requires a 5-7 day culture period, only at the end of which are results are obtained.

♦ One of the major limitations of a ^{3}H-thymidine uptake based two-way MLC is that it is not known whether the observed immune response is graft-versus-host or host-versus-graft, and as such cannot be used to predict GVHD. By staining the donor, i.e., the responder cells, in the two-way MLC with CFSE, only proliferation in this population is tracked. To predict HVGD, and ultimately graft rejection, the recipient cells are stained with CFSE. Thus, by loading only one of the participating donors in the two-way MLC the responder/stimulator interaction, which is observed in the one-way MLC is re-established. The CFSE-loaded cells are the responder cells since their proliferation can be tracked.

♦ Counterstaining of proliferating cells, which provides additional information, such as the phenotype, expression of activation markers and viability of the proliferating population.

In conclusion, the research described in this chapter indicates that the use of CFSE-labelling and flow cytometry to measure proliferation in a two-way MLC, in combination with the flow cytometric measurement of CD25 expression, provides an alternative, reliable and probably superior method to ^{3}H-thymidine uptake.



Maxwell, K, 2005, Mitosis, retrieved 21 February 2008, <www_kmaxwell_net-img-gallery-mitosis_jpg.htm>

Chapter 5
General Conclusion

Chapter 5. General Conclusion

Since the introduction of the one-way MLC in 1966 to assess histocompatibility between two individuals, very little has changed in its methodology, despite evidence that suggests procedures used for inactivation of the stimulator cells may significantly alter the outcome (Malinowski *et al.*, 1992). One of the primary objectives of the research undertaken in the current study was to evaluate cyclosporin A as an inactivator of the stimulator cells in the one-way MLC. Preliminary results indicated that cyclosporin A is comparable to mitomycin C and radiation in its inactivation of the stimulator cells. However it is also comparable to mitomycin C and radiation with respect to its deleterious effect on HLA-DR expression and cell viability. Since the primary activation signal for the alloreactive immune response depends on cell surface expression of HLA class II molecules, any procedure affecting HLA class II molecules on the cell surface, will also affect the outcome of the alloreactive immune response. Cyclosporin A, mitomycin C and radiation result in a significant reduction in and/or structural changes to HLA-DR molecules. Structural changes in HLA class II molecules common to both individuals prior to inactivation may amplify the observed alloreactive response regardless of the true degree of histocompatibility between donor and recipient, while a reduction of HLA class II molecules on the surface of the stimulator cells may result in a weakened allreactive immune response (Mann *et al.*, 1980; Malinowski *et al.*, 1992). Thus, it can be concluded that the results obtained from a one-way MLC in which one of the three strategies evaluated for

inactivation of the stimulator cells is used, may not be a true reflection of the true histocompatibilty between donor and recipient and are therefore unreliable.

Based on the conclusions reached above with respect to the procedures used to inactivate the stimulator cells in the one way MLC and the knowledge that immune reactions following organ tranplantation are bilateral due to the presence of immunocompetent "passenger cells" in the graft (Borel *et al.*, 1996, Sato *et al.*, 1999), subsequent efforts focused on evaluating and possibly improving the two-way MLC. One of the secondary objectives of the research undertaken was to investigate the potential use of IL-2 as a growth factor to improve the sensitivity and accelerate the time-course of the MLC. Preliminary results demonstrated that at concentrations of 5 IU/ml, IL-2 is weakly mitogenic, moderately increasing the magnitude of the response of PPD stimulated LPAs without driving the proliferation itself (ie. the difference in responses between systems with and without IL-2 are simply due to the increase in background counts). However when IL-2 was added to a series of two-way MLC at that concentration, some demonstrated a reduction in their level of proliferation. A previous study by Ting *et al*, (1984), demonstrated that at higher concentrations of IL-2 (15 IU/ml), suppressor T cells are induced to differentiate, a process largely dependent on the number of stimulatory cells in the MLC: the higher the numbers of stimulatory cells the greater the levels of IL-2 production and ultimately production of suppressor T cells. Thus, addition of exogenous IL-2 to an MLC in which high levels of IL-2 are already being produced as part of the alloreactive immune response may result in the production of suppressor T cells, which will inhibit

proliferation. Since the number of stimulatory cells in an MLC is not known, it can be concluded that the use of IL-2 to improve the sensitivity of the MLC and accelerate its time-course, may indeed have the opposite to the intended effect.

An interesting observation from the series of IL-2 experiments was that MLCs cultured 15 ml, non-pyrogenic, sterile tubes demonstrated considerably higher sensitivity than the corresponding 96-well flat-bottom culture plates. Cytokine analysis performed for one of these two-way MLC demonstrated that the microenvironment in the tube MLC, may be more conducive to the development of a Th1 response and subsequent proliferation. While the reasons for these differences have not been established, it is possible that the graeter numbers of cells in the tube system relative to the microplate system (as opposed to the cell densities/ concentrations, which are equivalent in both systems) may influence cell behaviour, favouring a Th1 reponse.

Another important objective was to establish a flow cytometric-based assay using CFSE to track proliferation. CFSE has been shown to correlate well with ^{3}H-thymidine uptake in the one-way MLC (Fulcher and Wong *et al.*, 1999; Popma *et al.*, 2000). The CFSE-based two-way MLCs demonstrated a significant increase in accumulation of daughter cells, with proliferation detected as early as Day 2 during the time-course of the MLC. CFSE-based proliferation can also be combined with counter-staining with monoclonal antibodies to phenotype the proliferating population. The CFSE-labelled cells were counterstained with

CD25 (IL-2R), the increase in which correlated with the increase in the number of daughter cells.

Clearly additional studies will be required to confirm and reinforce the findings of the current study. Future research will focus on the following: i) the comparative cytokine profiles in a larger series of two-way MLCs performed using cells cultured in either 15ml tissue culture tubes or 96 well micro-tissue culture plates as described in Chapter 3; ii) an extended comparison of the CFSE-/CD25-based flow cytometric two-way MLC with the conventional radiometeric (^{3}H-thymidine uptake) two-way MLC; and iii) evaluation of the two-way CFSE-/CD25-based MLC flow cytometric procedure in the setting of clinical organ transplantation by establishing compatibility indices (maximum number of daughter cells/CD25 expression which equates with an acceptable match).

In conclusion, a two-way MLC, performed in 15 ml, non-pyrogenic, sterile tubes may provide the optimum cytokine microenvironment for a Th1 alloreactive immune response, while tracking of proliferation/activation flow-cytometrically using CFSE-labelling and immunophenotyping with anti-CD25 of the responder cells, provides a more accurate and maybe even superior alternative to the standard one-way radiometric MLC. In addition, by reciprocal loading of cells with CFSE, the two-way
MLC can be used to predict either graft rejection or GVHD. This method is summarised in Figure 5.1.

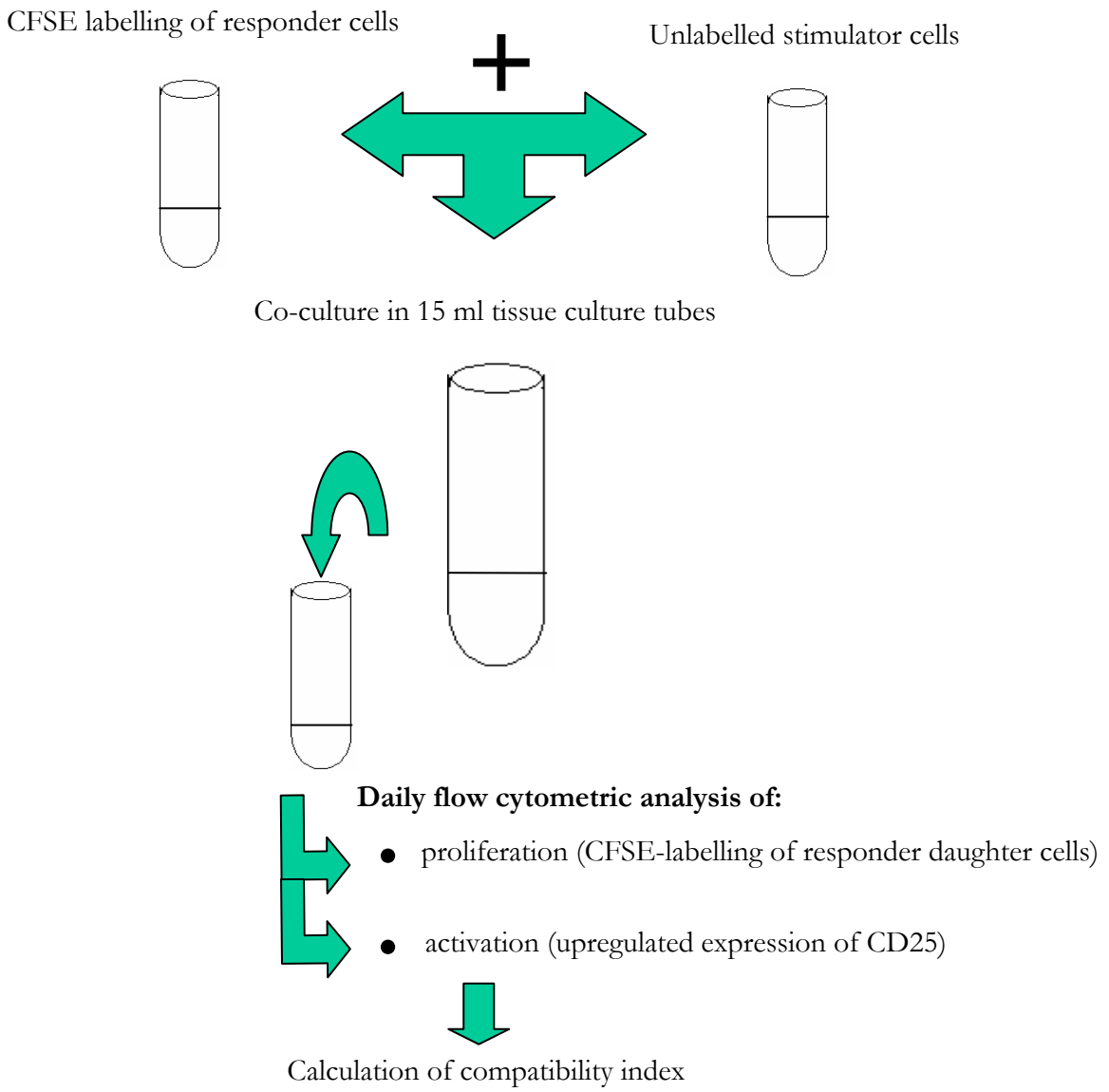
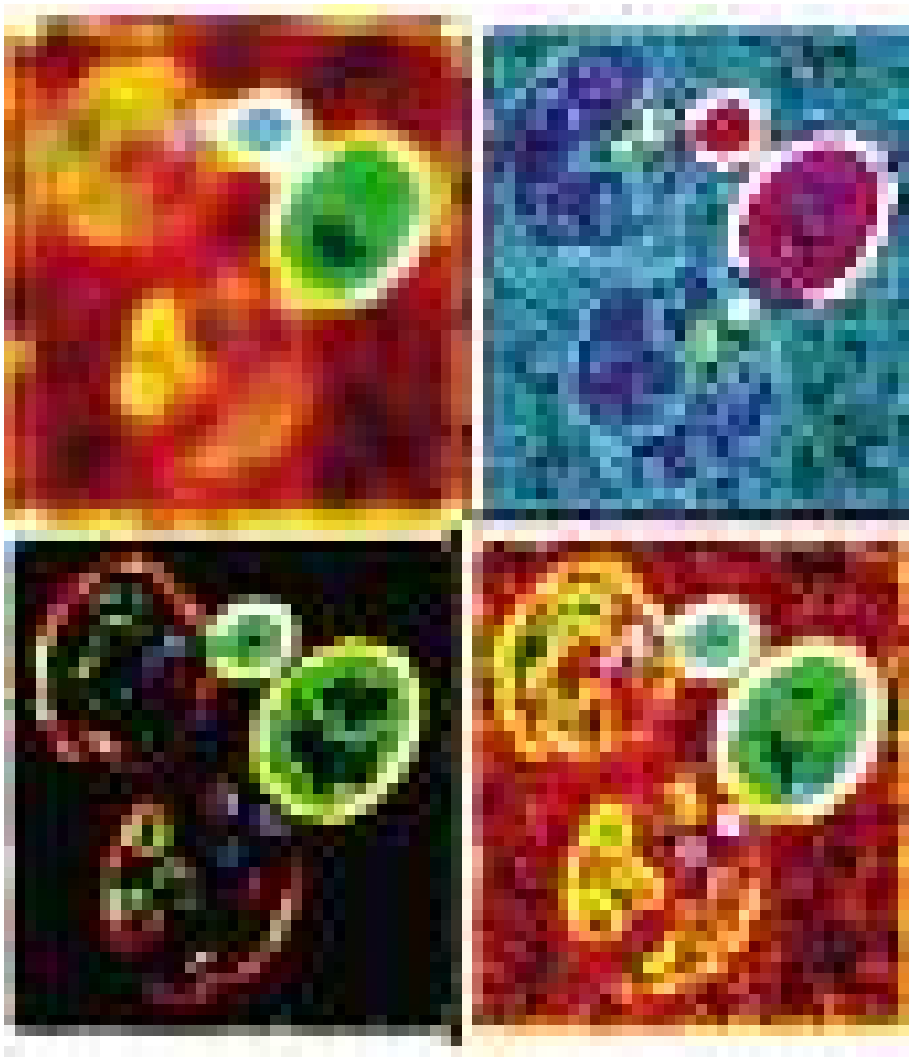


Figure 5.1 CFSE method



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Chapter 6
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