Characterization of the Immune Response to Alloantigens: An *In Vitro* Model System for Cognate Regulation of CD4 T cell Differentiation by CD8 T cells

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By

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ABSTRACT

It is accepted that CD4 T cells are required for the activation of naïve CD8 T cells. In this study, we have investigated the "reverse reaction" that of the effect of CD8 T cells have on the differentiation of CD4 T cells while present at the initiation of the immune response. Our experimental system consists of One Way Mixed Lymphocyte Reaction, MLR, where parental spleen cells homozygous at the Major Histocompatibility Complex, MHC, are activated by irradiated allogeneic spleen cells differing at the MHC loci. Differentiation of CD4 and CD8 T cells was assessed by their expression of distinct signature cytokines, IFN-γ and IL-4 by employing the single T cell ELISPOT assay. CD4 and CD8 T cell sorting and identification was achieved by Magnetically Activating Cell Sorting technology, MACs, and Flow-Cytometry, respectively. This methodology allowed us to explore the mechanism by which CD8 T cells regulate the differentiation of naïve CD4 T cells towards either a Th1(IFN-γ) or a Th2(IL-4) phenotype.

We have found that distinct CD4:CD8 T cell ratios of the spleens of different strains of mice correlate with a Th1(IFN-γ), a Th2(IL-4) or a mixed Th1(IFN-γ)/Th2(IL-4) type immunity when these cells are stimulated with spleen cells from mice differing at the MHC loci. In contrast, similar cultures depleted of CD8 T cells, invariably generate Th2(IL-4), CD4 T cells in all mouse strains tested. Both, unprimed and effector CD8 T cells suppress the Th2(IL-4) development in an antigen specific manner. They do so by means of an allo-antigen dependent secreted soluble factor. This factor is IFN-γ. Finally, the employment of the ELISPOT assay for enumerating and characterizing effector CD4 and CD8 T cells allowed us to verify, with contemporary methodology, the extraordinary high frequency of alloreactive T cells, a phenomenon described by Simonsen in 1957.

Thus, in these studies we have revisited the mechanism by which CD8 T cells were known to "suppress" the antibody response. Our findings indicate that CD8 T cells inhibit in a cognate manner the CD4 Th2 (IL-4) differentiation, the subset required for antibody development. Further on, by extrapolation our data may explain the well-known *in vivo* phenomena in which distinct naturally occurring CD4:CD8 T cell ratios from two different parental mouse strains, promote either Th1 immunity and graft rejection, or a Th2-type

response ineffective at clearing the graft, upon allo-transplantation of parental spleen cells into F1 mice. Consequently, a persistent Th2 response results in time in antibody-mediated generalised autoimmunity resembling Systemic Lupus Erythematosus.

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LIST OF ABBREVIATIONS

AFC	Antibody Forming Cells
aGVHD	Acute Graft Versus Host Disease
ALS	Anti-Lymphocytic Serum
ANOVA	Analysis of Variance
APCs	Antigen Presenting Cells
cGVHD	Chronic Graft Versus Host Disease
BCR	B Cell Receptor
BMT	Bone Marrow Transplants
^{51}Cr	Chromium-51
CTL	Cytotoxic T Lymphocyte
DAMPs	Danger Associated Molecular Patterns
DCs	Dendritic Cells
DRBC	Donkey Red Blood Cells
ELISPOT	Enzyme-linked Immunospot Assay
FACS	Fluorescence-Activated Cell Sorting
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GVHD	Graft Versus Host Disease
GVHR	Graft Versus Host Reaction
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IL	Interleukin
JAKs	Janus Kinases
LASU	Laboratory and Animal Services Unit
LCMV	Lymphocytic Choriomeningitis Virus

L major	Leishmania major
mAB	Monoclonal Antibody
MACS	Magnetic-Activated Cell Sorting
MHC	Major Histocompatibility Complex
MLC	Mixed Lymphocyte Culture
MLR	Mixed Lymphocyte Reaction
NO	Nitric Oxide
PBS	Phosphate-Buffered Saline
PBS-T	Phosphate-Buffered Saline with Tween 20
PE	Phycoerythrin
PFC	Plaque Forming Colonies
PHA	Phytohemagglutinin
r	Recombinant
TCR	T Cell Receptor
TNF	Tumour Necrosis Factor
RPMI	Roswell Park Memorial Institute
SCID	Severe Combined Immunodeficiency
SD	Standard Deviation
SLE	Systemic Lupus Erythematosus
SRBC	Sheep Red Blood Cells
ssDNA	Single Stranded Deoxyribose Nucleic Acid
STATs	Signal Transducer and Activator of Transcription
Th	T helper
TCR	T cell receptor
WBI	Whole Body Irradiation

CHAPTER 1

INTRODUCTION

1.1 The foundations of Immunology are anchored in the early research of Allogeneic Tissue Transplantation

At the beginning of the twentieth century, transplantation surgeons and tumour biologists working independently of each other began to lay the foundations of transplantation immunology and contributed significantly to immunology. Tumour biologists were attempting to understand the pathophysiology of tumours and thus transplanted allogeneic tumours into different hosts. E.E. Tyzzer and C.C. Little, two American geneticists, reported the reasons for the rejection of the transplanted tumours in 1916. They observed that tumours obtained from the Japanese waltzing mice, an inbred mouse strain, were accepted by all mice from that strain but regressed in mice from an unrelated strain. This observation prompted Tyzzer and Little to explore the role that genetic differences played in the regression of the tumour. Extensive research in this field eventually led to one of the most significant contributions of transplantation immunology to basic immunology: the discovery of the histocompatibility antigens, which were initially referred to as the transplantation antigens. Originally the major histocompatibility complex (MHC) locus was described in detail in mice as the H-2 locus (Gorer et al., 1948). Shortly after, a similar locus was described in humans as the human leukocyte antigens (HLA). In 1980, the Nobel Prize in Physiology or Medicine was given to Jean Dausset, George Snell and Baruj Benacerraf for their contributions to the discovery of the major histocompatibility system (Raju, 1999).

Knowledge of the existence of the MHC led to the discovery of major phenomena central to immunity: T cell recognition of MHC; the realisation of the existence of different T cell subsets, CD4 and CD8 T cells, recognising distinct MHC antigens; the role of class II MHC antigens in the generation of the antibody response originally described as immune response (Ir) genes; distinct pathways of antigen processing and presentation by either class I and class II MHC molecules and finally to the realisation that immune responses to foreign antigens are self MHC restricted.

The goal of the transplantation surgeons was to successfully transplant solid organs such as the kidney into genetically different recipients. During these early years of attempts at transplantation, an explanation as to why autografts were accepted, and allografts were rejected was a mystery to transplantation surgeons and tumour biologists alike. Peter Medawar, a zoologist at the time, was made aware of a British pilot who had crashed an aeroplane near Medawar's home during the early part of World War II (reviewed in Brent, 1997). The extensive burns of the pilot awoke in Medawar an appreciation for the potential lifesaving effect a successful skin allograft could have on the survival of a burn victim. This is particularly pertinent, as some burn victims may not necessarily have sufficient healthy skin available to undertake an autologous skin graft. Thus, began his interest in trying to understand the mechanisms behind the rejection of a skin allograft. Subsequently, his work on skin allografts in rabbits provided evidence of the immunologic nature of the rejection of allografts. Continued research into skin allografts by Medawar and his student Mitchison in 1953, demonstrated that immunity to tumour and skin allografts were transferrable. The idea that immunity could be transferred increased the knowledge of the cellular arm of the immune system and the critical role played by lymphocytes in immunity.

The concept of immunological tolerance made its appearance through the observations of R.D. Owen in 1945. He demonstrated that cattle dizygotic twins possessed both their own red blood cells and that of their twin. The cattle shared the same placenta allowing for the exchange of blood resulting in cellular chimerism. As a result, the cattle twins remained tolerant of each other's cells throughout their lives. A chance meeting between Medawar and H. P Donald, who was Director of the Agriculture Research Council's Animal Breeding and Genetics Research Organisation in Edinburgh, expanded of the concept of immunological tolerance. Donald had hoped to find a means to distinguish between monozygotic and dizygotic twins in cattle. Collaboration between Medawar and Donald led to a series of experimental skin grafts in cattle. Medawar hypothesised that if the cattle accepted the skin grafts from their twins they would be monozygotic while those that rejected the skin grafts from their twins would be dizygotic. The dizygotic twins however, were shown to accept each other's skin grafts initially despite their genetic differences but eventually rejected them over time. In contrast, the dizygotic twins rejected skin grafts

rapidly from parents and from siblings from a separate birth. Thus, the earlier findings of Owen (1945) and the findings of Medawar contributed to the recognition of the concept of immunologic tolerance.

Immunological reactivity of cells was found under a variety of experimental conditions. One such experiment was the inoculation of allogeneic lymphoid cells onto the chorioallantoic membranes of chick embryos. The unfortunate outcome of these experiments was the development of a graft versus host disease (GVHD), which occurs when the host cells inoculated into an immunocompromised recipient, mounts an immune response against the cells of the recipient. Burnet observed in 1959 that during a graft versus host reaction (GVHR) the proliferating cells tended to group together. This observation initiated his clonal selection theory, which has stood the test of time. This theory states that a single B cell specific for an antigen will proliferate and produce antibody exhibiting the same specificity. However, credit should be given to David Talmage who first reported on the specificity of antibodies (Talmage, 1957). Nossal and Lederberg successfully proved that the antibodies were specific in their action in 1958, which was eventually shown to be applicable to the T lymphocytes.

A selection of the phenomena mentioned above will be discussed in greater detail below.

1.2 Allograft rejection

1.2.1 Historical Perspectives

During the early years of the twentieth century transplantation was not as rigorously controlled as it is today. Numerous attempts were made to transplant a variety of organs and tissues some of which included spurs of young cocks and even a human tooth (Brent, 1997). Some researchers reported that their transplants were successful although no distinction was made between the different types of grafts undertaken. In addition, the use of inbred strains of mice had not yet made its appearance with the exception of the Japanese waltzing mice (Gates, 1925).

Grafts are classified as follows: autografts, are grafts transplanted into the same individual, such as a skin autograft from an unharmed portion of a burn victim's body to a burnt area of the same individual; allografts refer to grafts between different individuals of the same species while syngeneic grafts refer to grafts between animals of an inbred strain and of the same sex.

In 1908, Alexis Carrel, (reviewed in Brent, 1997) a surgeon, who had a keen interest in the transplantation of kidneys into dogs and cats, observed that autografts survived while the allografts invariably failed. He was highly perplexed by the short survival of the allografts. He believed that the recipients were responding to the donor organ and surmised that the use of sibling dogs and cats may prove successful. He, however, obtained results similar to his previous experiments.

At the time that normal tissue transplants were undertaken, tumour biologists were also performing tumour transplants in the hope of finding a cure for cancer. There are numerous tumour biologists that have contributed to this body of work. However, I wish to discuss the significant contributions made by J.B. Murphy at the Rockefeller Institute, New York. Murphy's initial attempts at the transfer of adult chicken tumours into different adult chicken hosts were not successful. However, he demonstrated in a series of experiments that rat tumours that were inoculated into chick embryos before day eight of incubation could establish themselves unhindered up until day eighteen. Interestingly, the rat tumours that were transplanted at day 15, began to regress between days 19 and 21 of development, which coincided with the development of the spleen (Murphy, 1913 and 1914a). In contrast, rat tumours that were inoculated into chicken embryos together with either the spleen or bone marrow of adult chickens at day 7 of embryogenesis regressed soon after inoculation. An influx of lymphocytes was observed around the tumour. Notably the chicken spleens rather than the bone marrow elicited a more rapid destruction of the rat tumours. Murphy surmised that the spleen and bone marrow of the adult chickens provided some form of defense against the rat tumour that was similar to that observed in the adult chickens inoculated with rat tumours (Murphy, 1914b). The finding that the spleen played a role in the regression of the tumours was augmented by the findings of Apolant (Brent,

1997) who showed that the life span of transplantable tumours increased if the spleen of the recipient was removed.

Murphy drew a general conclusion that the rejection or failure of the growth of the tumour allograft coincided with an increase in lymphocytes into the tumour area. He, however, did not conclude definitively that the lymphoid cells were the cause of the destruction of the rat tumour. Despite the lack of conviction on the role of the lymphocytes in tumour regression, the author wanted to determine what effect the removal of the lymphoid cells would have on the growth of the transplanted tumour (Murphy and Taylor, 1918). The authors subjected immunized 'white' mice to different doses of x-rays, which is known to reduce the number of circulating lymphocytes (Murphy and Norton, 1915). Briefly, mice were first immunized with allogeneic defibrinated blood beneath the skin. Ten days later both the immunized group of mice and a non-immunized group were inoculated with a mouse tumour from the same species as the donor of the defibrinated blood. Three weeks thereafter, they noticed that the transplanted tumours in 80% of the immunized mice had regressed while 90% of the non-immunized group developed tumours. These findings suggested that previous immunization provided immunity against a subsequent inoculation from the original donor. The mice in which the tumours had regressed were further divided into two groups, one receiving x-rays for seven days and a control group that did not receive x-rays. Both groups were again inoculated with the tumour. Tumours developed in mice that received x-rays. Murphy and Taylor thus, had demonstrated that the induced resistance to tumours could be ablated by the use of x-rays. These findings highlighted the importance of the lymphocytes in resisting tumour growth by depleting them using x-rays. Despite the thought-provoking findings of Murphy, his work remained largely unappreciated until the 1950's.

1.2.2 Immunological basis of allograft rejection

A brief note on why Medawar became involved in skin transplantation was given in 1.1 of the introduction. Collaboration between Gibson and Medawar resulted in what is considered today to be the first scientific report on human skin allografts (Gibson and Medawar, 1943). A series of skin grafts were undertaken on a burn victim using both

autografts, and allografts obtained from the patient's brother. Gibson did the surgeries while Medawar provided a histological description of the autografts and allografts over a period of 36 days after which the patient was discharged. The autografts were fully accepted with the innervation of blood and lymphatic vessels into the graft evident by day four. In contrast, the allografts that initially displayed epidermal outgrowths eventually showed signs of breakdown by day 15 and were completely rejected by day 23. In addition, the authors transplanted a second set of allografts, obtained from the brother, 15 days after the first allograft transplant. Notably the rejection of the second set allografts was more rapid than the first and did not display any epidermal outgrowths. This was a significant finding and illustrated one of the signatures of adaptive immunity, namely memory.

Subsequently, these findings were reproduced in outbred rabbits (Medawar, 1944). Medawar observed in rabbit skin autografts that although vascularization was accompanied by mild inflammation the grafts were always accepted. In contrast, he found that most of the rabbit skin allografts were rejected. A considerable influx of recipient lymphocytes and monocytes into the graft area was accompanied by intense inflammation. The rejection of the graft was usually complete within four days post transplantation. Medawar therefore concluded that rabbit skin allografts could not be successfully achieved. The influx of lymphocytes into the graft area was consistent with the earlier findings of Murphy (Murphy, 1914a and b). In these experiments Medawar's meticulous recording provided the first evidence of the 'mean survival times' of allografts, which refers to the period between the transplantation of the allograft to its complete destruction. The first set allografts had a mean survival time of 10 days while the second set allografts survived for only 6 days. Furthermore, the vigour with which the second set allografts were rejected was not observed when a second set allograft from a different donor was transplanted. This provided evidence for the specificity of allograft rejection. Notably, Medawar found that differences in sex had no significant effect on the survival times of the allografts. He did, however conclude that several differences in antigens between the recipient and the donor played a role in the rejection of the skin transplants in the rabbits. Medawar believed at the time that rejection of an allograft was due to an actively acquired immunity in which antibodies were the sole mediators of rejection. Notwithstanding, this partially correct

conclusion, it is now recognised that Medawar has made a significant contribution to understanding the immunologic basis for allograft rejection.

1.2.3 Linkage between allograft rejection and T cells

1.2.3.1 A historical perspective of the humoral and cell-mediated immune responses

To understand the controversy behind the humoral and cell-mediated immune responses, one needs to look at how researchers were influenced by the scientific concepts that were in vogue at the time. Eli Metchnikoff, led the charge for a cell as opposed to an antibody as the protector against pathogens. During his studies in 1884, on the digestive function of simple organisms he observed that amoeboid cells within these organisms engulfed foreign organisms using pseudopodia (Metchnikoff, 1905). He referred to these cells as phagocytes and questioned whether they also played a role in the protection of more complex organisms against pathogens. This led him to undertake experiments with this aim in mind. In one such experiment he inserted a thorn into a transparent starfish larva. The thorn was, subsequently surrounded by amoeboid cells. Consequently, he proposed his phagocytic theory, which suggested that phagocytes were important in the defense of an organism. This theory proved to be a catalyst for great objection by the humoralists who believed at the time that antibodies within the blood were the only means of defense against pathogens. They believed that leukocytes were only important for assisting in the circulation of bacteria around the body rather than playing any role in defense. In order to determine if phagocytes did indeed provide a defense mechanism against pathogens Metchnikoff turned to experiments with the water flea. The phagocytes proved essential for the survival of the water flea during feeding. They engulfed ingested spores that had pointed ends which could have ruptured the digestive tract causing water to enter. This would have resulted in the water flea bursting. This provided evidence of a non-specific defense mechanism for phagocytes. Thereafter, Metchnikoff wanted to determine if the phagocytes were important for actively acquired immunity. Robert Koch had earlier determined that the anthrax bacillus was responsible for anthrax. Metchnikoff vaccinated a group of rabbits against the anthrax bacillus. Thereafter he infected vaccinated and naïve rabbits. He reported the presence of the bacillus in the phagocytes in vaccinated animals while in the naïve rabbits

the bacillus remained in the fluid. He thus concluded that acquired immunity to anthrax was due to the phagocytes ingesting the bacteria.

The humoralists believed that humoral factors were the mediators of the immune response. This viewpoint stemmed from the centuries old belief by the Greeks who associated disease with changes in the four humours, namely blood, phlegm, yellow bile and black bile (Silverstein, 2003). Based on the findings of Von Behring and Kitasato in 1890, the humoralists believed that antitoxins in the serum of immune individuals were the main protectors against pathogens. The findings of experiments that were undertaken by Von Behring and Kitasato were reported in 1890. They demonstrated that animals could be immunized against tetanus toxin. If serum, from an animal that had recovered from the infection were inoculated into a non-infected animal, the non-infected animal would become resistant to a subsequent tetanus toxin exposure. The antitoxins, as they were referred to then, neutralized the tetanus toxins. Further studies by these authors using immune serum in children, stricken with diphtheria also proved successful. These findings constituted a large body of research citing antibodies as the only means by which acquired immunity was expressed.

Notably at the time, researchers who accepted the cell-mediated hypothesis used anthrax bacillus that was effectively cleared by the phagocytic cells. In contrast, the humoralists used serum which was successful in removing bacterial toxins. This clearly suggested that both the humoral and cell mediated responses could be important in mediating active immunity. However, it appeared inconceivable to either group that there may be some form of collaboration between the cellular and humoral-mediated immune responses. In 1903, Wright and Douglas tried to span the bridge in thinking between the humoral and cell-mediated response camps. They demonstrated that both antibodies and phagocytes played a role in the defense against bacteria. Their findings suggested that bacteria coated with serum factors (opsonised) were more effectively phagocytized (Wright and Douglas, 1903).

However, it was at this time that Paul Erlich's side-chain theory of antibody formation and function made its appearance. His findings augmented the argument in favour of the humoralists whose ideas proved dominant in the first half of the twentieth century. As a result of these two schools of thought it was to be expected that this would also extend to the mechanism by which allograft rejection occurred.

1.2.3.2 Humoral versus Cell-Mediated immune responses in allograft rejection

The tumour biologists in the early twentieth century did not attribute the rejection of allografts to antibodies. Their main observation was the influx of lymphocytes at the site of the graft. It was the work of Gorer who in 1942, revealed a role for antibodies in the destruction of malignant tissue transplants. His efforts were based on the hope of producing anticancer sera. He immunised resistant C57 black mice with leukemic cells. Thereafter, albino mice who were highly susceptible to the leukemic cells were inoculated with leukemic cells suspended in the sera from the immunised C57 black mice. The tumour cells were unable to establish themselves in the albino mice. Gorer demonstrated using agglutination assays that antibodies in the sera of the C57 black mice bound to the leukemic cells and this prevented the tumour cells from establishing themselves in the albino mice. Gorer further demonstrated that the protective effect against the leukemic cells was only achievable if serum was isolated from a donor that was previously immunized with leukemic cells (Gorer, 1942).

Amos and colleagues obtained similar findings in 1954 using normal tissue transplants (Amos et al., 1954). Inbred strains of mice received allogeneic skin grafts. After the complete regression of the grafts the sera of the mice that had received the allografts were tested for the presence of antibodies. With the aid of agglutination assays they observed the production of antibodies to both the red blood cells and the leukocytes of the donor strain.

While it was evident that antibodies were produced against both malignant and normal tissue transplants, the antibodies appeared to have a different effect on transplanted tumours and normal tissue transplants. The presence of antibodies against leukemic cells

appeared to be directly responsible in preventing the cancer from establishing in the host (Gorer, 1942). In contrast, antibody production observed after the transplantation of normal tissue did not appear to be associated with allograft rejection. This was reported by Medawar in 1948. Medawar demonstrated that adult skin epithelium could grow in serum that was not primed to contain antibodies to epithelial cells. He, however, observed that the survival of the skin of adult rabbits, placed in serum, which was specifically immunized against it, was not compromised. This finding suggested that antibodies in the serum did not retard the growth of the epithelium (Medawar, 1948). The finding, however, was not profound enough to change the thinking of the humoralists who believed that antibodies were important for the destruction of transplanted tissues.

Acknowledgement of the significance of the cell-mediated route of allograft rejection originated from the findings of N.A. Mitchison in 1953. Earlier experimental work used outbred strains of mice to demonstrate the passive transfer of immunity to tumours. Invariably, this led to contradictory results. Mitchison decided to revisit the passive transfer of transplantation immunity to a tumour using inbred strains of mice (Mitchison, 1953). With his initial experiments he showed that the rejection of a mouse lymphosarcoma occurred more rapidly in a non-susceptible mouse, when that mouse was previously exposed to the tumour, which it had rejected. This result was also observed in mice that were first immunized with tissue from an animal that had been exposed to the tumour. Subsequently, when the immunized mice received a tumour from the donor strain it rejected it much faster. These findings are consistent with the results Medawar obtained with his second set skin allografts, which were rejected faster than the first set allografts (Medawar, 1944). In addition, Mitchison observed that the transfer of the draining lymph nodes of immunised mice into normal syngeneic mice resulted in a more rapid rejection of the tumour. In contrast, the transfer of serum, peritoneal exudate and non-draining lymph nodes did not prove successful in increasing the speed at which the tumour was rejected. Thus, he concluded that transplantation immunity was transferred more efficiently with cells rather than with antibodies in serum.

Consequently, in 1953 Billingham, Brent and Medawar wanted to ascertain whether the above findings of Mitchison in 1953 also applied to normal tissue transplants. They once again used skin allografts and inbred strains of mice. The authors undertook a variety of experiments, which allowed them to make a distinction between actively acquired immunity and adoptively acquired immunity. In their first set of experiments mice received a skin allograft that completely disintegrated by the eleventh day. These mice subsequently received a second skin allograft on the opposite side of their bodies. The authors demonstrated that the second graft disintegrated faster than the first allograft. The authors referred to this type of response as an actively acquired immunity. In the second set of experiments, they transferred into a secondary host, various tissues and cells obtained from a primary host that had been actively immunized. The primary and secondary hosts were always from the same inbred strain. They found that the transfer of only the living draining lymph node tissue and to a lesser extent the spleen initiated the rapid disintegration of the skin allografts in the secondary host as observed in second set allografts. They referred to this type of immunity as being adoptively transferred. The immunity of the primary host was maintained for 30 days during which time it could still be transferred and affect a response. In contrast, large doses of blood, blood leukocytes and serum were ineffective in initiating a secondary response. Thus, the authors reported that the passive transfer of antibodies (which would confer passive immunity) was less likely to be associated with the rejection of skin allografts.

1.2.4 Link between Immunological Tolerance and Transplantation

In early studies, Billingham, Brent and Medawar had linked immunological tolerance to transplantation. They infected an embryo of a CBA/J mouse with lymphocytes from an adult A strain mouse. They did find that early exposure to alloantigens during embryogenesis made the offspring of the CBA/J mouse tolerant to a subsequent skin graft from an A mouse for the duration of their lives (Billingham, Brent and Medawar, 1953 and 1954). Thereafter, the tolerant mice were inoculated with serum from an animal immunised against A strain antigens in order to determine if antibodies in the serum would result in the rejection of allograft. The graft remained intact, demonstrating that the antibodies in the serum were ineffective in eliciting graft rejection.

Despite these findings there was still no consensus as to whether allograft rejection was due to cells or antibodies. It was in these circumstances that Billingham and Brent, in 1954 decided to further investigate the role of antibodies in allograft rejection in a more rigorous manner. In these experiments they tried to determine whether the dose of the serum and the mode of transmission of the serum had any effect on the outcome of allograft rejection. Secondary hosts were inoculated with serum from primary hosts that were immunized three times for maximal response. The serum in some cases was given to the secondary hosts before the transplantation of the allografts. These rigorous experiments failed to demonstrate a role for antibodies in graft rejection. These findings led them to argue strongly in favour of a cell-mediated response against allografts.

It was reported by Gorer in 1942, that leukemic cells in suspension required specific antibodies for clearance. It seems that host complement was activated, and the membrane attack complex of complement activation lysed the leukemic cells. In contrast, skin allografts and solid tumours required the action of lymphoid cells. These types of experiments shed light on the role of antibodies and /or cell-mediated immunity against allografts.

1.2.5 Major Histocompatibility Locus and Histocompatibility Antigens

Graft rejection has been shown to occur between individuals that are genetically different. Geneticists played a crucial role in determining which antigens present in the allograft were responsible for eliciting a humoral and/or a cell-mediated immune response in the recipient, which ultimately led to its rejection. Since these antigens were initially associated with the rejection of transplants they were referred to as the transplantation antigens. Currently they are referred to as the histocompatibility antigens. Below is a description of how the concept of histocompatibility antigens evolved.

1.2.5.1 The contributions of the geneticists

The work of C.C Little and E.E Tyzzer paved the way for the discovery of the genetic factors that elicited an immune response to allografts. The authors noted that tumours originating in an inbred strain of mice, the Japanese waltzing mouse was accepted in all

mice from that strain. However, the same tumour would regress in mice from a different strain. In 1916, Tyzzer and Little hypothesised that a 'large number of independently inherited factors' determined tumour susceptibility. Intrinsic to their analysis was the idea that there were multiple histocompatibility loci, which has subsequently been proven. Rejection occurred if there are any foreign histocompatibility antigens on the tumour.

In 1953, Snell and colleagues demonstrated that a difference in the H-2 alleles of the donor and host was the critical factor that determined susceptibility or resistance to an allogeneic tumour. They used 15 mouse strains, some of which were closely related and others that were not related. They first demonstrated that all mice from the same strain would accept a tumour from a donor of the same strain. The authors further demonstrated that the rejection of the tumour was shown to be less vigorous when the mouse strains were closely related than when they were distantly related. For example, BALB/c and the DBA/2 mice both exhibiting a H-2^d haplotype took a much longer time to reject a tumour from the other. In contrast, the C57BL/6, H-2^b, mice consistently rejected a tumour from the BALB/c, H-2^d, donor much more vigorously. Billingham, Brent and Medawar, demonstrated in 1953 the importance of H-2 in skin allograft rejection. Skin grafts between individuals of the same mouse strain were accepted while grafts between different strains of mice were rejected.

Interest in research into the MHC waned. However, after the discovery of the human MHC, referred to as the human leukocyte antigen (HLA) and evidence that the human HLA and the mouse MHC were strikingly similar, renewed interest in the MHC was generated. The significance of having all the HLA loci matching prior to a transplant have been demonstrated with grafts between monozygotic twins that are invariably accepted and grafts between unrelated individuals that are generally rejected (Kortholm, 1968).

As the mouse model is being used in this thesis only the H-2 of the mouse will be discussed in greater detail. The rejection of a tumour allograft (Snell, 1953) and a normal tissue allograft (Billingham, Brent and Medawar, 1953) has been shown to be due to differences in MHC antigens between donor and host. The expression of these antigens is controlled

by the MHC loci, which map in the H-2*K,* H-2*I*, H-2*D* and H-2*L* regions of chromosome 17. The *I* region is further divided into the *I-A* and *I-E* as well as the *I-B* and *I-C* regions which will not be discussed further. The products of the MHC loci can be identified serologically or by T cell reactivity. The H-2K and H-2D are specifically associated with acute graft rejection and the cytotoxic activity of T lymphocytes. They are important for the expression of MHC Class I molecules. These molecules are expressed on all nucleated cells of the body. Variations in the level of antibody production by different strains of mice are controlled by the immune response (Ir) loci. The Ir loci are critical for the expression of the MHC class II antigens, which are expressed on the antigen presenting cells (Klein, 1979 and Klein et al., 1981).

The MHC class I molecules are composed of a polymorphic α chain and non-polymorphic β_2 microglobulin. The MHC class II molecules are composed of a polymorphic α chain and a polymorphic β chain. The peptide binding regions of the MHC involve the $\alpha 1$ and $\alpha 2$ domains of the class I molecules and the $\alpha 1$ and $\beta 1$ domains of the class II molecules.

One of the most striking characteristics of the H-2 loci is their genetic polymorphism particularly in the peptide binding regions. Several alleles exist at a single H-2 locus within a given population (Klein, 1979). One possible explanation for this polymorphism of the H-2 loci is to ensure the survival of a species. It is suggested that the polymorphic nature of the MHC is essential in order to counteract the evasive techniques of pathogens. The polymorphism of the MHC in a population increases the number of antigenic determinants against which the immune system can respond. Pathogens must be presented to the T cells in order to elicit an immune response against them. The peptide binding site of the MHC molecule is specific for particular antigenic determinants of a pathogen. The MHC-peptide complex is presented to the T cells in order for an immune response to be generated against them. The resulting immune response may lead to the destruction and elimination of the pathogen. However, pathogens are able to mutate, thus eliminating the dominant antigenic determinants that are recognised by the MHC molecule. Therefore, if all the individuals within a species displayed the same MHC binding sites, then only specific antigenic

determinants can bind to the MHC molecule. The pathogen would be able to avoid detection, which could lead to the demise of that species.

1.2.5.2 Passenger Leukocytes express allo-MHC initiating alloresponses

Rapid allograft rejection is due to differences in MHC. Several studies were undertaken to determine the cells that were expressing the MHC molecules that initiated graft rejection. The concept of passenger leukocytes first made its appearance in Snell's review of 1957. Several studies reported a difference in immunogenicity between tissues that were taken from rats treated with cortisone and tissues taken from untreated rats when transplanted into allogeneic recipients. The cortisone treated tissue was far less immunogenic. It was suggested that the cortisone had reduced the number of leukocytes in the donor tissues, which implied that leukocytes were important for sensitising the recipient's T cells.

However, it was ten years later in 1967 that Steinmuller revisited this concept. The author found that skin grafts, obtained from mice that were made tolerant to allogeneic spleen cells, could immunize syngeneic recipients, allowing for a second set rejection of an allograft. Mice of the A strain were made tolerant by the injection of allogeneic spleens cells, (A x C3H) within 24 hours of birth. The authors believed the mice to be leukocyte chimaeras. The inoculation of the F1 cells into the neonatal mice reduced the risk of GVHD in the neonatal mice. Two months later the tolerized mice received a skin allograft from a C3H strain and syngeneic skin graft from an A strain on either side of the abdomen. Both grafts were fully accepted. The C3H and the A strain grafts were removed and transferred separately to non-tolerant A strain mice. The C3H skin allograft was destroyed within 15 days whereas, the A skin syngeneic graft was accepted. Both groups of mice then received a C3H skin allograft. As was expected the A strain group that had initially rejected the first C3H skin allograft rejected the second skin allograft more rapidly that the first transplant. However, the A strain that had received the isograft from the tolerant mice also rejected the C3H allograft with the same vigour as those that had received the second C3H skin allograft. This finding suggested to the author that the allogeneic cells in the tolerant A strain mice were transferred with the isografts in sufficient numbers to generate an immune response. A control group of A strain mice that received only one C3H allograft rejected

the graft much slower. These findings are consistent with the findings of Medawar who reported in 1944 that second set rejections occurred much faster than first set rejections. Steimuller surmised that since the neonatal mice were leukocyte chimaeras, the leukocytes from the original donor strain had migrated into the skin and had initiated an immune response in the syngeneic recipients.

In 1968, the work of Elkins and Guttmann contributed to the concept of the passenger leukocyte. Parental rat kidneys were transplanted into F1 hybrids that were tolerant to the parental kidneys. However, when parental lymphocytes were inoculated under the kidney capsule the destructive lesions of the kidney were observed. This was characteristic of a graft versus host reaction, which is discussed in greater detail in 1.3 of the introduction. However, when the recipient of the kidney had been irradiated prior to receiving the allogeneic kidney, the severity of the destructive lesions correlated with the dose of irradiation. The authors surmised that the circulating host leukocytes activated the donor spleen cells, which resulted in non-specific damage of the kidney parenchyma. When the host was irradiated prior to receiving an allogeneic kidney their leukocytes were destroyed and no longer provided the stimulus for the donor lymphocytes. The authors thus concluded that the circulating host leukocytes could provide the stimulus to activate the donor cells either in the donor organ or in the regional lymph nodes. They further suggested that removal of such leukocytes by irradiation could prove advantageous for transplantations.

Lafferty and colleagues expanded upon the concept of passenger leukocytes in 1975. They proposed that H-2 antigens expressed on the surface of cells are not strongly immunogenic on their own and additional stimuli are required from metabolically active and immunocompetent cells. In order to test their hypothesis, they cultured mouse thyroid lobes in medium for 12 days. Thereafter, the lobes were transplanted under the kidney capsule of allogeneic recipients in which the thyroid had been removed. Even though the thyroid cells of the donor expressed different H-2 antigens, the thyroid thrived in the recipient suggesting that parking the allograft in medium for a few days removes the metabolically active and immunocompetent cells that provided other stimuli required for activation.

Once it was determined that leukocytes expressing MHC antigens elicited an immune response, several studies were undertaken to search for the specific leukocyte expressing the MHC antigens. Lechler and Batchelor demonstrated using *in vivo* experiments that the dendritic cells of the donor expressed MHC molecules, which provided the trigger for allograft rejection (Lechler and Batchelor, 1982). Kidneys from F1 rats (As x Aug) that survived for a long period in the primary As host were transplanted into secondary As rat hosts. They continued to thrive in their secondary hosts. All nucleated cells express MHC class I molecules on their surfaces. Thus, the kidney cells with their MHC molecules should have elicited an immune response. The authors surmised that the cells that expressed both the incompatible MHC molecules and other stimulatory signals were absent reducing immunogenicity. In order to rescue the immune response, the authors inoculated small numbers of dendritic cells obtained from the donor at the time of retransplantation. The kidney allografts were subsequently rejected. This finding suggested that the dendritic cells expressed incompatible MHC molecules and other costimulatory signals, which led to the activation of the T cells.

1.2.5.3 T cells recognising MHC alloantigens

Although research had discovered the MHC antigens that elicited a rejection of a transplanted tumour or normal tissue transplants, the cells that were responding to these antigens had not yet being discovered. The importance of T lymphocytes in the production of antibodies and the killing of target cells was initiated with the work of Gowans in 1962. Following the discovery of the MHC antigens a series of experiments provided evidence that these transplantation antigens, served an additional role other than determining susceptibility or resistance to tumour and tissue transplants. Thus, follows below a discussion on the work of numerous researchers who demonstrated the association of T cell responses to MHC antigens in general and the recognition of different classes of MHC by two different subsets of T cells namely the CD4 and CD8 T cells.

Experiments undertaken by Kindred and Shreffler, tested the putative link between antibody production and H-2 antigens (Kindred and Shreffler, 1972). Their approach was to determine if the immune response to sheep red blood cells (SRBC) in nude mice could

be rescued by the inoculation of H-2 compatible thymus cells. Nude mice at the time had an H-2^d haplotype with a mutation at the Foxn1 gene which results in a genetic defect that prevents the development of a mature thymus. The lack of a functioning thymus, the site of T lymphocyte maturation (Miller, 1961), prevents these mice from mounting an immune response against foreign antigens. Furthermore, both thymus-derived and bone marrow-derived lymphocytes were known to be required for antibody production (Claman, 1966, Miller and Mitchell, 1968). Kindred and Shreffler backcrossed nude mice with BALB/c mice which have a H-2^d haplotype resulting in them displaying between 70 and 90% of the genes of the BALB/c strain. A suspension of 4 x 10^7 thymus cells was inoculated into the nude mice. There were three sets of donors of the thymus cells: the first set displayed an H-2^d haplotype and was derived from the BALB/c, DBA/2, and BALB/c x DBA/2 strains. The C57BL strain displaying an H-2^b haplotype and the CBA mice exhibiting a H-2^k haplotype were the other two thymus cell donors. After inoculation with the thymus cells the authors used haemagglutination assays to test the ability of the nude mice to produce antibodies against SRBC. The nude mice that had received thymus cells from the BALB/c, DBA/2 and the BALB/c x DBA/2 were able to produce antibodies against the SRBC. However, the mice that received thymus cells from the C57BL and CBA mice were unable to generate anti-SRBC antibodies. These findings demonstrated that H-2 compatibility was needed for thymocytes to rescue the immune response to SRBC in nude mice. Antibody production by the nude mice required that the H-2^d antigens recognised by the T cells were also expressed by the B cells. Thus, Kindred and Shreffler provided the first evidence of MHC restriction in B-T cell cooperation in 1972.

The findings reported by Kindred and Shreffler in 1972 demonstrated that antibody production required that the B cells express the same MHC molecules that T cells recognise as foreign. Work undertaken by Shearer in 1974 as well as the work of Zinkernagel and Doherty in 1974 provided evidence that the recognition of antigen by CTL, that we now know to be largely CD8 T cells, are restricted to a particular allele of MHC. Shearer cultured mouse spleen cells with trinitrophenyl-modified syngeneic spleen cells. Cytotoxic T cell activity was observed only against the trinitrophenyl-modified target cells and not against unmodified cells (Shearer, 1974). Zinkernagel and Doherty observed that cytotoxic

T cell activity against virus infected fibroblasts were MHC restricted (Zinkernagel and Doherty, 1974). Soon after Miller and colleagues demonstrated in 1975 that CD4 T cells that were responsible for antibody production and delayed-type hypersensitivity, responded to antigen in association with MHC class II encoded I-A and I-E gene products (Miller et al., 1975). However, the interaction between the T cell and the MHC-peptide complex was insufficient to activate the cells and required a second signal, as proposed by Lafferty and Cunningham in 1975. The second signal was later discovered to be the interaction between various costimulatory molecules.

1.2.5.4 Activation of CD4 T cells and CD8 T cells in response to allo-MHC: direct and indirect allorecognition

Nevertheless, there were instances when, despite the absence of passenger leukocytes, allografts were rejected. Thus, in 1982, Lechler and Batchelor proposed two distinct pathways for the activation of the T cells upon stimulation with allo-MHC, which did not necessarily occur independent of each other. The direct pathway occurred when the recipient's T cells recognized the intact MHC on the surface of the donor antigen presenting cells (APCs). There is no need for antigen processing and presentation by the host's APCs during this pathway. In contrast, the indirect pathway involved the processing and presentation of the alloantigens by the host's own APCs. It has been suggested that acute allorejection is due to the direct pathway (Lechler and Batchelor, 1982).

The robust response elicited by direct allo-recognition has been attributed to a high frequency of T cells specific for allo-MHC. The frequency of T cells specific for alloantigens, which is approximately 1 in 10^2, has been shown to be much higher than the frequency of T cells specific for a typical foreign antigen, which is of the order of 1 in 10^5 (Wilson, 1968). T cell activation is generally self-MHC restricted (Zinkernagel and Doherty, 1974). The T cell receptor (TCR) genes that encode the TCR that recognises MHC-peptide complex, involves random events resulting in an extensive range of specificities. Consequently, this results in a low frequency of T cells recognising a foreign peptide in the context of a given self-MHC. A model for the high frequency of T cells specific for allo-MHC and the way in which self-restricted T cells recognise allo-MHC-

peptide complexes have been described by Matzinger and Bevan in 1977.

It is suggested that the self-MHC and allo-MHC have specific structural similarities. T cells are positively selected in thymus based on their ability to recognise self-MHC. Therefore, self-restricted T cells, which have an inherent ability to recognise MHC molecules recognise allo-MHC in the same way they would recognise self-MHC. Thus, donor APCs express allo-MHC with a large variety of peptides to the T cells of the recipient. The peptides may have originated from proteins similar between for example different strains of mice. However, the interaction of these peptides with the allo-MHC appear foreign to the TCR of the recipient. Thus, T cells with different specificities would recognise the many different allo-MHC-peptide complexes eliciting a robust immune response.

The significance of the peptide in the context of MHC in eliciting a robust T cell immune response was reported by Heath and colleagues in 1991. The authors used a mutant T cell line, $T2K^b$, that expressed MHC class I molecules on the surface of cells, which were devoid of peptides processed endogenously. Alloreactive T cells specific for K^b did not display any cytotoxic activity in the ^{51}Cr release assay. However, MHC class I molecules of the $T2K^b$ cell line were able to bind with peptides that were added to the culture. Subsequently cytotoxic activity of the alloreactive T cells specific for K^b was observed. This finding suggested that peptide-MHC complex expressed on the surface of the cells were important for activation of T cells (Heath et al., 1991).

1.3 Graft versus host reactions and graft versus host disease

Bone marrow transplants (BMT) have proven to be beneficial in the treatment of haematological malignancies and more recently in the treatment of autoimmune diseases such as scleroderma (Sullivan et al., 2016). However, BMT are not without associated risks. Graft versus host disease (GVHD) occurs in 30-65% of allogeneic bone marrow recipients and is associated with a mortality of approximately 15% (Blazar et al., 2012). A GVHR is induced after the transfer of histoincompatible cells of a donor into an immunocompromised recipient (Champlin et al., 2000) or a recipient that cannot react to the donor cells for genetic reasons (Via et al., 1987). The latter case may occur when

parental cells are inoculated into the F1 offspring. The F1 offspring do not recognise the parental cells as being foreign (Via et al., 1987). In a GVHR the CD4 T cells and CD8 T cells of the donor recognise the genetically different MHC molecules on the recipient's APCs and are activated. Therefore, considerable effort is made to ensure that the recipient and the possible donors are screened and matched for HLA to decrease the risk of a GVHR after a BMT. In the ideal situation, a bone marrow transplant would occur between HLA genetically identical siblings. However, this occurs only in 30% of patients. Most transplant recipients receive bone marrow cells from either an HLA matched unrelated donor, a haploidentical donor or cord blood unit. The haploidentical donor is a family member with 6 to 8 HLA matches. The cord blood unit is the blood isolated from the umbilical cord and the placenta. However, despite rigorous HLA typing, bone marrow recipients may still develop GVHD (Blazar et al., 2013).

A GVHR may progress into either a lethal form known as the acute (a) GVHR or the chronic (c) form. These classifications are based on the pathology of the disease. A description of aGVHR and cGVHD and the factors determining the disease outcomes follow below.

1.3.1 Acute versus chronic graft versus host disease

1.3.1.1 Acute graft versus host reaction

In humans, the following clinical features characterize aGVHR: skin rashes, hepatitis and jaundice associated with the liver, as well as abdominal pain and diarrhoea (Jacobson and Vogelsang, 2007). Numerous mouse models have been utilised to demonstrate GVHD. In mice, aGVHR is characterised by cell engraftment of the donor cells, severe depletion of the host's lymphoid cells, in particular B cells, and extreme anemia, which may result in rapid death. Thus, aGVHR is often referred to as lethal GVHD (Pals et al., 1982). The aGVHR displays a highly inflammatory phenotype that has been likened to a cytokine storm (Ferrara et al., 2003). A cytokine storm is the dysregulation of the immune response resulting in extensive Th1 mediated cytokine production that causes tissue damage. Ferrara suggested that the aGVHR occurs in 3 phases: (1) tissue damage from conditioning, (2) donor T-cell activation and (3) inflammatory effectors.

Phase I: The first phase of aGVHR is tissue damage. Prior to a patient undergoing a tissue transplant, the patient undergoes a conditioning regimen. The conditioning regimen causes tissue damage that initiates the inflammatory response. The inflammatory response is characterized by the production of tumour necrosis factor (TNF)α, IL-1 and IL-6. The effects of the conditioning regimen in initiating the cytokine storm were demonstrated in mice (Xun et al., 1994). The authors used a severe combined immunodeficiency (SCID) mouse model to demonstrate the detrimental effects of radiation that was part of the conditioning regimen. The group of mice that underwent this regimen had increased levels of TNFα, IL-1 and IL-6 in their serum and colon compared to the group did not receive radiation. The authors surmised that the presence of cytokines was due to the release of danger associated molecular patterns (DAMPS) by cells during radiation that activated antigen-presenting cells (APC's) such as the dendritic cells (DCs) of the recipient. The TNFα and IL-6 resulted in colon tissue damage. Furthermore, the role of TNF in tissue damage was verified with the use of a human TNF receptor that antagonized the binding of TNF resulting in reduced tissue damage. In addition, they found that damage to the gastrointestinal tract triggered microbial invasion that further stimulated the APCs to produce TNFα, IL-1 and IL-6. The increase in TNFα and IL-1 leads to epithelial damage while the excessive amounts of IL-6 was associated with liver damage, a feature of aGVHR.

Phase II: The second phase of the cytokine storm that leads to aGVHR is the activation of donor CD4 and CD8 T cells through the recognition of host MHC molecules. This results in further production of cytokines such as interferon gamma (IFN-γ) and IL-2 by the donor T cells, which are evidence of a cell-mediated immune response. The increased production of IFN-γ has been shown to be an early indicator of aGVHR (Rus et al., 1995).

Phase III: The third phase involves the effects of the cytokines produced. The IFN-γ has numerous functions that further enhance the inflammatory response. The lytic activity of the CD8 T cells is augmented, which results in the depletion of the host cells. The IFN-γ enhances the microbicidal activities of macrophages that are associated with the first step as described above. Activated macrophages produce IL-12 that promotes a Th1

inflammatory response. Moreover, the IL-3 and GM-CSF produced by the T cells increases the macrophages in circulation. The macrophages in turn produce nitric oxide (NO) that damages target tissue. Furthermore, the NO inhibits target tissue repair by inhibiting proliferation of epithelial stem cells in the gut and skin (Nestel et al., 2000). The array of cytokines and their effects eventually leads to tissue damage associated with aGVHR.

1.3.1.2 Chronic graft versus host disease

In contrast, the clinical features of chronic GVHD in humans include lichenoid-oral or vaginal changes, dry eye syndrome, skin-discolouring, scleroderma-like disease, bronchiolitis obliterans and esophageal web formation (Lee et al., 2003). The clinical features of chronic GVHD abnormalities in mice include persistent lymphoid hyperplasia, and hypergammaglobulinaemia (Rolink et al., 1983). The persistent B cell stimulation eventually leads to the production of autoantibodies, which may manifest as autoimmune diseases such as sclerodermatous or the lupus-like autoantibody mediated disease depending on the murine model used (Blazar et al., 2012). The production of IL-4 by the alloreactive CD4 T cells which promotes B cell stimulation has been shown to be a signature cytokine of the development of cGVHD (Rus et al., 1995).

1.3.2 Experimental avenues leading to the discovery of graft versus host disease

1.3.2.1 Induction of tolerance to skin allografts

Murphy's interest in tumour transplants in chicken embryos provided early evidence of GVHD. In his experiments, as described earlier in the introduction, he observed that after transplanting the spleen cells of adult chickens onto the chorioallantoic membrane of chicken embryos, the spleens of these chicken embryos became enlarged. He further noticed the development of nodules on the chorioallantoic membrane. However, he suggested that growth factors were responsible for the formation of these nodules.

It was only in 1957 that Simonsen first recorded the enlargement of the spleen in chicken embryos as a consequence of a GVHR (Simonsen, 1957). Simonsen inoculated chicken embryos with adult spleen cells three days before hatching in the hope of tolerizing the chicks to the adult spleen cells. However, soon after hatching the chicks developed severe

haemolytic anemia and died. Simonsen believed that the chicks were tolerant to the donor cells as there was no evidence of antibodies against the donor cells. Further investigation revealed the enlarged spleens of the chicks. He reported that the donor cells had been activated by host antigens and proliferated, destroying and replacing the host lymphocyte population. He postulated that the recipient had to be young enough to be unable to respond to the donor cells and that the donor cells had to be mature enough to form antibodies in order for this phenomenon to occur. A cell-mediated immune response by the donor cells is currently known as the leading cause for a GVHR. A cell-mediated immune response by the donor cells, however, was not considered by Simonsen at the time.

In the same year that Simonsen reported his findings on GVHR in chicken embryos, Billingham and Brent reported similar findings using two inbred strains of mice. In his attempt to induce tolerance to allogeneic skin grafts he injected immunologically competent cells of adult mice into histoincompatible newborn mice. The newborn mice developed runt disease and eventually died two to three weeks later. Exfoliation of the skin and reduced flexibility were marked features of these mice, with considerable weight loss and the eventual enlargement of all lymph nodes as well as the spleen. They further observed that the donor cells persisted in the recipient. Notably the runt disease only arose with cells from adult spleens and not cells from the kidney or the skin. The fact that only spleen cells were effective provided evidence of the role of the lymphocytes on the development of runt disease in mice. The authors initially thought that the presence of a pathogen in the adult inoculum could have resulted in an infection, which led to death. However, they favoured the idea that the immunologically competent cells in the adult spleens mounted an immune response against the host's cells. In fact, they observed that the tissues that were most affected during runt disease were those to which the lymphocytes migrated. The migration of the lymphocytes through the lymphoid tissue was later demonstrated by Gowans and Knight in 1964 as described later in the introduction.

In a paper by Billingham and Brent in 1959, they provided conclusive evidence that runt disease in newborn mice was due to a GVHR. They inoculated newborn mice with cells from different histoincompatible donors with varying results. Spleen cells from adult F1,

(AxB) mice inoculated into parental (A) newborn mice did not result in runt disease. However, the inoculation of the spleen cells from the parental strain (A) into F1, (AxB) newborn mice induced runt disease. Furthermore, they noted that the inoculation of bone marrow, which contains fewer immunologically competent cells than spleen, resulted in a less severe form of runt disease in the newborn mice. The authors came to two conclusions: the first is that the donor cells that were inoculated were responsible for the runt disease that developed in the newborn mice and secondly that there was a dose effect between the number of immunologically competent cells and the severity of the disease.

GVHD was not only a phenomenon that occurred in mice and chickens but also in non-human primates such as the rhesus monkey. In 1961 Crouch and colleagues reported that monkeys receiving allogeneic bone marrow with critical contaminating T cells after irradiation also developed GVHD. Thus, it was that the phenomenon of GVHR was firmly demonstrated in a number of different animal species. Furthermore, this response of the donor cells against the host cells eventually leads to GVHD and potentially the death of the host.

1.3.2.2 Whole body irradiation and bone marrow transplantations

As early as 1915 Hektoen noted that with an unknown dose of irradiation, administered over several days, rats were unable to produce antibodies to SRBCs (Hektoen, 1915). This provided early evidence of the effects of irradiation on the immunological competent cells of the body. However, it was much later, after the atom bomb was deployed at the end of World War II that active investigation into the effects of radiation on the hematopoietic cells was undertaken at the Radiobiological Research Unit of the Atomic Energy Research Establishment in Harwell (United Kingdom) and the Radiobiologic Institute in Rijswijk (The Netherlands) (Brent, 1997). During these investigations the lethal dose for mice that resulted in the destruction of the bone marrow was determined. In order to counteract the effects of irradiation they transferred bone marrow from mice that differed at their major histocompatibility antigens into the irradiated mice. Although the transplant appeared initially to be protective, the mice developed extreme diarrhoea and weight loss and eventually died. Since allogeneic bone marrow was used in the transplant, the disease

manifestation was referred to as homologous disease. Homologous grafts were originally grafts transplanted from one individual to another of the same species. However, the homologous graft subsequently became known as the allograft.

The work on GVHD resulting in runt disease in mice, prompted Barnes and Loutit to undertake a series of studies, the findings of which were reported in 1958 (Barnes and Loutit, 1958). They explored the potential relationship between the runt disease observed in neonatal mice and the development of the homologous disease after bone marrow transplants in irradiated recipients. The transplanted cells were implicated in the development of runt disease. Barnes and colleagues inoculated irradiated adult mice with either allogeneic neonatal spleen cells or with adult bone marrow. The mortality rate was much higher when adult bone marrow cells were administered. Moreover, they found that bone marrow from an F1 into an irradiated parental strain increased the survival rate compared to the transfer of bone marrow from the parent into irradiated F1. These findings suggested that the inoculated cells were responsible for the development of the homologous disease in irradiated recipients as were they in neonatal mice and chicken embryos. Therefore, homologous disease was considered to be the same as the runt disease observed in mice and the GVHR observed in the chickens.

1.3.3 Factors determining the development of an acute graft versus host reaction and a chronic graft versus host disease

1.3.3.1 Different mouse strain combinations

In the GVHD murine model, the type of GVHD observed is greatly influenced by the combination of donor and recipient mouse strains used in the experiments. The early experiments undertaken by Billingham and Brent as reported in 1959, provided evidence of this in neonatal mice. They demonstrated that the type of GVHD was dependent on the degree of genetic differences between the donor and the recipients mouse strains. They first injected newborn mice of strain A (H-2^a) with spleen cells from adult C57 mice, (H-2^b), which were distant relatives of strain A. The newborn mice initially appeared normal but soon afterwards developed extreme diarrhoea with the cessation of their growth and died within two to three weeks of birth. In contrast, when the newborn mice of strain A were

injected with the spleen cells of adult C3H mice, (H-2^k), which were more closely related to strain A than the C57 mice, the newborn strain A mice survived for more than two months. During this time, however, they developed chronic GVHD. The authors suggested that the more distantly related the donor and host strains were, the more aggressive the response of the donor cells against the host cells.

The effect of the spleen cells of different combinations of mouse strains on the type of GVHD elicited, was further demonstrated in adult mice (Pals et al., 1982). A GVHR was induced when the spleen cells of adult C57BL/10 mice were injected into non-irradiated F1 (C57BL/10 x DBA/2) mice, (H-$2^{b/d}$). These F1 mice developed a lethal GVHR with a rapid decrease in the number of cells in the spleen, lymph nodes and thymus. Extreme anemia and excessive weight loss, features of aGVHR, were also evident in these mice. However, the mice did not die and two weeks later the lymphoid tissue of the F1 mice began to be repopulated by the donor cells. In contrast, the GVHR induced in F1 mice following the inoculation of spleen cells from adult DBA/2 mice, (H-2^d) was characterized by a slight initial cellular reduction followed by increased cell proliferation in the lymphoid tissue and specifically in the region in which the B cells were found. As the GVHR progressed an increase in B cell numbers correlated with an increase in IgG antibodies to nuclear antigens and double stranded DNA (dsDNA), which was absent in the F1 mice receiving the spleen cells of C57BL/10 mice. The spleens of the C57BL/10 mice, (H-2^b) and the DBA/2 mice (H-2^d) had an entirely different effect on the F1 recipient mice. It appeared that the T lymphocytes of the spleens of C57BL/10 mice were able to induce an acute GHVR resulting in the suppression of antibodies while the T lymphocytes in the spleens of the DBA/2 mice induced a chronic GVHD when inoculated into the same F1 mouse strain. The difference in T lymphocytes of these mouse strains is elaborated upon below.

1.3.3.2 T helper and T suppressor cells

From the above discussion it is clear that the T lymphocytes of the C57BL/10 mouse strain induce a different disease in the F1 mouse from that of the DBA/2 mouse strain. It was known at that time that T lymphocytes were subdivided into T helper (CD4 T cells) and T

suppressor cells (CD8 T cells) based on their Ly surface markers (Cantor and Boyse, 1975; Cantor et al., 1976). Furthermore, suppressor T cells and helper T cells reciprocally regulate the antibody and the cell-mediated immune responses, respectively, which is discussed in greater detail below (Ramshaw et al., 1977). Thus, the question arose as to whether the T suppressor cells in the C57BL/10 mouse were responsible for the development of the aGVHR in the F1 mouse strain while inhibiting the production of antibodies (Rolink et al., 1982). In order to explore this question, the authors inoculated the spleen cells from C57BL/10 mice into non-irradiated F1, (C57BL/10 x DBA/2) mice. the spleen cells of the F1 mice were removed at either day five or six and inoculated into non-irradiated secondary F1 recipients. The non-irradiated syngeneic F1 hosts receiving the day five T cells displayed a reduced number of cells in their lymphoid tissue, in particular, plasma cells and plasma cell precursors, which is a feature of aGVHR. All of these mice died within three weeks upon receiving the day five T cells. In contrast, all the F1 mice receiving the day six T cells developed cGVHD displaying excessive proliferation of their lymphoid tissue, which persisted for three months.

The authors then proceeded to evaluate the antibody promoting ability of the day five and day six T cells. Irradiated F1 mice received spleen cells depleted of T cells with varying numbers of either day five or day six T cells. Thereafter, they were injected with levan. Five days later the spleens of these mice were removed and their ability to generate plaque forming colonies (PFC) and antibodies against levan were determined. The spleen cells of irradiated syngeneic F1 mice receiving day five T cells were unable to generate PFC or mount an antibody response, which was indicated by the inability to produce of IgG antibodies. In contrast, the spleen cells of the F1 mice receiving T cells cultured for 6 days displayed a considerable increase in IgG antibodies when stimulated with levan. The authors concluded that the day five T cells were able to suppress the antibody response and were responsible for aGHVR while the day six T cells facilitated the antibody response and induced a cGHVD. Thus, day six cells were designated T helper cells that promoted cGVHD characterized by the robust antibody response while the day five cells were designated T suppressor cells that promoted the aGVHR that was characterized by the lysis of host cells and the absence of an antibody response. This is consistent with the findings

of Salvin who reported that the immune response to antigens evolves from an initial Th1 phenotype to a Th2 phenotype (Salvin, 1958).

1.3.3.3 Major Histocompatibility Antigens

Besides the contribution of the donor CD4 T cells and CD8 T cells in the induction of the different types of GVHD, the alloantigens of the host against which the donor T cells respond should also be considered. Differences in the MHC molecules between the donor and recipient are known triggers for activating the CD4 T and CD8 T cells. *In vitro* studies demonstrated that differences in MHC class II (I-A and /or I-E) antigens activated the alloreactive CD4 T cells. Similarly, the alloreactive CD8 T cells were activated by the recognition of differences in the MHC class I antigens, (K and D) (Rolink et al., 1983). Thus, the expression of the different MHC class I and class II molecules on the cells of the host can influence the activation of the alloreactive CD4 and CD8 T cells of the donor. In 1983, using these findings Rolink and colleagues undertook *in vivo* experiments to explore the genetic basis for the different disease outcomes. The authors used congenic C57BL/6 mice that differed at either the MHC class I and/or at the MHC class II loci. The mice used were the C57BL/6 wild type mouse, the B6 (bm1) mouse with a mutation at only the K locus and the B6 (bm12) mouse with a mutation at the I-A locus. The F1 strains prepared were F1 (bm1 x bm12) and the F1 (B6 x bm1) mice. Unfractionated spleen cells from the C57BL/6 mice were injected into the two different F1 mouse strains. A difference at both the K locus and the I-A locus was a requirement for the development of aGVHD while a difference at only the I-A locus was a requirement for the development of cGVHD.

An earlier experiment was undertaken with C57BL/10 mice and F1 mice that differed only across the I-E region. The recognition of only the I-E antigens on the surface of the F1 B cells and APC's by the B10.A(4R) mice was sufficient to generate a cGVHD characterized by autoantibodies (van Rappard-van Der Veen et al., 1982). These findings implied that the activation of both CD4 T cells and CD8 T cells were required for the development of aGVHD while activation of only CD4 T cells were a requirement for cGVHD.

1.3.3.4 Differences in CD8 T cells in the donor inoculum

From the above experiments the absence of alloreactive CD8 T cells gave rise to cGVHD. Thus, Via and Shearer, in 1987 undertook experiments to examine the effect of the CD8 T cells on inhibiting the development of cGVHD and ultimately preventing the development of the lupus-like disease, a consequence of cGVHD (Via et al., 1987). The authors inoculated F1 (C57BL/6 x DBA/2) mice with either the spleen cells of C57BL/6 mice or those of the DBA/2 mice. The spleens of the F1 mice that received the DBA/2 inoculum was found to have predominantly donor CD4 helper T cells and a near absence of donor CD8 T cells. In contrast the spleens of the F1 mice that received the C57BL/6 inoculum were found to have a predominance of the donor CD8 CTLs. The difference in the CD8 T cells in the donor inoculum of the two parental strains gave rise to different GVHD phenotypes in the F1 mice. The F1 mice receiving spleen cells from the DBA/2 mice displayed increased host cell proliferation and low numbers of the donor cells. The spleen cells in these F1 mice were devoid of CTL activity *in vitro*. The production of anti-DNA antibodies was also characteristic of these mice. The F1 mice that received spleen cells from the C57BL/6 mouse displayed a higher degree of chimerism with an increase in donor T cell that was predominantly of the CD8 T cell type. Furthermore, there was considerable reduction in the host lymphoid cells, specifically the B cells. The anti-F1 CTL activity of the spleen cells of the F1 mice that received the C57BL/6 spleen cells suggested that the reduction of the host's cells was due to the CTL activity. This result provided evidence of an aGVHR. The authors surmised that the lysis of the B cells would lead to a decrease in antibody production and that this may ultimately be a mechanism to prevent the development of the lupus-like autoimmune disease.

To further demonstrate the effects of the alloreactive CD8 T cells, the C57BL/6 inoculum was depleted of CD8 T cells using anti-Ly-2 antibody and complement prior to inoculation into the F1 mice. The F1 mice that received the CD8 T cell depleted C57BL/6 inoculum developed a similar GVH phenotype to the F1 mice that received the DBA/2 inoculum. Notably the inoculum of the parental strains contained comparable absolute numbers of CD4 T cells. Altogether these findings imply that the number of the CD4 T cells to that of the CD8 T cells may determine the type of GVHD that may develop in an allogeneic host

receiving donor T cells. Interestingly though, Tschetter demonstrated in 2000 that the aGVHR may evolve into a cGVHD that was characterized by antibodies to single stranded deoxyribose nucleic acid (ssDNA) and double stranded (ds) DNA antigens (Tschetter et al., 2000).

The findings of Via in 1987 was expanded upon by Tschetter in 2000. Tschetter demonstrated that there is a higher frequency of CD8 T cells specific for the F1 alloantigen in the C57BL/6 mouse strain than either in the BALB/c or the DBA/2 mouse strains by employing limiting dilution CTL assays. The C57BL/6 mouse strain had a CTL frequency of 1/1000 while the BALB/c and DBA/2 mouse strains both had a CTL frequency of 1/20,000 for their respective F1 cells in an *in vivo* mouse model. These findings further emphasize that the frequency of the CD8 T cells in the donor inoculum plays a pivotal role in the type of GHVD generated in a F1 recipient.

Thus, it was with this knowledge that we set out to determine the effects of varying numbers of the CD4 T cells relative to that of the CD8 T cells in the spleens of different mouse strains on the type of immune response generated against the same alloantigen.

In the above experiments genetic differences in C57BL/6 and DBA/2 mouse strains, unknown at the time, contributed to the different disease pathologies. It is now known that the mice with the H-2^b haplotype have a defective I-Eα gene and thus are unable to express the I-E molecule in the thymus (Flavell et al., 1985). The CD4 T cells are positively selected in the thymus based on their recognition of MHC class II, I-E or I-A molecules on the surface of the thymic epithelial cells. The lack of expression of I-E molecules results in a reduction in the positive selection of CD4 T cells. Consequently, a reduced number of CD4 T cells would migrate to the periphery. The expression of the MHC molecules in the thymus are important in determining the number of CD4 and CD8 T cells circulating in the periphery. Thus, the number of CD4 T cells to that of the CD8 T cells in the periphery in the C57BL/6 mouse strain would be anticipated to be lower than that of a mouse strain that does not have this defect. However, the CBA/J mouse strain that have intact I-A and I-E genes behave as a C57BL/6 mouse strain.

1.3.4 Lymphocytes are implicated in graft versus host reactions

Lymphocytes were demonstrated to play a key role in allograft rejection (Mitchison, 1953). It soon became apparent through a series of experiments that lymphocytes were also linked to the induction of a GVHR. The inoculation of histoincompatible spleen cells and not kidney cells into embryonic chickens and newborn mice resulted in splenomegaly and runt disease, respectively. These findings suggested that cells within the donor spleens could be responsible for the development of GVHD. Murphy observed both an enlarged spleen of the chicken embryo and an influx of lymphocytes after inoculation with adult chicken spleen cells.

However, it was Terasaki, a fellow in Medawar's laboratory who demonstrated that the lymphocytes were responsible for GVHR in chickens (Terasaki, 1959). In an effort to determine the type of cells in the donor inoculum responsible for the development of GHVR in chickens, he prepared a sample of lymphocytes with 95% purity as well as a monocyte population with 100% purity from the blood of adult chickens. Inoculating these two populations into separate chicken embryos resulted in a considerable increase in spleen size only when the lymphocytes were inoculated into the embryos. Thus, he was able to demonstrate that the lymphocytes rather than the monocytes were responsible for the development of GVHR in chickens.

Soon after the findings of Terasaki were reported in 1959, Billingham and colleagues reported in 1960 that cells in the thoracic duct lymph or lymphocytes from lymph nodes were also capable of inducing GVHR in rats. Moreover, since the findings of Gowans demonstrated in 1959 that thoracic duct cells were composed of 95% small lymphocytes it was assumed that small lymphocytes rather than large lymphocytes were responsible for GVHR. There remained, however, some disagreement in this regard, as some researchers believed that large lymphocytes were responsible for GVHR, since they were shown to proliferate in culture. Gowans and colleagues in 1962 took on the task of determining whether it was the large or small lymphocytes that were associated with the development of GVHD. The first step in this investigation was to tolerize newborn albino parental rats by injecting them with splenocytes from F1 rats. At age 6 weeks, the albino rats were

tolerant to a skin graft from the F1 rats. The authors were able to break the tolerance of the albino rats by inoculating them with small lymphocytes from syngeneic non-tolerant donors. These findings provided evidence that small lymphocytes were responsible for immune rejection and by inference for GVHR in rats. Further studies by Gowans in 1962 demonstrated that the small lymphocytes that caused the GVHR in the rats migrated to the spleens and subsequently developed into larger cells. This finding suggested that the large lymphocytes played a role in the immune response (Gowans, 1962).

1.3.5 Strategies to prevent Graft versus Host Disease

1.3.5.1 The removal of T lymphocytes from a bone marrow graft: a potential prophylactic measure against the development of GVHD

The profound clinical consequences of acute GVHR and chronic GVHD have motivated clinicians and researchers to find ways to prevent the development of these potential complications of BMT. T cells were identified as critical role players in the induction of a GVHR (Terasaki, 1959, Billingham et al., 1960). It was therefore hypothesised, that depletion of the T cells from allogeneic bone marrow, prior to inoculation into a host, may be a potential prophylactic measure against the development of GVHD. A series of investigations were conducted to test this hypothesis. In the first series of experiments, the T lymphocytes were depleted from the bone marrow of the donor mice using anti-theta antiserum and complement (Sprent et al., 1975). The development of GVHD was considerably reduced in these mice. The authors further, determined that the T lymphocytes circulating in the chimeras six months later were mainly of donor origin. In a second series of experiments, T lymphocytes were depleted in the donor using anti-lymphocytic serum (ALS) prior to whole body irradiation (WBI) (Norin et al., 1981). A control group of mice that underwent WBI only, prior to receiving allogeneic bone marrow developed GVHD and died within 45 days. In contrast, mice that received ALS before undergoing WBI and then receiving allogeneic bone marrow survived for more than 200 days (Norin et al., 1981). These findings provided evidence that T cell depletion from the bone marrow of a donor reduced the risk of GVHD in the recipient. However, despite the success in preventing GVHD, T cell depletion did not necessarily lead to successful engraftment of the allogeneic bone marrow.

Further research expanded on the data above. The effects of the removal of T cells from allogeneic bone marrow prior to inoculation on skin graft tolerance in the bone marrow recipient were determined. Lethally irradiated mice received allogeneic bone marrow either with or without T lymphocytes (Vallera et al., 1981). The depletion of T cells from the allogeneic bone marrow inoculum prevented the development of GVHD in the irradiated host. Furthermore, the recipients of the allogeneic bone marrow were able to accept skin grafts from the bone marrow donor. This phenomenon was also demonstrated in humans. Leukemic patients underwent radiation prior to receiving bone marrow depleted of T cells from HLA-identical siblings. They did not develop GVHD. However, their risk of graft failure increased considerably compared to those that received bone marrow containing T lymphocytes (Champlin et al., 2000; Marmont et al., 1991). Thus, it appeared that the T lymphocytes responsible for the development of GVHD were somehow also important for bone marrow maturation.

Studies emerged that demonstrated that T lymphocytes produced IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF) that were essential for bone marrow growth (Aversa et al., 1994). In these studies recombinant (r) GM-CSF was added to bone marrow depleted of T lymphocytes prior to injection into patients. The patients that received the rGM-CSF together with the bone marrow depleted of T lymphocytes had a significantly higher graft survival rate than patients that did not receive the rGM-CSF and had a lower risk of developing GVHD. It was thus suggested that the allogeneic bone marrow depleted of T cells should be supplemented with the specific cytokines required for successful engraftment.

The negative effects of the development of GVHD after BMT has been discussed above. However, there is a positive effect of GVHD. BMT are usually a last course of action for patients with leukemia. The main defence against the cancer cells is CTL activity. An acute GVHR in these patients is characterized by strong CTL activity. Therefore, depletion of T cells from the donor bone marrow inoculum may result in a relapse of the leukemia (Blazar et al., 2012). Therefore, total T cell depletion from the bone marrow inoculum may not be a viable strategy to prevent GVHD.

1.3.5.2 Predictors of the severity of GVHD after bone marrow transplantations

Although HLA-typing is mandatory in BMT, studies are undertaken to find ways to predict whether GVHD will occur and the severity of the manifestation. Early studies utilised the mixed lymphocyte reaction (MLR) to determine T cell alloreactivity. In a study undertaken by Bach and Kisken in 1967 the alloreactivity of the T cells of the different recipients were determined when stimulated with different donor T cells. The donor T cells were treated with mitomycin C and thus were unable to respond. The lymphocytes of a single donor and a single recipient were mixed and cultured for 4-5 days, after which tritiated thymidine, a radioactive labeled nucleoside was added. The incorporation of the tritiated thymidine into the dividing cells was measured by the precipitate that formed. The precipitate was counted with a scintillation counter and expressed as counts per minute. A high stimulation index indicated a high degree of alloreactivity between the cells of the donor and the recipient. Skin grafts were undertaken in varying combinations to determine if the stimulation index obtained in the mixed lymphocyte culture corresponded to the acceptance or rejection of the allografts.

Since the CTL response has been associated with acute GVHR, studies were undertaken to determine the effect of CTL frequency of donor bone marrow on the severity of GVHR. Thus, studies using the parent-into-F1 mouse model (Via et al., 1987) have suggested that the number of CTL precursors could be used to predict whether either an acute or a chronic GVHD develops. The cytotoxicity of the responder cells was determined by the Chromium-51 (^{51}Cr) release assay. In this assay ^{51}Cr is incorporated into target cells during incubation. Effector cells are subsequently added, and the chromium released is determined after a period of 4 hours using a gamma counter. Limiting dilution analysis was used to determine the frequency of CTL. A high number of CTL in the parental inoculum correlates with acute GVHR in the F1 mouse, while a low frequency correlates with chronic GVHD.

In human studies, conflicting evidence exists as to whether the number of CTL precursors can be used as a predictor of the severity of the GVHD. Some studies demonstrated that a high frequency of CTLp correlated with acute GVHR (Affaticati et al., 2000; Batchelor et al., 1990), while other studies suggested that the frequency of CTLp is not a good predictor

of the severity of the GVHD that developed after BMT (Wang et al., 2000). All the above human studies used limiting dilutions analysis to determine the frequency of the cytotoxic T precursor cells. Thus, it appears that a predictor for the development of GVHR and the severity of GVHD needs further investigation.

1.3.6 Parabiosis of parental mice to F1 offspring

The intense investigation into GVHD led some researchers to undertake interesting experiments involving the parabiosis of mouse strains that differed at the H-2. Parabiosis is the process of joining two individuals together to allow sharing of the blood supply. Suturing the skin of the individuals together promotes the formation of blood capillaries. Parabiosis provides an alternate method to the inoculation of allogeneic donor cells to an immunocompromised recipient for the study of GVHD. The blood chimerism in the parabionts permits the analysis of the effects of the circulating cells between the two animals.

In an early study, mice that originated from different strains formed parabiont pairs (Eichwald et al., 1959). When two genetically identical mice were joined they remained tolerant. However, a union between mouse strains that were completely H-2 incompatible led to the separation of the two mice. A third group of parabionts constituted a union between a parent and F1. Initially it was demonstrated that the GVHR induced in these mice would invariably lead to the death of both animals two to three weeks after the union. The death of the animals was referred to as parabiosis intoxication disease (Eichwald et al., 1959). However, later research provided evidence contrary to this. Not all H-2 incompatible unions end in death (Shaw et al., 1974). The parabiosis of the F1 (DBA/2 x C3H) with the parental DBA/2 mouse resulted in long-term survival of the pair despite H-2 incompatibility. The parental DBA/2 cells populated the entire lymphoid system of the F1 mouse. Furthermore, the F1 mouse displayed circulating red blood cells that were mainly of DBA/2 origin. Interestingly, the F1 were tolerant to a C3H and a DBA/2 skin graft, while the DBA/2 mouse showed no sign of rejecting the C3H skin allograft, although an antibody response against the C3H skin graft could have occurred. Despite the

unresponsiveness observed by the pair to a C3H skin graft, the cells of the pair were fully reactive against cells of the C3H mouse in a mixed lymphocyte reaction.

1.3.7 Autoimmunity: a consequence of chronic graft versus host disease

Chronic GVHD in the murine model resembles the antibody-mediated autoimmune disease, SLE in humans and is characterised by the presence of a number of different autoantibodies. These findings suggest that autoreactive B cells have been activated and produce antibodies to self-antigens. Several mouse models have been described in which the spontaneous development of SLE occurs such as the New Zealand strains and the Murphy Roths Large (MRL) strain (Borchers et al., 2000). GVHD can be induced in mouse models such as the parent-into-F1 model. In the latter model the donor CD4 T cells recognise allo-MHC class II, I-A and I-E, molecules on the B cells of the host. The immune response that is generated is a Th2 antibody response that is unable to eliminate host cells. The continuous presence of the host cells results in the persistent Th2 antibody response, which ultimately leads to the chronic form of the disease. The high frequency of the CD4 T cell repertoire, specific for allo-MHC (Wilson et al., 1968) results in the activation of a large number of CD4 T cells. As the host is not irradiated and the lymphocytes are still viable, the autoreactive B cells of the host can be activated. The B cells require two signals in order to differentiate into antibody producing cells. The first signal received by the autoreactive B cell is the binding of its B cell receptor (BCR) with its cognate ligand, a self-antigen. The alloreactive CD4 T cells of the donor interact with the MHC class II molecules expressed on the surface of the autoreactive B cells of the host thus providing the B cells with a second signal (Bretscher, 1973). A study undertaken by Morris and colleagues in 1990 demonstrated that the autoantibodies produced by the host B cells are from cognate T-B interactions (Morris et al., 1990). The activated B cells differentiate into plasma cells producing antibodies against the self-antigens. IgG anti-ssDNA antibodies are known to appear approximately 10 days after the induction of GVHR in the mouse. The lupus-specific autoantibodies such as the anti-dsDNA, and anti-Smith antibodies appear a month later (Puliaeva et al., 2009). The high frequency of alloreactive CD4 T cells may activate a large number of autoreactive B cells. This is evident by the presence of several different antibodies to self-antigens.

The presence of CD8 T cells was shown to inhibit the development of cGVHD and indirectly the autoimmune disease. The lysis of the host B cells appears to be one mechanism demonstrating how the CD8 T cells are able to accomplish this (Via et al., 1987).

1.3.8 Disease models related to CD4:CD8 T cell ratio

The CD4:CD8 T cell ratio has been shown to have practical applications in the treatment of human subjects with a variety of infective and oncological illnesses. One of the first observations of the importance of the CD4:CD8 T cell ratio in humans relates to humans infected with human immunodeficiency virus (HIV). In HIV, the CD4 T cell is preferentially infected and depleted by the HIV, which leads to an imbalance of the naturally occurring CD4:CD8 T cell ratio. The disturbance of the naturally occurring CD4:CD8 T cell ratio may lead to non-HIV related mortality and morbidity and other immune dysfunctions (Holmes et al., 2006). The non-HIV outcomes relates to greater risk of malignancy, cardiovascular and renal diseases. A low CD4:CD8 T cell ratio has been associated with a poor response to antiretroviral therapy (Rosado-Sanchez et al., 2017). In contrast, correction of the profoundly low CD4:CD8 T cell ratio may lead to an immunological disease called the immune reconstitution inflammatory syndrome (IRIS) (Smith et al., 2009). Research into ovarian cancer (Sato et al., 2005) and colorectal cancer (Diederichsen et al., 2003) in humans has explored the role of the CD4:CD8 T cell ratio as a prognostic indicator. A low CD4:CD8 T cell ratio in these individuals has been associated with a good clinical outcome. This suggests that a Th1 cell mediated response is generated which would be effective in killing the cancer cells.

1.4 The Mixed Lymphocyte Reaction

1.4.1 Principles of a Mixed Lymphocyte Reaction

The experimental system we use in our study is a one-way mixed lymphocyte reaction (MLR). The reaction occurs when lymphocytes from individuals that differ at their MHC are cultured together. Both sets of lymphocytes are activated when they recognise the opposing foreign MHC. The activated lymphocytes differentiate into large effector cells. In a one-way MLR only the responder lymphocytes are activated and differentiate into

effector cells. Different methods can be used to incapacitate the stimulator cells. Irradiation inactivates the stimulator cells while mitomycin C kills the stimulator cells.

1.4.2 The Discovery of the Mixed Lymphocyte Reaction

1.4.2.1 Cell types implicated in the MLR

The basis for the MLR originates from a technique developed by Nowel in 1960 that was subsequently used to culture lymphocytes. This technique involved the preparation of a culture of human blood leukocytes with a plant extract called phytohemagglutinin (PHA). Upon exposure of the leukocytes to the PHA, the leukocytes began to undergo mitosis, which was evident from the developing mitotic bodies. The general consensus was that the small lymphocytes in the leukocyte population were responding to the PHA and this method was subsequently used as a technique to culture lymphocytes. This technique prompted the development of the concept of the mixed lymphocyte reaction (MLR).

A year after the work by Nowel in 1960, Shrek and Donnelly embarked on an investigation of the morphology of the lymphocytes of patients with leukemia, without the use of PHA (Shrek and Donnelly, 1961). They observed the presence of "large cells with large nuclei and prominent nucleoli" in a single blood smear prepared five days after culture. The authors reported that this blood smear was derived from an *in vitro* culture that contained a mixture of blood from two different individuals. This report prompted Bain, Vas and Lowenstein to explore in 1964 whether they could reproduce the results obtained by Shrek and Donnelly with the *in vitro* mixing of leukocytes from unrelated pairs of human individuals in the absence of PHA. After five days of culturing the leukocytes from unrelated individuals the authors prepared stained blood smears of the cultures. They noticed that some of the leukocytes developed into large basophilic cells, which were at different stages of mitosis. The authors suggested that these large cells that were observed *in vitro* resembled cells that "developed *in vivo* in lymph nodes and spleens in response to the injection of soluble antigens and following skin homografts" (Bain et al., 1964). This suggestion provided the first evidence of the link between the mechanism of allograft rejection and the MLR. The authors noted that the large cells only appeared when leukocytes from one individual were cultured with the leukocytes from another individual.

There was no effect when leukocytes were cultured with the either the plasma, erythrocytes or platelets from another individual. Thus, it was that Bain and colleagues coined the phrases mixed leukocyte culture and mixed leukocyte reaction since only the mixing of the leukocytes could initiate this reaction. In 1967 Wilson obtained a similar result using the lymphocytes of rats, which is explained further below. Thus, the mixed leukocyte culture and the mixed leukocyte reaction soon became known as the mixed lymphocyte culture (MLC) and the mixed lymphocyte reaction (MLR)

1.4.2.2 Quantifying the proliferation of cells in the MLR

The effect of mixing the leukocytes of unrelated individuals in culture was evident by the increase in the size of cells observed in the blood smears (Bain, Vas and Lowenstein, 1964). In addition to the observation of the increase in cell size as an indicator of the MLR the authors quantified the effects by determining cell proliferation. Cell proliferation was visualised through the use of tritiated (^{3}H)-thymidime autoradiographs. As the proliferating cells were undergoing mitosis the radioactive thymidine was incorporated into the DNA of these cells. The authors found that the appearance of the large cells correlated with the uptake of radioactive thymidine. Notably they showed that the proliferation of the cells occurred only when leukocytes from different individuals were cultured together and not when leukocytes from one individual were cultured with the plasma, erythrocytes or platelets from another individual. However, there was some degree of variation in the leukocyte responses between the individual experiments. The reason for this observation became clearer with later studies from animal models discussed below (Wilson et al., 1967).

1.4.2.3 The Mixed Lymphocyte Reaction: a consequence of MHC disparity

A second important observation made by Bain, Vas and Lowenstein in 1964, was that a response only occurred when the leukocytes of unrelated individuals were cultured together and not when the leukocytes of monozygotic twins were cultured together. The results they observed by mixing the lymphocytes from dizygotic twins were variable. These findings implied that genetic differences between the individuals from whom the blood leukocytes were taken were partly responsible for the leukocyte reaction. The different responses

generated in the *in vitro* MLC appeared to reflect the results obtained in the *in vivo* skin transplantation experiments undertaken by Medawar in 1944. The lack of response observed when the lymphocytes of monozygotic twins were mixed together (Bain, Vas and Lowenstein, 1964) mirrored the acceptance of skin autografts in rabbits (Medawar, 1944). In contrast, the reaction observed when the lymphocytes of unrelated individuals were mixed together in the MLC reflected the rejection of allografts by a heterogeneous population of rabbits as described by Medawar in 1944. Based on their observations Bain, Vas and Lowenstein cautiously suggested that the MLR could be used to determine alloreactivity between donor and host and a lack of response may indicate histocompatibility between donor and host.

In the same year, 1964, that Bain, Vas and Lowenstein published their paper, Bach and Hirschhorn published their work on the culture of blood lymphocytes from three different individuals (Bach and Hirschhorn, 1964). The authors knew the immunological cross reactivity of the individuals beforehand. Their colleagues in the surgery department who were trying to determine donor and recipient combinations for kidney allografts provided this information. The surgeons had sensitised recipients with white cells from the three donors. Thereafter, the recipients received skin transplants from the other two donors. Two of the donors proved to be strongly cross-reactive. It was suggested at the time that these two individuals shared histocompatibility antigens. Thus, in 1964, Bach and Hirschhorn cultured the lymphocytes of the three donors in pairs, in all combinations. They compared the presence of large cells and the mitotic bodies in the blood smears of each combination. They found that the lymphocyte combination from the pair that was most cross-reactive displayed the lowest *in vitro* percentage of large cells and mitosis. The authors made a similar suggestion to that made by Bain and colleagues that the reaction of mixed cultures might be used to determine potential recipients of kidney allografts.

Numerous animal models were subsequently used to further explore the concept of the MLR. Using the lymphocytes of inbred strains of rats Wilson reported in 1967 that the vigour of the MLR was dependent on the degree of the MHC disparity. By this time, it was well appreciated that inbred strains of animals were homozygous and displayed a distinct

haplotype. Thus, the response of their lymphocytes to antigens would be uniform. The results Wilson obtained, attested to this. The rejection or acceptance of an allograft in rats is determined by the Ag-B histocompatibility gene locus. The authors first demonstrated that the response of the lymphocytes of individual rats from a single inbred strain mixed with the lymphocytes of the same F1 hybrid rats was consistent. However, the mixing of lymphocytes from individual rats from different strains that differed at the Ag-B locus, with the lymphocytes from the same F1-hybrid rats resulted in varying degrees of responsiveness. The combination of blood lymphocytes from rats that were more distantly related exhibited a stronger proliferative response, which was indicated by an increase in large cells and a higher level of thymidine incorporation. The conclusion from this study was that the greater the disparity of the MHC between the individuals from whom the lymphocytes were obtained the greater the number of large cells observed. The earlier studies of Bain reported in 1964, in which the mixing of human leukocytes produced varying degrees of responsiveness could thus be associated with the degree of MHC disparity between the human donors.

Secondary to the results obtained above, Wilson, as reported in 1967, examined the kinetics of the MLR. In the early stages of the MLR up until day four the reaction between the lymphocytes was minimal as observed by the low numbers of large cells. Thereafter, the response increased significantly peaking at day seven.

1.4.3 One-way Mixed Lymphocyte Reaction

At that time the authors were aware that F1-hybrid strains of animals did not mount an immune response against an allograft from the parental strains (Medawar, 1944). In 1967 Wilson and colleagues observed a similar unidirectional response in the MLR when the lymphocytes from DA male rats were cultured with the lymphocytes of F1, DA/BN male rats. All the lymphocytes undergoing mitosis were from the DA male rats and not from the F1 hybrid rats. This became a means to investigate the activity of just the responders within the mixed culture. The lymphocytes of the F1 hybrid rats were regarded as the stimulators. Subsequently this unidirectional reaction soon became known as a one-way MLR. There are other methods besides the use of F1 hybrids as stimulators that can be used to render

the stimulators incapable of activation and proliferation or kill the cells. These include the use of irradiation as well as the use of mitomycin C (Bach and Voynow, 1966), which binds to DNA by alkylation and interferes with mitosis (Verwij and Pinedo, 1990).

1.4.4 The Effector arm of the MLR

The role of lymphocytes in allograft rejection was extensively investigated in the 1950's and led to the idea that lymphocytes could be cytotoxic *in vitro*. This was demonstrated when blood lymphocytes from dogs that had received kidney allografts exerted a cytotoxic effect on the renal cells of the donors (Govaerts, 1960). Further evidence of the cytotoxic effect of lymphocytes was demonstrated in mice. Mouse lymphocytes sensitized with allogeneic cells surrounded their target cells with subsequent cytopathic changes and cell death (Rosenau and Moon, 1961).

Thus, by the time that the concept of the MLR emerged it was known that lymphocytes were responsible for rejecting allografts in vivo and that they were able to exert cytotoxic activity in vitro. Thus, research was undertaken to determine if the large cells produced in the MLR exerted an effector function. It was purported that if the cells generated in the MLR were functionally capable then the link between the MLR and the *in vivo* rejection of an allograft would be indisputable. Thus, in 1970 Hayry and Defendi reported their findings on the function of the large cells observed in the MLR. In order to determine this, the authors cultured the peripheral blood lymphocytes of either DBA/2 or C3H mice with the peripheral leukocytes of F1, DBA/2 x C3H mice. The lymphocytes sensitised *in vitro* were then incubated on day 6 with ^{51}Cr-labelled target cells. The lymphocytes killed the target cells, which was evident by the release of chromium. In addition to the effector function of the cells produced in the MLR, they also displayed specificity and memory in their function. Only target cells that displayed the priming antigen were destroyed.

Thus, the *in vitro* alloreactivity in the MLR became fully identified with *in vivo* allograft rejection. The MLR was synonymous with the immune reaction and the cytotoxic activity of the cells of the MLR was seen as the cellular effector arm of allograft rejection.

1.5 The Immunologically Competent Lymphocyte

1.5.1 The role of lymphocytes in immunity

In the early twentieth century tumour transplantation and healthy tissue transplantation were separate fields with little to no collaboration between investigators in these fields. In retrospect, had the researchers collaborated earlier, it is possible that they would have gained rapid understanding.

It was under these circumstances that Murphy undertook tumour transplantation experiments that implicated a role of lymphocytes in allograft rejection (Murphy, 1913). Murphy initially tried, albeit unsuccessfully, to transfer adult chicken tumours into adult chicken hosts. He did, however, demonstrate in a series of experiments that rat tumours inoculated into chick embryos before day eight of gestation could establish themselves unhindered. The chicken embryos appeared to be tolerant to the rat tumours. However, when the rat tumours were grown in chicken embryos in close proximity to secondary lymphoid tissue such as the spleen or bone marrow from adult chickens, their survival rate was considerably reduced. Although both the adult chicken spleens and bone marrow elicited a rapid influx of lymphocytes around the tumour, the adult chicken spleens resulted in a more rapid destruction of the rat tumours than the bone marrow grafts. Murphy surmised that the adult chicken spleens and bone marrow provided some type of defense against the rat tumour. Moreover, this defense appeared to be similar to that generated in adult chickens against adult chicken tumours. He drew the general conclusion that the rejection of the tumour allograft coincided with an increase in lymphocytes into the tumour area. He however, did not conclude that the lymphoid cells were the cause of the regression of the rat tumour.

Murphy's contemporary Apolant (Brent, 1997) provided further evidence of the role of the spleen cells in tumour regression by demonstrating that transplantable tumours survived if the spleen was removed. Furthermore, white mice that were initially resistant to a transplanted tumour became susceptible to it after exposure to radiation. The radiation depleted the circulating lymphocytes thereby rendering the mice susceptible. These

observations led Murphy to surmise that lymphocytes may have a role in preventing tumor growth.

Despite Murphy's findings there was a 50-year hiatus before lymphocytes became the subject of interest in graft rejection. One reason for this void in lymphocyte research was that most immunologists at that time believed that antibodies were the primary mediators of the acquired immune response. As late as the early 1960's lymphocytes were still regarded as hematopoietic stem cells. It was believed at the time that the lymphocytes entered the bone marrow and became the stem cells from which the erythrocytes and granulocytes originated.

Gesner and Gowans, undertook a series of experiments the findings of which were reported in 1962, to investigate this theory (Gesner and Gowans, 1962). It was known at the time that the transfer of bone marrow could rescue lethally irradiated animals. Thus, the authors surmised that if lymphocytes gave rise to bone marrow, then the inoculation of the lymphocytes into lethally irradiated animals should give rise to new bone marrow which would lead to the survival of the animal. Subsequently, they inoculated lethally irradiated CBA mice with either isologous thoracic duct cells or with isologous bone marrow cells. Thoracic duct cells contained lymphocytes that re-entered the blood stream. Only the mice that received the bone marrow cells survived and displayed extensive hematopoiesis in the red pulp of the spleen and the bone marrow. The mice that received isologous thoracic duct cells showed considerable increase in large lymphocytes particularly in the white pulp of the spleen but no evidence of hematopoiesis in bone marrow. These mice died within 9 to 12 days. The findings of this study suggested that thoracic duct cells or lymphocytes were not associated with hematopoiesis but may have some other yet to be determined role.

Gesner and Gowans thus wanted to determine the immunological function of the thoracic duct cells or the lymphocytes. They wanted to determine if the ability of lethally irradiated mice to reject an allograft can be restored by the inoculation of thoracic duct cells. Thus, the authors inoculated lethally irradiated mice with either rat bone marrow cells, or with rat bone marrow cells and isologous thoracic duct cells. The mice that received only rat

bone marrow cells showed signs of hematopoiesis and survived. In contrast, the mice that received both bone marrow and thoracic duct cells did not survive. The thoracic duct cells destroyed the allogeneic bone marrow cells. The inoculated thoracic duct cells were able to restore the ability of the lymphoid tissue to reject an allograft.

The work of Gowans and Knight proved to be critical in cementing the importance of the lymphocyte in graft rejection (Gowans and Knight, 1964). They used inbred strains of rats to demonstrate that "small" lymphocytes circulate from the blood to the lymph and back to the blood. In order to demonstrate this thoracic duct cells were obtained from rats and labelled with tritiated compounds. The large lymphocytes were labelled with tritiated thymidine while both the large and small lymphocytes were labelled with tritiated adenosine. Two groups of rats from the same inbred strain as the donors of the thoracic duct cells were transfused with labelled thoracic duct cells. At various time points the rats from one group were killed and with the aid of autoradiographs, the position of the labelled lymphocytes was determined. The thoracic ducts of the second group of rats were cannulated to determine if the labelled thoracic ducts cells injected into the blood stream entered the lymph nodes. The authors found that the small lymphocytes preferably trafficked from the blood through secondary lymphoid tissue such as the lymph nodes, the white pulp of the spleen and the Peyers's patches of the intestine. The preferential migration of the small lymphocytes through the spleens and the lymph nodes made it easier for researches to obtain large numbers of these cells to further investigate their role in immunity.

1.5.2 The thymus: the site of development for the immunologically active lymphocyte

Although a number of researchers were exploring the function of the thymus at the same time it was the work of J.F.A.P. Miller that provided the most notable contributions to the discovery of the importance of the thymus as a site for the development of the immunologically active lymphocyte. In his initial experiments in 1959, Miller embarked on a series of investigations to determine whether leukemia would develop in a low leukemic mouse strain such as the C3H mouse by the inoculation of leukemic tissue obtained from the high-leukemic mouse strain, the AK mouse. The results he obtained were

consistent with the original findings of Ludwik Gross in that the C3H strain developed leukemia. Miller was aware that lymphocyte leukemia occurred in the thymus and that adult thymectomy prevented the development of the cancer in both the low and the high leukemic strains of mice. These findings led him to explore the role of the thymus in the development of the virus-induced cancer (Miller, 1959). He undertook experiments in which low leukemic mice, C3Hf/G mice, were inoculated with leukemic tissue extracts at birth and then thymectomised three to four weeks later. None of these mice developed leukemia. However, grafting these thymectomised mice with syngeneic thymus tissue up to six months later lead to the development of leukemia. Miller postulated that the virus initially replicated in the thymus and then remained dormant post thymectomy in other tissues. Although this proved not to be the case, it became clear to Miller that the thymus played a role in the development of an immune response. A chance meeting with Medawar provided Miller with the insight to undertake neonatal thymectomies. Thus, neonatal mice were thymectomised and inoculated with the virus and the effect of the absence of a thymus at birth was assessed on the development of leukemia. An important finding from these experiments was the wasting disease observed in the thymectomised neonatal mice, which was accompanied by a considerable reduction in lymphocytes. Of critical importance was the finding that the mice thymectomised at birth and who received skin allografts at six weeks of age did not reject grafts. The grafts in fact, exhibited "luxuriant crops of hair". In stark contrast, mice that were thymectomised at three weeks of age did not show any impairment of the immune response to allografts. Thus in 1961 (Lancet) Miller hypothesised: *"and, contrary to the prevailing opinion, I postulated that during embryogenesis the thymus would produce the originators of immunologically competent cells many of which would have migrated to other sites at about the time of birth. This would suggest that lymphocytes leaving the thymus are specially selected cells"*.

A series of experiments followed to test experimentally his hypothesis (Miller, 1962) and to confirm his original findings on the acceptance of the allografts by the mice thymectomised at birth. Based on the findings of Gowans in 1962 who demonstrated that lymphocytes were critical role players in the rejection of allografts, Miller surmised that his thymectomised neonatal mice with their reduced lymphocyte population would accept

an allograft. He thus transplanted skin grafts from a variety of mouse strains with different degrees of histoincompatibility onto his thymectomised neonatal mice six weeks after birth. The mice accepted the allografts. The removal of the thymus soon after birth greatly impaired the immune response to the allograft. However, neonatal mice that were thymectomized at birth and then received syngeneic fetal thymuses two weeks later rejected skin allografts within fifteen days.

Independent work by Good and colleagues on the role of the thymus in immunity was published in the same year as the initial work described by Miller in 1962. The former group of authors thymectomized rabbits five days after birth and challenged them with BSA seven weeks later. The control group of rabbits that were not thymectomized readily produced antibodies to the BSA while none of the thymectomized rabbits produced antibodies (Good et al., 1962). The cumulative findings of Miller and Good provided evidence of the importance of the thymus in immunity.

1.5.3 Functional differences between thymus-derived and bone marrow-derived lymphocytes in the production of antibodies

Once the significance of lymphocytes in immunity became apparent cellular immunology advanced rapidly. Extensive research was undertaken to further the understanding of the role of these lymphocytes. During this time, it was initially thought that the thymus-derived lymphocytes were responsible for both cell mediated immunity and antibody production. This assumption was made when neonatally thymectomized mice were neither able to reject skin allografts or produce antibodies to Salmonella typhi H antigen. However, a series of experiments, as described below, thwarted this hypothesis that the thymus-derived lymphocytes were able to produce antibodies. Hereafter, thymus-derived lymphocytes will be referred to as T cells. In 1966 Claman and colleagues embarked on an investigation to determine if the thymus contained immunologically competent antibody forming cells (Claman et al., 1966). In their first set of experiments the authors inoculated thymocytes together with SRBC into irradiated syngeneic mice. The production of antibodies against the SRBC was not observed and the authors believed that more time was required for the thymocytes to reach maturity. Increasing the time unfortunately led to the demise of the

mice. The authors were aware that radiation sickness was common in individuals that were irradiated and that the transfer of bone marrow may prolong their lives. Thus, they inoculated the mice with thymocytes, SRBC and syngeneic bone marrow. What ensued was a robust anti-SRBC antibody response. As a result, the authors demonstrated that both the bone marrow and the thymus-derived lymphocytes were required for the development of an antibody response.

The findings of Claman in 1966 led Miller to revisit his initial hypothesis that the thymocytes were responsible for producing antibodies. Thus, followed a series of experiments by Miller and Mitchell in 1968 in this regard. The authors began by thymectomising newborn CBA mice, which were inoculated with either SRBC only, or a combination of SRBC and syngeneic thymocytes or thoracic duct cells three to five weeks later. They demonstrated that both syngeneic thymocytes and thoracic duct cells rescued the antibody response to SRBC. Furthermore, they were able to demonstrate that semi-allogeneic thymocytes and thoracic duct cells produced a similar result to that of the inoculum containing the syngeneic cells. The use of semi-allogeneic thymocytes and thoracic duct lymphocytes allowed the authors to determine the origin of the cells responsible for the production of antibodies. The neonatally thymectomised CBA mice were thus inoculated with thymocytes or thoracic duct cells from C57BL or F1 (CBA x C57BL) mice. The number of antibody producing cells was assessed by an *in vitro* plaque-forming cell (PFC) assay. The single cell suspension of the spleen cells of the neonatally thymectomised CBA mice was layered onto an agar plate together with SRBC and complement. Antibody producing cells specific for SRBC in the spleens of the CBA mice would produce antibody that bound to the SRBC, which would subsequently be lysed by complement. A clear zone or a plaque would form in the area of the lysed cell. Thus, using anti-H-2 isoantisera they were able to identify that the cells responsible for the formation of the PFC originated within the recipient. The anti-CBA serum reduced the number of PFC by 98-100% in comparison to the anti-C57BL serum that showed no reduction in PFC. The authors, surmised that the semi-allogeneic cells provided a helper function in the production of antibodies. Following on these experiments the authors then set out to determine the cell population responsible for the production of antibody to SRBC in the

CBA mice. Lethally irradiated thymectomised mice were inoculated with syngeneic bone marrow. Two weeks later the mice received F1, (CBA x C57BL) thoracic duct lymphocytes and SRBC. This resulted in the formation of anti-SRBC PFC. After their series of experiments Miller and Mitchell concluded that the thymocytes co-operated with the bone marrow in the production of antibodies and that the bone marrow contained cells responsible for the production of antibodies.

1.5.4 A morphological difference between thymus-derived and bone marrow lymphocytes

Clear evidence emerged that the thymocytes and bone marrow derived lymphocytes were functionally different, and this led Roitt in 1969 to designate the thymus-derived cells as T cells and the bone marrow derived lymphocytes as B cells (Roitt et al., 1969). Thus, it became essential to find a marker that could distinguish the T cells from the B cells. The theta (θ) antigen, which was initially described by Reiff and Allen, (1966) was found exclusively on thymocytes and the brain and also to a lesser extent expressed by peripheral lymphocytes. Subsequently Raff suggested in 1969 that θ could be used as a possible surface marker that could distinguish the T and B cells. Thymocytes were known to circulate through the lymph nodes and the spleen. Raff demonstrated that 80-90% of the lymphocytes from the lymph nodes and 30-50% of the spleen expressed the θ marker. A year later Raff (1970) was able to distinguish T cells from B cells using indirect immunofluorescence. He accomplished this by confirming the expression of θ on the surfaces of T cells only and the immunoglobulin expression on the surfaces of B cells only.

1.5.5 T cells as mediators of cytotoxicity

The cytotoxic role of the T cells was highlighted by the work of Cerottini and colleagues in 1970. At this time the GVHR was well described demonstrating an influx into and the repopulation of the spleens of the recipients by the donor cells which led to the destruction of the recipient's cells. Thus, the authors injected lethally irradiated DBA/2 mice with spleen cells from C57BL/6 mice. The cytotoxic T cells of the C57Bl/6 mice that had repopulated the spleens of the DBA/2 mice were able to lyse ^{51}Cr-labelled DBA/2 target

cells. The cytotoxic effect of the recipient spleen cells was abrogated when mixed with AKR anti-theta serum and complement. Theta is a marker expressed by T cells.

Thus, the cumulative work of many researches provided clear evidence that the bone marrow contained cells responsible for the production of antibodies while the thymus contained cells that assisted the bone marrow cells in the antibody production. Furthermore, the activated T cells could be cytotoxic.

1.5.6 Suppression and Promotion of the antibody response: the conflicting roles of T cells

Before the discovery that two distinct T cell subsets existed, the CD4 T cells and CD8 T cells (Cantor and Boyse, 1975), research emerged of the discordant roles of T cells in the production of antibodies. In their first set of experiments Gershon and Kondo used thymectomised, lethally irradiated and bone marrow reconstituted mice that received SRBC for 30 days. These mice were unable to produce antibody to SRBC during the 30 days and were considered tolerant. This finding suggested that the absence of thymocytes prevented the production of antibody by the bone marrow cells. Four days after the end of treatment the mice received thymocytes that reversed the initial effects. They were able to produce anti-SRBC antibodies (Gershon and Kondo, 1970). This result confirmed the earlier findings of Claman in 1966 and Mitchell and Miller in 1968 that both bone marrow cells and thymus cells are required for antibody production.

The authors undertook further experiments utilising the same protocol as above but with the addition of thymocytes at the initiation of the experiment (Gershon and Kondo, 1971). In this instance the mice were unable to produce antibodies even after receiving the second dose of thymocytes. The potential inhibition of the antibody response provided an alternate function for the thymocytes. They thus displayed a dual role; one of promoting the antibody response and one in suppressing the antibody response. The same authors in 1971 further cemented the idea that thymocytes were able to inhibit antibody production. They tolerized mice to SRBC and then adoptively transferred the spleen cells of the tolerized mice into syngeneic mice. The adoptively transferred lymphocytes prevented the production of

antibody in these mice. It was suggested that the adoptively transferred lymphocytes prevented the interaction between the recipient's thymocytes and their bone marrow cells. It was however only in their third paper in 1972 that Gershon used the term suppressor cell for the T cells suppressing the antibody response. Furthermore, they concluded that two populations of T cells might exist, one that suppresses the antibody response and one that enhances the antibody response.

1.5.7 The Discovery of Distinct T cell populations

T lymphocytes are composed of two distinct subsets, the CD4 T cell and CD8 T cell subsets that display a helper and a cytotoxic phenotype, respectively. This idea evolved during the early 1970's with the identification of the surface marker, Ly. The Ly antigens are expressed by T cells only and not by non-lymphoid cells. The Ly antigens are divided into three types, namely Ly-1, Ly-2 and Ly-3. Experiments with neonatally thymectomised mice showed a reduction in peripheral lymphocytes expressing the Ly antigens confirming that the expression of the Ly antigens are restricted to the thymus-derived cells.

T lymphocytes were known to be instrumental in different immune responses upon recognition of antigen such as the production of antibody and the rejection of allografts. In 1975 Cantor and Boyse wanted to determine if the different functions exhibited by T cells was reflective of the presence of different T cell subsets before exposure to antigen. Thus, using the surface marker Ly they explored if a single T lymphocyte population was heterogenous before stimulation. Using Ly antisera, they were able to show the existence of different T lymphocyte populations based on the expression of these Ly antigens. Depletion of Ly-2^+3^+ cells from the spleens of B6 mice inoculated into B6 x BALB/c mice, failed to generate cytotoxic T cells against BALB/c targets, while they produced PFCs to SRBCs upon immunisation with SRBCs. In contrast, depletion of Ly-1^+ cells from the spleen cells of B6 mice generated cytotoxic T cells against BALB/c targets, while they failed to produce PFCs to SRBCs upon immunisation with SRBCs. Thus, it was demonstrated that the Ly-1^+ T lymphocytes exhibited primarily a helper phenotype before antigen exposure whereas the Ly-2^+ T lymphocytes had a reduced helper phenotype with a dominant cytotoxic phenotype (Cantor and Boyse, 1975). The new nomenclature used

today denotes the Ly-1^+ cells as being the CD4 T cells while the Ly-2^+3^+ cells are considered to be the CD8 T cells. Thus, Cantor and Boyse demonstrated on the basis of the Ly-surface markers that T lymphocytes were grouped into two subsets with distinct functions. The Ly-1+, Ly-2+ and Ly3+ surface markers which are denoted as CD5, CD8α and CD8β by the current nomenclature of cluster differentiation. The CD5 marker is expressed by both CD4 and CD8 T cells but at a higher level of expression by the CD4 T cells. This would seem to clarify the findings of Cantor and Boyse that the Ly-1^+ cell is the CD4 T cell.

1.6 Immune Regulation

1.6.1 A historical perspective of Immune Regulation

The ability to modulate the immune response without any adverse effects has been extensively investigated in numerous situations such as in the hope of inducing tolerance to allografts. The benefits thereof are huge and ultimately would lead to improved quality of life and increased life expectancy. Some researchers believe that the concept of modulating the immune response dates as far back as the tenth century. The Chinese inhaled smallpox crusts from the lesions of infected individuals as a form of vaccination thereby, as we know it today, generating a primed state.

Despite the advancements made in establishing the dual roles of T lymphocytes in immunity there was limited research undertaken on the cell-mediated immune response of these lymphocytes. It was during this period that Parish and Liew undertook a series of experiments in 1972 to determine the relationship between the humoral (antibody) response and the cell mediated immune response of the T lymphocytes to the same antigen (Parish and Liew, 1972). They inoculated adult rats with different doses of cyanogen bromide digest of flagellin over a 28-day period. On day 28 the rats were challenged with 100µg of flagellin in saline which is considered an antibody-inducing dose. The cell-mediated immune response was determined by means of delayed type hypersensitivity (DTH) reactions. The reaction was observed when the footpads of the rats were injected with the antigen, which subsequently became swollen. Antibody production was determined by hemagglutination assays using SRBC coupled with flagellin. A low and a high dose of

antigen resulted in a DTH reaction upon challenge with the antibody-inducing dose of flagellin at day 28. During the DTH reaction a lack of antibody production was observed, while in contrast, a DTH reaction was absent during the antibody response that was generated with an intermediate dose. The authors thus concluded, that the generation of the cell-mediated and the antibody responses tended to be mutually exclusive. These findings expanded upon the original findings of Asherson and Stone who, in 1965 demonstrated that a DTH reaction could be prevented by first inoculating an animal with an antigen precipitated in alum or in a soluble form known to produce antibodies. Two weeks thereafter the animal was inoculated with the antigen in Freund's complete adjuvant known to induce DTH reaction. The secondary response was characterized as an antibody response. Asherson and Stone regarded this observation as the immune response deviating away from the DTH reaction (Asherson and Stone, 1965).

The above findings led to the hypothesis of immune class regulation, which proposed that the cell mediated and antibody responses to the same antigen were reciprocally regulated (Bretscher, 1974), which was subsequently successfully demonstrated by the work of Ramshaw and colleagues (Ramshaw et al., 1977). Based on the work of Cantor and Boyse in 1975, Ramshaw and colleagues used the anti-Ly and the anti-Ia sera to determine whether the T cells that suppressed the antibody response displayed a different phenotype from those that suppressed the cell-mediated response. Using the mouse model, they confirmed the presence of two distinct suppressor T cell subsets. They demonstrated that the suppressor T cell of the antibody response was Ly-1$^-$, Ly-2$^+$ and Ia$^+$. We now know that the T cell expressing Ly-2$^+$ is the CD8 T cell. In contrast, the T cells suppressing DTH or the cell-mediated immune response were Ly-1$^+$, Ly-2$^-$ and Ia$^-$, which are currently considered to be the CD4 T cells associated with an antibody response. Thus, it was shown that CD4 T cells and CD8 T cells reciprocally regulated the mutually exclusive cell-mediated and antibody responses of T lymphocytes, respectively.

1.6.2. The current concept of immune class regulation

The Th1/Th2 concept of reciprocal regulation of distinct classes of immunity by T cells and their cytokines is our contemporary explanation of immune class regulation. In 1986,

Mosmann and colleagues decided to further analyse the CD4 T cell subset using a panel of $Ly1^+$, $L3T4^+$, $Ly2^-$, Ia restricted T cell clones. They demonstrated by the stimulation with Concanavalin A (Con A) that T helper cells could be divided into two distinct subsets based on their expression of specific cytokines. The Th1 subset produced IL-2, IFN-γ, granulocyte macrophage colony stimulating factor (GM-CSF) and IL-3 while the Th2 subset produced IL-3, B cell stimulating factor 1 (IL-4) and IL-10. Different sources provide evidence of distinct functions exhibited by these two subsets. The Th1 group and not the Th2 subset have been shown to express delayed type hypersensitivity (DTH) upon transfer to naïve recipients (Cher et al., 1987). The Th2 subset, in contrast, is the dominant facilitator of B cell antibody production; facilitating the production of IgG1 (Isakson 1982) and IgE (Coffman 1986). Several articles demonstrate the Th1 subset's inability to provide help to B cell for antibody production. However, there is evidence of its role in the production of IgG2a (Snapper and Paul, 1987). Consequently, the Th1 subset is associated with DTH response while the Th2 subset is the main facilitator of antibody production. Furthermore, these functions tend to be mutually exclusive.

The Th1 and Th2 subsets and their unique pattern of cytokines displaying distinct functions are associated with reciprocal regulation of immunity. Evidence of this is through the antagonistic action of their signature cytokines, IFN-γ (TH1) and IL-4 (Th2). Gajewski and Fitch noted that the supernatants from cultures of Th1 clones would inhibit the proliferation of Th2 and not Th1 clones (Gajewski and Fitch, 1988). In contrast, Susan Swain and colleagues reported in 1990 that the addition of IL-4 to CD4 T cells stimulated with Concanavalin A (ConA) suppressed the generation of Th1 cells but enhanced the generation of Th2 cells *in vitro* (Swain et al., 1990).

IL-4 is essential for the activation and proliferation of B cells and was initially referred to as the B cell growth factor, (BCGF) (Howard, et al., 1982). The IL-4 stimulates the B cells to produce IgM and IgG1 antibodies (Boom et al., 1988). In contrast, a study undertaken by Snapper and Paul demonstrated the effects of IFN-γ on the production of murine IgG antibodies (Snapper and Paul, 1987). The authors demonstrated that the B cells from the spleens of DBA/2 mice stimulated with LPS produced large amounts of IgM and IgG2b

and low amounts of IgG1 and IgG2a. However, the addition of high concentrations of rIFN-γ inhibited the production of IgG1, IgG2b and IgE hallmarks of a Th2 immune response. In contrast, rIFN-γ stimulated the expression of IgG2a which has been associated with a Th1 immune response. The inhibitory effect of the rIFN-γ on the production of Th2 antibodies was abrogated by the addition of anti-rIFN-γ antibody. Thus, the cytokines produced by the Th1 and Th2 subsets suppressed the development of the opposing T helper phenotype while enhancing their own.

1.6.3. T helper subsets, their signature cytokines and transcription factors

The transcription factors, T-bet and GATA 3 have been identified as the signature transcription factors of the Th1 and Th2 subsets, respectively. Interaction of cytokines with their respective receptors on the surface of T cells initiates a signalling pathway that involves non-receptor tyrosine kinases called Janus kinases (JAKs) and transcription factors called signal transducers and activators of transcription (STATs). Before we describe the effects of the induction of the transcription factors, T-bet and GATA3 we will briefly discuss the signalling pathways initiated by the IFN-γ and IL-4.

The Th2 cytokines, IL-4 and IL-5 amongst others, bind to the type I cytokine receptors on the T cells while the type II cytokine receptors interact with type I and II interferons and IL-10 as well. The associated JAK tyrosine kinases are activated upon ligation of the receptors with the cytokines. Phosphorylation of the receptor chains occurs resulting in the recruitment of specific STAT proteins to the cytokine receptor. The STAT proteins are phosphorylated, and the dimers formed migrate to the nucleus binding to specific DNA sequences in the promoter regions of cytokine responsive genes and activate transcription factors such as the T-bet and GATA3 (Darnell, 1997). STAT 1 is associated with IFN-γ expression, while signalling through STAT 6 mediates IL-4 expression.

T-bet was isolated by Szabo and colleagues and its importance in the induction of the Th1 phenotype was revealed while searching for the transcription factors involved in the expression of the Th1 cytokines, IFN-γ, TNFβ and IL-2 (Szabo et al., 2000). At that time the region of the IL-2 promoter was characterized. Thus, using a yeast one-hybrid assay

the authors integrated the EGY48 yeast strain with the IL-2-promoter/HIS3 construct, which they had developed earlier. The yeast cells were then transfected with a library of Th1 cDNA fused to an activation domain. A novel protein was identified which they subsequently termed T-bet. The significant link between T-bet and the induction of the Th1 phenotype became apparent when the transcription factor was primarily expressed in Th1 cells and not Th2 cells. This link was further emphasized when naïve transgenic T cells cultures prepared under Th2 polarizing conditions switched to a Th1 phenotype in the presence of overexpressed T-bet. This was evident by the simultaneous loss of IL-4 and IL-5 expression and the activation of the expression of IFN-γ. To determine whether T-bet actually exerted an effect on the *IFNG* gene, EL4 cells, a murine tumour cell line, were transfected with T-bet and stimulated with PMA/ionomycin. The ensuing results led to the hypothesis that T-bet is a potent transactivator of IFN-γ. Many researchers argued that IL-12, a Th1 cytokine was responsible for the induction of T-bet. However, Zhu and colleagues demonstrated in 2000, using a mouse reporter strain for T-bet, that both IFN-γ and IL-12, which signals through STAT 4, were able to induce the expression of T-bet. Thus, T-bet the Th1 signature transcription factor is induced by both IFN-γ and IL-12 and in turn induces the expression of IFN-γ. Thus, IFN-γ has the ability of enhancing a Th1 response.

The signature transcription factor for the induction of a Th2 phenotype is GATA 3 as reported by Zheng and Flavell in 1997. Firstly, the authors observed a high level of expression of GATA3 in the naïve CD4 T cells at the periphery, which is probably due to its requirement in T cell development within the thymus. This contrasts greatly to the expression of T-bet which is inducible in newly activated CD4 T cells primed towards a Th1 phenotype. Furthermore, they noted differences in the level of expression of GATA 3 in naïve CD4 T cells primed towards either a Th1 or a Th2 phenotype. The level of the transcription factor remained high as the cells differentiated towards a Th2 phenotype whereas it decreased considerably in the Th1 cells. Moreover, GATA 3 was shown to promote the Th2 phenotype by being a potent transactivator of the *IL-4* promoter.

What then is the regulatory role of the transcription factors in the current thinking on immune class regulation? One-way in which the concept of immune class regulation can be reconciled, is through the mutual antagonistic action of GATA 3 and IL-12. This would occur if GATA3 inhibits the expression of the IL-12 receptor, independent of the presence of IL-4, preventing the development of a Th1 phenotype. On the other hand, IL-12 signalling through STAT4 inhibits the expression of GATA3, a critical component of Th2 development. However, the introduction of GATA 3 after the development to a Th1 phenotype is unable to prevent the expression of IFN-γ in these cells (Ouyang et al., 1998). The T-bet repression of IL-4 expression was found not to be due to a direct effect since it did not repress the *IL-4* promoter transactivation (Szabo et al., 2000).

Another interesting theory is one of competition. As the immune response evolves from a Th1 to a Th2 immune response as demonstrated by Salvin in 1958, the level of IL-10 increases. The Th1 cytokine, IFN-γ and IL-10 produced by Th2 cells compete for the same cytokine receptors, cytokine receptor II, on the surface of cells.

1.6.4 Factors that influence the Th1/Th2 phenotype of CD4 T cells

1.6.4.1 Antigen Dose

The dose of the antigen was one of the first factors identified that influenced the type of immune response generated. In 1958 Salvin inoculated guinea pigs with diphtheria toxin or OVA protein in incomplete Freund's adjuvant (Salvin, 1958). The guinea pigs displayed delayed type hypersensitivity (DTH), a Th1 immune response, which transitioned into an antibody response over a certain period of time. The dose of the antigen determined the rate at which the transition from DTH to antibody response occurred. A low dose of antigen promoted a DTH response and resulted in a significant delay in the generation of the antibody response. In contrast, a high dose of antigen elicited a rapid antibody response with a near imperceptible DTH. Several studies using different antigens such as *L. major* (Menon and Bretscher, 1998), BCG (Power et al., 1998), simian immunodeficiency virus (SIV) (Dittmer et al., 1996) and in neonatal mice infected with murine leukemia virus (Sarzotti et al., 1996) have reported similar findings to that of Salvin in 1958. In each of

the studies above a low dose of antigen was associated with a Th1 immune response while a high dose of antigen generated a Th2 immune response.

1.6.4.2 The number of responding CD4 T cells

A study reported by Ismail and Bretscher demonstrated that the number of responding CD4 T cells had a significant effect on the Th1/Th2 response generated against an antigen (Ismail and Bretscher, 2001). Lethally irradiated mice were reconstituted with different numbers of syngeneic spleen cells. Thereafter the mice were immunized with a dose of SRBC known to elicit a mixed Th1/Th2 immune response. The mice that received a low number of spleen cells and more specifically a low number of CD4 T cells generated a predominant Th1 immune response, while higher numbers of CD4 T cells generated a predominant Th2 immune response to SRBC.

1.7 The interaction between the CD4 T cell and CD8 T cell

1.7.1 CD4 T cell help required for activation of naïve CD8 T cells

As described earlier naïve CD8 T cells are activated through signalling of their TCR with the MHC class I-peptide complex and the interaction with costimulatory molecules on the surface of T cells and APCs. The CD8 T cells take on a cytotoxic phenotype and are referred to as cytotoxic T lymphocytes (CTLs) once activated. Different stimuli such as intracellular viruses and bacteria as well as transformed cells and major and minor histocompatibility molecules may induce the development of the CTLs. The literature provides extensive evidence of the role of CD4 T cells in the activation of the CD8 T cell. The type of antigen has been shown to determine whether the CTL response generated would be helper dependent or helper independent. This was largely based on the inflammatory nature of the antigen. It was demonstrated that for the generation of a CTL response to a non-inflammatory antigen such as ovalbumin, CD4 T cell help was a requirement (Bennett et al., 1998). The CD4 T cell help was also demonstrated for a CTL response against allo-MHC (Schoenberger et al., 1998, Via et al., 1987). In contrast, the CTL response to inflammatory antigens such as the ectromelia virus (Buller et al., 1987) and the influenza virus (Lui and Mullbacher, 1989) has been shown to be independent of CD4 T cell help. However, there are studies demonstrating that the generation of CTL

responses against inflammatory antigens still require CD4 T cell help. A study by Kast and colleagues in 1986 has demonstrated that the cooperation of both the CD4 and CD8 T cell is required for the elimination of a lethal Sendai virus.

It appears that with certain inflammatory antigens CD4 T cell help is not a requirement. However, on secondary exposure of the antigen the initial CD4 T cell help was shown to be critical for the establishment of memory cells as assessed by a secondary challenge (Shedlock and Shen, 2003; Sun and Bevan, 2003). The primary CTL responses that did not require CD4 T cell help generated impaired CTL responses to secondary exposure to antigen. Thus, it appears that although the primary CTL response may not always require CD4 T cell help, it is critical for a robust effective secondary CTL response.

It has been suggested that the help afforded to the CD8 T cell by the CD4 T cell is in the form of the IL-2 cytokine. This idea evolved in the context of an *in vitro* system in which cytolytic T lymphocyte precursors with a Ly-2^{+} marker required the addition of IL-2 in order to proliferate (Erard et al., 1985). However, it was demonstrated in IL-2 knockout mice that islet cell allografts were rejected (Steiger et al., 1995). This led to the suggestion that a soluble factor may not be the only requirement in the generation of CTLs. Thus, it was suggested that cell-to-cell contact could be a requirement. The interaction between the CD40L expressed on activated CD4 T cells and the CD40 expressed by mature dendritic cells was demonstrated as being a possible mechanism (Bennett et al., 1998). The interaction between the CD40L and the CD40 presumably licenses the dendritic cell to activate the CD8 T cells.

1.7.2. Linked recognition of CD4 and CD8 T cells: a requirement for CD8 T cell activation

The CD40-CD40L interaction led to the suggestion that linked recognition may be a requirement for providing help. A study was undertaken to explore the need for linked recognition in the generation of CTLs (Keene and Forman, 1982). The authors demonstrated that unprimed CTLs specific for the Qa-1^{b} alloantigen, needed to be primed in vivo with two kinds of epitopes in order to generate a secondary CTL response in vitro.

They observed that female B6.Tla^{a} (Qa-1^{a}) stimulated with congenic spleen cells from female B6 (Qa-1^{b}) donors could not mount an anti-Qa-1^{b} CTL response. The inability of these cells to generate a CTL response was not due to a deficiency in CTL precursor cells. Priming the B6.Tla^{a} mice with allogeneic spleen cells from A.BY mice resulted in anti-Qa-1 CTL activity in vitro. Thus, the allogeneic cells provided some sort of help. Thus the authors decided to use male B6 (Qa-1^{b}) expressing the H-Y antigens to prime the female B6.Tla^{a} (Qa-1^{a}) mice. The result was that the female B6.Tla mice spleen cells generated a CTL response specific to the Qa-1 molecule.

1.7.3 The role of CD8 T cells in suppressing the antibody response

Effector CD8 T cells kill infected and transformed cells via two pathways. Killing of the target cells is mediated by the production of cytolytic granules such as perforin and granzyme (Badovinac et al., 2000; Lobe et al., 1986, Podack et al., 1991) or by the Fas/FasL (ligand) interaction (Kataoka et al., 2001; Russell and Ley, 2002). CD8 T cells also produce IFN-γ (Fong and Mosmann, 1990). In order to exert its regulatory effect, CD8 T cells require CD4 T cell help to become fully activated effector cells as described in 1.7 (Bennett et al., 1998; Shedlock and Shen, 2003; Sun and Bevan, 2003).

As described in 1.5.6 of the introduction several studies demonstrated that T cells were able to either suppress or stimulate the antibody response (Gershon and Kondo, 1970; Gershon et al., 1972). A study by Basten and colleagues in 1974 provided further evidence of T cells suppressing the antibody response. Mice were rendered tolerant to fowl immunoglobulin by the inoculation of a high dose of deaggregated immunoglobulin, which was known to elicit a tolerant state. Tolerance at that time was considered to be an absence of an antibody response to the antigen, although a cell-mediated immune response may have occurred. Tolerant spleen cells were transferred into irradiated syngeneic hosts. The host mice were unable to mount an antibody response against the fowl immunoglobulin when challenged with the antigen, demonstrating the successful transfer of tolerance. The role of the T cells in suppressing the antibody response was confirmed when spleen cells of the tolerant donor were treated with anti-Thy1 antibody prior to being adoptively transferred. The syngeneic recipients of these spleen cells were able to mount an antibody

response upon challenge with the fowl immunoglobulin comparable to that of the controls. Notably the transfer of a high enough number of, tolerant spleen cells, 25 x 10^7, was able to significantly reduce the PFC in normal mice when challenged with the antigen. This finding suggests that a high number of suppressor T cells were required to inhibit antibody production in mice that had not been irradiated. To test whether B cells were tolerant, the tolerant spleens were treated with anti-Thy1 serum to remove the T cells and then transferred with normal T cells into the syngeneic hosts. A strong antibody response to fowl immunoglobulin was generated in these mice. This suggested that tolerance operated only at the T cell level.

In addition to the *in vivo* studies demonstrating the T cell suppression of the antibody response, *in vitro* studies were also undertaken (Rich and Pierce, 1974). The authors demonstrated that mouse spleen cells activated with a non-specific plant mitogen, Con A produced a highly immunosuppressive factor in the supernatant. The addition of this supernatant to a culture containing mouse spleen cells and SRBC inhibited the antibody response to SRBC. These findings suggest that a soluble factor obtained from Con A-activated T cells is able to suppress the antibody response.

After the discovery of two distinct T cell populations, the CD4 and CD8 T cells (Cantor and Boyse, 1975) it soon became apparent that the delayed type hypersensitivity reaction and antibody responses were reciprocally regulated by the CD4 and CD8 T cell, respectively (Bretscher, 1972; Ramshaw et al., 1977). Thus, although suppression of the immune response can occur in either direction, our study focuses on the effect of CD8 T cells on CD4 T cell differentiation. Therefore, in addition to its ability to lyse infected target cells the CD8 T cell gained prominence in the 1970's as the suppressor cell of the antibody response.

Susceptibility to *Leishmania (L) major* is attributed to an ineffective antibody response. A study undertaken by Herath and colleagues in 2003 demonstrated the means by which CD8 T cells suppressed the antibody response and ensured resistance to the intracellular pathogen. Three groups of BALB/c mice were inoculated with 2 x 10^6, of *L major*

promastigotes, a dose which would render the mice susceptible to the pathogen. At the time of infection one group of mice received a single injection of CD4 monoclonal antibody (mAb). The transient depletion of the CD4 T cells appeared to provide protection against the pathogen. Reducing the number of responding CD4 T cells has been shown to generate a cell-mediated immune response (Ismail and Bretscher, 2001). The second group of mice received a single dose of CD4 mAb and anti-CD8 mAb twice a week for the duration of the infection. The third group was the control group that were only infected with the pathogen. The first group of mice in which the CD4 T cells were transiently depleted, were able to clear the infection. In contrast the control group and the group in which the CD8 T cells were depleted were unable to clear the infection. One month after the initiation of the infection the cells of the lymphoid organs were restimulated with the pathogen for 6 days. In the first group the number of CD8 T cells increased considerably compared to the control group. The authors also found that in the absence of the CD8 T cells a reduction in both the IFN-γ production as well as the frequency of IFN-γ -producing CD4 T cells was observed. The authors suggested that the CD8 T cells allows for an increase in IFN-γ and more importantly regulates the increase of IFN-γ by the CD4 T cells that confers protection.

Moskophidis and colleagues demonstrated in 1992, the role of CD8 T cells in suppressing the development of an antibody response against the lymphocytic choriomeningitis virus (LCMV) virus. In this study the authors used transgenic mice displaying a TCR specific for the 32-42 LCMV gp1 epitopes in association with H-2D^{b}. The mice were infected with a high dose of the LCMV strain. A strong CD8 CTL immune response ensued with no detectable antibody forming cells (AFC). It could be suggested that the high dose allowed for a strong CTL response, which was sufficient to clear the virus and inhibited the evolution of the immune response into an antibody mode. A low dose of the virus resulted in an antiviral antibody response. Furthermore, to confirm their findings that the CD8 T cells suppressed the antibody response, the authors depleted the mice of CD8 T cells using rat anti-CD8 monoclonal antibody. After the depletion of the CD8 T cells the spleen cells of the transgenic mice exhibited AFC upon infection with LCMV. The authors concluded that the CD8 CTLs were responsible for the suppression of the antibody response against LCMV.

Effector CD8 T cells are able to mount a cell mediated immune response to intracellular pathogens and are able to suppress the antibody response to intracellular pathogens. However, do the CD8 T cells suppress an antibody response to exogenous antigens? In 1992, Tuttosi and Bretscher demonstrated the ability of CD8 T cells to suppress the antibody response to SRBC an exogenous antigen. In order to generate a strong antibody response, mice were immunized initially with a 0.1% suspension of SRBC. A day later they were inoculated with a 100-fold higher dose. The spleen cells from these mice were restimulated in culture with SRBC. To determine the DTH of the spleen cells, the cells were injected into the footpads of the mice and the swelling characteristic of DTH was assessed. The IgG response was also assessed. The spleen cells that were originally generating an antibody response had switched to a cell-mediated immune mode. Thus, it appeared that the antibody response was being suppressed. The authors set out to determine which cells were suppressing the antibody response to the SRBC. They depleted the spleen cells of CD4 T cells using the anti-CD4 GK1.5 antibody. The remaining cells, which contained the CD8 T cells consistently, inhibited the antibody production to SRBC. Thus, CD8 T cells have been shown to suppress the antibody response to an exogenous antigen.

Several studies have demonstrated the role of CD8 T cells in suppressing the antibody response to alloantigens in the GHVD mouse model (Rolink and Gleichmann, 1983; Rus et al., 1995; Tschetter et al., 2000; Via et al., 1987). Two different GVH phenotypes developed in F1 mice inoculated with parental lymphocytes. Lymphocytes from the C57BL/6 mouse generated an acute GVHR in the F1 mice, while lymphocytes from a DBA/2 mouse generated a chronic GHVD characterised by an antibody-mediated autoimmune disease that resembles systemic lupus erythematosus (SLE) in humans in the F1 mouse. The different GVH phenotypes observed in the F1 mice was attributed to differences in the frequency of the CD8 T cells in the parental inoculum (Tschetter et al., 2000; Via et al., 1987). Further investigation into differences in the frequency determined that the frequency of CTL precursors in the spleens of the DBA/2 strain were relatively low i.e. 1/20000 in comparison to the frequency in the spleen of the C57BL/6 strain, 1/1000. (Tschetter et al., 2000) The authors thus concluded that the presence or near absence of CD8 T cells in the donor inoculum determined whether an acute GVH reaction

or whether a chronic GVHD occurred. Furthermore, to confirm the role of the CD8 T cells in generating an acute GVH reaction in the F1 strain, the CD8 T cells were depleted from the C57BL/6 inoculum using anti-Ly-2^{+} serum prior to injection into the F1 mice (Via et al., 1987). Subsequently, the F1 mice developed a chronic GVHD similar to the GVHD observed when lymphocytes from DBA/2 strain were inoculated into the F1 strain. Furthermore, Rolink and Gleichmann demonstrated in 1983 that only CD4 T cells are required to generate the chronic GVHD, while both the CD4 and CD8 T cell are necessary for an acute GVHR. Thus, we can conclude that the CD8 T cells present in the inoculum are able to suppress the antibody response promoted by the CD4 T cells in chronic GHVD.

1.8. The mechanism of antibody suppression by the CD8 T cell

As interest in suppressor T cells gained momentum, extensive research was undertaken to determine the mechanism by which antibody suppression was accomplished. Research into the mechanism suggested that there were two ways in which the antibody response could be suppressed. One way in which the antibody response can be suppressed is by lysing the cells which are critical for antibody production. A second way in which the antibody response can be suppressed is via the production of soluble factors that exert a suppressive effect on the development of a Th2 immune response.

The lack of an antibody response observed in various studies (Basten et al., 1974; Rich and Pierce, 1974), suggested that B cells were suppressed resulting in no antibody production. A later study by Via and colleagues in 1987, using the GVHD mouse model reported a significant decrease in B cells in F1 mice that developed an acute GVHR. This was in contrast to, the F1 mice that developed chronic GVHD in which an increase in B cells and autoantibodies were observed. The authors suggested that the antibody response was absent in the acute GVHR due to the cytotoxic activity of the donor CD8 T cells, which lysed the B cells. It is well documented that an alloresponse occurs due to MHC disparity between donor and host cells. The CD8 T cells of the donor recognise differences in the MHC class I molecules on the surface of the host B cells resulting in the killing of the B cells. One of the ways in which effector CD8 T cells lyse target cells is through the Fas/FasL pathway (Shustov et al., 1988). The latter authors demonstrated using the GVHD mouse model that

there was a correlation between the lysis of host spleen cells and the Fas/FasL expression on effector CD4 and CD8 T cells. Effector CD8 and CD4 T cells expressed increased levels of FasL in mice displaying aGVHR, compared to the CD4 and CD8 T cells of mice displaying cGHVD that expressed similar levels of FasL to mice in which a GVHR was not induced. The upregulation of FasL expression was dependent on the presence of IFN-γ produced by both the CD4 and CD8 T cells. The authors suggested that the dependence on IFN-γ in the expression of FasL may be due to the Fas-encoding gene having an IFN-γ responsive element in its promoter (Shustov et al., 1988). A study undertaken by Rozendaal and colleagues in 1989 was consistent with the findings of Shustov and colleagues in that direct contact between donor and host cells is required to suppress the antibody response. Rozendaal and colleagues reported that the use of a double chamber system that separated the donor cells from the hosts did not elicit suppression of the antibody response.

However, a study undertaken by Herath and colleagues in 2003 demonstrated the significance of a soluble factor produced by CD4 and CD8 T cells in conferring protection against *L major* parasites. Resistance to the parasites requires a cell mediated immune response. The authors suggested that the CD8 T cells produced a soluble factor IFN-γ, that increased the number of IFN-γ producing CD4 T cells which helped to protect mice infected with the parasite in a cell mediated immune response (Herath et al., 2003).

The study by Basten in 1974 demonstrated that suppression of the antibody response was specific. The authors transferred tolerant spleen cells to irradiated syngeneic mice together with normal T cells, the tolerizing antigen and donkey red blood cells (DRBC). The mice were unable to produce an antibody response to the tolerizing antigen. However, a concurrent development of a normal antibody response to DRBC occurred (Basten, 1974). In contrast to the *in vitro* system of Rich and Pierce in 1974, the soluble factor produced by Con-A- activated T cells was able to suppress the antibody response of T cells activated with SRBC. These findings suggest that the suppression of the antibody response is “non-specific”. This led to extensive research into the specificity of suppression (Rich and Pierce, 1974).

Studies have demonstrated that the *I*-region of the H-2 gene complex of the mouse regulates the type of immune response generated against different antigens. Thus in 1976 evidence emerged that a distinction could be made between the T helper and the T suppressor cells based on their specific *I*-region expression (Murphy et al., 1976; Okumura et al., 1976). It was believed that the T cells associated with B cell antibody production expressed surface markers that were coded by the *I*-A and *I*-E sub-regions. In contrast, T suppressor cells expressed surface markers that were coded by a newly identified locus the *I*-J region. The authors suggested that this region occurred between the *I*-A and *I*-E regions (Murphy et al., 1976). Further research into the mechanism of antibody suppression led to the identification of an antigen specific suppressive T cell factor that expressed the same surface markers as the suppressor T cells. The I-J region was also responsible for the expression of these molecules (Tada et al., 1976). However, this turned out not to be the case. After the characterization of the mouse histocompatibility complex (Steinmetz, 1982), the *I-J* region was not found to be between the *I*-A and *I*-E regions as initially reported. The area between the *I-A* and *I-E* regions was only two kilobases which was part of the I-Eβ (Kronenberg et al., 1983). These findings had a devastating effect on research into CD8 *I-J* suppressor T cells and ultimately led to the decline in research into the CD8 T cell as the suppressors of the antibody response.

CHAPTER 2

2.1 RATIONALE

The studies of this thesis arose from the interest of our laboratory in the aetiology of generalised, antibody mediated autoimmunity initiated by the immune response to transplantation antigens. They are based on findings made several decades ago in a model system of tolerance and autoimmunity in chickens made chimaeric during embryonic development (Havele, 1982). Chimeras were generated by injecting donor stem cells obtained from the embryonic spleens of inbred chickens differing at the MHC locus, into the embryo of the recipient. The chimaeric birds that developed by embryonic manipulation belonged to two major categories:

(i) Chimaeras developed at an early stage of development (up to 15 days, the full period of gestation being 21 days) displayed permanent chimaerism and complete unresponsiveness at both the cell mediated and humoral level against the donor antigens over the life period of approximately four years. These birds appeared to be tolerant in a way that is analogous to the unresponsiveness displayed by the immune system to self-antigens.
(ii) Chimaeras generated at about day 17 of gestation were unresponsive at the cell-mediated level but they produced specific anti-donor strain allo-antibodies detected as early as 10 days post hatching. With time, they developed a severe autoimmune syndrome characterized by the production of multiple autoantibodies directed against self tissue antigens.

Subsequent to these observations van Rappard-van Der Veen and colleagues reported in 1982, a remarkably similar observation in the parent-to-F1 mouse model mice inoculated with histoincompatible lymphocytes differing only in the I-A and I-E locus (identical in the K and D loci) developed a severe syndrome of antibody mediated autoimmune syndrome resembling the human condition of systemic lupus erythematosus (SLE). In subsequent years, Via and colleagues studied systematically the aetiology of Graft versus Host Reaction, GVHR. The advancement of technology allowed them to explore the requirements for, CD4 or CD8 T cells in the donor inoculum in causing Graft versus Host Rejection, GVHR or an autoimmune syndrome resembling the human condition of SLE,

which they named Graft versus Host Disease (GVHD). Graft versus host autoimmune disease could be initiated upon injection of DBA/2 parental lymphocytes into C57BL/6 x DBA/2, F1, hosts, while a lethal GVH disease occurs upon administration of C57BL/6 lymphocytes into the same F1 mice. Furthermore, depletion of CD8 T cells from the C57BL/6 inoculum before administration into F1 mice converts the lethal GVH phenotype to chronic autoimmune disease. Via and Shearer have argued that the development of the autoimmune phenotype involving extensive lymphoproliferation is the outcome of a deficient CD8 T cell activation unable to control the antibody autoimmune state.

From these collective observations it became important to us to delineate the role of CD8 T cells in regulating the cell mediated or antibody response exploring an *in vitro* experimental system of one-way mixed lymphocyte response.

2.2 HYPOTHESIS

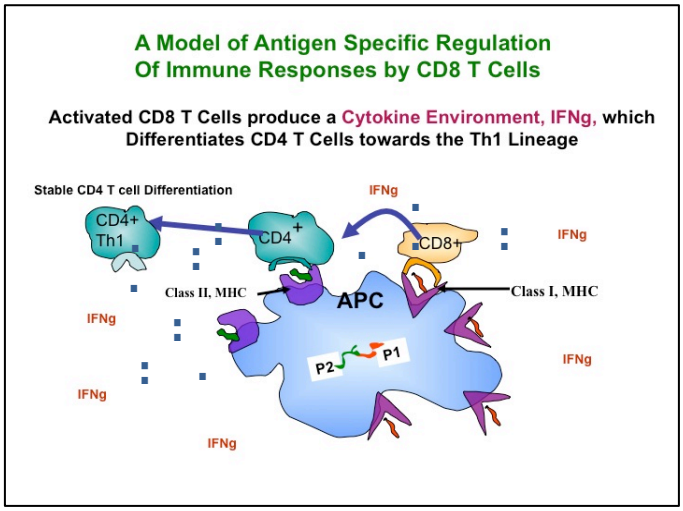
We hypothesised that the generation of CD4, Th1(IFN-γ) or Th2(IL-4) immunity depends on both:

i) *the ratio of CD4 T cells:CD8 T cells specific for antigen at the initiation of the immune response and*

ii) on the ability of antigens to co-ordinately induce both CD4 T cells and CD8 T cells.

The MHC class I and class II alloantigens expressed on the APCs of the F1 mouse strain have the potential for being dually recognized by both CD8 T cells and CD4 T cells, respectively. Therefore, according to the above model, CD4 Th1/Th2 immunity is subjected to the cognate regulation by CD8 T cells. Our model suggests that efficient induction of CD8 T cells at the initiation of the immune response results in increased and stable CD4/Th1-type immunity with concomitant inhibition of Th2-type specific immune responses. We believed that the mechanism by which the CD8 T cells exert their regulatory function on CD4 T cell differentiation is through the production of the soluble factor, IFN-γ that provides a particular cytokine environment that is conducive for enhancing a Th1 immune response (Figure 2.1A). In contrast, in the absence of efficient CD8 T cell activation, and therefore, an absence of the critical cytokine environment, IFN-γ, alloantigen stimulates a Th2 response (Figure 2.1B).

A



B

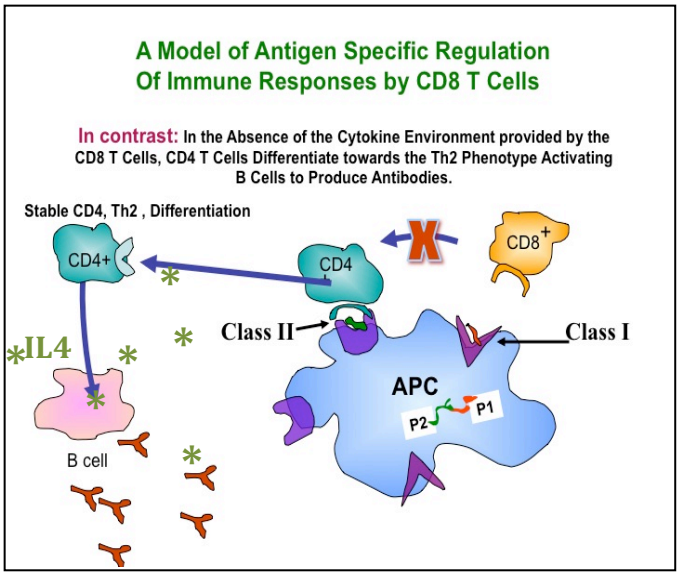


Figure 2.1: A Three-Cell Model of Antigen Specific Regulation of Immune Responses to Alloantigens by CD8 T cells

(A) The CD8 T cells provide the cytokine environment, IFN-γ, that drive CD4 T cell differentiation towards the Th1 mode.

(B) In the absence of the cytokine environment provided by the CD8 T cells, the CD4 T cells would predominantly differentiate to Th2 phenotype characterized by IL-4 production.

2.3 AIMS

There are three fundamental aims to this study.

The ***first aim*** was to analyze the role of the naturally occurring CD4:CD8 T cell ratio in adult mice in determining the class of immunity, Th1 (IFN-γ) or Th2 (IL-4), generated upon allo-MHC stimulation.

The ***second aim*** of the study was to determine the regulatory role of the CD8 T cells in the differentiation of naïve CD4 T cells towards a Th1 (IFN-γ) phenotype and away from a Th2 (IL-4) phenotype.

The ***third aim*** of the study was to determine the mechanism by which the CD8 T cells exert their regulatory role on the differentiation of naïve CD4 T cells.

CHAPTER 3
MATERIALS AND METHODS

3.1 Mice

BALB/c, *H-2*^{d}, C57BL/6, *H-2*^{b}, CBA/J, *H-2*^{k}, C3H, *H-2*^{k}, DBA/2J, *H-2*^{d}, and DBA/1J, *H-2*^{q}, all 8-12-week-old female mice were used as the Responders in our study. The Stimulators were irradiated spleen cells of heterozygous (BALB/c x C57BL/6), F1, (*H-2*$^{d/b}$) female mice. To illustrate antigen specificity, we used 8-12-week-old C3H mice with an H-2^{k} haplotype. The BALB/c, C57BL/6 and C3H mice were obtained from the Charles River Laboratories (Montreal, Quebec). The CBA/J, DBA/1 and DBA/2 mice were obtained from Jackson Laboratory (Bar Harbour, Maine, USA). All mice were housed at the Health Sciences, Laboratory and Animal Services Unit (LASU), University of Saskatchewan. All our experiments were conducted under a protocol approved by the University of Saskatchewan's Animal Research Ethics Board that adhered to the Canadian Council on Animal Care guidelines for humane animal use.

3.2 Media and Solutions

3.2.1 Roswell Park Memorial Institute Culture Medium

The culture medium was prepared with Roswell Park Memorial institute (RPMI-1640) medium (Gibco, Grand Island, N.Y.), 10% heat inactivated characterized Fetal Bovine Serum (FBS) (Thermo Scientific), 100U/ml of penicillin-streptomycin (Gibco, Grand Island, N.Y.), 0.8 mM sodium pyruvate and 0.5M 2-mercaptoethanol (Sigma). This medium was also used in the blocking step of the ELISPOT assay.

3.2.2 Phosphate Buffered Saline

A 10x stock of phosphate buffered saline (PBS) was prepared by combining 80.0 g/L NaCl, 2.0 g/L KCl, 11.5 g/L $Na_2HPO_4 \bullet 12\ H_2O$ and 2.0 g/L KH2PO4 in distilled deionized water (ddH2O). The pH was adjusted to 7.2. A 1x working stock of PBS was obtained by diluting the stock in ddH2O.

3.2.3 Phosphate Buffered Saline-Tween 20

PBS-Tween (PBST) was prepared by adding 0.05% v/v Tween 20 (Fisher Scientific, USA)

to a working solution of PBS. PBST was used for the washing steps in the development stage of the enzyme-linked immunospot (ELISPOT) assay.

3.2.4 Magnetic Activated Cell Sorting buffer

Magnetic Activated Cell Sorting (MACS) Buffer consisted of PBS supplemented with 2mM NaEDTA.

3.2.5 Fluorescence Activated Cell Sorting buffer

Fluorescence Activated Cell Sorting (FACS) Buffer consisted of PBS supplemented with 2mM NaEDTA

3.2.6 Carbonate/Bicarbonate Buffer for Coating ELISPOT Plates

- *Preparation of Solution A:* 8.4g NaHCO3 + 100mL H_2O
- *Preparation of Solution B:* 10.6g Na_2CO3 + 100mL H_2O

A mixture of 45.3mL of A + 18.2mL of B + 936.5mL H_2O was prepared. The pH of the solution was adjusted to 9.6 using A or B.

3.2.7 NBT/BCIP Developing Solution for ELISPOT assay

ELISPOT plates (Millipore) were developed by applying a solution of nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate toluidine (NBT/BCIP), (Roche), substrate diluted 1:100 in an alkaline (pH 9.5) substrate buffer consisting of 0.1M Tris-HCl, 0.1M NaCl and 0.05M $MgCl_2$.

3.2.8 Recombinant Interferon Gamma and Anti-Interferon Gamma antibody

The dose of recombinant interferon gamma (rIFN-γ) (BioLegend) needed in our experiment was determined by a bioassay. Previously in our laboratory the bioactivity of IFN-γ was determined by employing a bioassay which measured the protection provided by the IFN-γ to L929 cells, a rat fibroblast cell line, when infected with endomyocarditis, EMC, virus. Briefly, 3 x 10^4 L929 cells/well were incubated in 96 well flat-bottomed plates for 24 hours, in the presence of different dilutions of a given supernatant. Thereafter, media was suctioned, and the cultures were infected with 100-fold higher titre of EMC virus in

media. The titre of the virus was predetermined using the Karber assay to define the dose which caused the death of 50% of the cells. The reciprocal dilution which protects 50% of the L929 cells from destruction by the virus was designated as 1 unit of IFN-γ bioactivity. Recombinant, purified IFN-γ was used as a standard in all assays. Based on the findings of these experiments we determined that 2000 units were required. A unit of IFNg activity equalled that of the international unit as calculated by the commercial supplier. Thus, we used 10ul/well of 0.1ng/ml rIFN-γ in our experiments. The standard amount of neutralizing anti-IFN-γ monoclonal antibody (XMG1.2, BioLegend) was chosen based on its ability to neutralise 2000 units of rIFN-γ activity.

3.3 Preparation of Spleen Single Cell Suspensions

Mice were euthanized via cervical dislocation and the spleens were aseptically removed and placed in complete RPMI medium on ice. Spleens were gently ground through a sterile wire mesh using the rubber end of a sterile syringe. The cells were separated into a single suspension by continuous pipetting. Thereafter, they were placed in a 15ml centrifuge tube (Costar) for two minutes in order to allow large particles to settle. The cells present in the supernatant were transferred to a fresh 15 mL centrifuged tube and centrifuged at 500x*g* for 7 minutes. The cell pellet was resuspended in 5ml of complete RPMI medium per spleen. The number of leukocytes present in the suspension was determined using a haemocytometer and by counting the viable white cells at a 1:5 dilution in Trypan blue. Viable white cells were finally adjusted to a concentration of 10^7 cells/ml.

3.4 Mixed Lymphocyte Reaction

The Mixed Lymphocyte Reaction (MLR) occurs when lymphocytes obtained from individuals that differ at their MHC are mixed together. The small lymphocytes in the *in vitro* system are activated when they recognise the foreign MHC. Subsequently these small lymphocytes differentiate into large cells that exhibit an effector function against target cells that express the specific foreign MHC. The MLR is fully discussed in the Introduction section 1.3. We used three different culture preparations in our study that represented the MLR. These include our Control Cultures, CD8 T cell Depleted Cultures and CD4 T cell Depleted Cultures.

3.4.1 Control Cultures

The basic experimental system we employed is One-Way Mixed Lymphocyte Reaction. The stimulators in our MLR were always irradiated in order to prevent their proliferation and their participation in the reaction. In particular, Responder spleen cells from different inbred strains of mice, homozygous at the MHC locus, were cultured with irradiated spleen cells from different strains of mice under defined conditions of Responder and Stimulator cell density. The strain combination of Responder and Stimulator spleen cells is indicated in particular Figure Legends in the Results section. We refer to these cultures as our Control Cultures.

For optimal cell interaction between Responder and Stimulator spleen cells and dependent on the aim of the experiment as described in the legends of the figures we used 10^7cells per well in 6-well plates (9.5cm^2 growth area), 4 X 10^6 cells per well in 12-well plates (3.8 cm^2 growth area) and 2 x 10^6 cells per well in 24-well plates (1.88cm^2 growth area) for the responders. Stimulator spleen cells were used at a 1:1 ratio with the responder spleen cells.

3.4.2 CD8 T cell Depleted Cultures

Depletion of $CD8^+$ T cells from the spleens of mice was achieved by employing the rat monoclonal antibody TIB-211 (American Type Culture Collection) specific for a non-polymorphic part of the Ly2 antigen. The number of spleen cells used is dependent on the culture plate used: see 3.4.1 of Materials and Methods and detailed in particular Figure legends. The spleen cells were incubated for one hour on ice with a ten-fold higher titre of TIB 211 antibody in ascites fluid than that required to obtain maximal complement dependent lysis of the splenic T cells. The spleen cells were then incubated with 1/20 dilution of Low-Tox-M rabbit complement (Cedarlane) at 37°C. The depletion of the CD8 T cell was verified by flow cytometry: see 3.8 of Materials and Methods. Equivalent numbers of Control cultures depleted of $CD8^+$ T cells were cultured with identical numbers of Stimulator cells as in the Control cultures. The number of Responder and Stimulators spleen cells per CD8 T cell depleted culture varied as per the plate type used (a 6-well plate, a 12-well plate or a 24-well plate) and is expanded on in the result section.

3.4.3 CD4 T cell Depleted Cultures

A similar procedure as that which was followed to deplete $CD8^+$ T cells from the spleens of mice was used to deplete the $CD4^+$ T cells from the spleens of mice. Monoclonal antibody GK1.5 and Low Tox-M rabbit complement were used to deplete the $CD4^+$T cells from the spleens. Depletion of the $CD4^+$T cells from the spleens was verified by flow cytometry. Thus, equivalent numbers of Control cultures, which were depleted of $CD4^+$ T cells, were cultured with identical numbers of Stimulator cells as in the Control cultures.

3.4.4 Transwell Membrane Cultures

The transwell-membrane plates (Costar, USA) consisted of 12-well plates in the lower chamber while the upper chamber consisted of 24-well plate inserts. The membrane separating the two chambers had a pore size of 0.4 μm that only allowed molecules to pass through and not cells. The cultures prepared in the upper and lower chambers of the transwell-membrane plates varied and are detailed in the Figure legends in the results sections.

3.5 Irradiation of Stimulator Spleen Cells

The spleen cells of the F1, (BALB/c x C57BL/6) and C3H mice, which, were used as the stimulators in our experiments were irradiated with 1000-1500 Rads from a Cobalt 60 (University of Saskatchewan). This abrogated the stimulator cells' ability to replicate and ensured that a one-way mixed lymphocyte reaction was obtained.

3.6 Sorting out of unprimed CD8 T cells from the spleens of mice for use in Reconstruction assays

Naïve CD8 T cells were isolated from spleen cells of mice for use in Reconstructed Cultures as detailed in particular Figure legends. The isolation of these cells occurred in two steps:

3.6.1 T cell enrichment of the spleen cells using Nylon wool

Nylon wool columns (Polysciences, Inc.) were used for T cell enrichment of the mouse spleen cells. The columns were prepared the day prior to the cell isolation. The columns

were rinsed with warm RPMI culture medium and then filled with 7ml RPMI culture medium. Columns were incubated overnight at 37°C and 5% CO_2. The following day the spleen cells of the mice were centrifuged at 500 x *g* for 7 minutes and resuspended in 2ml of warm RPMI culture medium. The columns were washed once with 5ml warm medium. Thereafter, cells were transferred to columns and incubated for one hour at 37°C and 5% CO_2. After incubation, the columns were washed twice with 5 mL of warm RPMI culture medium. Cells were centrifuged at 500 x *g* for 7 minutes. The pellet was resuspended in 5ml per spleen in RPMI culture medium.

3.6.2 *Magnetic Activated Cell Sorting*

After T cell enrichment, CD8 T cells were sorted by negative selection using Magnetic Activated Cell Sorting (MACS) monoclonal antibodies to CD4 T cells (CL012) Miltenyi Biotec. The cell number of the T cell enriched spleen cells was determined using Trypan blue (Biomedical Ltd) and a haemocytometer. The spleen cells were centrifuged at 500 x *g* for 7 minutes. The pellet was then, resuspended in MACS buffer (90µl per 10^7 cells). Monoclonal antibodies to CD4 T cells were added at 10µl per 10^7 cells. Thereafter cells were incubated for 30 minutes at 4°C. The cells were separated in MACS columns placed in a MACS separator. The flow through which consisted of the unlabelled CD8 T cell population was collected. Cells were centrifuged at 500x*g* for 7 minutes. The pellet was resuspended in 2ml of complete RPMI medium. Cells were counted using Trypan blue and sorting of the CD8 T cells was verified using flow cytometry: see 3.8 of Materials and Methods.

3.7 Assessment of the Immune Responses by ELISPOT assay

An ELISPOT assay was used to enumerate the number of antigen specific effector T cells producing the signature cytokines, IFN-γ and IL-4 of a Th1 or Th2 response, respectively.

- *ELISPOT plate preparation*

On the first day of the ELISPOT assay, 96-well cellulose ester filtration plates (Millipore) were coated with either purified rat anti-mouse anti-IFN-γ (1µL/mL of 1 mg/mL) or purified rat anti-mouse anti-IL-4 (2µL/mL of 0.5 mg/mL) antibody (BD Pharmingen) in carbonate/bicarbonate buffer and incubated overnight at 4°C.

- *Plating of effector T cells*

On the following day, the coated plates were blocked with 100µl/well of complete RPMI medium for 1 hour at 37°C in a 5% CO_2 incubator. The cultures that had been prepared on day 0 were harvested on day 5 to assess the IFN-γ and IL-4 production by the effector cells. Cells were subsequently plated on the ELISPOT plate in duplicate on three different two-fold cell dilutions with plus or minus fresh nominal F1 antigen or an unrelated alloantigen to assess specificity of the response. The number of cells per well ranged between 3-4 x 10^3 cells at the highest concentration. Plates were incubated at 37°C in a 5% CO_2 incubator for 16 to18 hours.

- *Plate development and counting*

The plates were subsequently washed 4 times with PBST solution, 3 times with water and once with PBS. Thereafter 100µl per well of biotinylated rat anti-mouse anti-IFN-γ monoclonal antibody (2µl/ml in 1ml PBS, BD Pharmingen) or 100µl per well of biotinylated rat anti-mouse anti-IL-4 monoclonal antibody (2µl/ml in 1ml PBS, BD Pharmingen) were added to the appropriate wells and incubated at room temperature for 2 hours. The plates were washed with PBST seven times and once with PBS to remove unbound secondary antibody. Thereafter 100µl per well (0,2µg/ml) streptavidin-alkaline phosphatase conjugate in PBS (Cedarlane, Ontario) was added and subsequently incubated at room temperature for 1hour. Plates were washed 8 times, with water. A color reaction developed with the addition of 100µl per well NBT/BCIP (Roche; 200µl per 10ml PBS). Plates were incubated for approximately 15 minutes in a dark area at room temperature. With the appearance of spots, plates were washed with cold water to stop the enzymatic reaction. Plates were left to dry overnight. Spots were counted by direct visualization and were expressed as number of spots per 10^7 input cells per culture. Antigen-dependent cytokine producing cells were enumerated by counting cytokine spots in antigen positive wells. The number of spots in antigen negative wells were counted and then subtracted from the number of spots obtained in the antigen positive wells.

Generally, 0.1% (3 -5 x 10^3 cells) of the culture was plated per ELISPOT well. Thus, the total number of spots per 10^7 cells input per culture at day 0 was 100% of the culture.

3.8 Flow Cytometric Analysis

We determined the CD4 T cell and CD8 T cell percentages employing PE-anti-mouse CD4 (L3/T4) (CL012) and FITC anti-mouse CD8a (Ly2) (CL169) antibodies (Cedarlane Ontario) and flow cytometry at a cell concentration of approximately one million cells per sample. The cells were incubated at 4°C for 30 minutes. Cells were washed twice, centrifuging at 850 x g for two minutes. Cells were resuspended in 500µl FACS buffer for analysis. Flow cytometry was undertaken using an BD Accuri C6 cytometer or BD EPIC XL Cytometer (Becton Dickinson) and analyzed using BD Accuri C6 software or Flow Jo software (Tree Star). To verify that the percentages obtained for CD4 T cells and CD8 T cells, APC anti-mouse CD3 monoclonal antibody (BioLegend) (CL17) was used. Employing the forward and side scatter ells were gated on the lymphocyte population. Thereafter, APC anti-mouse CD3 antibodies was gated in the lymphocyte population. The CD4 T cell and the CD8 T cell percentages were obtained by gating on the anti-CD3 population.

3.9 Statistical Analysis

Significance was set at $p < 0.05$ and assessed by t tests or by analysis of variance (ANOVA) with post hoc analyses relying on Tukey's multiple comparison test (GraphPad Prism 7). Data are represented as mean ± standard deviation (SD) of the mean from duplicate cultures. The minimal variability and very strong consistent results between cultures allowed us to use duplicate cultures.

RESULTS

The results section is divided into three chapters, which report on the findings of the three main aims of our study.

CHAPTER 4

Analysis of the role of the naturally occurring CD4:CD8 T cell ratios of the spleens of adult BALB/c, C57BL/6, C3H, CBA/J, DBA/1 and DBA/2, mice in determining the Th1 (IFN-γ) /Th2 (IL-4) phenotype of the immune response when stimulated with allo-MHC as assessed at day 5 of culture

Preamble

The first chapter assesses variations in the ratio of the number of CD4 T cells to that of CD8 T cells (subsequently referred to as the CD4:CD8 T cell ratio) that occur in the spleens of different strains of mice. Subsequently we assessed whether different CD4:CD8 T cell ratios were associated with the generation of immune responses of different Th1(IFN-γ)/ Th2 (IL-4) phenotypes. We used an *in vitro* system of direct alloreactivity to assess the cytokine production of the effector T cells. We initially set out to determine the CD4:CD8 T cell ratio of the BALB/c and C57BL/6 mouse strains as they are considered to be the antibody (Th2) and the cell-mediated (Th1) strains, respectively. We hypothesised that a relatively low CD4:CD8 T cell ratio would characterize the C57BL/6 strain comparatively to a relatively high ratio that would characterize the BALB/c mouse, putatively explaining their propensity to generate a cell mediated immune response (Th1) or antibody response (Th2), respectively. We determined, that the relatively high CD4:CD8 T cell ratio of the spleens of BALB/c mice correlated with a mixed Th1(IFN-γ)/Th2(IL-4) immune response upon allo-MHC stimulation and that a relatively low ratio in C57BL/6 mice correlated with a predominant Th1 (IFN-γ) response. Subsequently we tested if the above findings were consistent amongst other mouse strains such as the CBA/J, C3H, DBA/1 and DBA/2 mice. We consistently found that a relatively high CD4:CD8 T cell ratio of the spleens of mice tends towards a mixed Th1 (IFN-γ)/Th2(IL-4) immune response while a relatively low ratio tends towards a predominant Th1(IFN-γ) immune response upon allo-MHC stimulation.

4.1 Correlation between the CD4:CD8 T cell ratio and the phenotype of the immune response, Th1 or Th2, generated by the spleen cells of different strains of adult mice upon allo-MHC stimulation

4.1.1 The naturally occurring CD4:CD8 T cell ratio of the spleens of adult BALB/c and C57BL/6 mice are different

The findings of Via in 1987 have demonstrated that the frequency of the CD8 T cells in the spleens of C57BL/6 and DBA/2 mice were different, whereas the frequency of the CD4 T cells in the spleens of the two mouse strains were comparable. This indicated to us that the CD4:CD8 T cell ratio is different in the C57BL/6 and DBA/2 mice. Furthermore, this difference in the frequency of the CD8 T cells of the two strains of mice correlated with a distinctly different immune response when their lymphocytes were injected into F1, (C57BL/6 x DBA/2) mice. The administration of DBA/2 lymphocytes consisting of a low frequency of CD8 T cells resulted in an antibody mediated autoimmune disease in the F1 mice. In contrast, the administration of the lymphocytes of the C57BL/6 mice consisting of a higher frequency of CD8 T cells resulted in acute graft rejection and in the inhibition of the antibody response. The authors reported that the suppression of the antibody response was due to the lysis of recipient B cells expressing allo-MHC by the cytotoxic activity of the CD8 T cells.

We wished to determine whether BALB/c and C57BL/6 spleen cells also displayed different CD4:CD8 T cell ratios and what effect the difference would have on the type of immune response generated against the irradiated spleen cells of F1, (BALB/c x C57BL/6) mice. Consequently, our first step was to systematically characterize the naturally occurring CD4:CD8 T cell ratio of the spleens in these two strains of mice. Naïve mice were euthanized by cervical dislocation and their spleens were aseptically removed. We then determined the CD4 T cell and CD8 T cell percentages in the spleens of individual, adult BALB/c and C57BL/6 mice employing flow cytometry (Figure 4.1). In naïve BALB/c mice there were approximately twice as many CD4 T cells as CD8 T cells as illustrated by the flow cytometric results in Figure 4.2A. In contrast, the percentage of the CD4 T cells and the CD8 T cells of the C57BL/6 mice were similar as shown in Figure 4.2B**.** These

findings corresponded to a CD4:CD8 T cell ratio of approximately 2:1 and 1:1 in the spleens of BALB/c and C57BL/6 mice, respectively. Furthermore, the difference in the CD4:CD8 T cell ratio of the spleens of the BALB/c and C57BL/6 mice, is statistically significant as indicated in Figure 4.2C.

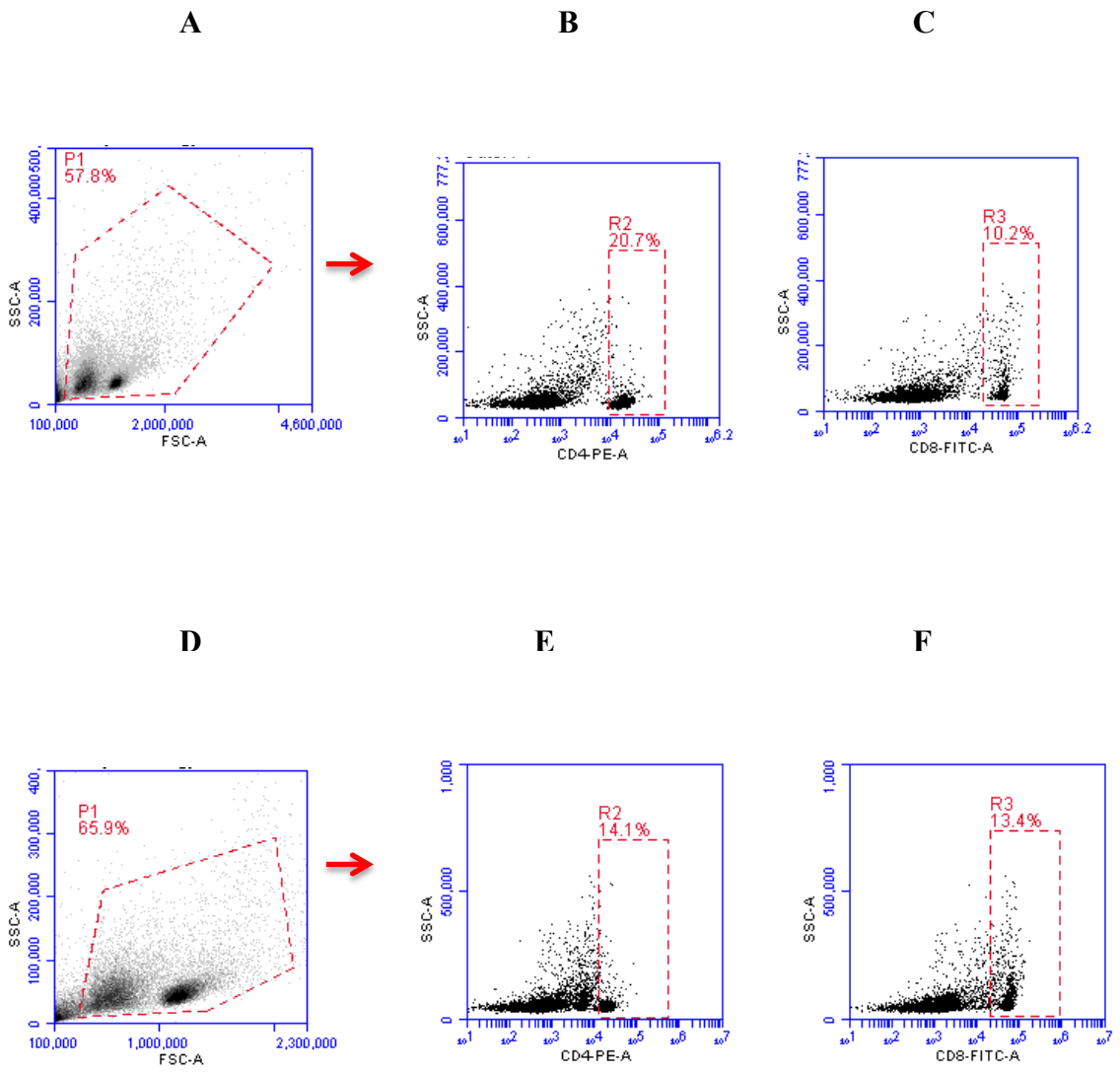
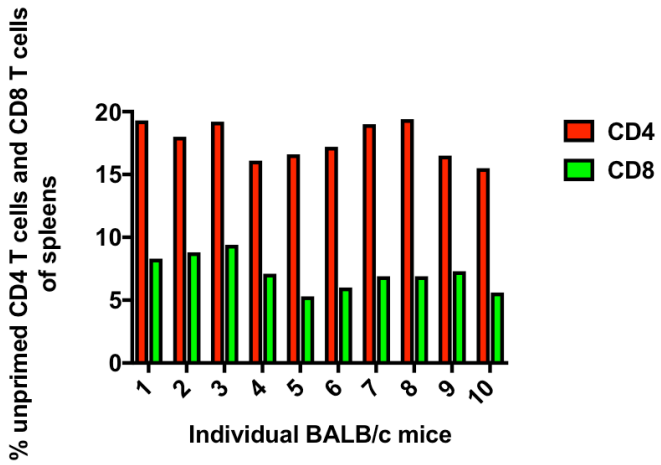
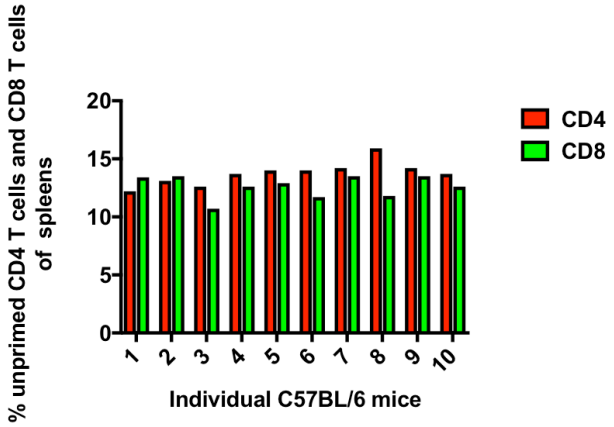


Figure 4.1: Characterization of the CD4 T cell and the CD8 T cell percentages of the spleens of unprimed, adult BALB/c and C57BL/6 mice. Spleen cells of naïve, adult (8-12-week-old) BALB/c (**A, B** and **C**) and C57BL/6 (**D, E** and **F**) mice were stained with PE-anti-mouse CD4 (L3/T4) and FITC anti-mouse CD8a (Ly2) antibodies. We firstly gated the lymphocyte population, based on forward and side scatter (**A** and **D**). Thereafter the percentages of CD4 T cells (**B** and **E**) and CD8 T cells (**C** and **F**) were determined from the gated lymphocyte population, P1. Data show one representative experiment of at least ten independent experiments per mouse strain.

A



B



C

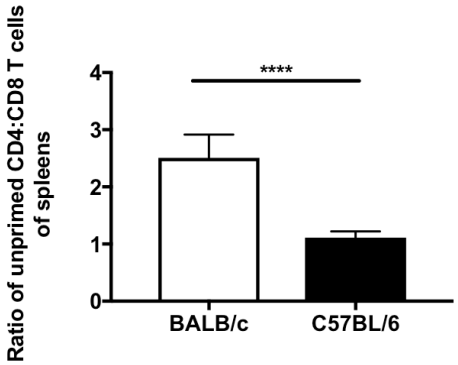


Figure 4.2

Figure 4.2: Significant difference between the naturally occurring CD4:CD8 T cell ratio among spleen cells of adult BALB/c and C57BL/6 mice. Unprimed spleen cells of individual, adult BALB/c and C57BL/6 mice (8-12-week-old) were stained with PE-anti-mouse CD4 (L3/T4) and FITC anti-mouse CD8a (Ly2) antibodies. The percentage of CD4 T cells and CD8 T cells of BALB/c (**A**) and C57BL/6 (**B**) mice were assessed via flow cytometry as described in Figure 4.1. The CD4:CD8 T cell ratio of the 10 BALB/c and 10 C57BL/6 mice was compared (**C**). Data are represented as mean ± standard deviation (SD) of the mean from duplicate cultures. Data are representative of three independent experiments with the spleens from ten mice per strain. Statistical significance was assessed using unpaired students' *t* test. **** $p < 0.0001$

4.1.2 The CD4:CD8 T cell ratio are similar among the spleen cells and the cells of the inguinal lymph nodes

We chose spleens as the secondary lymphoid tissue in our study. This was based on their size and the ease with which we could harvest them from the mice. Once we had established that a significant difference existed between the naturally occurring CD4:CD8 T cell ratios in the spleens of the BALB/c and C57BL/6 mice we needed to determine whether a similar CD4:CD8 T cell ratio was also evident in other secondary lymphoid tissue. Thus, we set out to determine the CD4:CD8 T cell ratio of the inguinal lymph nodes of the two strains of mice. These lymph nodes were chosen because they were easily harvested. Using flow cytometry, we found that there was no difference between the naturally occurring CD4:CD8 T cell ratio of the spleens and the inguinal lymph nodes of the BALB/c and C57BL/6 mice see Figure 4.3. Although the absolute percentages of CD4 T cells and CD8 T cells in the inguinal lymph nodes were higher than that of the spleens, the CD4:CD8 T cell ratio remained the same in these two strains of mice. We infer that the spleens are representative of other secondary lymphoid tissue such as the inguinal lymph node. Furthermore, the naturally occurring CD4:CD8 T cell ratio in the peripheral blood was comparable to that in the spleens of the BALB/c and C57BL/6 mice (Pinchuk and Filipov, 2008). We therefore, felt it valid to use the spleen cells of the BALB/c and C57BL/6 mice in all our experiments.

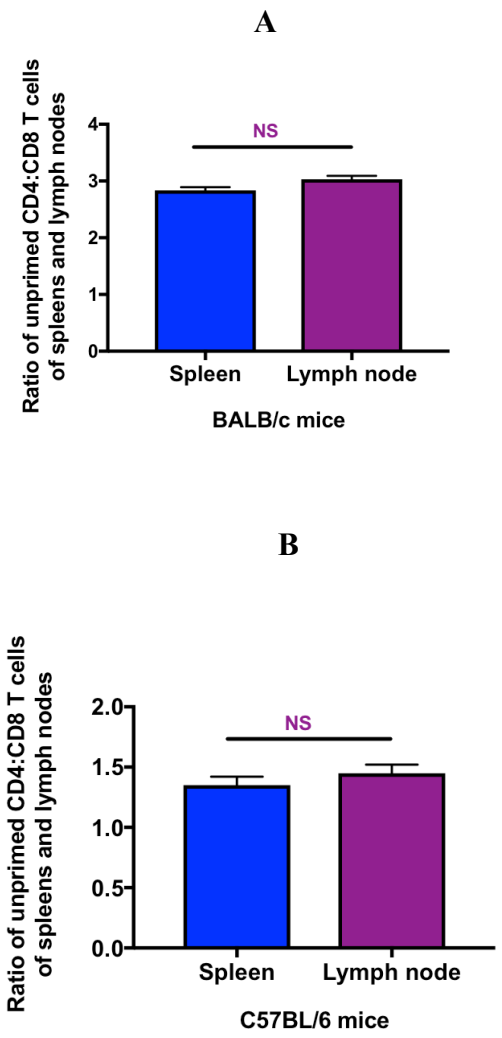
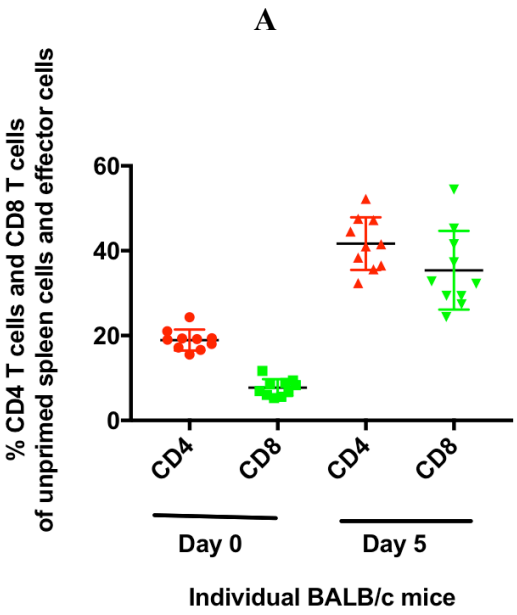


Figure 4.3: The CD4:CD8 T cell ratio are similar among the spleen cells and the cells of the inguinal lymph nodes. Unprimed cells of spleens and inguinal lymph nodes of adult BALB/c and C57BL/6 mice (8-12-week-old) were stained with PE-anti-mouse CD4 (L3/T4) and FITC anti-mouse CD8a (Ly2) antibodies. Employing flow cytometry, we assessed the percentages of CD4 T cells and CD8 T cells of BALB/c (**A**) and C57BL/6 (**B**) mice. Data are representative of two independent experiments with spleen cells and inguinal lymph nodes from four mice per strain. Statistical significance was assessed using paired students' *t* test. NS: not significant

4.1.3 Expansion of CD4 T cells and CD8 T cells of the spleens of adult BALB/c and C57BL/6 mice assessed at day 5 of culture

The work of Wilson, reported in 1967, employing the MLR demonstrated, that the mixing of blood lymphocytes from rats that differ at the MHC resulted in an increase in large cells. Further studies demonstrated that the incorporation of thymidine into the DNA of the cells indicated that the cells were undergoing mitosis, which resulted in an increase in cell number (Bain, Vas and Lowenstein, 1964). We observed a similar increase in large cells in our MLR. The basic experimental system we employed was a one-way MLR (Bach and Voynow, 1966; Wilson et al., 1967). In particular, ten million spleen cells from either, BALB/c mice, homozygous at the MHC loci, H-2^d, or C57BL/6 mice, homozygous at the MHC loci, H-2^b were cultured with ten million irradiated spleen cells from heterozygous, H-$2^{d/b}$ (BALB/c x C57BL/6), F1 mice for 5 days at 37°C and 5% CO_2 in 6-well plates. We employed flow cytometry to determine the frequency of the CD4 T cells and CD8 T cells in the 5-day cultures. Expansion of the spleen cells of BALB/c mice resulted in similar percentages of the CD4 T cells and the CD8 T cells, see Figure 4.4A. In contrast, although both the CD4 T cells and the CD8 T cells of the spleens of C57BL/6 mice expanded in the day 5 cultures, the percentage of the CD8 effector T cells was higher than the CD4 effector T cells as illustrated in Figure 4.4B. There is a significant difference in the CD4:CD8 T cell ratio of the expanded cells of the BALB/c and C57BL/6 mouse strains as illustrated in Figure 4.5.


A
% CD4 T cells and CD8 T cells
of unprimed spleen cells and effector cells
60
40
20
0
CD4
CD8
CD4
CD8
Day 0
Day 5
Individual BALB/c mice

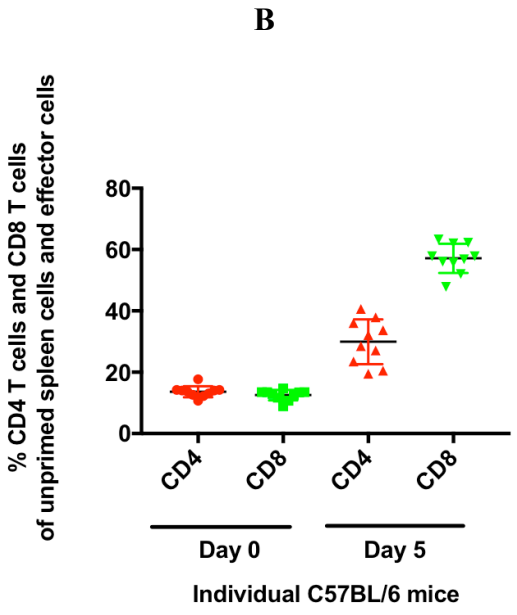

B
% CD4 T cells and CD8 T cells
of unprimed spleen cells and effector cells
80
60
40
20
0
CD4
CD8
CD4
CD8
Day 0
Day 5
Individual C57BL/6 mice

Figure 4.4

Figure 4.4: Expansion of CD4 T cells and CD8 T cells of the spleens of adult BALB/c and C57BL/6 mice upon allo-MHC stimulation assessed at day 5 of culture. Ten million spleen cells of either individual BALB/c (**A**) or C57BL/6 (**B**) mice were cultured with an equivalent number of irradiated F1, (BALB/c x C57BL/6) spleen cells and RPMI complete medium and incubated for 5 days at 37°C and 5% CO_2 in 6-well plates. At day 5, cells were harvested and stained with PE-anti-mouse CD4 (L3/T4) and FITC anti-mouse CD8a (Ly2) antibodies. Employing flow cytometry, we assessed the percentage of CD4 T cells and CD8 T cells at day 0 (unprimed T cells) and day 5 (Effector T cells) of BALB/c (A) and C57BL/6 (B) mice. Data are representative of five independent experiments.

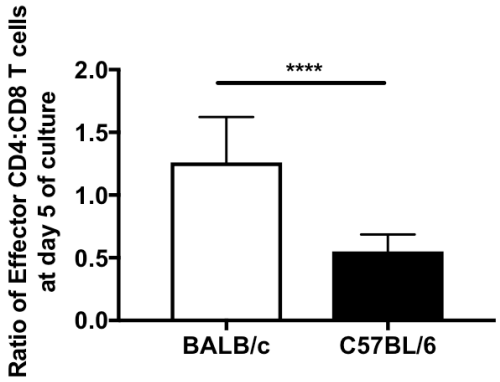


Figure 4.5: Significant difference in the CD4:CD8 T cell ratio of the spleen cells of the adult BALB/c and C57BL/6 mice assessed at day 5 of culture. Ten million spleen cells of either individual BALB/c or C57BL/6 mice were cultured with an equivalent number of irradiated F1, (BALB/c x C57BL/6) mice spleen cells and RPMI complete medium and incubated for 5 days at 37°C and 5% CO_2 in 6-well plates. At day 5, cells were harvested and stained with PE-anti-mouse CD4 (L3/T4) and FITC anti-mouse CD8a (Ly2) antibodies. Flow cytometry was employed to assess the percentage of CD4 T cells and CD8 T cells of the spleens of BALB/c and C57BL/6 mice at day 5 of culture. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of five independent experiments per mouse strain. Statistical significance was determined by unpaired students' *t* test. **** $p < 0.0001$

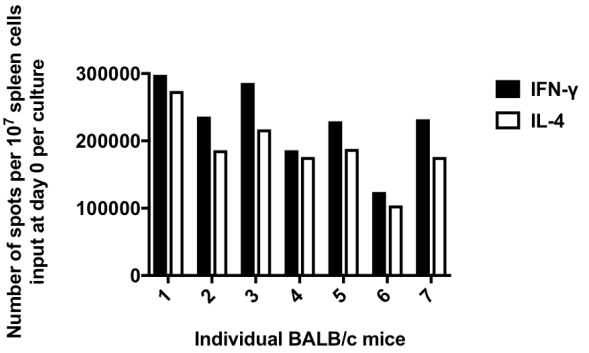
4.1.4 The naturally occurring CD4:CD8 T cell ratio of the spleens of BALB/c and C57BL/6 mice is associated with a particular type of immune response, Th1 /IFN-γ or Th2/ IL-4, generated upon stimulation with allo-MHC

We had previously determined that the CD4:CD8 T cell ratio of the spleens of the BALB/c and the C57BL/6 mice is different. We thus wanted to assess whether the different CD4:CD8 T cell ratios observed in spleens of BALB/c and C57BL/6 mice were associated with the generation of immune responses of different Th1(IFN-γ)/Th2(IL-4) phenotypes upon stimulation with allo-MHC. Our *in vitro* system allowed us to explore the differential development of T cells through quantitative interactions initiated by antigen and through cytokine molecules produced by lymphocytes in an orderly fashion. Thus, five days after culturing the spleen cells with irradiated F1 (BALB/c x C57BL/6) spleen cells, we harvested and restimulated the cells with the nominal antigen and assessed their allo-MHC-dependent IFN-γ and IL-4 production employing the ELISPOT assay. Differences in the MHC between the spleen cells of the BALB/c mouse and the irradiated spleen cells of the F1 mice generated a mixed Th1 (IFN-γ) /Th2 (IL-4), immune response (Figure 4.6A). In contrast, the MHC disparity between the spleen cells of the C57BL/6 mice and the irradiated spleen cells of the F1 mice generated a predominantly Th1 (IFN-γ) immune response (Figure 4.6B). We found that there was a significant difference in the production of IL-4 between the spleen cells of these two mouse strains when activated under identical conditions. The spleen cells of the BALB/c mice produced significantly higher levels of IL-4 than the spleen cells of the C57BL/6 mice (Figure 4.6C). The level of IFN-γ production appeared to be similar between the two mouse strains. Thus, the IFN-γ:IL-4 ratio of the two mouse strains was significantly different (Figure 4.7). We thus concluded that the relatively high CD4:CD8 T cell ratio of the spleens of the BALB/c mice is associated with a mixed Th1(IFN-γ)/Th2(IL-4) immune response upon stimulation with allo-MHC while the relatively low CD4:CD8 T cell ratio of the spleens of the C57BL/6 mice is associated with a predominant Th1(IFN-γ) immune response upon allo-MHC stimulation.

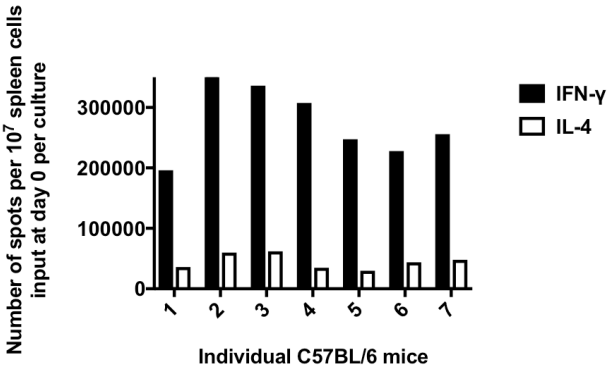
The MHC disparity between donor and host cells is a known activator of alloreactive T cells (Rolink et al., 1983, van Rappard-van der Veen, et al., 1982). Alloreactive T cells can

recognise allo-MHC directly without the need for processing the antigen, thus reducing the time of activation and proliferation of effector T cells (Letchler and Batchelor, 1982). The large number of spots observed on our ELISPOT plate after only 5 days with allo-MHC stimulation provides strong evidence for direct allorecognition and further verifies the observation of a high frequency of T cell alloreactivity. The frequency of T cells specific for allo-MHC is approximately 1 in10^2, whereas the frequency of T cells specific for a typical foreign antigen is of the order of 1 in 10^5 (Wilson et al., 1968).

A



B



C

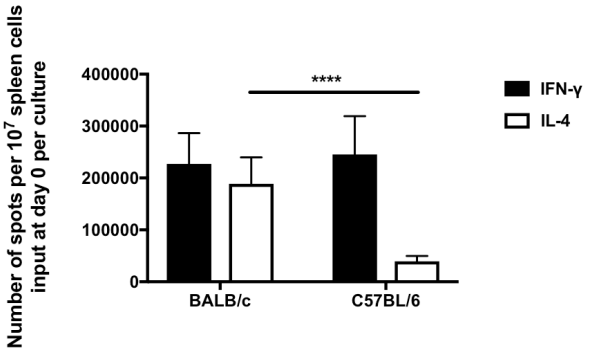


Figure 4.6

Figure 4.6: IFN-γ and IL-4 production of the Effector T cells of spleens of adult BALB/c and C57BL/6 mice assessed at day 5 of culture. Ten million unprimed spleen cells of either individual, adult BALB/c (**A**) or individual adult C57BL/6 (**B**) mice were cultured with an equivalent number of fresh, irradiated F1, (BALB/c x C57BL/6) spleen cells and incubated for 5 days at 37°C and 5% CO_2 in 6-well plates. On day 5, culture equivalent effector cells, typically 3-5 x 10^3 cells/well at the highest concentration, were assessed for IFN-γ and IL-4 production in our ELISPOT assay. The comparison between the IFN-γ and IL-4 producing cells in individual BALB/c and C57BL/6 mice is depicted in **C**. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the spleen cells from two to three mice per experiment. Data are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture. The p values were obtained with two-way analysis of variance (ANOVA) and Tukey's post-test for multiple comparisons.

**** $p < 0.0001$

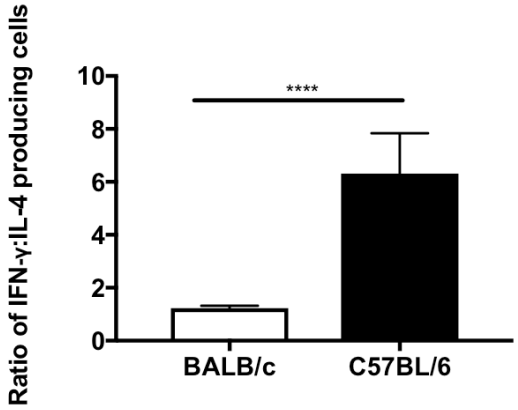


Figure 4.7: Significant difference between the ratio of IFN-γ:IL-4 producing cells of spleens of adult BALB/c and C57BL/6 mice. The figure represents the re-plotting of the data of Figure 4.6. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the spleen cells from two to three mice per experiment. Statistical significance was determined by unpaired students' *t* test. **** $p < 0.0001$

4.1.5 The CD4:CD8 T cell ratio of the spleens of CBA/J, C3H, DBA/2 and DBA/1 strains of mice affects is associated with a particular type of immune response, Th1/IFN-γ or Th2/IL-4, generated upon stimulation with allo-MHC

Once we had determined that the CD4:CD8 T cell ratio of the spleens of the BALB/c and C57BL/6 mice is associated with a particular type of immune response generated against allo-MHC we wanted to determine if this finding was consistent in other strains of mice. We used the CBA/J and C3H strains of mice that display a H-2^k MHC haplotype as well as the DBA/2 and the DBA/1 strains of mice, which display a H-2^d and a H-2^q MHC haplotype, respectively. Our first step was to characterize the CD4:CD8 T cell ratio of the spleens of the different strains of mice. Employing flow cytometry, we found that the spleen cells of the CBA/J and the C3H mice exhibited a CD4:CD8 T cell ratio below 2 while the spleens of the DBA/2 had a ratio above 2 (Figure 4.8 and Figure 4.9). The spleen cells of the DBA/1 mouse strain had a CD4:CD8 T cell of 2. We thus wanted to determine the type of immune response the spleen cells of the above-mentioned strains of mice would display upon stimulation with allo-MHC. Five days after culturing the spleen cells with irradiated F1, (BALB/c x C57BL/6) we harvested the cells and determined the expansion of cells in the different strains of mice using flow cytometry (Figure 4.10A and 4.10B). Thereafter spleens cells were restimulated with the nominal antigen and their allo-MHC-dependent IFN-γ and IL-4 production was assessed by employing the ELISPOT assay (Figure 4.10C and Figure 4.11). We found that the spleen cells of the CBA/J and C3H strains of mice that displayed a CD4:CD8 T cell ratio below 2 generated a predominant Th1 (IFN-γ) immune response, albeit, more so in the CBA/J mice than in the C3H strain. In contrast, the spleen cells of the DBA/1 and DBA/2 mouse strains that exhibited a CD4:CD8 T cell ratio of 2 and above 2, respectively generated a mixed Th1 (IFN-γ)/ Th2 (IL-4) immune response upon stimulation with allo-MHC.

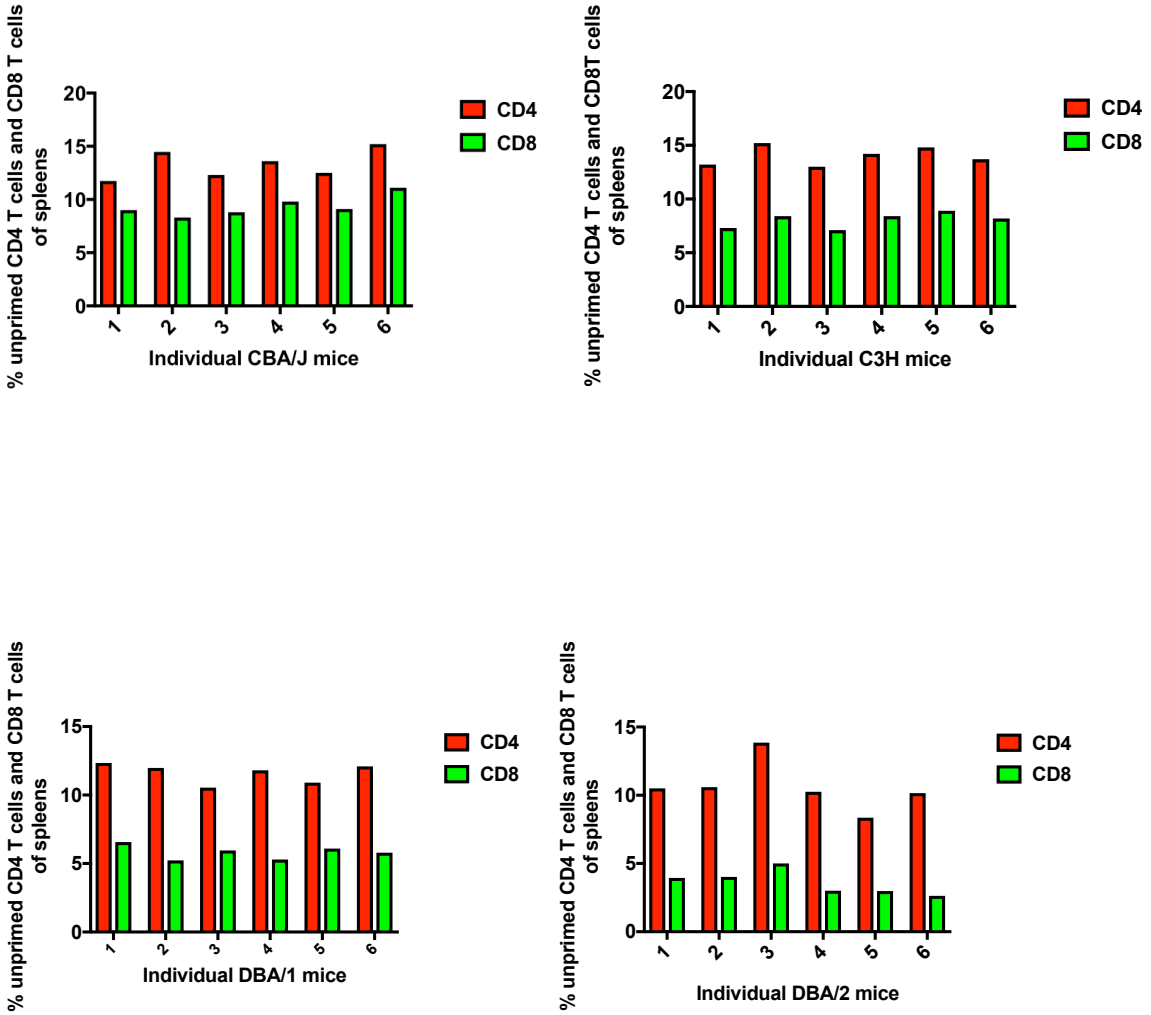


Figure 4.8: Variations in the CD4:CD8 T cell ratio of the spleens of individual, adult CBA/J, C3H, DBA/2 and DBA/1 mice. Unprimed cells of the spleens of the different strains of mice were stained with PE-anti-mouse CD4 (L3/T4), FITC anti-mouse CD8a (Ly2) and APC anti-mouse CD3 antibodies. Flow cytometry was employed to assess the percentage of CD4 T cells and CD8 T cells of the spleens of the different strains of mice at day 0. Data are representative of two independent experiments with three mice per strain per experiment.

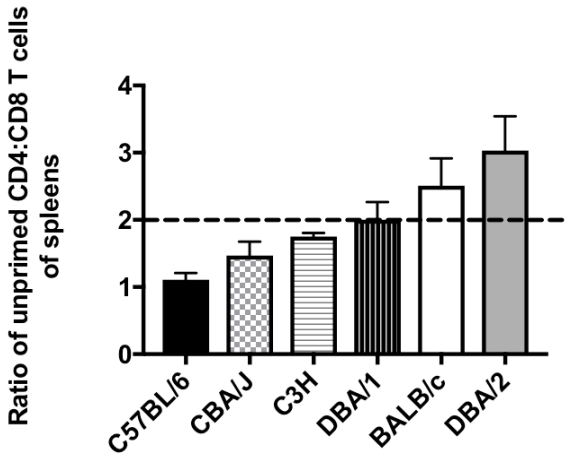
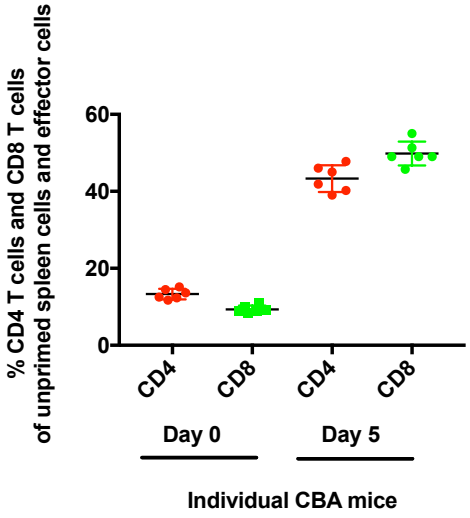
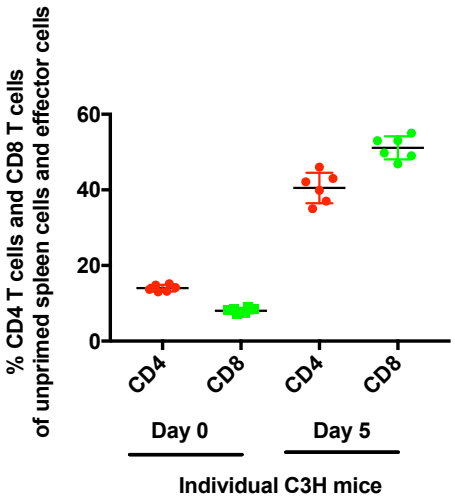
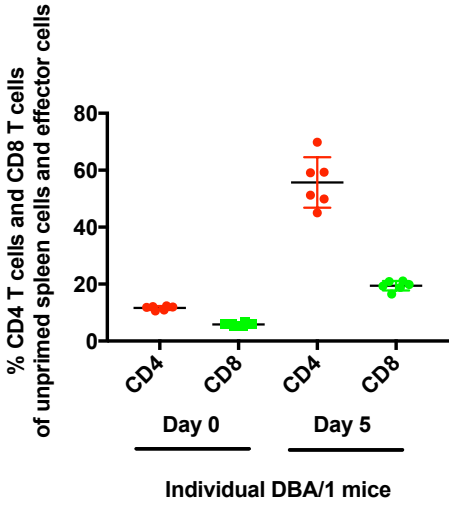


Figure 4.9: Comparison of the naturally occurring CD4:CD8 T cell ratios of different strains of mice. The figure represents the re-plotting of the data of Figure 4.5 and Figure 4.8. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of two independent experiments with the spleen cells from three individual, CBA/J, C3H, DBA/1 or DBA/2 mice per experiment and three independent experiments with the spleen cells from two to three individual, BALB/c and C57BL/6 mice per experiment.


% CD4 T cells and CD8 T cells
of unprimed spleen cells and effector cells
60
40
20
0
CD4
CD8
CD4
CD8
Day 0
Day 5
Individual CBA mice


% CD4 T cells and CD8 T cells
of unprimed spleen cells and effector cells
60
40
20
0
CD4
CD8
CD4
CD8
Day 0
Day 5
Individual C3H mice


% CD4 T cells and CD8 T cells
of unprimed spleen cells and effector cells
80
60
40
20
0
CD4
CD8
CD4
CD8
Day 0
Day 5
Individual DBA/1 mice

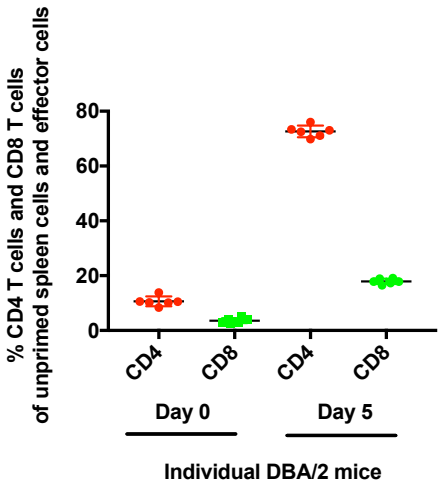
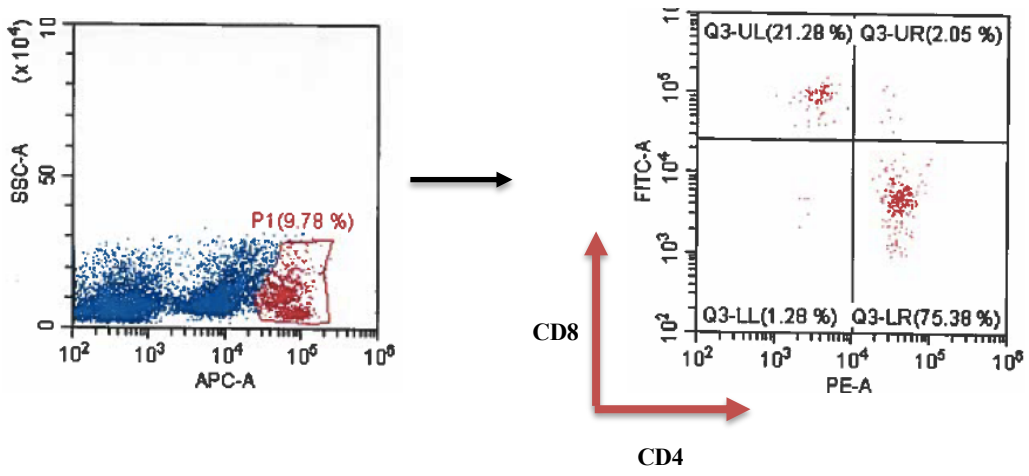
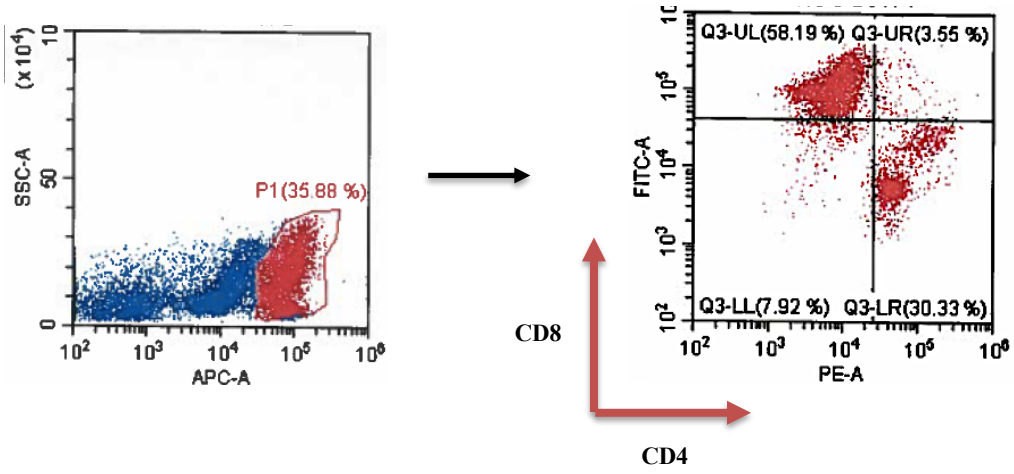

% CD4 T cells and CD8 T cells
of unprimed spleen cells and effector cells
80
60
40
20
0
CD4
CD8
CD4
CD8
Day 0
Day 5
Individual DBA/2 mice

Figure 4.10A



(I) DBA/2 mouse strain



(II) CBA/J mouse strain

Figure 4.10B

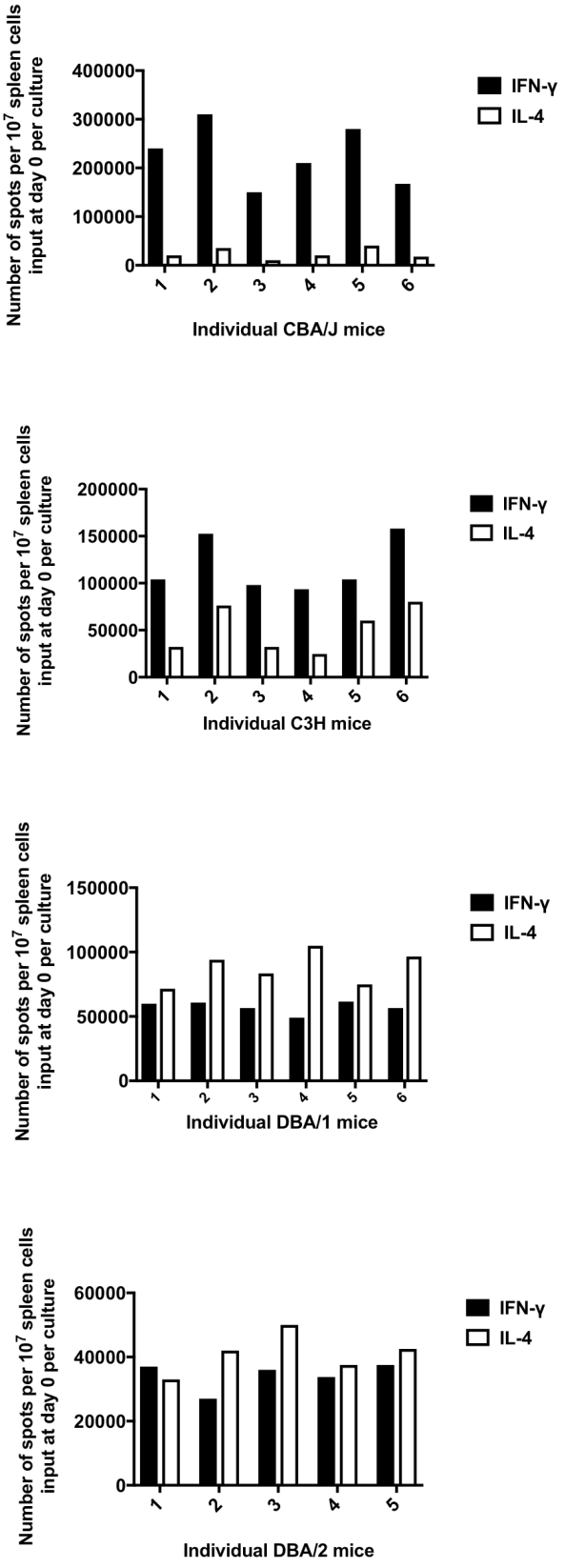

Number of spots per 10^7 spleen cells input at day 0 per culture
400000
300000
200000
100000
0
1
2
3
4
5
6
Individual CBA/J mice
IFN-γ
IL-4
Number of spots per 10^7 spleen cells input at day 0 per culture
200000
150000
100000
50000
0
1
2
3
4
5
6
Individual C3H mice
IFN-γ
IL-4
Number of spots per 10^7 spleen cells input at day 0 per culture
150000
100000
50000
0
1
2
3
4
5
6
Individual DBA/1 mice
IFN-γ
IL-4
Number of spots per 10^7 spleen cells input at day 0 per culture
60000
40000
20000
0
1
2
3
4
5
Individual DBA/2 mice
IFN-γ
IL-4

Figure 4.10C

Figure 4.10: The IFN-γ and IL-4 production of the Effector T cells of spleens of individual, adult CBA/J, C3H, DBA/2 and DBA/1 mice were assessed at day 5 of culture. (**A**) Four million unprimed spleen cells of either individual adult CBA/J, or C3H, or DBA/1 or DBA/2 mice were each cultured with an equivalent number of fresh, irradiated F1, (BALB/c x C57BL/6) spleen cells and incubated for 5 days at 37°C and 5% CO_2 in 12-well plates. (**A**) On day 5, effector cells of the different strains of mice were stained with PE-anti-mouse CD4 (L3/T4), FITC anti-mouse CD8a (Ly2) and APC anti-mouse CD3 antibodies. Flow cytometry was employed to assess the percentage of CD4 T cells and CD8 T cells of the spleens of the different strains of mice at day 5. (**B**) Flow cytometry data show one representative experiment of two independent experiments of the DBA/2 (**I**) and CBA/J (**II**) mouse strains. (**C**) Culture equivalent effector cells, typically 3-5 x 10^3 cells/well at the highest concentration, were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Data are representative of two independent experiments with two or three individual mice per experiment and are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture.

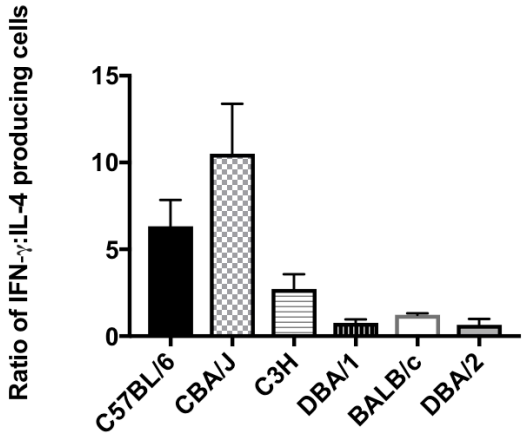
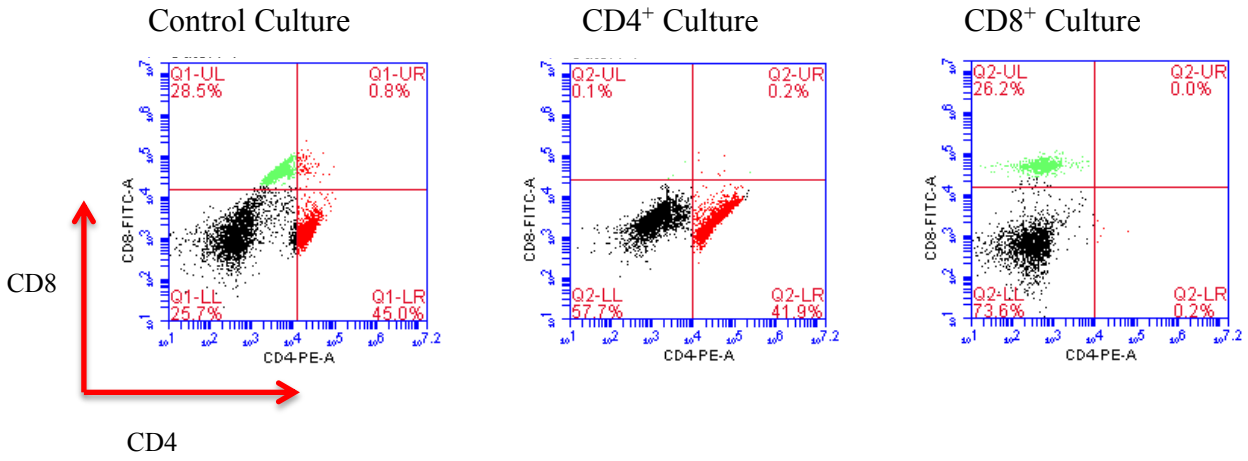


Figure 4.11: Comparison of the ratio of IFN-γ:IL-4 producing cells in different strains of mice. The figure represents the re-plotting of data from Figures 4.7 and 4.10C. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of two independent experiments with the spleen cells from five or seven mice per strain.

4.1.6 Characterization of the Phenotype of Effector T cells of the spleens of BALB/c mice producing IFN-γ and IL-4 Cytokines: IL-4 is produced exclusively by CD4 T cells

The findings presented in Figure 4.6 indicate that the BALB/c mouse generates a mixed Th1 (IFN-γ)/Th2 (IL-4) upon stimulation with allo-MHC, as opposed to the C57BL/6 mouse that generates a predominantly Th1 (IFN-γ) immune response. Consequently, we characterized the phenotype of the T cell that produces the IL-4 in BALB/c cultures activated *in vitro* with fresh irradiated F1, (BALB/c x C57BL/6) spleen cells. On day 5, cultures were sorted by negative selection with MACS monoclonal antibodies to CD8 positive T cell fractions and CD4 positive T cell fractions prior to their assessment for alloantigen-dependent cytokine production by the ELISPOT assay. To confirm the successful selection of the CD4 T cell and CD8 T cell populations flow cytometry was employed (Figure 4.12A). We found that, while IFN-γ, was produced by both CD4 T cells and CD8 T cells, IL-4 was produced exclusively by the CD4 T cells (Figure 4.12B).

A



B

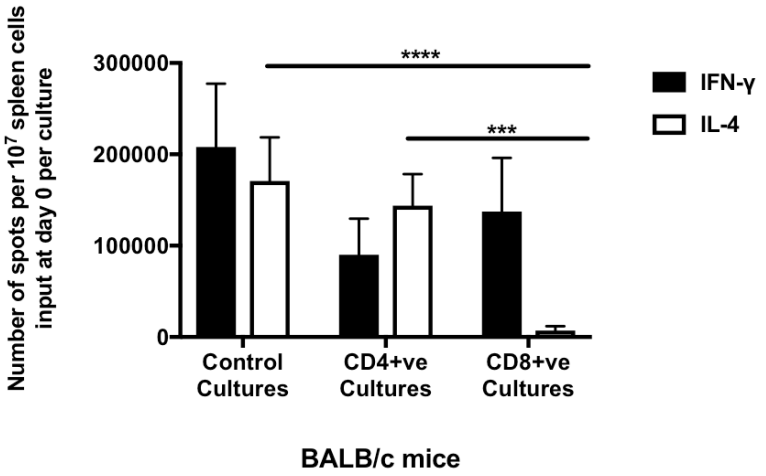


Figure 4.12

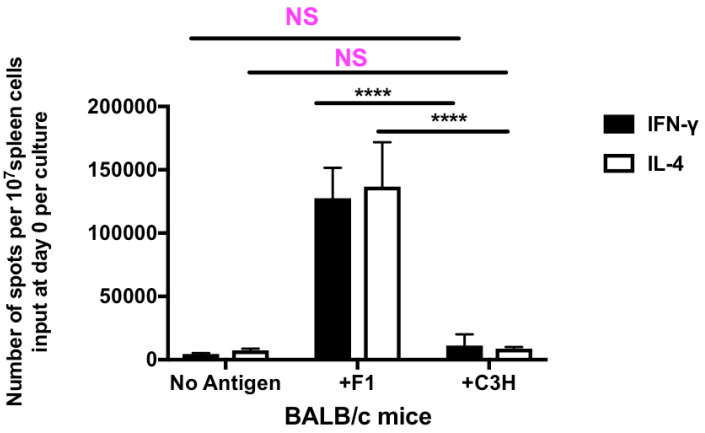
Figure 4.12: Characterization of the phenotype of Effector T cells producing IFN-γ and IL-4 cytokines: IL-4 is produced exclusively by CD4 T cells

Ten million unprimed spleen cells of BALB/c mice were cultured with an equivalent number of fresh, irradiated F1, (BALB/c x C57BL/6) mouse spleen cells and incubated for 5 days at 37°C and 5% CO_2 in 6-well plates. On day 5, cultures were fractionated by negative selection using MACS monoclonal antibodies to CD4 positive T cell fractions and CD8 positive T cell fractions prior to their assessment for allo-MHC dependent cytokine production by the ELISPOT assay. Fractionation was verified using Flow Cytometry. (**A**) Flow cytometry data show one representative experiment of three independent experiments. (**B**) Culture equivalent effector cells, typically 3-5 x 10^3 cells/well at the highest concentration, were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Data are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the spleen cells from six mice per strain. The p values were obtained with ANOVA and Tukey's post-test for multiple comparisons. *** $p = 0.0001$, ****$p < 0.0001$

4.1.7 The production of IFN-γ and IL-4 cytokines by Effector T cells is antigen-dependent and antigen specific

From this point on we continued our experiments using only our initial choice of mouse strains, the BALB/c and C57BL/6 mice. The next step was to determine if the immune response to allo-MHC that we observed for the BALB/c and C57BL/6 mice was dependent on the priming antigen as well as being able to discriminate between various antigens. In order to achieve this objective, we primed the spleen cells of either the BALB/c or the C57BL/6 mice with the irradiated F1 spleen cells for 5 days. Thereafter, the production of IFN-γ and IL-4 was ascertained either, with or without the restimulation with the nominal F1 antigen, to determine if the immune response generated was antigen dependent. Furthermore, the cells were also restimulated with unrelated allo-MHC cells that were from the freshly, irradiated spleen cells of a C3H mouse strain that exhibits the H-2^k haplotype. In the absence of the antigen or when stimulated with an unrelated antigen the spleen cells of the BALB/c mice (Figure 4.13A) and C57BL/6 mice (Figure 4.13B) produce negligible amounts of IFN-γ and IL-4. However, restimulation with the nominal F1 antigen, resulted in a mixed Th1 (IFN-γ) / Th2 (IL-4) immune response by the spleen cells of the BALB/c mice and a predominant Th1 (IFN-γ) immune response by the spleen cells of the C57BL/6 mice as initially observed in Figure 4.6C. This suggests that the immune response of the spleen cells of the BALB/c and the C57BL/6 mice to allo-MHC is antigen-dependent and antigen-specific.

A



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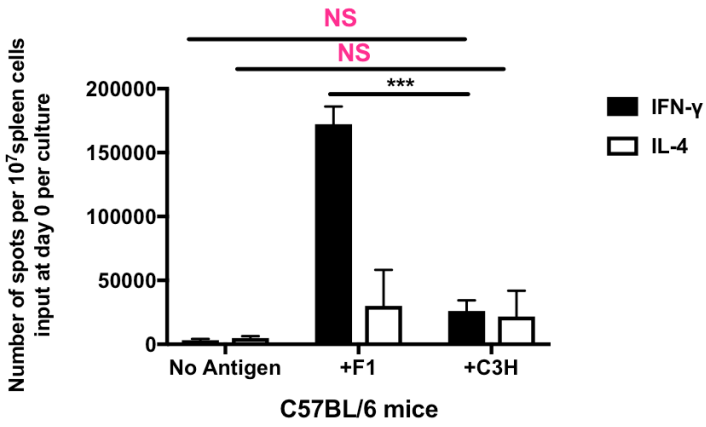


Figure 4.13

Figure 4.13: The production of IFN-γ and IL-4 in response to allo-MHC stimulation is antigen-dependent and antigen-specific. Ten million unprimed spleen cells of either adult BALB/c (A) or C57BL/6 (B) mice were cultured with an equivalent number of fresh, irradiated F1, (BALB/c x C57BL/6) spleen cells for 5 days at 37°C and 5% CO_2 in 6-well plates. On day 5, culture equivalent effector cells, typically 3-5 x 10^3 cells/well at the highest concentration, were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Primed cells received either no antigen, were restimulated with the nominal antigen, F1, or were restimulated with an unrelated antigen, the irradiated spleen cells of C3H mice. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the spleen cells from six mice per strain. The p values were obtained with ANOVA with Tukey's post-test for multiple comparisons. *** $p = 0.0001$, **** $p < 0.0001$**,**
NS not significant

4.1.8 Individual spleen cells versus pooled spleen cells

(I) No difference was observed in the percentages of CD4 T cells and CD8 T cells of individual spleens and pooled spleen cells of BALB/c and C57BL/6 mice

Due to the many varied objectives we wished to achieve in a single experiment, spleen cells from a single mouse would have been insufficient to successfully accomplish this. Thus, we decided to pool spleen cells from two or more mice per experiment. In order to justify the use of pooled spleen cells we first compared the unprimed CD4:CD8 T cell ratio of spleen cells from individual mice with that of pooled spleen cells using flow cytometry. There was no difference in the CD4 T cells and CD8 T cells of individual and pooled spleens of both BALB/c (Figure 4.14A) and C57BL/6 (Figure 4.14B) mice.

(II) The production of IFN-γ and IL-4 by the cells of individual spleens of BALB/c and C57BL/6 mice is equivalent to the IFN-γ and IL-4 production of pooled spleen cells upon restimulation with fresh irradiated F1 (BALB/c x C57BL/6) spleen cells

The next step was to compare the allo-MHC-dependent production of the IFN-γ and IL-4 by the individual spleens and the pooled spleens when cultured under identical conditions. As described above we cultured ten million unprimed spleen cells of either the individual spleens or the pooled spleen of both mouse strains with equivalent numbers of irradiated F1 allo-MHC for 5 days. Employing the ELISPOT assay we assessed the production of the IFN-γ and IL-4. A comparison of individual spleen cells versus pooled spleen cells indicated no difference between the frequencies of the IFN-γ or IL-4 producing cells after stimulation with allo-MHC in either, BALB/c (Figure 4.15A) or C57BL/6 (Figure 4.15B) mice. This result justified our use of pooled spleen cells in subsequent experiments.

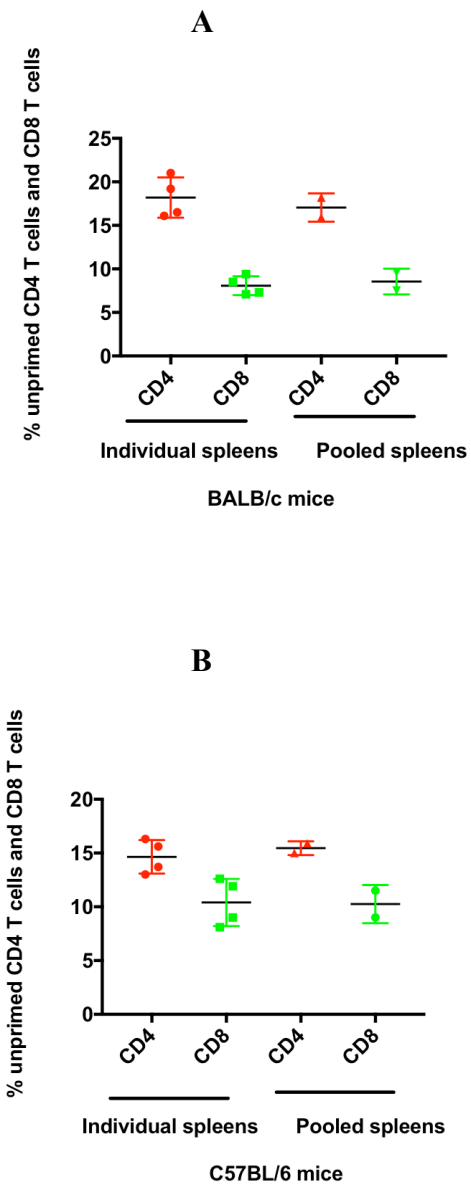
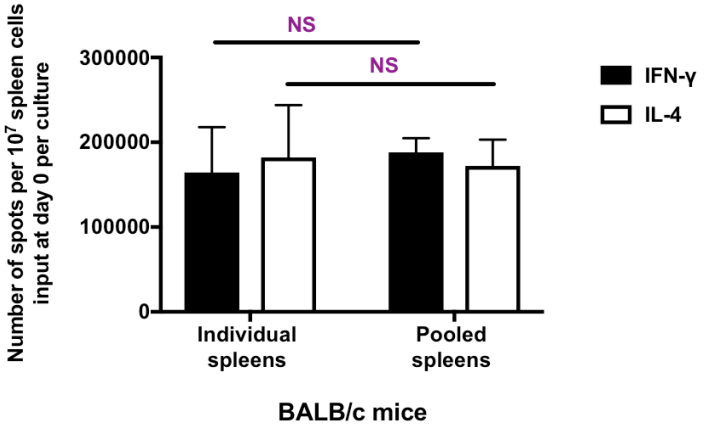


Figure 4.14: No difference in the percentages of CD4 T cells and CD8 T cells of individual spleens and pooled spleen cells of BALB/c and C57BL/6 mice. Unprimed cells of individual and pooled spleens of adult BALB/c (**A**) and C57BL/6 (**B**) mice (8-12-week-old) were stained with PE-anti-mouse CD4 (L3/T4) and FITC anti-mouse CD8a (Ly2) antibodies. The percentage of CD4 T cells and CD8 T cells were assessed via flow cytometry. Data are representative of two independent experiments with two individual mice per experiment.

A



B

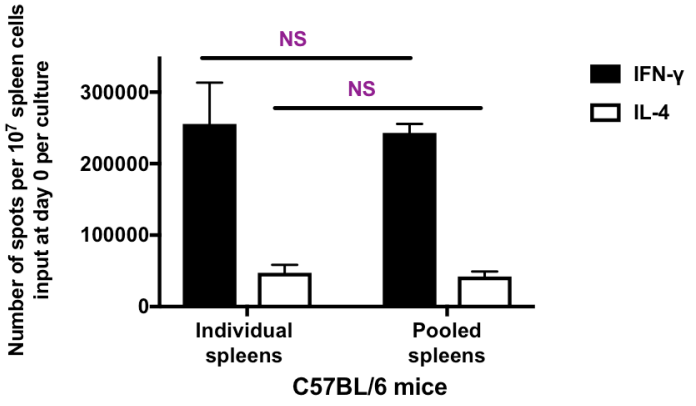


Figure 4.15

Figure 4.15: The production of IFN-γ and IL-4 by the cells of individual spleens of BALB/c and C57BL/6 mice is equivalent to the IFN-γ and IL-4 production of pooled spleen cells at day 5 of culture upon restimulation with F1 (BALB/c x C57BL/6) spleen cells. Ten million unprimed spleen cells of either individual spleens or pooled spleen cells of either BALB/c (**A**) or C57BL/6 (**B**) mice were cultured with an equivalent number of fresh, irradiated F1, (BALB/c x C57BL/6) spleen cells and incubated for 5 days at 37°C and 5% CO_2 in 6-well plates. On day 5, culture equivalent effector cells, typically 3-5 x 10^3 cells/well at the highest concentration, were assessed for IFN-γ and IL-4 in our ELISPOT assay. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of two independent experiments with the spleen cells from two individual mice per experiment. The p values were obtained with ANOVA and Tukey's post-test for multiple comparisons. NS: not significant

CHAPTER 5

The role of CD8 T cells in promoting the differentiation of naive CD4 T cells towards a Th1 (IFN-γ) mode and the concomitant inhibition of the CD4 Th2 (IL-4) subset

Preamble

The second section of the results explores the role of the CD8 T cell in the differentiation of naïve CD4 T cells to either a Th1 (IFN-γ) or a Th2 (IL-4) subset. The importance of CD8 T cells in enhancing a Th1 immune response has been demonstrated in various studies. In the GVHD mouse model the development of an acute GVHR in F1 mice was dependent on a high frequency of CD8 T cells in the parental inoculum of C57BL/6 mice injected into the F1 (C57BL/6 x DBA/2) mice (Tschetter et al., 2000; Via et al., 1987). In contrast, the near absence of CD8 T cells in the inoculum of DBA/2 mice resulted in an antibody mediated autoimmune disease. Herath has demonstrated in 2003 the significance of the CD8 T cells in providing protection against an intracellular pathogen, *L major* (Herath et al., 2003). The presence of the CD8 T cells enhanced a Th1 immune response, characterized by the presence of IFN-γ which was required for protection, while the absence of CD8 T cells resulted in a Th2 immune response, which rendered the individual mice susceptible to the pathogen. These findings argue in favour of a regulatory role for CD8 T cells in determining the type of immune response, Th1 or Th2, generated.

We thus set out to explore in our *in vitro* system the effect of the CD8 T cells on the differentiation of the naïve CD4 T cell into either a Th1 (IFN-γ) or a Th2 (IL-4) producing cell in the spleens of BALB/c and C57BL/6 mice upon allo-MHC stimulation. Undertaking *in vitro* experiments allowed us to manipulate cultures in order to research on the mechanism of the regulatory role of the CD8 T cells on CD4 T cell differentiation. According to our model in Figure 2.1, differentiation of unprimed CD4 T cells towards a stable Th1 phenotype is critically dependent on the concomitant induction of CD8 T cells by allo-MHC. In contrast, the absence of the CD8 T cells in the initial priming of lymphocytes by allo-MHC greatly facilitates the differentiation of CD4 T cells towards the Th2 lineage. To test our hypothesis, we used two approaches. The indirect approach examined the immune response of the naïve CD4 T cells upon stimulation with allo-MHC

in the absence of CD8 T cells. Spleen cells were depleted of CD8 T cells by using anti-CD8 TIB 211 antibody and rabbit complement as described in the Material and Methods. In the direct approach the original CD4:CD8 T cell ratio of CD8 T cell depleted cultures was reconstructed as close as possible to the original ratio by the addition of sorted naïve CD8 T cells. The ELISPOT assay was used to assess the production of IFN-γ and IL-4 at the single cell level in cultures in which the CD8 T cells were either present or absent or in cultures to which different numbers of CD8 T cells were added.

5.1 CD8 T cells regulate the differentiation of the CD4 T cell to either a Th1(IFN-γ) or a mixed Th1/Th2 (IFN-γ /IL-4) immune response

5.1.1 The absence of CD8 T cells at the initiation of the immune response to allo-MHC results in the CD4 T cells displaying a Th2 (IL-4) phenotype (Indirect Approach)

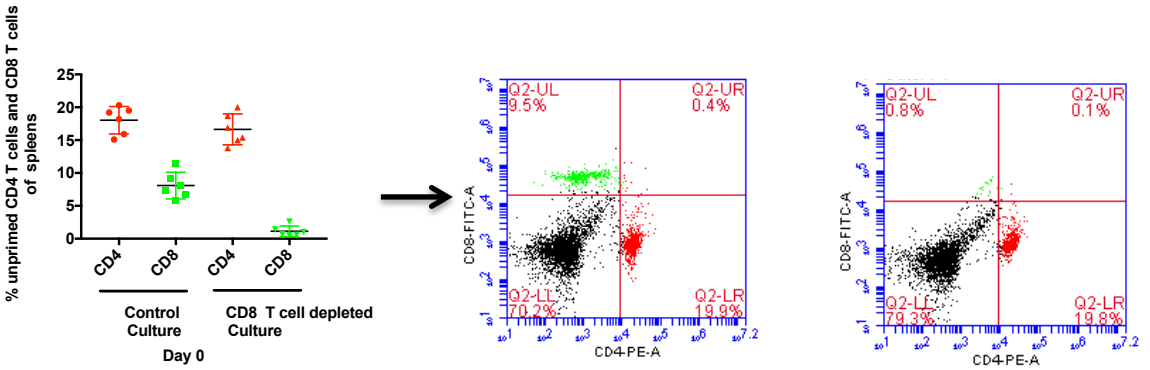
In the indirect approach the differentiation of the CD4 T cell, in response to stimulation with allo-MHC was determined in the absence of CD8 T cells. The CD8 T cells were depleted from the spleen cells using anti-CD8 TIB 211 antibody and Low Tox-M rabbit complement. Flow cytometry verified the depletion of CD8 T cells from the spleen cells of the BALB/c mice (Figure 5.1A) and the C57BL/6 mice (Figure 5.2A) at day 0 and that this was maintained after 5 days (Figure 5.1B and Figures 5.2B) in culture. The control cultures of the spleens of BALB/c mice contained both CD4 and CD8 T cells and resulted in a mixed Th1(IFN-γ) /Th2 (IL-4) immune response. Similarly, the control cultures of the spleens of C57BL/6 mice produced a predominant Th1 (IFN-γ) immune response as illustrated in Figure 4.6. However, it is evident that in the absence of CD8 T cells, spleen cells acquired a Th2 (IL-4) phenotype in response to allo-MHC which is depicted by the predominant production of IL-4 in both mouse strains (Figure 5.1C and Figure 5.2C). The predominant IL-4 production in the CD8 T cell depleted cultures of both mouse strains equate to a significant difference in the IFN-γ:IL-4 ratio of both BALB/c (Figure 5.3A) and C57BL/6 (Figure 5.3B). The fact that both types of culture are stimulated under identical conditions and the only difference between responder cells is the presence or absence of

CD8 T cells argues favourably for a regulatory role for the CD8 T cell in the acquisition of the Th1 (IFN-γ) or the Th2 (IL-4) phenotype by naïve CD4 T cells.

In addition, we wanted to ascertain whether these findings were consistent across other strains of mice. Thus, the spleen cells of CBA/J, C3H, DBA/2 and DBA/1 mice were depleted of CD8 T cells as described above. CD8 T cell depleted spleens were cultured with irradiated allo-MHC for 5 days at 37°C and 5% CO_2. We found that the depletion of the CD8 T cells from the spleens of mice resulted in a predominant Th2 (IL-4) immune response in all strains of mice tested, CBA/J, C3H, DBA/2 and DBA/1 (Figure 5.4). Thus, we can conclude that in the absence of CD8 T cells, CD4 T cells differentiate into a Th2 (IL-4) subset upon stimulation with allo-MHC.

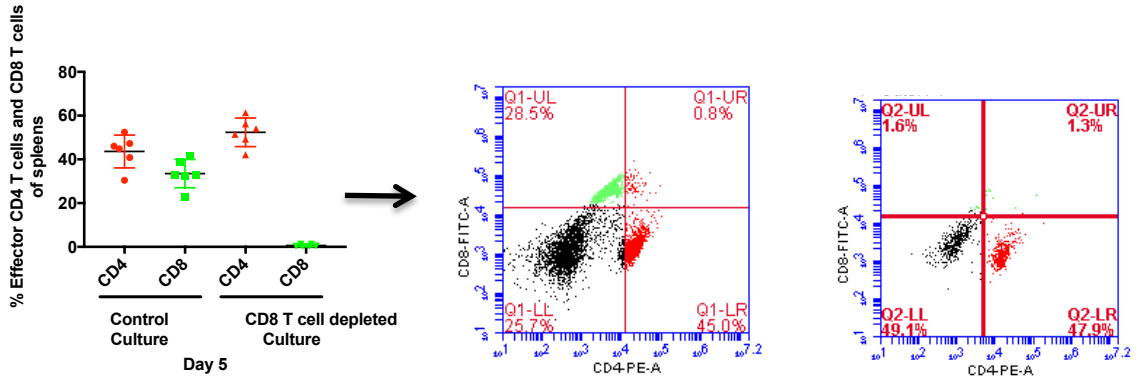
We also decided to determine if the immune response of the CD4 T cells in the CD8 T cell depleted cultures stimulated with allo-MHC in the BALB/c and C57BL/6 mice was also antigen-dependent and antigen-specific. The CD8 T cell depleted cultures were restimulated on day 5 with the nominal F1 antigen or an unrelated antigen, the irradiated spleen cells of the C3H mouse. CD8 T cell depleted cultures also received no antigen stimulation in the ELISPOT plate. We found that the immune response of the CD4 T cells to allo-MHC in the CD8 T cell depleted cultures of both BALB/c (Figure 5.5A) and C57BL/6 (Figure 5.5B) mice were also antigen-dependent and antigen-specific.

A



Control Culture CD8 T cell Depleted Culture

B



Control Culture CD8 T cell Depleted Culture

C

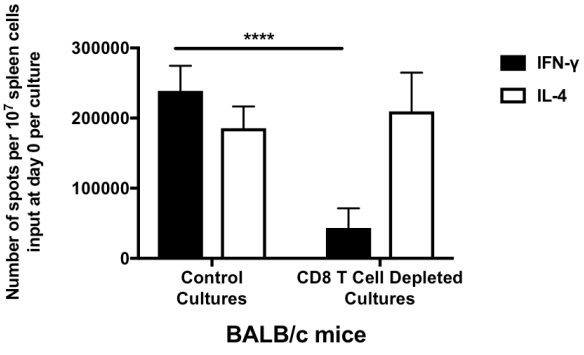
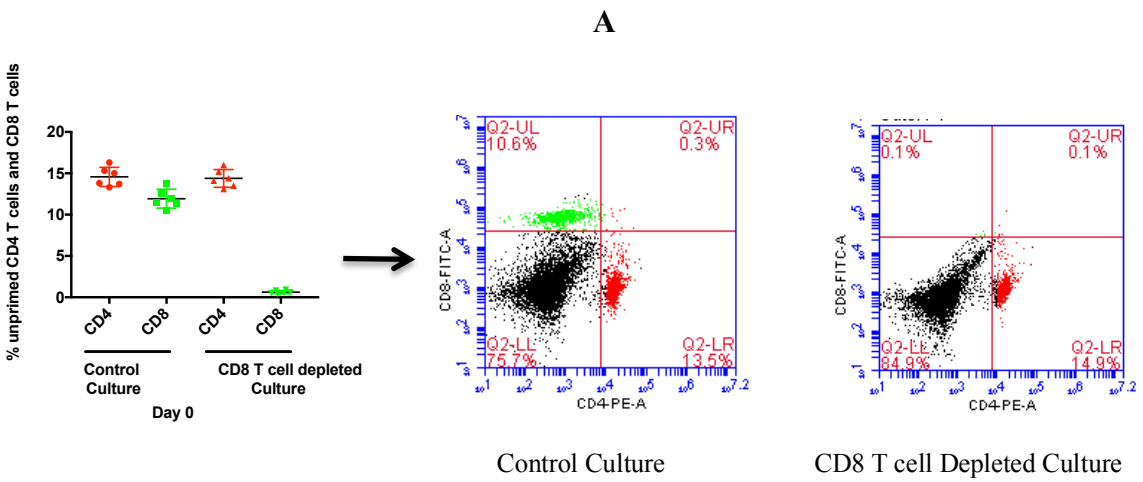
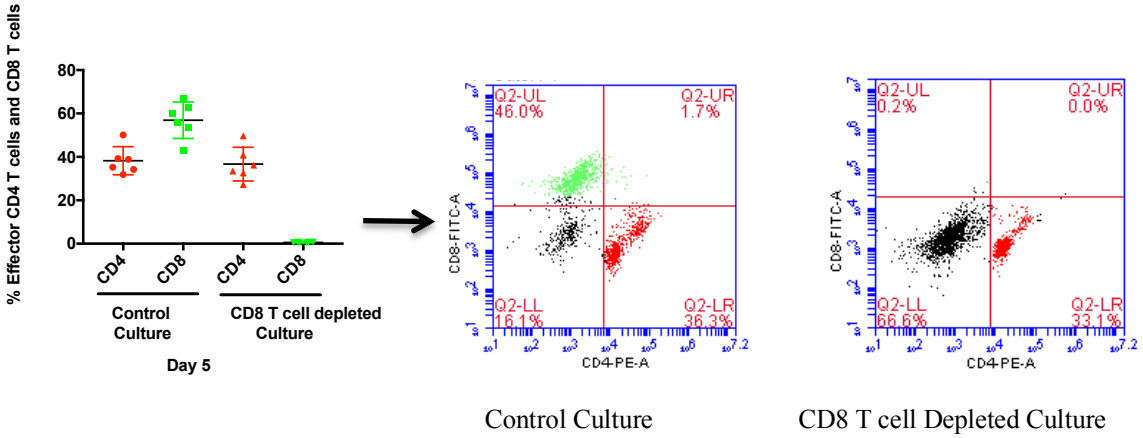


Figure 5.1

Figure 5.1: CD8 T cells regulate the immune response of the spleen cells of BALB/c mice away from a Th2 mode towards a mixed Th1/Th2 mode. Ten million spleen cells from BALB/c mice or equivalent numbers of CD8 T cell depleted spleen cells, were cultured with ten million irradiated F1, (BALB/c x C57BL/6), spleen cells for 5 days, at 37°C and 5% CO_2 in 6-well plates. Cultures were depleted of CD8 T cells by using anti-CD8 TIB 211 antibody and Low Tox-M rabbit complement. Percentages of CD4 T cells and CD8 T cells were assessed by flow cytometry on day 0 (**A**) and day 5 (**B**). Flow cytometry data show one representative experiment of three independent experiments. (**C**) On day 5, culture equivalent effector cells, typically 3-5 x 10^3 cells/well at the highest concentration, were assessed for IFN-γ and IL-4 production in our ELISPOT assay. ELISPOT data are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the spleen cells from two individual mice per experiment. The p values were obtained with ANOVA and Tukey's post-test for multiple comparisons.

****$p < 0.0001$


A
% unprimed CD4 T cells and CD8 T cells
20
15
10
5
0
CD4
CD8
CD4
CD8
Control Culture
CD8 T cell depleted Culture
Day 0
Q2-UL 10.6%
Q2-UR 0.3%
Q2-LL 75.7%
Q2-LR 13.5%
CD8-FITC-A
CD4-PE-A
Q2-UL 0.1%
Q2-UR 0.1%
Q2-LL 84.9%
Q2-LR 14.9%
CD8-FITC-A
CD4-PE-A
Control Culture
CD8 T cell Depleted Culture


B
% Effector CD4 T cells and CD8 T cells
80
60
40
20
0
CD4
CD8
CD4
CD8
Control Culture
CD8 T cell depleted Culture
Day 5
Q2-UL 46.0%
Q2-UR 1.7%
Q2-LL 16.1%
Q2-LR 36.3%
CD8-FITC-A
CD4-PE-A
Q2-UL 0.2%
Q2-UR 0.0%
Q2-LL 66.6%
Q2-LR 33.1%
CD8-FITC-A
CD4-PE-A
Control Culture
CD8 T cell Depleted Culture

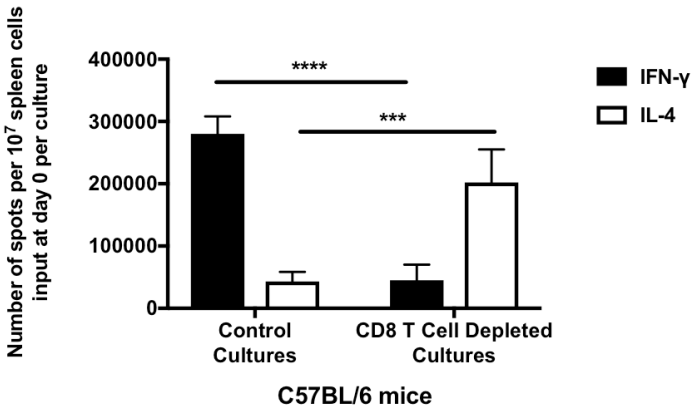
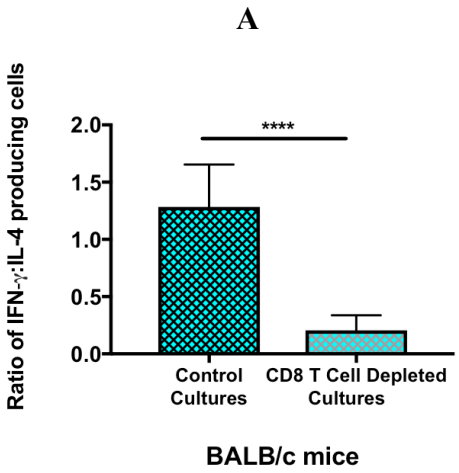

C
Number of spots per 10^7 spleen cells input at day 0 per culture
400000
300000
200000
100000
0
****
***
IFN-γ
IL-4
Control Cultures
CD8 T Cell Depleted Cultures
C57BL/6 mice

Figure 5.2

Figure 5.2: CD8 T cells regulate the immune response of the spleen cells of C57BL/6 mice towards a Th1 mode. Ten million spleen cells from the C57BL/6 mouse or equivalent numbers of CD8 T cell depleted spleen cells, were cultured with irradiated F1, (BALB/c x C57BL/6), spleen cells for 5 days, at 37°C and 5% CO_2 in 6-well plates. Cultures were depleted of CD8 T cells by using anti-CD8 TIB 211 antibody and Low Tox-M rabbit complement. Percentages of CD4 T cells and CD8 T cells were assessed by flow cytometry on day 0 (**A**) and day 5 (**B**). Flow cytometry data show one representative experiment of three independent experiments. (**C**) On day 5, culture equivalent effector cells, typically 3-5 x 10^3 cells/well at the highest concentration, were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the spleen cells from two individual mice per experiment. The p values were obtained with ANOVA and Tukey's post-test for multiple comparisons.

*** $p = 0.0001$, ****$p < 0.0001$



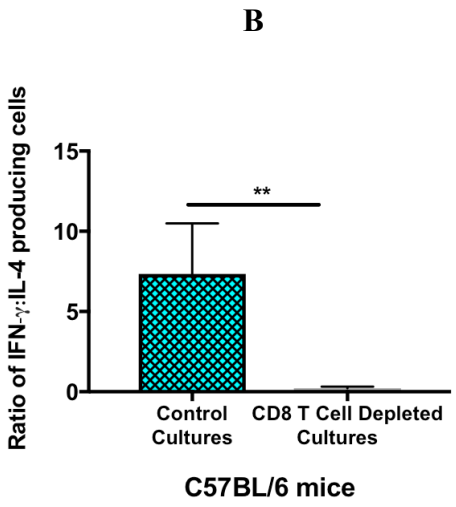


Figure 5.3: Significant difference in the ratio of IFN-γ:IL-4 producing spleen cells in the control cultures and the CD8 T cell depleted cultures of BALB/c and C57BL/6 mice. The figure represents the re-plotting of the data of BALB/c mice in Figure 5.1C and the C57BL/6 mice in Figure 5.2C. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the spleen cells from two individual mice per experiment. Statistical analysis was determined by unpaired student's t test. ** $p = 0.0039$; **** $p < 0.0001$

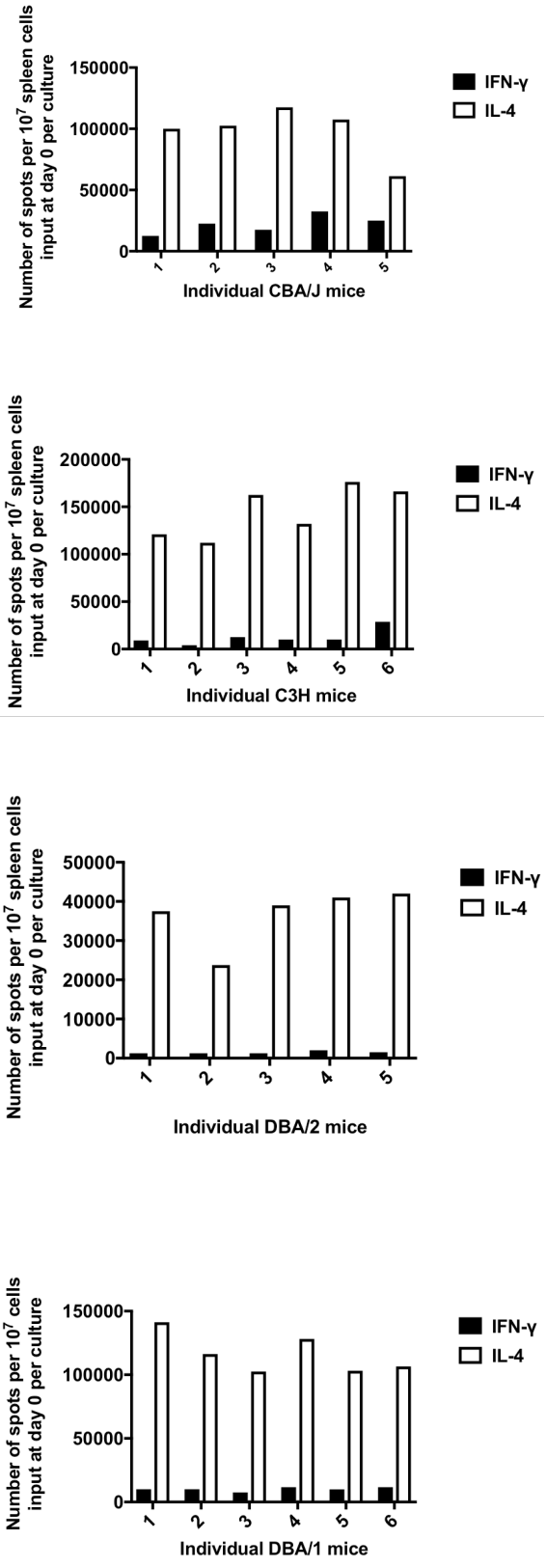
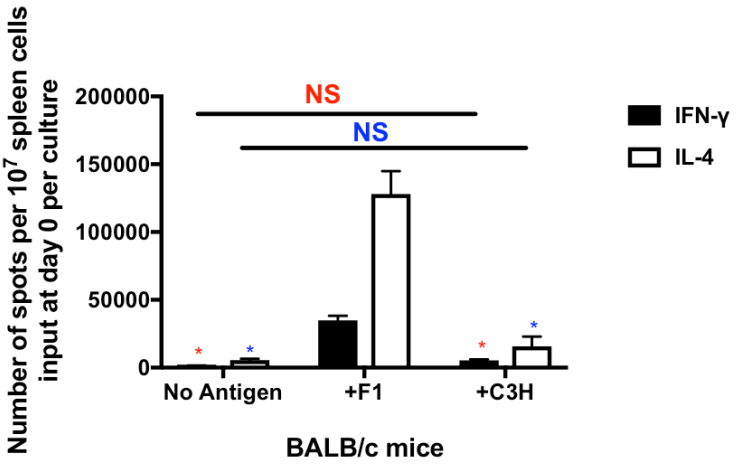

Number of spots per 10^7 spleen cells input at day 0 per culture
150000
100000
50000
0
1
2
3
4
5
Individual CBA/J mice
IFN-γ
IL-4
Number of spots per 10^7 spleen cells input at day 0 per culture
200000
150000
100000
50000
0
1
2
3
4
5
6
Individual C3H mice
IFN-γ
IL-4
Number of spots per 10^7 spleen cells input at day 0 per culture
50000
40000
30000
20000
10000
0
1
2
3
4
5
Individual DBA/2 mice
IFN-γ
IL-4
Number of spots per 10^7 cells input at day 0 per culture
150000
100000
50000
0
1
2
3
4
5
6
Individual DBA/1 mice
IFN-γ
IL-4

Figure 5.4

Figure 5.4: CD8 T cells regulate the immune response towards a Th1 mode and away from a Th2 phenotype in the CBA/J, C3H, DBA/2 and DBA/1 strains of mice. Four million spleen cells depleted of CD8 T cells, were cultured with four million irradiated F1, (BALB/c x C57BL/6), spleen cells at 37°C and 5% CO_2 for 5 days. Cultures were depleted of CD8 T cells by using anti-CD8 TIB 211 antibody and Low Tox-M rabbit complement. On day 5, culture equivalent effector cells, typically 3-5 x 10^3 cells/well at the highest concentration, were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Data are representative of two independent experiments with spleen cells from two or three individual mice per experiment and are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture.

A


Number of spots per 10^7 spleen cells input at day 0 per culture
200000
150000
100000
50000
0
NS
NS
IFN-γ
IL-4
No Antigen
+F1
+C3H
BALB/c mice

B

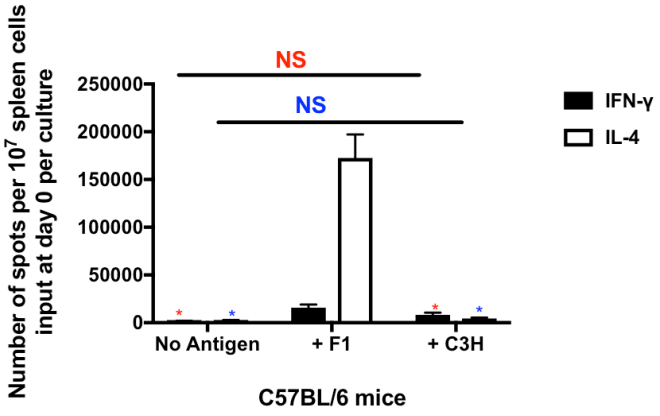

Number of spots per 10^7 spleen cells input at day 0 per culture
250000
200000
150000
100000
50000
0
NS
NS
IFN-γ
IL-4
No Antigen
+ F1
+ C3H
C57BL/6 mice

Figure 5.5

Figure 5.5: The production of IFN-γ and IL-4 by Effector T cells in CD8 T cell depleted cultures is alloantigen-dependent and alloantigen-specific. Ten million spleen cells depleted of CD8 T cells of either BALB/c (**A**) or C57BL/6 (**B**) mice were cultured with an equivalent number of fresh, irradiated F1, (BALB/c x C57BL/6) spleen cells for 5 days, at 37°C and 5% CO_2 in 6-well plates. On day 5, culture equivalent effector cells, typically 3-5 x 10^3 effector cells/well at the highest concentration, were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Primed cells received no antigen, or were restimulated with the nominal antigen, irradiated F1 spleen cells or restimulated with an unrelated alloantigen, irradiated spleen cells of C3H mice. Data are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the spleen cells from two individual mice per experiment. The p values were obtained with ANOVA and Tukey's post-test comparing for multiple comparisons.

NS not significant

5.1.2 The reconstruction of the CD4:CD8 T cell ratio in CD8 T cell depleted cultures with sorted unprimed CD8 T cells switches the immune response from a Th2 to a Th1 mode in C57BL/6 mice or to a mixed Th1/Th2 phenotype in BALB/c mice (Direct Approach)

We further tested the regulatory role of CD8 T cells in the decision-making process of switching the Th2 (IL-4) immune response to a Th1 (IFN-γ) mode by reconstructing the CD4:CD8 T cell ratio of CD8 T cell depleted cultures to that of normal controls (as closely as possible) and comparing the IFN-γ and IL-4 producing cells in the reconstructed cultures. We obtained a sorted CD8 T cell population from the spleens of the BALB/c (Figure 5.7A) and C57BL/6 (Figure 5.8A) mice as described in Materials and Methods. The production of IFN-γ and IL-4 by the control cultures, CD8 T cell depleted cultures and reconstructed CD8 T cell depleted cultures were assessed by ELISPOT assay on day 5 of culture. We found that the CD8 T cells were able to quantitatively switch the immune response from a Th2 (IL-4) immune response to a mixed Th1(IFN-γ)/Th2(IL-4) mode, through the restoration of the IFN-γ in the CD8 T cell depleted cultures of the BALB/c mice (Figures 5.7B and C). Similarly, the addition of CD8 T cells to the CD8 T cell depleted cultures of C57BL/6 mice at day 0 was able to switch the immune response from a predominant Th2 (IL-4) immune response to a predominant Th1 (IFN-γ) mode (Figures 5.8B and C). Thus, we found that the immune response observed in the control cultures was restored in the reconstructed CD8 T cell depleted cultures. The findings in Figure 5.7 and Figure 5.8 confirms the regulatory role of CD8 T cells in the differentiation of naïve CD4 T cells towards a mixed Th1/Th2 immune response in BALB/c mice and towards a predominant Th1 immune response in C57BL/6 mice.

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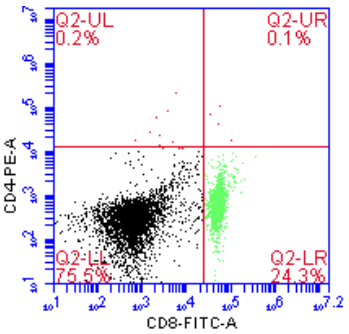

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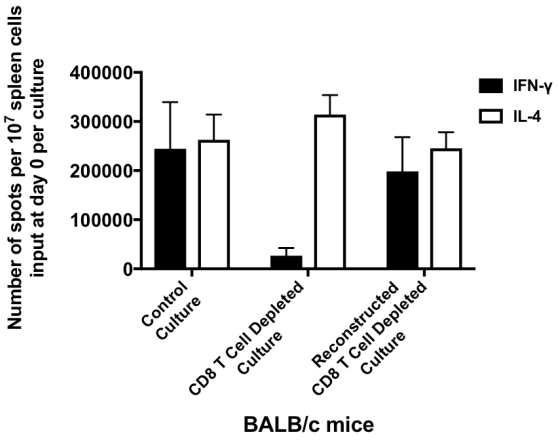

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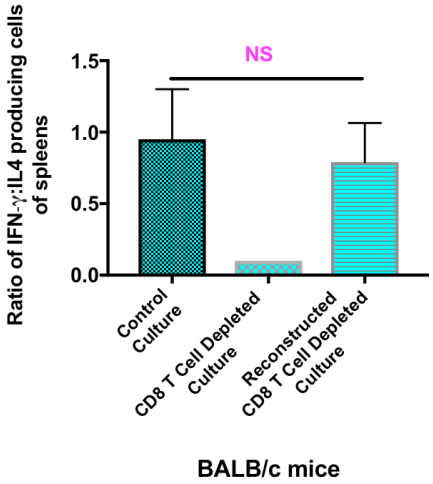

Figure 5.6

Figure 5.6: The presence of CD8 T cells results in a switch from a Th2 to a mixed Th1/Th2 immune response in reconstructed CD8 T cell depleted cultures of the BALB/c mice. Ten million spleen cells from BALB/c mice and equivalent numbers of CD8 T cell depleted spleen cells were cultured with ten million irradiated F1, (BALB/c x C57BL/6), spleen cells at 37°C and 5% CO_2 for 5 days in 6-well plates. A specific number of sorted CD8 T cells, representing their natural percentage in normal spleens, were added to the cultures at day 0 in order to reconstruct the naturally occurring CD4:CD8 T cell ratio. Sorted $CD8^+CD4^-$ T cells were obtained using the non-adherent fraction of the Nylon wool column for T cell enrichment. Subsequently CD8 T cells were obtained by negative selection using MACS monoclonal antibodies to CD4 T cells. (A) Verification of sorted CD8 T cells was assessed by flow cytometry. Flow cytometry data show one representative experiment of three independent experiments. (B) On day 5, culture equivalent effector cells, typically 3-5 x 10^3 cells/well at the highest concentration, were assessed for IFN-γ and IL-4 production in our ELISPOT assay. (C) Comparison of the IFN-γ:IL-4 ratio of the control, CD8 T cell depleted and reconstructed CD8 T cell depleted cultures. Data are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the pooled spleen cells from three mice per experiment. Statistical analysis was determined by unpaired student's t test.
NS: not significant

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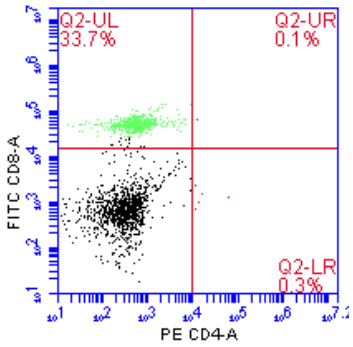

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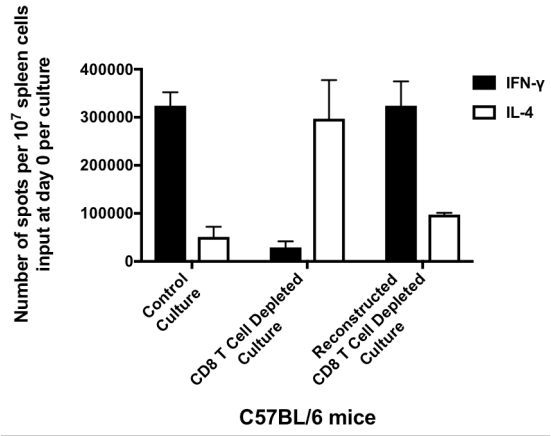

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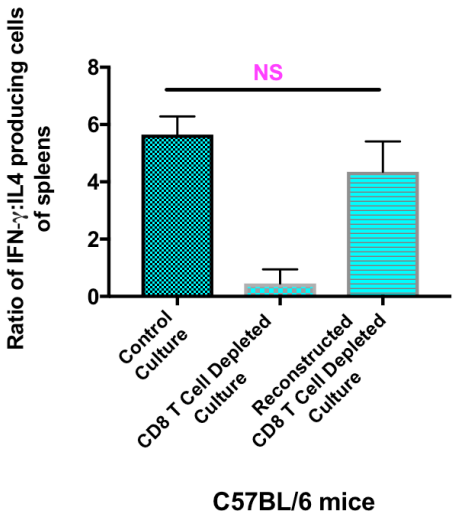

Figure 5.7

Figure 5.7: The presence of CD8 T cells results in a switch from a Th2 to a Th1 immune response in reconstructed CD8 T cell depleted cultures of the C57BL/6 mice. Ten million spleen cells from C57BL/6 mice and equivalent numbers of CD8 T cell depleted spleen cells were cultured with irradiated F1, (BALB/c x C57BL/6), spleen cells for 5 days, at 37°C and 5% CO_2 in 6-well plates. A specific number of sorted CD8 T cells, representing their natural percentage in normal spleens, were added to the cultures at day 0 in order to reconstruct the naturally occurring CD4:CD8 T cell ratio. Sorted $CD8^+CD4^-$ T cells were obtained using Nylon wool for T cell enrichment and thereafter, negative selection using MACS monoclonal antibodies to CD4 T cells. Verification of sorted CD8 T cells was assessed by flow cytometry. (**A**) Flow cytometry data show one representative experiment of three independent experiments. (**B**) On day 5, culture equivalent Effector cells were assessed for IFN-γ and IL-4 production in our ELISPOT assay. (**C**) Comparison of the IFN-γ:IL-4 ratio of the Control, CD8 T cell depleted and Reconstructed CD8 T cell depleted cultures are depicted. ELISPOT data are plotted as the number of spots per 10^7cells input per culture. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the pooled spleen cells from three mice per experiment. Statistical analysis was determined by unpaired student's t test. NS: not significant

5.1.3 The reconstruction of the CD4:CD8 T cell ratio in CD8 T cell depleted cultures with sorted unprimed CD8 T cells switches the CD4 Th2 (IL-4) immune response to a mixed Th1(IFN-γ)/Th2 (IL-4) phenotype

Immune regulation as described in the introduction states that the CD8 T cell inhibits the antibody response (Bretscher, 1974; Ramshaw et al., 1977). Our work assessed the differentiation of naïve CD4 T cells by cognate regulation of CD8 T cells at the level of cytokines produced. Our findings in Figures 5.6 and 5.7 show that the addition of sorted unprimed CD8 T cells to CD8 T cell depleted cultures resulted in an increase in the production of IFN-γ and a decrease in IL-4 in both the BALB/c and the C57BL/6 cultures. We, however, wished to determine the effect the addition of sorted CD8 T cells would have on the production of cytokines by the CD4 T cells. We wanted to determine whether the increase in IFN-γ observed in the reconstructed CD8 T cell depeted cultures occured at the CD4 T cell level and was not only due to the addition of CD8 T cells which is known to produce IFN-γ. Thus at day 5 of culture we obtained $CD4^+CD8^-$ T cell populations from the control cultures, the CD8 T cell depleted cultures and the reconstructed CD8 T cell depleted cultures by negative selection using MACS monoclonal antibodies to CD8 T cells. The CD4 T cells in the reconstructed CD8 T cell depleted cultures of the BALB/c spleen cells (Figure 5.8) showed a decrease in IL-4 and an increase in IFN-γ. Similarly, the CD4 T cells in the reconstructed CD8 T cell depleted cultures showed a decrease in IL-4 and an increase in IFN-γ (Figure 5.9). Our findings demonstrate that the addition of sorted unprimed CD8 T cells to the reconstructed cultures causes a switch from a predominant Th2 (IL-4) response to a mixed IFN-γ /IL-4 response by the CD4 T cell.

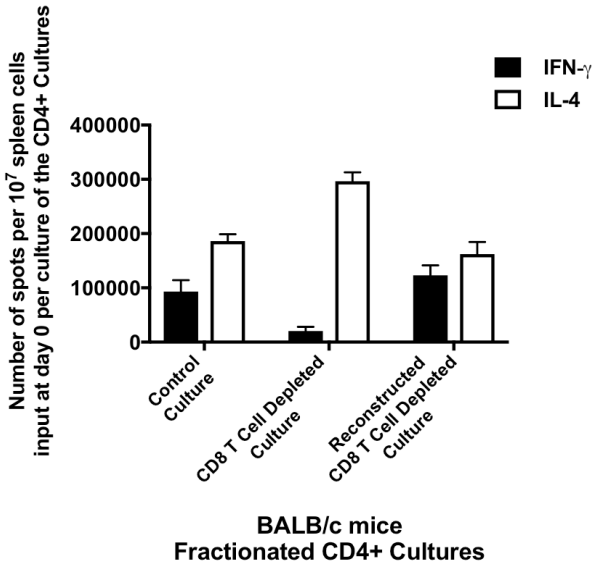

Figure 5.8: The presence of CD8 T cells switches the CD4 Th2 (IL-4) response to a mixed Th1 (IFN-γ)/Th2 (IL-4) immune response in reconstructed CD8 T cell depleted cultures in BALB/c mice. Ten million spleen cells from BALB/c mice and equivalent numbers of CD8 T cell depleted spleen cells were cultured with ten million irradiated F1, (BALB/c x C57BL/6), spleen cells at 37°C and 5% CO_2 for 5 days in 6-well plates. A specific number of sorted, unprimed CD8 T cells were added to the CD8 T cell depleted cultures at day 0 in order to reconstruct the naturally occurring CD4:CD8 T cell ratio. On day 5, control and reconstructed cultures were fractionated into $CD4^+CD8^-$ T cell populations by negative selection using MACS monoclonal antibodies to CD8 T cells prior to their assessment for allo-MHC dependent cytokine production by the ELISPOT assay. Culture equivalent Effector cells of the $CD4^+CD8^-$ T cell populations were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Data are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the pooled spleen cells from three mice per experiment.

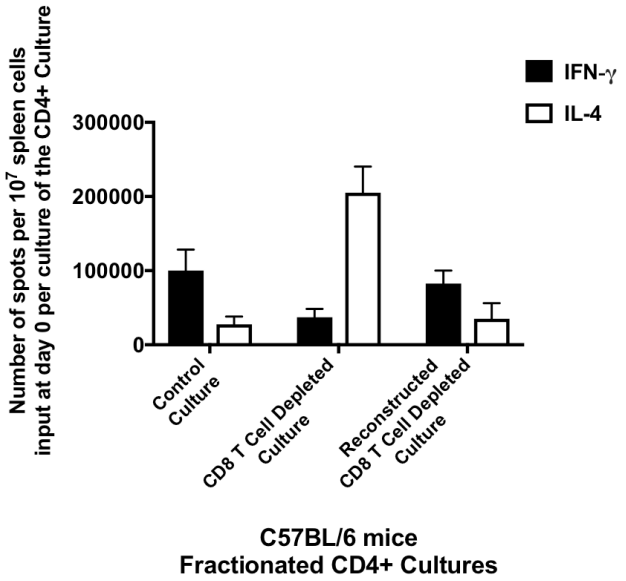

Figure 5.9: The presence of CD8 T cells switches the CD4 Th2 (IL-4) response to a mixed Th1 (IFN-γ)/Th2 (IL-4) in reconstructed CD8 T cell depleted cultures in C57BL/6 mice. Ten million spleen cells from C57BL/6 mice and equivalent numbers of CD8 T cell depleted spleen cells were cultured with ten million irradiated F1, (BALB/c x C57BL/6), spleen cells for 5 days, at 37°C and 5% CO_2 in 6-well plates. A specific number of sorted, unprimed CD8 T cells were added to the CD8 T cell depleted cultures at day 0 in order to reconstruct the naturally occurring CD4:CD8 T cell ratio. On day 5, control and reconstructed cultures were fractionated into $CD4^+$ $CD8^-$T cell populations by negative selection using MACS monoclonal antibodies to CD8 T cells prior to their assessment for allo-MHC dependent cytokine production by the ELISPOT assay. Culture equivalent Effector cells of the $CD4^+CD8^-$ T cell populations were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Data are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the pooled spleen cells from three mice per experiment.

CHAPTER 6

Revisiting the mechanism by which CD8 T cells exert their regulatory role on the differentiation of naïve CD4 T cells by employing the transwell membrane plate

Preamble

The prominence of CD8 T cells as suppressor cells of the antibody response arose in the 1970's. Extensive research into the mechanism by which the CD8 T cells exerted their suppressor effect gave rise to two possible pathways. Several studies employing the GVHD mouse model demonstrated that direct cell-to cell contact was required in which the donor cells lysed the host cells. Host B cells differing at the MHC class I molecule, were lysed by donor CD8 T cells (Via et al., 1987) by means of the Fas/FasL pathway (Kataoka et al., 2001; Shustov et al., 1998) or through the production of cytolytic granules. B cells are critical for antibody production. Thus, a significant reduction in B cells would abrogate an antibody response. In contrast, studies using the GVHD mouse model (Shand, 1976) demonstrated, using the double chamber, the role of a soluble factor in suppressing the antibody response upon stimulation with allo-MHC. However, the soluble factor was not identified in the above study. Similarly, the antibody response to the intracellular pathogen, *L major* was shown to be suppressed by means of a soluble factor. In this study IFN-γ was identified as the soluble factor, which was produced by both the CD8 and CD4 T cells (Herath et al., 2003). Conflicting results in the specificity of suppression were reported. Studies demonstrated that CD8 suppressor T cells produced antigen specific molecules coded by the *I-J* region in the MHC (Murphy et al., 1976; Tada et al., 1976). However, studies emerged that disproved these findings (Kronenberg et al., 1983; Steinmetz, 1982). This ultimately led to the decline in research into the CD8 T cell's role as the suppressor of the antibody response.

Thus, we wanted to revisit, in the third section of the results, the concept of the CD8 T cells as suppressors of the Th2 antibody response and provide a mechanism by which the CD8 T cells exert this role on the differentiation of the naïve CD4 T cell. In previous experiments we observed the effect of CD8 T cells on CD4 T cells while they were in direct contact with each other. However, studies have demonstrated that high levels of IFN-γ

characterized the acute rejection while chronic GVHD was characterized by IL-4 (Rus et al., 1995). Therefore, based on our model in Figure 2.1, we hypothesised that CD8 T cells exerted their regulatory role on the differentiation of naïve CD4 T cells through the production of the soluble factor, IFN-γ. In order to assess this, we employed transwell-membrane plates with a pore size of 0.4μm that only allowed molecules to pass through and not cells. In addition, we employed a neutralizing antibody to assess whether the soluble factor was indeed IFN-γ.

6.1 The effect of the addition of IFN-γ on the production of cytokines by CD8 T cell depleted cultures

IFN-γ, the signature cytokine of the Th1 immune response has been shown to suppress the Th2 (IL-4) immune response (Gajewski and Fitch, 1988). Based on our model we hypothesized that IFN-γ is the soluble factor that is produced by CD8 T cells upon stimulation with allo-MHC at the initiation of the immune response that drives the differentiation of a naïve CD4 T cells towards a Th1 mode. We decided thus, to first test the effects of the addition of IFN-γ to a CD8 T cell depleted culture in the lower chamber of a transwell-membrane plate. In the lower chamber of the transwell membrane plate four million spleen cells depleted of CD8 T cells using anti-CD8 TIB211 antibody and complement, were cultured with an equivalent number of irradiated F1 spleen cells for 5 days. In the upper chamber, which was equivalent to the growth area of a 24-well plate, we added either RPMI complete medium or 10μl/well recombinant (r) IFN-γ, or 10μg/well anti-IFN-γ antibody, or 10μl/well of rIFN-γ and 10μg/well anti-IFN-γ antibody as depicted in Figure 6.1. On day 5, culture equivalent Effector cells were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Recombinant (r) IFN-γ added to the upper chamber of the transwell membrane plate resulted in switch from a predominant Th2 (IL-4) to a predominant Th1 (IFN-γ) immune response by the CD8 T cell depleted cultures in the lower chamber of the BALB/c (Figure 6.1A) and the C57BL/6 (Figure 6.1B) mice. These findings suggest that IFN-γ was able to migrate through the membrane and bring about this effect. The addition of the neutralising anti-IFN-γ antibody together with rIFN-γ to the upper chamber abrogated the effects of the rIFN-γ. The anti-IFN-γ antibody neutralised the rIFN-γ and thus it did not have any effect on the immune response of the CD8 T cell

depleted cultures in the lower chamber. Thus, the CD8 T cell depleted cultures in this instance exhibited an immune response similar to that observed when only RPMI medium was added. These findings augmented the IFN-γ's role in causing a switch from a predominant Th2 (IL-4) to a predominant Th1 (IFN-γ) immune response in the CD8 T cell depleted cultures.

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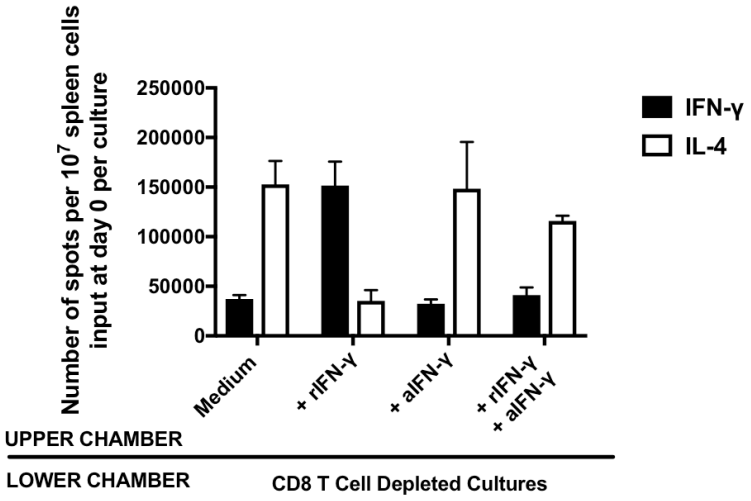
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IFN-γ
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UPPER CHAMBER
LOWER CHAMBER
CD8 T Cell Depleted Cultures
BALB/c mice

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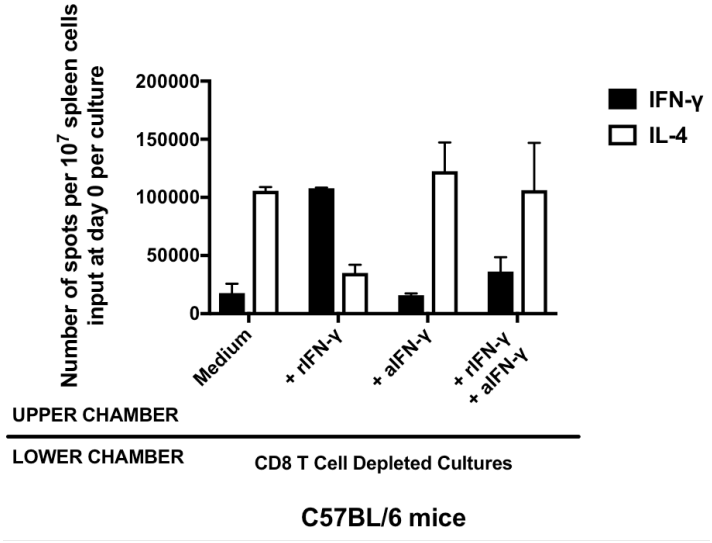
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+ aIFN-γ
+ rIFN-γ + aIFN-γ
UPPER CHAMBER
LOWER CHAMBER
CD8 T Cell Depleted Cultures
C57BL/6 mice

Figure 6.1

Figure 6.1: IFN-γ switches the CD4 Th2 (IL-4) immune response to a Th1 (IFN-γ) immune response in CD8 T cell depleted cultures.

Four million spleen cells depleted of CD8 T cells of either BALB/c (**A**) or C57BL/6 (**B**) mice were cultured with an equivalent number of irradiated F1, (BALB/c x C57BL/6) spleen cells in the lower chamber of 12-well transwell membrane plates for 5 days, at 37°C and 5% CO_2. In the upper chamber, we added either RPMI complete medium or 10µl/well rIFN-γ, or 10µg/well anti-IFN-γ antibody, or 10µl/well of rIFN-γ plus 10ug/well anti-IFN-γ antibody. On day 5, culture equivalent Effector cells of the CD8 T cell depleted cultures in the lower chambers were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Data are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the pooled spleen cells from three mice per experiment.

6.2. The effect of the CD4 and CD8 T cells in control cultures in switching the CD4 Th2 (IL-4) immune response to a mixed Th1 (IFN-γ)/Th2 (IL-4) immune response in CD8 T cell depleted cultures

Subsequently we tested the effect the cytokines, produced by the control cultures of BALB/c and C57BL/6 mice that contained both CD4 T cells and CD8 T cells, would have on the cytokine production in a CD8 T cell depleted cultures of the same mouse strain. Four million spleen cells depleted of CD8 T cells of either the BALB/c mice (Figure 6.2A) or the C57BL/6 mice (Figure 6.2B) were cultured with equivalent numbers of irradiated F1, (BALB/c x C57BL/6) spleen cells in the lower chamber of the 12-well transwell membrane plate. In the upper chamber, which was equivalent to the growth area of a 24-well plate, two million spleen cells of either BALB/c mice (Figure 6.2A) or C57BL/6 mice (Figure 6.2B) were cultured with equivalent numbers of irradiated F1 spleen cells. On day 5 of culture, the frequency of the IFN-γ and IL-4 producing cells of the CD8 T cell depleted cultures in the lower chamber of the two mouse strains were assessed by ELISPOT assay.

The control cultures of the spleens of BALB/c mice have been shown to produce both IFN-γ and IL-4 upon stimulation with allo-MHC as seen in Figure 4.6. In Figure 6.2A the control culture of the BALB/c mice in the upper chamber, produced both IFN-γ and IL-4 which resulted in an increase in IFN-γ production by the CD8 T cell depleted culture in the lower chamber but appeared to have no effect on the production of IL-4. The control cultures of the spleens of C57BL/6 mice produce predominantly IFN-γ as depicted in Figure 4.6. This resulted in a significant change in the IFN-γ production by the CD8 T cell depleted culture in the lower chamber as observed in Figure 6.2B. Furthermore, the predominant production of IFN-γ by the control culture spleen cells of the C57BL/6 mice in the upper chamber resulted in a significant decrease in the production of IL-4 by the CD4 T cells, which was not evident when the control cultures of the BALB/c mice were added to the upper chamber. These findings suggest that IFN-γ has an inhibitory effect on the production of IL-4 by the CD8 T cell depleted culture, which is consistent with the findings reported by Gajewski and Fitch in 1988.

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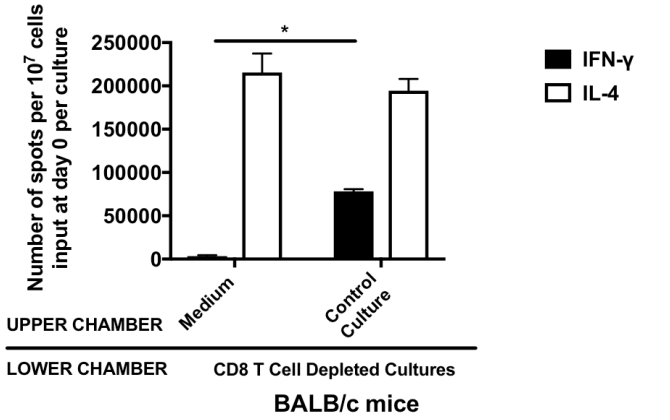
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Control Culture
UPPER CHAMBER
LOWER CHAMBER
CD8 T Cell Depleted Cultures
BALB/c mice

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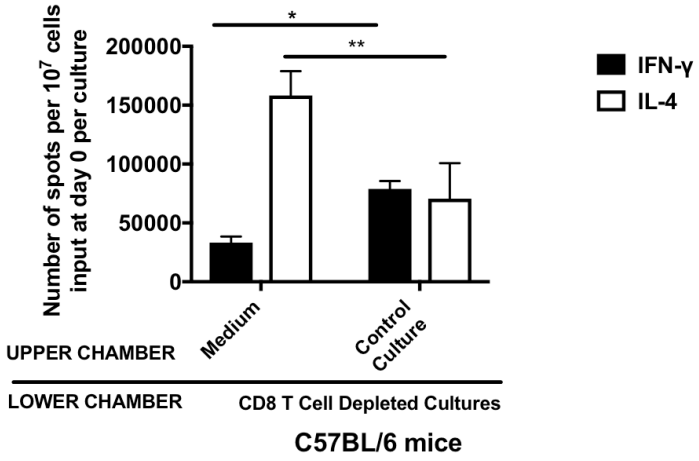
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IFN-γ
IL-4
Medium
Control Culture
UPPER CHAMBER
LOWER CHAMBER
CD8 T Cell Depleted Cultures
C57BL/6 mice

Figure 6.2

Figure 6.2: The effect of the control culture in switching the CD4 Th2 (IL-4) immune response to a mixed Th1 (IFN-γ)/Th2(IL-4) immune response in CD8 T cell depleted cultures. Four million spleen cells depleted of CD8 T cells of either BALB/c (**A**) or C57BL/6 **(B**) mice were cultured with equivalent numbers of irradiated F1, (BALB/c x C57BL/6) spleen cells in the lower chambers of 12-well transwell membrane plates at 37°C and 5% CO_2 for 5 days. In the upper chamber we added either RPMI complete medium or two million spleen cells of either BALB/c (**A**) or C57BL/6 (**B**) mice cultured with equivalent numbers of irradiated (BALB/c x C57BL/6), F1 spleen cells. On day 5, culture equivalent Effector cells of the CD8 T cell depleted cultures in the lower chambers were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Data are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the pooled spleen cells from three mice per experiment. The p values were obtained with ANOVA and Tukey's post-test comparing for multiple comparisons.

*p = 0.0148; **p = 0.0016

6.3 CD8 T cells require CD4 T cell help to differentiate into IFN-γ producing Effector cells

We had demonstrated in Figure 6.2 that the control culture with both CD4 and CD8 T cells in the upper chamber of a transwell membrane was able to increase the production of IFN-γ of the CD8 T cell depleted culture in the lower chamber. The next step was to determine if unprimed CD8 T cells in the absence of CD4 T cells added to the upper chamber had any affect on cytokine change in the CD8 T cell depleted culture in the lower chamber. Numerous studies have provided evidence for CD4 T cell help being a requirement for the activation and differentiation of CD8 T cells to effector and memory cells (Bennet et al., 1998, Bos and Sherman, 2010; Shedlock and Shen, 2003; Sun and Bevan, 2003). We therefore wished to determine if the unprimed CD8 T cells in our system required CD4 T cell help. Thus, we prepared cultures containing CD4 T cells and CD8 T cells and fractionated cultures with either $CD4^+CD8^-$ T cells or $CD4^-CD8^+$ T cells only in 6-well plates for BALB/c (Figure 6.3A) and C57BL/6 (Figure 6.3B). The CD4 T cells and CD8 T cells were depleted using complement together with either anti-CD4 GK1.5 antibody or anti-CD8 TIB 211 antibody, respectively. The CD4 T cells and CD8 T cells of both strains of mice were cultured with irradiated (BALB/c x C57BL/6), F1 spleen cells. As observed in Figure 6.3 the CD8 T cell depleted cultures were able to produce IL-4, while the CD4 T cell depleted cultures were unable to produce IFN-γ or IL-4 upon stimulation with the F1 alloantigen. We had previously demonstrated in Figure 4.12 that CD8 T cells are able to produce IFN-γ upon allo-stimulation. Thus, our findings in Figure 6.3 suggests that the CD8 T cells require CD4 T cell help in both strains of mice in order to produce IFN-γ upon stimulation with alloantigen.

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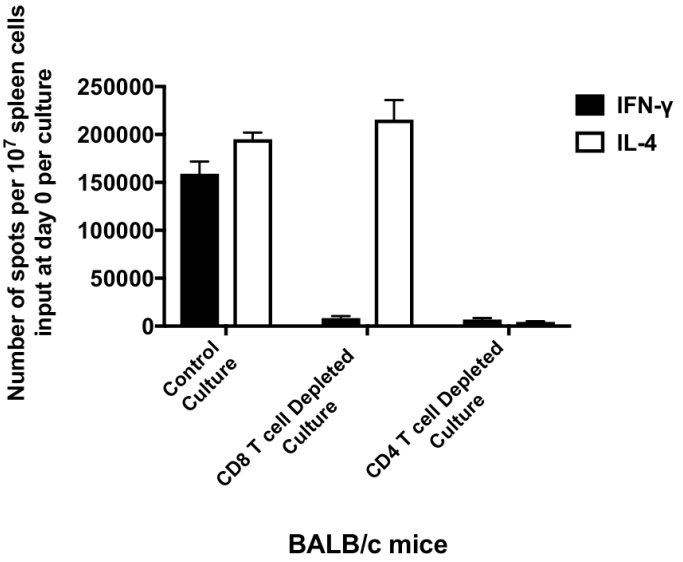
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Control Culture
CD8 T cell Depleted Culture
CD4 T cell Depleted Culture
BALB/c mice

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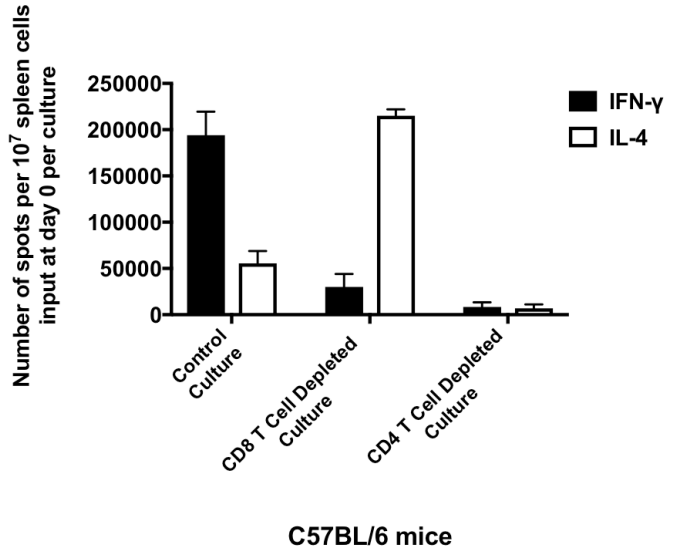
Number of spots per 10^7 spleen cells input at day 0 per culture
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IFN-γ
IL-4
Control Culture
CD8 T Cell Depleted Culture
CD4 T Cell Depleted Culture
C57BL/6 mice

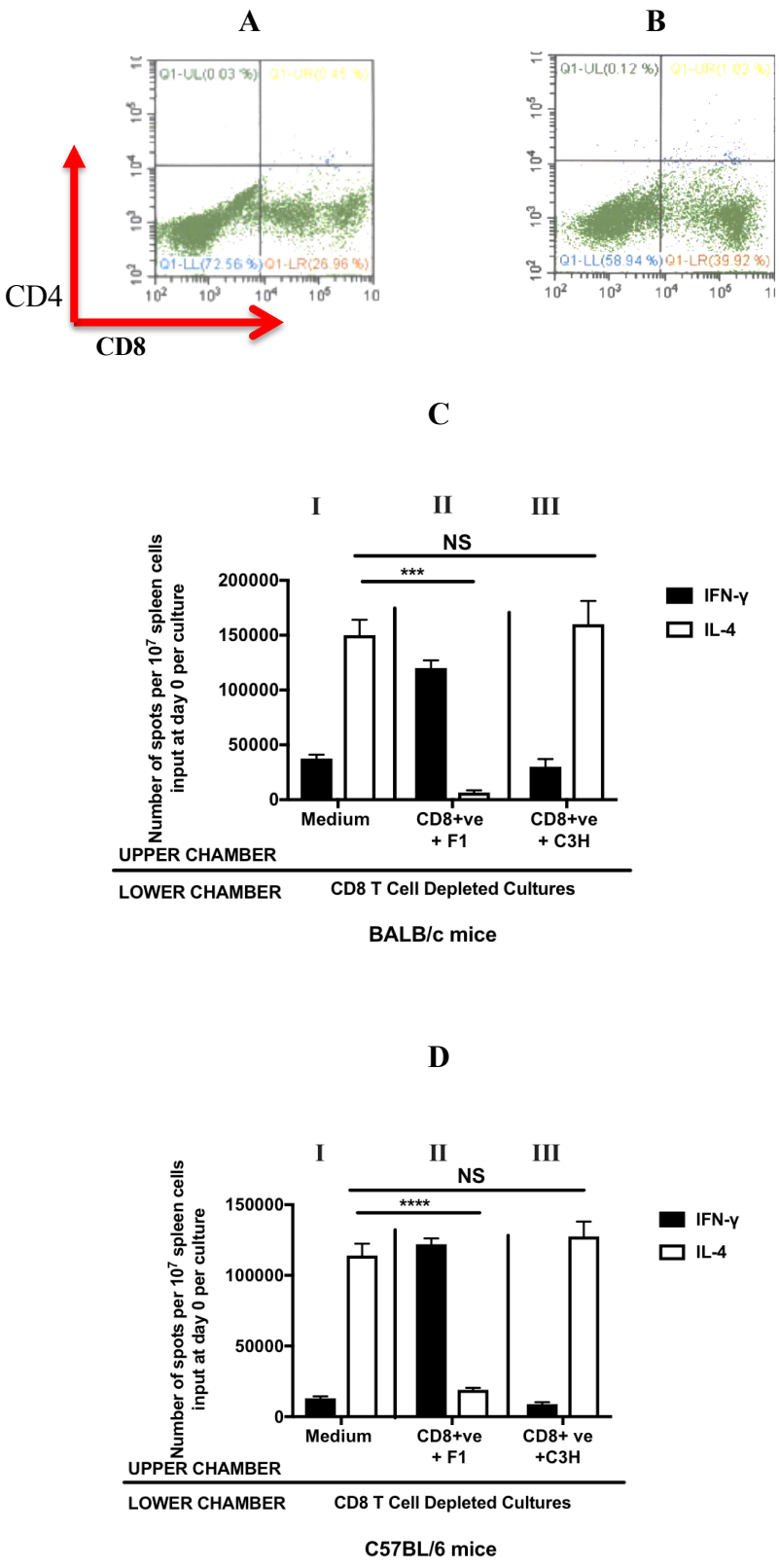
Figure 6.3

Figure 6.3: CD8 T cells require CD4 T cell help in order to produce IFN-γ upon stimulation with allo-MHC

Ten million spleen cells from BALB/c (**A**) and C57BL/6 (**B**) mouse strains and equivalent numbers of CD8 T cell depleted spleen cells and equivalent numbers of CD4 T cell depleted spleen cells were cultured with irradiated F1, (BALB/c x C57BL/6) spleen cells for 5 days, at 37°C and 5% CO_2 in 6-well plates. Cultures were depleted of CD8 T cells using anti-CD8 TIB 211 antibody and Low Tox-M rabbit complement. Cultures were depleted of CD4 T cells by using anti-CD4 GK1.5 antibody and Low Tox-M rabbit complement. On day 5, culture equivalent Effector cells were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Data are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the pooled spleen cells from three mice per experiment.

6.4 Effector CD8 T cells switch the CD4 Th2 (IL-4) immune response to a mixed Th1(IFN-γ)/Th2 (IL-4) immune response

Our findings in Figure 6 clearly indicate that unprimed CD8 T cells require cognate CD4 T cells to activate CD8 T cells. Thus, we set out to determine the role of effector CD8 T cells and their secretory products on CD4 T cell differentiation. In order to determine this, we first generated Effector CD8 T cells as per our normal protocol for control cultures. At day 5 of culture we sorted effector CD8 T cells employing negative selection with MACS monoclonal antibodies to CD4 T cells. Flow cytometry was used to verify the successful sorting of the CD8 T cells of the spleen cells of the BALB/c (Figure 6.4A) and the C57BL/6 (Figure 6.4B) mouse strains. Thereafter, spleen cells depleted of CD8 T cells were cultured with equivalent numbers of irradiated F1 spleen cells in the lower chamber of 12-well transwell membrane plates. We added 2 x 10^5 effector CD8 T cells with either equivalent numbers of the nominal, F1 antigen or with the irradiated spleen cells of C3H mice, an unrelated antigen to the upper chamber in both BALB/c (Figure 6.4C) and C57BL/6 (Figure 6.4D) mouse strains. The presence of the Effector CD8 T cells that were restimulated with the nominal antigen in the upper chamber resulted in a switch in the CD8 T cell depleted culture in the lower chamber from an almost exclusive Th2 (IL-4) to an almost exclusive Th1 (IFN-γ) response. In addition, the effector CD8 T cells cultured with the unrelated alloantigen, irradiated spleen cells of C3H mice, did not result in a change to the production of the cytokines produced by the CD8 depleted culture in the lower chamber. These findings suggest that the effector CD8 T cells are able to switch the CD4 Th2 (IL-4) immune response of CD8 T cell depleted cultures to a Th1 (IFN-γ) immune response in an antigen specific manner. Furthermore, since the membrane only allows soluble factors to pass through we hypothesised that the means by which the Effector CD8 T cells exerted their effect on the CD4 T cell was through the production of a soluble factor.


A
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Q1-UL(0.03 %)
Q1-UR(0.45 %)
Q1-LL(72.56 %)
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Q1-UL(0.12 %)
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Q1-LR(39.92 %)
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CD8+ve + C3H
UPPER CHAMBER
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CD8 T Cell Depleted Cultures
BALB/c mice
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Number of spots per 10^7 spleen cells input at day 0 per culture
IFN-γ
IL-4
Medium
CD8+ve + F1
CD8+ ve +C3H
UPPER CHAMBER
LOWER CHAMBER
CD8 T Cell Depleted Cultures
C57BL/6 mice

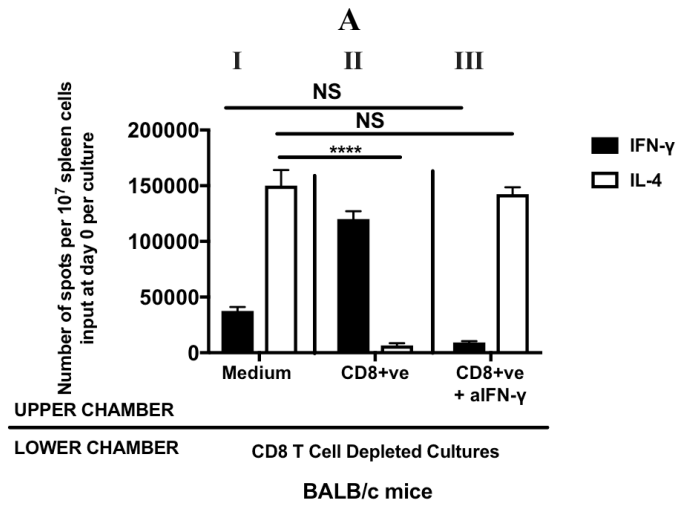
Figure 6.4

Figure 6.4: Effector CD8 T cells switch the CD4 Th2 immune response to a Th1 immune response in an antigen specific manner.

Effector CD8 T cells were prepared as per our standard protocol for control cultures five days before addition to transwell membrane plates. On day 5 purified $CD8^{+}CD4^{-}$ T cells were obtained using negative selection with MACS monoclonal antibodies to CD4 T cells. Flow cytometry was used to verify the sorted CD8 T cells of BALB/c (**A**) and C57BL/6 (**B**) mouse strains. Four million CD8 T cell depleted spleen cells of either BALB/c (**C**) or C57BL/6 (**D**) mice were cultured with equivalent numbers of irradiated F1 spleen cells in the lower chamber of 12-well transwell membrane plates for 5 days, at 37°C and 5% CO_2. In the upper chamber we added either RPMI complete medium (**I**), 2 x 10^5 Effector CD8 T cells of either BALB/c (A) or C57BL/6 (B) with an equivalent number of irradiated F1 spleen cells (**II**) or 2 x 10^5 Effector CD8 T cells with an unrelated antigen, the irradiated spleen cells of C3H mice (**III**). On day 5, culture equivalent Effector cells of the CD8 T cell depleted cultures in the lower chambers were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Data are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the pooled spleen cells from three mice per experiment. The p values were obtained with ANOVA and Tukey's post-test for multiple comparisons. ***p= 0.0008, **** $p < 0.0001$, NS not significant

6.5 Identification of IFN-γ as an essential factor produced by CD8 T cells that is required to switch a predominant Th2 (IL-4) response to a Th1 (IFN-γ) immune response

We have demonstrated in Figure 4.12 that the CD8 T cells of the spleens of the BALB/c mouse strain produce IFN-γ and not IL-4 upon stimulation with alloantigens. Thus, we surmised that the CD8 T cells of the BALB/c and C57BL/6 mouse strains would produce IFN-γ upon allo-MHC stimulation, which migrate across the membrane of the 12-well transwell membrane plate. The IFN-γ would thus exert an inhibitory effect on the development of a Th2 immune response by the CD4 T cells in the lower chamber resulting in a switch from a Th2 (IL-4) to a predominant Th1 (IFN-γ) response. Four million spleen cells depleted of CD8 T cells from BALB/c mice (Figure 6.5A) and from C57BL/6 mice (Figure 6.5B) were cultured with equivalent numbers of irradiated F1 spleen cells in the lower chamber of the 12-well transwell membrane plate. 2 x 10^5 Effector CD8 T cells were cultured with equivalent numbers of irradiated F1 spleen cells in the upper chamber with or without 20μg/well neutralising anti-IFN-γ antibody. The presence of the effector CD8 T cells in the upper chamber resulted in a switch from a Th2 (IL-4) immune response to a Th1 (IFN-γ) immune response in the CD8 T cell depleted cultures in the lower chamber. The addition of the neutralizing anti-IFN-γ antibody to the upper chamber prevented effector CD8 T cells exerting the switch in the CD8 T cell depleted cultures. We thus can conclude that CD8 T cells inhibit the Th2 (IL-4) response of CD4 T cells specific for allo-MHC through the production of IFN-γ.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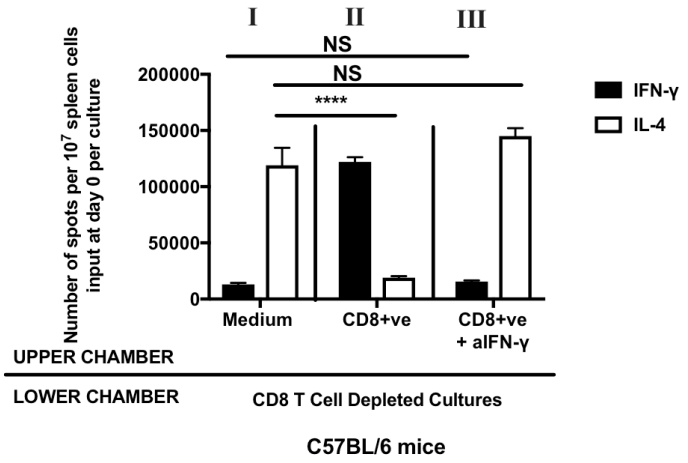
Number of spots per 10^7 spleen cells input at day 0 per culture
IFN-γ
IL-4
Medium
CD8+ve
CD8+ve + aIFN-γ
UPPER CHAMBER
LOWER CHAMBER
CD8 T Cell Depleted Cultures
BALB/c mice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Number of spots per 10^7 spleen cells input at day 0 per culture
IFN-γ
IL-4
Medium
CD8+ve
CD8+ve + aIFN-γ
UPPER CHAMBER
LOWER CHAMBER
CD8 T Cell Depleted Cultures
C57BL/6 mice

Figure 6.5

Figure 6.5: Effector CD8 T cells switch CD4 Th2 (IL-4) immune response to a Th1 (IFN-γ) phenotype by producing IFN-γ.

Effector CD8 T cells were prepared as per our standard protocol for control cultures 5 days prior to addition to the transwell membrane plates. On day 5, purified Effector $CD8^+CD4^-$ T cells were obtained using negative selection with MACS monoclonal antibodies to CD4 T cells. Four million CD8 T cell depleted spleen cells of either BALB/c (A) or C57BL/6 (B) mice were cultured with equivalent numbers of irradiated (BALB/c x C57BL/6), F1 spleen cells in the lower chamber of 12-well transwell membrane plates at 37°C and 5% CO_2 for 5 days. In the upper chamber we added either RPMI complete medium (**I**), 2 x 10^5 sorted Effector CD8 T cells of either BALB/c (A) or C57BL/6 (B) with the nominal F1 spleen cells (**II**) or 2 x 10^5 sorted Effector CD8 T cells with 20ug/well of neutralizing anti-IFN-γ antibody plus the nominal F1 spleen cells (**III**). Flow cytometry was employed to verify the sorted CD8 T cells. On day 5, culture equivalent Effector cells of the CD8 T cell depleted culture in the lower chamber were assessed for IFN-γ and IL-4 production in our ELISPOT assay. Data are plotted as the number of spots per 10^7 spleen cells input at day 0 per culture. Data are represented as mean ± SD of the mean from duplicate cultures. Data are representative of three independent experiments with the pooled spleen cells from three mice per experiment. The p values of the number of alloantigen specific IFN-γ and IL-4 producing cells were obtained with ANOVA with Tukey's post-test for multiple comparisons. **** $p < 0.0001$, NS not significant

CHAPTER 7
DISCUSSION

Preamble

Resistance to some infections and the successful elimination of some intracellular pathogens or the rejection of tumours often requires a Th1, cell-mediated immune response. The generation of a Th2 immune response proved ineffective in clearing certain pathogens (Dittmer et al., 1996). Several studies provide evidence for the crucial role of cytotoxic T lymphocytes (CTLs), which are largely effector $CD8^+$ T cells, in the protection against intracellular pathogens (Sun and Bevan, 2003) and viral infections (Hatziioannou et al., Jin et al., 1999; Schmitz et al., 1999). Infiltration of cytotoxic T lymphocytes into breast and colon cancer tissue correlates with a good prognosis (Ziai et al., 2018). Although their mode of killing infected target cells is through the secretion of cytolytic granules (Badovinac et al., 2000; Lobe et al., 1986, Podack et al., 1991) and the Fas-FasL interaction (Kataoka et al., 2001; Russell and Ley, 2002), CD8 CTLs also produce IFN-γ (Fong and Mosmann, 1990), which is the signature cytokine of a Th1 immune response (Mosmann et al., 1986), suggesting a role for CD8 T cells in enhancing the Th1 immune response.

In addition to their ability to kill target cells, CD8 T cells are able to suppress the antibody response of the CD4 T cell according to the theory of immune regulation proposed by Bretscher in 1974 and its subsequent demonstration by Ramshaw and colleagues in 1977. Subsequently, the CD8 T cell gained prominence as the suppressor cell of the CD4 antibody response and was followed by extensive research into the mechanism by which it exerted its suppressor effects. Systemic lupus erythematosus (SLE), a human antibody-mediated autoimmune disease has been attributed to the inability of the CD8 T cells to suppress the persistent antibody response (Filaci et al., 2001). The suppressor function of CD8 T cells was also observed in the GVHD mouse model (Rus et al., 1995; Via et al., 1987).

A substantial effort was made to understand the mechanism by which CD8 T cells suppressed the antibody response. Several studies demonstrated that suppression was

executed by the cytotoxic activity of the CTL's (Rozendaal et al., 1989; Shustov et al., 1998, Via et al., 1987). The CTL's lysed B cells, which resulted in a decrease in antibody production (Via et al., 1987). However, other studies suggested that CD8 T cells produced a soluble effector molecule that suppressed the antibody response (Herath et al., 2003; Rich and Pierce, 1974; Shand, 1976). Furthermore, conflicting reports emerged as to whether the soluble factor was antigen-specific (Takemori and Tada; 1975) or whether it acted in a non-specific manner (Rich and Pierce, 1974).

The aim of this thesis was thus to re-examine the role of the CD8 T cell in the inhibition of the CD4 Th2 (IL-4) antibody response and to revisit the mechanism by which CD8 T cells exerts its suppression. The study employed an allo-MHC specific *in vitro* system where the differential development of CD4 and CD8 T cells could be observed through quantitative interactions initiated by allo-MHC and through the production of cytokines, IFN-γ and IL-4, by the T cells. We found that the naturally occurring and controlled manipulation of CD4:CD8 T cell ratios were predictive of whether a Th1 (IFN-γ) or Th2 (IL-4) immune response was generated against allo-MHC. Furthermore, the presence of CD8 T cells at the initiation of the immune response against allo-MHC inhibits the differentiation of the CD4 T cell to a Th2 (IL-4) phenotype. Moreover, we determined that the mechanism by which the CD8 T cells exerted their suppressor effects on the Th2 subset was through the production of a soluble factor, IFN-γ, which has been reported by others to enhance the Th1 immune response and concomitantly inhibit the Th2 immune response (Gajewski et al., 1999).

7.1 The relationship between the CD4:CD8 T cell ratio of spleens of various mouse strains and the tendency of CD4 T cells to differentiate to either a Th1 (IFN-γ) or a Th2 (IL-4) subset in response to allo-MHC

A naïve CD4 T cell has the potential to differentiate into distinct T cell subsets with specific functions and cytokine production. Numerous factors have been shown to play a role in determining the type of CD4 T cell subset that is generated upon antigen stimulation. There is evidence that the nature of the antigen (Scott et al., 1987, Scott et al., 1988) and the dose of the antigen (Dittmer et al., 1996; Menon and Bretscher, 1998; Power et al., 1998; Salvin,

1958; Sarzotti et al., 1996) independently influence the type of immune response, Th1 or Th2 generated. The cytokine milieu has also been implicated as a determinant of the immune response that is generated but this will be elaborated upon later in section 7.4 of the discussion. A low antigen dose is associated with a Th1 immune response while a high dose elicits a Th2 immune response. However, the antigen dose required to elicit an immune response varies between different strains of mice (Menon and Bretscher, 1998). This observation suggests that there are other factors, in addition to the antigen dose that play a key role in generating either a predominant Th1 or a predominant Th2 immune response. The work by various researchers highlighted a possible role for the CD4:CD8 T cell ratio in determining a Th1/Th2 immune response to allo-MHC (Rus et al., 1995; Via et al., 187). They demonstrated that lymphocytes from distinct parental strains containing comparable numbers of CD4 T cells but significantly different numbers of CD8 T cells generated either a Th1 (cell-mediated) or a Th2 (antibody-mediated) immune response when inoculated into F1 mice. Furthermore, data from a later study revealed that a low number of responding CD4 T cells present at the initiation of the immune response were shown to generate a Th1 immune response against SRBC, while a high number of CD4 T cells generated a Th2 immune response to the same antigen (Ismail and Bretscher, 2001). Moreover, work done in our laboratory demonstrated that the spleens cells of neonatal C57BL/6 and BALB/c mice displayed a CD4:CD8 T cell ratio, 3-5-fold higher than their adult counterparts and that this high ratio correlated with a predominant Th2 (IL-4) immune response upon stimulation with allo-MHC (Warner, 2015). Although the immune response of neonatal mice is biased towards a Th2 immune response (Adkins et al., 2000; Barrios et al., 1996), a low antigen dose has been shown to elicit a Th1 immune response (Sarzotti et al., 1996). The collective findings above of the different experiments suggested that the CD4:CD8 T cell ratio might have some significance in determining the Th1/Th2 immune response to antigens.

We initially set out to explore the role of the CD4:CD8 T cell ratio in the spleens of the adult C57BL/6 and BALB/c mice. The C57BL/6 mouse mounts a strong Th1 immune response against viral antigens and is generally considered by researchers to be a ‘cell-mediated’ mouse. In contrast, the BALB/c mouse is considered to be the ‘antibody’ mouse

often generating a strong Th2 immune response to antigen. Based on our hypothesis, we anticipated that a relatively low CD4:CD8 T cell ratio would characterize the C57BL/6 strain while a relatively high CD4:CD8 T cell ratio would characterize the BALB/c mouse. This difference in CD4:CD8 T cell ratio would explain the antibody behaviour (Th2) or cell mediated immunity (Th1) in each of the respective mouse strains. We subsequently demonstrated that the spleen cells of the C57BL/6 mouse strain had a CD4:CD8 T cell ratio of approximately 1, while the spleen cells of the BALB/c mouse strain had a ratio greater than 2.5 (Figure 4.2) Our findings are consistent with the findings of Pinchuk and Filipov (2008) who demonstrated a higher CD4:CD8 T cell ratio in the BALB/c than C57BL/6 mouse strains. Furthermore, the latter authors demonstrated that the CD4:CD8 T cell ratio was consistent across both the spleens and the peripheral blood mononuclear cells (PBMCs) of the BALB/c and C57BL/6 mice. We in turn demonstrated that the CD4:CD8 T cell ratio was similar in the spleens and the inguinal lymph nodes of two mouse strains (Figure 4.3). Thus, we concluded that the CD4:CD8 T cell ratio was consistent between secondary lymphoid organs and in the peripheral blood of the BALB/c and C57BL/6 mice.

We suspect that the difference in the CD4:CD8 T cell ratio between the BALB/c and C57BL/6 mouse strains originates from a defective I-Eα gene of the C57BL/6 mouse strain (Flavell et al., 1985). The I-Eα gene is critical for the expression of MHC class II, I-E molecules on the surface of cells. Consequently, the defective I-Eα gene of the C57BL/6 mouse results in a lower expression of MHC class II, I-E molecules in the thymus, during the development of T cells. Single positive CD4 T cells are positively selected based on their recognition of the MHC class II molecules of the thymic epithelium. Fewer MHC class II molecules ultimately lead to fewer CD4 T cells being positively selected in the thymus. This leads to fewer CD4 T cells in the periphery of the C57BL/6 mice, which likely explains in part why a lower CD4:CD8 T cell ratio exists in the C57BL/6 than the BALB/c mouse strain. This also suggests that differences in the expression of MHC class I and II molecules in the thymus, affects the CD4:CD8 T cell ratio at the periphery which may ultimately lead to different immune responses to the same antigen.

Once we had determined that there was a significant difference in the naturally occurring CD4:CD8 T cell ratio of the spleens between BALB/c and C57BL/6 mouse strains, we set out to determine if these differences correlated with either a Th1 or Th2 immune response. The Th1 and Th2 immune responses are characterized by the presence of the signature cytokines IFN-γ and IL-4, respectively (Mosmann et al., 1986). We found that the high CD4:CD8 T cell ratio in the BALB/c mouse strain correlated with a mixed Th1(IFN-γ /Th2 (IL-4) immune response, while the relatively low ratio CD4:CD8 T cell ratio of the spleens of C57BL/6 mouse strain produced a predominant Th1 (IFN-γ) immune response (Figure 4.6 and 4.7). These findings were consistent across other mouse strains where subtle variations in the CD4:CD8 T cell ratios (Figures 4.8 and 4.9), was associated with variations in the type of immune response generated (Figure 4.10C). The spleen cells of the CBA/J and C3H expressed a CD4:CD8 T cell ratio below 2 and subsequently generated a predominant Th1 (IFN-γ) immune response against allo-MHC. Notably the CBA/J and C3H mice, both displaying a H-2^k haplotype are closely related with a common ancestry (Snell et al., 1953). In contrast, the spleen cells of the DBA/1 and DBA/2 mice expressed a CD4:CD8 T cell ratio of 2 and more, respectively, and generated a mixed Th1 (IFN-γ) / Th2 (IL-4) immune response against allo-MHC. The DBA/1 and DBA/2 mice also have a close relationship, which may explain the similarity in immune response (Snell et al., 1953).

We found that the CD4:CD8 T cell ratio is the same in spleens and lymph nodes as well as in the blood (Pinchuk and Filipov, 2008). Our accumulative findings in both adult and neonatal studies (Warner, 2015), suggest that the CD4:CD8 T cell ratio is a determining factor in the differentiation of CD4 T cells. The differentiation of naïve CD4 T cells towards a Th1 phenotype is dependent on the concomitant induction of CD8 T cells by the same antigen, driven by a low CD4:CD8 T cell ratio. In contrast, inefficient induction of CD8 T cells due to a high CD4:CD8 T cell ratio results in the naïve CD4 T cell differentiating into a Th2 subset. Moreover, the type of immune response generated has been shown to be antigen-dependent and antigen specific (Figure 4.13).

Our data suggest that the CD4:CD8 T cell ratio may predict whether a Th1(IFN-γ) or Th2 (IL-4) immune response is generated against allo-MHC. This prediction may have relevance to the field of transplantation immunity. An important complication associated with bone marrow transplantations (BMT) in humans is graft versus host disease (GVHD). A graft versus host reaction (GVHR) occurs when donor T cells recognise and mount an immune response against allo-MHC on the surfaces of the host's antigen presenting cells. A prerequisite for the development of a GVHR is the presence of immunologically competent cells within the donor inoculum and the inability of the host to respond to the donor cells (Billingham, 1959). Recipients of bone marrow transplants undergo a conditioning regimen which includes chemotherapy and/or radiation prior to the transplant (Xun et al., 1994). This regimen destroys the recipient's immune cells and renders the individual incapable of responding to the donor cells (Ferrara et al., 2003). The GVHR can result in either an acute or a chronic form of disease. The acute GVHR is characterized by a Th1(IFN-γ) immune response, while the chronic form is characterized by a Th2 (IL-4) immune response (Rus et al., 1995). Currently HLA-typing is mandatory prior to BMT to reduce the risk of GVHD. Despite the rigorous HLA-typing GHVD does occur.

Several studies were undertaken that set out to determine a possible predictor of GVHD in BMT. The frequency of the CTL precursors (CTLp) has been suggested as a possible predictor of the severity of the GVHD in humans (Affaticati et al., 2000; Batchelor et al., 1990) and in the mouse model (Via et al., 1987). In the above studies limiting dilution analysis was undertaken to determine the frequency of the CTLp. The authors observed that a higher frequency of CTLp results in an acute GVHR while a low frequency results in the chronic form of the disease. However, there were studies that contradicted this finding. Wang and colleagues reported in 2000 that the frequency of CTLp was not a good predictor of the severity of GVHD. We, however, suggest that whether an acute GVHR or a chronic GVHD manifests after a BMT, can be determined by the CD4:CD8 T cell ratio in the donor tissue. A low ratio correlates with a predominant Th1 (IFN-γ) immune response which is characteristic of the acute GVHR (Rus et al., 1995). In contrast, a relatively high ratio correlates with a mixed Th1 (IFN-γ)/Th2(IL-4) immune response suggestive of a chronic GVHD.

7.2 The role of CD8 T cells in promoting the differentiation of naive CD4 T cells to a Th1 (IFN-γ) subset and the concomitant inhibition of a CD4 Th2 (IL-4) response

As background to our finding that CD8 T cells have a role in promoting the differentiation of a naïve CD4 T cells to a Th1 subset and bring about concomitant inhibition of the CD4 Th2 subset, we will briefly review pertinent work from the 1970's. In the early 1970's it became evident that T cells played a primary role in cell-mediated and antibody responses and that these responses were mutually exclusive (Parish and Liew, 1972). According to a theory of immune regulation (Bretscher, 1974; Ramshaw et al., 1977), the suppressor T cell of the antibody response is the CD8 T cell, while the CD4 T cell is able to inhibit the cell-mediated response. Several *in vivo* and *in vitro* studies confirmed these findings (Herath et al., 2003; Kataoka et al., 2001; Pickel and Hoffmann, 1977; Rolink and Gleichman, 1983; Tschetter et al., 2000; Via et al., 1987).

Evidence of the CD8 T cells role in suppressing the antibody response was demonstrated in the GVHD mouse model and was referred to as allosuppression. In 1977, Pickel and Hoffman demonstrated that spleen cells of mice undergoing a GVHR were unable to generate PFC upon stimulation with SRBC *in vitro*. Prior to testing spleen cells for antibody production, the mice were injected with allogeneic spleen cells to establish a GVHR. The authors tentatively identified the CD8 T cell as the suppressor of the antibody response. Independent *in vivo* studies demonstrated that an antibody response could occur in mice in which a GVHR was induced (Rus et al., 1995; Via et al., 1987). The generation of the antibody response was however, dependent on the frequency of CD8 T cells present in the inoculum of the allogeneic cells. If the inoculum of the donor contained a high frequency of CD8 T cells an acute GVHR occurred in the recipient mice with no antibody production observed. In contrast, if the inoculum of allogeneic cells had a significant reduction in CD8 T cells the recipient mice developed an antibody mediated autoimmune disease resembling lupus in humans. These findings suggest that without the regulatory role of CD8 T cells, CD4 T cells differentiate into the phenotype that is favourable for the development of a Th2 antibody-mediated disease. The importance of the CD8 T cells in suppressing the antibody response has also been reported in humans. Individuals with

active SLE, the antibody mediated autoimmune disease, appear to have an impaired CD8 T cell suppressor function (Filaci et al., 2001)

According to our model illustrated in Figure 2.1, the presence of CD8 T cells at the initiation of the immune response is critical in the differentiation of the naive CD4 T cell to a Th1 (IFN-γ) subset. In contrast, the absence of CD8 T cells results in the differentiation of the naive CD4 T cells to a Th2 (IL-4) subset upon stimulation with allo-MHC. In our study the spleen cells of different strains of mice generated varying Th1/Th2 phenotypes upon stimulation with allo-MHC (Figures 4.6 and 10C). We attributed this variation in Th1/Th2 phenotypes to the differences in the CD4:CD8 T cell ratio of the spleens of the different strains of mice. The deliberate depletion of CD8 T cells from the spleens of all the strains of mice used in the study, (BALB/c, C57BL/6, C3H, DBA/2 and DBA/1) elicited a near exclusive Th2 (IL-4) immune response upon stimulation with allo-MHC (Figures 5.1 and 5.2 and 5.4). Our results are consistent with those of Via, that were reported in 1987. The latter authors demonstrated that an inoculum of spleen cells that had originally generated a Th1 immune response in recipient mice generated a Th2 antibody mediated autoimmune disease in the recipient mice when the inoculum was depleted of CD8 T cells. We are confident to conclude that the CD8 T cell is pivotal in determining the type of immune response that is generated when a naïve CD4 T cell is exposed to an allo-antigen.

We have evaluated further the defining role of the CD8 T cell in the differentiation of the CD4 T cell, by reconstructing the original CD4:CD8 T cell ratio in the CD8 T cell depleted cultures of BALB/c and C57BL/6 mice. The addition of sorted unprimed CD8 T cells to the CD8 T cell depleted cultures reversed the immune response to the original Th1/Th2 phenotype (Figures 5.6 and 5.7). In addition, we demonstrated that the presence of CD8 T cells in the reconstructed CD8 T cell depleted cultures had a direct effect on the cytokine production of the CD4 T cells. In the CD8 T cell depleted cultures; the CD4 T cells produced IL-4. However, with the addition of CD8 T cells, the CD4 T cell showed a reduction in the production of IL-4 and an increase in the production of IFN-γ in both the BALB/c and C57BL/6 mice (Figures 5.8 and 5.9). These findings are consistent with the

findings of Herath who reported in 2003 that the presence of CD8 T cells increased the number of IFN-γ producing CD4 T cells in response to *L major* parasites.

Our findings argue in favour of a role for the CD8 T cells to drive the differentiation of the CD4 T cell towards a Th1 (IFN-γ) subset and inhibit the differentiation of the naive CD4 T cell into a Th2 (IL-4) subset in response to allo-MHC. Thus, the inhibition of the Th2 (IL-4) immune response by the CD8 T cell results in the inhibition of the antibody response. The Th2 signature cytokine, IL-4 (Mosmann et al., 1986; Swain et al., 1990) is essential for the activation and proliferation of B cells and was initially referred to as the B cell growth factor, (BCGF) (Howard, et al., 1982; Swain et al., 1988). The IL-4 stimulates the B cells to produce IgM and IgG1 antibodies (Boom et al., 1988) and IgE (Coffman et al., 1987). We have demonstrated that the addition of unprimed CD8 T cells to the CD8 T cell depleted cultures caused a switch from a predominant Th2 (IL-4) immune response to a mix Th1(IFN-γ)/Th2 (IL-4) response at the CD4 T cell level (Figures 5.9 and 5.10). The CD4 T cells increased their production of IFN-γ and decreased their production of IL-4. The reduction in IL-4 reduces B cell activation. We can thus conclude, that the CD8 T cell suppresses the Th2 antibody response indirectly, by inhibiting the production of IL-4, which in turn is critical for B cell activation and the production of IgM, IgG1 and IgE antibodies. Notably, IFN-γ, the signature cytokine of a Th1 immune response promotes the production of IgG2a.

7.3 The mechanism by which the CD8 T cells promotes the differentiation of the CD4 T cell into a Th1 (IFN-γ) phenotype and suppresses a Th2 (IL-4) immune response against allo-MHC

The model that we have proposed in Figure 2.1, describes that the presence of CD8 T cells drive the differentiation of naïve CD4 T cells towards a Th1 (IFN-γ) phenotype and away from a Th2 (IL-4) phenotype upon allo-MHC stimulation, while the absence of the CD8 T cells results in a CD4 Th2 (IL-4) phenotype. To complete the model, we embarked on a series of experiments in which we proposed that the regulatory role of the CD8 T cell on the differentiation of the CD4 T cell is mediated by a soluble factor, the cytokine, IFN-γ.

Researchers proposed two possible mechanisms by which antibody suppression occurred; either through the cytotoxic activity of CTLs or by means of a soluble factor produced by effector cells. Several studies have demonstrated that the presence of CD8 T cells is a determining factor in the suppression of the antibody response to intracellular pathogens (Herath et al., 2003) and to alloantigens (Kataoka et al., 2001; Rolink and Gleichmann, 1983; Tschetter et al., 2000; Via et al., 1987). The presence of CD8 T cells during an *L major* infection greatly enhanced resistance to and the clearance of the pathogen. In contrast, the absence of the CD8 T cells resulted in an antibody response, which rendered the individual mice susceptible to the pathogen (Herath et al., 2003). In the allo-response, studies have demonstrated the role of CTLs in the manifestation of the acute GVHR (Kataoka et al., 2001; Rozendaal et al., 1989; Via et al., 1987), which is characterized by a significant reduction in host B cells numbers (Via et al., 1987). Differences between MHC class I molecules on the surface of host and donor B cells, stimulates the donor CD8 CTLs to lyse the host B cells. As B cells are critical for the production of antibody, a reduction in host B cells reduces the potential for a Th2 antibody response against allo-MHC. Notably CD4 T cell activation is also a requirement for the development of an acute GVHR (Rolink and Gleichmann, 1983).

Effector CTLs are known to lyse target cells via two pathways; either by the secretion of cytolytic granules or by the Fas-FasL pathway. In a study undertaken by Kataoka and colleagues in 2001 they reported an increase in FasL mRNA expression in the spleen, small intestines and liver in mice in which an acute graft versus host reaction occurred which was not evident in in the chronic GVHD. This finding suggested that there was a correlation between the expression of FasL and the type of GVHR observed. The authors thus suggested that the CTLs lysed the B cells via the Fas-FasL pathway, which abrogated the antibody response. The findings of Kataoka in 2001 expanded upon the earlier findings of Rozendaal and colleagues in 1989, which reported that the suppression of the antibody response was by the cytotoxic activity of CTL and not through a soluble factor. This however, was contrary to the findings of Shand who in 1976 demonstrated using a double chamber with a semi-permeable membrane that a soluble factor is a possible means by which allo-suppression occurs. However, they did not volunteer a possible candidate.

Studies were also undertaken that demonstrated that antigen specific molecules were instrumental in the suppression of the antibody response (Takemori and Tada; 1975) that were seemingly coded by the *I-J* region located between the *I-A* and *I-E* regions of the MHC (Taniguchi et al., 1976). Research soon emerged that made this particular mechanism unlikely (Kronenberg et al, 1983; Steinmetz, 1982). This ultimately led to a decline in research into the mechanism of the suppressor function of CD8 T cells. As the exact mechanism by which CD8 T cells exert their regulatory role remained undetermined, our intention was to revisit the mechanism by which CD8 T cells suppress the CD4 Th2 (IL-4) immune response to allo-MHC.

We proposed an alternate mechanism by which CD8 T cells inhibits the Th2 antibody response in the GVHD mouse model. The cytokine environment has been shown to be important in determining the type of T cell subset that is generated upon stimulation with an antigen (Gajewski and Fitch, 1988; Gajewski et al., 1989, Swain et al., 1990). IFN-γ is considered to be the signature cytokine of the Th1 immune response (Mosmann et al., 1986). Numerous studies have reported the presence of IFN-γ during the acute GVHR and its absence from chronic GVHD (Kataoka et al., 2001; Rus et al., 1995; Shustov et al., 1998). IFN-γ has a stimulatory effect on macrophages, by increasing their microbicidal activity and upregulating MHC class I and II molecules on APCs. Moreover, IFN-γ has been shown to inhibit the production of IL-4 (Gajewski and Fitch, 1988; Gajewski et al., 1989).

While investigating the cytokine profile during the cell cycle of newly activated CD4 T cells in 1998, Bird and colleagues demonstrated that IFN-γ was produced within hours of the first cell division. However, the secretion of IL-4 was evident only after a minimum of three cell divisions. These findings are consistent with the much earlier findings of Salvin in 1958. He reported that a cell-mediated, DTH, response to an antigen such as diphtheria toxoid is initially present and over a period of time transitions into an antibody response. The rate of transition is dose dependent. Contrary to this, Rus and colleagues reported in 1995, using a parent into F1 model in which both CD4 and CD8 T cells were present, that

the first cytokine produced in mice in which GVHR was induced was IL-4, irrespective of whether the mice eventually developed acute GVHR, characterized by IFN-γ, or chronic GVHD characterized by IL-4.

In our model we propose that higher numbers of responding CD8 T cells at the initiation of the immune response would lead to increased IFN-γ production, that drives the differentiation of the naïve CD4 T cell towards a Th1 (IFN-γ) phenotype. Similarly, if few CD8 T cells are present, that is a high CD4:CD8 T cell ratio, with reduced IFN-γ production, the naïve CD4 T cell differentiates into a Th2 (IL-4) subset. We have determined that CD8 T cells produce IFN-γ upon stimulation with allo-MHC and do not produce IL-4 as illustrated in Figure 4.12. This is consistent with the findings of Fong and Mosmann in 1990, which demonstrated that murine CD8 T cells clones produce IFN-γ and not IL-4.

The mechanism of suppression, which we propose is partly based on the current concept of immune class regulation. The Th1 and Th2 immune responses are reciprocally regulated by the antagonistic action of their signature cytokines, IFN-γ and IL-4, respectively. IFN-γ is known to have an inhibitory effect on the production of the Th2 cytokine, IL-4 and to concomitantly enhance a Th1 immune response (Gajewski and Fitch, 1988; Snapper and Paul, 1987). We subsequently demonstrated using transwell-membrane plates that antigen-specific activated CD8 T cells in the upper chamber produce IFN-γ that is able to migrate across the membrane and suppresses the production of IL-4 by CD8 T cell depleted cultures stimulated with allo-MHC in the lower chamber (Figure 6.4). Notably, in both the BALB/c and C57BL/6 mice the spleen cells in the CD8 T cell depleted cultures generated a predominant Th1 (IFN-γ) immune response. The spleen cells of BALB/c mice initially generated a mixed Th1/Th2 immune response with the unprimed CD8 T cells (Figure 4. 7). However, we assume that the primed CD8 T cells added in the above experiments (Figure 6.4) produced large amount of IFN-γ at the initiation of the immune response, which inhibited the production of IL-4. This is consistent with the findings of Snapper and Paul in 1987. Furthermore, to verify that IFN-γ was the soluble factor responsible for the switch from a Th2(IL-4) to a Th1(IFN-γ) immune response, we introduced a neutralising

anti-IFN-γ antibody (Figure 6.5). When anti-IFN-γ antibody was added to the upper chamber the switch from a Th2 (IL-4) to a Th1 (IFN-γ) immune response was inhibited in both BALB/c and C57BL/6 mouse strains. The fact that rIFN-γ alone can cause a switch in the immune resonse, we are thus able to conclude that IFN-γ was the soluble factor produced by the CD8 T cells that drives the CD4 T cell response towards a Th1 (IFN-γ) mode and concomitantly inhibits an antibody response to allo-MHC.

7.4 Implications for Transplantation Immunity: Graft Rejection or Antibody-Mediated Autoimmune Diseases

The findings of our *in vitro* model demonstrate that CD8 T cells creates a specific cytokine milieu that drives the differentiation of naïve CD4 T cells towards a Th1 (IFN-γ) immune response with the concomitant inhibition of a Th2 (IL-4) immune response upon stimulation with allo-MHC. The CD8 T cells in our study produced IFN-γ, which is known to augment a Th1 cell-mediated immune response and inhibit a Th2 immune response (Gajewski and Fitch 1988). The Th1 immune response has been associated with an acute GVHR, which leads to the destruction of host tissue. Thus, in our system the presence of the CD8 T cells and their production of IFN-γ would result in the rejection of an allograft. In contrast, the absence of CD8 T cells or in the presence of reduced numbers of CD8 T cells the CD4 T cell would differentiate into a Th2 subset. A persistent Th2 antibody immune response would occur due to ineffective immune response to clear host cells. The high frequency of alloreactive CD4 T cells of the donor would activate the autoreactive B cells of the host in a cognate T-B interaction (Morris et al., 1990). This would result in generalised autoimmunity (Bretscher, 1973). Thus, the CD8 T cell plays an indirect role in inhibiting development of an antibody-mediated autoimmune disease such as lupus in the GVHD mouse model.

7.5 Conclusion

Distinct CD4:CD8 T cell ratios of the spleens of different strains of mice is associated with a Th1(IFN-γ), a Th2(IL-4) or a mixed Th1/Th2 type immunity when these cells are stimulated with spleen cells of mice differing at the MHC locus in an *in vitro* model of alloreactivity. Furthermore, we have demonstrated that this ratio is consistent between

spleens and inguinal lymph nodes. In the absence of CD8 T cells, CD4 T cells from the spleens of the different strains of mice differentiate into a predominantly Th2(IL-4) subset. Naïve and Effector CD8 T cells suppress the Th2(IL-4) development in an antigen specific manner by means of a secreted soluble factor. Effector CD8 T cells switch the Th2(IL-4) response to a Th1 phenotype by producing IFN-γ. Thus, the naturally occurring CD4:CD8 T cell ratio promotes either Th1 immunity required for graft rejection, or Th2-type responses ineffective for attacking the graft. Thus, a persistent Th2 response by donor cells against host cells results in time in antibody-mediated generalised autoimmunity, resembling Systemic Lupus Erythematosus. Thus, in these studies we have revisited the mechanism by which CD8 T cells were known to act as "suppressors" of the antibody response. Our studies indicate that CD8 T cells inhibit in a cognate manner the CD4 Th2(IL-4) differentiation, a CD4 subset required for antibody development.

CHAPTER 8

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