ABSTRACT

Stem cell transplantation and high-dose chemotherapy is the most effective treatment for certain cancers such as leukemia and lymphoma. It is reported from the leukemia and lymphoma society that there are about 28,540 new cases of leukemia and 54,370 cases of non-Hodgkin lymphoma are diagnosed each year in the United States. Allogeneic stem cell transplantation has been widely studied due to the lack of HLA (Human Leukocyte Antigen)-matched donors. However, the application of allogeneic stem cell transplantation is restricted by the development of severe graft-versus-host disease (GvHD), which is believed to be caused by donor T cells. Depletion of donor T cells abrogates GvHD, while the chance of viral infection and leukemia relapse is increased due to the delay of immuno-reconstitution after extensive T cell depletion. Selective depletion of alloreactive T cells which mediate GvHD from donors, while retaining T cells with reactivity to pathogens and third party antigens is an alternative strategy that may protect the patient from opportunistic infections and shorten the immunodeficiency post transplantation. However, the success of this strategy depends on the extent of alloreactive T cell depletion and rem-
aining anti-viral and third party reactivity. One of the main challenges associated with inefficient alloreactive T cell depletion is the stimulation and detection of potential alloreactive T cells.

This study is focusing optimizing the condition for generating alloreactive T cells \textit{in vitro}; selection of the optimum combination of activation antigens to define alloreactive T cells; and applying immunomagnetic cell separation method to maximize depletion of alloreactivity with the preservation of anti-third-party reactivity for clinical application.
Dedicated to my parents
ACKNOWLEDGMENTS

I would like to thank Dr. Jeffrey Chalmers and Dr. Sherif Farag for their guidance during the whole development of this work.

In addition, I would like to express my appreciation to the research group members, specially Ioana Lozonschi and Ying Xiong, for their contributions, Dr. Xiaodong Tong, Dr. Oscar Lara and Dr. Huading Zhang, for their suggestions to the completion of this work.

Special acknowledgements to Dr. Lynn O’donnell for her valuable comments in assay development.

I would like to thank Mr. Bryan McElwain at the Ohio State University Analytical Cytometry Laboratory for his expertise on flow cytometry analysis.

Also specially thanks to all the blood donors, Ying Xiong, Xiaodong Tong, Dr. Farag, Dr. O’donnell, Hillary Stone, Bhavya Mehta, Mike Mollet, Oscar Lara, and Cassandra Cook.
VITA

November 6, 1978      Born - Jining, China

July, 2000         B.S. Chemical Engineering,
Dalian University of Technology, China

August, 2000 -August, 2001     Research Associate,
Dalian University of Technology, China

September, 2001-present     Graduate Research Associate
Chemical and Biomolecular Engineering
The Ohio State University

PUBLICATIONS

Liu CB, Shao M, et al. High Concentration Ethanol Continuous Fermentation Using

High Concentration Ethanol Continuous Fermentation Using Yeast Flocs. B.S.
Thesis. July, 2000, China

FIELD OF STUDY

Major Field:                          Chemical Engineering
Specialization:                   Biochemical Engineering
# TABLE OF CONTENTS

ABSTRACT.............................................................................................................. ii  
DEDICATION ......................................................................................................... iv  
ACKNOWLEDGMENTS ......................................................................................... v  
VITA........................................................................................................................ vi  

LIST OF TABLES ................................................................................................... xi  
LIST OF FIGURES ............................................................................................... xiii  

CHAPTERS:  

1. INTRODUCTION ................................................................................................. 1  

   1.1 Definition...................................................................................................... 1  
   1.2 Research motivation...................................................................................... 2  
   1.3 T cell depletion: the development and limitation ........................................... 4  
   1.4 Alloreactive T cell depletion ....................................................................... 5  
   1.5 The significance of optimizing alloreactive T cell generation ....................... 8  
   1.6 Optimize generation of alloreactive T cells *in vitro* ................................... 9  
   1.7 Activation markers...................................................................................... 10  
   1.8 Cell separation methods ........................................................................... 15  
   1.9 Immunomagnetic cell separation technology ........................................... 16  
   1.10 Quadrupole Magnetic Flow Sorter (QMS)............................................... 18  
   1.11 Definition and measurements of magnetophoretic mobility ....................... 21  
   1.12 Dissertation organization........................................................................... 24  

2. OPTIMIZE GENERATION OF ALLOREACTIVE T CELLS ............................ 26  

   2.1 Motivation .................................................................................................. 26  
   2.2 Material and Methods ................................................................................. 28  
      Cell Preparation ........................................................................................... 28  
      Cytokine pretreatment of recipient cells ....................................................... 29  
      One-way mixed lymphocytes reaction (MLR)............................................. 29  
      Depletion of CD25⁺ Cells.......................................................................... 30  
      Cell labeling for flow cytometry.................................................................. 30  
      Flow cytometry analysis............................................................................. 31  
      Quantification of MHC and activation antigen expression ....................... 31
Statistical study.................................................................................................................. 32

2.3 Results and Discussions .......................................................................................... 32
Kinetics of activated antigen expression........................................................................... 32
Effect of IL-2 and IL-15 on activation antigen expression ............................................ 36
Pretreatment of stimulator cells with IFN-γ and TNF-α ...................................................... 37
Pre-depletion of CD4+CD25+ T regulatory cell before MLR ............................................. 42
Scale-up of mixed lymphocytes reaction ........................................................................ 46

2.4 Discussion.............................................................................................................. 53

3. SELECTION OF TARGET ACTIVATION ANTIGENS FOR ALLOGRAFT ACTIVITY DEPLETION .................................................................................. 58

3.1 Motivation ............................................................................................................... 58
3.2 Material and Methods ............................................................................................ 60
Cell Preparation .................................................................................................................. 60
One-way mixed lymphocytes reaction .............................................................................. 60
Cell labeling for flow cytometry ...................................................................................... 60
Alloreactive T cell depletion ............................................................................................. 61
Statistical study .................................................................................................................. 62
QMS separation .................................................................................................................. 62
Limiting dilution assay ...................................................................................................... 62
BrdU proliferation assay ..................................................................................................... 63

3.3 Results ..................................................................................................................... 64
Selection of activation antigens ......................................................................................... 64
Co-expression of activation antigens ................................................................................. 68
Expression of CD4, CD8, CD45RA and CD45RO on alloreactive T cell subsets ................. 78
Depletion of cells expressing activation markers using QMS ............................................ 81
Determine the functional significance of activation antigen expressing cells ................. 86
Determine the anti-third party reactivity of residual cells after alloreactive T cell depletion .................................................................................................................. 88

3.4 Discussion.................................................................................................................. 93

4. A NEW STRATEGY FOR AUGMENTED ACTIVATION OF ALLOREACTIVE T CELLS: DENDRITIC CELL STIMULATION ......................................................... 97

4.1 Motivation ............................................................................................................... 97
4.2 Introduction of dendritic cells ..................................................................................... 98
4.3 Material and Method ............................................................................................... 100
Isolation of PBMCs .......................................................................................................... 100
Generation of Dendritic Cells ......................................................................................... 100
Dendritic cell stimulated mixed lymphocytes reaction (DC MLR) .................................. 101
5. QUANTIFICATION OF THE ANTIGEN EXPRESSION LEVEL AND ITS EFFECT ON MAGNETIC SEPARATION ........................................................... 117

5.1 Motivation ............................................................................................ 117

Definition and measurements of magnetophoretic mobility .......................... 118
Structure of activation antigens .................................................................... 120
Quantification of antibody binding capacity (ABC) ................................ 123

5.2 Material and Methods ........................................................................... 125

Cell Preparation ....................................................................................... 125
Pretreatment of recipient cells .................................................................. 126
One-way mixed lymphocytes reaction...................................................... 126
Cell labeling for flow cytometry............................................................... 127
Magnetic cell labeling for CTV or QMS................................................... 127
Flow cytometry analysis........................................................................... 127
Magnetophoretic mobility analysis by CTV ............................................. 128

5.3 Results .................................................................................................. 128

Quantification of activation antigen expression on alloreactive T cells .... 128
Optimize the magnetic labeling process.................................................... 129
Increase the magnetophoretic mobility of “dim” expression cell .............. 131

5.4 Discussion............................................................................................. 135

6. COMPARISON OF DIFFERENT CELL FREQUENCY ANALYSIS APPROACHES.......................................................... 137

6.1 Motivation ............................................................................................ 137

Limiting dilution assay ............................................................................. 138
Flow cytometry ....................................................................................... 140

6.2 Material and Methods ........................................................................... 142

Cell Preparation ....................................................................................... 142
One-way mixed lymphocytes reaction...................................................... 142
Cell labeling for flow cytometry............................................................... 143
Alloreactive T cell depletion .................................................................... 143
Limiting dilution assay ........................................................................... 144
BrdU proliferation assay ........................................................................... 145
6.3 Results ............................................................................................................................ 146
  Comparison of different gating strategies on cell analysis by flow cytometric assay .................. 146
  Comparing flow analysis versus limiting dilution assay ........................................... 151
6.4 Discussions ............................................................................................................ 159

7. CONCLUDING REMARKS AND FUTURE DIRECTIONS ................................. 161

LIST OF REFERENCES ............................................................................................... 165
LIST OF TABLES

Table 2.1 Specific activation of each activation antigen after 4 days’ MLR (“*” represents significant increase by T-test analysis) .................................................. 37
Table 2.2 The effect of pre-treating recipient cells on alloreactive T cell activation (“*” representing statistically significant increase) ......................... 40
Table 2.3 The number of activation antigen expressed on activated T cells on the 4th day of MLR (“*” representing statistically significant increase) ...... 40
Table 2.4 T-test analysis of the effect of CD25+ cell predepletion on alloreactive T cell activation .................................................................................................. 46
Table 2.5 Volumetric transfer coefficient of Wave bioreactor® and spinner flask50
Table 3.1 Specific activation of alloreactive T cells expressing different activation antigens .............................................................................................................. 68
Table 3.2 Distribution of activation antigens on alloreactive T cells ............... 72
Table 3.3 Alloreactive T cell fractions represented by each activation antigen... 77
Table 3.4 Priority sequence of activation antigens on representing alloreactive T cells .............................................................................................................. 77
Table 3.5 T test analysis of antigen combinations on representing alloreactive T cells .............................................................................................................. 78
Table 3.6 Fractions of T cells expressing CD4 and CD8 following MLR ........ 80
Table 3.7 Expression of CD45RA and CD45RO on alloreactive T cells .......... 81
Table 3.8 Expression of CD45RA and CCR7 on CD25+CD69+CD38+HLADR+ T cells following MLR or autologous control .................................................. 81
Table 3.9 Determination of residual alloreactivity in different cell subsets ...... 88
Table 3.10 Log depletion of alloreactivity achieved by depleting different cell subsets .............................................................................................................. 88
Table 3.11 Preserved anti-third party reactivity after selective depletion of alloreactive T cells .............................................................................................................. 92
Table 4.1 Specific antigen expression on mature dendritic cells .................. 104
Table 4.2 Percentage of alloreactive T cell represented by different antigen combinations .............................................................................................................. 107
Table 4.3 Priority sequence of activation antigens on representing DC stimulated alloreactive T cells .............................................................................. 109
Table 4.4 T test results of antigen combinations on representing alloreactive T cells .............................................................................................................. 110
Table 4.5 Comparison of activation antigen expression pattern on DC stimulated alloreactive T cells and PBMC stimulated alloreactive T cells.................. 113
Table 6.1 Comparison of T cell frequency analyzed by LDA and flow cytometric assay ........................................................................................................ 153
Table 6.2 Comparison of alloantigen reactive T cell frequency analyzed by LDA and flow cytometry ................................................................. 154
Table 6.3 Comparison of LDA and flow cytometry in determination of alloreactivity depletion................................................................. 158
LIST OF FIGURES

Figure 1.1 Composition of quadrupole magnetic sorter (QMS) ......................... 20
Figure 1.2 Geometry of quadrupole magnetic sorter (QMS) ............................ 21
Figure 1.3 Cell Tracking Velocimetry ............................................................... 24
Figure 2.1 CD25 activation kinetics in mixed lymphocytes reaction .................. 34
Figure 2.2 CD69 activation kinetics in mixed lymphocytes reaction .......... 34
Figure 2.3 CD71 activation kinetics in mixed lymphocytes reaction ............. 35
Figure 2.4 OX40 activation kinetics in mixed lymphocytes reaction .......... 35
Figure 2.5 HLA-DR activation kinetics in mixed lymphocytes reaction .... 36
Figure 2.6 The increase folds of MHC II expression on CD14+ cells by pretreatment stimulators with IFN-γ and TNF-α ................................................. 41
Figure 2.7 The increase folds of MHC I expression on CD14+ cells by pretreatment stimulators with IFN-γ and TNF-α ................................................. 41
Figure 2.8 CD4+CD25+ cell fraction in PBMC before and after CD25+ cell depletion .................................................................................................... 44
Figure 2.9 CD3+CD25+ specific activation before or after CD25+ T cell depletion .................................................................................................... 45
Figure 2.10 CD3+CD71+ specific activation before or after CD25+ T cell depletion .................................................................................................... 45
Figure 2.11 CD3+OX40+ specific activation before or after CD25+ T cell depletion .................................................................................................... 46
Figure 2.12 Alloreactive T cell activation under different culture condition ...... 48
Figure 2.13 Alloreactive T cell activation under different culture condition ...... 48
Figure 2.14 Wave bioreactor® ............................................................................ 49
Figure 2.15 Mixing process of the 20L Wave bioreactor® ................................. 51
Figure 2.16 Wave bioreactor® System 20/50EH ................................................ 52
Figure 3.1 Fraction of T cells expressing activation antigens after 4 days’ MLR 66
Figure 3.2 Fraction of T cells expressing antigens after 4 days’ autologous stimulation .............................................................................................. 67
Figure 3.3 Co-expression of CD71, OX40 and HLADR with CD25 and CD69 on CD3 positive cells following mixed lymphocytes reaction .......... 70
Figure 3.4 Co-expression of CD71, OX40, HLADR, CD38, CD25 and CD69 by sequential gating ..................................................................................... 71
Figure 3.5 Distribution of alloreactive T cell subsets by antigen expression ..... 76
Figure 3.6 CD25+ T cell depletion by QMS....................................................... 83
Figure 3.7 CD69+ T cell depletion by QMS....................................................... 84
Figure 3.8 CD71+ T cell depletion by QMS....................................................... 84
Figure 3.9 OX40+ T cell depletion by QMS ...................................................... 85
Figure 3.10 HLADR+ T cell depletion by QMS.................................................. 85
Figure 3.11 Residual alloreactivity in different cell subsets............................... 87
Figure 3.12 Residual alloreactivity and anti-third party reactivity of cells after CD25+ cell depletion .................................................................................. 90
Figure 3.13 Residual alloreactivity and anti-third party reactivity of cells after CD25+ and CD69+ cell depletion ................................................................. 91
Figure 3.14 Residual alloreactivity and anti-third party reactivity of cells after CD25+, CD69+ and HLADR+ cell depletion .................................................. 91
Figure 3.15 Linearity of BrdU proliferation assay ............................................. 92
Figure 3.16 Flow cytometric analysis of cell subsets before and after selective alloreactive T cell depletion ........................................................................ 93
Figure 4.1 Specific antigen expression on dendritic cells................................. 104
Figure 4.2 Expression of activation antigens on DC stimulated alloreactive T cells .................................................................................................................. 109
Figure 4.3 Expression of CD25 on DC and PBMC stimulated alloreactive T cells ........................................................................................................ 111
Figure 4.4 Comparison of alloreactive T cell activation in DC MLR and normal MLR ........................................................................................................ 112
Figure 5.1 Structure of CD25 and IL-2 complex ............................................. 120
Figure 5.2 Structure of CD69 molecule ........................................................... 121
Figure 5.3 Structure of CD71 and transferrin complex .................................... 121
Figure 5.4 Structure of HLA-DR3 molecule.................................................... 122
Figure 5.5 Structure of CD38 molecule ........................................................... 123
Figure 5.6 Fluorescence intensity distribution of QuantiBRITE Beads ............ 124
Figure 5.7 Standard calibration curve for ABC quantification ......................... 125
Figure 5.8 Comparison of expression of different activation antigens on alloreactive T cells ........................................................................ 129
Figure 5.9 The effect on primary antibody concentration of magnetic mobility of cells ........................................................................................................ 130
Figure 5.10 The effect on secondary antibody concentration of magnetic mobility of cells ...................................................................................................... 130
Figure 5.11 CD25 expression a. before MLR b. after QMS depletion c. after MLR and before QMS depletion................................................................. 132
Figure 5.12 CD69 expression on T cells a. Before MLR b. After 4 days’ MLR without pretreatment c. After 4 days’ MLR with pretreatment........ 133
Figure 5.13 a. One-step magnetic labeling b. Two-step magnetic labeling ..... 134
Figure 6.1 Comparison of two gate strategies for flow cytometry analysis .... 148
Figure 6.2 Comparison of CD25$^+$ T cell activation (on the 3$^{rd}$ and 4$^{th}$ day of MLR) analyzed by two lymphocyte gating strategies ................................. 150
Figure 6.3 Comparison of CD69$^+$ T cell activation (on the 3$^{rd}$ and 4$^{th}$ day of MLR) analyzed by two lymphocyte gating strategies ........................................ 150
Figure 6.4 Comparison of HLADR$^+$ T cell activation (on the 3$^{rd}$ and 4$^{th}$ day of MLR) analyzed by two lymphocyte gating strategies ........................................ 151
Figure 6.5 Flow cytometric analysis of T cell fraction in peripheral blood mononuclear cells .................................................................................... 153
Figure 6.6 Flow cytometric analysis of CD25, CD69 and HLADR expression on alloantigen activated T cells .............................................................. 155
Figure 6.7 Expression of CD25, CD69, HLADR and CD38 on cells before and after alloreactive T cell depletion ......................................................... 157
CHAPTER 1

INTRODUCTION

1.1 Definition

Transplantation is the process of taking cells, tissues, or organs, called a graft, from one individual and placing them into a (usually) different individual. A graft transplanted from one individual to the same individual is called an autologous graft. A graft transplanted between two genetically identical or syngeneic individuals is called a syngeneic graft. A graft transplanted between two genetically different individuals of the same species is called an allogeneic graft. A graft transplanted between individuals of different species is called a xenogeneic graft. The molecules that are recognized as foreign on allografts are called alloantigens. The lymphocytes and antibodies that react with alloantigens are described as being alloreactive (Abbas et al, 2003).

Bone marrow transplantation or stem cell transplantation is a medical procedure involving the transplantation of hematopoietic stem cells. Stem cell transplantation and high-dose chemotherapy is the most effective treatment for certain cancers such
as leukemia, lymphoma and myeloma. There are two main types of stem cell transplantation, autologous stem cell transplantation and allogeneic stem cell transplantation. Autologous stem cell transplantation involves the isolation and transfer of stem cells from an individual to the same individual. Allogeneic stem cell transplantation involves two genetically distinct people, the donor and the recipient. When a transplant involves two genetically distinct people, there is possibility that the cells from the graft will react against the host. This reaction causes graft versus host disease (GvHD). In order to avoid rejection of the transplanted graft or severe GvHD, the donor should have the identical human leukocyte antigens (HLA) as the recipient. The human leukocyte antigen system (HLA) is the group of genes in the human major histocomptibility complex (MHC) that encodes the cell-surface antigen-presenting proteins. The proteins encoded by HLA are expressed on the outer surface of cells that are unique to that person. The immune system uses this HLA system to differentiate self cells and non-self cells (Wikipedia encyclopedia). There are many HLA antigens. The more disparate human leukocytes antigens existing between the donor and the recipient, the higher level of alloreactions will be induced in the transplant setting.

1.2 Research motivation

It is reported from the leukemia and lymphoma society that there are about 34,810 new cases of leukemia, 63,740 new cases of non-Hodgkin lymphoma and 15,980 new cases of myeloma were diagnosed in the United States in 2005. It is
reported from the leukemia and lymphoma society that there are approximately 9,000 people in North America undergo stem cell transplantation for leukemia, lymphoma or myeloma each year. There are two main types of stem cell transplantation, autologous stem cell transplantation and allogeneic stem cell transplantation. Allogeneic stem cell transplantation is the only curative options for many patients with leukemia or lymphoma. For allogeneic stem cell transplantation, in order to avoid rejection of the transplanted stem cells or severe GvHD, the donor should be HLA-matched with the recipient. However, only 25-30% of patients have human leukocyte antigen (HLA)-matched sibling donors. For patients without a suitable family donor, the chance of finding HLA-matched unrelated donor is 10%-50% (Beatty et al, 1995).

It is this lack of suitable HLA matches for a significant fraction of the human population that has lead to the use of partially HLA-matched or HLA-mismatched allogeneic stem cell transplantation. However, the development of severe graft-versus-host disease (GvHD), which is believed to be caused by donor T cells, can be fatal. Consequently, the depletion of donor T cells abrogates GvHD (Aversa et al, 1998; Ruggeri et al, 2002), has been and continues to be studied as a solution. However, T cells are one of the principle cells in immune system which recognize foreign antigens from infectious microbes and function to destroy these microbes or infected cells. Therefore, complete T cell depletion delays the immune reconstitution, which results in increased chances of opportunistic infection and leukemia relapse. Selective depletion of alloreactive T cells which mediate GvHD from donors, while
retaining T cells with reactivity to pathogens and third party antigens is a strategy that may protect the patient from opportunistic infections and shorten the immunodeficiency post transplantation (Koh et al, 1999; Guimond et al, 2002, André-Schmutz et al, 2002). However, the success of this strategy depends on the extent of alloreactive T cell depletion and remaining anti-viral and third party reactivity. The objective of this study is to develop the methodology to perform alloreactive T cell depletion using a flow-through magnetic cell separation system for clinical application.

1.3 T cell depletion: the development and limitation

Allogeneic hematopoietic stem cell transplantation is the only curative therapy available for many patients with hematological malignancies. Although the allogeneic hematopoietic stem cell transplantation has been developed for twenty years, GvHD is still the major problem which needs to be overcome for successful HLA-mismatched stem cell transplantation (Goker et al ,2001; Nagler et al,2001; Vigorito et al ,2001; Ho et al ,2001; Goerner et al ,2002). The pathophysiology of GvHD is still not clearly described; however, the immuno-competent T cells from the donor stem cell graft have been indicated as the major source to mediate the GvHD (Hertenstein et al, 1998). It has also been reported that current treatment of GvHD includes down-regulation of T lymphocytes by inhibiting cellular proliferation (methotrexate), inhibition of de novo purine synthesis (mycophenolate mofetil), suppression of IL-2 secretion by blocking calcineurin activity (cyclosporine, FK-506), interfering with
downstream growth signaling pathways (sirolimus), reduction of T-cell responsiveness by blocking the IL-2 receptor (daclizumab) and T cell depletion from the donor graft (Ho et al, 2001). Of these various approaches, T cell depletion is the most effective method to prevent GvHD. It has been reported that the depletion of T cells to a median of $3 \times 10^4$ CD3$^+$ cells/kg of the recipient body and the infusion of a median of $10 \times 10^6$ purified CD34$^+$ cells/kg of the recipient body will eliminate GvHD completely (Aversa et al, 1998).

Although sufficient depletion of T cells from the donor graft can abrogate GvHD and the engraftment of T-cell depleted graft can be facilitated by the infusion of a sufficiently high dose of CD34$^+$ stem cells, disadvantages still exist with this strategy. Examples include: delayed immune reconstitution, viral-infections and leukemia relapse. Typically, the regeneration of the immune system after allogeneic stem cell transplantation is a slow process after the transplantation; NK cells, the earliest lymphoid cells to appear, typically take 2 to 3 weeks, followed by B cells (3 to 6 months) and T cells (3 to 12 months) (Ho et al, 2001). The functional operation of these cells takes even longer: patients, after allogeneic stem cell transplantation, are susceptible to viral infection, particularly cytomegalovirus (CMV) and Epstein-Barr virus (EBV) for years (Ho et al, 2001).

### 1.4 Alloreactive T cell depletion

Several of the currently investigated alloreactive T cell depletion approaches include blocking T cell costimulatory pathways (Gribben et al, 1996; Guinan et al,
1999), using inhibitory cytokines (Groux et al, 1996; Boussiotis et al, 2001) to suppress the allo-antigen reactivity of T cells, and negative depletion of alloreactive T cells \textit{in vitro}. In this study, negative depletion of alloreactive T cells using a magnetic cell sorting system will be developed. The potential advantages of this strategy include relatively simple operation and high depletion efficiency.

During HLA-mismatched stem cell transplantation, the alloantigens presented by major or minor histocompatibility complex elicit activation of alloreactive T cells (Abbas, 2003). Mixed lymphocyte reaction (MLR) is used as a standard model to elicit the response of the alloreactive T cells to foreign molecules. The MLR is induced by the culture of peripheral blood mononuclear cells (PBMC) from one individual with PBMC from another individual. In order to simplify the analysis, the PBMC from one individual can be treated with either $\gamma$-irradiation or the antimitotic drug mitomycin C to abrogate their proliferations (this population becomes the model recipient). This treated PBMC population is then mixed with PBMC from a different individual (this population becomes the model donor). Such a model system is called a one-way MLR.

During alloreactive T cell activation, several specific activation antigens will sequentially appear on the surface of the T cells which are regulated by different activation pathways (Fehse et al, 2000). It is these surface antigens that can be(are) targeted for the depletion of alloreactive T cells, two of the most commonly used antigens for depletion are CD25 and CD69. In one such study, CD69 was used as an activation marker to deplete alloreactive T cells from the donor using magnetic cell
sorting column (Miltenyi biotech), the proliferative capacity to the original recipient was depleted to 11.5±9.9% of the original while 77.8±20.9% of the third party reactivity was preserved (Koh et al, 1999) In this study, triplicate cultures were performed with the same number of CD69⁺ depleted or non-depleted cells with the same number of recipient or third-party cells, and the average ³H uptakes by the cultures was measured as proliferative capacity of CD69⁺ depleted or non-depleted cells to the recipient or third party (Koh et al, 1999). In another study, a combination of CD25⁺ and CD69⁺ cells were depleted using magnetic cell sorting column (Miltenyi biotech), and lower alloreactivity was obtained, compared with either CD25⁺ or CD69⁺ cells depletion alone (Fehse et al, 2000). In a third study, CD4⁺hiCD38⁺ cells, were depleted using fluorescence activated cell sorting (FACS), ≥1-2 log₁₀ depletion of alloreactivity was achieved by flow cytometry analysis (Martins et al, 2004). In addition, other activation markers were considered as potential markers for alloreactive T cell depletion such as CD71, CD40L and OX40 (Fehse et al, 2000).

As stated previously, other strategies have been used to achieve alloreactive T cell depletion in order to prevent GvHD. CD95/CD95L-mediated activation-induced cell death was applied to selectively eliminate alloreactive T cells in allogeneic murine model system, ≥80% decrease of proliferative and lytic responses were achieved (Hartwig et al, 2002). Immunotoxin anti-Tac(Fv)-PE38 and anti-Tac(Fv)-PE38KDEL were used to target the IL-2 receptor on alloreactive T cells,
7.6±1.4% against-host activity was left while 64.2±5% third party reactivity was preserved (Mavroudis et al, 1996).

Another strategy based on photodynamic elimination of T cells has been reported to achieve 2-3 log\(_{10}\) depletion of alloreactive T cells (Guimond et al, 2002). In this study, alloreactive T cells were selectively killed by 4,5-dibromorhodamine methyl ester (TH9402), a photosensitizer can be retained in T lymphocytes during activation, which becomes highly cytotoxic with visible light (Guimond et al, 2002). In a clinical Phase I trial of alloreactive T cell depletion using clinical-grade CD25 specific immunotoxin, acute GvHD development was found to be related directly to the extent of alloreactivity depletion (André-Schmutz et al, 2002). After 2-3 log\(_{10}\) depletion of CD3\(^+\)CD25\(^+\) cells from donors using immunotoxin, less than 1% anti-host alloreactivity (The depletion of alloreactivity was determined by the comparison of \(^3\)H uptakes by primary mixed lymphocytes reaction and secondary MLR after selective alloreactive T cell depletion.) was reported in 12 out of 16 patients, and no cases of severe GvHD (greater than grade II) arose from the 12 patients (André-Schmutz et al, 2002).

1.5 **The significance of optimizing alloreactive T cell generation**

The development of GvHD after allogeneic stem cell transplantation is related to the degree of alloreactive T cell depletion from the graft (André-Schmutz et al, 2002). As indicated in section 1.4, the degree of alloreactivity depletion achieved by available methods is normally less than 3 log\(_{10}\), which may not be sufficient for the
high degree removal of alloreactive T cell to abrogate the development of GvHD. The possible reason for insufficient depletion of alloreactive T cells by previous methods is that some potential alloreactive T cells escape the activation, identification or depletion process.

The specific objective of this study is to optimize the generation and detection of alloreactive T cells in vitro and develop a flow-through magnetic cell separation system to maximize the depletion of alloreactive T cells while retaining the maximum third-party reactivity for clinical application.

1.6 Optimize generation of alloreactive T cells in vitro

In order to achieve the objective of depleting maximum number of alloreactive T cells while retaining the maximum third-party reactivity for clinical application, it is important to optimize the in vitro process for generating, identifying and depleting alloreactive T cell. The maximum number of potentially alloreactive T cells in the sample must first be stimulated to express the maximum number of target activation antigen(s) before selective depletion.

It has been reported that pretreatment of stimulator cells with TNF-α and IFN-γ may increase the expression of MHC class I and II molecules on antigen presenting cells (Koh et al, 1999), which will enhance the alloreactive T cell activation. In addition, adding the cytokines such as IL-2 may also increase the expression of activation antigens on the alloreactive T cells (Guimond et al, 2002). It was found that a subset of CD25+ T cells, CD4−CD25high T cells, can induce T cell anergy
(Dieckmann et al, 2001; Ermann et al, 2001) and have the ability to suppress GvHD (Hoffmann et al, 2002; Taylor et al, 2002). Removal of these CD4^+CD25^{high} T cells maybe detrimental, on the other hand, these cells may suppress the activation of alloreactive T cells in MLR.

CD25 and CD69 are the most commonly targeted antigens for alloreactive T cell depletion, and simultaneous targeting these two and several other alleged activation antigens such as CD71, HLA-DR, OX40, CD40L, CD38, CD58, CD152, CD122, CD95 and CD103 may improve the alloreactive T cell depletion (Fehse et al, 2000). Since the activation of different antigens may be regulated by different pathways, the expression kinetics of each activation antigen will be different. Therefore, better definition of the kinetics of different activation antigen expressions in MLR may suggest the optimum duration of MLR, optimum antigen combination, and depletion strategy (sequentially depletion based on expression kinetics of different activation antigens).

1.7 Activation markers

The potential activation antigens expressed on alloreactive T cells in this study include CD25, CD69, CD95, CD71, CD40L, OX40, HLADR, CD38, CD152, CD103 and CD122. The property of each activation antigen is described in the following.

The CD 95 receptor was discovered in 1989 and its ligand was discovered in 1993 as major initiators of apoptosis (Matiba et al, 1997). The CD95 receptor was found to be expressed on a high number of cells from different tissues of the immune
system and other organs (Leithäuser et al, 1993), and its main function is to trigger apoptosis (Trauth et al, 1989; Yonehara et al, 1989). It has been shown that mature human T cells are sensitive to agonistic anti-CD95 mAb only after prolonged in vitro activation, so the T cell activation sensitizes T cell to the CD95-mediated apoptosis pathway (Dhein et al, 1995). Previous studies showed T-cell death that occurs by suicide is mediated by release of a soluble variant of CD95L from the same activated T cell (Dhein et al, 1995). The cytotoxic T cells are able to kill CD95⁺ cells by employing perforin and CD95L (Matiba et al, 1997).

The OX40 molecule is a 50-kD glycoprotein that is expressed on recently activated CD4⁺ T cells (Tittle et al, 1997), and can be expressed on T cells activated during a mixed lymphocyte reaction (MLR) (Paterson et al, 1987). OX40 is a member of the tumor necrosis factor (TNF) receptor family, and this family plays a critical role in either activation or programmed cell death of lymphocytes (Bazzoni et al, 1996). It has also been reported that the OX40 molecule could be found on activated T lymphocytes involved in recognizing autoantigen(s) at the site of inflammation in autoimmunity (Weinberg et al, 1994; 1996 a; 1996 b). Recently, there were studies showed that OX40 was highly expressed on donor T cells isolated from peripheral blood lymphocytes (PBL), spleen, and other target organs during acute GvHD and these T cells were alloreactive (Tittle et al, 1997).

CD71, the transferrin receptor, has a low expression on normal resting lymphocytes (Schwarting et al, 1989) while it is expressed on most cells upon activation (Jefferies et al, 1984). The transferrin receptor is essential for iron transport
into proliferating cells (Judd et al, 1980), such as mitogen-activated (Larrick et al, 1979) and alloantigen-activated (Phillips et al, 1984) lymphoblasts. The transferrin receptor is also present on early erythroid cells but is lost as reticulocytes differentiate into mature erythrocytes (Loken et al, 1987). Therefore the CD71 receptor can be used to discriminate activated lymphocytes from resting lymphocytes (Larrick et al, 1979; Phillips et al, 1984) and for the analysis of metabolic activation or cellular proliferation (Judd et al, 1980).

CD40L (CD154) is a surface molecule expressed by recently activated T cells and plays a crucial regulatory role in a diversity of immunological processes (Schonbeck et al, 2001). Expression of CD40L, regulated primarily by signaling through the T cell receptor, can be detected on T cells very early (1-2 hours) after activation, peaking 6 hours after activation and declining over the following 16-24 hours (Castle et al, 1993). It has been found that the expression of CD40L is only on T lymphocytes, not on other cell types (Hosono et al, 2003).

CD69 antigen is a type II integral membrane protein belonging to the family of C-type lectin receptors (Testi et al, 1994). CD69 is one of the earliest cell surface molecules to be expressed upon T cell activation, appearing as soon as 1 hour after exposure to a potent stimulus such as phytohaemaggkytinin (Testi et al, 1989). CD69 is also involved in early events of monocytes and platelets activation. CD69 contributes to T cell activation by Ca$^{2+}$ influx, synthesis of different cytokines and their receptors, induction of the expression of c-myc and c-fos protooncogenes (Ziegler et al, 1994). CD69 contributes to platelet activation by Ca$^{2+}$ influx,
hydrolysis of arachidonic acid, and aggregation (Ziegler et al, 1994). CD69 also plays a functional role in redirected lysis mediated by activated NK cells (Ziegler et al, 1994). It is expressed constitutively on platelets and thymocytes but in other cell types, including T cells, B cells, NK cells and neutrophils, it is absent at resting levels and inducible only upon cellular activation (Cebran et al, 1993). The CD69 expression on T lymphocytes can be upregulated by cell proliferation and the increased secretion of IL-2 and IFN-γ (Koh et al, 1999).

HLA-DR is a human class II molecule of the major histocompatibility complex (MHC). HLA-DR is a transmembrane glycoprotein composed of an α chain (36 kD) and a β subunit (27kD) expressed primarily on antigen presenting cells: B cells, monocytes, macrophages, and the thymic epithelial cells. HLA-DR is also expressed on activated T cells (BD pharmingen).

CD25, α chain of IL-2 receptor, is expressed on activated lymphocytes (T and B) and monocytes (BD pharmingen). The expression level of CD25 on the donor T cells after MLR is highly increased. Previous study suggests that alloreactive T cells can be depleted by sorting all the CD25+ cells. However, there is a relatively high remaining alloreactivity (up to 20%), even after depleting both CD25+ and CD69+ cells from MLR (Fehse et al, 2000). It suggests that there are several different activation pathways for the alloreactive T cell activation. Moreover, there is a small T cell population, regulatory T cell, express CD25 constitutively (Sakaguchi et al, 2001; Ng et al, 2001; Baecher-Allan et al, 2001).
CD122, β chain of the IL-2 receptor and IL-15 receptor, is expressed on T cells, B cells, monocytes, myeloid precursors, and NK cells (BD pharmingen). The subunit (p55, CD25), common γ subunit, and the β subunit of IL-2/IL-15 receptor forms a high-affinity, signaling complex for IL-2/IL-15 which can be expressed by activated T and B lymphocytes.

The CD38 antigen is expressed on activated T lymphocytes, natural killer (NK) lymphocytes, myeloblasts, and erythroblasts (Dörken et al, 1989; Terstappen et al, 1991; Giorgi et al, 1986; Landay et al, 1984; Nicholson et al, 1989; Terstappen et al, 1990; Tedder et al, 1990). The antigen is expressed during the early stages of T- and B-lymphocyte differentiation, is lost during the intermediate stages of maturation, and then reappears during the final stages of maturation (Salazar-Gonzalez et al, 1985; Tedder et al, 1985).

CD152 is expressed on recently activated CD4⁺ and CD8⁺ T cells and binds to CD80 and CD86 present on antigen presenting cells (APC) with high avidity (Abbas et al, 2003). The function of CD152 is to inhibit T cell activation by counteracting signals delivered by CD28. Experimentally, graft rejection is reduced or delayed by blocking costimulatory signals, such as with a soluble form of CD152 that prevents the B7 molecules on APCs from interaction with T cell CD28 (Abbas et al, 2003).

CD103, 175 kDa, is a type I transmembrane molecule, CD103 is expressed on PHA-stimulated peripheral blood lymphocytes and it is rarely expressed on resting peripheral blood lymphocytes (BD Pharmingen).
1.8 Cell separation methods

Cell separation methods can be categorized on the basis of different fundamental principles/properties by which the separation is achieved. Examples of these principles/properties include: physical properties such as size or density and immunologically differentiable surface properties, such as specific surface markers, i.e. the CD series.

For physical properties, different cell subsets can be separated using correlative techniques based on their size, density, electric charge, and adhesion properties. For example, peripheral blood mononuclear cells are routinely separated from other cells in peripheral blood by a density gradient. Macrophage cells can be removed from leukocytes by using the selective adhesion properties of the macrophage to plastics (Buckley et al, 2000). Free-flow electrophoresis can fractionate erythrocytes based on electric charge (Ramírez et al, 2003).

Immunological based separation technology typically uses antibodies which bind to specific cell surface markers. Various technologies have been developed based on immunological specialty, including: affinity chromatography (Subramanian, 1998), fluorescence activated cell sorting (FACS) (Martins et al, 2004), cell lysis using immunotoxins (Guimond et al, 2002), and magnetic activated cell sorting (MACS) (Sun et al, 1998).
1.9 Immunomagnetic cell separation technology

Immunomagnetic cell separation has been used widely in biological research and clinical application. The fundamental principle of immunomagnetic separation is the interaction between cell subsets and a magnetic field, where the target cell subpopulation has been labeled with magnetic micro or nano particles. A variety of approaches/methodologies are used to achieve the actual separation exploiting the magnetic force operating on the targeted cell population. The advantage of this methodology is relative ease of operation, low cost and wide application to many fields.

In order to separate the target cell population from cell mixtures, there are two main approaches: positive selection and negative depletion. In positive selection, the target cell population is magnetically labeled and subsequently collected in the magnetic positively separated cell fraction. Alternatively, in negative depletion, undesired cells are labeled with magnetic particles, and the desired, non-magnetic target cell population is collected in the non-magnetic cell fraction.

The magnetic particles or beads used to label target cells have a wide size range, from several nanometers to micrometers. Large magnetic particles with diameter from 1 µm to 5 µm, usually have a high magnetic moment; therefore, large magnetic particles are used in low magnetic gradient field. Dynal beads are examples of such large magnetic particles and are produced by Dynal (Oslo, Norway) with size ranging from 2.8 µm to 4.5 µm.
Small magnetic particles, with diameter ranging from 50 nm to 300 nm, are also referred to as colloidal beads, or magnetic nanoparticles. A high gradient magnetic field is required for separation using these small magnetic particles. An example of small magnetic particle application is MACS® family of separation columns (MiniMACS through CliniMACS; Miltenyi Biotec, Germany). These columns are packed with small steel spheres and the columns are held by a device which surrounds the column with a magnetic filed, a large magnetic energy gradient is created between the small spheres within the column. The cell suspension including magnetically labeled targeted cells flow through these columns and the magnetically labeled cells are attached and retained in the high magnetic energy gradient regions between the individual steel spheres while the unlabeled cells, ideally, flow through (Zborowski et al, 1996) (Miltenyi Biotech). After removing the MACS column from magnetic gradient field, the magnetic conjugated cells are then eluted.

In contrast to the batch, retention system, such as the MACS® technology, flow-through magnetic cell separation systems have been developed in the laboratories of Chalmers and Zborowski. Quadrupole magnetic flow sorter (QMS) (Sun et al, 1998; Chalmers et al, 1998) and the Dipole magnetic flow sorter (DMFS) (Moore et al, 1998) have been successfully developed and tested. The QMS was developed for high-throughput negative depletion or positive selection of cell populations (Chalmers et al, 1998), while the DMFS was designed to fractionate positive labeled cells based on their cell surface antigen expression, correspondingly, based on the magnetophoretic mobility of the labeled cells.
Compared with fluorescence activated cell sorting (FACS), the high throughput of QMS (10^6 - 10^7 cells/s) is a big advantage, since 10^{10} cells are needed to be processed for clinical-scale stem cell transplantation. Moreover, it is easy to scale-up for QMS and the operation of QMS is relatively simple and low cost.

1.10 Quadrupole Magnetic Flow Sorter (QMS)

Quadrupole magnetic flow sorter (QMS) is a continuous flow through magnetic cell sorting system based on a quadrupole magnetic field (Figure 1.1). The magnetic field strength, \( S_m \), inside the quadrupole field is axially symmetric and defined by:

\[
S_m = \frac{B_0^2}{\mu_0 r_0} \rho
\]  

where \( B_0 \) is the maximum magnetic field intensity in the flow region, \( \mu_0 \) is the magnetic permeability of free space, \( \rho \), equal to \( r/r_0 \), is the dimensionless radial distance from the quadrupole field axis, \( r \) is the distance from the field axis, and \( r_0 \) is the outer cylinder inner wall radius. Since the magnetic field strength \( (S_m) \) is proportional to the distance from the field axis \( (r) \), the magnetic field strength \( (S_m) \) in the quadrupole system is only in the radial direction.

The magnetically labeled cells move in the radial direction in the QMS system due to the application of the radial magnetic field. The radial velocity of the labeled cells in magnetic field is expressed by:

\[
v_m = m \cdot S_m = m \cdot \frac{B_0^2}{\mu_0 r_0} \rho
\]

where \( m \) is the cell’s magnetophoretic mobility.
A separation process can be described as introducing the mixture of magnetically labeled and unlabeled cells into the QMS separation system though inlet $a'$. The carrier buffer, without cells, is pumped into the QMS separation system through inlet $b'$. If the cell suspension flows through the magnetic energy gradient in the QMS system, the magnetically labeled cells with sufficiently high magnetophoretic mobility migrate past the outlet splitting cylinder (shown as $r_{OSC}$ in Figure 1.2) and exit from outlet $b$, while the nonmagnetically labeled cells exit from outlet $a$.

There are several parameters evaluating the performance of QMS separation process such as throughput, purity ($P$), recovery ($F$), the resolving power ($X$), and the dilution factor ($Y$).

\[ P_E = \frac{N_{E,lg}}{N_{E,lg} + N_{E,ng}} \]  

\[ F_E = \frac{N_{E,lg}}{N_{F,lg}} \]  

\[ X = \frac{m_1}{m_1 - m_0} \]  

\[ Y = \frac{Q(a')}{Q(b)} \]

where $N_{E,lg}$ and $N_{E,ng}$ represents the number of target cells and non-target cells in the enriched fraction respectively. $N_{F,lg}$ is the number of target cells in the feed. $m_1$ is the average magnetophoretic mobility of the magnetically labeled cells, $m_0$ is the "cutoff" mobility of unlabeled cells. And $Q(a')$, $Q(b)$ is the volumetric flow rate through inlet $a'$ and outlet $b$, respectively.
Based on the mode of magnetic sorting process (negative depletion or positive selection) and the requirement of the separation efficiency, the separation process with QMS can be optimized so that a very high purity of target cells is obtained, with, as a consequence, a lower overall recovery, or optimized in such a way that very high recovery of the target cell is obtained, with a lower final purity.

Figure 1.1 Composition of quadrupole magnetic sorter (QMS)
1.11 Definition and measurements of magnetophoretic mobility

Cell separations with immunomagnetic cell separation systems are based on the difference in magnetic force operating on magnetically labeled cells in contrast to the lack of force (or in some cases opposite force) operating on the nonmagnetically labeled cells in the magnetic field. In some systems this force results in a combination of movement as well as a resistive force holding the labeled cells to a surface. Therefore, it is desirable to maximize the magnetic force, and in some systems, the movement difference of magnetically labeled cells and nonmagnetically labeled cells.
Magnetophoretic mobility was defined in order to quantify the movement of magnetic cells in a non-uniform magnetic field, which can be expressed as follows,

\[
m = \frac{v_c}{S_m} = \frac{ABC \cdot \beta \cdot F_b}{3 \cdot \pi \cdot D_c \cdot \eta \cdot S_m} = \frac{\Delta \chi \cdot V_m}{3\pi D_c \eta} ABC \beta
\]

where \( V_m \) represents the volume of one magnetic nanoparticle, \( \Delta \chi \) represents the difference in magnetic susceptibility between the paramagnetic nanoparticle and the suspending fluid, \( D_c \) is the diameter of magnetic cells, \( \eta \) is the viscosity of suspending fluid, \( ABC \), antibody binding capacity, represents the number of primary antibody labeled on each cell, and \( \beta \), magnification factor, is the number of secondary antibody labeled per primary antibody.

The magnetophoretic mobility of magnetically labeled cells can be experimentally measured by an instrument developed in Chalmers and Zborowski’s lab on a cell-by-cell basis (McCloskey et al, 2001; McCloskey et al, 2000; Nakamura et al, 2001; Moore et al, 2000; Chalmers et al, 1999). This equipment is referred to as Cell Tracking Velocimetry (CTV) (Figure 1.3), and consists of a microscope, CCD camera, lighting system, computer and particle tracking program. A specifically designed, constant magnetic energy gradient was developed to quantify the movement of magnetically labeled cells. CTV works by subjecting the cells to a magnetic energy gradient and recording the motion in a computer. The recorded images are analyzed using a computer algorithm. Finally, the magnetic mobility of each tracked cell, and the distribution of the magnetic mobility for the total cell population are calculated. Based on the distribution of cell magnetophoretic mobility, a model was developed.
and applied to predict the performance and operation parameters of QMS (Williams et al, 1999).

A new CTV system is being developed in the laboratories of Chalmers to incorporate the measurement of size distribution and magnetophoretic mobility of cells by quantifying the settling velocity and magnetophoretic velocity of cells at the same time. From the simultaneous measurement, dot plots can be developed by presenting the cell size and magnetophoretic mobility on X and Y axis respectively. Then different cell population can be presented in different regions on the dot plots, the performance of cell sorting using QMS can be predicted more accurately.
1.12 Dissertation organization

The outlines of Chapter 2 through Chapter 5 are as follows:

Chapter 2 describes different strategies for optimizing the alloreactive T cell generation in vitro. The activation kinetics of alloreactive T cell subsets expressing activation antigens including CD25, CD69, CD71, HLADR, and OX40 was studied. The effect of different cytokines on increasing alloreactive T cell activation was tested.
The scale-up of mixed lymphocytes reaction for clinical application was also discussed in Chapter 2.

Chapter 3 discusses the identification of alloreactive T cells by using multiple activation antigens. The expression of activation antigens including CD25, CD69, CD71, CD95, OX40, HLADR, CD38, CD103, CD122, and CD152 on alloreactive T cells was analyzed by FACSARia, a nine-parameter flow cytometer. The immune functional significance of alloreactive T cell subsets on inducing alloreactivity was determined by limiting dilution assay. The degree of alloreactivity depletion achieved by targeting multiple activation antigens and single activation antigen was compared.

A new strategy to optimize the generation and depletion of alloreactive T cells by recipient monocytes-derived dendritic cells was investigated in Chapter 4. The expression of activation antigens on dendritic cell activated alloreactive T cells was studied. Moreover, the degree of alloreactivity depletion with this strategy was measured.

In Chapter 5, the antibody binding capacity (ABC) of different activation antigens expressed on alloreactive T cells was determined. The effect on increasing ABC on magnetophoretic mobility of target cells and the performance of magnetic cell separation was discussed.

Flow cytometric assay and limiting dilution assay were compared in Chapter 6 of their advantages and disadvantages in the quantification of specific cell population.
CHAPTER 2

OPTIMIZE GENERATION OF ALLOREACTIVE T CELLS

2.1 Motivation

Allogeneic stem cell transplantation is the only curative option for many patients with hematological malignant diseases such as acute leukemia, chronic leukemia and refractory non-Hodgkin’s lymphoma. Graft versus host disease (GvHD) is the main limitation of allogeneic stem cell transplantation, which is induced by a T cell subpopulation from the graft, alloreactive T cells. The activation of alloreactive T cells requires the presentation of alloantigens by antigen presenting cells (APCs). Those APCs with alloantigen could be recognized by donor T cells, then a proportion of donor T cells will be activated and proliferate. The activated and proliferating T cells will attack recipient (stimulator) cells and cause severe graft versus host disease (GvHD). This population of donor T cells is called as alloreactive T cells, and the activity of alloreactive T cells against recipient cells is called as alloreactivity. The selective depletion of alloreactive T cells can abrogate graft versus host disease (GvHD) while preserving the anti-third party reactivity of
the graft. The incidence of GvHD development after transplantation is correlated to the degree of alloreactive T cell depletion, the less possibility of GvHD development (André-Schmutz et al, 2002). In order to eliminate GvHD after allogeneic stem cell transplantation, it is required to deplete the maximum number of alloreactive T cells from the donor. Therefore, it is critical to optimize the stimulation, identification and depletion of all alloreactive T cell subsets.

Different strategies were investigated in order to optimize the generation of alloreactive T cells in vitro, which leads to the higher degree of alloreactivity depletion (Solomon et al, 2002; Amrolia et al, 2005). Brenner et al (2003) have demonstrated that more CD25+ alloreactive T (10-15% T cells)cells can be generated by using HLA-mismatched host Epstein-Barr virus (EBV)-transformed lymohoblastoid cell lines (LCLs) as stimulators (Brenner et al, 2003). It has been reported that the generation of host-reactive donor lymphocytes can be optimized (22-34% T cells were activated) by using OKT-3 expanded CD3+ lymphocytes as simulators (Barrett et al, 2002).

In this study, standard mixed lymphocyte reactions (the cell source is peripheral blood mononuclear cells) were performed to generate alloreactive T cells. In order to optimize the culture condition for generating more alloreactive T cells in vitro, four different aspects were studied. First, it is indicated that multiple activation antigen may be expressed on alloreactive T cells and the expression of these activation antigens on alloreactive T cells might be regulated by different pathways. The activation kinetics of alloreactive T cell subsets express different activation antigens
were studied in order to determine the optimum culture duration for mixed lymphocytes reaction (MLR). Secondly, T cell growth factors, IL-2 and IL-15, were studied for their effect on increasing number of alloreactive T cells being generated. The optimum doses of IL-2, IL-15 and their combinations were also determined. Thirdly, the recipient cells were treated with IFN-γ and TNF-α before MLR and the treatment effect was determined. IFN-γ and TNF-α have been reported to have the function of increasing MHC molecule expression on antigen presenting cells (APCs). Therefore, theoretically, the treatment can amplify the immune responses induced by APCs and increase the number of generated alloreactive T cells. Finally, CD4⁺CD25⁺ cells have immunosuppressant function to inhibit the alloreactive T cell activation, and the CD4⁺CD25⁺ T cells is important to suppress graft versus host reactivity after transplantation. Therefore, the CD25⁺ cells were pre-depleted before MLR, and the effect of pre-depletion on improve alloreactive T cell activation was studied.

2.2 Material and Methods

Cell Preparation

In this study, human peripheral leukocyte packs from American Red Cross were used as cell source, which is different from the apheresis product used in clinical application. The difference in cell composition between leukocyte packs and apheresis product may have effect on the application of the results to clinical study.

Human peripheral leukocytes were ordered from the American Red Cross, Central Ohio Region. The peripheral blood mononuclear cell (PBMC) fraction was isolated.
and separated by Ficoll-Hypaque density gradient centrifugation (PBMCs and other components from human peripheral leukocytes have different density). The cells were washed with phosphate buffered saline (PBS) twice and resuspended in AIM-V® medium supplemented with streptomycin sulfate (50 µg/ml), gentamicin sulfate (10 µg/ml), L-glutamine (2 mM), and 10% pooled human AB Serum. The number of peripheral blood mononuclear cells was determined using a hemacytometer.

**Cytokine pretreatment of recipient cells**

The PBMC fraction was resuspended in AIM-V® medium supplemented with streptomycin sulfate (50 µg/ml), gentamicin sulfate (10 µg/ml), L-glutamine (2 mM), and 10% pooled human AB Serum at a concentration of 5x10^6 cells/well. The cells were treated with 500, 1000, 2000 U/ml IFN-γ or 500, 1000, 2000 U/ml TNF-α or combinations of IFN-γ and TNF-α for 24 hours. Then the cells were washed with PBS once and resuspended in AIM-V supplemented with streptomycin sulfate (50 µg/ml), gentamicin sulfate (10 µg/ml), L-glutamine (2 mM), and 10% pooled human AB Serum. The cells can be irradiated (30 Gy) and used as stimulators for MLR.

**One-way mixed lymphocytes reaction (MLR)**

A one-way mixed lymphocytes reaction (MLR), involving two unrelated HLA non-identical donors (A and B), was set up in this study. The responder PBMC (A) were incubated at 37°C with the same number of irradiated (30 Gy) stimulator PBMC (B*). All cultures were performed in 12-well tissue culture plate (BD) at a concentration of 3x10^6 cells/ml with a final volume of 2 ml. For control stimulations,
the responder PBMC (A) were incubated with the same number of irradiated (30 Gy) autologous PBMC (A*) at a concentration of $3 \times 10^6$ cells/ml with a final volume of 2 ml.

**Depletion of CD25$^+$ Cells**

Aliquots of responder cells and stimulator cells were suspended in labeling buffer (PBS+0.5% BSA+2mM EDTA) at a concentration of $10^7$ cells/80 µl. Then 20 µl anti-CD25 PE (BD Pharmingen) was added into the cell suspension and the cell mixture was incubated at 4 °C for 30 minutes. Then the cells were washed twice with labeling buffer. After washing, 20 ul anti-PE MACS beads (Miltenyi Biotech) were added to the sample tubes, 80 µl labeling buffer was added to keep the total volume at 100 µl. The sample tubes were then incubated in the dark at 4 °C for 30 minutes. After labeling, the cells were washed twice, and then the cell samples were suspended in 2 ml labeling buffer. Then the cell suspension was passed through Mini MACS system (Miltenyi Biotech), the negative fraction (non-magnetic cells) was collected, washed, resuspended, counted and titrated for mixed lymphocytes reaction.

**Cell labeling for flow cytometry**

Cells were harvested from tissue culture plates or flasks, washed twice with PBS, and counted. Aliquots of $0.5 \times 10^6$ or $1 \times 10^6$ cells were transferred to the 75x12 mm polypropylene tubes. Appropriate antibodies for the specific experiment were added to the cell suspension as recommended by the manufacturer. The total incubation volume was brought up to 100 µl. Then the sample tubes with antibodies were
incubated in the dark at 4 °C for 30 minutes. After labeling, the cells were washed twice. Basically, 2 ml of labeling buffer (PBS+0.5% BSA+2 mM EDTA) was added into each sample tube and the sample tubes were centrifuged at 300 g, 4 °C for 8 minutes. After centrifugation, the supernatant was discarded and another wash step was repeated. 1 ml fixing solution (2% paraformaldehyde solution) was added for 1x10^6 cells. The samples can then be stored at 4 °C in the dark for up to one week.

**Flow cytometry analysis**

A four-color flow cytometry system, FACSCalibur, was used in this study. The FACSCalibur system is a dual-laser and four-color benchtop system. The dyes such as FITC, PE, PI, PerCP, 7AAD and PE-Cy5 can be excited by the 488 nm blue laser and APC can be excited by the 633 nm red laser.

Unstained and appropriate single fluorescence stained samples were run first to adjust the voltage setting and compensation of the flow cytometer. Then the isotype control samples and tested samples were processed by CellQuest, 10,000 events were collected for most samples.

**Quantification of MHC and activation antigen expression**

The QuantiBRITE® beads were used in order to quantify the number of activation antigens and MHC molecules expressed on different cell subsets. The QuantiBRITE® beads are composed of four beads populations with a different, known number of PE molecules bound to each population. A standard curve can be made by plotting log_{10} (geometry mean of Fluorescence intensity) versus log_{10} (mean PE molecules per bead)
of each beads population. By fitting the geometry mean fluorescence intensity of the specific antibody labeled on cells to the standard calibration curve, the expression of surface antigens on cells can be quantified (Note: The QuantiBRITE® assay has to be performed at the same time that the cells are analyzed.).

Statistical study

The T test analysis was performed to determine the statistical significance of different treatments and culture conditions. JMP software was used to perform all the statistical analysis.

2.3 Results and Discussions

Kinetics of activated antigen expression

Based on our current understanding, the expression of activation antigens on activated T cells under stimulation is different for different activation antigens due to the regulation of different pathways, and the maximum expression of each of these different activation antigens is most probably also unique. In order to empirically find the optimum culture duration for achieving the maximum number of alloreactive T cells expressing those allo-activated antigens, in vitro mixed lymphocytes reaction (MLR) cultures were performed. After 24 hours, 48 hours, 72 hours, 96 hours and 120 hours, the cells from MLR were removed, (Note: sufficient number of wells in a 12-well tissue culture plates were started at time equal to zero to provide an unanalyzed sample at each time point), labeled with antibody for CD3, a pan T cell
marker, and antibodies for allo-induced activated antigens including CD25, CD69, CD71, OX40 and HLADR, and analyzed on a FCM. In Figure 2.1-2.5, the expression kinetics for CD25, CD69, CD71, OX40 and HLADR are presented, respectively.

To understand more accurately the activation process, the results presented in Figures 2.1-2.5 are in the form of specific activation, which is the difference in the percentage of activated antigen expressing cells in lymphocytes from the MLR culture and from the autologous control. (Note: the lymphocytes gates of flow cytometry analysis are based on Forward scatter and Side Scatter.) As outlined previously, the autologous control was conducted by culturing the same number of donor PBMC and γ-irradiated donor PBMC (30 Gy) together at the cell concentration of 3x10^6 cell/ml supplement with 50 U/ml IL-2.

T-test analysis (N >= 6 for most culture time) was performed to determine if the activation of alloreactive T cells following different duration of MLR can be significantly increased. In Figure 2.1 to Figure 2.5, the columns with “*” represent the culture time for MLR following which significant high fraction of alloreactive T cells can be activated. It is indicated from Figure 2.1-2.5 and Table 2.1 that the activation of CD25^+ and CD71^+ alloreactive T cells are significant improved after three days’ MLR; the highest fraction of OX40^+ alloreactive T cells are activated after two day’s MLR; the optimum culture time for stimulating HLADR^+ alloreactive T cells is 3 days; and there is no significant difference in CD69^+ alloreactive T cell activation between three and four days’ MLR. Overall, significant high fraction of alloreactive T
cells expressing these activation antigens can be stimulated after three or four days’ MLR. Four days of MLR was selected for further study because the highest fraction CD25+ T cells (The commonest studied alloreactive T cell subsets) were generated after four-day MLR.

Figure 2.1 CD25 activation kinetics in mixed lymphocytes reaction

Figure 2.2 CD69 activation kinetics in mixed lymphocytes reaction
Figure 2.3 CD71 activation kinetics in mixed lymphocytes reaction

Figure 2.4 OX40 activation kinetics in mixed lymphocytes reaction
Effect of IL-2 and IL-15 on activation antigen expression

IL-2 is a T cell growth factor, plays an important role in T cell proliferation and differentiation, and is reported to be secreted by T cells upon antigen recognition (Abbas et al, 2003). The second growth factor is IL-15 which has many similar functions with IL-2 and shares $\beta$ and $\gamma$ chains of IL-2 receptor for signal-transducing (Giri et al, 1994); however, the difference of IL-2 and IL-15 in regulating T cell activation is not well-defined and one of the goals of this set of experiments was to determine if the specific activation of cells expressing activation antigens can be increased by adding IL-15 in the MLR cultures. Table 2.1 compares the effect of adding IL-2, IL-15, or a combination of IL-2 and IL-15 to MLR culture on improving the specific activation of allo-activated cells. As previously presented study, the specific activation was measured relative to a control culture and the five surface
antigens used to identify activation, CD25, CD69, CD71, OX40, and HLADR, were determined by the FCM analysis. The actual values presented are a mean of four studies with a standard deviation. T-test analysis was performed to test if treating MLR with IL-2 and IL-15 can increase the alloreactive T cell activation significantly, the results indicate that only the activation of CD25⁺ alloreactive T cells can be increased by treating MLR with 50U/ml IL-2 (p=0.05). However, the nonspecific activation of cells expressing activation antigens is increased with the addition of IL-2 and IL-15, which makes the selection of activation antigens representing alloreactive T cells very difficult; therefore, it was decided to perform MLR without IL-2 and IL-15 for cell subsets selection.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific activation of alloreactive T cells (%; Mean ± SD, N=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD25</td>
</tr>
<tr>
<td>0 U/ml IL-2</td>
<td>2.76 ± 1.7</td>
</tr>
<tr>
<td>50 U/ml IL-2</td>
<td>5.34 ± 0.9*</td>
</tr>
<tr>
<td>10ng/ml IL-15</td>
<td>4.42 ± 1.95</td>
</tr>
<tr>
<td>15ng/ml IL-15</td>
<td>4.06 ± 1.85</td>
</tr>
<tr>
<td>50U/mlIL-2 +10ng/ml IL-15</td>
<td>5.07 ± 1</td>
</tr>
</tbody>
</table>

Table 2.1 Specific activation of each activation antigen after 4 days’ MLR (“*” represents significant increase by T-test analysis)

Pretreatment of stimulator cells with IFN-γ and TNF-α

IFN-γ is a homodimeric protein produced mainly by T cells and natural killer cells that have been activated by antigens, mitogens, or alloantigens. IL-2, bEGF, and EGF
induce the synthesis of IFN-γ, among other things. IFN-γ has antiviral and antiparasitic activities and also inhibits the proliferation of a number of normal and transformed cells. IFN-γ is a modulator of T cell growth and functional differentiation which acts synergistically with IL-1 and IL-2 and appears to be required for the expression of IL-2 receptors on the cell surface of T lymphocytes, thus influences cell-mediated mechanisms of cytotoxicity. IFN-γ also stimulates the expression of CD4 on T helper cells. Moreover, IFN-γ regulates the expression of MHC class II genes and is the only interferon that stimulates the expression of these proteins (Abbas et al, 2003).

TNF-α is secreted by macrophages, monocytes, neutrophils, T-cells and NK-cells following their stimulation by bacterial lipopolysaccharides. Cells expressing CD4 secrete TNF-α while CD8+ cells secrete little or no TNF-α. TNF-α shows a wide spectrum of biological activities. It causes cytolysis and cytostasis of many tumor cell lines in vitro. In leukocyte and lymphocyte progenitors, TNF-α stimulates the expression of class I and II HLA and differentiation antigens, and the production of IL-1, colony stimulatory factors, IFN-γ, and arachidonic acid. TNF-α enhances the proliferation of T-cells induced by various stimuli in the absence of IL-2. Some subpopulations of T-cells only respond to IL-2 in the presence of TNF-α (Abbas et al, 2003).

Based on the above descriptions, both IFN-γ and TNF-α have the potential ability to enhance MHC-associated molecular expression. Such an enhancement would amplify the recognition phase of immune responses by increasing expression of the ligands.
that T cells recognize. Theoretically, pretreatment of stimulator cells with IFN-γ and TNF-α will increase the MHC expression which in turn would amplify the immune responses such as alloreactive T cell activation in a MLR. Consequently, the effect of pre-treating stimulators with IFN-γ and TNF-α on MHC I and MHC II expression was studied. In Figure 2.6, the MHC II molecules expressed on CD14⁺ cells were increased significantly (N=2, p<0.05) after the treatment with IFN-γ for 24 or 48 hours. The MHC I molecules expressed on CD14⁺ cells were not increased significantly (N=4) after the treatment with IFN-γ and TNF-α for 24 or 48 hours as shown in Figure 2.7. In both Figure 2.6 and Figure 2.7, the error bars represent standard deviation.

The effect of pre-treating stimulator cells with IFN-γ and TNF-α on increasing the alloreactive T cells activation, as measured by percentage of cells specifically activated, is shown in Table 2.2. The actual values presented are a mean of six studies with a standard deviation. The T-test analysis was performed to determine if alloreactive T cell activation can be significantly increased by the pretreatments. HLADR⁺ alloreactive T cell activation can be increased significantly by pre-treating stimulator cells with 2000 U/ml IFN-γ (p=0.03). The effect of pre-treating stimulator cells with IFN-γ and TNF-α on increasing activation antigen expression on alloreactive T cells is presented in Table 2.3. The actual values presented are a mean of three studies with a standard deviation. The T-test analysis was performed to test if the activation antigen expression on alloreactive T cells can be increased significantly by the pretreatments. It was concluded that pretreatment of stimulators with 500 U/ml
IFN-γ for 24 hours before MLR is significant in increasing the expression of CD69 on alloreactive T cells (p=0.05).

<table>
<thead>
<tr>
<th>Alloreactive T cells</th>
<th>CD25⁺</th>
<th>CD69⁺</th>
<th>CD71⁺</th>
<th>OX40⁺</th>
<th>HLADR⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean+SD (%), N=6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non treat 500U IFN-γ/ml</td>
<td>3.67±2.3</td>
<td>4.89±2.95</td>
<td>2.31±1.68</td>
<td>3.36±3.85</td>
<td>1.74±1.66</td>
</tr>
<tr>
<td>1000U IFN-γ/ml</td>
<td>4.83±2.81</td>
<td>6.16±3.62</td>
<td>2.77±0.71</td>
<td>5.83±3.18</td>
<td>2.22±1.25</td>
</tr>
<tr>
<td>2000U IFN-γ/ml</td>
<td>3.86±2.2</td>
<td>5.00±4.02</td>
<td>2.23±0.55</td>
<td>4.56±3.27</td>
<td>3.08±1.58</td>
</tr>
<tr>
<td>5000U IFN-γ/ml</td>
<td>7.42±6.96</td>
<td>6.39±5.96</td>
<td>3.70±2.50</td>
<td>7.09±6.54</td>
<td>4.17±2.49*</td>
</tr>
<tr>
<td>TNF-α/ml</td>
<td>4.16±2.30</td>
<td>4.39±4.15</td>
<td>1.76±0.82</td>
<td>5.27±3.09</td>
<td>2.94±2.43</td>
</tr>
</tbody>
</table>

Table 2.2 The effect of pre-treating recipient cells on alloreactive T cell activation ("*" representing statistically significant increase)

<table>
<thead>
<tr>
<th>Molecule number</th>
<th>CD25</th>
<th>CD69</th>
<th>CD71</th>
<th>OX40</th>
<th>HLADR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean+SD (%), N=3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non treat 500U IFN-γ/ml</td>
<td>2571±935</td>
<td>8886±5123</td>
<td>1204±442</td>
<td>1623±868</td>
<td>5459±2931</td>
</tr>
<tr>
<td>1000U IFN-γ/ml</td>
<td>3314±918</td>
<td>16851±6209</td>
<td>1171±565</td>
<td>2186±1383</td>
<td>5733±2465</td>
</tr>
<tr>
<td>2000U IFN-γ/ml</td>
<td>2629±993</td>
<td>16535±6318</td>
<td>1155±420</td>
<td>1965±1251</td>
<td>5808±2501</td>
</tr>
<tr>
<td>5000U IFN-γ/ml</td>
<td>3741±767</td>
<td>11504±4706</td>
<td>1722±929</td>
<td>1847±1002</td>
<td>4801±1540</td>
</tr>
<tr>
<td>TNF-α/ml</td>
<td>2601±1110</td>
<td>12770±3995</td>
<td>1177±375</td>
<td>2125±1211</td>
<td>5345±2665</td>
</tr>
</tbody>
</table>

Table 2.3 The number of activation antigen expressed on activated T cells on the 4th day of MLR ("*" representing statistically significant increase)
Figure 2.6 The increase folds of MHC II expression on CD14\(^+\) cells by pretreatment stimulators with IFN-\(\gamma\) and TNF-\(\alpha\)

Figure 2.7 The increase folds of MHC I expression on CD14\(^+\) cells by pretreatment stimulators with IFN-\(\gamma\) and TNF-\(\alpha\)
Pre-depletion of CD4^+CD25^+ T regulatory cell before MLR

Starting in the late 1960s and early 1970s, the regulatory lymphoid cells have been shown to suppress a variety of immune functions including antibody responses (Gershon et al, 1974; Tada et al, 1974), cell proliferation towards mitogens or allogeneic lymphocytes (Anacerio et al, 1979; Rich et al, 1974), and the induction of cytolytic lymphocytes (Hirano et al, 1976). Approximately 6% of the CD4^+ cells present in the peripheral blood of human adults are CD4^+CD25^+ T regulatory cells, the function of which is to suppress the activation and proliferation of CD4^+ and CD8^+ T cells by inhibiting the IL-2 secretion (Dieckmann et al, 2001). It has been also observed that IFN-γ production was inhibited by CD4^+CD25^+ T regulatory cells (Shevach et al, 2001). It has been demonstrated that in a mouse bone marrow transplantation model CD4^+CD25^+ cells can prevent graft-versus-host disease (GvHD) by blocking major histocompatibility complex (Taylor et al, 2002; Hoffmann et al, 2002; Cohen et al, 2002; Trenado et al, 2003; Jones et al, 2003). Furthermore, T regulatory cells polyclonally expanded (CD4^+CD25^+) with anti-CD3 and IL-2 were demonstrated to effectively prevent GvHD (Taylor et al, 2002). In human peripheral blood, the T regulatory cells are less common than in murine lymph node or spleen; also, it has been show that only the 1%-2% brightest CD25 expressers have the suppression effect (Baecher-Allan et al, 2001). Finally, it has been reported that in vitro expanded human CD4^+CD25^+ T regulatory cells can block allogeneic dendritic-cell stimulated MLR almost completely (Godfrey et al, 2004).
These crucial, complex, and multifunctional properties of CD4⁺CD25⁺ T regulatory cells pose unique challenges in alloreactive T cell depletion strategies. Specifically, the blocking of the secretion IL-2 and IFN-γ, inhibiting the alloantigen stimulated CD4⁺ and CD8⁺ T cell activation, and retaining the anti-tumor and graft-versus-leukemia function are important, positive functions that one wishes to preserve in a transplant. However, in order to maximize the alloantigen induced alloreactive T cell activation and activation antigen expression, it may be desirable to deplete the CD4⁺CD25⁺ T regulatory cells prior to an in vitro MLR.

In this study, a strategy that depletes CD25⁺ T cells before MLR was applied to improve the alloreactive T cell activation. The preliminary in vitro MLR studies were performed to compare alloantigen induced T cell activation in MLR with or without CD25⁺ T cells depletion. Figure 2.8 compares the fraction of CD4⁺CD25⁺ cells in PBMC before and after CD25⁺ cell depletion. It is shown in Figure 2.9-2.11 that the specific cell activation in MLR with and without pre-depletion of CD25⁺ T cells from donors, recipients or both. In those figures, “ A ”, “ B ” represents donor and recipient respectively, “ * ” represents the cells were irradiated before MLR, “ A- ” and “ B- ” represents that CD25⁺ T cells were depleted from that individual before MLR. In all three surface antigens investigated (CD25, CD71 and OX40), the depletion of CD25⁺ T cells from both donor and recipient cell population before MLR increased the specific activation of allo-antigen induced activated cells. However, the T-test comparison of the effect of each pre-depletion condition on alloreactive T cell activation (shown in Table 2.4) indicates that the effect of pre-depleting CD25⁺ cells
on increasing alloreactive T cell activation is not significant. In this study, CD25<sup>+</sup> cells were pre-depleted instead of CD4<sup>+</sup>CD25<sup>+</sup> cells; <1 log<sub>10</sub> depletion of CD4<sup>+</sup>CD25<sup>+</sup> cells was achieved with Mini MACS; only there replicate experiments were performed independently; all these may explain why the effect of CD25<sup>+</sup> cell pre-depletion on increasing alloreactive T cell activation is not significant.

![Figure 2.8 CD4<sup>+</sup>CD25<sup>+</sup> cell fraction in PBMC before and after CD25<sup>+</sup> cell depletion](image)

Figure 2.8 CD4<sup>+</sup>CD25<sup>+</sup> cell fraction in PBMC before and after CD25<sup>+</sup> cell depletion
Figure 2.9 CD3^+CD25^+ specific activation before or after CD25^+ T cell depletion

Figure 2.10 CD3^+CD71^+ specific activation before or after CD25^+ T cell depletion
Figure 2.11 CD3⁺OX40⁻ specific activation before or after CD25⁺ T cell depletion

<table>
<thead>
<tr>
<th></th>
<th>CD25</th>
<th>CD71</th>
<th>OX40</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB⁻</td>
<td>0.97</td>
<td>0.81</td>
<td>0.14</td>
</tr>
<tr>
<td>A-B⁻</td>
<td>0.70</td>
<td>0.96</td>
<td>0.01</td>
</tr>
<tr>
<td>A-B⁻</td>
<td>0.73</td>
<td>0.85</td>
<td>0.12</td>
</tr>
<tr>
<td>A-B⁻</td>
<td>0.56</td>
<td>0.27</td>
<td>0.01</td>
</tr>
<tr>
<td>A-B⁻</td>
<td>0.58</td>
<td>0.19</td>
<td>0.16</td>
</tr>
<tr>
<td>A-B⁻</td>
<td>0.84</td>
<td>0.25</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Table 2.4 T-test analysis of the effect of CD25⁺ cell predepletion on alloreactive T cell activation

**Scale-up of mixed lymphocytes reaction**

The final goal of this study is to develop methodology and technology for clinical scale depletion of the *in vitro* generated alloreactive T cells. Therefore, the optimum
MLR condition will be adapted to process clinical apheresis samples. For normal donors, it is expected to collect $2 \times 10^8$ to $6 \times 10^8$ mononuclear cells per kg donor body (based on the data from the apheresis lab of James Cancer Hospital in the Ohio State University); consequently, $1.2 \times 10^{10}$ to $3.6 \times 10^{10}$ mononuclear cells from a donor with 60 kg’s weight. With the current culture condition for MLR ($3 \times 10^6$ cells/well), scaling-up of the MLR to accommodate this many cells would probably require several liters of volume.

One preliminary experiment was performed to compare the alloreactive T cell activation following the MLR in 12-well tissue culture plates (2ml) with cell concentration of $1 \times 10^6$, $3 \times 10^6$ and $5 \times 10^6$ cells/ml with MLR in T25 (5ml of culture volume) and T75 (15ml of culture volume) tissue culture flasks with cell concentration of $3 \times 10^6$ cells/ml (Figure 2.12). In addition, a MLR in 250ml (100ml culture volume) spinner flask with cell concentration of $3 \times 10^6$ cells/ml was also conducted (Figure 2.13). The difference existing in cell activation during scaling-up the MLR from 12-well tissue culture plate (2ml) to T75 flask (15ml) and 250ml spinner flask (100ml); it can be speculated that the activation of alloreactive T cells is sensitive to the culture condition. It is necessary to establish a robust and optimized culture system for clinical-scale alloreactive T cell activation.
Wave bioreactor® is a new, disposable cell culture system. As shown in Figure 2.14, it consists of a rocker platform, a single-use cell culture bag and an aeration system
(Singh et al, 1999). The rocker platform induces the wave motion to the liquid inside the cell bag to improve bulk mixing and off-bottom suspension of cells. The rocking motion also generates waves at the liquid-gas interface, which enhance gas transfer dramatically. The sterilized, disposable cell bag has the advantages including ease of use, elimination of cleaning and sterilization and reducing risk of cross-contamination (Pierce et al, 2004). Temperature control can be achieved by placing the entire unit inside a cell culture incubator or by heating the underside of the culture chamber. The wave motion promotes excellent bulk liquid movement and minimizes any temperature gradients.

Figure 2.14 Wave bioreactor®
Compared with other cell culture devices such as spinner flasks and roller bottles, the wave motion of liquid in the Wave bioreactor® chamber generates a larger mass transfer surface than static culture which results in a much greater volumetric transfer coefficient ($k_{La}$). Table 2.5 shows the comparison of volumetric transfer coefficient in Wave bioreactor® and spinner flask (Singh et al, 1999).

<table>
<thead>
<tr>
<th>Liquid(ml)</th>
<th>$k_{La}$ in Wave Bioreactor</th>
<th>$k_{La}$ in spinner</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1.61 hr$^{-1}$ (in 2L bag)</td>
<td>1.94 hr$^{-1}$</td>
</tr>
<tr>
<td>500</td>
<td>3.5 hr$^{-1}$ (in 2L bag)</td>
<td>1.15 hr$^{-1}$</td>
</tr>
<tr>
<td>1000</td>
<td>3 hr$^{-1}$ (in 2L bag)</td>
<td>0.9 hr$^{-1}$</td>
</tr>
<tr>
<td>10000</td>
<td>4 hr$^{-1}$ (in 20L bag)</td>
<td>0.4 hr$^{-1}$ (estimated)</td>
</tr>
<tr>
<td>100000</td>
<td>4 hr$^{-1}$ (in 200L bag)</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.5 Volumetric transfer coefficient of Wave bioreactor® and spinner flask

The mixing in Wave bioreactor® is achieved by wave agitation induced by a rocking motion. The agitation system provides good nutrient distribution, off-bottom suspension, and excellent oxygen transfer without damaging shear fluid or gas bubbles (Singh et al, 1999). As shown in Figure 2.14, the mixing process in Wave bioreactor® is very fast, the fully mixing in 20L Cellbag could be achieved in 6.5 seconds (Singh et al, 2004). Compared with traditional stir-tank bioreactor, the gentle and efficient mixing way of Wave bioreactor® is very important to alloreactive T cell activation process because cell-cell attachment is required during the activation process.
Wave bioreactor® has been used in T cell expansion, with a cell density up to 88 \times 10^6 \text{ cells/ml} and a high cell viability (Wave Biotech). Xcyte Therapies® used disposable 20L Cellbag™ (as shown in Figure 2.16) with a working volume of 10L for the GMP manufacture of autologous activated T cells for clinical trials, obtaining a density of 17.9 \times 10^6 \text{ cells/ml}, purity of 98%, viability of 92.3% and high expression of CD25 (Hami et al, 2003).
As stated above, wave bioreactor® could be applied in the scaling-up of mixed lymphocytes reaction for the generation of alloreactive T cells \textit{in vitro} based on the properties of the process. Compared with traditional stirred tank bioreactor, the wave bioreactor is much lower cost and less labor required. Cross-contamination could be avoided by using the disposable plastic sterile cell bag. Unlike traditional stirred tank bioreactor, there is no stir bar inside the wave bioreactor so that it provides a more gentle fluid mixing way and more uniformed distribution, which is necessary during the alloreactive T cell activation since it requires the contact between T cells and antigen presenting cells (APCs) which expressing alloantigens. Moreover, the wave bioreactor is supplied with perfusion controllers, which makes supplying fresh medium and removing old medium much easier.
2.4 Discussion

GvHD is still a major limitation to the success of allogeneic stem cell transplantation. The incidence of GvHD can be prevented by T-cell depletion, while the extreme depletion of T cells leads to delayed immune reconstitution, increased rates of viral infections and relapse. Selective depletion of alloreactive T cells is an alternative way to reduce the incidence of GvHD while preserving the cells with anti-virus reactivity (Cavazzana-Calvo et al, 1990; Mavroudis DA et al, 1996). The incidence of GvHD development is related to the degree of alloreactive T cell depletion from donors (André-Schmutz et al, 2002). Therefore, it is important to generate the maximum number of potential alloreactive T cells and identify these alloreactive T cells. CD25 and CD69 are the commonest activation antigens used to target alloreactive T cells. In this study, the activation of T cells expressing CD25, CD69, CD71, OX40 and HLADR activation antigens were studied and different strategies were applied to optimize mixed lymphocytes reaction for the generation of these allo-activated T cell in vitro.

Mixed lymphocytes reaction (MLR) is a common method to detect the donor allo-antigen response towards recipient prior to transplantations. In this study, the allo-antigen reactive (alloreactive) T cells were generated in vitro by MLR. It is shown that there are more T-cells expressing CD25, CD69, CD71, OX40 and HLADR with allo-stimulation. Therefore, CD25, CD69, CD71, OX40 and HLADR can be used as surface antigens to identify potentially alloreactive T cells. The
activation kinetics of different alloreactive T cell subsets expressing different activation antigens were studied to determine that optimum time for alloreactive T cell stimulation. Peak level of CD25, CD69, CD71 and HLADR expression occurred between three and four days of MLR. The co-expression of CD25, CD69, CD71, OX40 and HLADR on alloreactive T cells was not studied due to the unavailability of multiple-flow cytometric analysis. Therefore, the accurate optimum-culture-time for alloreactive T cell activation could not be determined by comparing alloreactive T cells expressing CD25, CD69, CD71, OX40 and HLADR following different culture time. Three and four days of MLR were reported as the optimum culture time for alloreactive T cell activation (Fehse et al, 2000, Koh et al, 1999, Barrett et al, 2002).

In this study, 4-day MLR was determined as the culture time for later study because more CD25+ (the commonest surface antigen to define alloreactive T cells) cells were generated after 4-day MLR. For clinical application, the chance of opportunistic infections and contamination can be reduced by performing MLR for three days instead of four days. Therefore, the co-expression of potential activation antigens on alloreactive T cells following 3 or 4-day MLR should be studied in future to determine the optimum MLR culture time for clinical application.

The number of MHC II molecules expressed on antigen presenting cells can be increased by the treatment with IFN-γ so that the immune response induced by these APCs can be amplified. Of all the activation antigen studied (CD25, CD69, CD71, OX40 and HLADR), the activated cell fraction expressing HLADR can be increased significantly by the 2000 U/ml IFN-γ pretreatment. Moreover, it was concluded the
number of CD69 expressing on alloreactive T cells can be increased by the pretreatment of stimulator cells in MLR with 500 U/ml IFN-\(\gamma\). As discussed in Chapter 5, there is a fraction of alloreactive T cells express low level of activation antigens. The big challenge for high degree alloreactive T cell depletion is to deplete these cells with low level antigen expression. Since more antibodies for CD69 (e.g. anti-CD69 fluorochrome or magnetic beads conjugates) can be labeled on CD69 expressing alloreactive T cells with the up-regulation of CD69 on these cells, the pretreatment strategy should be applied to optimize the depletion of CD69\(^+\) alloreactive T cells by magnetic activated cell sorting in the future.

IL-2, the T cell growth factor, is used commonly to increase the alloreactive T cell activation from MLR (Guimond et al, 2002; Fehse et al, 2000; Koh et al, 1999). IL-2 and IL-15 were studied for their effect on increasing alloreactive T cells activation, however, it is suggested from the data that the specific activation of alloreactive T cells expressing CD69, CD71, OX40 and HLADR can not be increased significantly. IL-2 treatment only improves that specific activation of CD25\(^+\) alloreactive T cells. However, the treatment of IL-2 and IL-15 for MLR induces high level expressing of activation antigens on non-allo-activated T cells. The non-specific activation of alloreactive T cells induced by IL-2 was also addressed by Barrett et al (Barrett et al, 2002). One of the major objectives of this study is to improve the detection of alloreactive T cells by multiple activation antigens (The expression of multiple activation antigens on alloreactive T cells will discussed in Chapter 3 in
detail), the non specific expression of activation antigens will confuse the study. Therefore, IL-2 and IL-15 treatment will not be applied in future study.

Different strategies were applied to improve the activation of alloreactive T cells in vitro. Brenner et al have demonstrated that the generation of CD3⁺CD25⁺ alloreactive T cells can be increased (about 15%) by stimulating the mixed lymphocytes reaction with HLA-mismatched host Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) (Brenner et al, 2003). It has been reported that CD25 up-regulation on responder cells were increased (22-34%) by using OKT-3 expanded CD3⁺ lymphocytes as simulators (Barrett et al, 2002). Compared with these strategies, the cytokine treating strategy applied in this study is not sufficient in increasing alloreactive T cell activation. It was found in our study and was also reported by Barrett that IL-2 can induce nonspecific activation of alloreactive T cells (Barrett et al, 2002). It is indicated from these strategies that optimized stimulator-cell-source is important to increase the alloreactive T cell activation. Therefore, dendritic cells, the most potent antigen presenting cells, were determined to be used as stimulator cells for alloreactive T cell activation. The effect of using dendritic cells to stimulate alloreactive T cells will be discussed in Chapter 4.

Moreover, only single activation antigen, mainly CD25, was used to target and deplete alloreactive T cells in most of current approaches. In this study, it is indicated that some alloreactive T cells expressing CD69, CD71, OX40 and HLADR. Therefore, the depletion of CD25⁺ cells following MLR may not be sufficient to achieve high degree of alloreactive T cell depletion. The effect of applying multiple activation
antigens on increasing the detection and depletion of alloreactive T cells was studied and discussed in Chapter 3.
CHAPTER 3

SELECTION OF TARGET ACTIVATION ANTIGENS FOR ALLOREACTIVITY DEPLETION

3.1 Motivation

The development of GvHD is the major limitation to allogeneic stem cell transplantation, which is the only curative treatment for many patients with hematological malignancies. Pre-depletion of T cells from the donor before the transplantation can abrogate GvHD. However, there are some severe disadvantages associated with T cell depletion such as delayed immune reconstitution, increased incidence of leukemia relapse and viral infections, and loss of donor versus leukemia activity (GVL). An alternative strategy to reduce the incidence of GvHD while persevering anti-third party reactivity is selective depletion of alloreactive T cells mediating GvHD before the transplantation. The commonly used methods for alloreactive T cell depletion include fluorescence-activated cell sorting, magnetic-activate cell sorting and immunotoxin treatment, which are based on differentiating alloreactive T cells from other cells using special cell surface antigens. CD25 and CD69 are the most commonly used activation antigens for alloreactive T cell depletion. It has been reported that higher alloreactive T cell depletion efficiency
can be achieved by depleting both CD25+ and CD69+ cells; however, there is still relatively high proliferative reactivity left after the depletion of CD25+ cells, CD69+ cells or both (Koh et al, 1999). Besides CD25 and CD69, other activation antigens such as CD71, OX40 and HLADR have been proposed as potential targets for alloreactive T cell depletion (Fehse et al, 2000). It has been reported that CD4hiCD38+ cells, were depleted using fluorescence activated cell sorting (FACS), and ≥1-2 log₁₀ depletion of alloreactivity was achieved by flow cytometry analysis (Martins et al, 2004).

In this study, the expression of potential activation antigens including CD25, CD69, CD71, CD40L, OX40, HLADR, CD38, CD58, CD152, CD122 and CD103 on alloreactive T cells were studied as potential candidates for depletion of alloreactive T cells with the goal of achieving a selective depletion of alloreactivity greater than 3 log₁₀. The activation antigens upregulated during alloreactive T cells activation were selected; the priority sequence of these activation antigens on representing alloreactive T cells was determined; and the immune functions of each cell subsets expressing different activation antigens were studied. The optimum combination of activation antigens for improving alloreactivity depletion was determined. Finally, the anti-third party reactivity of residual cells after the selective depletion was also measured.
3.2 Material and Methods

Cell Preparation

Human peripheral leukocytes were ordered from the American Red Cross, Central Ohio Region. The peripheral blood mononuclear cell (PBMC) fraction was isolated and separated by Ficoll-Hypaque density gradient centrifugation. The cells were washed with PBS twice and resuspended in AIM-V supplemented with streptomycin sulfate (50 µg/ml), gentamicin sulfate (10 µg/ml), and L-glutamine (2 mM). The number of peripheral blood mononuclear cells was determined using a hemacytometer.

One-way mixed lymphocytes reaction

A one-way mixed lymphocytes reaction (MLR), involving two unrelated HLA non-identical donors (A and B), was used in this study. The responder PBMC (A) were incubated at 37°C with the same number of irradiated (30 Gy) stimulator PBMC (B*). All cultures were performed in 12-well tissue culture plate (BD) at a concentration of 3x10^6 cells/ml with a final volume of 2 ml. For control stimulations, the responder PBMC (A) were incubated with the same number of irradiated (30 Gy) autologous PBMC (A*) at a concentration of 3x10^6 cells/ml with a final volume of 2 ml.

Cell labeling for flow cytometry

Cells were harvested from tissue culture plates or flasks, washed twice with PBS, and counted. 0.5x10^6 or 1x10^6 cells were transferred to the 75x12 mm polypropylene
tubes. Appropriate antibodies for the specific experiment were added to the cell suspension as recommended by the manufacturer. The total incubation volume was brought up to 100 µl. Then the sample tubes with antibodies were incubated in the dark at 4 °C for 30 minutes. After labeling, the cells were washed twice. Basically, 2 ml of labeling buffer (PBS+0.5% BSA+2 mM EDTA) was added into each sample tube and the sample tubes were centrifuged at 300 g, 4 °C for 8 minutes. After centrifugation, the supernatant was discarded and another wash step was repeated. If the samples were not to be analyzed the same day, 1 ml fixing solution (2% paraformaldehyde solution) was added for 1x10^6 cells. The samples could be stored at 4 °C in the dark for up to one week. FACSAria flow cytometer and FACSDiva software were used to collect 100,000 events for each sample.

**Alloreactive T cell depletion**

Cells were harvest after four days’ mixed lymphocytes reaction and washed once with PBS. Four cell aliquots with 2x10^7 cells/ml were prepared. The first cell aliquot was labeled with 50 µl anti CD25-PE; the second cell aliquot was labeled with 50 µl anti CD25-PE and 50 µl anti CD69-PE; the third cell aliquot was labeled with 50 µl anti CD25-PE, 50 µl anti CD69-PE and 50 µl anti HLADR-PE; the fourth cell aliquot was labeled with 50 µl anti CD25-PE, 50 µl anti CD69-PE, 50 µl anti HLADR-PE and 50 µl anti CD38-PE at 4 °C for 30 minutes in the dark. Then the cell samples were washed with labeling buffer twice and labeled with 100 µl anti-PE microbeads at 4 °C for 30 minutes in the dark. After labeling, the cells were washed with labeling buffer
twice and resuspended in 4 ml labeling buffer. The cell suspension were passed through MACS LD column surrounded by SuperMACS (Miltenyi Biotech), the nonmagnetic cell fraction was collected as used for setting up limiting dilution assay.

**Statistical study**

The T test analysis was performed to determine the statistical significance of presented results. JMP software was used to perform all the statistical analysis.

**QMS separation**

For QMS separations, the magnetically labeled cell samples were suspended in labeling buffer at 3x10⁶ cells/ml. The QMS was operated with only inlet a’ and outlet a, the flow rates for both inlet and outlet were 1.5 ml/min.

**Limiting dilution assay**

Cells (A) were cultured in a 96-well U bottom tissue culture plate at a final volume of 100 µl and stimulated with 10⁵ irradiated (30 Gy) feeder cells(B*). In the control culture, the cells were stimulated with 10⁵ autologous irradiated feeder cells (A*). The concentrations of responder cells ranged from 10000 to 100 cells/well for non-depleted cell sample including four dilutions of 10000, 1000, 100, 10 cells/well. 24 replicate wells were set up for each dilution. The culture was incubated at 37 °C with 5% CO₂, and 50 µl culture medium was add to each well on the 6th and 12th day for feeding. On the 14th day, the culture was tested for proliferating cell frequency by BrdU incorporation assay. 14-day culture for limiting dilution assay was determined
in order to stimulate maximum potential proliferative cells (LDA protocol from Bone Marrow Transplantation Lab of The Ohio State University James Cancer Hospital), however, the linearity of cell growth on the 14th culture was not tested. The proliferating cell frequency corresponds to the T cell frequency.

The cell frequency was calculated by Taswell method (Chapter 6.1). Briefly, the mean and standard deviation of BrdU incorporation by the 24 control cultures at each cell concentration were calculated. The mean+3SD value of these control cultures were used as baselines, if the BrdU incorporation of one test well was greater than the baseline, the culture well was consider as positive proliferating response. Then the fraction of wells with negative proliferation response at each cell concentration can be obtained. Poisson description was applied in describing the relation between the average number of cells tested per replicate culture and the number of negatively responding cultures per group. Plot the linear relationship between negative well faction and ln (average cell number/well), and the slope is proliferating cell frequency.

**BrdU proliferation assay**

On the 13th day of limiting dilution assay, the cells were labeled with BrdU at a concentration of 10 mM and incubated at 37 °C with 5% CO₂ for 18-24 hours. On the 14th day, the cells were centrifuge at 300 g for 10 minutes and the labeling medium was removed. The cells were dried using a hair-dryer for approximately 15 minutes. 200 µl/well FixDenat solution was next added to the cells and the suspension was
incubated for 30 minutes at 37°C. After incubation, the FixDenat solution was removed, 100 µl/well anti-BrdU-POD solution was added and the suspension was incubated for 90 minutes at 37°C with 5% CO₂. Then the extra anti-BrdU-POD solution was removed and each well was rinsed with 200 µl-300 µl washing solution for three times. After washing, 100 µl/well substrate solution was added to each well. Finally, the absorbance at 370 nm was measured when the color was developed. The reported linear range of this assay is comparable to that of the ³H incorporation assay, which is form 100 cells/well to 10,000 cells/well (Roche Applied Science). However, our experiment data indicates the linear range of this assay is from 50 cells/well to 1,000 cells/well. Therefore, it is insufficient to determine the proliferative capability of cells from the absorbance which indicating BrdU incorporation by cells. It was decided to combine BrdU incorporation assay and limiting dilution assay to determine the frequency of proliferating cells by Taswell method (Chapter 6.1). It is more accurate to compare proliferating cell frequency than compare the BrdU incorporation.

3.3 Results

Selection of activation antigens

The expression of activation antigen, CD25, CD69, CD71, CD40L, CD95, OX40, HLADR, CD38, CD58, CD152, CD122 and CD103, on alloreactive T cells was studied. It is shown in Figure 3.1 that an example of T-cell fractions expressing these activation antigens after 4 days’ MLR; and Figure 3.2 shows an example of T-cell
fractions expressing these antigens after 4 days’ autologous control stimulation. The comparison of Figure 3.1 and Figure 3.2, and Table 3.1 indicates that CD25, CD69, CD71, OX40, HLADR and CD38 are upregulated dramatically with the activation of alloreactive T cells. In Table 3.1, it shows the statistical analysis (N≥3) of the specific activation of alloreactive T cells expressing these activation antigens (specific activation=fraction of T cells expression activation antigens after 4-day MLR - fraction of T cells expressing activations after 4-day autologous control stimulation).

In Table 3.1, “*” indicates the up-regulation of the activation antigen on alloreactive T cell subset is significant by T test analysis (p<0.05). Therefore, CD25, CD69, CD71, OX40, HLADR and CD38 are selected to define alloreactive T cells in this study.
Figure 3.1 Fraction of T cells expressing activation antigens after 4 days’ MLR
Figure 3.2 Fraction of T cells expressing antigens after 4 days’ autologous stimulation
<table>
<thead>
<tr>
<th>Percent of activated T cells</th>
<th>Mean (%)</th>
<th>SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁻CD25⁺ (*)&amp;</td>
<td>9.37</td>
<td>5.06</td>
</tr>
<tr>
<td>CD3⁻CD69⁺ (*)&amp;</td>
<td>5.49</td>
<td>2.64</td>
</tr>
<tr>
<td>CD3⁻CD71⁺ (*)&amp;</td>
<td>9.01</td>
<td>4.61</td>
</tr>
<tr>
<td>CD3⁻CD38⁺ (*)&amp;</td>
<td>4.15</td>
<td>0.97</td>
</tr>
<tr>
<td>CD3⁻OX40⁺ (*)&amp;</td>
<td>4.62</td>
<td>1.58</td>
</tr>
<tr>
<td>CD3⁻HLADR⁺ (*)&amp;</td>
<td>4.74</td>
<td>1.73</td>
</tr>
<tr>
<td>CD3⁻CD58⁻</td>
<td>1.54</td>
<td>1.30</td>
</tr>
<tr>
<td>CD3⁻CD103⁺</td>
<td>0.39</td>
<td>0.26</td>
</tr>
<tr>
<td>CD3⁻CD152⁺</td>
<td>1.15</td>
<td>0.94</td>
</tr>
<tr>
<td>CD3⁻CD122⁺</td>
<td>2.15</td>
<td>1.26</td>
</tr>
<tr>
<td>CD3⁻CD40L⁺</td>
<td>1.62</td>
<td>1.16</td>
</tr>
</tbody>
</table>

Table 3.1 Specific activation of alloreactive T cells expressing different activation antigens

**Co-expression of activation antigens**

In order to select activation antigen combinations for alloreactive T cells depletion, the co-expression between other activation antigens and CD25 and CD69 was studied. Figure 3.3 shows the co-expression of CD71, OX40 and HLADR with CD25 or CD69 analyzed by four-color flow cytometry assay (FACS Calibur). Figure 3.3 demonstrates that there are T cell populations expressing activation antigens including CD71, OX40 and HLADR, which are CD3⁻CD25⁻CD69⁻ (Note, to be an event in these figures, the cell was first selected in the Forward vs Side scatter plot and it had to be positive for CD3). Therefore, targeting only CD25 and CD69 antigens is not likely to deplete all of the activated T cells expressing those activation antigens. The co-expression between those activation antigens may be why there is still
alloreactivity left after depleting both CD25⁺ and CD69⁺ cells from a donor. In order to define optimum cell subsets for alloreactivity depletion, the co-expression between CD71, OX40, CD38, HLADR, CD25 and CD69 were studied using seven-color flow cytometry assay (FACSARia). Figure 3.4 shows the sequential gating of cells expressing activated antigens including CD71, OX40, CD38, HLADR, CD25 and CD69. In Figure 3.4, dot plot “a” shows CD25 expression on lymphocytes; dot plot “b” shows CD69 expression on the CD3⁺CD25⁻ cells; dot plot “c” shows HLADR expression on the CD3⁺CD25⁺CD69⁻ cells; dot plot “d” shows CD38 expression on the CD3⁺CD25⁺CD69⁻HLADR⁻ cells; dot plot “e” shows OX40 expression on the CD3⁺CD25⁺CD69⁻HLADR⁻CD38⁻ cells; and dot plot “f” shows CD71 expression on the CD3⁺CD25⁺CD69⁻HLADR⁻CD38⁻OX40⁻ cells. From Figure 3.4, it is clear that there are distinct cell populations existing which express different activation antigens including CD25, CD69, CD38, CD71, OX40 and HLADR. By analyzing the flow cytometric dot plots with different gate strategies, the cell fractions expressing different activation antigen combinations were determined as shown in Table 3.1. For example, 5.97% of the alloreactive T cells express CD25, CD69, CD71, OX40, HLADR and CD38; and approximately 36% of the alloreactive T cells expressing only a single activation antigen. It can be calculated from Table 3.2 that the fraction of alloreactive T cells expressing two or more activation antigens and the fractions of alloreactive T cells expressing either CD25, or CD69, or CD71, or OX40, or HLADR, or CD38 uniquely. As show in Figure 3.5, 63.43% of the alloreactive T cells express two or more activation antigens, 8.25% of the alloreactive T cells express only CD25,
19.92% of the alloreactive T cells express only CD69, 4.98% of the alloreactive T cells express only HLADR, 1.98% of the alloreactive T cells express only CD38, 1.10% of the alloreactive T cells express only OX40, and 0.35% of the alloreactive T cells express only CD71. The cells express single activation antigen can only be depleted by targeting their unique activation antigens.

Figure 3.3 Co-expression of CD71, OX40 and HLADR with CD25 and CD69 on CD3 positive cells following mixed lymphocytes reaction
Figure 3.4 Co-expression of CD71, OX40, HLADR, CD38, CD25 and CD69 by sequential gating
<table>
<thead>
<tr>
<th>Antigen expressed by cell subsets</th>
<th>Cell fraction</th>
<th>Mean (N=6)</th>
<th>SD (N=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25/CD69/CD71/OX40/HLADR/CD38</td>
<td>5.97%</td>
<td>2.41%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD69/CD71/OX40/CD38</td>
<td>0.95%</td>
<td>0.43%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD69/CD71/HLADR/CD38</td>
<td>4.37%</td>
<td>2.05%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD71/OX40/HLADR/CD38</td>
<td>0.44%</td>
<td>0.30%</td>
<td></td>
</tr>
<tr>
<td>CD69/CD71/OX40/HLADR/CD38</td>
<td>0.13%</td>
<td>0.12%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD69/OX40/HLADR/CD38</td>
<td>3.79%</td>
<td>2.74%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD69/CD71/CD38</td>
<td>2.74%</td>
<td>1.59%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD69/OX40/CD38</td>
<td>1.25%</td>
<td>0.91%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD69/HLADR/CD38</td>
<td>4.90%</td>
<td>3.37%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD71/OX40/CD38</td>
<td>0.16%</td>
<td>0.13%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD71/HLADR/CD38</td>
<td>0.79%</td>
<td>0.49%</td>
<td></td>
</tr>
<tr>
<td>CD25/OX40/HLADR/CD38</td>
<td>0.85%</td>
<td>0.38%</td>
<td></td>
</tr>
<tr>
<td>CD69/CD71/OX40/CD38</td>
<td>0.00%</td>
<td>0.00%</td>
<td></td>
</tr>
<tr>
<td>CD69/CD71/HLADR/CD38</td>
<td>0.21%</td>
<td>0.23%</td>
<td></td>
</tr>
<tr>
<td>CD69/OX40/HLADR/CD38</td>
<td>0.12%</td>
<td>0.12%</td>
<td></td>
</tr>
<tr>
<td>CD71/OX40/HLADR/CD38</td>
<td>0.13%</td>
<td>0.15%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD69/CD38</td>
<td>5.68%</td>
<td>4.60%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD71/CD38</td>
<td>0.91%</td>
<td>0.72%</td>
<td></td>
</tr>
<tr>
<td>CD25/OX40/CD38</td>
<td>0.87%</td>
<td>0.39%</td>
<td></td>
</tr>
<tr>
<td>CD25/HLADR/CD38</td>
<td>0.82%</td>
<td>0.70%</td>
<td></td>
</tr>
<tr>
<td>CD69/CD71/CD38</td>
<td>0.03%</td>
<td>0.03%</td>
<td></td>
</tr>
<tr>
<td>CD69/OX40/CD38</td>
<td>0.06%</td>
<td>0.07%</td>
<td></td>
</tr>
<tr>
<td>CD69/HLADR/CD38</td>
<td>0.54%</td>
<td>0.41%</td>
<td></td>
</tr>
<tr>
<td>CD71/OX40/CD38</td>
<td>0.00%</td>
<td>0.01%</td>
<td></td>
</tr>
<tr>
<td>CD71/HLADR/CD38</td>
<td>0.10%</td>
<td>0.06%</td>
<td></td>
</tr>
<tr>
<td>OX40/HLADR/CD38</td>
<td>0.21%</td>
<td>0.31%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD38</td>
<td>2.49%</td>
<td>2.37%</td>
<td></td>
</tr>
<tr>
<td>CD69/CD38</td>
<td>0.91%</td>
<td>0.67%</td>
<td></td>
</tr>
<tr>
<td>CD71/CD38</td>
<td>0.03%</td>
<td>0.03%</td>
<td></td>
</tr>
<tr>
<td>OX40/CD38</td>
<td>0.64%</td>
<td>0.84%</td>
<td></td>
</tr>
<tr>
<td>HLADR/CD38</td>
<td>0.25%</td>
<td>0.11%</td>
<td></td>
</tr>
<tr>
<td>CD38</td>
<td>1.98%</td>
<td>1.02%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD69/CD71/OX40/HLADR</td>
<td>1.89%</td>
<td>0.68%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD69/CD71/HLADR</td>
<td>1.81%</td>
<td>1.70%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD69/OX40/HLADR</td>
<td>1.23%</td>
<td>0.80%</td>
<td></td>
</tr>
<tr>
<td>CD25/CD71/OX40/HLADR</td>
<td>0.32%</td>
<td>0.12%</td>
<td></td>
</tr>
<tr>
<td>CD69/CD71/OX40/HLADR</td>
<td>0.23%</td>
<td>0.14%</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 Distribution of activation antigens on alloreactive T cells (Continued)
In Table 3.2, the alloreactive T cells were divided into different subsets by the expressed activation antigens. The fraction of alloreactive T cells represented by each activation antigen can be calculated from data shown in Table 3.2, for example, the cell fraction represented by CD25 is the sum of all CD25 expressed cell factions (e.g. the cell subset expressing only CD25, the cell subset expressing CD25 and CD69, the cell subset expressing CD25, CD69 and HLADR, and the cell subset expressing all

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25/CD69/HLADR</td>
<td>2.46%</td>
<td>0.36%</td>
</tr>
<tr>
<td>CD25/CD71/HLADR</td>
<td>0.50%</td>
<td>0.52%</td>
</tr>
<tr>
<td>CD25/OX40/HLADR</td>
<td>0.26%</td>
<td>0.16%</td>
</tr>
<tr>
<td>CD69/CD71/HLADR</td>
<td>0.34%</td>
<td>0.21%</td>
</tr>
<tr>
<td>CD69/OX40/HLADR</td>
<td>0.19%</td>
<td>0.13%</td>
</tr>
<tr>
<td>CD71/OX40/HLADR</td>
<td>0.36%</td>
<td>0.35%</td>
</tr>
<tr>
<td>CD25/HLADR</td>
<td>1.39%</td>
<td>0.50%</td>
</tr>
<tr>
<td>CD69/HLADR</td>
<td>3.03%</td>
<td>1.32%</td>
</tr>
<tr>
<td>CD71/HLADR</td>
<td>0.43%</td>
<td>0.50%</td>
</tr>
<tr>
<td>OX40/HLADR</td>
<td>0.29%</td>
<td>0.28%</td>
</tr>
<tr>
<td>HLADR</td>
<td>4.98%</td>
<td>2.28%</td>
</tr>
<tr>
<td>CD25/CD69/CD71/OX40</td>
<td>0.30%</td>
<td>0.23%</td>
</tr>
<tr>
<td>CD25/CD69/OX40</td>
<td>0.54%</td>
<td>0.25%</td>
</tr>
<tr>
<td>CD25/CD71/OX40</td>
<td>0.04%</td>
<td>0.04%</td>
</tr>
<tr>
<td>CD69/CD71/OX40</td>
<td>0.01%</td>
<td>0.01%</td>
</tr>
<tr>
<td>CD25/OX40</td>
<td>0.30%</td>
<td>0.20%</td>
</tr>
<tr>
<td>CD69/OX40</td>
<td>0.23%</td>
<td>0.13%</td>
</tr>
<tr>
<td>CD71/OX40</td>
<td>0.01%</td>
<td>0.01%</td>
</tr>
<tr>
<td>OX40</td>
<td>1.10%</td>
<td>0.94%</td>
</tr>
<tr>
<td>CD25/CD69/CD71</td>
<td>1.36%</td>
<td>1.59%</td>
</tr>
<tr>
<td>CD25/CD71</td>
<td>0.47%</td>
<td>0.72%</td>
</tr>
<tr>
<td>CD69/CD71</td>
<td>0.11%</td>
<td>0.12%</td>
</tr>
<tr>
<td>CD71</td>
<td>0.35%</td>
<td>0.51%</td>
</tr>
<tr>
<td>CD25/CD69</td>
<td>5.24%</td>
<td>1.87%</td>
</tr>
<tr>
<td>CD69</td>
<td>19.92%</td>
<td>7.98%</td>
</tr>
<tr>
<td>CD25</td>
<td>8.25%</td>
<td>3.82%</td>
</tr>
</tbody>
</table>
other activation antigen combinations with CD25). Table 3.3 shows the fraction of alloreactive T cells represented by each activation antigen calculated from data shown in Table 3.2, and T test analysis was done to compare if the alloreactive T cell faction represented by each activation antigen is significantly different, then these activation antigens were divided into different groups by T test analysis (p<0.05). For example, as shown in Table 3.3, the alloreactive T cell fraction represented by CD69 is significantly higher than that represented by HLADR.

The priority sequence of these activation antigens was determined based on the fractions of alloreactive T cells represented by each activation antigen and different antigen combinations. For the selection of the activation antigen with the first priority, the alloreactive T cell faction represented by each activation antigen was compared (Table 3.3); 70.36% of alloreactive T cells are represented by CD69, which is higher than the fraction of alloreactive T cells represented by any other single activation antigen; However, since there is no significant difference in representing alloreactive T cells between CD25 and CD69, CD25 was selected as the first activation antigen to be targeted in this study due to its common usage. The second antigen to be targeted is CD69; 89.17% alloreactive T cells can be selected by targeting both CD25 and CD69, which is higher than the percentage of alloreactive T cells selected by targeting any other two activation antigens. The third antigen to be targeted is HLADR; 95.90% alloreactive T cells can be selected by targeting CD25, CD69 and HLADR, which is higher than the percentage of alloreactive T cells selected by targeting any other combinations of three activation antigens. Similarly, the fourth and fifth activation
antigens to be targeted in sequence were determined to be CD38 and OX40. As shown in Table 3.4, 98.55% of the alloreactive T cells can be targeted by CD25, CD69, HLADR and CD38, while 99.61% of the alloreactive T cells can be selected by targeting five antigens CD25, CD69, HLADR, CD38 and HLADR simultaneously.

Some problems will arise with targeting more activation antigens for alloreactive T cell activation; such as economic issue (antibody is expensive especially for large scale application) and nonspecific loss of other cells (HLADR is constitutively expressed on macrophage and dendritic cells). Therefore, it is important to determine if the fraction of alloreactive T cells targeted by more activation antigens is significantly increased. The T test analysis was done to compare the fractions of alloreactive T cell selected by single activation antigen, or two, or three, or four, or five activation antigens (shown in Table 3.5). It in indicated from Table 3.5 that the fraction of alloreactive T cells represented by two-antigen combination (CD25 and CD69) is significantly higher that that represented by single antigen (CD25); and the fraction of alloreactive T cells represented by three-antigen combination (CD25, CD69 and HLADR) is significantly higher that that represented by two-antigen combination (CD25 and CD69); however, there is no significant difference in representing alloreactive T cells among three-antigen combination (CD69, CD25 and HLADR), four-antigen combination (CD69, CD25, HLADR and CD38), and five-antigen (CD69, CD25, HLADR and CD38 and OX40).

As stated above, flow cytometric analysis indicates that the alloreactive T cells selective by four-antigen combination in not significant higher than that selected by
three-antigen combination. However, it is important to compare the residual alloreactivity after alloreactive T cell depletion targeted by different activation antigen combinations. To test the difference, alloreactive T cells were selectively depleted by targeting the antigen combination 1 to 4 shown in Table 3.4, and the residual alloreactivity and third party reactivity were determined (Discussed in section “Determine the functional significance of activation antigen expressing cells”).

![Pie chart showing distribution of alloreactive T cell subsets by antigen expression]

Figure 3.5 Distribution of alloreactive T cell subsets by antigen expression
Activation antigens | Alloreactive T cell fraction | Statistically different group
--- | --- | ---
CD69 | 70.36% | 1
CD25 | 63.18% | 1
HLADR | 47.56% | 2
CD38 | 42.20% | 2
CD71 | 25.43% | 3
OX40 | 22.79% | 3

Table 3.3 Alloreactive T cell fractions represented by each activation antigen

<table>
<thead>
<tr>
<th>Priority</th>
<th>Activation antigens</th>
<th>Activation antigen combinations</th>
<th>Alloreactive T cell fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD25</td>
<td>CD25</td>
<td>63.18%</td>
</tr>
<tr>
<td>2</td>
<td>CD69</td>
<td>CD25+CD69</td>
<td>89.17%</td>
</tr>
<tr>
<td>3</td>
<td>HLADR</td>
<td>CD25+CD69+HLADR</td>
<td>95.90%</td>
</tr>
<tr>
<td>4</td>
<td>CD38</td>
<td>CD25+CD69+HLADR+CD38</td>
<td>98.55%</td>
</tr>
<tr>
<td>5</td>
<td>OX40</td>
<td>CD25+CD69+HLADR+CD38+OX40</td>
<td>99.61%</td>
</tr>
</tbody>
</table>

Table 3.4 Priority sequence of activation antigens on representing alloreactive T cells
<table>
<thead>
<tr>
<th>Activation antigens</th>
<th>Activation antigens</th>
<th>Difference</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25+CD69+HLADR</td>
<td>CD25</td>
<td>32.18%</td>
<td>2.771e-11</td>
</tr>
<tr>
<td>+CD38+OX40</td>
<td>CD25</td>
<td>30.79%</td>
<td>6.969e-11</td>
</tr>
<tr>
<td>CD25+CD69+HLADR</td>
<td>CD25</td>
<td>27.55%</td>
<td>6.691e-10</td>
</tr>
<tr>
<td>+CD38</td>
<td>CD25+CD69</td>
<td>19.58%</td>
<td>3.521e-7</td>
</tr>
<tr>
<td>CD25+CD69+HLADR</td>
<td>CD25+CD69+HLADR+CD38+OX40</td>
<td>12.61%</td>
<td>1.715e-4</td>
</tr>
<tr>
<td></td>
<td>CD25+CD69+HLADR+CD38</td>
<td>11.22%</td>
<td>6.013e-4</td>
</tr>
<tr>
<td></td>
<td>CD25+CD69</td>
<td>7.97%</td>
<td>9.950e-3</td>
</tr>
<tr>
<td></td>
<td>CD25+CD69+HLADR+CD38+OX40</td>
<td>4.63%</td>
<td>0.117</td>
</tr>
<tr>
<td></td>
<td>CD25+CD69+HLADR+CD38</td>
<td>3.24%</td>
<td>0.267</td>
</tr>
<tr>
<td></td>
<td>CD25+CD69+HLADR+CD38+OX40</td>
<td>1.39%</td>
<td>0.631</td>
</tr>
</tbody>
</table>

Table 3.5 T test analysis of antigen combinations on representing alloreactive T cells

Expression of CD4, CD8, CD45RA and CD45RO on alloreactive T cell subsets

T cells consist of functionally distinct populations, helper T cells and cytotoxic T lymphocytes. In response to antigenic stimulation, helper T cells secret cytokines and stimulate the activation and proliferation of T cells and other cells, cytotoxic T cells kill cells that produce foreign antigens directly. CD4 is expressed on helper T cells constitutively, while CD8 is expressed on cytotoxic T cells. The ratio of CD4⁺ cells versus CD8⁺ cells in normal T cell population is 2:1. It is indicated by Edinger et al. that CD4⁺ T cells mainly mediate GVHD (Edinger et al, 2003). Martin et al. reported 1-2 log₁₀ depletion of alloreactivity can be achieved by depleting CD4⁺CD38⁺ cells
Martins et al, 2004). The percentage of CD4+, CD8+, CD4+CD8+ alloreactive T cells expressing different combinations of activation antigens was obtained by nine-parameter flow cytometric analysis (six independent replicates). It is indicated from Table 3.6 that for some alloreactive T cell subsets, the ratio of CD4+ cells versus CD8+ cells is still close to 2:1; however, for some alloreactive T cell subsets such as CD25+ and CD38+ alloreactive T cells, the percentage of CD4+ cells is much higher than that of CD8+ cells; for alloreactive T cell subsets such as CD69+ alloreactive T cells, the percentage of CD8+ cells and that of CD4+ cells is similar. From the study reported by Edinger et al., CD25+ and CD38+ alloreactive T cells might be the major inducer of GvHD (Edinger et al, 2003). Therefore, it is important to determine that alloreactivity induced by cell subsets expressing different activation antigens.

CD45RA is a surface marker for naïve cell, and CD45RO is a surface marker for memory cells. It has been suggested that chronic GvHD is associated with a preponderance of effector memory cells (CD45RA-CCR7+) (Yamashita et al, 2004). Table 3.7 shows the distribution of CD45RA and CD45RO on different alloreactive T cell subsets (Summary of four independent experiments), about 60-70% of alloreactive T cells expressing different activation antigens are CD45RA-. Table 3.8 compares the fraction of CD45+CCR7+ cells in T cells expressing activation antigens (CD25, CD69, HLADR and CD38) following MLR and autologous control culture (Summary of three independent experiments), it is indicated from Table 3.8 that the fraction of CD45+CCR7+ cells expressing activation antigens (CD25, CD69, HLADR and CD38) is increased significantly (p=0.01) with the activation of
alloreactive T cells. Therefore, the depletion of CD25⁺, CD69⁺, HLADR⁺ and CD38⁺ alloreactive T cell may decrease the incidence of GvHD development after allogeneic stem cell transplantation.

<table>
<thead>
<tr>
<th>Expressed antigen</th>
<th>CD8⁺</th>
<th>CD4⁺</th>
<th>CD4⁺CD8⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25/69/DR/CD38</td>
<td>0.28</td>
<td>0.51</td>
<td>0.21</td>
</tr>
<tr>
<td>CD25/69/DR</td>
<td>0.36</td>
<td>0.44</td>
<td>0.19</td>
</tr>
<tr>
<td>CD25/69/38</td>
<td>0.38</td>
<td>0.43</td>
<td>0.18</td>
</tr>
<tr>
<td>CD25/DR/38</td>
<td>0.16</td>
<td>0.64</td>
<td>0.19</td>
</tr>
<tr>
<td>CD69/DR/38</td>
<td>0.42</td>
<td>0.40</td>
<td>0.18</td>
</tr>
<tr>
<td>CD25/69</td>
<td>0.25</td>
<td>0.65</td>
<td>0.10</td>
</tr>
<tr>
<td>CD25/DR</td>
<td>0.29</td>
<td>0.59</td>
<td>0.12</td>
</tr>
<tr>
<td>CD25/38</td>
<td>0.35</td>
<td>0.50</td>
<td>0.15</td>
</tr>
<tr>
<td>CD69/DR</td>
<td>0.49</td>
<td>0.41</td>
<td>0.10</td>
</tr>
<tr>
<td>CD69/38</td>
<td>0.42</td>
<td>0.48</td>
<td>0.10</td>
</tr>
<tr>
<td>HLADR/CD38</td>
<td>0.25</td>
<td>0.56</td>
<td>0.19</td>
</tr>
<tr>
<td>CD25</td>
<td>0.17</td>
<td>0.76</td>
<td>0.07</td>
</tr>
<tr>
<td>CD69</td>
<td>0.50</td>
<td>0.47</td>
<td>0.03</td>
</tr>
<tr>
<td>HLADR</td>
<td>0.42</td>
<td>0.51</td>
<td>0.06</td>
</tr>
<tr>
<td>CD38</td>
<td>0.19</td>
<td>0.76</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 3.6 Fractions of T cells expressing CD4 and CD8 following MLR
Expressed antigen (N=4) | CD45RA⁺ | CD45RO⁺
---|---|---
CD3 | 74.44% | 25.56%
CD25 | 35.28% | 64.72%
CD69 | 38.50% | 61.50%
HLADR | 48.51% | 51.49%
CD38 | 48.93% | 51.07%
CD25/CD69 | 33.26% | 66.74%
CD25/HLADR | 31.68% | 68.32%
CD25/CD38 | 22.79% | 77.21%
CD69/HLADR | 39.04% | 60.96%
CD69/CD38 | 36.15% | 63.85%
HLADR/CD38 | 39.23% | 60.77%
CD25/CD69/HLADR | 36.68% | 63.32%
CD25/CD69/CD38 | 29.96% | 70.04%
CD25/HLADR/CD38 | 30.95% | 69.05%
CD69/HLADR/CD38 | 46.36% | 53.64%
CD25/CD69/HLADR/CD38 | 43.32% | 56.68%

Table 3.7 Expression of CD45RA and CD45RO on alloreactive T cells

<table>
<thead>
<tr>
<th>(N=3)</th>
<th>CD3⁺CD25⁺CD69⁺CD38⁺HLADR⁺</th>
<th>CD45RA⁻CCR7⁺</th>
<th>CD45RA⁺CCR7⁺</th>
<th>CD45RA⁻CCR7⁻</th>
<th>CD45⁺CCR7⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLR</td>
<td>4.79%</td>
<td>27.97%</td>
<td>9.84%</td>
<td>57.40%</td>
<td></td>
</tr>
<tr>
<td>Autologous control</td>
<td>15.15%</td>
<td>50.08%</td>
<td>11.93%</td>
<td>22.84%</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.8 Expression of CD45RA and CCR7 on CD25⁺CD69⁺CD38⁺HLADR⁺ T cells following MLR or autologous control

**Depletion of cells expressing activation markers using QMS**

Preliminary depletion of cells expressing activation antigens including CD25, CD69, CD71, OX40 and HLADR was performed using QMS, and the results are...
presented in Figure 3.6-3.10. The log depletion of specific cells was calculated by Equation 3.1.

\[
\log_{10}(\text{depletion}) = -\log_{10}\left(\frac{\text{number of alloreactive Tcells after depletion}}{\text{number of alloreactive Tcells before depletion}}\right)
\]  

Figure 3.6 indicates that about 3 log\text{\textsubscript{10}} depletion of CD25\textsuperscript{+} T cells can be achieved by using QMS to depletion CD25\textsuperscript{+} cells. Moreover, about 1-1.5 log\text{\textsubscript{10}} depletion of cells expressing CD69, CD71, OX40 or HLADR was also achieved with the depletion of CD25\textsuperscript{+} cell by QMS, which is due to the co-expression of activation antigens on alloreactive T cells. As discussed previously, there are different alloreactive T cell subsets existing which express different combinations of activation antigens, therefore, the cells co-express CD25 with other activation antigens (CD69, CD71, OX40 and HLADR) can be depleted by the selective depletion of CD25\textsuperscript{+} cells. However, as indicated in Figure 3.6, only 1-1.5 log\text{\textsubscript{10}} depletion of cells expressing other activation antigens can be achieved with the depletion of CD25\textsuperscript{+} cells, which is because there are CD25\textsuperscript{-} cells expressing CD69, CD71, OX40 and HLADR exsiting. Similarly, it is also shown in Figure 3.7-3.10 that about 2-3 log\text{\textsubscript{10}} depletion of cells expressing other single activation antigen can be achieved by depleting these cells by QMS, at the same time, only about one log\text{\textsubscript{10}} depletion of cells expressing other activation antigens can be achieved. Therefore, this study indicates that higher degree depletion of alloreactive T cells can be achieved by targeting multiple activation antigens simultaneously.
For most alloreactive T cell subsets expressing specific activation antigens, 2-3 log_{10} depletion of these cells could be achieved using QMS by current operation parameters. As shown in Equation 3.1, the log_{10} depletion of alloreactive T cells achieved by QMS is related to the number of alloreactive T cells before and after depletion. The performance of QMS is stable, however, the number of alloreactive T cells stimulated after four-day MLR from different donors is different, and this will cause the main variability of log_{10} depletion of alloreactive T cells achieved by QMS.

Figure 3.6 CD25^{+} T cell depletion by QMS
Figure 3.7 CD69⁺ T cell depletion by QMS

Figure 3.8 CD71⁺ T cell depletion by QMS
Figure 3.9 OX40+ T cell depletion by QMS

Figure 3.10 HLADR+ T cell depletion by QMS
**Determine the functional significance of activation antigen expressing cells**

Although there are distinct cell subsets existing which express different activation antigens, it is important to determine the functional significance of cells expressing each activation antigen in order to define the optimum cell subsets to be targeted for alloreactive T cell depletion. The priority sequence of activation antigens on representing alloreactive T cells was determined by flow cytometric analysis of activation antigen expression on allo-activated T cells after four-day MLR. It is indicated from the flow cytometric analysis that CD25, CD69, HLADR and CD38 are the activation antigens with the first to fourth priority respectively. Therefore, cells subsets expressing different combinations of activation antigens including CD25, CD69, HLADR and CD38 were depleted, and the alloreactivity of the remaining cells after depletion were measured by BrdU proliferation assay in order to determine the activation antigens needed to be targeted for $3\log_{10}$ depletion of alloreactivity.

In Figure 3.11, the alloreactivity of different cell subsets was compared (Cell concentration is 100,000 cells per well). It is indicated in Figure 3.11 that the residual alloreactivity is decreasing gradually with depletion of more cells expressing different activation antigens. The residual cells after CD25$^+$, CD69$^+$ and HLADR$^+$ cell depletion have very low alloreactivity, which is similar to the alloreactivity induced by autologous control. Table 3.9 shows the frequency of alloreactive T cells in different residual cell subsets and the degree of alloreactivity depletion after targeting different combinations of activation antigens for alloreactive T cell depletion. Table
3.10 summarizes the alloreactivity depletion results of 6 experiments. It is indicated from Table 3.10 that more depletion of alloreactivity can be achieved by targeting multiple activation antigens for depletion and 3log depletion of alloreactivity can be achieved by targeting CD25, CD69 and HLADR simultaneously for alloreactive T cell depletion.

Figure 3.11 Residual alloreactivity in different cell subsets
Table 3.9 Determination of residual alloreactivity in different cell subsets

<table>
<thead>
<tr>
<th>Depleted cells</th>
<th>Proliferating cell frequency</th>
<th>Log depletion of alloreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells before alloreactive T cell depletion</td>
<td>1 in 2992</td>
<td></td>
</tr>
<tr>
<td>Residual cells after CD25+ cell depletion</td>
<td>1 in 57358</td>
<td>1.28</td>
</tr>
<tr>
<td>Residual cells after CD25+ and CD69+ cell depletion</td>
<td>1 in 392278</td>
<td>2.11</td>
</tr>
<tr>
<td>Residual cells after CD25+,CD69+, and HLADR+ cell depletion</td>
<td>&lt;1 in 2616081 &gt;2.94</td>
<td></td>
</tr>
<tr>
<td>Residual cells after CD25+,CD69+, HLADR+, and CD38+ cell depletion</td>
<td>&lt;1 in 2616081 &gt;2.94</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.10 Log depletion of alloreactivity achieved by depleting different cell subsets

<table>
<thead>
<tr>
<th>Depleted cells</th>
<th>Log depletion of alloreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>CD25+ cells</td>
<td>1.28</td>
</tr>
<tr>
<td>CD25+CD69+ cells</td>
<td>2.11</td>
</tr>
<tr>
<td>CD25+CD69+HLADR+ cells</td>
<td>&gt;2.94</td>
</tr>
<tr>
<td>CD25+69+HLADR+CD38+ cells</td>
<td>&gt;2.94</td>
</tr>
</tbody>
</table>

**Determine the anti-third party reactivity of residual cells after alloreactive T cell depletion**

Besides log depletion of alloreactivity, the anti-third party reactivity of cells after selective depletion of alloreactive T cells is another important parameters to evaluate the depletion strategy. Anti-third party reactivity refers to the immune reactivity of cell products towards foreign antigens from the third party other than the recipient. The incidence of viral infections after transplantation is correlated to the anti-third
party reactivity; the more preserved anti-third party reactivity, the less chance of viral infections.

Figure 3.12 compares the residual anti-host reactivity and anti-third party reactivity of CD25-depleted cells. It is indicated from Figure 3.12 that the anti-host reactivity is decreased to the level of autologous response, while there is still high anti-third party reactivity after selective depletion of CD25$^+$ cells. Figure 3.13 and 3.14 shows the residual anti-host reactivity and anti-third party reactivity of CD25/CD69-depleted cells and CD25/CD69/HLADR-depleted cells respectively. Figure 3.12 to Figure 3.14 indicates that the anti-third party reactivity of residual cells may not decrease with targeting more activation antigens for selective depletion of alloreactive T cells. However, as shown in Figure 3.15, the linear range of BrdU proliferation assay only covers 50 cells/well to 1000 cells/well. Therefore, the difference in BrdU incorporation can not be detected by absorbance at the cell concentration greater than 1000 cells/well (The normal range of cell concentration for our detection is 1000 to 100,000 cells/well) and no accurate conclusion can be drawn from Figure 3.12 to 3.14.

The preserved anti-third party reactivity can not be determined by only comparing the BrdU incorporation (detected by absorbance) of cells before and after selective depletion of alloreactive T cells. It was decided to combine BrdU incorporation assay and limiting dilution assay to determine the frequency to proliferative cells reacting toward third-party stimulation before and after selective depletion of alloreactive T cells. Table 3.11 shows the preserved anti-third party
reactivity of three independent experiments after selective alloreactive T cell depletion. It is indicated from Table 3.11 that high anti-third party reactivity was lost during the selective depletion, which may mainly caused by nonspecific loss of non alloreactive T cells during the depletion process. As shown in Figure 3.16, the non-T cells expressing targeted activation antigens were depleted, and there are some cells without activation antigens were also retained in the MACS magnetic column nonspecifically. The cells after four-day MLR become sticky and might be nonspecifically retained on MACS column easily. This problem can be solved by using QMS instead of Miltenyi MACS system because the design of QMS provides a better control flow system.

![Graph](image)

**Figure 3.12** Residual alloreactivity and anti-third party reactivity of cells after CD25⁺ cell depletion
Figure 3.13 Residual alloreactivity and anti-third party reactivity of cells after CD25$^+$ and CD69$^+$ cell depletion

Figure 3.14 Residual alloreactivity and anti-third party reactivity of cells after CD25$^+$, CD69$^+$ and HLADR$^-$ cell depletion
Figure 3.15 Linearity of BrdU proliferation assay

<table>
<thead>
<tr>
<th>Depleted cells</th>
<th>Preserved anti-third party reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>CD25&lt;sup&gt;+&lt;/sup&gt; cell depletion</td>
<td>56.23</td>
</tr>
<tr>
<td>CD25&lt;sup&gt;+&lt;/sup&gt; and CD69&lt;sup&gt;+&lt;/sup&gt; cell depletion</td>
<td>15.14</td>
</tr>
<tr>
<td>CD25&lt;sup&gt;+&lt;/sup&gt;,CD69&lt;sup&gt;+&lt;/sup&gt; and HLADR&lt;sup&gt;+&lt;/sup&gt; cell depletion</td>
<td>13.80</td>
</tr>
<tr>
<td>CD25&lt;sup&gt;+&lt;/sup&gt;,CD69&lt;sup&gt;+&lt;/sup&gt;, HLADR&lt;sup&gt;+&lt;/sup&gt; and CD38&lt;sup&gt;+&lt;/sup&gt; cell depletion</td>
<td>11.22</td>
</tr>
</tbody>
</table>

Table 3.11 Preserved anti-third party reactivity after selective depletion of alloreactive T cells
3.4 Discussions

GvHD is one of the major limitations to the success of allogeneic stem cell transplantation. GvHD can be abrogated by extreme depletion of T cells from the donor graft; however, severe problems are associated with T-cell depletion such as delayed immune reconstitution and increase incidence of viral infection. Selective depletion of alloreactive T cells is an alternative strategy which has been studied widely for preventing GvHD and preserving the anti-third party reactivity for the transplants recipient after allogeneic stem cell transplantation. The commonest approach for alloreactive T cell depletion is to target activation antigens, single activation antigen is selected to be targeted in most of the current alloreactive T cell depletion methodologies. Currently, CD25 and CD69 are used commonly as surface markers to deplete alloreactive T cells, however, high alloreactivity are still remain in

Figure 3.16 Flow cytometric analysis of cell subsets before and after selective alloreactive T cell depletion
cells after CD25+ and CD69+ cell depletion (Feshe et al, 2000; Koh et al, 1999). One of the main reasons for this inefficient depletion of alloreactive T cells is the limited detection of alloreactive T cells by single or double activation antigens. In this study, the expression of activation antigen, CD25, CD69, CD71, CD40L, CD95, OX40, HLADR, CD38, CD58, CD152, CD122 and CD103, on alloreactive T cells was studied, in order to find the optimum combination of activation antigens for alloreactive T cell depletion.

CD25, CD69, CD71, OX40, HLADR, and CD38 were selected to represent alloreactive T cells due to their up-regulation on alloreactive T cells after MLR. The co-expression of CD25, CD69, CD71, OX40, HLADR, and CD38 on alloreactive T cells were determined by a nine-parameter flow cytometric analysis. The alloreactive T cell faction represented by each activation antigen and antigen combination (two, three, four or five-antigen combination) was obtained. For our definition of alloreactive T cells (express activation antigens CD25, CD69, CD71, CD38, OX40 or HLADR), the data indicates that 13% percent of alloreactive T cells can not be selected by targeting CD25 and CD69. Koh et al. reported that 11.5±9.9% of proliferative reactivity towards original recipient was left after selective depleting CD25+ and CD69+ cells from the cells after 3-day MLR by Miltenyi MACS. It was concluded that sufficient high degree depletion of alloreactive T cells can not be obtained by targeting single or two activation antigens. Moreover, the distribution of CD4, CD8, CD45RO, CD45RA on alloreactive T cells expressing these antigens (CD25, CD69, CD71,OX40, HLADR and CD38) was determined by a nine-parameter
flow cytometric analysis. It is indicated that the fraction of CD45<sup>−</sup>CCR7<sup>+</sup> cells in alloreactive T cells expressing activation antigens including CD25, CD69, CD71, OX40, HLADR and CD38 is increased dramatically after 4-day MLR, CD45RA<sup>−</sup>CCR7<sup>+</sup> is the surface characteristic of effector memory cells, which have been reported as the major mediator of chronic GvHD. Therefore, in order to achieve a high degree of alloreactive T cell depletion and abrogate GvHD, it is necessary to perform the alloreactive T cell depletion by targeting multiple activation antigens simultaneously.

In order to select the optimum activation antigen combination for sufficient alloreactive T cell depletion, the priority sequence of activation antigen on representing alloreactive T cells was determined by comparing the T cell fraction selected by all different antigen combinations. CD25, CD69, HLADR, CD38, OX40 and CD71 were determined as the activation antigen with the first, second, third, fourth, fifth, and sixth priority on representing alloreactive T cells respectively. The alloreactive T cells after 4-day MLR were selective depleted by targeting CD25, CD25/CD69, CD25/CD69/HLADR respectively, and the residual proliferative activity towards original recipient was measured by limiting dilution assay. ≥ 3 log<sub>10</sub> depletion of allo-proliferative reactivity towards original recipient was achieved by depleting CD25<sup>+</sup>, CD69<sup>+</sup> and HLADR<sup>+</sup> cells from cells generated from 4-day MLR. Compared with alloreactivity depletion achieved by other available strategies (Guimond et al, 2002, Martins et al, 2004; Fehse et al, 2000, Koh et al, 1999, Amorlia et al, 2005; Barrett et al, 2002), ≥ 3 log<sub>10</sub> depletion of alloreactivity is relative high.
The depletion of alloreactivity achieved by depleting CD25\(^+\), CD69\(^+\), HLADR\(^+\) and CD38\(^+\) cells can not be determined due to the limitation of cell number. However, there is high possibility that the depletion of alloreactivity can be increased by targeting more activation antigens for depletion.

Furthermore, the anti-third party reactivity after selective alloreactive T cell depletion was measured. Compared with literature-reported preserved anti-third party reactivity (≥70\%), the residual anti-third party reactivity after selective depletion of alloreactive T cells in this study is low. The main reason for this low preserved anti-third party reactivity is that a portion of non alloreactive T cells can be depleted nonspecifically by current operating parameters. Miltenyi Super MACS system was used for selective alloreactive T cell depletion in this study, cells can be nonspecifically retained in the MACS separation column easily due to its design. Compared with MACS, QMS has a better flow control system so that QMS might provide better performance for alloreactive T cell depletion, especially the recovery of non alloreactive T cells.
CHAPTER 4

A NEW STRATEGY FOR AUGMENTED ACTIVATION OF ALLOREACTIVE T CELLS: DENDRITIC CELL STIMULATION

4.1 Motivation

One challenge for sufficient alloreactive T cell depletion is to stimulate maximum potential alloreactive T cells for depletion. In this study, a new approach using recipient derived dendritic cells as stimulators to augment the alloreactive T cell activation was investigated.

Primary mixed lymphocytes reaction (MLR) is the commonest method studied for the generation alloreactive T cells in vitro, culturing donor lymphocytes with allogeneic recipient lymphocytes together. Different strategies were investigated in order to optimize the generation of alloreactive T cells in vitro, which leads to the higher degree of alloreactivity depletion. Brenner et al (2003) have demonstrated that the generation of CD3+CD25+ alloreactive T cells can be increased by stimulating the mixed lymphocytes reaction with HLA-mismatched host Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) (Brenner et
al, 2003). It has been reported that CD25 up-regulation on responder cells were increased (22-34%) by using OKT-3 expanded CD3$^+$ lymphocytes as simulators (Barrett et al, 2002). In this study, recipient monocyte-derived dendritic cells (DCs), the most potent antigen presenting cells, were used as stimulators to optimize the alloreactive T cell activation. The expression of potential activation antigens on DCs stimulated alloreactive T cells was determined by a nine-parameter flow cytometric analysis. The priority sequence of activation antigens on representing alloreactive T cell subsets was determined. The residual alloreactivity and anti-third party reactivity after depleting different alloreactive T cell subsets were measured.

**4.2 Introduction of dendritic cells**

Dendritic cells (DCs) are the most potent of all professional antigen presenting cells including dendritic cells, macrophages and B lymphocytes (Abbas et al, 2003). DCs start out in an immature state in peripheral tissues where they detect regulatory signals with high endocytic activity and low T-cell activation potential. When a stimulatory threshold is reached, immature dendritic cells terminally differentiate into mature dendritic cells that migrate to lymph nodes where they recruit and stimulate naive T cells (Dietz et al, 2000).

The maturation of dendritic cells reduces the high rate of antigen uptake and increases the expression of antigen presenting molecules, costimulatory molecules, and the secretion of cytokines and chemokines on cell surface, which greatly enhance mature DCs’ ability of activating T-cells. CCR7, a chemotactic receptor that induces
the dendritic cells to travel through the blood stream to the spleen or through the
lymphatic system to a lymph node, is upregulated on mature DCs. The maturation of
dendritic cells changes the expression of adhesion molecules and receptors which are
involved in their migration, homing and cell–cell interactions. Therefore, dendritic
cells’ maturation is critical to the initiation of immune responses (Dietz et al, 2004).

There are three main types of human DCs which are originated from pluripotent
stem cells; interstitial DCs residing in the skin and lymphoid organs and two subsets
of blood DCs, the CD11c+ myeloid and CD11+ plasmacytoid DC (Shortman et al,
2002). Circulating blood DCs are rare cell population, accounting for less than 1% of
human peripheral blood mononuclear cells, and these cells are difficult to maintain in
culture. The current cell sources for in vitro DCs generation are CD34+ hematopoietic
stem cells and blood monocytes. The most commonly used method for generating
mature DCs in vitro with full T cell stimulatory capacity is to culture CD14+ monocytes with GM-CSF and IL-4 for 5-7 days, and then stimulate these cells with
microbial, proinflammatory or T cell-derived mediators (Dauer et al, 2003).

Dauer et al. developed a method for fast DCs generation from human monocytes
in 48 hours. In order to make the alloreactive T cell generation process shorter, which
will decrease the incidence of viral infections in clinical application, the fast DCs
generation method developed by Dauer et al. was applied into this study, and the
generated mature DCs were used as stimulators to optimize the alloreactive T cell
activation.
4.3 Material and Method

Isolation of PBMCs

Human peripheral leukocytes were ordered from the American Red Cross, Central Ohio Region. The peripheral blood mononuclear cell (PBMC) fraction was isolated and separated by Ficoll-Hypaque density gradient centrifugation. The cells were washed with PBS twice and resuspended in RPMI supplemented with streptomycin sulfate (50 µg/ml), gentamicin sulfate (10 µg/ml), L-glutamine (2 mM), and 2% pooled human AB Serum. The number of peripheral blood mononuclear cells was determined using a hemacytometer.

Generation of Dendritic Cells

Monocytes were isolated from PBMCs by Miltenyi Midi MACS system with targeting CD14. Briefly, $10^8$ cells were transferred to a 75x12 mm polypropylene tube and the volume of cell suspension was adjusted to 80µl. Then 50µl anti-CD14 Microbeads were added to the sample tubes and these tubes were incubated in the dark at 4 °C for 30 minutes. After labeling, the cells were washed twice with labeling buffer, then the cell samples were suspended in 2ml labeling buffer for magnetic separation. The CD14+ monocytes were selected by passing the labeled cell suspension through LS column (Miltenyi Biotech) surrounded by Miltenyi Midi MACS separator. Then the LS column was taken off from the Midi MACS separator and the CD14+ monocytes were washing off by 4 ml labeling buffer. The number of CD14+ monocytes was determined using a hemacytometer. These CD14+ monocytes
were resuspended in RPMI supplemented with streptomycin sulfate (50 µg/ml),
gentamicin sulfate (10 µg/ml), L-glutamine (2 mM), and 2% pooled human AB
Serum at a concentration of 1.5x10^6 cells/ml. The CD14^+ monocytes were treated with
1000 U/ml GM-CSF and 500 U/ml IL-4 (Peprotech, NJ) for 24 hrs, then the cells
were treated with 10 ng/ml IL-1β, 1000 U/ml TNF-α and 1 μM PGE2 for another 24
hrs. The generated dendritic cells were stained with antibodies for specific surface
receptors including CD83, CD86, CD80, CD40, CD14, MHCII and CCR7, in order to
test the maturation of dendritic cells from monocytes.

**Dendritic cell stimulated mixed lymphocytes reaction (DC MLR)**

The generated dendritic cells were washed twice with PBS, irradiated to 30Gy,
then resuspended in AIM-V supplemented with streptomycin sulfate (50 µg/ml),
gentamicin sulfate (10 µg/ml), and L-glutamine (2 mM) at a concentration of 1x10^6
cells/ml. Since dendritic cells have a higher capability in antigen presentation than
peripheral blood mononuclear cells and relative small number of dendritic cells can be
generated from recipient-monocytes, it was decided to mix the irradiated dendritic
cells from the recipient with peripheral blood mononuclear cells from the donor with
the ratio of 1:3 at the concentration of 2x10^6 cells/ml for dendritic cell stimulated
mixed lymphocyte reaction (DC MLR). However, the effect of higher ratio of
dendritic cells to peripheral blood mononuclear cells on increasing alloreactive T cell
activation was not studied. As for the autologous control of DC MLR, the irradiated
dendritic cells from the recipient were mixed with peripheral blood mononuclear cells from the recipient with the ratio of 1:3 at the concentration of $2 \times 10^6$ cells/ml.

**Flow cytometry analysis**

$0.5 \times 10^6$ or $1 \times 10^6$ cells were harvested from tissue culture plates, washed twice with PBS, and transferred to the 75x12 mm polypropylene tubes. Appropriate antibodies for the specific experiment were added to the cell suspension as recommended by the manufacturer. The total incubation volume was brought up to 100 µl. Then the sample tubes with antibodies were incubated in the dark at 4 °C for 30 minutes. After labeling, the cells were washed twice with 2 ml of labeling buffer (PBS + 0.5% BSA + 2 mM EDTA). The samples were analyzed by FACSARia system and FACSDiva software, 100,000 events were collected for most samples.

**Statistical study**

The T test analysis was performed to determine the statistical significance of presented results. JMP software was used to perform all the statistical analysis.

**Proliferation assays**

Responder cells (A) were cultured in a 96-well U bottom tissue culture plate at a final volume of 100 µl and stimulated with $2 \times 10^4$ allogeneic stimulator DC cells (DC$_b^*$) which had been previously irradiated (30 Gy). In the autologous control culture, the responder cells (A) at the corresponding cell number were cultured with $10^5$ irradiated (30 Gy) autologous cells (A$^*$). The concentrations of responder cells
ranged from 10000 to 10 cells/well for non-depleted cell sample including four dilutions of 10000, 1000, 100, 10 cells/well. For depleted cell sample, the responder cell concentrations ranged from $10^6$ to 100 cells/well including four dilutions of $10^6$, $10^5$, $10^4$, $10^3$ cells/well. 24 replicate wells were set up for each dilution. The culture was incubated at 37 °C with 5% CO$_2$, and 50 µl culture medium was fed for each well on the 6$^{th}$ and 12$^{th}$ day. On the 14$^{th}$ day, the cell proliferation was tested by BrdU incorporation assay. As described in Chapter 3, the proliferating cell frequency was determined by Taswell method by combining limiting dilution assay and BrdU incorporation assay, therefore, the linearity of BrdU incorporation assay has no effect on cell frequency calculation.

4.4 Results

Generation of Dendritic Cells

After 48 hrs culture, about 40-50% of cells are mature dendritic cells, which are developed from monocytes with the stimulation of cytokines. The special surface receptors for mature dendritic cells including CD40, CD83, CD86, MHC II and CCR7 are up-regulated and the antigens for monocytes such as CD14 are absent. Table 4.1 shows the summary of antigen expression on mature dendritic cells. It is indicated from Table 4.1 that about 80-90% of the DCs population (around 60% of total cells) has the characteristic of mature dendritic cells, which are CD40$^+$CD83$^+$CD14$^+$CD80$^+$CD86$^+$HLADR$^+$CCR7$^+$. Figure 4.1 shows expression of different surface antigens on mature dendritic cells.
Table 4.1 Specific antigen expression on mature dendritic cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>DCs</th>
<th>CD40&lt;sup&gt;+&lt;/sup&gt;CD83&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD14&lt;sup&gt;+&lt;/sup&gt;CD80&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD86&lt;sup&gt;+&lt;/sup&gt;HLADR&lt;sup&gt;+&lt;/sup&gt;</th>
<th>FSC&lt;sup&gt;+&lt;/sup&gt;CCR7&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>66.08%</td>
<td>80.96%</td>
<td>81.27%</td>
<td>69.01%</td>
<td>81.30%</td>
</tr>
<tr>
<td>2</td>
<td>65.93%</td>
<td>94.47%</td>
<td>96.54%</td>
<td>92.42%</td>
<td>90.07%</td>
</tr>
<tr>
<td>3</td>
<td>59.99%</td>
<td>91.61%</td>
<td>89.89%</td>
<td>90.23%</td>
<td>97.85%</td>
</tr>
<tr>
<td>Mean</td>
<td>64.00%</td>
<td>89.01%</td>
<td>89.23%</td>
<td>83.89%</td>
<td>89.74%</td>
</tr>
<tr>
<td>SD</td>
<td>3.47%</td>
<td>7.12%</td>
<td>7.66%</td>
<td>12.93%</td>
<td>4.48%</td>
</tr>
</tbody>
</table>

Figure 4.1 Specific antigen expression on dendritic cells

**Antigen co-expression on DC stimulated alloreactive T cells**

By similar definition as Chapter 3, the alloreactive T cells stimulated by dendritic cells was identified by the up-regulation of CD25, CD69, CD71, OX40, HLADR and CD38 on cells following dendrite cell stimulated MLR. The expression pattern of activation antigens on alloreactive T cells stimulated by dendritic cells was
determined by a nine-parameter flow cytometric analysis (Twelve replicated experiments were performed independently). The fractions of cell subsets expressing different activation antigen combinations was determined by performing flow cytometric analysis with different gating strategies (shown in Table 4.2). From the data shown in Table 4.2, the fraction of alloreactive T cells expressing multiple activation antigens and single activation antigen can be calculated. There is about 35% alloreactive T cells expressing only single activation antigen and 65% alloreactive T cells expressing more than one activation antigen. The distribution of alloreactive T cells expressing multiple and single activation antigen is shown in Figure 4.2. As Figure 4.2 shows, 15.16% of alloreactive T cells express only CD25, 6.32% of alloreactive T cells express only CD38, 5.13% of alloreactive T cells express only HLADR, 4.74% of alloreactive T cells express only CD71, 3.31% of alloreactive T cells express only CD69, and 0.99% of alloreactive T cells express only OX40; these cells only can be depleted by targeting their unique activation antigens. Furthermore, the fraction of alloreactive T cells selected by targeting different antigen combinations can be calculated from data shown in Table 4.2. For example, the cell fraction selected by CD25, 73.82%, is the sum of all cell subsets expressing CD25 (e.g. the cell subset expressing CD25 only, the cell subset expressing CD25 and CD69 only, the cell subset expressing CD25, CD69 and HLADR only, and so on). The fractions of alloreactive T cells selected by each single activation antigen were compared; similarly, the fractions of alloreactive T cells selected by each two-activation antigen combination, each three-activation antigen combination, each
four-activation antigen combination, and each five-antigen combination were also compared respectively. Then the priority sequence of activation antigens was obtained based on the comparisons mentioned above. As shown in Table 4.3, CD25 is the activation antigen with the first priority; 73.81% of the alloreactive T cells can be selected by targeting CD25, which is higher than the percentage of alloreactive T cells represented by other activation antigens. The activation antigen with the second priority is HLADR; 84.03% of the alloreactive T cells can be selected by targeting both CD25 and HLADR, which is higher than the percentage of alloreactive T cells expressing any other two activation antigens. Similarly, CD38, CD71 and CD69 were determined as the surface antigens with the third, fourth and fifth priority on representing alloreactive T cells respectively.

As stated previously in Chapter 3, it is important to determine if the selected alloreactive T cell fraction can be increased significantly by targeting the cells with more activation antigens. The T test analysis was performed to compare the fraction of alloreactive T cells represented by one, two, three, four or five activation antigens (These activation antigen combinations were determined by priority sequence as shown in Table 4.3). As the T test analysis result shown in Table 4.4, there is significant difference in representing alloreactive T cell fraction between single antigen (CD25), two-antigen combination (CD25 and HLADR), three-antigen combination (CD25, HLADR and CD38) and four-antigen combination (CD25, HLADR, CD38 and CD71); on the other hand, there is no significant difference in representing alloreactive T cell fraction between four-antigen combination (CD25,
HLADR, CD38 and CD71) and a five-antigen combination (CD69, CD25, HLADR, CD38 and CD71). The antigen-priority-sequence information from can be used to select antigen combinations for alloreactive T cell depletion. Moreover, the antigen-priority-sequence needs to be confirmed by testing the depletion of alloreactivity achieved by targeting different activation antigen combinations.

<table>
<thead>
<tr>
<th>Antigen combinations</th>
<th>Alloreactive T cell fraction (N=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>CD25/CD69/CD71/OX40/HLADR/CD38</td>
<td>0.67%</td>
</tr>
<tr>
<td>CD25/CD69/CD71/OX40/CD38</td>
<td>0.06%</td>
</tr>
<tr>
<td>CD25/CD69/CD71/HLADR/CD38</td>
<td>0.60%</td>
</tr>
<tr>
<td>CD25/CD71/OX40/HLADR/CD38</td>
<td>2.20%</td>
</tr>
<tr>
<td>CD69/CD71/OX40/HLADR/CD38</td>
<td>0.00%</td>
</tr>
<tr>
<td>CD25/CD69/OX40/HLADR/CD38</td>
<td>1.63%</td>
</tr>
<tr>
<td>CD25/CD69/CD71/CD38</td>
<td>0.11%</td>
</tr>
<tr>
<td>CD25/CD69/OX40/CD38</td>
<td>0.05%</td>
</tr>
<tr>
<td>CD25/CD69/CD38/HLADR</td>
<td>1.09%</td>
</tr>
<tr>
<td>CD25/CD71/OX40/CD38</td>
<td>0.09%</td>
</tr>
<tr>
<td>CD25/CD71/HLADR/CD38</td>
<td>9.93%</td>
</tr>
<tr>
<td>CD25/OX40/HLADR/CD38</td>
<td>1.42%</td>
</tr>
<tr>
<td>CD69/CD71/OX40/CD38</td>
<td>0.00%</td>
</tr>
<tr>
<td>CD69/CD71/HLADR/CD38</td>
<td>0.02%</td>
</tr>
<tr>
<td>CD69/OX40/HLADR/CD38</td>
<td>0.02%</td>
</tr>
<tr>
<td>CD71/OX40/HLADR/CD38</td>
<td>0.10%</td>
</tr>
<tr>
<td>CD25/CD69/CD38</td>
<td>0.45%</td>
</tr>
<tr>
<td>CD25/CD71/CD38</td>
<td>0.98%</td>
</tr>
<tr>
<td>CD25/OX40/CD38</td>
<td>0.09%</td>
</tr>
<tr>
<td>CD25/HLADR/CD38</td>
<td>4.19%</td>
</tr>
<tr>
<td>CD69/CD71/CD38</td>
<td>0.00%</td>
</tr>
<tr>
<td>CD69/OX40/CD38</td>
<td>0.00%</td>
</tr>
<tr>
<td>CD69/HLADR/CD38</td>
<td>0.13%</td>
</tr>
<tr>
<td>CD71/OX40/CD38</td>
<td>0.00%</td>
</tr>
<tr>
<td>CD71/HLADR/CD38</td>
<td>0.82%</td>
</tr>
</tbody>
</table>

Table 4.2 Percentage of alloreactive T cell represented by different antigen combinations (Continued)
<table>
<thead>
<tr>
<th>Combination</th>
<th>Percent 1</th>
<th>Percent 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>OX40/HLADR/CD38</td>
<td>0.21%</td>
<td>0.47%</td>
</tr>
<tr>
<td>CD25/CD38</td>
<td>1.60%</td>
<td>0.92%</td>
</tr>
<tr>
<td>CD69/CD38</td>
<td>0.28%</td>
<td>0.35%</td>
</tr>
<tr>
<td>CD71/CD38</td>
<td>0.24%</td>
<td>0.34%</td>
</tr>
<tr>
<td>OX40/CD38</td>
<td>0.05%</td>
<td>0.06%</td>
</tr>
<tr>
<td>HLADR/CD38</td>
<td>1.07%</td>
<td>1.08%</td>
</tr>
<tr>
<td>CD38</td>
<td>6.32%</td>
<td>3.43%</td>
</tr>
<tr>
<td>CD25/CD69/CD71/OX40/HLADR</td>
<td>0.73%</td>
<td>1.00%</td>
</tr>
<tr>
<td>CD25/CD69/CD71/HLADR</td>
<td>1.04%</td>
<td>1.16%</td>
</tr>
<tr>
<td>CD25/CD69/OX40/HLADR</td>
<td>3.07%</td>
<td>5.59%</td>
</tr>
<tr>
<td>CD25/CD71/OX40/HLADR</td>
<td>0.81%</td>
<td>0.61%</td>
</tr>
<tr>
<td>CD69/CD71/OX40/HLADR</td>
<td>0.01%</td>
<td>0.01%</td>
</tr>
<tr>
<td>CD25/CD69/HLADR</td>
<td>5.04%</td>
<td>4.84%</td>
</tr>
<tr>
<td>CD25/CD71/HLADR</td>
<td>3.86%</td>
<td>2.64%</td>
</tr>
<tr>
<td>CD25/OX40/HLADR</td>
<td>2.73%</td>
<td>3.78%</td>
</tr>
<tr>
<td>CD69/CD71/HLADR</td>
<td>0.06%</td>
<td>0.08%</td>
</tr>
<tr>
<td>CD69/OX40/HLADR</td>
<td>0.06%</td>
<td>0.12%</td>
</tr>
<tr>
<td>CD71/OX40/HLADR</td>
<td>0.07%</td>
<td>0.15%</td>
</tr>
<tr>
<td>CD25/HLADR</td>
<td>11.00%</td>
<td>8.89%</td>
</tr>
<tr>
<td>CD69/HLADR</td>
<td>0.91%</td>
<td>1.12%</td>
</tr>
<tr>
<td>CD71/HLADR</td>
<td>1.22%</td>
<td>1.22%</td>
</tr>
<tr>
<td>OX40/HLADR</td>
<td>0.49%</td>
<td>1.24%</td>
</tr>
<tr>
<td>HLADR</td>
<td>5.13%</td>
<td>3.33%</td>
</tr>
<tr>
<td>CD25/CD69/CD71/OX40</td>
<td>0.21%</td>
<td>0.40%</td>
</tr>
<tr>
<td>CD25/CD69/OX40</td>
<td>0.18%</td>
<td>0.23%</td>
</tr>
<tr>
<td>CD25/CD71/OX40</td>
<td>0.22%</td>
<td>0.24%</td>
</tr>
<tr>
<td>CD69/CD71/OX40</td>
<td>0.00%</td>
<td>0.01%</td>
</tr>
<tr>
<td>CD25/OX40</td>
<td>0.46%</td>
<td>0.50%</td>
</tr>
<tr>
<td>CD69/OX40</td>
<td>0.01%</td>
<td>0.02%</td>
</tr>
<tr>
<td>CD71/OX40</td>
<td>0.06%</td>
<td>0.16%</td>
</tr>
<tr>
<td>OX40</td>
<td>0.99%</td>
<td>2.69%</td>
</tr>
<tr>
<td>CD25/CD69/CD71</td>
<td>0.41%</td>
<td>0.68%</td>
</tr>
<tr>
<td>CD25/CD71</td>
<td>1.97%</td>
<td>1.61%</td>
</tr>
<tr>
<td>CD69/CD71</td>
<td>0.03%</td>
<td>0.05%</td>
</tr>
<tr>
<td>CD71</td>
<td>4.74%</td>
<td>5.41%</td>
</tr>
<tr>
<td>CD25/CD69</td>
<td>1.68%</td>
<td>1.35%</td>
</tr>
<tr>
<td>CD69</td>
<td>3.31%</td>
<td>1.82%</td>
</tr>
<tr>
<td>CD25</td>
<td>15.16%</td>
<td>4.56%</td>
</tr>
</tbody>
</table>
Figure 4.2 Expression of activation antigens on DC stimulated alloreactive T cells

Table 4.3 Priority sequence of activation antigens on representing DC stimulated alloreactive T cells

<table>
<thead>
<tr>
<th>Priority</th>
<th>Activation antigens</th>
<th>Activation antigens combinations</th>
<th>Alloreactive T cell fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD25</td>
<td>CD25</td>
<td>73.81%</td>
</tr>
<tr>
<td>2</td>
<td>HLADR</td>
<td>HLADR+CD25</td>
<td>84.03%</td>
</tr>
<tr>
<td>3</td>
<td>CD38</td>
<td>CD25+HLADR+CD38</td>
<td>90.93%</td>
</tr>
<tr>
<td>4</td>
<td>CD71</td>
<td>CD25+HLADR+CD38+CD71</td>
<td>96.82%</td>
</tr>
<tr>
<td>5</td>
<td>CD69</td>
<td>CD25+HLADR+CD38+CD71+CD69</td>
<td>99.09%</td>
</tr>
<tr>
<td>Activation antigens</td>
<td>Activation antigens</td>
<td>Difference</td>
<td>p-Value</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>---------------------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CD25+HLADR+CD38+CD71+CD69</td>
<td>CD25</td>
<td>25.43%</td>
<td>2.529e-15</td>
</tr>
<tr>
<td>CD25+HLADR+CD38+CD71</td>
<td>CD25</td>
<td>22.10%</td>
<td>4.011e-13</td>
</tr>
<tr>
<td>CD25+HLADR+CD38</td>
<td>CD25</td>
<td>17.25%</td>
<td>9.094e-10</td>
</tr>
<tr>
<td>CD25+HLADR+CD38+CD71+CD69</td>
<td>HLADR+CD25</td>
<td>15.08%</td>
<td>2.985e-8</td>
</tr>
<tr>
<td>CD25+HLADR+CD38+CD71</td>
<td>HLADR+CD25</td>
<td>11.76%</td>
<td>5.600e-6</td>
</tr>
<tr>
<td>HLADR+CD25</td>
<td>CD25</td>
<td>10.35%</td>
<td>4.600e-5</td>
</tr>
<tr>
<td>CD25+HLADR+CD38+CD71+CD69</td>
<td>CD25+HLADR+CD38</td>
<td>8.18%</td>
<td>9.330e-4</td>
</tr>
<tr>
<td>CD25+HLADR+CD38</td>
<td>HLADR+CD25</td>
<td>6.90%</td>
<td>4.640e-3</td>
</tr>
<tr>
<td>CD25+HLADR+CD38+CD71</td>
<td>CD25+HLADR+CD38</td>
<td>4.86%</td>
<td>4.251e-2</td>
</tr>
<tr>
<td>CD25+HLADR+CD38+CD71+CD69</td>
<td>CD25+HLADR+CD38+CD71</td>
<td>3.32%</td>
<td>1.606e-1</td>
</tr>
</tbody>
</table>

Table 4.4 T test results of antigen combinations on representing alloreactive T cells

**Comparison of cell activation under the stimulation of dendritic cells and PBMCs**

It is indicated from the plots of Forward Scatter versus Side Scatter (Figure 4.3) that more cells are become bigger and more granular after dendritic cell stimulated mixed lymphocytes reaction (DC MLR). As Figure 4.3 shows, the percentage of CD25⁺ alloreactive T cells was increased by performing DC MLR instead of peripheral blood mononuclear cells (PBMCs) stimulated mixed lymphocytes reaction (Normal MLR). Furthermore, the expression of CD3 is down regulated in a portion of CD25⁺ cells after DC MLR.
Figure 4.3 Expression of CD25 on DC and PBMC stimulated alloreactive T cells

As stated in Chapter 3, the alloreactive T cells are also defined by the up-regulation of activation antigens including CD25, CD69, CD71, OX40, HLADR and CD38 in this study. More T cells can become allo-activated and express activation antigens (CD25, CD69, CD71, OX40, HLADR and CD38) by dendritic cell-stimulation instead of peripheral blood mononuclear cell-stimulation. The fraction of alloreactive T cells in T cells after dendritic cell stimulated MLR (DC
MLR, N=12) and peripheral blood mononuclear cells stimulated MLR (Normal MLR, N=6) is compared. As shown in Figure 4.4, the fraction of alloreactive T cells in T cells after DC MLR is significantly higher than that after normal MLR (p<0.05).

![Figure 4.4 Comparison of alloreactive T cell activation in DC MLR and normal MLR](image)

The expression patterns of activation antigens on alloreactive T cells stimulated by dendritic cells and peripheral blood mononuclear cells are different. DC stimulated alloreactive T cell subsets expressing each activation antigen are compared with that stimulated by PBMC, Table 4.5 shows the T test analysis of this comparison. As Table 4.5 shows, more CD25+, HLA-DR+ and CD71+ alloreactive T cells are stimulated by dendritic cells, however, the DC stimulated alloreactive T cells
expressing CD69 is decreased significantly. This explains why the priority sequences of activation antigens on represent DC stimulated alloreactive T cells and PBMC stimulated alloreactive T cells are different, for example, HLADR is the activation antigen with the second priority to represent DC stimulated alloreactive T cells, while CD69 is the activation antigen with the second priority to represent PBMC stimulated alloreactive T cells.

<table>
<thead>
<tr>
<th>In T cells</th>
<th>Normal MLR(N=6)</th>
<th>DC MLR(N=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean    SD</td>
<td>Mean    SD</td>
</tr>
<tr>
<td>CD25⁺ cell fraction</td>
<td>20.76%  5.54%</td>
<td>33.55%  14.75%</td>
</tr>
<tr>
<td>CD69⁺ cell fraction</td>
<td>23.05%  5.41%</td>
<td>9.55%   10.66%</td>
</tr>
<tr>
<td>CD71⁺ cell fraction</td>
<td>7.99%   2.08%</td>
<td>15.29%  9.99%</td>
</tr>
<tr>
<td>OX40⁺ cell fraction</td>
<td>7.65%   3.70%</td>
<td>10.02%  9.07%</td>
</tr>
<tr>
<td>HLADR⁺ cell fraction</td>
<td>14.20%  3.58%</td>
<td>28.66%  14.36%</td>
</tr>
<tr>
<td>CD38⁺ cell fraction</td>
<td>14.41%  7.71%</td>
<td>17.57%  12.22%</td>
</tr>
</tbody>
</table>

Table 4.5 Comparison of activation antigen expression pattern on DC stimulated alloreactive T cells and PBMC stimulated alloreactive T cells

4.5 Discussions

GvHD is still the main limitations to allogeneic stem cell transplantation. Depletion of T cells from the donor graft can eliminate GvHD; however, there are severe problems associated with T-cell depletion such as delayed immune reconstitution, increase incidence of viral infection and relapse. An alternative approach to prevent GvHD is selective depletion of alloreactive T cells, the anti-third
party reactivity of the transplants can be preserved by apply this strategy. In order to achieve sufficient alloreactive T cells depletion and eliminate GvHD after allogeneic stem cell transplantation, the maximum number of potential alloreactive T cells has to be stimulated and detected. The portion of antigen presenting cells in peripheral blood mononuclear cells is not high enough to make an efficient stimulation of alloreactive T cells. Different strategies were applied to improve the activation of alloreactive T cells \textit{in vitro}. Brenner et al optimized the generation of CD3$^+$CD25$^+$ alloreactive T cells by stimulating the mixed lymphocytes reaction with HLA-mismatched host Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) (Brenner et al, 2003); and Barrett et al has reported that CD25 up-regulation on responder cells were increased (22-34%) by using OKT-3 expanded CD3$^+$ lymphocytes as simulators (Barrett et al, 2002). In this study, recipient-monocyte-derived dendritic cells were used as stimulator cells to optimize the activation of alloreactive T cells.

Dendritic cells are the most potent antigen presenting cells, which are essential in inducing various immune reactions. It takes 5 to 7 days to generate mature dendritic cells commonly, we applied a fast dendritic cell generation method (Dauer et al, 2003) to generate mature dendritic cells in 48 hours in this study. The fast DC generation method makes the whole process of alloreactive T cell generation and depletion shorter, which is crucial to the transfer of this process into clinical application. 40-50% of the stimulator cells used in this study is mature dendritic cells. The potential alloreactive T cells stimulated by recipient-monocyte-derived dendritic cells (46.62±15.60% of T cells) are much more than that stimulated by peripheral blood
mononuclear cells (32.41±5.36% of T cells). Compared with the optimized alloreactive T cell activation process (22-34%) of T cells were stimulated to express CD25) reported by Barrett et al. (2002), the up-regulation of CD25 (33.55±14.75% of T cells) on T cells after DC stimulation is competitive. Moreover, multiple activation antigens were studied for their expression on DC stimulated alloreactive T cells, up to 46.62±15.60% of DC stimulated T cells can be detected and selected potentially, which is a great improvement by comparing with literature (Guimond et al, 2002, Martins et al, 2004; Fehse et al, 2000, Koh et al, 1999, Amorlia et al, 2005; Barrett et al, 2002; André-Schmutz et al, 2002). As described in Chapter 3, the co-expression pattern of activation antigens on DC stimulated alloreactive T cells was studied, and the priority sequence of activation antigens on representing alloreactive T cells was determined. Compared with PBMC stimulated alloreactive T cells, the CD69 expression on DC stimulated alloreactive T cells in down-regulated.

The most effective allogeneic stimulator cells for selective depletion of alloreactive T cells have not been defined. Unselected PBMC (Koh et al, 1999), OKT-3 activated recipient lymphocytes (Barrett et al, 2002), keratinocytes (Van Dijk et al, 1999), and recipient EBV-transformed LCLs (Amorlia et al, 2005) have been reported as stimulator cells to optimize generation of alloreactive T cells. In this study, dendritic cells were chosen as stimulators and it is indicated that dendritic cells have great capability in alloreactive T cell stimulation, and more potential alloreactive T cells can be detected and selected by using multiple activation antigens. Since monocytes only account 3-9% (Abbas et al, 2003) of human leukocytes, therefore, it
is a challenge to generate enough dendritic cells for alloreactive T cells stimulation in clinical-scale application. Granulocyte-monocyte colony-stimulating factor (GM-CSF), a cytokine promotes the maturation of bone marrow cells into dendritic cells and monocytes, can be used to treat patients in order to collect more monocytes.
CHAPTER 5

QUANTIFICATION OF THE ANTIGEN EXPRESSION LEVEL
AND ITS EFFECT ON MAGNETIC SEPARATION

5.1 Motivation

Immunomagnetic cell separation is based on the difference in magnetic force operating on a magnetically labeled cell in contrast to the lack of force (or in some cases opposite force) operating on a nonmagnetically labeled cell in the magnetic field. In some systems this force results in a combination of movement as well as a resistive force holding the labeled cells to a surface. Therefore, it is desirable to maximize the magnetic force, and in some systems, the movement difference of magnetically labeled cells and nonmagnetically labeled cells. It has been shown that this force correlates to the efficiency of magnetic labeling on the target cell, which is related to the number of targeted antigen expressed on cell surface and the number of antibodies labeled on the targeted antigens (McCloskey et al, 2000; Comella et al, 2001).
In this study, the expression of activation antigens on alloreactive T cells was quantified and the effect on increasing the magnetophoretic mobility of the magnetically labeled, activated T cells was discussed. In addition, improving the magnetic separation of alloreactive T cells was discussed.

**Definition and measurements of magnetophoretic mobility**

In order to quantify the movement of magnetic cells/particles in a non-uniform magnetic field, magnetophoretic mobility, \( m \), was defined (Reddy et al, 1995; McCloskey, 2001) by:

\[
m = \frac{v_c}{S_m}
\]  

where \( v_c \) represents the velocity of magnetic particle in the magnetic field, and \( S_m \) correlates to the magnetic energy gradient of the field.

The magnetic force acting on a magnetically labeled cell is proportional to the number of magnetic particles labeled on the cells and the magnetic force acting on each particle. For a two-step magnetic labeling, the magnetic force can be expressed as,

\[
F_m = (n_1 \theta_1 \lambda_1) \cdot (n_2 \theta_2 \lambda_2) \cdot \eta_3 \cdot F_b
\]  

where \( n_1 \) is the number of antigen molecules expressed on each cell, \( \theta_1 \) is the fraction of antigen molecules bound by the primary antibody, and \( \lambda_1 \) is the valence of the primary antibody binding, which depends on the specificity of the antigen-antibody binding. In a manner similar to the primary antibody, \( n_2 \), \( \lambda_2 \) and \( \theta_2 \) refer to the properties of the secondary antibody. \( \eta_3 \) represents the number of magnetic
nanoparticles conjugated on each secondary antibody and $F_b$ is the magnetic force acting on each magnetic nanoparticle. $n_1 \theta_1 \lambda_1$ also has analogies to the concept of antibody bind capacity, $ABC$, for a specific antigen expressed on cells. $n_2 \theta_2 \lambda_2 \eta_3$ is equal to the number of magnetic nanoparticles conjugated per primary antibody bound on the cell surface, which can be expressed by $\beta$ as a magnification factor. Thus, the magnetic force acting on each cell, $F_m$, can be expressed as:

$$F_m = ABC \cdot \beta \cdot F_b$$  \hspace{1cm} (5.3)

The magnetophoretic mobility can be solved based on the force balance on a magnetically labeled cell as follows,

$$m = \frac{V_m}{S_m} = \frac{ABC \cdot \beta \cdot F_b}{3 \pi D \eta \cdot S_m} = \frac{\Delta \chi \cdot V_m \cdot ABC \beta}{3 \pi D \eta}$$  \hspace{1cm} (5.4)

where $V_m$ represents the volume of one magnetic nanoparticle, $\Delta \chi$ represents the difference in magnetic susceptibility between the paramagnetic nanoparticle and the suspending fluid. The magnetophoretic mobility of magnetically labeled cells can be experimentally measured by an instrument developed in Chalmers and Zborowski’s lab on a cell-by-cell basis (McCloskey et al, 2001; McCloskey et al, 2000; Nakamura et al, 2001; Moore et al, 2000; Chalmers et al, 1999).

It is indicated from Equation 5.4 and pervious studies (Chalmers et al, 1998; McCloskey et al, 2000; Serke et al, 1998) that the magnetophoretic mobility of a magnetically labeled cell can be increased by upregulating the number of targeted antigens on the cells ($ABC$) and improving the labeling efficiency of primary and secondary antibodies to the targeted antigens (McCloskey et al, 2000).
**Structure of activation antigens**

The performance of magnetic cell separation by QMS depends on the magnetophoretic mobility of the magnetically labeled cells. The antigen-antibody binding properties such as binding valence and binding affinity are important to optimize the magnetophoretic mobility of target cells. These properties are related to the structure of antigens and their antibodies. The structure of activation antigens including CD25, CD69, CD71, HLADR and CD38 are discussed as follows.

CD25 is the α chain of the IL-2 receptor with a molecular weight of 55kDa. There is no CD25 expressed on resting T cells, however, CD25 is upregulated on activated T cells. The binding affinity of CD25 with IL-2 receptor is $10^{-8}$M (Berard et al, 1999). Figure 5.1 shows the structure of CD25 and IL-2 complex (RCSB Protein Data Bank).

![Figure 5.1 Structure of CD25 and IL-2 complex](image)

CD69 is a type II integral membrane protein belonging to the family of C-type lectin receptors (Testi et al, 1994). CD69 is up regulated on T cells during early
activation. As shown in Figure 5.2 (RCSB Protein Data Bank), CD69 is a disulfide-linked homodimer.

Figure 5.2 Structure of CD69 molecule

CD71, the transferrin receptor, is upregulated on cells upon activation and proliferation. It is essential for iron transport into proliferating cells. Figure 5.3 shows the structure of CD71 and transferrin complex (RCSB Protein Data Bank).

Figure 5.3 Structure of CD71 and transferrin complex
HLA-DR is a human class II molecule of the major histocompatibility complex (MHC). HLA-DR is a transmembrane glycoprotein composed of an α chain (36 kD) and a β subunit (27 kD), which is expressed primarily on antigen presenting cells: B cells, monocytes, macrophages, and the thymic epithelial cells. HLA-DR is also expressed on activated T cells. The structure of human HLA-DR3 molecule is shown in Figure 5.4(RCSB Protein Data Bank).

As shown in Figure 5.5 (RCSB Protein Data Bank), CD38 is a single chain type II transmembrane molecule composed of 300 amino acids. The CD38 antigen is expressed on activated T lymphocytes, and natural killer (NK) lymphocytes.
Quantification of antibody binding capacity (ABC)

As stated previously, the ABC is an important parameter to maximize during magnetic cell labeling. The ABC of cells depends on the cell property and antigen characteristics, and the ABC of cells can be quantified using Quantum Simply Cellular® (QSC) assay or QuantiBRITE® assay (Comella, 2001). The Quantum Simply Cellular (QSC) beads contain different populations of beads, each population having different, calibrated quantities of goat-anti-mouse IgG conjugated to the bead surface. Therefore, to determine the ABC of a cell population, the antibody targeting the specific cell surface marker of interest must be of mouse origin. Next, the QSC beads, labeled with the antibody fluorochrome conjugate of interest, should be analyzed at the same time that the labeled cells are analyzed. The antibody binding capacity (ABC) of the antigen expressed on cells can be then quantified by comparing the fluorescence intensity of cells and that of QSC beads.
The QuantiBRITE beads are composed of four populations of beads with a different, known number of PE molecules bound to each population, the range being typical for lymphocyte surface antigens. The fluorescence intensity histogram of a typical QuantiBRITE “kit” is shown in Figure 5.6, demonstrating the four distinct populations of different PE molecules. A standard curve (Figure 5.7) is subsequently made by plotting log_{10}(geometry mean of Fluorescence intensity) versus log_{10}(mean PE molecules per bead) of each beads population. By fitting the geometry mean fluorescence intensity of the specific antibody labeled on cells to the standard calibration curve, the antibody binding capacity (ABC) of the cells can be quantified (Note: The QuantiBRITE assay has to be performed at the same time that the cells are analyzed.).

Figure 5.6 Fluorescence intensity distribution of QuantiBRITE Beads
Calibration Curve

\[ y = 1.0054x - 1.1775 \]

\[ R^2 = 0.9998 \]

Figure 5.7 Standard calibration curve for ABC quantification

It has been reported that Quantum Simply Cellular assay over-estimate the ABC. A number of factors have been suggested for the discrepancy, including labeling conditions such as temperature, incubation time, and concentration (Comella, 2001; Serke et al, 1998). Therefore, QuantiBRITE assay was used in this study.

5.2 Material and Methods

Cell Preparation

Human peripheral leukocytes were ordered from the American Red Cross, Central Ohio Region. The peripheral blood mononuclear cell (PBMC) fraction was isolated and separated by Ficoll-Hypaque density gradient centrifugation. The cells were washed with PBS twice and resuspended in AIM-V supplemented with streptomycin sulfate (50 µg/ml), gentamicin sulfate (10 µg/ml), L-glutamine (2 mM), and 10%
pooled human AB Serum. Size distribution of the peripheral blood mononuclear cells was determined using a Coulter Counter Multisizer II® (Beckman Coulter, Miami, FL). The number of peripheral blood mononuclear cells was determined using a hemacytometer.

**Pretreatment of recipient cells**

Cells were cultured in RPMI supplemented with streptomycin sulfate (50 µg/ml), gentamicin sulfate (10 µg/ml), L-glutamine (2 mM), and 10% pooled human AB Serum at a concentration of 5x10^6 cells/ml. Different doses of IFN-γ (500-2000 U/ml) and TNF-α (500-2000 U/ml) were added into the culture. After 24 hours, the cells were harvested, washed and irradiated (30 Gy). Then the cells were ready for the mixed lymphocytes reaction.

**One-way mixed lymphocytes reaction**

A one-way mixed lymphocytes reaction (MLR), involving two unrelated HLA non-identical donors (A and B), was used in this study. The responder PBMC (A) were incubated at 37°C with the same number of irradiated (30 Gy) stimulator PBMC (B*). All cultures were performed in 12-well tissue culture plate (BD) at a concentration of 3x10^6 cells/ml with a final volume of 2 ml. For control stimulations, the responder PBMC (A) were incubated with the same number of irradiated (30 Gy) autologous PBMC (A*) at a concentration of 3x10^6 cells/ml with a final volume of 2 ml.
Cell labeling for flow cytometry

Cells were harvested from tissue culture plates or flasks, washed twice with PBS, and counted. 0.5x10⁶ or 1x10⁶ cells were transferred to the 75x12 mm polypropylene tubes. Appropriate antibodies for the specific experiment were added to the cell suspension as recommended by the manufacturer. The total incubation volume was brought up to 100 µl. Then the sample tubes with antibodies were incubated in the dark at 4 °C for 30 minutes. After labeling, the cells were washed twice with labeling buffer (PBS+0.5% BSA+2 mM EDTA). If the samples were not to be analyzed at the same day, 1 ml fixing solution (2% paraformaldehyde solution) was added for 1x10⁶ cells. The samples can then be stored at 4 °C in the dark for up to one week.

Magnetic cell labeling for CTV or QMS

After the cells were labeled with appropriate fluorescence conjugated antibodies, the cell samples were washed twice with labeling buffer. Then appropriate antibody-magnetic beads conjugates were added into the sample tubes. The total volume was kept at 100 µl or 200 µl based on the amount of antibody added. The sample tubes were then incubated in the dark at 4 °C for 30 minutes. After labeling, the cells were washed twice, then the cell samples were suspended in labeling buffer at different concentration based on the requirement of future experiments.

Flow cytometry analysis

Unstained and appropriate single fluorescence stained samples were run first to adjust the voltage setting and compensation of the flow cytometer. Then the isotype
control samples and tested samples were processed by CellQuest; 10,000 events were collected for most samples. For the ABC study, 10,000 QuantiBRITE beads (BD Pharmingen) were acquired and used as standard.

**Magnetophoretic mobility analysis by CTV**

After magnetic labeling, the cell samples were suspended in labeling buffer and pumped into the CTV channel, and, 20-30 sets of images were generally recorded. The images were analyzed using the image processing software from which, the magnetophoretic mobility distributions of cells were calculated.

**5.3 Results**

**Quantification of activation antigen expression on alloreactive T cells**

The expression of different activation antigens (CD25, CD69, CD71, OX40 and HLADR) on alloreactive T cells was quantified by flow cytometric analysis.

Figure 5.8 compares the number of CD3, CD25, CD69, CD71, OX40, and HLADR molecules expressed on alloreactive T cells. Compared with CD3, a pan T cell surface antigen, the expression of activation antigens on alloreactive T cells is low. The average number of CD25, CD71, OX40 and HLADR expressed on alloreactive T cells are less than 10,000 molecules/ cell, which is about 4-5 folds lower than the number of CD3 molecules expressed on alloreactive T cells. The expression of CD69 molecule on alloreactive T cells is relative high with about 15,000 molecules/ cell.
Figure 5.8 Comparison of expression of different activation antigens on alloreactive T cells

**Optimize the magnetic labeling process**

The performance of magnetic cell separation depends on the difference in magnetophoretic mobility between magnetically labeled and unlabeled cells. Successful control of the magnetophoretic mobility of magnetically labeled cells is one main objective of magnetic cell sorting using QMS. Theoretically, the higher is the difference in magnetophoretic mobility between magnetically labeled and unlabeled cells; the better is the magnetic cell separation.

In order to maximize the magnetophoretic mobility of magnetically labeled alloreactive T cells, HLADR was chosen as a target antigen for labeling, and a single experiment was performed to compare different labeling conditions. Figure 5.9 indicates that increase the concentration of the primary antibody, at a constant
secondary antibody concentration (300 µl/ml anti-PE magnetic beads) may result in the HLADR+ having a higher magnetophoretic mobility. Moreover, as indicated in Figure 5.10, the magnetic mobility of HLADR+ cells may also be increased with a fixed concentration of anti-HLADR PE and higher concentration anti-PE magnetic beads used during labeling.

It is indicated from this study that in a two-step labeling process, the magnetic mobility of target cells may be optimized by increasing ABC of specific surface antigens (for alloreactive T cells, in some cases, this involves the use of specific media additives, i.e. IFN-γ, and the concentration of the primary antibody and secondary antibody for labeling. The requirement of magnetic cell separation, depletion efficiency and target cell recovery, is important to optimize parameters for magnetic cell labeling process.

![Primary Ab Saturation Curve](image)

**Figure 5.9** The effect on primary antibody concentration of magnetic mobility of cells
Increase the magnetophoretic mobility of “dim” expression cell

One of the fundamental parameters to determine the performance of immunomagnetic cell separation (such as QMS) is the difference in magnetophoretic mobility between magnetically labeled cells and unlabeled cells. Figure 5.11 shows the dot plots of CD25 expression before MLR, after MLR before CD25$^+$ cell depletion and after CD25$^+$ cell depletion using QMS respectively. Key to future clinical applications is the ability to not only remove all the “bright” cells but also “dim” cells; i.e. the low expressing cells remaining in Figure 5.11 b. Therefore, increasing the magnetophoretic mobility of “dim” expressing cells is crucial for improving the performance of QMS separation. Since the number of target antigens expressed on cell surface is very low for the “dim” expressing cells, in order to
increase the magnetophoretic mobility of those cells, it is required to label more magnetic beads or beads with a higher “field interaction parameter”.

Figure 5.11 CD25 expression a. before MLR b. after QMS depletion c. after MLR and before QMS depletion

A number of strategies to increase magnetophoretic mobility of “dim” cells will now be presented. Firstly, and most fundamentally, increasing the density of the expression of the targeted surface antigen on the targeted cells would potentially remove all the alloreactive T-cells (i.e. make all the cells bright). In the process of generating alloreactive T cell by MLR, it has been shown previously that pretreatment of stimulator cells with IFN-γ and TNF-α before MLR increases the expression of some activated antigens such as CD69. (as described in Chapter 2) In Figure 5.12, histogram a, b and c show the expression of CD69 before MLR, and after 4 days’ MLR without stimulator pretreatment, after 4 days’ MLR with 500 U/ml IFN-γ
pretreatment of stimulators, respectively. The CD69 expression can be improved significantly by pretreating stimulators with 500 U/ml IFN-γ.

Secondly, by applying different magnetic labeling protocols, the amount of magnetic beads/particles labeled on cell surface could be increased so that the magnetophoretic mobility of target cells could be increased. As shown in Figure 5.13, the most commonly used labeling protocols include one-step labeling and two-step labeling. In one-step labeling process, the target antigens on cell surface are labeled with magnetic particle conjugated antibodies directly; while in two-step labeling process, the target antigens on cell surface are labeled with fluorescence conjugated antibodies and magnetic particle conjugated antibodies sequentially. The amount of magnetic particles labeled on target cell surface with different labeling protocols is different. It is reported that the magnetophoretic mobility of CD3⁺ cells could be increased to about 10-20 folds by using two-step labeling protocol (anti-CD3 PE, followed by anti-PE Imag-DM) instead of one-step labeling protocol (anti-CD3
Imag-DM) (Tong, 2005). Figure 5.14 (a) shows the increased fold of CD3$^+$ cells’ magnetophoretic mobility by using two-step labeling protocol instead of one-step labeling protocol; and Figure 5.14 (b) compares the magnetophoretic mobility of CD3$^+$ cells by using one-step labeling protocol and two-step labeling protocol.

Figure 5.13 a. One-step magnetic labeling b. Two-step magnetic labeling

Figure 5.14 (a). Increased fold of CD3$^+$ cells’ magnetophoretic mobility by using two-step labeling protocol than one-step label protocol (b). Comparison of magnetophoretic mobility of CD3$^+$ cells by using one-step labeling protocol and two-step labeling protocol
Thirdly, the co-expression between different activation surface antigens on activated cells could be used to increase the magnetophoretic mobility by labeling those activation antigens simultaneously. The strategy of two-step QMS separation could be investigated by using different separation parameters (such as flow rate, flow rate ratio in different outlet and inlet) in two steps for “bright” cells and “dim” cells respectively.

Fourthly, the use of antibody-magnetic conjugates with a higher field interaction parameter also can increase the magnetophoretic mobility of targeted cell population (Zhang et al, 2005).

5.4 Discussion

Immunomagnetic cell separation technique has been used widely in biological research and clinical application, the separation is based on the difference in magnetic force operating on magnetically labeled cells in contrast to the lack of force operating on the nonmagnetically labeled cells in the magnetic field. In order to improve the separation efficiency, it is desirable to maximize the movement difference (inflected by magnetophoretic mobility) of magnetically labeled cells and nonmagnetically labeled cells.

The antibody binding capacity (ABC) of target cells for specific antigen is correlated to the amount of magnetic-conjugated antibodies labeled on target cells, which is important for optimizing the magnetophoretic mobilities of these targeted magnetically labeled cells. In this study the ABC of activation antigens (including
CD25, CD69, CD71, OX40 and HLADR) expressed on alloreactive T cells was quantified by QuantiBRITE assay. Compared with other common antigens targeted for cell separation (e.g. CD3), the expression level of activation antigens (CD25, CD69, CD71, OX40 and HLADR) on alloreactive T cells is low. In order to improve the alloreactive T cell depletion efficiency, different methods were discussed in this study to increase the magnetophoretic mobility of magnetically labeled alloreactive T cells. It is indicated that pretreatment of recipient cells with IFN-γ before mixed lymphocyte reaction can increase the ABC of CD69 expressed on alloreactive T cells. Furthermore, the ABC can be increased by increasing the concentration of antibody during labeling process.

It is suggested from this study that optimizing the magnetic labeling process for alloreactive T cells is necessary for improve the performance of QMS in alloreactive T cell depletion (e.g. depletion efficiency and cell recovery), and it is feasible to increase the magnetophoretic mobilities of magnetically labeled alloreactive T cells by increasing ABC of activation antigen (e.g. CD69) and optimizing the magnetic labeling process (e.g. antibody combinations, antibody concentrations)
CHAPTER 6

COMPARISON OF DIFFERENT CELL FREQUENCY ANALYSIS APPROACHES

6.1 Motivation

Alloreactive T cell depletion or T cell depletion prior to allogeneic stem cell transplantation abrogates graft versus host disease (GvHD) associated with the transplantation. It has been reported that the incidence of GvHD development after transplantation is correlated to the degree of alloreactivity depletion. Therefore, it is crucial to accurately, quantitatively determine the degree of alloreactivity depletion achieved by an alloreactive T cell depletion process. Flow cytometry is a powerful tool in research and clinical application for the diagnosis, isolation and study of specific cell types and disease conditions. The determination of specific cell types by flow cytometric analysis is based on detecting the unique surface antigen expressed on the targeted cells. For example, CD3, a pan T cell antigen, can be used to differentiate T cells from other cell types. Beyond surface marker identification, functional assays can also be used to detect and quantify specific cell types. For example, the limiting dilution assay (LDA) was developed to quantify the frequency
of immuno-reactive cells in a homogeneous cell suspension. In order to determine the frequency of cells with different immune functions, various experimental methods have been developed by combining limiting dilution assay and relevant detection methods.

In this chapter, flow cytometric analysis with different gating strategies were compared; and limiting dilution assay and flow cytometric analysis were compared for their quantification of T cell and alloreactive T cell frequency.

**Limiting dilution assay**

The limiting dilution assay (LDA) was developed to quantify the frequency of immuno-reactive cells in a homogeneous cell suspension. The basis of LDA is to quantify immuno-reactive cells by detecting an all-or-nothing (positive or negative) immune response in each individual culture within groups of replicate cultures that vary in the dose of cells to be tested. Taswell developed a statistical method for the analysis of experimental data from limiting dilution assays in the early 1980s. The single hit model was assumed that every single immuno-reactive cell can generate a detectable positive response while other cells and culture factors are at non-limiting doses (Taswell, 1981). When all dilutions of the tested cells are homogeneous suspensions, the Poisson probability distribution can be used to correlate the number of tested cells in each replicate culture group and the average number of immuno-reactive cells in the culture as follows (Taswell,1981),

$$P_c(X) = \frac{(\varphi X)^c e^{-\varphi X}}{c!}$$  \hspace{1cm} 6.1
where $X$ represents the average number of tested cells in each culture group and $c$ represents the number of immuno-reactive cells in the tested cell populations. $P_c(X)$ represents the probability that the culture receives any number $c$ of immune responsive cells. For the single-hit model, a positive immune response is not quantitative (i.e. a distinction can not be made to distinguish between the presence of one or more than one immune-reactive cell). However, in the absence of an immune responsive cell, no immune response can occur. Therefore, the Poisson equation has to be applied in describing the relation between the average number of cells tested per replicate culture and the number of negatively responding cultures per group as follows,

$$X_{\text{neg}} \times \exp(X) = e^{-\phi X} \tag{6.2}$$

In order to quantify the immuno-reactive cell frequency accurately, Taswell chose four different statistical methods including weighted averaging, least squares, likelihood maximization, and $\chi^2$ minimization to estimate the frequency of immuno-reactive cells in a cell population. When the estimated results by different statistical methods were compared with original experimental data, it was shown that the $\chi^2$ minimization method has the minimum bias and variance; maximum accuracy and precision (Taswell, 1981).

Various experimental methods have been developed by the combination of limiting dilution assay and related detection methods. For example, the quantification of T Helper cell frequency can be made by measuring the IL-2 secretion doses on the basis of limiting dilution assay. If the IL-2 secreted by the culture is greater than mean plus
3 times standard deviation of the control culture, then that culture is defined as positive. The frequency of T helper cell can be calculated using $\chi^2$ minimization analysis based on the number of positive culture in each dilution of cell suspension. By culturing cell suspension (cells to be measured) with $^{51}$Cr-labeled target cells together, the frequency of cytotoxic T cells in the cell suspension can be quantified on the basis of $^{51}$Cr release. If the culture with $^{51}$Cr release exceeds the mean spontaneous release by 3 times standard deviations, the culture could be considered as positive. Finally, the frequency of proliferating T cells can be quantified by measuring the $^3$H or BrdU incorporation by the cultures at various dilutions of the tested cell suspension.

**Flow cytometry**

Flow cytometry was developed to quantify cellular subsets by optical means. The cytometer typically measures several parameters simultaneously for each cell including forward scatter intensity which is approximately proportional to cell diameter; side (90 degrees relative to the incident laser beam) scatter intensity which is approximately proportional to the degree of granularity of the cells; and fluorescence intensities at several wavelengths which is either intrinsic to the cells or as a result of the labeling of the cells with specific chemical dyes. Light scatter (forward and side scatter) can be used to exclude dead cells, cell aggregates and cell debris, and it can also be used to distinguish specific cell subpopulation from a cell mixture. Lymphocytes can be differentiated from other peripheral blood mononuclear
cells in a blood leukocyte sample. Antibodies, conjugated to fluorescent molecules, can be used to target specific cell surface antigens. Intracellular components including DNA, specific nucleotide sequences in DNA or mRNA can also be measured by flow cytometry.

With the continued development of flow cytometry hardware, software and fluorescent dyes, it is now possible to perform multiple parameter flow cytometric analysis more accurately and quantitatively. It has been reported that up to 11 distinct fluorescence’s signals, besides forward scatter and side scatter can be measured simultaneously (Baumagarth et al, 2000). Such multiple-color flow cytometry analysis can be used to define new cell subsets and quantify rare cell populations more accurately.

In order to enhance the accuracy of multiple-color flow cytometric analysis, several factors need to be considered including hardware, software and fluorochromes. The BD FACSDiVa or FACSARia software/hardware platforms can digitize the light signals from the system at a rate of 10 million times per second into 16384 discrete levels. Therefore, the use of logarithmic amplifiers is not required and subsequent, well documented inaccuracies introduced by the logarithmic amplifiers can be eliminated (Baumagarth et al, 2001). In addition, BD FACSDiVa provides an improved compensation method using an automatic matrix inversion removing the need to “compensate” before the actual analysis begins. In regard to the choice of fluorochromes, using “bright” fluorochromes for the low-expressed and
non-identified cell subsets has been suggested (BD Bioscience). The FACSARia flow
cytometer supplement with FACSDiVa software was used to perform multiple-color
flow cytometric assay in this study.

6.2 Material and Methods

Cell Preparation

Human peripheral leukocytes were ordered from the American Red Cross, Central
Ohio Region. The peripheral blood mononuclear cell (PBMC) fraction was isolated
and separated by Ficoll-Hypaque density gradient centrifugation. The cells were
washed with PBS twice and resuspended in AIM-V supplemented with streptomycin
sulfate (50 µg/ml), gentamicin sulfate (10 µg/ml), and L-glutamine (2 mM). Size
distribution of the peripheral blood mononuclear cells was determined using a Coulter
Counter Multisizer II® (Beckman Coulter, Miami, FL). The number of peripheral
blood mononuclear cells was determined using a hemacytometer.

One-way mixed lymphocytes reaction

A one-way mixed lymphocytes reaction (MLR), involving two unrelated HLA
non-identical donors (A and B), was used in this study. The responder PBMC (A)
were incubated at 37°C with the same number of irradiated (30 Gy) stimulator PBMC
(B*). All cultures were performed in 12-well tissue culture plate (BD) at a
concentration of 3x10^6 cells/ml with a final volume of 2 ml. For control stimulations,
the responder PBMC (A) were incubated with the same number of irradiated (30 Gy)
autologous PBMC (A*) at a concentration of 3x10^6 cells/ml with a final volume of 2 ml.

**Cell labeling for flow cytometry**

Cells were harvested from tissue culture plates or flasks, washed twice with PBS, and counted. 0.5x10^6 or 1x10^6 cells were transferred to the 75x12 mm polypropylene tubes. Appropriate antibodies for the specific experiment were added to the cell suspension as recommended by the manufacturer. The total incubation volume was brought up to 100 µl. Then the sample tubes with antibodies were incubated in the dark at 4 °C for 30 minutes. After labeling, the cells were washed twice. Basically, 2 ml of labeling buffer (PBS+0.5% BSA+2 mM EDTA) was added into each sample tube and the sample tubes were centrifuged at 300 g, 4 °C for 8 minutes. After centrifugation, the supernatant was discarded and another wash step was repeated. If the samples were not to be analyzed on the same day, 1 ml fixing solution (2% paraformaldehyde solution) was added for 1x10^6 cells. The samples can then be stored at 4 °C in the dark for up to one week. The samples labeled with viability dye 7AAD were analyzed at the same day of labeling sample. FACSARia flow cytometer and FACSDiva software was used to collect 100,000 events for each sample.

**Alloreactive T cell depletion**

Cells were harvested after four days’ mixed lymphocytes reaction and washed once with PBS. The cell aliquots with 2x10^7 cells/ml were prepared, and labeled with 50 µl PE conjugated antibodies for CD25; CD25 and CD69; CD25, CD69 and
HLADR; CD25, CD69, HLADR and CD38 respectively at 4 °C for 30 minutes in the dark. Then the cell samples were washed with labeling buffer twice and labeled with 100µl anti-PE MACS microbeads at 4 °C for 30 minutes in the dark. After labeling, the cells were washed with labeling buffer twice and resuspended in 4 ml labeling buffer. The cell suspension were passed through MACS LD column mounted in a SuperMACS (Miltenyi Biotech), the nonmagnetic cell fraction was collected and used for setting up limiting dilution assay.

**Limiting dilution assay**

I. Quantification of alloreactive T cell frequency

Responder (also referred as donor) cells (A) were cultured in a 96-well U bottom tissue culture plate at a final volume of 100 µl with $10^5$ irradiated (30 Gy) allogeneic stimulator (also referred as recipient) cells(B*). In the control culture, the responder cells (A) at the corresponding cell number were cultured with $10^5$ irradiated (30 Gy) autologous cells (A*). The concentrations of responder cells ranged from $10^4$ to 10 cells/well for non-depleted cell sample including four dilutions of $10^4$, $10^4$, $10^2$ and 10 cells/well. For depleted cell sample, the responder cell concentrations ranged from $10^6$ to 1000 cells/well including eight dilutions of $10^6$, $10^5$, $10^4$ and $10^3$ cells/well. 24 replicate cultures were set up for each dilution. The culture was incubated at 37 °C with 5% CO$_2$, and 50 µl culture medium was fed for each well on the 6th and 12th day. On the 14th day, the culture was tested for proliferating cell frequency by BrdU
incorporation assay. It is assumed that the proliferating cell frequency corresponds to the host-specific alloreactive T cell frequency.

II. Quantification of T cell frequency

Cells (A) were cultured in a 96-well U bottom tissue culture plate at a final volume of 100 µl and stimulated $10^5$ irradiated (30 Gy) feeder cells (B*). In the control culture, only $10^5$ irradiated feeder cells (B*) were cultured. The concentrations of responder cells ranged from $10^3$ to 1 cells/well for non-depleted cell sample including four dilutions of $10^3$, $10^2$, 10 and 1 cells/well. 24 replicate cultures were set up for each dilution. The culture was incubated at 37 °C with 5% CO$_2$, and 50 µl culture medium was fed for each well on the 6th and 12th day. On the 14th day, the culture was tested for proliferating cell frequency by BrdU incorporation assay. The proliferating cell frequency corresponds to the T cell frequency.

**BrdU proliferation assay**

On the 13th day of limiting dilution assay, the cells were labeled with BrdU at a concentration of 10 mM and incubated at 37 °C with 5% CO$_2$ for 18-24 hours. On the 14th day, the cells were centrifuged at 300 g for 10 minutes and the labeling medium was removed. The cells were dried using a hair-dryer for approximately 15 minutes. 200 µl/well FixDenat solution was next added to the cells and the suspension was incubated for 30 minutes at 37°C. After the incubation, the FixDenat solution was removed, 100 µl/well anti-BrdU-POD solution was added and the suspension was incubated for 90 minutes at 37°C with 5% CO$_2$. Then the extra anti-BrdU-POD
solution was removed and each well was rinsed with 200 µl-300 µl washing solution for three times. After the washing, 100 µl/well substrate solution was added to each well. Finally, the absorbance at 370 nm was measured when the color was developed.

6.3 Results

Comparison of different gating strategies on cell analysis by flow cytometric assay

I. Effect of fluorescent dyes for viability and nuclei dye on analysis

Setting up appropriate gates is crucial for analyzing different cell populations by flow cytometry. The gates set up by traditional gating strategy are based on parameters including Forward Scatter and Side Scatter. These two parameters are related to different properties of cells including size (Forward Scatter) and granularity (Side Scatter). For cells from human peripheral blood, the healthy lymphocyte gate (R1) was empirically set as shown in Figure 6.1 “g” (Koh et al, 1999; Givan et al, 2001).

With the availability of FACS Aria, a sever-color benchtop flow cytometer, it is possible to develop a new gating strategy to analyze nuclei, viability and other surface antigens of cells simultaneously.

A newly developed far-red dye, DRAQ5, has the ability to penetrate the outer membrane of cells, which can be used to discriminate nucleated cells from debris (Givan et al, 2001). 7AAD is a fluorescent dye for DNA analysis, which is permeable to only dead cells (Givan et al, 2001). By labeling cells with DRAQ5 and 7AAD, the live, nucleated cells can be selected without applying the empirical gating strategy.
The comparison of two gating strategies (empirical strategy and new strategy) is shown in Figure 6.1. Figure 6.1 “a” “b” and “c” shows the histogram of the single color positive control for DRAQ5, 7AAD and PE-Cy7 respectively, which were used to set up appropriate gates for analysis. Figure 6.1 “d” indicates that 82.94% of the events collected by flow cytometry are cells (DRAQ5+) (the DRAQ5 gate, R1 (red dots), was set up by single color control as shown in Figure 6.1 “a”). As Figure 6.1 “e” shown, 7AAD was used to differentiate live cells from dead cells, and 56.02% of the events are live cells (7AADDRAQ5+) (the 7AAD gate, R2 (green dots), was set up by single color control as shown in Figure 6.1 “b”). Figure 6.1 “f” shows that there are 54.35% of the events are live T cells (7AADDRAQ5+CD3+) (the CD3 gate, R3 (blue dots), was set up by single color control as shown in Figure 6.1 “c”). Figure 6.1 “g” presents the lymphocytes gate based on Forward Scatter and Side Scatter (R1 (red dots) is the empirically healthy lymphocytes region), and 51.65% of the events are included in the empirical gate. As shown in Figure 6.1 “h”, there are 49.86% of the events are healthy CD3+ T cells based on the empirical gate shown in Figure 6.1 “g”. Figure 6.1 “i” shows the expression of 7AAD and DRAQ5 on the empirically gated CD3+ T cells, it is indicated that there are 1.74% of the events are either debris or dead cells, and these events were considered as healthy CD3+ T cells by the empirical gating strategy). Overall, I suggest that the new gating strategy provides a more accurate method to define cell populations.
II. Comparison of two flow gating strategies: Forward scatter/Side scatter gate versus Side scatter/CD45\(^{+}\) gate

In this study, two different gating strategies for lymphocytes were compared for the quantification of alloreactive T cell activation. By using the first strategy, the lymphocytes were gated based on their Forward Scatter and Side Scatter. By using the
second strategy, the lymphocytes were gated based their Side Scatter and CD45 expression. CD45 is a surface antigen expressed on leukocytes, therefore, the CD45+ gate include all the peripheral blood mononuclear cells. Figure 6.2, 6.3 and 6.4 show the comparison of CD25+, CD69+ and HLADR+ alloreactive T cell activation on the third and fourth day of mixed lymphocytes reaction analyzed by these two different gating strategies.

It is indicated from these figures that a difference exists in quantification of alloreactive T cells between these two strategies. In these figures, the activated cell percentage represents the percent of activated T cells expressing different activation antigens in lymphocytes. A difference exists in lymphocytes fraction gated by these two strategies. In order to quantify the activated T cells accurately, viability dye 7AAD, CD45 (surface antigen for leukocytes) and CD3 (surface antigen for T cells) can be stained to determine the percentage of CD3+ T cells in leukocytes, and the cell number can be counted by flow cytometer (Brando et al, 2001), then the number of activated T cells expressing different activation antigens can be quantified.
Figure 6.2 Comparison of CD25⁺ T cell activation (on the 3rd and 4th day of MLR) analyzed by two lymphocyte gating strategies.

Figure 6.3 Comparison of CD69⁺ T cell activation (on the 3rd and 4th day of MLR) analyzed by two lymphocyte gating strategies.
CD3⁺HLADR⁺ cell activation

![Graph showing CD3⁺HLADR⁺ cell activation](image)

Figure 6.4 Comparison of HLADR⁺ T cell activation (on the 3rd and 4th day of MLR) analyzed by two lymphocyte gating strategies

**Comparing flow analysis versus limiting dilution assay**

I. Determine T cell frequency

The number of T cells in peripheral blood mononuclear cells was quantified by flow cytometric assay and limiting dilution assay (LDA); respectively, in this study. Table 6.1 shows the comparison of T cell frequency in peripheral blood mononuclear cells quantified by these two methods. During the flow analysis, the cells were only stained by CD3 antibody without viability dye such as 7AAD, and the peripheral blood mononuclear cells was gated based on forward scatter and side scatter expression. Figure 6.5 presents the percentage of CD3⁺ cells analyzed by flow cytometry. As shown in Table 6.1, the T cell frequency determined by LDA is slightly lower than that determined by flow cytometric assay. For the determination
of T cell frequency by LDA, the result is very sensitive to the culture environment (the detection is based on T cell growth in 14 days of culture). Therefore, the possibility of underestimating frequency of specific cell population by LDA is higher. It has been reported that the frequency of CD8\(^+\) antigen specific cell was underestimated by as much as 500 folds with LDA (MiMichael et al, 1998).

Comparing these two methods, flow cytometric analysis is simple, fast and sensitive. In order to increase the accuracy of flow cytometric analysis, viability dye such as 7AAD and nucleated dye such as DRAQ\(^5\) and DAPI can be used. Limiting dilution assay can be combined with related detection methods to quantify the frequency of immune competent cells with specific immune reactivates. For example, the frequency of proliferating cells can be determined by measuring the BrdU or \(^{3}\)H incorporation; the frequency of cytotoxic cells can be determined by measuring the \(^{51}\)Cr release from \(^{51}\)Cr pre-labeled targeted cells, the frequency of Helper T cells can be determined by measuring the secretion of IL-2. Limiting dilution assay is important for determining the frequency of cells with specific immune functions because it is easy to be combined with other detection methods.

In conclusion, experimental requirements are crucial to determine which method will be used. In order to quantify the percent of T cells in a cell mixture, flow cytometric analysis is a good option. However, limiting dilution assay (functional test) is better for testing the safety of the T-cell-depleted cell product for stem cell transplantation.
Table 6.1 Comparison of T cell frequency analyzed by LDA and flow cytometric assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency</td>
<td>1 in 3 (33.33%)</td>
<td>1 in 4 (25.00%)</td>
<td>1 in 9 (11.11%)</td>
</tr>
<tr>
<td>P</td>
<td>0.837</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Chi Square</td>
<td>2.773</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>d.f.</td>
<td>6</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>95% range</td>
<td>2~4</td>
<td>2~7</td>
<td>5~15</td>
</tr>
<tr>
<td>Flow analysis CD3⁺ (%)</td>
<td>38.17</td>
<td>24.68</td>
<td>18.02</td>
</tr>
</tbody>
</table>

II. Determine alloreactive T cell frequency

In this study, the frequency of alloantigen reactive T cell was determined by the combination of limiting dilution assay and BrdU incorporation assay. As shown in
Figure 6.6, the percent of activated T cells expressing activation antigens CD25, CD69 and HLADR in peripheral blood mononuclear cells (PBMC) was determined by flow cytometric analysis with 7AAD as viability dye. Table 6.2 shows the comparison of alloreactive T cell frequency analyzed by flow cytometric assay and limiting dilution assay. As shown in Table 2, the frequency of alloreactive T cells is much lower than the activated T cell percentage determined by flow cytometric analysis. The first reason is that some cells expressing activated antigen such as CD25, CD69 and HLADR probably are not alloreactive. Secondly, the result of LDA is very sensitive to the culture condition and MiMichael et al (1998) reported that the frequency of alloantigen-reactive T cell was underestimated by LDA (MiMichael et al, 1998). The alloantigen reactive T cell frequency determined by LDA in this study is similar as reported with a frequency range of 1/1000 to 1/10000 (Hernandez-Fuentes et al, 2002; Bishara et al, 1994).

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency</td>
<td>3138</td>
<td>1928</td>
<td>2992</td>
</tr>
<tr>
<td>(0.03%)</td>
<td>0.03%</td>
<td>0.05%</td>
<td>0.03%</td>
</tr>
<tr>
<td>P</td>
<td>0.184</td>
<td>0.259</td>
<td>0.305</td>
</tr>
<tr>
<td>Chi Square</td>
<td>0.98</td>
<td>2.702</td>
<td>2.373</td>
</tr>
<tr>
<td>d.f.</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>95% range</td>
<td>1407–7003</td>
<td>1184–3138</td>
<td>1870–4789</td>
</tr>
<tr>
<td>Flow analysis CD25⁺ (%)</td>
<td>4.65</td>
<td>17.23</td>
<td>8.42</td>
</tr>
<tr>
<td>Flow analysis CD69⁺ (%)</td>
<td>7.35</td>
<td>11.19</td>
<td>3.78</td>
</tr>
<tr>
<td>Flow analysis HLADR⁺ (%)</td>
<td>4.39</td>
<td>5.02</td>
<td>4.23</td>
</tr>
</tbody>
</table>

Table 6.2 Comparison of alloantigen reactive T cell frequency analyzed by LDA and flow cytometry
III. Frequency of alloantigen reactive T cells after alloreactive T cell depletion

In this study, the log depletion of alloreactivity after immunomagnetic depletion of cell subsets expressing different combinations of activation antigens including CD69, CD25, HLADR and CD38 was determined by LDA and flow cytometric assay respectively. Figure 6.7 shows the expression of CD25, CD69, HLADR and CD38 on cells before and after alloreactive T cell depletion. The frequencies of alloantigen reactive T cells after and before alloreactive T cell depletion were also determined by LDA. Table 6.3 compares LDA and flow cytometric assay for analyzing the log depletion of alloreactivity achieved after alloreactive T cell depletion respectively. It
is indicated from the LDA results that residual alloreactivity in cells after alloreactive T cell depletion is decreased gradually with targeting more activation antigens for depletion. Briefly, 1.28 log depletion of alloreactivity was achieved after depleting CD25$^+$ cells after four days’ mixed lymphocyte reaction; 2.11 log depletion of alloreactivity was achieved after targeting both CD25 and CD69 for depletion; and by depleting CD25$^+$, CD69$^+$ and HLADR$^+$ cells, 2.94 log depletion of alloreactivity was obtained. However, the flow cytometric assay only provides the percentage of cells expression each activation antigen before and after depletion, the immune functions of each cell expressing different activation antigens can not be determined. The log depletion of alloreactivity calculated based on flow cytometry assay only reflects the degree of cell depletion, which is not an indication of immune functions of residual cells. Therefore, the log depletion of alloreactivity calculated on the basis of flow cytometric assay is similar for different depletions.
Figure 6.7 Expression of CD25, CD69, HLADR and CD38 on cells before and after alloreactive T cell depletion (Continued)
Figure 6.7 (Continued)

<table>
<thead>
<tr>
<th>Cells</th>
<th>Log depletion of alloreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDA</td>
</tr>
<tr>
<td>Cells before alloreactive T cell depletion</td>
<td>1.28</td>
</tr>
<tr>
<td>Residual cells after CD25⁺ cell depletion</td>
<td>2.11</td>
</tr>
<tr>
<td>Residual cells after CD25⁺ and CD69⁺ cell depletion</td>
<td></td>
</tr>
<tr>
<td>Residual cells after CD25⁺, CD69⁺ and HLADR⁺ cell depletion</td>
<td>2.94</td>
</tr>
<tr>
<td>Residual cells after CD25⁺, CD69⁺, HLADR⁺ and CD38⁺ cell depletion</td>
<td>2.94</td>
</tr>
</tbody>
</table>

Table 6.3 Comparison of LDA and flow cytometry in determination of alloreactivity depletion
6.4 Discussions

In order to develop a successful strategy for selective alloreactive T cell depletion to abrogate GvHD and decrease rates of viral infections and relapse after allogeneic stem cell transplantation, it is critical to develop effective assays quantifying the recipient-tolerance and third party reactivity of cell product accurately.

In this study, limiting dilution analysis (LDA) was compared with flow cytometric assay to quantify the frequencies of T cell and alloantigen reactive T cells. Both LDA and flow cytometric assay show similar results in quantifying the frequency of T cells in peripheral blood, while LDA provides a more sensitive detection of alloantigen reactive T cells.

The cell-frequency detection by flow cytometric assay is based on specific surface antigen expression on different cell types, which is simple, fast and with high reproducibility. However, after alloreactive T cell depletion, it is important to determine the specific immune reactivity of the cell product towards original stimulator (recipient), third-party stimulator, and infectious virus such as Cytomegalovirus (CMV) and Epstein-Barr Virus (EBV). The residual alloreactivity and third-party reactivity of cell product can be determined by proliferation assay which quantifies the $^3$H or BrdU incorporation by proliferating cells. Both $^3$H and BrdU incorporation assays have detecting limitations; the linear range of both assays is from 100 to 10,000 cells/well as reported (Roche). In order to determine the alloreactivity and third-party reactivity of cell product after selective depletion more
accurately, LDA, an assay developed to quantify immuno-reactive cell frequency, needs to be applied in the secondary stimulation of cell product after depletion. LDA can be combined with proliferation assay and cytotoxicity assay to determine the frequency of alloreactive cells, third-party reactive cells, CMV reactive cells, EBV reactive cells and recipient-specific cytotoxic T lymphocytes precursor (CTLp) in the cell product after selective depletion.

Another important issue is to define the cell source of alloreactive T cells detected in the secondary stimulation after selective depletion, either from inefficient immunomagnetic cell depletion or cells that escape initial activation in primary MLR. A MSCV based vector carrying an enhanced green fluorescent protein (eGFP) marker gene can be used to mark alloantigen-specific T cells in MLR (Pollack et al., 1998). Following selective alloreactive T cell depletion and secondary stimulation, the donor T cells that co-express activation antigen as well as eGFP can be detected by flow cytometry; detection of such cells indicate that these activated cells escaped magnetic depletion and were not newly activated cells. It is important to note that the accuracy of this method is related to the transduction efficiency of the vector.

In summary, flow cytometric assay is an efficient method to detect the frequency of T cells before and after T cell depletion for stem cell transplantation, while LDA and relevant immune functional assay are necessary to detect the frequency to various cells towards recipient, third-party and virus after selective depletion of alloreactive T cells.
CHAPTER 7

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Selective depletion of alloreactive T cells is a widely studied strategy to abrogate GvHD associated with allogeneic stem cell transplantation, while preserving the anti-third party reactivity to decrease the incidence of viral infection, relapse and graft failure after the transplantation. One of the main challenges for successful alloreactive T cell depletion is to stimulate and detect the maximum amount of alloreactive T cells. In this study, various methods were studied in order to optimize the alloreactive T cell activation.

Mixed lymphocyte reaction, the commonest way to generate alloreactive T cells, was optimized from the aspect of culture duration and cytokine treatment. The data indicates the optimum culture duration for alloreactive T cell generation is 4-day; IL-2 and IL-15 treatment of MLR does not increase the alloreactive T cell activation significantly, and nonspecific up-regulation of activation antigens can be caused by IL-2 treatment; IFN-γ pretreatment of stimulator cells can increase the CD69 expression on alloreactive T cells.
In this study, six activation antigen including CD25, CD69, CD71, OX40, HLADR, and CD38 were used to define alloreactive T cells, it is found that 13% of alloreactive T cells were missing by targeting only CD25 and CD69 based on this activation-antigen definition (CD25 and CD69 the most commonly used activation antigen for alloreactive T cell depletion). The priority sequence of activation antigen on representing alloreactive T cells was determined by comparing the alloreactive T cell fraction represented by each activation-antigen-combination. CD25, CD69, HLADR and CD38 were determined as the activation antigen with the first, second, third and fourth priority respectively. The depletion data suggested that $\geq 3 \log_{10}$ depletion of alloreactivity can be achieved by targeting CD25, CD69 and HLADR for immunomagnetic alloreactive T cell depletion by Miltenyi Super MACS.

A new strategy to optimize the stimulation of alloreactive T cells by recipient-monocyte-derived dendritic cells was studied. The up-regulation of activation antigens including CD25, HLADR and CD71 on alloreactive T cells stimulated by DC was significantly improved by comparing with that stimulated by PBMC. The DC stimulated up-regulation of CD25 on alloreactive T cells is competitive to other reported optimizing methods (Barrett et al, 2002). It is suggested that a higher degree of alloreactivity depletion can be achieved by targeting DC stimulated alloreactive T cells with multiple activation antigens (No literature report on alloreactive T cell depletion by targeting three or more activation antigens is available).

The process of alloreactive T cell depletion needs to be optimized. Miltenyi Super MACS system was mainly used in this study for alloreactive T cell depletion due to the ease of sterilizing. However, it was found that a high portion of non-alloreactive T cells were depleted during this MACS depletion process, which has a severe effect on
the anti-third party reactivity of cell products after depletion. There reason for this nonspecific depletion might be that the depletion columns retain nonmagnetically labeled cells nonspecifically due to its design (The cells after 4-day MLR become bigger, more granular and stickier). Compared with Miltenyi MACS system, QMS provides a better flow system; the depletion channel of QMS is bigger and there is no steel spheres packed inside the channel (The Miltenyi depletion columns are packed with small steel spheres inside); the flow rate of samples in QMS depletion channel can be controlled (The flow rate of depleting samples in Miltenyi MACS system is only due to gravity.) In order to achieve alloreactive T cell depletion with high depletion efficiency and recovery by the QMS, the magnetically mobilities of magnetically labeled alloreactive T cells need to be increased (Note: the expression of activation antigens on alloreactive T cells is low). Therefore, the magnetic labeling process for alloreactive T cells needs to be optimized by adjusting antibodies for labeling, concentration of antibodies for labeling and labeling protocol (one-step labeling or two-step labeling process).

Besides flow cytometric assay, limiting dilution assay and proliferation assay, in order to obtain an accurate estimate of cells product at the respect of functional significance, it is necessary to develop and apply more assays to determine the frequency of recipient-specific cytotoxic T lymphocytes precursors, CMV reactive cells and EBV reactive cells. In order to determine if the alloreactive T cells detected after selective depletion is due to inefficient immunomagnetic cell depletion or from cells that escape initial activation in primary MLR, an eGFP tracking assay can be applied (Discussed in Chapter 6).

As for clinical application, Wave® bioreactor is a good option for scaling-up the mixed lymphocytes reaction. Cross-contamination could be avoided by using the
disposable plastic sterile cell bag. The gentle fluid mixing way provided by Wave® bioreactor is necessary during the alloreactive T cell activation since it requires the contact between T cells and antigen presenting cells (APCs) which expressing alloantigens. The perfusion mode of operation is available for Wave® bioreactor, which makes feeding process much easier. Moreover, compared with traditional stirred tank bioreactor, the wave bioreactor is much lower cost and less labor required.

Finally, it is necessary to consider economic issues during scaling-up the process for clinical application. It is indicated from the internal report of my colleague that $2,000 to $6,000 will be needed to perform a CD3⁺ T cell depletion for 10¹⁰ cells (10¹⁰ cells will be needed for a single stem cell transplantation). During CD3⁺ T cell depletion, only a single antibody for CD3 is needed, however, for alloreactive T cell depletion, multiple antibodies will be needed in order to perform an efficient depletion. Therefore, the antibody-cost for clinical-scale alloreactive T cell depletion will be more than that for T cell depletion. From the economic point of view, optimizing the labeling process to decrease the necessary amount of antibody is very important for clinical application.
LIST OF REFERENCES

Abbas AK, Lichtman AH. Cellular and Molecular Immunology 5th Edition 2003

AB Dietz, PA Bulur et al. Maturation of human monocyte-derived dendritic cells studied by microarray hybridization. BBRC. 2000; 275, 731-738


BD FACSDiVa Option White Paper, BD Bioscience
BD pharmingen product description for anti-human HLA-DR PE

BD pharmingen product description for anti-human CD25 PE


Buckley CD, Amft N, et al. Persistent introduction of the chemokines receptor CXCR4 by TGF-B1 on synovial T cells contributes to their accumulation within the rheumatoid synovium. J Immunol 2000; 165:3423-9


Comella K. Immunomagnetic Separations: Effects of Antibody Concentration, Effects of Antibody Concentration, and Quantitation of Antibody Binding Capacity, M.S. Thesis 2001; The Ohio State University

Cytokines online pathfinder encyclopedia


168


Matiba B, Mariani SM, et al. The CD95 system and the death of a lymphocyte. Immunology 1997; 9: 59-68


Paterson DJ, Jeffries WA, et al. Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T blasts. Mol Immunol 1987; 24:1281

Pierce LN, Shabram PW. Scalability of a Disposable Bioreactor from 25L-500L Run in Perfusion Mode with a CHO-Based Cell Line: A Tech Review. Bioprocessing Journal 2004;1 4


RCSB Protein Data Bank, http://www.rcsb.org/pdb/


Roche BrdU proliferation assay manual


Singh V. Disposable bioreactor for cell culture using wave-induced agitation. Cytotechnology 1999; 30:149-158


Subramanian G. Bioseparation and Bioprocessing. 1998


Tong X. Experimental data 2005


Weinberg AD, Wallin JJ, et al. Target organ specific upregulation of the MRC OX-40 marker and selective production of Th1 lymphokine mRNA by encephalitogenic T helper cells isolated form the spinal cord of rats with EAE. J Immunol 1994; 152:4712


