The *in vivo* molecular and cellular interactions involved in peptide induced graft tolerance

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Declaration of Originality

I hereby declare that this thesis is my own composition, describes my own work except where specifically stated and has not been submitted in any other application for a higher degree.

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Abstract

Intranasal (i.n.) administration of the class II restricted peptide, NAGFNSNRANSSRSS, encoded by the *Dby* gene (HY-A^b*Dby*) can induce tolerance to male skin grafts and haematopoietic cells in high responder B6 ($H2^b$) female mice (Chai et al., 2004). i.n. administration of the single class II peptide is able to induce tolerance not only in B6 recipients that express two additional MHC Class I epitopes but also in (B6xCBA)F_1 mice that express a further class II and two class I restricted HY peptide epitopes, a total of five.

The aim of this project was to explore the mechanism(s) by which i.n. HY-A^b*Dby* peptide induces tolerance to male grafts. To do this gene expression in antigen specific T cells was analysed during both the tolerance induction phase and during the linked suppression phase, after male skin grafting.

During tolerance induction HY-specific cells were found to upregulate the genes encoding FoxP3, PD-1, CTLA4 , IFNγ and IL-13. This suggests that i.n. peptide induced a regulatory T cell population that may also modulate antigen presenting DCs.

Following challenge with male cells, antigen specific $CD8^+$ cells from rejecting mice were found to upregulate genes involved in cell killing such as granzyme B, FasL and cathepsin B. $CD8^+$ cells from tolerant mice were not found to upregulate these genes. Furthermore, after male skin grafting antigen specific $CD8^+$ T cells accumulated the graft draining lymph nodes.

Taken together, the results presented in this thesis suggest that i.n. peptide induces a regulatory population of $FoxP3^+$ $CD4^+$ T cells that can prevent transplantation rejection by modulating dendritic cell function and preventing antigen specific $CD8^+$ T cell from migrating from the graft draining lymph node.

Abbreviations

7-AAD	7-Amino-actinomycin D
ACAID	Anterior chamber-associated immune deviation
AIRE	Autoimmune regulator
ALPS	Autoimmune Lymphoproliferative Syndrome
AP1	Activator protein 1
APCs	Antigen Presenting cells
APC	Allophycocyanin
BAFF	B cell activating factor
BLIMP	B-lymphocyte-induced maturation protein-1
CCR4	Chemokine (C-C motif) receptor 4
cDNA	Copy DNA
CFSE	Carboxyfluorescein succinimidyl ester
CLN	Cervical Lymph nodes
CMTMR	(5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine)
COX-2	Cyclooxygenase-2
CR2	Complement receptor 2
CRP	C-reactive protein
CSC	Clinical Sciences Centre
CSF	Colony stimulating factor
Ct	Cycle Threshold
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DISC	Death Inducing Signalling Complex
DLN	Draining Lymph Node
DMEM	Dulbecco's Modified Eagle Medium
DN	Double Negative
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease
DP	Double Positive
EAE	Experimental Autoimmune Encephalitis
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
Fos	FBJ osteosarcoma oncogene
FoxP3	Forkhead box P3
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
GITR	Glucocorticoid-induced tumour necrosis factor receptor
GP130	Glycoprotein 130
GPR	Global Pattern Recognition
HA	Autosomal minor Histocompatibility antigens
HBSS	Hank's Buffered Salt Solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HK-1	Hemokinin 1
HLA	Human leukocyte antigen
HY	Minor Histocompatibility antigens encoded on the Y chromosome
HY-A^b*Dby*	H2-Ab restricted peptide encoded by the DBY gene
i.n.	Intranasal
i.v.	Intravenous
IBD	Inflammatory Bowel Disease
ICAM	Intracellular adhesion molecules
ICOS	Inducible co-stimulatory molecule
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin

IPEX	Immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance
IQA	Immunoquant Array
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LCMV	Lymphocytic Choriomeningitis Virus
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph node
LPS	Lipopolysacharride
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
Mar	Marilyn cells/mice
MBP	Myelin Basic Protein
Mins	Minutes
MHC	Major Histocompatibility Complex
MLR	Mixed Lymphocyte Reaction
Mmp	Matrix Metalloproteinase
MMTV	Mouse mammary tumour virus
MRC	Medical Research Council
NDLN	Non-draining lymph node
NFAT	Nuclear factor of activated T-cells
NFKB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer Cells
NOD	Non-obese diabetic mice
NOS	Nitric Oxide synthase
OCT	Optimal Cutting Temperature compound
OSM	Oncostatin M
OVA	Ovalbumin
OX2	NAD(P)H dehydrogenase quinone 2 (Ox2)
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PD1	Programmed Death 1

PDL	Programmed Death Ligand
PE	Phycoerythrin
PECAM	Platelet/endothelial cell adhesion molecule
PerCP	Peridinin chlorophyll protein
PI-Treg	Peptide Induced Regulatory T cell
PLP	Proteolipid protein
PMA	Phorbol myristate acetate
pMHC	Peptide in the context of the major histocompatibility complex
Q-PCR	Quantitative -Polymerase Chain Reaction
RA	Retinoic acid
RCLB	Red Cell Lysis Buffer
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute culture media
RT	Reverse Transcription
SCID	Severe Combined Immunodeficiency
SOCS	Suppressors of cytokine signalling
SP	Single Positive
STAT	Signal Transducers and Activators of Transcription protein
TARC	Thymus and Activation Regulated Chemokine
TCR	T cell receptor
TGF	Transforming Growth Factor
TLR	Toll-like receptor
TNF	Tumour Necrosis Factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
TYK	Tyrosine Kinase
VCAM	Vascular cell adhesion molecule-1
WT	Wild type

Contents Page

Title Page	1
Declaration of Originality	2
Acknowledgments	3
Abstract	4
List of Abbreviations	5
Table of Contents	9
List of Figures	14
List of Tables	16

Chapter 1: Introduction

1.1 Immunological Tolerance	18
1.1.2 Mechanisms of T cell Tolerance	18
1.1.3 Central Tolerance	18
1.1.4 Peripheral Tolerance	21
1.2 Regulatory T cells (Tregs)	25
1.2.1 FoxP3	26
1.2.2 Treg function	27
1.2.3 Naturally occurring regulatory T cells	29
1.2.4 Inducible Regulatory T cells	29
1.2.5 Treg associated cell surface molecules	31
1.2.6 Migration of Tregs	35
1.2.7 How do peripheral Tregs suppress effector cells?	37
1.2.8 Other types of Tregs	39
1.2.9 Gene expression in Tregs	40

1.3 Dendritic cells and tolerance 43
1.3.1 DC function 43
1.3.2 DCs provide signal three to T cells 44
1.3.3 Indolamine 2,3 dioxygenase 45

1.4. Transplantation tolerance 48
1.4.1 Minor H Antigens 48
1.4.2 The HY model 49
1.4.3 Linked suppression 53
1.4.4 Tolerance induced by intranasal administration of HY peptides 54

1.5 Aims 57
1.6 Preliminary microarray work 58

Chapter 2: Materials and Methods

2.1 Cell Culture Reagents 62

2.2. In Vivo Experiments 63
2.2.1 Animals 63
2.2.2 Adoptive transfers 63
2.2.3 Intranasal peptide 64
2.2.4 Flow Cytometery sorting 64
2.2.5 Skin Grafting 64
2.2.6 *In Vivo* Killing assay 65

2.3 In Vitro Assays 66
2.3.1 Cell freezing 66
2.3.2 Depletion of Thy1.1 66
2.3.3 Enrichment of Thy1.2 66
2.3.4 Skin digestion for lymphocytes 66
2.3.5 IFNg capture assay 67

2.3.6 Transmigration Assay 67

2.4. Flow Cytometry 68
2.4.1 General Method 68
2.4.2 Naïve T cell staining 68
2.4.3 Regulatory T cell staining 68
2.4.4 Tetramer Staining 69
2.4.5 Seven Colour FACS 69

2.5. Microscopy 70

2.6 Molecular Experiments 71
2.6.1 RNA extraction from cell pellets 71
2.6.2 RNA extraction from Skin 71
2.6.3 cDNA synthesis 72
2.6.4 Immunoquant array and Global Pattern Recognition 72
2.6.5 Quantitative PCR TaqMan Assay 73
2.6.6 Quantitative PCR Sybr Green Assay 73

Chapter 3: Tolerance Induction Phase

3.1 Introduction 75
3.2 Developing methods for adoptive transfers 76
3.3 Results 82
3.4 Peptide alone 82
3.5 Peptide +LPS 85
3.6 Naïve Marilyn Cells 87
3.7 Gene Expression 88
3.8 Gene expression in antigen specific T cells following i.n. peptide alone 89
3.9 Gene expression in antigen specific T cells following i.n. peptide + LPS 91

3.10 Protein expression of FoxP3 by intracellular staining 93
3.11 Induction of tolerance after adoptive transfer of Marilyn cells 95
3.12 Discussion 96
3.13 Analysis of gene expression 97
3.14 Genes expressed following treatment with peptide alone 99
3.15 Genes expressed following treatment with peptide +LPS 108
3.16 Conclusions 116

Chapter 4: Linked suppression phase

4.1 Introduction 118
4.2 Genes expressed in antigen specific $CD8^+$ T cells during linked suppression 120
4.3 $CD8^+$ HY-D^bUty tetramer $^+$ cells after skin grafting 120
4.4 Tolerance to Haematopoietic cells 120
4.5 Gene expression in $CD8^+$ T cells from tolerant or rejecting mice 124
4.6 Discussion: Gene expression in HY peptide specific $CD8^+$ T cells 128
4.7 Cells from tolerant mice 128
4.8 Cells from rejecting mice 133
4.9 Conclusions: Gene expression in $CD8^+$ T cells during linked suppression 138

4.10 Genes expressed in antigen specific $CD4^+$ T cells during linked suppression 140
4.11 IFNγ capture assay 140
4.12 Marilyn transfer and immunisation with male cells 148
4.13 Discussion: Gene expression in HY peptide specific $CD4^+$ T cells 152
4.14 Conclusions: Gene expression in HY peptide specific $CD4^+$ T cells during linked suppression 156

Chapter 5: The Migration of antigen specific T cells following i.n. peptide and male skin grafting

5.1 Introduction 157

5.2 CBA mice do not reject male cells or male skin grafts after transfer of $CD8^+$ T cells from C6 transgenic mice 158

5.3 Migration of Matahari T cells following skin grafting 160

5.4 Expression of chemokine receptors on $CD8^+$HY-D^b*Uty*$^+$ T cells 170

5.5 In Vitro migration of $CD8^+$HY-D^b*Uty* tetramer$^+$ cells 173

5.6 Do regulatory T cells enter the graft? 176

5.7 Discussion: Does i.n. peptide alter the migration of antigen specific T cells? 179

Chapter 6: Conclusions 182

6.1 Conclusion 195

6.2 Further work 195

References

References 196

Appendix I

S.1 Gene expression in antigen specific T cells following i.n. peptide alone 226

S.2 Gene expression in antigen specific T cells following i.n. peptide +LPS 230

S.3 Reproducibility of the IQA 232

Appendix II

S.4 Raw data: Significant genes in $CD4^+Thy1.2^+$ Marilyn cells after i.n. HY-A^b*Dby* peptide during induction of linked suppression 233

S.5 Raw data: Significant genes in $CD4^+Thy1.2^+$ Marilyn cells after i.n. HY-A^b*Dby* peptide during graft rejection 236

List of Figures

Figure 1.1 Gene encoding the HY epitopes 52

Figure 1.2: Preliminary microarray data 59

Figure 3.1: Adoptive transfer of Marilyn cells into a B6 Ly5.1 recipient 77

Figure 3.2: Ly5.1 phenotype of Marilyn cells 77

Figure 3.3: Flow cytometric analysis of CFSE labelled Marilyn cells following adoptive transfer into B6 and B6 Thy1.1 recipient strains, before and after sorting 78

Figure 3.4: Enrichment of $Thy1.2^+$ cells by depletion of Thy1.1 cells using microbeads 81

Figure 3.5: Enrichment of $Thy1.2^+$ cells by microbead positive selection 81

Figure 3.6: Flow cytometric analysis of adoptively transferred CFSE labelled $Thy1.2^+$ Marilyn cells 84

Figure 3.7: Flow cytometric analysis of adoptively transferred CFSE labelled Thy1.2+ Marilyn cells 5 days after i.n. peptide + LPS 86

Figure 3.8 Flow cytometry profiles of naïve T cells before and after sorting 87

Figure 3.9 FoxP3 protein expression 94

Figure 3.10 i.n. HY-A^b*Dby* peptide can still induce tolerance to male skin grafts after transfer of Marilyn cells. 95

Figure 4.1 $CD8^+$ HY-D^b*Uty* $tetramer^+$ cells from 4 female mice 22 days after male grafting. 122

Figure 4.2 i.n. HY-A^b*Dby* can induce tolerance to CFSE labelled male spleen cells 122

Figure 4.3 $CD8^+$ D^b*Uty*$^+$ cells in spleen and lymph nodes following administration of male cells 123

Figure 4.4 Naïve $CD8^+$ T cells. 123

Figure 4.5: Gating strategy for IFNγ capture assay 141

Figure 4.6 Spleen cells from unimmunised B6 female secrete little IFNγ when stimulated with HY-A^b*Dby* peptide 141

Figure 4.7 Sensitivity of the Cytokine capture assay 143

Figure 4.8 WT B6 spleen cells were mixed at different ratios with Marilyn cells and stimulated with 20ug/ml HY-A^b*Dby* for 4h. 144

Figure 4.9 CD4$^+$IFNγ$^+$ cells from cervical lymph nodes 147

Figure 4.10 B6 mice were given i.n. Ab peptide+LPS and five days later spleen and lymph nodes were taken and restimulated with 20ug/ml HY-A^b*Dby* peptide for 4 hours 147

Figure 4.11. Induction of tolerance to haematopoietic cells grafts following adoptive transfer of Marilyn cells 149

Figure 4.12 Expansion of CD4$^+$Thy1.2$^+$ Marilyn T cells after *in vivo* transfer male cells 149

Figure 4.13 Similarities in gene expression in antigen specific CD4$^+$ T cells during the linked suppression phase 154

Figure 5.1 Female CBA mice do not consistently reject CFSE labelled male cells after transfer of C6 cells 159

Figure 5.2 22 days after male skin graft, Thy1.2$^+$Vβ8.3$^+$ HY-D^b*Uty* specific Matahari cells could not be detected in the graft draining lymph nodes, the spleen or the skin 161

Figure 5.3 22 days after male skin graft CD8$^+$ HY-D^b*Uty* specific Matahari cells in the draining and non draining lymph nodes, spleen and the skin. 164

Figure 5.4 Microscopy of DLN,NDLN and Skin 22 days after skin grafting 166

Figure 5.5 Chemokine receptor molecules expressed by CD8$^+$ Tetramer$^+$ cells 172

Figure 5.6 *In vitro* migration assay 175

Figure 5.7 Expression of *Ifnγ, Cd4, FoxP3, Cd8* in RNA preparations from male skin grafts 24 days after grafting 177

Figure 6.1. Tolerance induction phase 189

Figure 6.2 Rejection of male skin grafts or cells 191

Figure 6.3 A. Linked suppression is mediated by regulatory T cells 193

Figure 6.3 B. Linked suppression is mediated by tolerogenic DCs 194

List of Tables

Table 1.1 Molecules associated with Treg function 33
Table 1.2 Studies looking at gene expression in regulatory T cells by DNA microarray 42
Table 1.3 The top 149 changed genes from the microarray analysis were analysed using Ingenuity software 60
Table 2.1 TCR transgenic mice used in this project. 63
Table 2.2 Antibodies used in multi-colour FACS experiments. 70
Table 2.3 Sequences of Primers used for 'in house' quantitative PCR assay 74
Table 3.1: Comparison of cells treated with CFSE before or after CD4 separation 79
Table 3.2: Comparison of 2 RNA extraction kits 82
Table 3.3: Cell recoveries after adoptive transfer and i.n. peptide alone 84
Table 3.4: Cell recoveries after adoptive transfer and i.n. peptide +LPS 86
Table 3.5 Gene expression in antigen specific $CD4^+$ T cells during the tolerance induction phase 90
Table 3.6 Gene expression in antigen specific T cells following in. peptide + LPS 92
Table 3.7 Genes significantly changed in antigen specific T cells after treatment with either i.n. HY-A^b*Dby* alone or with LPS 98
Table 4.1. Genes that are significantly changed in $CD8^+$ HY-D^b*Uty*$^+$ T cells from tolerant mice 125
Table 4.2. Genes that are significantly changed in $CD8^+$ HY-D^b*Uty*$^+$ T cells from rejecting mice 126
Table 4.3 Genes that are significantly either up or downregulated in $CD8^+$ HY-D^b*Uty*$^+$ cells from either rejecting mice or tolerant mice 127
Table 4.4 Genes significantly changed in $CD4^+$ $Thy1.2^+$ Marilyn cells after i.n. HY-A^b*Dby* peptide at the time of induction of linked suppression 150
Table 4.5 Genes significantly changed in $CD4^+$ $Thy1.2^+$ Marilyn cells at the time of graft rejection 151
Table S.1 Genes that were consistently up or downregulated in both experiments 228
Table S.2 Key for Table S.1 and S.5. 229
Table S.3: Summary of the different conditions of each quantitative array experiment 229

Table S.4 Summary of the results of each experiment 230
Table S.5 Genes that were consistently up or downregulated in four experiments 231
Table S.6 Genes significantly changes in CD4+ Thy1.2+ Marilyn cells after i.n. HY-AbDby peptide at the time of induction of linked suppression 233
Table S7 Genes significantly changed in $CD4^+$ $Thy1.2^+$ Marilyn cells at the time of graft rejection 236

Chapter 1 : Introduction

1.1 Immunological Tolerance

Transplantation of solid organs and stem cells is a common treatment for organ failure and leukaemia. Improved immunosuppressive regimens have greatly increased the success of solid organ transplantation by reducing the incidence of transplant rejection. However, immunosuppression has considerable side effects such as an increased risk of certain cancers (Zafar et al., 2008) and increased risk of infections (Mueller, 2008) which can reduce the life of both the transplanted organ and the recipient. In addition, improved immunosuppression has greatly improved early graft survival but has had a limited effect in preventing chronic rejection (Pascual et al., 2002). Therefore it is still desirable to be able to re-programme the immune system to tolerate transplanted organs and cells.

Immunological tolerance can be defined as a controlled unresponsiveness to specific antigens without overall immunity being impaired. An understanding of how tolerance is induced and subsequently causes non-responsiveness to grafts could contribute to the design of treatments that will allow the induction of antigen specific tolerance to organ and haematopoietic stem cell transplants in the clinic.

Immunological tolerance can be divided into two broad areas: Central, involving thymic education of T cells, and peripheral, involving non-responsiveness of mature cells in the circulation and tissues.

1.1.2 Mechanisms of T cell Tolerance

1.1.3 Central Tolerance

One of the most fundamental aspects of our immune systems is it ability to differentiate self from non self. This was first identified by one of the early pioneers of immunological research; Paul Erlich. In 1901 he first suggested that there must be mechanisms that prevent the immune system from self-reactivity or ‘horror autotoxicus’ (reviewed by Silverstein, 2001). It is now known that as T cells develop in the thymus they are

exposed to a series of selective processes that minimise the risk of self reactivity in the periphery while still enabling an effective immune response. More recently it has been shown that this includes special selection criteria that allows the development of regulatory T cells.

T cell progenitors develop in the bone marrow and enter the thymus through the cortico-medullary junction (reviewed by Takahama, 2006). Firstly the progenitors populate the cortex of the thymus. At this stage the progenitors do not express either CD4 or CD8 and are therefore called double negative T cells (DN). Once in the thymus DN cells proliferate and rearrange their TCRβ genes and TCRα genes and develop to become double positive (DP) cells, that is they express both CD4 and CD8 (Goldrath and Bevan, 1999).

These DP cells in the cortex are highly motile until they interact with cortical epithelial cells (cTECs) that present self peptides as peptide-MHC (pMHC) complexes (Bousso et al., 2002). Thymocytes that bind to pMHC receive maturation signals that trigger them to become mature single positive (SP) thymocytes (expressing either CD4 or CD8) and to upregulate CCR7 which allows them to migrate into the medulla (Ueno et al., 2004). This process is known as positive selection. It allows T cells that recognise and bind to peptide-MHC with low avidity to progress to the next stage of development. If the T cells cannot recognise and bind peptide-MHC they will be unable to carry out their function in the periphery. In the thymus if the developing thymocytes do not bind peptide-MHC, and do not receive survival/maturation signals, they will undergo apoptosis. Only 3-5% of developing lymphocytes survive this selection process and proceed to the medulla (Egerton et al., 1990, Goldrath and Bevan, 1999).

The medulla is a specialised environment that allows both clonal deletion and the development of regulatory T cells (Tregs) to take place.

Clonal deletion was first described by Kappler and Marrack in 1987 (Kappler et al., 1987). Using a model of endogenous superantigen they showed that self reactive T cells were deleted in the thymus. It is now thought that deletion occurs at the DP to SP transition (Baldwin et al., 2005, Zhan et al., 2003) although the exact mechanism is not known.

Positive selection of self-reactive regulatory T cells (Tregs) also occurs in the medulla. High affinity interactions between the TCR and pMHC can lead to the expression of

FoxP3 by the developing thymocyte (Sakaguchi, 2004, Fontenot et al., 2005, Watanabe et al., 2005). Once in the periphery these cells can prevent autoimmunity. Naturally occurring (educated in the thymus) Tregs will be discussed in further detail below.

The medulla contains epithelial cells (mTECs) and dendritic cells (DCs), both of these are specialised to induce tolerance.

mTECs express co-stimulatory molecules such as CD80 and CD86. They can also express tissue specific antigens (TSAs), which are proteins that would normally be expressed in another parts of the body or at different times in development. This is sometimes called promiscuous gene expression by cells of the thymus. Collectively mTECS express about 3000 additional genes or 5-10% of all currently known genes (Kyewski and Derbinski, 2004). These self antigens are presented in the thymus by MHC molecules in order to induce tolerance to self proteins. Developing thymocytes that bind with high avidity to these self proteins will be deleted from the T cell repertoire; clonal deletion (Palmer, 2003) .

The DCs in the thymus also express co-stimulatory molecules such as CD40, CD80 and CD86 cell and have been shown to cross present TSAs to the developing thymocytes (Gallegos and Bevan, 2004).

The expression of TSAs is partly dependent on expression of the autoimmune regulator (AIRE) gene. AIRE regulates the expression of a subset of TSAs in the thymus (Anderson et al., 2002, Kyewski and Derbinski, 2004). The exact mechanism by which AIRE can induce the expression of other genes is not known. Since there are few structural similarities between the TSAs that are induced by AIRE expression, Kyewski *et al.*, have suggested that it may regulate gene expression by an epigenetic mechanism involving gene methylation (Tykocinski et al., 2008). Mutations in the AIRE gene result in Autoimmune polyglandular syndrome type I (APS1) also known as Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (Nagamine et al., 1997, Consortium, 1997) and a similar disease in mice (Anderson et al., 2002, Liston et al., 2003, Kuroda et al., 2005). The mutations in the AIRE gene results in some TSAs not being presented by mTECs to developing thymocytes. Therefore these thymocytes are not deleted and once in the periphery cause autoimmune disease (Liston et al., 2003). Nevertheless, mTECS from AIRE deficient mice do express some TSAs such as C-reactive protein, (CRP) and glutamic acid decarboxylase (GAD67) (Anderson et al.,

2002) suggesting that there are other unidentified mechanisms that lead to the expression of TSAs.

How central tolerance develops at the molecular level is still not clear. For example: a developing thymocyte binding to self antigen with high affinity in the medulla can either express FoxP3 and become regulatory or undergo apoptosis. It is known that both processes require co-stimulatory signals and the thymic medullary environment (Buhlmann et al., 2003, Gao et al., 2002, Salomon et al., 2000, Kumanogoh et al., 2001, Serra et al., 2003). SP mature in the medulla for approximately 12 days (Egerton et al., 1990) before the selected, self tolerant cells are exported to the periphery.

1.1.4 Peripheral Tolerance

Clonal deletion of self reactive T cells in the thymus is not sufficient to prevent autoimmune disease, there are self reactive T cells in the periphery that must be controlled. Once in the periphery there are several mechanisms whereby peripheral tolerance can be mediated: deletion, ignorance, anergy and regulation.

Deletion

Deletion can also occur in the periphery where it is thought to be a homeostatic mechanism that regulates the immune response and is driven by either high dose of antigen or withdrawal of IL-2. The former, also known as activation induced cell death, involves the binding of the death receptor Fas to FasL on the surface of T cells and results in the induction of apoptosis. The importance of deletion in maintaining peripheral tolerance can be seen in mice that have defects in this pathway. MRL/*lpr* and *gld* mice have mutations in either Fas or FasL and this results in a lupus like syndrome (Watanabe-Fukunaga et al., 1992, Sobel et al., 1993, Suda et al., 1993). In humans defects result in this pathway results in Autoimmune Lymphoproliferative Syndrome (ALPS) (Fisher et al., 1995). Therefore Fas binding FasL has physiological role in preventing autoimmunity.

Alternatively, signalling through the mitochondrial pathway by proteins that express a BH3 domain such as Bim can also kill T cells. This has been shown to be the dominant pathway in a model of systemic tolerance in DO11.10 (DO11) TCR transgenic mice specific for a peptide from ovalbumin (OVA) (Barron et al., 2008).

Ignorance

If a T cell never encounters an antigen it cannot be activated by it. When antigens that are expressed at low levels or are not presented to T cells often enough to cause activation or induce tolerance, this results in T cell ignorance. However, presentation of such antigens in a proinflammatory environment can lead to T cell activation. For example in immune privileged tissues such as the eye, naïve T cells cannot circulate here as they do not express the right adhesion molecules. Trauma to one eye can release antigens that are not usually accessible to T cells into the circulation. This results in T cell activation in the draining lymph node and generation of effector T cells that can now infiltrate into both eyes and cause inflammation. This is known as Sympathetic Ophthalmia (Castiblanco and Adelman, 2009).

Anergy

Anergy can be defined as a tolerance mechanism in which lymphocytes become inactivated following antigen encounter. Schwartz (Schwartz, 2003) divided anergy into two types which are functionally different and have been shown to have a different molecular basis: Clonal Anergy and Adaptive Tolerance.

T cell clonal anergy is primarily an *in vitro* phenomenon and can be induced by 6-12 hours of strong TCR signal in the absence of co-stimulation, or by a low affinity ligand in the presence of co-stimulation (Mirshahidi et al., 2001). At the molecular level it is mediated by a block in either the MAP kinase pathway signalling (reviewed by Schwartz, 2003) or calcium/calmodulin/calcineurin (Jenkins et al., 1990). These anergic T cells have a block in IL-2 transcription and defects in AP1 response elements (Kang et al., 1992) so they cannot proliferate in response to antigen stimulation.

Clonal anergy can be reversed by exposure to PMA and Ionomycin, this induces IL-2 production and normal proliferation (Mondino et al., 1996, Fields et al., 1996, Quill et al., 1992). Exogenous IL-2 can also reverse the anergic state (Essery et al., 1988) and more recently anti OX-40 antibody has been shown to reverse anergy (Bansal-Pakala et al., 2001).

Adaptive tolerance was first described by Rammensee and colleagues (Rammensee et al., 1989). They transferred cells from Mls-1^a mice into Mls-1^b mice and followed the $V\beta6^+$ positive T cells that are specific for the Mls-1^a superantigen. These cells persisted in the host but did not proliferate. When, re-stimulated *in vitro* the antigen specific T cells could upregulate the IL-2 receptor, CD25, but did not secrete IL-2.

Further experiments by Rocha and Von Boehmer (Rocha and Von Boehmer, 1991) showed that TCR transgenic HY specific $CD8^+$ T cells transferred into athymic syngeneic male mice (that express the HY antigen) expanded rapidly and then declined in numbers. If the persisting cells were re-stimulated *in vitro* they did not proliferate. Furthermore, if these cells were transferred to a female athymic mouse they recovered the ability to proliferate (Rocha et al., 1993). This suggests that these cells need the presence of antigen to remain unresponsive.

This early model of adaptive tolerance relied on homeostatic expansion of T cells in an immuno deficient host. This is now known to be an antigen independent expansion so the results should be regarded with caution (Surh and Sprent, 2000).

The adoptive transfer model of tolerance first described by Jenkins group (Pape et al., 1998) is more physiological. In these experiments TCR transgenic $CD4^+$ T cells specific for OVA were transferred into an unirradiated syngeneic mouse. In response to intravenous (iv) administration of soluble peptide, the antigen specific T cells underwent a limited expansion followed by cell death. The persisting cells were unresponsive to antigen both *in vitro* and *in vivo,* even when antigen was given with incomplete Freunds Adjuvant. Intracellular staining showed that these cells did not synthesise either IL-2 or TNFα. In vitro they secreted 10-20 fold less IL-2 than naïve cells. BrdU labelling showed that they underwent cell division by day 5 but their expansion was 3-6 fold less than naïve T cells. The anergic state lasted up to 3 weeks after antigen challenge but was gone by 7 weeks. The unresponsive state could be extended if the soluble antigen was injected every week, showing that the antigen needs to be present for these cells to remain anergic.

Adaptive tolerance was also shown in developmental T cell models, indicating that it may have a role central tolerance too. Using a radiation induced bone marrow chimera model, where I-E molecules and MMTV superantigens were only expressed in host tissues and not the bone marrow derived cells, Ramsdell and Fowlkes (Ramsdell et al.,

1989) showed that antigen specific T cells that had not been deleted in the thymus were unresponsive in the periphery. In this model clonal deletion was incomplete and self reactive T cells persisted in the periphery, however, these T cells did not lead to autoimmunity. *In vitro* these cells were unresponsive in MLR or when directly stimulated with plate bound anti-Vβ antibodies. These cells did not secrete IL-2. Adaptive tolerance could be reversed if the cells were stimulated with IL-7 in vitro for 1 week. The presence of antigen was again shown to be crucial for maintaining this tolerant state since if these cells were transferred into an irradiated host that lacked the I-E molecule (Ramsdell and Fowlkes, 1992) anergy could be reversed.

Although both clonal anergy and adaptive tolerance are characterised by a block in both synthesis and responsiveness to IL-2 there are some important differences. In most models of adaptive tolerance, anergic cells secrete limited amounts of cytokines (except IL-10), require antigen to be present and anergy cannot be reversed by the addition of IL-2. This is not the case for clonal anergy where cells only need to be stimulated once, but for a long time, and anergy can be reversed by adding IL-2 (Mondino et al., 1996, Fields et al., 1996). At the molecular level, biochemical experiments have shown that clonal anergy is mediated by a block in signalling in the Ras/MAP kinase pathway (Schwartz, 2003) whereas adaptive tolerance is mediated by a block in calcium or tyrosine signalling (Utting et al., 2000).

Anergic T cells fell out of favour with the irrepressible rise of regulatory T cell research (see below). The main difference between these tolerance mechanisms is that T cell anergy is a cell intrinsic state, whereas regulatory T cells prevent other cells from responding. However, it is becoming clear that regulatory T cell themselves fit the description of an anergic T cell (hyporesponsive to antigen and have a block in IL-2 transcription) so the two should not necessarily be seen as separate mechanisms.

1.2 Regulatory T cells (Tregs)

Suppressor or Regulatory T cells have a controversial history. As early as 1969 Nishizuka and Sakakura (Nishizuka and Sakakura, 1969) demonstrated that if female mice were given a thymectomy at 2-4days of age, then they would become infertile as a result of autoimmune destruction of the ovaries. Furthermore, Penhale and colleagues (Penhale et al., 1973) showed that removing the thymus and giving sublethal irradiation to adult rats resulted in thyroiditis.

Both experiments suggested that there were T cells in the thymus that could prevent autoimmune disease. Research in the early 1980s seemed to show that suppressor T cells acted by producing antigen specific soluble factors encoded by the I-J region of the MHC or by Ig genes (Dorf and Benacerraf, 1984). However, as a result of advances in molecular techniques it was later shown that the TCR genes were encoded separately from Ig genes and that there was no evidence of an I-J region identifiable within the MHC (reviewed by Shevach, 2000). In addition, the identification of Th1 and Th2 subsets at this time resulted in the idea that suppressor T cells may just be a part of this spectrum of T cell functions and not a separate linage. So research into suppressor T cells fell out of favour.

Nevertheless, some success was achieved in the 1980s when attempts were made to find a cell surface marker that would identify suppressor/regulatory cells. Sakaguchi et al. (Sakaguchi et al., 1985) demonstrated that transfer of $CD5^{high}CD4^{+}$ T cells could prevent type 1 diabetes in NOD mice. Powrie and Mason (Powrie and Mason, 1990) used a rat model to show the existence of Tregs. They used congenitally athymic rats, and found that transfer of $CD45RB^{high}$ $CD4^{+}$ T cells caused a severe wasting disease in these animals. However, if unseparated $CD4^{+}$ cells were transferred or if $CD45RB^{low}CD4^{+}$ cells were transferred there was no disease, showing that $CD45RB^{low}$ cells could directly prevent autoimmunity.

However, it wasn't until 1995 that the breakthrough came: when Sakaguchi et al. demonstrated that CD25 (the IL-2 receptor α chain) could be used as a surface marker for regulatory T cells (Sakaguchi et al., 1995). CD25 is expressed by 5-10% of

peripheral $CD4^+$ T cells and <1% peripheral $CD8^+$ T cells in normal humans and mice. These $CD25^+CD4^+$ T cells are also $CD5^{high}CD45RB^{low}$, fitting with the existing findings that cells with this phenotype were regulatory. Sakaguchi et al. transferred BALB/c spleen cells depleted of $CD25^+CD4^+$ T cells into BALB/c athymic nude mice. This resulted in autoimmune disease that affected many organs including the stomach, thyroid, ovaries, adrenal glands and islets of langerhans. Larger doses of $CD25^+CD4^+$ depleted cells results in lethal autoimmune disease. However, co-transfer of a small number of $CD25^+CD4^+$ T cells with the $CD25^-$ cells prevented autoimmune disease (Sakaguchi et al., 1995).

These experiments were important in establishing that despite negative selection in the thymus, the normal immune system contains $CD4^+$ T cells that are potentially autoreactive and can cause disease. These cells are controlled by $CD4^+$ regulatory T cells some of which express CD25. If $CD25^+CD4^+$ T cells are deleted or not present, then tolerance to self antigens breaks down (Sakaguchi, 2004).

However, CD25 is not a very selective marker since all T cells upregulate CD25 upon activation. Further attempts have been made to define a suppressive population within the $CD25^+CD4^+$ population. The cell surface markers $CD103^+$ is expressed by a subset of regulatory cells which are specialised for interacting with epithelial cells (Lehmann et al., 2002). $CD62^{hIgh}CCR7^+$ Tregs have also been described. These cells can migrate to secondary lymphoid tissues and delay diabetes (Szanya et al., 2002). More recently, folate receptor 4 (FR4) has been suggested as being expressed by a highly effective Treg subset that can prevent allograft rejection (Yamaguchi et al., 2007). These additional marker have limited effectiveness as they can be expressed by activated T cells, or in the case of FR4 are expressed as a spectrum of intensities, therefore isolating $FR4^{high}$ cells can be subjective.

1.2.1 FoxP3

Immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance (IPEX) syndrome was first described in a family in which 17 males had died in infancy (Powell et al., 1982). The symptoms (diarrhoea, diabetes mellitus, hemolytic anemia, destruction of the thyroid gland) seemed to be consistent with an overactive immune system, yet there were normal T and B cell numbers.

Nearly 10 years later Scurfy mice were described. These mice were found to have an X linked disease that caused scaliness, crusting of the eyes and ears and resulted in early death (average 24 days). This was found to be due to hyperactive T cells and the over production of cytokines (Godfrey et al., 1991). The gene defect in Scurfy mice was found to be in the FoxP3 gene that encodes the transcription factor Scurfin. In Scurfy mice a frameshift mutation prevents the forkhead domain being transcribed (Brunkow et al., 2001).

A number of studies then demonstrated that mutations in the FoxP3 gene were the cause of human IPEX syndrome (Chatila et al., 2000, Wildin et al., 2001, Bennett et al., 2001).

FoxP3 was subsequently shown to be specifically expressed in regulatory T cells (Hori et al., 2003, Fontenot et al., 2003). Both peripheral $CD25^+CD4^+$ T cells and thymocytes were found to express FoxP3. Retroviral gene transfer of FoxP3 into naïve T cells converted them into regulatory cells that expressed CD25, CTLA4, CD103 and GITR, and could suppress proliferation (Hori et al., 2003, Fontenot et al., 2003). Mice in which the FoxP3 gene was specifically deleted were shown to have hyperactive T cells similar to those found in Scurfy mice. Furthermore, in chimeric mice reconstituted with stem cells derived from both WT and FoxP3 deficient mice, regulatory T cells only developed from the WT cells, suggesting that FoxP3 is a key gene in the development of regulatory T cells (Fontenot et al., 2003). Therefore the symptoms in IPEX humans and Scurfy mice are not due to an overactive immune system but lack of regulation by Tregs.

1.2.2 Treg function

Tregs can suppress the proliferation of other $CD4^+$ and $CD8^+$ T cells. This can be demonstrated *in vitro* by co-culture of $CD25^-CD4^+$or $CD8^+$ cells with regulatory $CD25^+CD4^+$, the degree of suppression is measured by the inhibition of T cell proliferation.

In order for $CD25^+CD4^+$ T cells to be inhibitory, they need to be activated through their TCR either by specific antigen or a polyclonal stimulus, since it was demonstrated that irrelevant antigens could not stimulate suppression (Takahashi et al., 1998, Thornton and Shevach, 1998). Compared to the concentration of antigen need to stimulate naïve

T cells, Tregs require 10-100 fold lower concentrations of antigen to be activated (Takahashi et al., 1998). Once stimulated Tregs act in a non-specific fashion, that is they can suppress T cells expressing different TCRs and even $CD8^+$ T cells (Takahashi et al., 1998).

In vitro, regulatory T cells do not proliferate unless stimulated by TCR in the presence of IL-2 or CD28, and this is accompanied by a loss in suppressive activity (Takahashi et al., 1998). Withdrawal of either IL-2 or CD28 reverts the cells to an anergic state (Takahashi et al., 1998, Thornton and Shevach, 2000). This observation demonstrated that Tregs could be expanded *in vitro* with IL-2, allowing their limited numbers to be increased for experimental purposes.

More recently it has been shown that Tregs can target the APCs as well as responder T cells; by modulating the APCs Tregs can prevent the priming and differentiation of effector T cells. (Misra et al., 2004). Therefore this may have influenced the results of these *in vitro* experiments. Some T cell proliferation assays have been done in the presence of APCs, in which the APCs and the responder cells could be the targets of Tregs. However, some of the experiments used soluble CD3 or plate/bead bound antibodies to stimulate Tregs. In these assays only the responder T cells are the Treg targets. It remains to be elucidated if these would change any of the fundamentals of Treg biology that have been established using these *in vitro* proliferation assays.

Tregs are thought to suppress the proliferation of other T cells either by cytokine secretion or cell to cell contact. For Tregs *in vitro* it seems that cell to cell contact is the crucial factor as supernatants from activated Tregs or Tregs and $CD4^+$ T cells fail to suppress the response of $CD4^+$ T cells (Takahashi et al., 1998). Furthermore, if Tregs and effector T cells are separated by a semi permeable membrane then suppression cannot take place (Takahashi et al., 1998, Thornton and Shevach, 1998). However, this does not necessarily rule out the existence of a inhibitory cytokine that requires close proximity between the Treg and responder cell.

The role of cytokines in the effector functions of Tregs is not clear – several experiments seem to give conflicting results. Generally, IL-10 and TGFβ have been shown to be important although this may depend on the model and the way in which Tregs are induced. For example, in a murine model of irritable bowel disease (IBD) transfer of $CD25^-CD45RB^{high}CD4^+$ into SCID mice results in IBD. This can be prevented by co-

transfer of $CD25^+$ or $CD45RB^{low}$ $CD4^+$ T cells. However if these cells are transferred with anti IL-10 monoclonal antibody then there is no suppression and the mice develop IBD (Asseman et al., 1999). However other papers have shown that regulatory T cells from mice deficient in IL-10 can still prevent autoimmune disease (Suri-Payer and Cantor, 2001). It seems that in some models IL-10 is the main mechanism of suppression, whilst in others other cytokines or cell-cell contact maybe more important.

It is now recognised that Tregs are a diverse population, which can be broadly split into either naturally occurring or peripherally induced.

1.2.3 Naturally occurring regulatory T cells

These cells are generated in the thymus during the double positive to single positive transition stage. This has been shown by transferring thymocytes depleted of $CD4^+CD25^+$ cells, into syngeneic nude mice, this leads to autoimmune disease (Itoh et al., 1999). Furthermore, $CD4^+CD25^+$ cells express FoxP3, and FoxP3 deficiency abrogates their function (Hori et al., 2003, Fontenot et al., 2003). The TCR expressed by these cells is specific for self antigens (Jordan et al., 2001, Bensinger et al., 2001). Therefore the thymus produces both pathogenic self reactive T cells that have escaped negative and positive selection and regulatory cells that control response to self antigens in the periphery.

1.2.4 Inducible Regulatory T cells

There are now many examples in the literature of regulatory cells being generated from mature $CD4^+$ cells in the periphery. These cells may act in a similar way to naturally occurring Tregs, and express FoxP3, but may differ in the expression of some markers such as CD25. Tregs can be induced or 'converted' from peripheral $CD4^+$ T cells when antigen is presented under certain conditions, such as:

Multiple stimulations with a low peptide dose

In order to induce Tregs *in vivo* is has been shown that antigen dose is important. For example, T cells from the DO11.10 (DO11) TCR transgenic mice were transferred into

host mice, and treated with OVA peptide by either oral or intravenous (i.v.) administration. This treatment induced $CD4^+CD25^+$ Tregs in the host. However, the number depends on the dose of antigen. A low dose of peptide led to a higher number of antigen specific $CD4^+CD25^+$ cells. These Tregs were shown to be defective in IL-2 expression and could mediate suppression *in vitro* (Thorstenson and Khoruts, 2001).
A number of other studies have used chronic antigen to induce tolerance. Anderson et al administered 10 doses of myelin basic protein (MBP) intranasally (i.n.) with 3-4 day intervals between doses. They found this induced a population of $CD4^+$ T cells which did not produce IL-2 and expressed CTLA4 (Anderson et al., 2006). Verginis et al found that giving female mice either 1μg or 10μg of male HY peptide per day for 14 days via a sub-cutaneous minipump induced tolerance to male skin grafts and CFSE labelled male cells regardless of the dose (Verginis et al., 2008).

In the presence of certain cytokines

Cytokines have a crucial role both in the induction of Tregs and in their function. There is some evidence to suggest that stimulation of mature CD4+ T cells with TGFβ is sufficient to convert them into Tregs, for example in a model of house dust mite asthma (Chen et al., 2003), and in a model of T cell dependent antibody production (Zheng et al., 2002). This is supported by *in vitro* work which showed that addition of TGFβ to Tregs in culture allowed them to expand and prevented the down regulation of FoxP3 that otherwise accompanied this expansion (Chai et al., 2008).

Oral or mucosal routes of antigen

Both of these routes have been shown to induce $FoxP3^+$ regulatory T cells. In a mouse model of asthma, oral antigen could prevent effector T cell developing in the absence of naturally occurring regulatory T cells (Mucida et al., 2005). Antigen presentation by a subset of $CD103^+$ DCs in the mesenteric lymph node has been shown to induce FoxP3 expression in naïve T cells (Coombes et al., 2007).

T cell expansion under lymphopenic conditions

It has also been shown that peripheral Tregs can be induced in lymphopenic conditions in the gut, following oral antigen (Sun et al., 2007).

In the above conditions naive T cells will differentiate into Tregs rather than effector T cells. Unlike naturally occurring Tregs, inducible Tregs may be specific for self antigens such as MBP Ac1-9 (Anderson et al., 2006) or newly encountered antigens such as male specific peptide (Verginis et al., 2008). There are several types of peripheral Tregs, even some $CD8^+$ T cell subsets have been shown to have suppressor activity (Kiniwa et al., 2007, Liu et al., 2004). This project is concerned with inducible $CD4^+$ Tregs and how they acquire and exert their inhibitory effects.

1.2.5 Treg associated cell surface molecules

Ligation of other molecules either on the surface of Tregs or on other cells associated with Tregs has also been shown to be important for their function (Table 1.1).

CD28 and CTLA4 both bind to CD80 and CD86 expressed on APCs. In naïve T cells CD28 provides co-stimulatory signal that promotes IL-2 secretion, T cell expansion and prevents anergy and death, whereas CTLA4 provides naïve T cells with a negative signal which attenuates T cell response (Salomon and Bluestone, 2001).

However, in Tregs these molecules have been shown to have different roles. Unlike naïve T cells, Tregs do not need CD28 co-stimulation for activation since Tregs from CD28 deficient mice are equally as potent *in vitro* as those from WT mice (Takahashi et al., 2000). Nevertheless CD28 does seem to have a role in the thymus and/or survival in the periphery as CD28 deficient mice have fewer Tregs (Salomon et al., 2000). CD28 also appears to be important for the conversion of naïve $CD4^+$ T cells into Tregs both *In vitro* (Chen et al., 2003, Kretschmer et al., 2005, Walker et al., 2005) and *in vivo* (Liang et al., 2005, Guo et al., 2008).

In contrast to naïve T cells which only express CTLA4 after activation, Tregs express CTLA4 constitutively (Takahashi et al., 2000, Salomon et al., 2000, Read et al., 2000). This molecule is important to the function of these cells as blocking with monoclonal antibodies can inhibit Treg mediated suppression (Read et al., 2000) and cause an autoimmune disease similar to that caused by depleting Tregs (Takahashi et al., 2000).

Similarly CTLA4 deficient mice develop a lethal lymphoproliferative disease that can be inhibited by addition of WT cells (Bachmann et al., 1999). Therefore it has been suggested that CTLA4 ligation may cause T cells to exert suppression. One possible mechanism of suppression is through the upregulation of IDO in the APC. Back signalling through CD80 and CD86 has been shown to upregulate the expression of IDO (Grohmann et al., 2002, Orabona et al., 2005). As discussed below, the upregulation of IDO can prevent T cell proliferation.

Molecule	Function	Reference
CTLA4	Negative regulator of immune responses Constitutively expressed on $CD4^+CD25^+$ T cells Tregs deficient in CTLA4 cannot suppress in both in vivo and in vitro assays, and cannot down regulate CD80 and CD86 on DCs.	(Takahashi et al., 2000) (Wing et al., 2008)
CD28	Constitutively expressed by Tregs, provides co-stimulatory signal. Required for the development of naturally occurring Tregs in the thymus and is essential for the differentiation of Tregs from naive T cells.	(Sansom and Walker, 2006) (Guo et al., 2008)
ICOS	Co-stimulatory receptor expressed on activated T cells and $FoxP3^+$ cells involved in the survival and expansion of T cells during the effector phase of the immune response. Role in controlling autoimmunity and in mucosal tolerance models.	(Burmeister et al., 2008) (Herman et al., 2004) (Miyamoto et al., 2005)
GITR	Expressed at high levels on $CD4^+CD25^+$ T cells. Co-Stimulation with anti-GITR agonistic antibodies leads to increased proliferation of both $CD25^+$ and $CD25^-$ T cells and increased production of IL-10.	(McHugh et al., 2002) (Kanamaru et al., 2004)
PD1	Binding to its ligands gives a negative signal to T cell. May be required for the conversion of naïve $CD4^+$ T cells to Tregs in the periphery – DC subsets express PD-L1.	(Keir et al., 2008) (Wang et al., 2008)
NP1	Neuropilin 1 is expressed on $CD4^+CD25^+$ Tregs but not naïve T cells. Promotes longer interaction with immature DCs	(Sarris et al., 2008)
CD103	CD103 is an integrin expressed at mucosal sites. Expression on dendritic cells (but not on T cells) is essential for the development of Tregs in a model of Colitis.	(Annacker et al., 2005) (Leithäuser et al., 2006)
GPR83	G protein-coupled receptor 83 has been shown to be upregulated on Tregs but not on recently activated T cells. The significance of this is controversial. Hasan et al suggested that this could be important of the induction of Tregs in the periphery. However GPR83 deficient mice have shown no defects in either naturally occurring or inducible Tregs.	(Hansen et al., 2006) (Lu et al., 2007)
FR4	Naturally occurring Tregs express high amounts of folate receptor 4. $CD25^+CD4^+FR4^{hi}$ cells can suppress graft rejection.	(Yamaguchi et al., 2007)
RA	Retinoic acid can act indirectly to promote TGFβ dependent conversion of mature $CD4^+$ T cells into Tregs.	(Hill et al., 2008)

Table 1.1 Molecules associated with Treg function. Binding of CTLA4, CD28, ICOS, GITR or PD1 provide second a second signal to the T cells that can be either stimulatory or inhibitory. The other molecules have all been shown to modulate either naturally occurring or induced Tregs either directly or indirectly through other T cells or DCs.

Tregs also have increased expression of GITR (glucocorticoid-inducible TNF receptor), ICOS and PD1 which modulate co-stimulatory second signals. However these molecules are also expressed on activated effector T cells which can make dissecting their function difficult.

GITR is expressed at high levels of Tregs (Kanamaru et al., 2004). Studies using agonist anti-GITR antibodies (Kanamaru et al., 2004, Ronchetti et al., 2004, Esparza and Arch, 2005), $GITR^{-/-}$ mice (Stephens et al., 2004) or soluble GITR ligand (Ji et al., 2004) have shown that signalling through this pathway provides a strong co-stimulatory signal to both naïve T cells and Tregs. Stimulation of GITR leads to an increased proliferation of both $CD25^{+}$ and $CD25^{-}$ T cells and production of large amounts of the immunosuppressive cytokine, IL-10 (Kanamaru et al., 2004).

ICOS is a CD28 homologue that also provides a co-stimulatory signal, although it is thought that this molecule plays a role in the later stages of an immune response. Some studies have shown that ICOS has a non-essential role in stimulating Tregs (Golovina et al., 2008, Guo et al., 2008). However, it may contribute to the overall survival and growth of both Tregs and effector T cells (Burmeister et al., 2008).

PD1 is an inhibitory receptor expressed by activated peripheral T cells. It has an important role in peripheral tolerance as $PD1^{-/-}$ mice develop severe autoimmune disease (Khoury and Sayegh, 2004). PD1 can bind two ligands which are broadly expressed (Keir et al., 2006). It's exact role on Tregs is not known, however recent evidence suggests that DCs presenting antigen, signal through PDL1 to convert naïve $CD4^{+}$ T cell into $FoxP3^{+}$ Tregs (Wang et al., 2008).

Several other molecules have been proposed to have putative roles in the function of regulatory T cells. Neuropilin 1 is thought to increase the contact time between Tregs and DCs (Sarris et al., 2008). CD103 has a proposed role in mucosal tolerance models (Annacker et al., 2005, Leithäuser et al., 2006) and folate receptor 4 is proposed as a new surface marker that could be selective for $CD4^{+}CD25^{+}$ Tregs (Yamaguchi et al., 2007). It is not yet clear if these molecules are essential for Treg function.

1.2.6 Migration of Tregs

Like other T cell subsets, Tregs need to be able to migrate and interact with other cells types to exert their suppression. Naïve T cells circulate through the secondary lymphoid organs until they are activated when they can leave the circulation by migrating through the post-capillary venules (Fabbri et al., 1999). Migration across post-capillary venules and into the tissue involves three distinct phases: tethering, rolling and extravasation. Tethering involves the slowing of the lymphocyte and weak attachment to the vascular wall, this is mediated by the expression of selectin molecules such as L-selectin (CD62L) (Lasky et al., 1992). As the lymphocyte slows it will roll across the surface of the vasculature. This exposes the lymphocyte to chemokines which can bind to G-protein coupled chemokine receptors expressed by the lymphocyte (Rot and von Andrian, 2004). This leads to firm adhesion to the vascular wall which is mediated by the binding of integrins such as LFA-1, to members of the endothelial immunoglobulin superfamily such as ICAM-1, ICAM-2 and VCAM-1 (Springer, 1995). Finally the lymphocyte passes into the tissue via one of two routes: paracellular (at the junction of two endothelial cells) (Burns et al., 1997) or the transcellular route (through the endothelial cell) (Feng et al., 1998). The paracellular route it thought to be the standard method of crossing the endothelium and this involves the interaction of junctional adhesion molecules such as ICAM-1, ICAM-2 and PECAM-1 (Nourshargh and Marelli-Berg, 2005).

Tregs have been shown to express L-selectin (CD62L) that allows them to home to lymph nodes and also migrate within the extravascular tissues during acute and chronic inflammation (Khan and Kubes, 2003). Both $CD62L^+$ and $CD62L^-$ Tregs can express FoxP3, but there is some evidence to suggest that the $CD62L^+$ population maybe more potent suppressors (Fu et al., 2004).

In vivo, it has been reported that $CD4^+CD25^+CD62L^+$ T cells expand in the graft draining lymph node of tolerant allograft recipients. Furthermore, if anti-CD62L monoclonal antibodies are given, this will prevent lymphocyte homing and will lead to early rejection of the graft (Ochando et al., 2005).

Several studies have looked at the expression of chemokine receptors by regulatory T cells and there have been conflicting results. There is some evidence that Tregs express CCR7 and respond to the chemokines CCL19 and CCL21 (Fu et al., 2004, Iellem et al., 2001). Whereas others have found that Tregs are hyporesponsive to CCL21 induced migration (Flanagan et al., 2004). This is most likely to be because Tregs are a heterogeneous population. However a number of studies have shown that Tregs express a combination of CCR4 (Iellem et al., 2001), CCR5 (Bystry et al., 2001, Wysocki et al., 2005), CCR8 (Iellem et al., 2001) and CCR6 (Kleinewietfeld et al., 2005). In general they have been found to migrate in response to CCL17 and CCL1 (Colantonio et al., 2002). APCs and activated T and B cells are a good source of chemokines, in particular mature DCs secrete CCL17 (Kleinewietfeld et al., 2005) and mature T cells can secrete CCL1 (Selvan et al., 1997). This suggests that these cells may attract Tregs.

This seems to be confirmed by imaging experiments using 2 photon laser scanning microscopy of the pancreatic lymph node of diabetic mice (Tang et al., 2006). It was reported that Tregs behave in a similar way to $CD4^+CD25^-$ cells. Both subsets home to the T cell zone and cluster at the T cell-B cell boundary, both subsets swarm and form static conjugates with APCs presenting cognate antigen. Tregs formed long lasting complexes with DCs in the draining lymph node but no stable interactions between Tregs and effector T cells were seen (Tang et al., 2006).

Despite there being very few differences in the individual chemokine receptors and selectins expressed by Tregs a number of researchers have reported impaired mobility in tolerant T cells. As described above, Ochando *et al.*, found tolerant T cells accumulated in the graft draining lymph node following MHC mismatched transplantation, and this depended on the expression of CD62L (Ochando et al., 2005). Similarly, using a model of negative vaccination to xenoantigens, Mirenda *et al* found that tolerant T cells were retained in the draining lymph node where they encountered antigen (Mirenda et al., 2005). In models of transplantation tolerance it has been shown that tolerant T cells were

retained within the skin graft (Graca et al., 2002). The authors demonstrated that the limited mobility was reversible by re-transplanting the skin graft onto T-cell depleted hosts (Graca et al., 2002). Taken together these results suggest that Tregs have a limited migratory capacity compared to effector T cells, and this seems to be important for their functions. However, they express similar selectins and chemokine receptors to effector T cells.

1.2.7 How do peripheral Tregs suppress effector cells?

The mechanisms by which Tregs are induced and suppress other T cells is still controversial. Depending whether the Tregs are from humans or mice, if they are tested *in vivo* or *in vitro* and what model of tolerance is involved leads to different mechanisms.

As discussed above, Tregs may directly inhibit other T cells by secreting cytokines such as TGFβ and IL-10. For example in a mouse model of colitis, $CD4^+$ T cells that expressed the dominant negative TGFβ receptor and therefore could not respond to TGFβ, could not be controlled by Tregs (Fahlén et al., 2005). Similarly in tumour specific $CD8^+$ T cells that could not respond to TGFβ, were also resistant to suppression by Tregs (Chen et al., 2005). In some models both IL-10 and TGFβ have been shown to mediate suppression (Huang et al., 2005).

Tregs may also inhibit other T cells by cell to cell interaction. It has been proposed that this could be by direct T to T interaction (Thornton and Shevach, 2000), although the molecules involved in this are not yet clear. Another candidate for mediating this interaction is CTLA4 as some reports have shown that CTLA4 ligation with antibody could induce TGFβ production in murine $CD4^+$ T cells (Chen et al., 1998). However, this remains controversial since more recent reports using a neutralising TGFβ antibody have demonstrated that CTLA4 could suppress independently of TGFβ secretion (Sullivan et al., 2001).

Tregs may also inhibit indirectly through intermediate cells such as APCs or mast cells. In particular binding of CTLA4 to CD80 and CD86 expressed by DCs could

induce the expression of the immunomodulatory enzyme indolamine 2,3 dioxygenase (Grohmann et al., 2002) (see below).

In addition, recent work has suggested Treg suppression could be mediated by mast cells. Mast cells can act as APCs and express co-stimulatory molecules. Lu et al (Lu et al., 2006) demonstrated that mice made tolerant to allogeneic skin grafts by i.v. infusion of allogeneic cells (donor-specific transfusion) and anti-CD154 needed mast cells. They found that cells infiltrating the graft upregulated FoxP3 and IL-10. Mast cell markers (Mcpt1, Mcpt5, Tph1 and Fcer1a – a high affinity IgE receptor) were also upregulated in the tolerant group. Furthermore, they could not induce tolerance in mast cell deficient mice. These Tregs made a large amount of IL-9, which is a mast cell growth and activation factor. Neutralisation of IL-9 was found to accelerate allograft rejection in tolerant mice. The researchers concluded that IL-9 was the functional link through which Tregs recruit and activate mast cells.

Another possibility is that Tregs can affect the migration of effector T cells. As described above Tregs themselves may have impaired trafficking ability, which seems to be relevant to their function. They may also alter the migration of effector T cells to prevent them responding to antigen. There is some evidence to support this: it has been shown that hyporesponsive T cells localised at the site of tolerance induction (Mirenda et al., 2005). Also, using a TCR transgenic mouse model of diabetes, Tregs were found to inhibit $CD4^+$ T cell activation and also suppress tissue infiltration. This limited migration capacity was associated with a failure of naïve $CD4^+$ T cells to differentiate into effector T cells and upregulate CXCR3 (Sarween et al., 2004).

Alternatively, Tregs might simply compete with other $CD4^+$ T cells for antigen and be more efficient. This hypothesis is pertinent for naturally occurring Tregs as their repertoire has been selected to be specific for self antigens (Hsieh et al., 2004, Cozzo et al., 2003). These Tregs could then control self reactive T cells in the periphery.

Following suppression by Tregs, target T cells upregulated a distinct set of genes. These genes were unique to Treg interaction as they were not found in

cells which had been anergised, deprived of IL-2 or treated with TGFβ (Sukiennicki and Fowell, 2006). Investigating the molecular changes in these cells could yield clues of how they were induced.

There appears to be several mechanisms by which Tregs can inhibit effector T cells and this depends on the intensity of the TCR stimulation, the site of regulation and the cytokines present at the time of activation.

1.2.8 Other types of Tregs

In addition to $CD4^+CD25^+$ Tregs a number of other suppressive CD4 T cells have been described:

Tr1 cells

Tr1 cells are anergic T cells that can also suppress other T cell populations. They are induced by the priming of naïve T cells with antigen in the presence of IL-10 (Groux et al., 1997). These cells secrete large amounts if IL-10, TGFβ and IL-5, small amounts of IFNγ and IL-2 and no IL-4 (Groux et al., 1997). Tr1 cells do not constitutively express FoxP3 but upon activation its expression can be induced so it is similar to $CD4^+CD25^-$ T cells (Vieira et al., 2004, Levings et al., 2005). It is thought that Tr1 cells mediate suppression by secretion of IL-10 which can prevent APCs from upregulating co-stimulatory molecules and proinflammatory cytokines and can also act on T cells to inhibit IL-2 and TNFα secretion (Pestka et al., 2004). Although activated in an antigen specific fashion these cells can suppress naïve and memory T cells *in vitro* and *in vivo*. (Groux et al., 1997, Barrat et al., 2002, Bacchetta et al., 1994, Levings et al., 2005).

Th3 cells

These $CD4^+CD25^-$ cells result from oral exposure to antigen. These cells have been demonstrated in Multiple Sclerosis patients given oral treatment with myelin

basic protein (MBP) and proteolipid protein (PLP). This treatment increased the number of antigen specific T cells that were able to secrete the immunosuppressive cytokine TGFβ (Fukaura et al., 1996). Th3 clones isolated from mice made tolerant to MBP by oral antigen have been shown to suppress the induction of experimental autoimmune encephalitis (EAE) by secretion of TGFβ (Chen et al., 1994). However, IL-10 can also be important in the suppressive effect of these cells. In a model of transplantation tolerance it was found that intratracheal administration of allogeneic splenocytes 7 days before a cardiac transplant was able to prolong the survival of the cardiac graft in mice but this was dependent on IL-10 rather than TGFβ (Aramaki et al., 2005). As with Tr1 cells, these cells were activated in an antigen specific fashion but act on both $CD4^+$ and $CD8^+$ T cell in an non-antigen specific fashion (Weiner et al., 1994).

Peptide induced Tregs (PI-Treg)

Repeated administration of a specific antigen can also lead to suppressive T cells that are $CD25^-FoxP3^-CTLA4^+$ and can suppress proliferation of naïve T cells *in vivo*. In a mouse model of EAE, tolerance could be induced by i.n. soluble MBP Ac1-9 peptide. Tolerance was dependent on $CD4^+$ regulatory T cells and secretion of IL-10 (Sundstedt et al., 2003). These PI-Tregs secreted very little IFNγ but high amounts of IL-10 and no IL-2. Analysis of the gene expression in these antigen specific cells showed high levels of T-bet, normally associated with Th1 differentiation, egr-2 which may control expression of Fas/FasL and E3 ubiquitin ligases (Anderson et al., 2006).

1.2.9 Gene expression in Tregs

Several studies have looked at gene expression in regulatory T cells by microarray (reviewed in Table 1.2). All showed that Tregs have a distinct gene expression profile and this was likely to be due to the balance of signals through

different pathways such as TGFβ and IL-2 not just to signalling by FoxP3. Generally, most of the papers found that the significantly changed genes in regulatory T cells tended to be enriched for genes encoding cells surface proteins, extracellular proteins and cell cycle related genes. There was also some evidence to suggest that $FoxP3^+$ cells might express more tissue homing receptors (Fontenot et al., 2005).

None of these studies have looked at induced Tregs except the paper from David Wraith's group (Anderson et al., 2006) which is described above. The regulatory cells described in this paper failed to produce IL-2 and responded to antigen by secreting IL-10 which prevented the proliferation of naïve T cells (Sundstedt et al., 2003). These cells did not express FoxP3 (Vieira et al., 2004) and so it is not surprising that their results differ from what has been found in gene expression studies from naturally occurring Tregs. Very little is known about the gene expression in induced $FoxP3^+$ regulatory cells.

Ref	Treg	Main Findings
(McHugh et al., 2002)	Naturally occurring $CD4^+CD25^+$	GITR and SOCS increased on $CD25^+$ cells
(Huehn et al., 2004)	Naturally occurring $\alpha_E^+CD25^+$ Vs $\alpha_E^+CD25^-$	CD44, ICOS, Granzyme B, FucTVII, Integrin β, CD54, CCR2, CXCR3, CCR6, Ki67 – upregulated in α_E^+ $CD25^-$ cells
(Herman et al., 2004)	Naturally occurring $CD4^+CD25^+CD69^+$ Vs effector T cells	Tregs express GITR, CD103, Nrp-II, IL-10, CTLA4. LN Tregs have a different profile to pancreatic Tregs.
(Fontenot et al., 2005)	$CD4^+FoxP3^{gfp+}CD25^+$ T cells Vs other subsets	Tregs have increased amounts of IL-10, CD103, Klrgl, Nrp1, GITR, ICOS, Fg12, Gpr83 and CTLA4. They have decreased amounts of FasL, Sema4a, IL-4, Pde3b and Pde7a. $CD25^{lo}FoxP3^{gfp+}$ have upregulated tissue homing receptors.
(Anderson et al., 2006)	PI-Tregs	High levels of T-beta and Erg-2
(Gavin et al., 2007)	Naturally occurring thymic and peripheral $CD4^+FoxP3^{gfp+}$	$CD4^+FoxP3^{gfp+}$ have distinct molecular profile – express FgL2, CD73, CD39, TRAIL, CTLA4
(Hill et al., 2007)	Treg like cells generated in different conditions	Regulatory T cells have a distinct gene signature but this is due to interplay of signalling by FoxP3, TGFβ and IL-2

Table 1.2 Studies looking at gene expression in regulatory T cells by DNA microarray.

1.3 Dendritic cells and tolerance

1.3.1 DC function

DCs have several important functions in the immune system. They take up and process antigen in the tissues, migrate to the secondary lymphoid tissues where they present the processed antigen to T cells. This allows T cells to be exposed to many different antigens at different locations in the periphery. DCs also provide additional signals to T cells that shape the subsequent immune response.

Work in the 1970s and 1980s showed that DCs were more potent stimulators of T cells than B cells or macrophages (Steinman and Witmer, 1978, Nussenzweig et al., 1980, Inaba and Steinman, 1984). In 1985 Schuler and Steinman (Schuler and Steinman, 1985) isolated langerhans cells (LCs, a special type of DC found in the skin and mucosa) and cultured them. When freshly isolated, these cells had high expression of MHC but were poor stimulators of proliferation. Once cultured for 2 days, the cells matured and became potent stimulators in the same assay. Later experiments by Romani et al showed that once cultured, the LCs became less efficient at antigen processing (Romani et al., 1989).

The migratory capacity of DCs was first shown by Austyn at al (Austyn et al., 1988) who transferred fluorescently labelled DCs IV into recipient mice. The transferred cells were found to migrate to the spleen between 3 and 24h after transfer. The authors suggested that DCs have homing receptors similar to those of T cells. Furthermore Larsen et al (Larsen et al., 1990) were able to show that LCs could migrate from skin grafts and mature in the process. As these cells mature they have been shown to lose the ability to process antigen (Reis e Sousa et al., 1993).

These, and other results, led to a widely accepted theory of DC function. In this model immature DCs in the periphery sample antigen and migrate to the secondary lymphoid organs where they mature. Maturation is accompanied by a downregulation in antigen uptake and processing and an increase in the capacity to prime T cells. Mature DCs have decreased expression of FcgRs and

increased expression of MHC, LFA1, CD80 and CD86 (Schuler and Steinman, 1985, Larsen et al., 1992, Inaba et al., 1994) making them potent T cell stimulators. In this model only mature DCs can prime T cells.

The maturation process was found to be regulated by exogenous signals. TNF, LPS and CD40L have all been shown to decrease antigen sampling and increase the ability of DCs to stimulate T cell proliferation (Sallusto and Lanzavecchia, 1994, Winzler et al., 1997). This has also been shown in vivo: following systemic administration of LPS, MHC II expressing DCs were found to migrate (Roake et al., 1995, De Smedt et al., 1996). Typically these molecules that signal DC maturation are released at time of inflammation or stress, otherwise known as 'danger' signals.

1.3.2 DCs provide signal three to T cells

DCs present antigen in the context of MHC to T cells. This binding of TCR and pMHC is known as signal one. Signal two refers to the binding of other molecules on the cell surface that provide the T cell with additional positive or negative signals that can modify the strength of the response. Typically though signal two refers to the binding of CD28 to CD80 and CD86 expressed by the DC. This provides a strong positive signal that promotes proliferation (although it should be noted that CTLA4 also binds to CD80 and CD86 with high affinity and sends a negative signal to the T cell preventing proliferation) (Keir and Sharpe, 2005). The third signal refers to signals from the DC that shape the type of T cell response. For example, IL-12 secretion promotes Th1 or CTL differentiation (Trinchieri, 2003), while Notch ligand expression promotes the generation of Th2 cells (Amsen et al., 2004, Tu et al., 2005).

As described above, if antigen is presented in the absence of co-stimulation, this will result in anergy or deletion of the T cell. If antigen is presented with co-stimulatory signals then this will lead to T cell expansion, differentiation and the development of memory cells. This fits well with the model of DC maturation described above. For example if antigen is presented by immature DCs that do

not express CD80/CD86 then they cannot stimulate T cell proliferation and this results in anergy/deletion or the development of regulatory cells (Steinman and Nussenzweig, 2002, Steinman et al., 2003). Conversely, if antigen is presented by mature DCs that express high amounts of MHC and CD80/86 then this will lead to T cell proliferation.

However this view of immature DCs being 'tolerogenic' and mature DCs as being 'immunogenic' has been criticised for being too simplistic. It has been shown that some immature DC subsets cause immunity rather than tolerance. In a model that used transfer of naïve OVA-peptide specific $CD8^+$ T cells into OVA-peptide transgenic mice that express peptide only in thymus and skin epithelial tissue, antigen presentation by LCs was shown to initiate a chronic skin disease whereas presentation of the same antigen by APCs from other tissues was tolerogenic (Mayerova et al., 2004).

Also, DCs that induce tolerance can have a mature phenotype, that is, express high levels of co-stimulatory molecules (Albert et al., 2001, Menges et al., 2002). In fact, co-stimulation is needed for the induction of tolerance in some models (Perez et al., 1997, Probst et al., 2005).

1.3.3 Indolamine 2,3 dioxygenase

There are at least two ways in which DCs can induce tolerance. In the absence of danger signals, presentation of antigen by immature DCs will be tolerogenic. This has been shown in models of $CD8^+$ T cell tolerance, where presentation of LCMV derived epitopes results in tolerance if presented by immature DCs but expansion and effector differentiation if presented by mature DCs (Probst et al., 2005). Similar results have been shown in $CD4^+$ and $CD8^+$ OVA specific T cells (Bonifaz et al., 2002). However, there also seems to be mechanisms by which certain subsets of mature DCs can induce tolerance, such as the expression of the enzyme Indolamine 2,3 dioxygenase (IDO).

Munn and colleagues were the first to describe IDO expression as a regulatory mechanism which prevented the rejection of allogeneic foetuses. They gave

pregnant mice a pharmacological IDO inhibitor, 1-MT and found that this led to T cell mediated rejection (Munn et al., 1998).

Since then IDO has been proposed as a major mechanism by which DCs induce tolerance in T cells. Many studies have shown that overexpression of IDO resulted in immunosuppression and tolerance both *in vitro* and *in vivo*. For example – transfer of the IDO gene into cell lines could suppress allospecific T cell responses *in vitro* (Mellor et al., 2002). *In vivo* overexpression of IDO in lung transplants prevented acute allograft rejection (Swanson et al., 2004) and IDO gene transfer into pancreatic cells from NOD mice was associated with prolonged survival when these cells were transferred into NOD/SCID recipients (Alexander et al., 2002). In addition, IDO inhibition by 1-MT could abrogate the tolerance effects if IDO (Grohmann et al., 2002, Munn et al., 1998).

IDO catalyses the breakdown of the essential amino acid tryptophan into N-formyl-kynurenine which is then converted into other metabolites. T cells need tryptophan to proliferate, therefore if IDO is expressed and active the local environment becomes deficient in tryptophan and T cells cannot proliferate in response to antigen (Grohmann et al., 2000). In addition, the tryptophan metabolites can also be cytotoxic to T cells (Mellor and Munn, 2004).

Several papers have now shown that soluble CTLA4 Ig fusion protein could induce the expression of IDO in CD80/86 expressing DCs (Grohmann et al., 2002, Mellor et al., 2003, Fallarino et al., 2003, Munn et al., 2004, Mellor et al., 2004). In a mouse model of islet cell transplantation, it has been shown that the tolerogenic effects of CTLA-4 Ig protein could be reversed by the administration of 1-MT (Grohmann et al., 2002). As Tregs constitutively express CTLA4, they could also induce the expression of IDO in mouse DCs *in vitro* (Fallarino et al., 2003).

This has changed the existing dogma of how DCs function. As described above, it was thought that immature DCs were tolerogenic and mature DCs were immunogenic. However, IDO expression in immature DCs can be induced by $CTLA4^+$ Tregs. These DCs can mature and maintain their IDO expression during the maturation process (Munn et al., 2004). Therefore it has been suggested that

only difference between tolerogenic and immunogenic DCs is the expression of IDO (reviewed by Mellor and Munn, 2004). Furthermore, It also seems that IDO^+ DCs can activate Tregs in tumour draining lymph nodes to exert their suppressive effects (Sharma et al., 2007, Munn and Mellor, 2007).

There may also be other immunmodulatory molecules expressed by tolerogenic DCs such as the enzyme Arginase 1 (Munder et al., 1999). This enzyme competes with nitric oxide synthase (NOS) for its substrate L-arginine which it converts into ornithine and urea. NOS converts L-arginine into nitric oxide which is proinflammatory. It is thought that the upregulation of arginase I represents a immunomodulatory mechanism as this prevents the synthesis of the proinflammatory mediator (Bronte and Zanovello, 2005). Indeed it has been shown that Arginase 1 is upregulated in DCs conditioned with IL-10 (Nolan et al., 2004).

1.4 Transplantation tolerance

Inducing tolerance to transplanted tissues and cells began with the work of Peter Medawar and his colleagues (Billingham and Medawar, 1951). They were able to utilise central tolerance mechanisms to induce tolerance to transplanted organs. They found that if mice were given allogeneic bone marrow at birth, they would accept skin grafts from the bone marrow donor strain but not from an unrelated mouse strain. It is now known that this is because the treated mice will have T cells and antigen presenting cells (APCs) derived from both their own and the donor bone marrow stem cells. Therefore the T cells in the thymus will be selected on their ability to recognise both donor and recipient MHC expressed on thymic APCs. Inducing tolerance in mature mice (and humans) is much more difficult and mechanisms controlling peripheral tolerance have to be manipulated. Therefore inducing antigen specific tolerance in the clinic is still an aspiration, some 58 years after Peter Medawar's early work.

Immunological destruction of transplanted cells and tissues is due to the recognition of major and minor histocompatibility antigens. MHC mismatched grafts are rejected quickly without immunosuppression and this can be mediated by both T and B cells (Afzali et al., 2008, Terasaki and Cai, 2008).

MHC matched transplant can also be rejected; this is due to differences in the minor histocompatibility (H) antigens.

1.4.1 Minor H Antigens

Minor H antigens are small peptides derived from polymorphic proteins. Work by Rammensee's group showed that they are the result of these proteins being processed and presented at the cell surface of APCs by MHC class I and class II molecules where they are recognised by either $CD4^+$ or $CD8^+$ T cells (Falk et al., 1990). In contrast to the MHC, minor H antigens are poor at eliciting an antibody mediated response but can be the targets of T cell mediated rejection following

MHC matched transplantation (Beck et al., 1993) and can cause GvHD following stem cell transplantation (Goulmy et al., 1996, Goulmy, 1996). Minor antigens can also be expressed by leukemic cells. Recognition of these minor antigens is desirable as donor T cells will destroy malignant cells, this is known as the graft versus leukaemia (GvL) effect (reviewed by Bleakley and Riddell, 2004).

Several minor antigens have now been described in both humans and mice (Simpson et al., 2002). Originally it was thought that these were single dominant genes. However, extensive mapping studies using congenic strains of mice that only differ by small fragments of DNA, revealed that this 'gene' was actually a group of several genes, located close together so inherited as a group (Davis and Roopenian, 1990). Within each locus at least one gene would encode a cytotoxic determinant that could give rise to a CTL response, and a helper determinant that could elicit $CD4^+$ helper T cell response. Both the cytotoxic and helper determinants are required for graft rejection (Roopenian, 1992).

In humans, minor antigens have been identified using T cells clones derived from bone marrow transplant recipients. Recipients of minor antigen mismatched stem cell grafts undergoing either grafts versus host disease (GvHD) or host versus graft (HvG) will generate T cells targeting these minor antigens. These can be expanded *in vitro*, cloned and used to screen minor H antigen expressing EBV cell lines. The minor antigens HA-1, HA-2, HA-4 and HA-5 were all identified in this way – they are processed and presented to T cells in complex with HLA-A2 (van Els et al., 1990, van Els et al., 1992).

1.4.2 The HY model

In this study, transplantation tolerance will be investigated using the male specific minor H antigen model, HY. These are minor H antigens encoded by ubiquitously expressed Y chromosome genes. HY was first identified more than 50 years ago by Eichwald and Silmser (Eichwald and Silmser, 1955, Eichwald and Silmser, 1981). They demonstrated that in some strains of mice, females could reject male skin grafts whilst males always accepted male grafts,

suggesting that rejection was controlled by factors encoded on the Y chromosome. $H2^b$ strains of mice were found to rapidly reject male grafts, whereas $H2^d$ and $H2^K$ were poor responders. Using congenic strains of mice that differ by small amounts of genetic material, including the MHC coding region on chromosome 17, B10.BR ($H2^k$) and B10.D2 ($H2^d$), Bailey *et al*., were able to show that the genes controlling male graft rejection (at the time these were called the Immune response (Ir) genes) must be encoded within the H2 region (Bailey, 1971, Bailey and Hoste, 1971).

Soon after Zinkernagel and Doherty demonstrated that presentation of peptides from LCMV were MHC restricted (Zinkernagel and Doherty, 1974), Gordon *et al*. demonstrated that the HY antigens were presented by MHC; this explained why the Ir genes mapped to the H2 region (Gordon et al., 1975).

Work then focussed on identifying the peptides and genes that encode the HY antigens. Much of this was determined using mutant sex-reversed (*Sxr*) mice. This mutation is caused by the transfer of the duplicated sex determining *Sxr* region of the Y chromosome to the X chromosome during male meiosis in a carrier parent (Singh and Jones, 1982, McLaren and Monk, 1982). Male carriers of the mutation produce 4 progeny in equal ratios: X/X Females, Sex reversed infertile X/X^{Sxra} males, X/Y non carriers and X/Y^{Sxra} carrier males (Cattanach et al., 1971). Using HY specific T cell clones, it was shown that X/X^{Sxra} males were HY^+ (Simpson et al., 1981), therefore the genes encoding HY must be present in the Sxr^a region.

Experiments using further variants on these mutant mice and cell lines in which the X chromosome had been inactivated narrowed the search a region on the short arm of the Y chromosome which included the genes *Sry, Zfy-1, Zfy-2, Uty, Smcy* and *Ube1y-1* (Simpson et al., 1997). Furthermore, X inactivation experiments showed that there was likely to be several genes encoding the HY antigens (Scott et al., 1991).

This was used as the starting point for a series of transfection studies that aimed to identify the genes encoding the peptides. A series of cosmids containing genes from the short arm of the Y chromosome were created and transfected

into P1Htr cells that also expressed the $H2^k$ MHC Class I molecule ($P1K^k$) (Scott et al., 1992). These were then tested for HY antigen expression using an $H2^k$-restricted HY-specific (HY-K^k) T cell clone. Cosmid MEM14 was found to cause HY-K^k specific T cells to respond. The cosmid contained 40kb fragment of DNA which included most of the *Smcy* gene. The cosmid was digested into 2 restriction fragments that were subcloned and transfected into the $P1K^k$ cells. The 9kb MEM14C fragment from the 3' end of the gene was found to confer expression of the antigen as the transfectants were recognised by the HY-K^k specific T clone. Further subcloning revealed that 1-2kb fragment conferred the epitope. The amino acid sequences of the exons in this fragment from the X and Y homologues of this gene (*Smcx* and *Smcy*) were compared and 6 octamer peptide sequences were identified that conformed to the H2-K^k binding motif and differed between the X and Y copies. One peptide (TENSGKDI) was identified as the peptide that the HY-K^k specific T clone responded to (Scott et al., 1995).

The human HLA-B7 restricted HY peptide was identified from the *SMCY* gene by eluting peptides from HLA-B7 MHC molecules and analysing them by microcapillary liquid chromatography and mass spectrometry using an HY-specific HLA-B7 restricted T cell clone to identify peptides expressing the epitope (Wang et al., 1995).

Using a similar series of transfections that defined the HY-K^k Smcy epitope, the HY-D^b epitope was identified soon after. The HY specific H2-D^b restricted T cell clone recognised the peptide WMHHNMDLI which is encoded by the *Uty* gene (Greenfield et al., 1996). This was followed by the identification of another HY epitope in the *Smcy* gene, KCSRNRQYL restricted by H2-D^b (Markiewicz et al., 1998) (Figure 1.1). The final peptide to be identified in the *Smcy* gene was RRLRKTLL. This is the H2-D^k restricted HY epitope (Millrain et al., 2005).

The class II restricted peptides were identified using DCs to process and present peptides from COS cells that had been transfected with cDNA constructs expressing the known ubiquitously expressed Y chromosome genes plus an additional invariant chain sequences. Two peptides were identified, both of which were encoded within the *Dby* gene. NAGFNSNRANSSRSS was found to be the

H2-A^b restricted HY epitope, while REEALHQFRSGRKPI was found to be H2-E^k restricted epitope (Scott et al., 2000).

The genes encoding the peptides are shown in Figure 1.1.

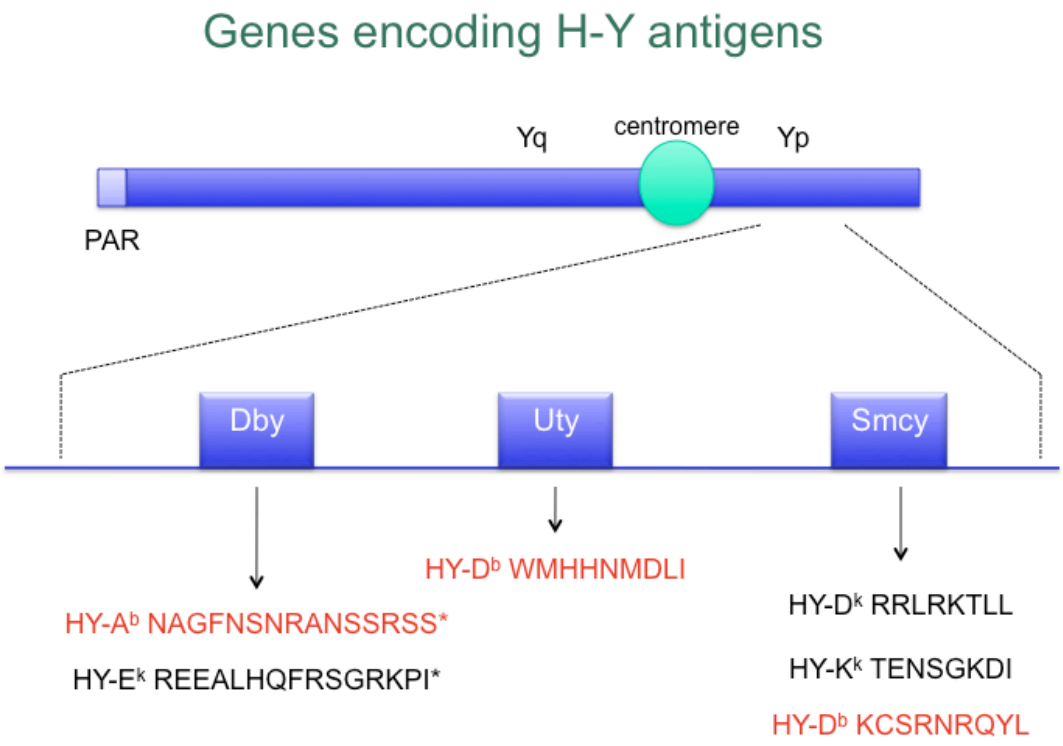


Figure 1.1 Gene encoding the HY epitopes. Three genes – *Dby, Uty* and *Smcy*-encoded on the short arm of the mouse Y chromosome give rise to immunogenic peptides that can cause rejection in syngeneic female recipients. The peptide sequences shown in red are $H2^b$ restricted while the peptides shown in black are $H2^k$ restricted. The two peptides encoded by the *Dby* gene (indicated with *) are presented by MHC class II molecules to $CD4^+$ T cells.

HY is similar to the autosomal minor antigens that have been described e.g. H3 and H4. In all cases mapping has shown that each locus is comprised of individual genes encoding either the CD4 or the CD8 restricted epitopes (Roopenian and Davis, 1989, Davis and Roopenian, 1990). Both of these epitopes are needed for graft rejection (Roopenian 1992). Indeed in studies where there is only one CD8 restricted peptide difference between the recipient and the donor graft tolerance rather than rejection results. (Antoniou et al., 1996).

The evidence that the HY antigens can contribute to the rejection of human renal transplants is controversial (Billingham and Hings, 1981, Ellison et al., 1994, Oliver, 1974, Opelz and Terasaki, 1977, Pfeffer and Thorsby, 1982). However, it has been shown to have a role in the rejection of cardiac transplants (Crandall et al., 1988, Kawauchi et al., 1993) liver transplants (Candinas et al., 1995), stem cell transplants (Goulmy et al., 1977, Goulmy et al., 1978) and risk of GvHD following stem cell transplant. (Atkinson et al., 1986, Gale et al., 1987).

HY was also used to study transplantation tolerance. Billingham *et al.,* (Billingham and Silvers, 1960, Billingham et al., 1965) have shown that tolerance to HY skin grafts could be induced in high responder mice if neonates were injected with syngeneic or allogenic male cells. More recently, Chai *et al.,* have shown that tolerance to HY antigens could be induced in high responder mice by i.n. administration of the MHC class II restricted HY peptides (Chai et al., 2004). The process by which this causes transplantation tolerance will be explored in this thesis.

1.4.3 Linked suppression

Linked suppression is the process by which tolerance to one set of antigens can spread to other unrelated antigens, but only if they are presented to the T cell by the same APC. For example, CBA ($H2^k$) mice could be made tolerant to multiple minor H antigen mismatched B10.BR ($H2^k$ on B10 background) skin grafts if they are given $CD4^+$ and $CD8^+$ specific monoclonal antibodies at the time of grafting. After the monoclonal treatment these mice were tolerant of B10.BR but would reject third party skin grafts, even if these grafts were

placed in the same graft bed as the B10.BR graft. For example transgenic CBK mice that express the $H2K^b$ class I molecule on a B10 background were rejected, because the $H2K^b$ molecule is strongly immunogenic. However, about half of the CBA mice that were tolerant to B10.BR would accept grafts from (B10.BR x CBK)F_1 mice. Mice that were tolerant to (B10.BR x CBK) F_1 skin grafts would then also accept CBK skin grafts despite $H2K^b$ expression. This is because $H2K^b$ had been presented on the same cells as the B10.BR minor antigens to which they were already tolerant (Waldmann and Cobbold, 1998). This shows that through linked suppression tolerance can be spread to other antigens

1.4.4 Tolerance induced by intranasal administration of HY peptides

Intranasal peptide administration has been used to induce tolerance in models of allergy caused by house dust mite (Hoyne et al., 1996) and birch pollen (Repa et al., 2008), and autoimmune disease such as EAE (Anderton and Wraith, 1998) and rheumatoid arthritis (Broere et al., 2008).

In EAE models, oral and i.n. peptide antigen can induce tolerance and ameliorate disease. The mechanism of tolerance induction was dependent on the dose of antigen – high doses resulted in deletion (Chen et al., 1995) while low doses could induce a population of regulatory T cells (Chen et al., 1994) (Whitacre et al., 1991, Friedman and Weiner, 1994).

In models of EAE, tolerance could not be induced by i.n. peptide in IL-$10^{-/-}$ mice, suggesting that this cytokine is crucial for the function of these induced regulatory T cells (Massey et al., 2002).

Furthermore, there is evidence that that i.n. peptide can induce linked suppression to other antigens. For example i.n. administration of the immunodominant epitope of Der P1 could induce tolerance to another four peptides (Hoyne et al., 1997). And i.n. administration of peptide 139-151 of proteolipid protein (PLP) could induce tolerance to two other myelin basic protein (MBP) peptides (Anderton and Wraith, 1998). The mechanism by which this occurred is not known but it was usually the immunodominant peptide that induced tolerance to other peptides.

Previous work in this laboratory using the HY model has shown that i.n. administration of the MHC class II restricted HY peptides to female mice resulted in transplantation tolerance and linked suppression (Chai et al., 2004).

Tolerance to male skin grafts could be induced in 90-100% of high responder strain ($H2^b$) or ($H2^b$ x $H2^k$)F_1 female mice, by i.n. HY-A^b*Dby*. These recipients were still able to reject multiple minor H antigen mismatched grafts. Linked suppression was demonstrated as i.n. HY-A^b*Dby* induced tolerance in the F1 recipients to 1 additional class II and 4 additional class I peptide epitopes. However, i.n. administration of the HY-E^k *Dby* peptide to ($H2^b$ x $H2^k$)F_1 female mice resulted in tolerance in half the recipients (Chai et al., 2004).

Intranasal peptide could also induce tolerance to male bone marrow. Female mice were pre-treated with the HY-A^b*Dby* peptide or PBS, then given low dose irradiation followed by male bone marrow cells. The male bone marrow engrafted in the peptide treated mice creating a chimeric female recipient with a proportion of male derived T cells, but not the PBS treated mice. Peptide pre-treatment also caused prolonged survival of syngeneic CFSE labelled male spleen cells and these mice were subsequently tolerant of a male skin graft (Chai et al., 2004).

During the tolerance induction phase following i.n. peptide administration, *in vitro* investigation of cytokine production by the endogenous HY-A^b peptide specific $CD4^+$ T cells showed that the there were no detectable cells from the cervical lymph nodes producing either IL-2 or IFNγ in response to irradiated female cells pulsed with either HY-A^b*Dby* or HY-D^b*Uty* peptide (Chai et al., 2004).

After skin grafting, and during the induction of linked tolerance in female mice that had been pre-treated with peptide, both HY-A^b*Dby* specific $CD4^+$ T cells and HY-D^b*Uty* $CD8^+$ T cells were shown to produce IFNγ but not IL-2 in response to male peptide. Surprisingly IL-10 was found not to have a crucial role in this model as tolerance could still be induced in IL-$10^{-/-}$ mice (Chai et al., 2004).

How i.n. peptide induces graft tolerance is not known. However, tolerance could be transferred to neonatal females by $CD4^+CD25^+$ and $CD25^-$

splenocytes (Chai et al., 2004). This and the results above suggests that skin graft tolerance is not simply deletion of the antigen specific T cells but involves a population of antigen specific regulatory cells. If the peptide is presented by DCs in the draining lymph nodes, in absence of co-stimulatory signals this could lead to the induction of anergy or suppressive properties in the antigen specific cells. This project will analyse these antigen specific cells and describe the molecular changes that take place within them.

1.5 Aims

Using i.n. peptide induced antigen specific tolerance in the HY model the aims of this project are to:

- investigate gene expression in antigen specific $CD4^+$ T cells during *in vivo* tolerance induction
- investigate gene expression in antigen specific $CD4^+$ and $CD8^+$ T cells in tolerant mice following male skin graft i.e. during the induction of linked suppression
- examine the migration of HY antigen specific T cells after male skin grafting

1.6 Preliminary microarray work

Prior to the experiments described in this thesis, some preliminary microarray work was carried out in the lab by Diane Scott, Caroline Addey and Jian-Guo Chai in collaboration with the MRC Harwell.

In order to look at gene expression during the tolerance induction phase (before skin grafting) cells from female TCR transgenic Marilyn mice were used. $CD4^+$ T cells from these mice are specific for the male class II restricted peptide NAGFNSNRANSSRSS (HY-A^b*Dby*). These cells were labelled with CFSE and transferred into female B6 recipients. The recipients were then given either 3 doses i.n. HY-A^b*Dby* peptide, or one dose of i.n. HY-A^b*Dby* peptide plus LPS. Five days later the CFSE labelled antigen specific T cells were sorted from the recipient cells by flow cytometry. RNA was extracted from the sorted cells and gene expression was then analysed by an 'in house' microarray from the Sanger Institute, Cambridge. Figure 1.2 shows the gene expression in both conditions (peptide alone and peptide +LPS) compared to naïve cells from Marilyn mice. Of the ten thousand genes compared a shortlist of 149 genes were found to be significantly changed between conditions. This included gene with non-immunological functions. The change in expression of genes that were of special interest is shown in Figure 1.2.

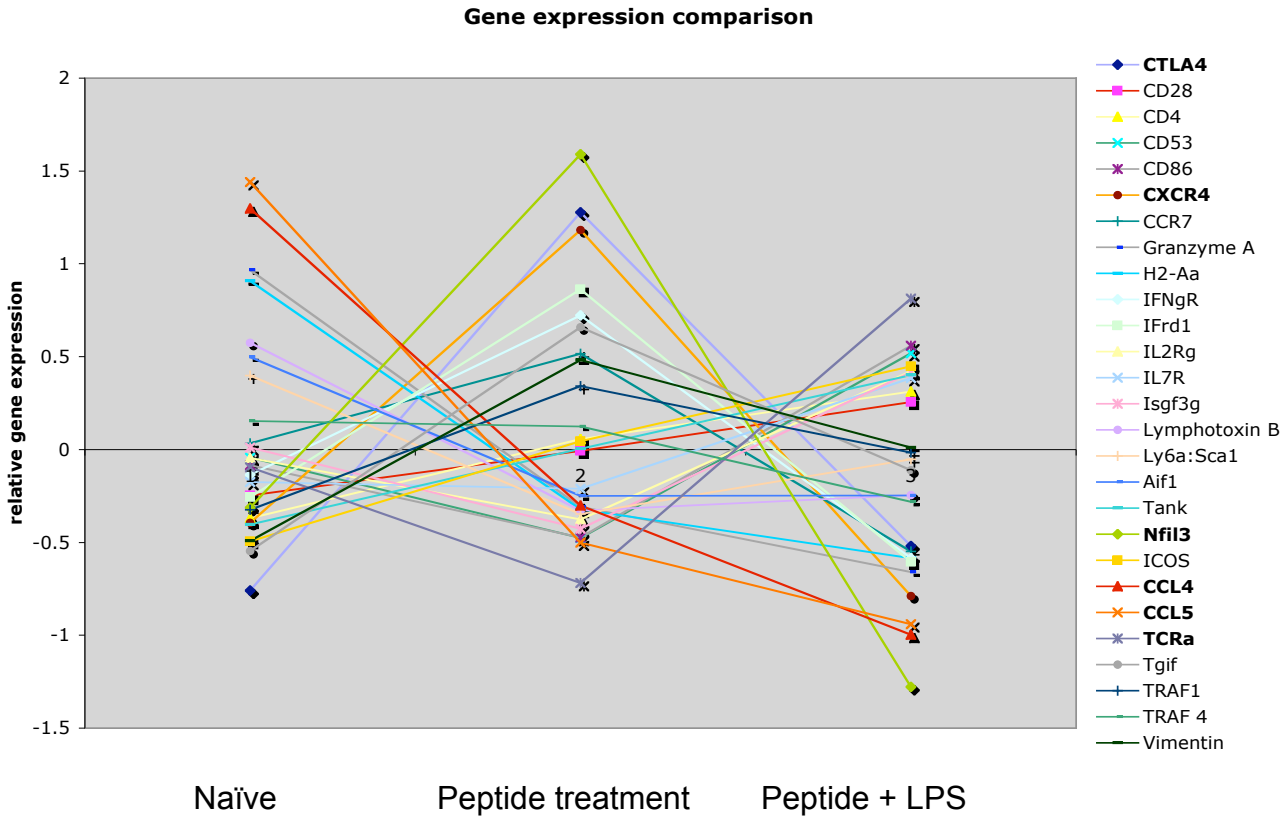


Figure 1.2: CD4$^+$ Cells from female Marilyn mice (specific for the male class II restricted peptide NAGFNSNRANSSRSS) were labeled with CFSE and transferred into female B6 recipients. The recipients were then given either 3 doses of i.n. NAGFNSNRANSSRSS peptide or one dose of peptide + LPS. Five days later the CFSE labeled antigen specific T cells were isolated by flow cytometry. RNA was extracted from the sorted cells. Gene expression analysis was performed by the Harwell microarray facility using a mouse immunology oligo array supplied by the Sanger Institute, Cambridge, with primers for 4200 genes. The resulting data was analyzed by Dr Peter Underhill using MAANOVA software (Churchill, 2004). Using all F tests, 39 genes were identified as being significantly changed between the conditions. Using the F1 test alone, 149 genes were identified. These included genes with non-immunological functions. The change in expression of 27 genes of interest are shown.

These data gave the indication that there were significant changes in gene expression and these were worth investigating further. In order to gain the best possible information from the microarray data I did further work to analyse the data using Ingenuity software. Table 1.3 shows a summary of the pathways in which several genes were found to be changed.

	Peptide treated	Peptide + LPS treated
T cell receptor signalling		
Cd4	↑	↑
Zap-70	↑	↑
Cd28	↓	↑
Ctla4 (Cd152)	↑	↓
Nfkia	↑	↓
Lymphocyte migration		
Cxcr4	↑	↓
Itgal	↑	↑
Itgb1	↑	↑
Actb	↓	↑
Cyba	↓	↑
Rac2	↓	↑
Cd44	↓	↑
NFKB signalling		
Lta	↓	↑
Zap70	↑	↑
Nfkbia	↑	↓
Interferon signalling		
Ifngr1	↑	↓
Isgf3g	↓	↑
Antigen presentation		
Ia associated invariant chain	↓	↓
H2-Aa	↓	↓
H2-T23	↓	↑
H2-Ebl	↑	↓
Chemokine signalling		
Ccl4	↓	↓
Ccl5 (Rantes)	↓	↓
Cxcr4	↑	↓
Others		
Tgif (Tgf beta signalling)	↑	↓
Erg1	↑	↓
C2 (complement cascade)	↓	↑
Bax (Bcl2 associated protein)	↑	↓

Table 1.3 The top 149 changed genes from the microarray analysis were analysed using Ingenuity software. This program identifies if there are genes that belong to the same functional group (for example genes involved in antigen processing and presentation) and can give an indication that a specific pathway or function is up or down regulated compared to expression in naïve $CD4^+$ T cells.

The results of the ingenuity analysis highlight some of the differences between gene expression found under tolerising and immunising conditions. For example the genes involved in lymphocyte migration, in particular the chemokine receptors *Cxcr4* which was found to be upregulated in tolerised cells but down regulated in immunised cells compared to it expression in

naïve T cells. The protein CXCR4 is expressed by naïve T cells and allows them to circulate through lymphoid tissue (reviewed by Bachmann et al., 2006). This may indicate there are difference in both the maturation of T cells and migratory ability of antigen specific T cells following i.n. peptide treatment. Also *Nfkbia* (gene encoding the transcription factor NFκB) was found to be upregulated following treatment with peptide alone and downregulated following peptide +LPS. As this gene is a transcription factor the downstream effects of this gene could be very significant.

In this thesis I use a Q-PCR array to examine gene expression in antigen specific T cells in both tolerising and immunising conditions. The results of these microarray experiments provided the basis when selecting genes to be tested by Q-PCR.

Chapter 2 Materials and Methods

2.1 Cell Culture Reagents

Complete RPMI

RPMI 1640 Medium (Gibco, Paisley, UK) supplemented with 10% foetal calf serum (FCS; Autogen Bioclear, Calne, United Kingdom), HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; 10 mM), penicillin (100 IU/mL) and 100 µg/mL streptomycin, and 5 x 10^{-5} M 2-mercaptoethanol and 2 mM L-glutamine (all Gibco).

DMEM

DMEM (Gibco) supplemented with 2mM Glutamate, 5ml 100U/ml Streptomycin and Penicillin, 1mM Sodium Pyruvate (Gibco), 20mM HEPES, 1% non essential amino acids (Sigma), 50mM 2-mercaptoethanol and 20% FCS

HBSS

HBSS (Gibco) supplemented with 100 IU/mL penicillin, 100µg/mL streptomycin and 5% FCS.

PBS

Phosphate buffered saline was made up from tablets (Oxoid Limited, Basingstoke, UK) and autoclaved for 10minutes at 115°C.

2.2 *In Vivo* Experiments

2.2.1 Animals

TCR transgenic Marilyn and MataHari mice (originally obtained from Olivier Lantz, Institut Curie, Paris), C6, Ly5.1 and Thy1.1 mice were bred in the biological services unit at the Medical Research Council Clinical Sciences Centre (CSC). Table 2.1 shows further details of the transgenic mice. C57BL/6 and CBA mice were from Harlan Olac, (Bicester, United Kingdom). All procedures were conducted in accordance with the Home Office Animals Act 1986.

Name	Ref	TCRβ chain	MHC restriction	Binds Peptide	
Marilyn	(Lantz et al., 2000)	Vβ6	$H2^b$	NAGFNSNRANSSRSS	Class II
MataHari	(Valujskikh et al., 2002)	Vβ8.3	$H2^b$	WMHHNMDLI	Class I
C6	(Douek et al., 1996)	Vβ11	$H2^k$	TENSGKDI	Class I

Table 2.1 TCR transgenic mice used in this project.

2.2.2 Adoptive transfers

Unless otherwise stated, in all experiments single cell suspensions were obtained by pushing dissected lymph nodes and spleen through a cell strainer and washed in supplemented HBSS. In the adoptive transfer experiments single cell suspensions from the spleen and lymph nodes of Marilyn mice were washed in HBSS and resuspended in 1ml PBS. 5,6-carboxyflourescein succinimidyl ester (CFSE) (Molecular Probes, Cambridge, United Kingdom) was added at a final concentration of 5mM, and cells were incubated in the dark at room temperature for 10 min. Cells were washed twice in PBS, counted and then injected i.v. into recipient mice. An average of approximately $45x10^6$ Marilyn cells were transferred per recipient.

2.2.3 Intranasal peptide

HY^{Ab}*Dby* peptide: NAGFNSNRANSSRSS was synthesized by the Central Research Resources unit of the CSC using 9-fluorenylmethyloxycarbonyl–

protected amino acids and (2-(1-H-benzotriazol-2-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation chemistry. Synthetic peptides were made up as 5mg/ml stocks in PBS and filter sterilized.

Mice were anaesthetised with Isoflurane. Peptide treated mice were given 100μg HY^{Ab}*Dby* peptide intranasally (i.n.) on three consecutive days. Mice treated with peptide + LPS were given one dose of 100μg HY^{Ab}*Dby* peptide and 4μg LPS (Sigma, Poole, UK) i.n.

2.2.4 Flow Cytometery sorting

The spleen and lymph nodes of recipient mice were dissected, washed in HBSS, incubated for 10 minutes at room temperature in red cell lysis buffer (RCLB: Puregene, Flowgen, Ashbydelazouch, UK). $CD8^+$ T cells and B cells were depleted by incubating cells at 4ºC for 20 min on a rotor with 50μl of anti-CD8 and anti-B220 Dynabeads (Dynal, Invitrogen Ltd, Paisley, UK). The cells were applied to a magnet and the non-binding negative fraction was collected and washed in PBS. Cells were then stained with either 2μl Anti Thy1.2) or Ly5.2 PE (both from BD Biosciences, Pharmingen, Cowley, UK) for 20mins on ice. Cells were then washed again in PBS and resuspended in RPMI supplemented with 5% FCS. Cells were then sorted and $CFSE^+$ and PE^+ double positive cells were collected.

2.2.5 Skin Grafting

Skin grafting was conducted using the method of Billingham and Medawar (Billingham and Medawar, 1951) using tail skin grafted onto the lateral thorax.

Mice were anaesthetised using with a mixture of 200μg Medetomide (Domitor, Pfizer, Sandwich, Kent), 150μg Ketamine (Ketaset, Fort Dodge, Southampton), 60μg Meloxicam (Metacam, Boehringer Ingelheim, Bracknell, UK) and 3638μl dH_2O. Each mouse given around 0.4ml depending on weight. Once the mice had lost the pedal reflex, the lateral thorax was shaved and sprayed with antiseptic. A small area of skin (approx $1cm^2$) on the lateral thorax was cut away and full thickness tail skin from donor mice was placed on the area. The area was then dressed using gauze soaked in Vaseline and

covered with a plaster cast. Anaesthesia was reversed by Atipamezole (Anti-Sedan, Pfizer).

The mice were kept in a warming box at 30°C until completely recovered. 7 to 8 days after grafting the plaster casts were carefully removed and the grafts were observed every 2 to 3 days and scored as rejected when less than 10% viable tissue remained. The results analysed using SPSS software.

2.2.6 *In vivo* killing assay

Single cell suspensions made from the spleens of syngeneic WT male and female mice were harvested, washed and resuspended in 1ml PBS. Cells were then stained with CFSE at room temperature for 10mins in the dark. Female splenocytes were labelled with a low concentration of CFSE (final concentration of 0.5mM) and male cells were labelled with high concentration of CFSE (final concentration 5mM). Cells were then washed twice with PBS counted and mixed at a ratio of 1:1. The ratio was checked by FACS and adjusted accordingly if necessary. Between 5-10 million cells were given intravenously (i.v.) to each recipient.

24hours after injection, the mice were tail bled and the blood was collected into blood buffer (PBS with 10mM EDTA (Sigma) and 100μ/ml Heparin (Multiparin)). The cells were incubated with RCLB for 10 minutes at room temperature, washed in PBS and FACS analysed to get the starting ratios.

One week later mice the mice were bled again and the cells were treated in the same way as described above then analysed by FACS. The ratio of male to female cells was calculated and compared to the starting ratio.

In some experiments the fluorescent orange dye CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) (Molecular Probes) was used instead of CFSE. Briefly, $2x10^6$ splenocytes/ml in complete RPMI supplemented with 10μM or 100μM CMTMR were incubated at 37°C for 15mins, followed by incubation in fresh complete RPMI for another 15mins at 37°C. Cells were then washed twice in PBS, counted and mixed at a ratio of 1:1 and given by IV. Mice were then bled 24 hours and 7 days later as described above.

2.3 *In Vitro* Assays

2.3.1 Cell freezing

Sorted cells were transferred to sterile Eppendorf tubes (Eppendorf, Cambridge, UK), spun down and snap frozen by dropping into LN_2. Frozen cell pellets were then stored at -80°C

2.3.2 Depletion of Thy1.1

In order to decrease the sorting time, in some experiments $Thy1.1^+$ cells were depleted by adding 4μl anti-Thy1.1 FITC antibody (BD biosciences) and incubating for 20mins on ice. Cells were washed in PBS then 10μl anti-FITC microbeads were added (Miltenyi Biotech, Surrey, UK). Cells were incubated again on ice for 20mins. They were then washed in buffer: 500ml PBS supplemented with 2ml 0.5M EDTA (Sigma) and 5ml FCS (Autogen Bioclear) and separated using an MS column (Miltenyi Biotech). Cells were then stained with anti-Thy1.2 PE (BD biosciences), and sorted as detailed above.

2.3.3 Enrichment of Thy1.2

In some experiments $Thy1.2^+$ cells were enriched before sorting to reduce the sorting time. Cells were incubated with 15μl anti-Thy1.2 microbeads (Miltenyi biotech) on ice for 20mins. They were then washed in buffer (see above) and separated using an MS column (Miltenyi biotech). The positive fraction was washed and stained with anti Thy1.2 PE (BD biosciences), and sorted as detailed above.

2.3.4 Skin digestion for lymphocytes

Skin grafts were cut into small pieces and digested with 0.125% (v/v) trypsin (Invitrogen) at 37°C for 1h. Viable cells were enriched by ficoll-paque (GE Healthcare, Amersham Bucks) centrifugation and washed in PBS before bseing stained.

2.3.5 IFNγ capture assay

The cytokine secretion assay (Miltenyi Biotec) was carried out according to the manufacturers instructions. Briefly, cells were stimulated *in vitro* as detailed in the text. Cells were then washed with cold buffer (500ml PBS supplemented 4ml 0.5M EDTA and 0.5% FCS) and incubated with the catch reagent on ice for 5mins followed by 45mins at 37°C. Cells were washed in cold buffer and labelled with 10μl IFNγ detection antibody PE, 1μl anti-CD4 FITC and 1μl anti-B220 APC. Before analysis 0.5μg/ml 7-AAD (BD biosciences) was added to exclude dead cells. In order to analyse the results, firstly the lymphocytes were gated then dead cells (7-AAD^+) were excluded and after this the $B220^+$ cells were excluded from the analysis.

2.3.6 Transmigration assay

Female C57BL/6 endothelial cells (a gift from Federica Marelli-Berg) were grown in supplemented DMEM (see above). Endothelial cells were stimulated with 600U/ml IFNγ (Pepro Tech, Peterborough, United Kingdom) for 72 hours then trypsinised.

Transwells with 3.0μm pore size in a 6 well plate (Corning Transwell collagen-coated membrane inserts, Sigma) were coated in 2% gelatine (Sigma). The endothelial cells were washed and resuspended at $1x10^6$/ml. 2ml DMEM medium was added to each well, and 800μl of the endothelial cell suspension. The endothelial cells were then incubated on the transwell overnight at 37°C. The following day, the endothelial cells examined under the microscope to check that they formed a confluent monolayer across the transwell.

Single cell suspensions were made from the spleen, graft draining and non-graft draining lymph nodes of rejecting and tolerant mice. The red cells were lysed and $CD4^+$ and $B220^+$ cells were depleted using Dynabeads as described above to give a $CD8^+$ T cell enriched population. Cells were resuspended at 2.5 $x10^6$/ml in supplemented RPMI.

The endothelial cell coated transwells were washed with PBS and 800μl ($2x10^6$ cells) from the $CD8^+$ T cell-enriched population were added to the upper chamber of the transwells in complete RPMI. The cells were incubated at 37°C for 24 hours.

The cells in the upper and lower chambers were carefully harvested into FACS tubes, counted and stained with 0.25μl Db*Uty* Tetramer PE and 1μl CD8 APC in 10μl complete RPMI per sample for 20mins at 37°C. Cells were then washed in PBS and analysed by flow cytometry.

2.4 Flow Cytometry

2.4.1 General Method

Throughout the project, unless otherwise stated, the same protocol was followed for FACS. Cells were passed through a filter to form a single cell suspension in PBS. Directly conjugated antibodies were added to the sample at approximately 0.4μg/10x10^6 cells. The cells were then incubated at 4°C for 20minutes then washed twice in PBS. Data was acquired with Cell Quest (BD biosciences) but further analysis was done using FlowJo (Treestar, Oregon, USA).

2.4.2 Naïve T cells staining

Both naïve Marilyn $CD4^+$ T cells and naïve WT B6 $CD8^+$ T cells were used as controls for the gene expression studies. Lymphocytes were isolated from lymph nodes and spleen of the mice and incubated with the following antibodies:

$CD4^+$ T cells - anti-Vβ6 FITC, anti-CD4 PerCP, anti-CD45RB PE and anti-CD25 APC (all BD biosciences) for 20mins on ice, washed with PBS and sorted as described above for $CD4^+V\beta6^+CD45RB^+CD25^-$ population.

$CD8^+$ T cells - anti-CD44 FITC, anti-TCRβ PE, anti-CD62L APC (all BD bioscience) and anti-CD8 Pacific Blue (e-bioscience) for 20mins on ice, washed with PBS and sorted as described above for $CD8^+TCR^+CD62L^{high}CD44^{low}$ population.

2.4.3 Regulatory T cell staining

45x10^6 Marilyn (Thy1.2) cells were transferred into each Thy1.1 recipients without CFSE staining. The recipients then received 3x 100μg doses of

HY^{Ab}*Dby* i.n. Five days after the first dose, cells from the spleen and lymph nodes were taken and CD8^{+} T cells and B cells were depleted by incubating cells at 4ºC for 20 min on a rotor with 50µl of anti-CD8 and anti-B220 Dynabeads (Dynal). The cells were separated on a magnet and the negative (non-binding) fraction was collected and washed in PBS. Cells were then incubated with the following antibodies: CD25 PE, CD4 PerCP and Thy1.2 APC (all from BD biosciences) for 15minutes at 4ºC. Cells were washed again in PBS and incubated with 1ml of CytoFix/Cytoperm solution (BD Biosciences) for 1 hour at 4ºC. Cells were washed with perm/wash solution (BD Biosciences) and stained with Anti FoxP3 antibody (BD biosciences) before being analysed by FACS.

2.4.4 Tetramer staining

Mice were tail bled into blood buffer, then incubated with RCLB for 10mimutes at room temperature. Cells were then washed in 10%FCS RPMI and incubated with 10µl 10%FCS RPMI with 0.25µl Db*Uty* tetramer PE (Proimmune, Oxford, UK) and 0.5µl anti CD8 APC (BD Biosciences) for 20mins at 37ºC. Cells were then washed in PBS and analysed by FACS. The red cells were lysed and the cells were then stained in the same way as described above.

2.4.5 Seven colour FACS

Cells from the spleen, draining and non-draining lymph nodes of peptide treated and control (no treatment) were isolated and pooled. Some cells were set aside for single staining and unstained controls. The remaining cells were washed in HBSS, incubated for 10 minutes at room temperature in 3mls RCLB. The cells were then washed again in complete RPMI and stained with 0.25ul Tetramer PE in 10µl complete RPMI at 37°C 20mins. Without washing, the other antibodies listed below, except streptavidin conjugated APC-Cy7, were added. Cells were then incubated for 20mins at 4°C, washed in PBS and incubated with 1µl of Streptavidin conjugated APC Cy7 for 20mins at 4°C. Cells were washed in 10% RPMI and analysed using Cyan (Dako, Ely, UK)

and Summit software (Dako). This was done at the flow cytometry laboratory at St Mary's Campus, Imperial College London.

	Channel	Dye	Antibody	Manufacturer
1	FL1	FITC	Cxcr4	BD Biosciences
2	FL2	PE	Db*Uty* Tetramer	ProImmune
3	FL4	PE-Cy5	Cd4	e Bioscience
4	FL5	PE-Cy7	Ccr7	e Bioscience
5	FL6	Pacific Blue	Cd8	e Bioscience
6	FL8	APC	Cxcr3	R&D Systems
7	FL9	Streptavidin conjugated APC Cy7	Biotin conjugated anti-CD11a (LFA-1)	e Bioscience

Table 2.2 Antibodies used in multi-colour FACS experiments.

2.5 Microscopy

Skin, spleen and lymph nodes were suspended in OCT (Raymond Lamb, Eastbourne, UK) and snap frozen in Isopentane (Fisher Scientific). Tissues were stored at -80°C before cutting into 5mm sections using a cryostat. Tissue sections were suspended on poly L lysine coated slides (CA Hendley, Essex, UK). Slides were then fixed and permeabilised in Acetone (BHD, VWR, Leicester) at -20°C for 10 minutes, washed in PBS then blocked using PBS supplemented with 10% FCS for 20mins at 4°C. They were then stained with CD8-Biotin (ebioscience) 10μg/ml in PBS supplemented with 3%FCS, for 1 hour at room temperature followed by 3 washed in PBS. The slides were then stained with 1μl in 50μl Streptavidin FITC (BD bioscience) and Thy1.2 PE 10μg/ml in 3% PBS for 1 hour at room temperature. They were then washed three times in PBS and mounted using vectashield with DAPI (Vector labs, Burlingame, California).

2.6 Molecular Experiments

2.6.1 RNA extraction from cell pellets

RNA was extracted from either frozen cell pellets or from cells immediately after sorting using either RNAqueous-4PCR followed by a DNase I step (all from Ambion, Huntingdon, UK) or PicoPure kit (Arcturus, Molecular Devices Ltd, Wokingham, UK) with a 'on column' DNase I digestion step using DNAse I from Qiagen (West Sussex, UK). In both cases the manufacturers instructions were followed. 1μl of RNase inhibitor (Qiagen) was also added to RNA preparations. RNA quantity and quality was analysed on the Nanodrop 1000. Samples were stored at -80ºC.

2.6.2 RNA extraction from skin

Skin grafts from culled mice were cut into 4 pieces and snap frozen by dropping into liquid nitrogen. The skin was stored at -80°C. RNA was extracted by adding 1ml Trizol (In Vitrogen) and homogenising the sample on ice using electrical homogeniser. Samples were then incubated at room temperature for 5mins, then 200μl of Chloroform were added. Samples were vigorously shaken and incubated for a further 3 minutes. They were then centrifuged for 15mins at 12,000g and the colourless aqueous was carefully transferred into a fresh tube. RNA was then precipitated by adding 500μl isopropanol and incubated at room temperature for 10mins. This was followed by a centrifugation 12,000g for 10min at 2-8ºC. The RNA is then precipitated in the bottom of the tube. The supernatant was removed and RNA was washed by adding 1ml 75% Ethanol. Samples were vortexed and then centrifuged at 7,500g for 5mins at 2-8ºC. The supernatant was then removed and the RNA pellet left to air dry at room temperature. When all the alcohol had evaporated RNA was dissolved in 30μl RNase free water (Qiagen).

RNA preparations were then cleaned using Qiagen mini columns with and 'on column' DNase I digestion step using DNAse I (Qiagen). 1μl RNase inhibitor (Qiagen) was also added to samples. RNA quantity and quality was assessed using the Nanodrop 1000. Samples were stored at -80ºC.

2.6.3 cDNA synthesis

cDNA was made from 100ng RNA using the MessageSensor RT kit (Ambion) using 10mM random hexamer primers (Ambion). The manufactures' instructions were followed except with longer incubation times – samples were incubated at 43ºC for 60mins followed by 95ºC for 10mins. Samples were either used straight away for the IQA or stored at -20ºC.

2.6.4 Immunoquant Array (IQA) and Global Pattern Recognition (GPR)

Custom 384 well plates containing frozen primers were obtained from Bar Harbour Biotechnology (Bar Harbour, Maine, USA). A master mix was prepared containing 2016μl Power Sybr green master mix (Applied Biosystems), 1814.4μl DEPC treated water (Ambion) and 201.6μl cDNA diluted 1:10 with DEPC treated water (Ambion). 10μl was added to each well on the plate. Plates were run on the 7900HT sequence detection system using 384 well block (Applied Biosystems). Standard cycling conditions were used (95ºC 10mins followed by 40 cycles of 95 ºC for 15sec and 60 ºC for 1 min).

The Ct values for the genes were then exported into global pattern recognition (GPR) software algorithm. This software compares the Ct values of each gene and determines whether the differences are statistically significant. It also takes into account biological replicates to give a statistically robust result.

Firstly, genes are analysed to see whether they qualify as normalisers. Normalisers have similar Ct values for both the control and experimental sample (that is, they are unchanged by the experimental conditions). They must also have a Ct value less than 37.5 because Ct values above this are from low copy number genes and can be unreliable.

The second phase of the analysis looks at each gene in turn to determine if the change in Ct is significant. Each gene is compared to every normaliser in succession and the ΔCt is calculated ($\Delta C_{T\ Gene} = C_{T\ Gene} - C_{T\ Normaliser}$). For each gene – normaliser pair, the ΔCt for the control and experimental group are compared by a two-tailed unpaired Student's T test. A P value of less than 0.05 is considered significant.

In earlier version of the software after all the comparisons were made, a score was generated: For example comparing gene expression of Marilyn cells from peptide treated vs. naïve cells, FoxP3 was found to be significantly different when compared to 67 of the 75 normalisers. The score was calculated as 67/75= 0.89. The results were then given in rank order. Scores of over 0.4 are regarded as significant.

During the course of the project a newer version of the software was developed which gave a P Value instead of the score. In this case all P values less than 0.05 are considered significant.

The fold change in gene expression was then calculated using the 10 best normalisers (genes that vary little in expression between control and experimental groups). As fold change is calculated after the ranking has been done, genes that have high scores may not always be the genes with the greatest change.

2.6.5 Quantitative PCR TaqMan Assay

The amount of FoxP3, CD8 and 18S mRNA in skin preparations was analysed using a TaqMan Assay (Applied Biosystems). The manufactures instructions were followed – briefly, for each reaction 0.5μl of 20x TaqMan gene expression mix, 5μl 2x TaqMan master mix (Applied Biosystems), 1μl cDNA and 3.5μl DEPC treated H_2O. All samples were run in triplicate for 10mins at 95ºC followed by 40 cycles of 95 ºC for 15sec and 60 ºC for 1 minute. Relative quantification was calculated using RQ manager software (Applied Biosystems) with 18S as a housekeeping gene and untreated tail skin samples as calibrators.

2.6.6 Quantitative PCR Sybr Green Assay

The amount of CD4, IFNγ and 18S mRNA in skin preparations was analysed using in house primers and Sybr green (Applied Biosystems). For each reaction a mix was made of 5μl 2x Power Sybr Green master mix, 0.5μl 10mM forward primer, 0.5μl 10mM reverse primer (for sequences see table 2.3), 1μl cDNA and 3μl DEPC treated H_2O. A standard curve was also run by converting 400ng RNA to cDNA and diluting the cDNA to a final concentration of 50, 10, 5, 1, 0.1 and 0.01ng. Standard curve, unknown samples and non-

template controls were all run in triplicate for triplicate for 10mins at 95ºC followed by 40 cycles of 95 ºC for 15sec and 60 ºC for 1 minute. Relative quantification was calculated using the ΔΔCt method using 18S as a housekeeping gene and untreated tail skin as calibrators.

Gene	Forward Primer	Reverse Primer
18S rRNA	CCGCAGCTAGGAATAATGGAAT	CGAACCTCCGACTTTCGTTCT
Cd4	CCGTACAAAAGTCCACCAAAGAC	TCCCATCACCTCACAGGTCAA
Ifnγ	TCTTCTTGGATATCTGGAGGAACTG	GCAATACTCATGAATGCATCCTTT

Table 2.3 Sequences of Primers used for 'in house' quantitative PCR assay. Sequences taken from (Akilesh et al., 2003).

Chapter 3 : Tolerance Induction Phase

3.1 Introduction

Intranasal HY-A^b*Dby* peptide is known to induce tolerance to male skin grafts in female mice. The first part of this project will look at the tolerance induction phase to investigate the changes in gene expression that take place in antigen specific CD4$^+$ T cells that are responding to the initial peptide challenge.

To analyse gene expression in antigen specific T cells under tolerising (peptide alone) or immunising (peptide + LPS) conditions, cells from TCR transgenic mice specific for HY-A^b*Dby* peptide (Marilyn) were used. Marilyn cells were labelled with CFSE and adoptively transferred into WT syngeneic B6 female recipients that were then treated intranasally (i.n.) with HY-A^b peptide +/- LPS. Five days later the Marilyn T cells were sorted from the recipient spleen and lymph nodes, RNA was extracted and gene expression was analysed using a quantitative PCR array. This was thought to be a suitable time point because mice given male skin grafts 5 days after i.n. peptide have been shown to be tolerant to the male graft (Dr JG Chai, personal communication). Also differences in cytokine secretion by antigen specific T cells have been described at this time point. It has been shown that more cells from the cervical lymph nodes of female mice treated with i.n. HY-A^b*Dby* peptide + LPS secrete IFNγ and IL-2 in response to *in vitro* stimulation with peptide than if mice are treated with HY-A^b*Dby* peptide alone. No IL-2 or IFNγ secreting cells were detected in WT mice given i.n. peptide alone (Chai et al., 2004).

3.2 Developing methods for Adoptive Transfers

Choice of recipient

To distinguish between the transferred Marilyn cells and the recipient cells it was necessary to chose another marker, in addition to CFSE. In these experiments the Ly5 (CD45 expressed on all nucleated mouse blood cells) and Thy-1 (expressed on mouse T cells) markers were used.

Firstly, $Rag^{-/-}$ Marilyn T cells were transferred into a Ly5.1 recipient which was then given i.n. HY-A^{b}*Dby* peptide + LPS (Figure 3.1). This showed that CFSE labelled Marilyn cells were negative for Ly5.2. Therefore Marilyn cells could only be differentiated on basis of the CFSE staining. Following this result, the Ly5 phenotype of the Marilyn mice was investigated using anti Ly5.1 and anti Ly5.2 antibodies (Figure 3.2). The results showed that Marilyn cells were $Ly5.1^{+}$, and this was confirmed following contact with Dr Olivier Lanz from whom the transgenic line originated.

Next CFSE labelled Marilyn $Ly5.1^{+}CD4^{+}$ T cells ($27x10^{6}$) were adoptively transferred into WT B6 $Ly5.2^{+}$ recipients. 24 hours later the mice received HY-A^{b}*Dby* + LPS. Five days later cells from the spleen and lymph nodes were analysed. Most of the CFSE labelled cells divided up to 6 times. However, the sorting the gate was placed in the wrong position and only cells that had divided 6 times or more were recovered, this meant the cell recovery was only 125,000 cells (Figure 3.3A).

Finally Marilyn T cells ($Thy1.2^{+}$) were adoptively transferred into a Thy1.1 recipient. Figure 3.3.B shows the transfer of $100x10^{6}$ CFSE labelled Marilyn cells into a Thy1.1 recipient. Thy-1 is only expressed on T cells and since in this experiment the $CD4^{+}$ T cells were not isolated before sorting, there is a population of CFSE positive Thy1.2 negative cells, that were gated out when the cells were sorted. Again the cells had divided approximately 6 times. The gate in this experiment was widened to collect all dividing cells and a total of $1.4\ x10^{6}$ cells were recovered. From these results it was decided that Thy1.1 recipients would be used in future experiments as the separation of recipient/donor cells was very clear and the CFSE divisions were easy to see and the transfer gave a good yield of cells.

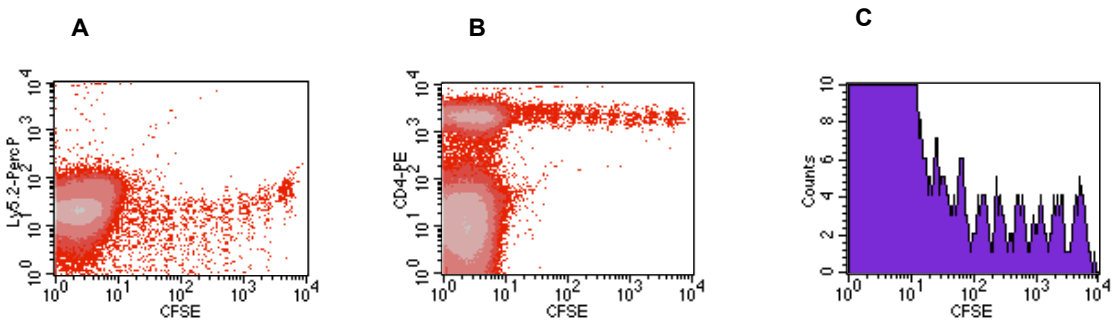


Figure 3.1: Adoptive transfer of Marilyn cells into a B6 Ly5.1 recipient. 24h after transfer, recipients were treated i.n. with peptide plus LPS. Splenocytes were taken at day 5 for sorting. (A) CFSE and Ly5.2 staining of cells. (B) CD4 staining against CFSE staining (C) histogram of CFSE staining of the cells. All graphs show profiles before sorting.

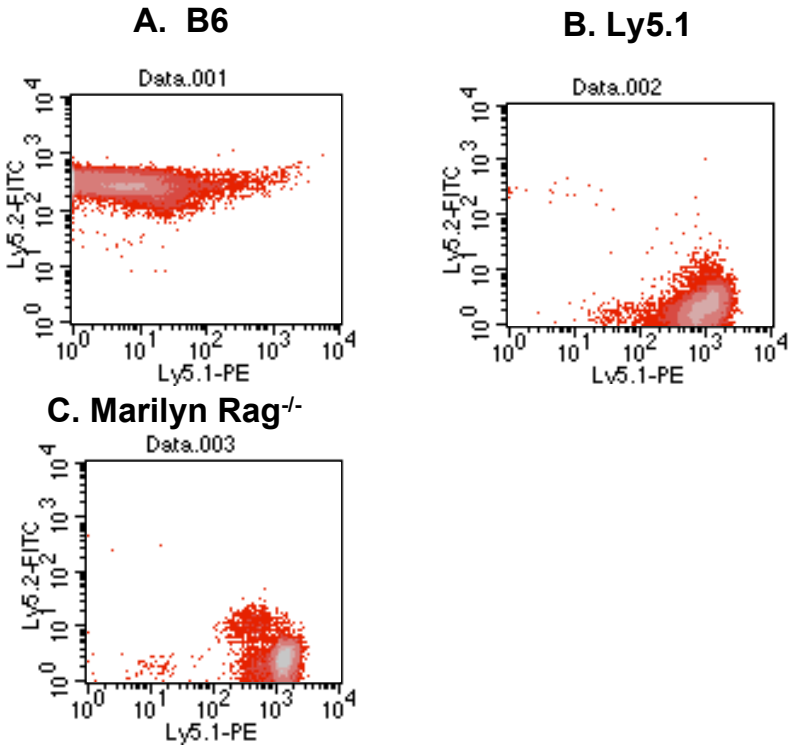


Figure 3.2: Ly5.1 phenotype of Marilyn cells. Splenocytes from the strains indicated were stained with anti-Ly5.1 PE and anti-Ly5.2 FITC. (A) B6 (B) B6 Ly5.1 (C) Marilyn Rag$^{-/-}$

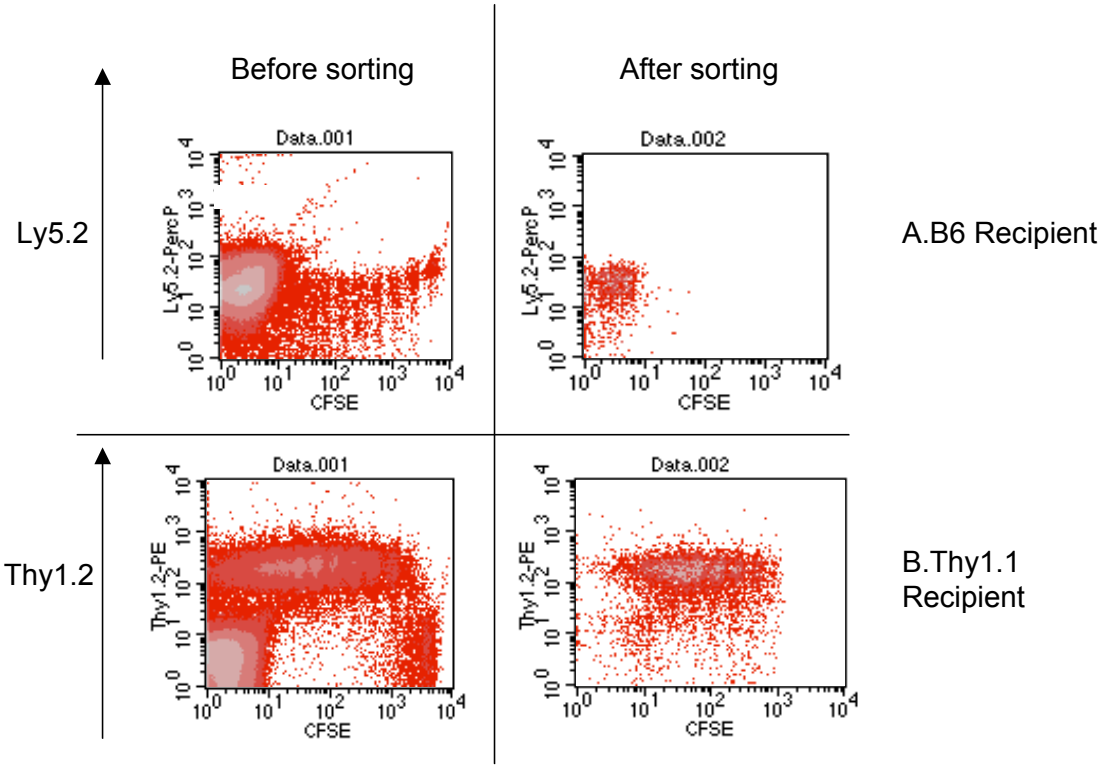


Figure 3.3: Flow cytometric analysis of CFSE labelled Marilyn cells following adoptive transfer into B6 and B6 Thy1.1 recipient strains, before and after sorting. Recipients received HY-A^{b}*Dby* peptide + LPS i.n. 24h after cell transfer, cells from the spleen and lymph nodes were taken at day 5 for sorting. (A) Marilyn cells transferred into a B6 recipient (B) Marilyn cells transferred into a Thy1.1 recipient. In both cases cells that had not divided as identified by the CFSE profile were excluded from the sorting.

CFSE staining and isolation of $CD4^+$ Marilyn T cells

To determine whether it was better to either stain all cells with CFSE and then select the $CD4^+$ T cells, or to isolate the $CD4^+$ T cells first and label only these cells with CFSE, both approaches were tested; the results are shown in Table 3.1.

	1. CFSE → CD4 separation	2. CD4 separation → CFSE
Cell recovery	5.6×10^6	4×10^6
Viability	84%	56%
Purity*	92% $CD4^+$	-
Function**	4 rounds of division	4 rounds of division

Table 3.1: Comparison of cells treated with CFSE before or after CD4 separation

*Assessed by flow cytometry using anti-CD4 PE and anti CD8 PerCP

** The cells were stimulated with anti-CD3 and anti-CD28 dynabeads for 3 days and analysed by flow cytometry. The number of divisions was estimated from the CFSE profile

The cell recovery and viability was lower using the second method ($CD4^+$ separation followed by CFSE staining). The purity of these cells could not be assessed, as the CFSE staining was too bright. Both methods yielded cells which were functionally similar following stimulation with anti-CD3 and anti-CD28 beads. As the first method (CFSE followed by $CD4^+$ separation) gave better cell recovery, viability and a high purity it was decided that this would be the best strategy for the adoptive transfers.

In later experiments the $CD4^+$ separation step was no longer included. This was because some $CD4^+$ T cells may be lost during the separation process and as the number of cells recovered was limited, all possible causes of loss were minimised. Without the separation step cells could be transferred into the recipient faster. Although this resulted in other cell types being labelled with CFSE and transferred into recipient mice, these cells could be gated out during the subsequent cell sorting and only the $Thy1.2^+$ divided CFSE-labelled cells were collected.

Enriching the Thy1.2$^+$ population

5 days after i.n. HY-A^b*Dby* the Thy1.2$^+$ Marilyn cells were separated from Thy1.1$^+$ recipient cells by Flow cytometric sorting. To decrease the time taken to sort the Thy1.2$^+$ Marilyn cells, two approaches were tested.

The first was to deplete the recipient Thy1.1 cells with microbeads before the sorting (Figure 3.4). A mixture of Thy1.2$^+$ Marilyn cells and Thy1.1$^+$ cells were separated using microbeads and the resulting positive and negative fractions were analysed by FACS. The Thy1.2$^+$ population was enriched from 8% to 14%. However, after enrichment there was still a considerable number of unwanted Thy1.1 cells present.

The second approach involved positively selecting the Marilyn Thy1.2$^+$ cells with microbeads. It was first investigated whether this approach would interfere with further cells sorting i.e. whether having a microbead attached to the cell would prevent other Thy1.2 antibodies from binding. Preliminary data (Figure 3.5) showed that anti-Thy1.2 conjugated with PE antibody could still bind cells. Following positive selection using Thy1.2 microbeads, 96% of the positive fraction was Thy1.2$^+$. However there was still an unacceptably large population (51%) remaining in the unselected (negative) fraction. This could be improved by the addition of more microbeads or by using more separation columns. However, since the number of cells recovered from the adoptive transfers was very low, it was decided that the sorting would be done by flow cytometry alone. Although this would take longer it would give the maximum recovery of cells.

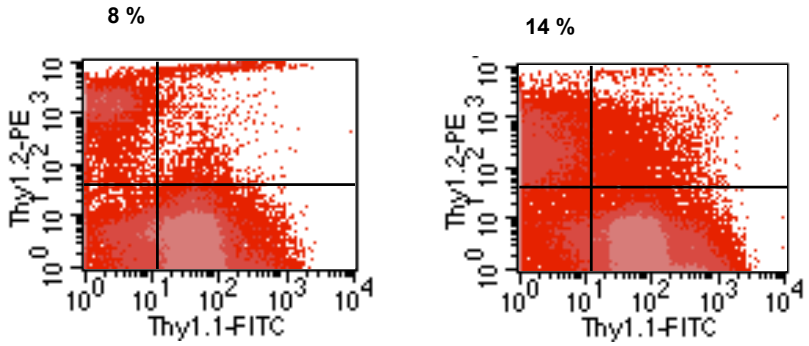


Figure 3.4: Enrichment of Thy1.2$^+$ cells by depletion of Thy1.1 cells using microbeads

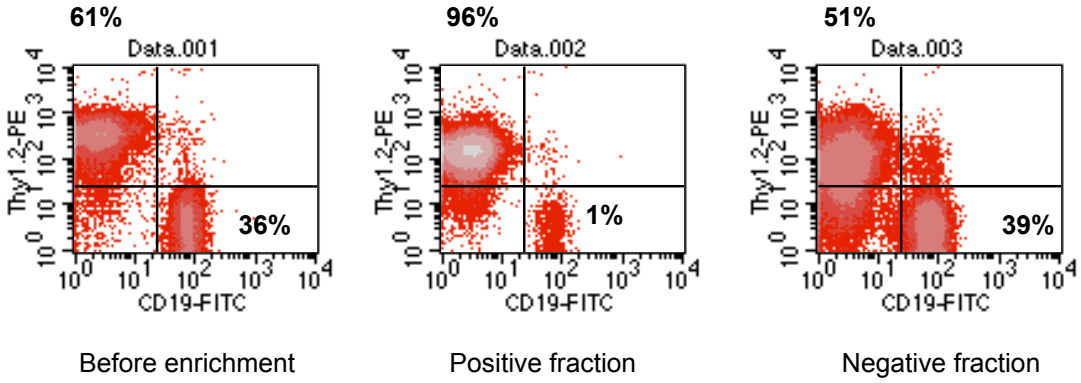


Figure 3.5: Enrichment of Thy1.2$^+$ cells by microbead positive selection. Before enrichment 61% were Thy1.2$^+$, after enrichment the positive fraction contained 96% Thy1.2$^+$ cells however 51% of the cells in the negative fraction were also Thy1.2$^+$.

RNA extraction

As limited numbers of cells were recovered from the adoptive transfers, it was important to optimise the conditions for RNA extraction. To determine which protocol gave the highest quantity of high quality RNA for amplification, spin column kits from 2 manufacturers were compared (Table 3.2) using different numbers of $CD4^+$ T cells from WT B6 mice.

It was an essential requirement that the RNA extraction method should include a DNase treatment to break down any contaminating DNA, so it cannot be later amplified in the Q-PCR. The 4PCR kit has a DNase I step after the RNA extraction and the PicoPure kit has an on-column DNase I digestion step.

	4PCR kit		PicoPure kit	
No. of cells	ng/μl	260/280nm	ng/μl	260/280nm
$1x10^6$	10.5	2.1	188.79*	1.96*
$0.5 x10^6$	6.4	2.88	26.02	1.98
$0.1 x 10^6$	0	-0.01	0.86	4.37
$0.05 x 10^6$	0.15	5.4	3.4	1.68

Table 3.2: Comparison of 2 RNA extraction kits. RNA extracted from frozen $CD4^+$ T cell pellets except * which were isolated from fresh $CD4^+$ T cells. The 260/280nm ratio gives an indication of DNA or protein contamination and is 1.9 –2.1 in pure RNA samples.

It was found that the Picopure kit gave a higher yield of RNA with a higher purity at all cell numbers used. This method was used to extract RNA from the sorted cell recovered from the adoptive transfers.

Having established optimal procedures, the adoptive transfers were carried out.

3.3 Results

3.4 Peptide alone

9 transfers of CFSE labelled Marilyn cells into $Thy1.1^+$ recipient mice, that were then treated i.n. with peptide alone, were performed. Figure 3.6 shows

an example of the flow cytometry profiles both before and after cell sorting. Cells were gated and sorted on the basis of the CFSE and Thy1.2^{+} staining. This yielded a high purity population – increased from 1.6% before sorting to 92% after sorting.

The cell recoveries are summarised in Table 3.3. There is no clear correlation between the number of Marilyn cells transferred and the number of cells recovered five days later. The number of cells recovered ranged from 3,570 to 1.35 x10^{6}. This is probably due to experimental factors and experience. However the cell recoveries were smaller than after peptide + LPS (Table 3.4) where the mean number of cells recovered was approximately 700,000 while the mean number after peptide alone was 275,000. This has also been shown by others, where tolerant T cells undergo a more limited expansion compared to WT cells (Verginis et al., 2008).

Following peptide treatment the antigen specific cells divided, as seen by their CFSE profiles. Surprisingly, the number of divisions was greater than when peptide was given with LPS (Figure 3.7). Following peptide + LPS cells divided up to 7 times but highest proportion of cells divided 5 times. Following i.n. peptide alone antigen specific T cells divided up to 8 times but the highest proportion of cells divided 7 or 8 times.

No. of Recipients	No of Marilyn cells given i.v. (x10^6) per B6 recipient	No. of i.n. peptide treatments	No of Marilyn cells recovered
3	20	1	15,000
2*	22	3	1.35 x 10^6
4	23	3	93, 205
2	25	3	235, 000
2	29	3	36, 890
2	30	3	514, 000
2	42	3	62,000
2	65	3	150,000
1	141	3	3, 570

Table 3.3: Cell recoveries after adoptive transfer of CFSE-labelled Thy1.2$^+$ Marilyn cells into female Thy1.1$^+$ recipients given 3 doses of i.n. HY-A^b*Dby* peptide alone. Cells were sorted 5 days after the first peptide treatment. The number of CFSE$^+$Thy1.2$^+$ Marilyn cells is given.
*only CD4+ Marilyn cells were transferred.

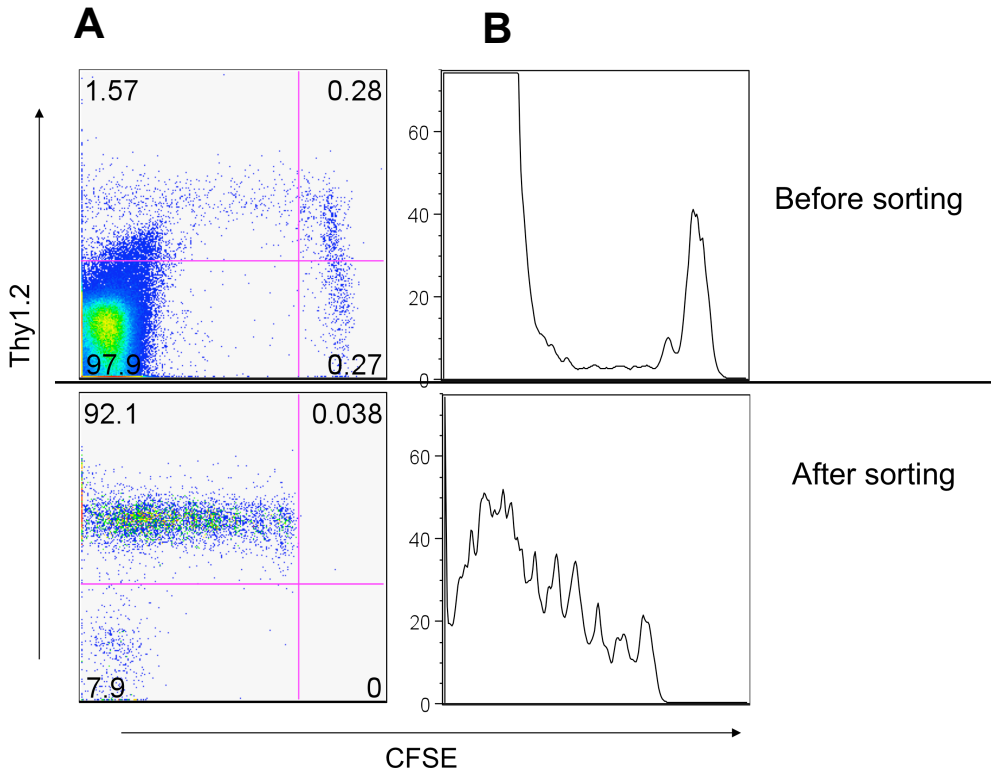


Figure 3.6: Flow cytometric analysis of adoptively transferred CFSE labelled Thy1.2$^+$ Marilyn cells into mice treated with 3 doses of HY-A^b*Dby* peptide alone. Cells were sorted 5 days after the first dose (A) before sorting 1.57% of the population were CFSE labelled Thy1.2$^+$ Marilyn cells. After assorting this was enriched to 92.1% of the population. (B) Histograms of CFSE staining. Cells that had not divided were excluded from the sorting.

3.5 Peptide +LPS

In total 9 adoptive transfers were done using either Thy1.1^{+}, Ly5.1^{+} or B6 recipient mice treated with i.n. HY-A^{b} peptide + LPS (Table 3.4). An example of the gates used for sorting is given in Figure 3.7.

Isolating CFSE^{+} Thy1.2^{+} cells by cell sorting resulted in high purity populations, in all transfers this was over 90%. The numbers of Marilyn cells recovered (Table 3.4) was variable and ranged from 25,000 to 1.5 x 10^{6}. It did not depend on the number of naïve Marilyn cells transferred. Other factors such as choice of donor/recipient genotype, the number of lymph nodes identified and dissected, the number of washing steps and the position of the gate when sorting all played a role in improving cell yield.

Recipient Strain	No. of Marilyn cells given i.v. (x10^6) per B6 recipient	No. of Marilyn cells recovered
Thy1.1	20	25, 000
B6	27*	125,000
Ly5.1	29	1.5 x 10^6
Thy1.1	30	646, 000
Ly5.1	31*	860,000
Thy1.1	42	18, 000
Thy1.1	44	1.3 x 10^6
Thy1.1	56	400,000
Thy1.1	100	1.4 x 10^6

Table 3.4: Cell recoveries after adoptive transfer of CFSE-labelled Thy1.2$^+$ Ly5.2$^+$ Marilyn cells into B6 female recipients. 24 hours after transfer, recipients were then given HY-A^b*Dby* peptide + LPS. Cells were sorted 5 days after peptide plus LPS treatment. The number of CFSE$^+$Thy1.2$^+$ or CFSE$^+$Ly5.2$^+$ cells is given.
* only CD4+ Marilyn cells were transferred.

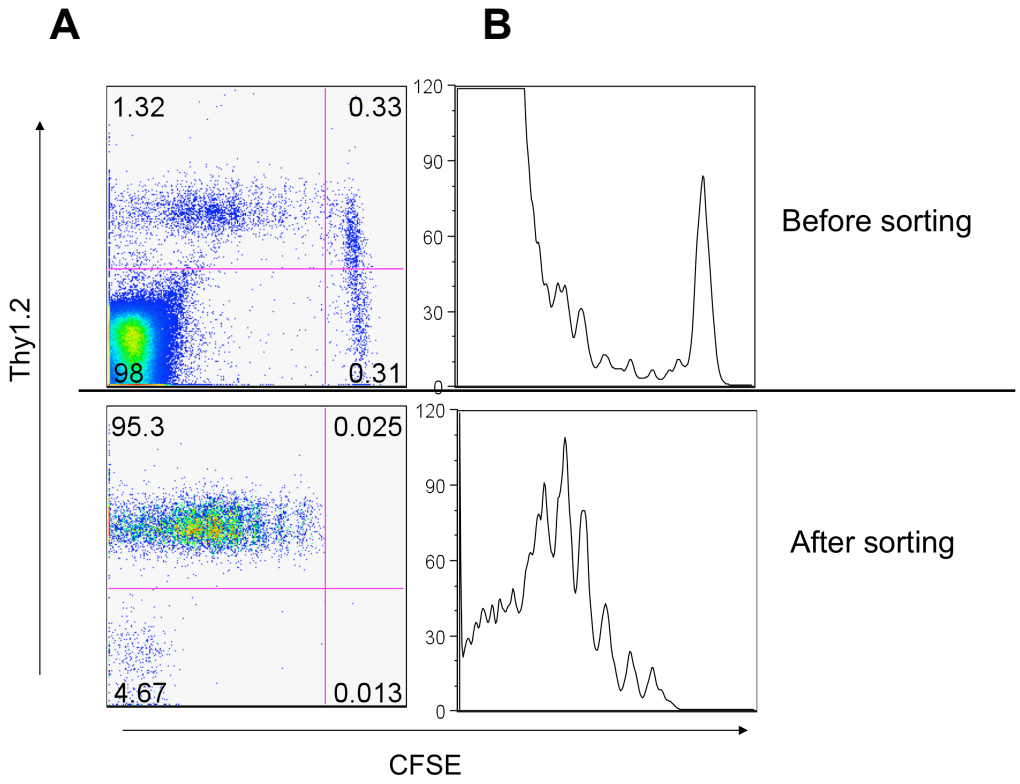


Figure 3.7: Flow cytometric analysis of adoptively transferred CFSE labelled Thy1.2+ Marilyn cells 5 days after i.n. peptide + LPS. (A) Before sorting 1.32% of gated cells were CFSE positive, Thy1.2+ Marilyn cells. After sorting 95.3% of the gated cells were Marilyn cells. (B) Histogram of CFSE staining. Cells that had not divided were excluded from the sorting.

3.6 Naïve Marilyn cells

Naïve antigen specific cells were used as the controls for these experiments. Cells were isolated from the spleen and lymph nodes of untreated $Rag^{-/-}$ Marilyn mice and sorted for their expression of $V\beta6^{+}$ (the beta chain of the transgenic T cell receptor), $CD4^{+}$ $CD25^{-}$ $CD45RB^{high}$(Figure 3.8).

RNA was extracted from recovered $CFSE^{+}$ $Thy1.2^{+}$ from the naïve, peptide alone and peptide + LPS treated mice and gene expression was analysed by quantitative PCR array.

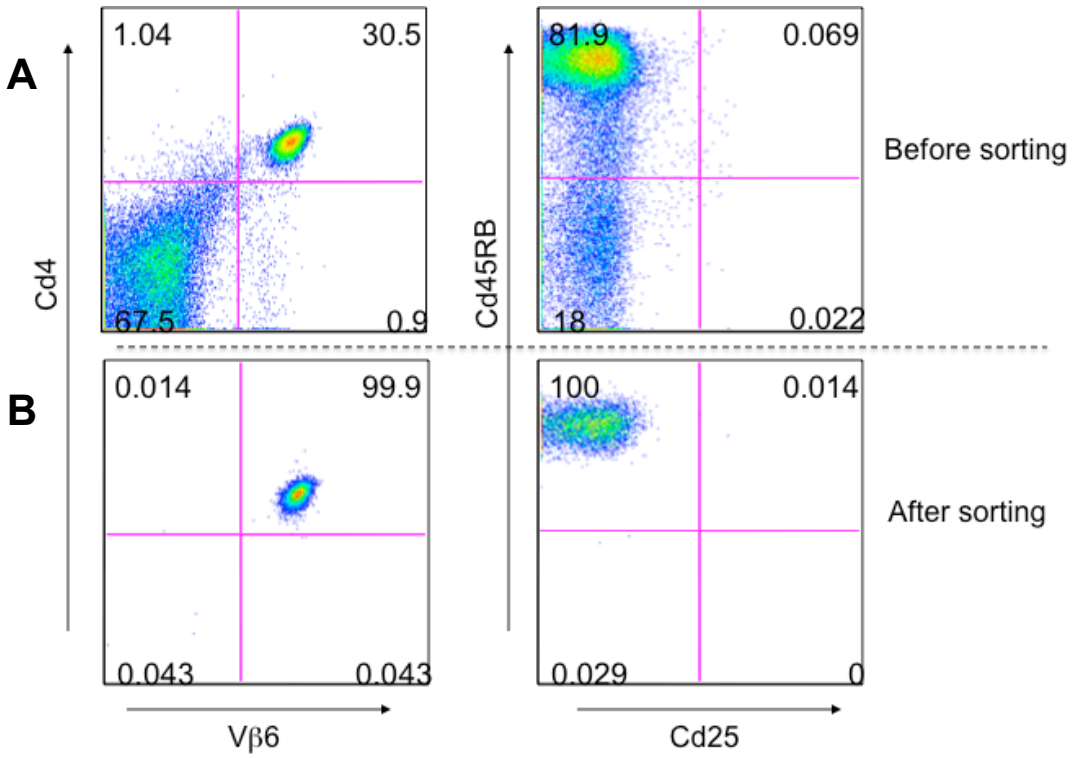


Figure 3.8 Flow cytometry profiles of naïve T cells before and after sorting. (A) Before sorting (B) after sorting 99% were naïve TCR positive cells (CD4+, Vβ6+, CD45RB+ and CD25-).

3.7 Gene expression

Real time quantitative PCR is a sensitive technique for quantifying gene expression. However, a large body of literature has criticised many aspects of the technique, in particular the reliance on housekeeping genes to normalise the experimental samples. Many of the commonly used house keeping genes such as 18S, β-Actin and GADPH can be changed by the experimental conditions and are not suitable for normalisation (Bustin et al., 2005). It has been shown that using an inappropriate housekeeping gene can affect whether the expression of a gene is judged to have changed.

To circumvent this problem, a PCR array can be used, which normalises to a range of different genes therefore avoiding the reliance a single one. The array used in these experiments is called Immunoquant array (IQA) or StellaArray and has been developed by our collaborators at the Jackson laboratories and Bar Harbour Biotech. Data were analysed using the Global Pattern Recognition software which normalises each gene to all the other expressed genes, then identifies significant changes in biological replicates.

Since each experiment yielded a small number $CFSE^+$ $Thy1.2^+$ Marilyn T cells, cell preparations were pooled and RNA was extracted from these pools. This was advantageous as it minimised intra animal variation. Each pool was analysed on one plate. There were three pools (and therefore three plates) for each condition, and there were three conditions: peptide alone, peptide plus LPS, and naïve cells.

The IQA results show the genes that were significantly up or downregulated compared to the naïve samples. The global pattern recognition software also calculates the fold change in the expression of these genes with respect to a selected number of normaliser genes. In each case RNA from mice treated with peptide +LPS and RNA from mice treated with peptide alone was compared to RNA from naïve cells.

3.8 Gene expression in antigen specific T cells following i.n. peptide alone

The recovery of Marilyn $CD4^+$ T cells 5 days after i.n. peptide alone was consistently low, and the amount of RNA was consequently also low and generally less good quality. The gene array experiment was done twice (see appendix) a summary of the results is given in Table 3.5. 26 genes were found to be significantly changed, 4 genes (*Sfpi1, Ly6a/e, Ccr9* and *Jun.1*) were down regulated.

Foxp3 was significantly changed (p=0.005) with a 184 fold increase in expression in the peptide samples (it's expression was undetectable in the naïve sample). The gene *Ctla4* was also found to be significantly upregulated (p=0.02, expression increased 27 fold).

Pdcd1 encodes the protein Programmed death 1 (PD1) which is an important inhibitory molecule, this gene was also found to be upregulated (p=0.05, increased 17 fold). Also upregulated were the cytokine genes *Ifng* (68 fold), *Il21* (264 fold), *Il13* (8 fold) and the receptors *Il15ra* (92 fold) and *Il18r* (70 fold). Some genes involved in the migration of T cells were also upregulated *Ccr4* (29 fold) and *Ccl3* (15 fold).

	Gene	p-value	Fold Change
1	*Tyk2*	0.002	685
2	*Il18r1*	0.004	70
3	*Foxp3*	0.005	184
4	*Ifng.ET*	0.007	68
5	*Il15ra2*	0.012	92
6	*Pax6*	0.016	37
7	*Sfpi1*	0.019	-14
8	*Ctla4*	0.019	27
9	*Ptgs2.ET*	0.021	133
10	*Ccr4*	0.023	29
11	*Cd44.1*	0.024	7
12	*Tra1.1*	0.025	10
13	*Mmp13.ET*	0.027	175
14	*Cd5.1*	0.031	4
15	*IgG1.1*	0.038	36
16	*Il13*	0.039	8
17	*Ox2*	0.042	14
18	*Ly6a/e*	0.043	-8
19	*Nfil3*	0.043	4
20	*Lag3*	0.044	34
21	*Baffr.1*	0.045	39
22	*Pdcd1.1*	0.046	17
23	*Ccr9*	0.047	-15
24	*Jun.1*	0.050	-10
25	*Tac4*	0.056	11
26	*Ccl3*	0.057	15

Table 3.5 Gene expression in antigen specific $CD4^+$ T cells during the tolerance induction phase. Cells from TCR transgenic Marilyn mice were transferred into Thy1.1 recipients that then received i.n. HY-A^b*Dby*. Five days later $Thy1.2^+$ $CFSE^+$ Marilyn cells were sorted, Cells were pooled and RNA was extracted and analysed by quantitative PCR array. Down regulated genes are shown in blue.

3.9 Gene expression in antigen specific T cells following i.n. peptide + LPS

Following i.n. peptide+ LPS 43 genes were found to be significantly changed; 7 were downregulated and 36 were upregulated (Table 3.6). Among the significantly upregulated genes were the tyrosine kinase gene *Tyk2* (increased 1201 fold, p=0.001), the Il-18 receptor gene *Il18r1* (increased 107 fold, p=0.003) and *Cox2* (increased 226 fold, p=0.008). Of particular interest were the genes *Cxcr3* (increased 14 fold, p=0.02), *Ccr4* (increased 49 fold, p=0.007) and *Ccr6* (increased 125 fold, p=0.004) which encode proteins involved in the migration of T cells.

In contrast to the gene expression in samples from mice treated with peptide alone the *FoxP3* gene was not found to be significantly upregulated. However, the regulatory genes *Ctla4* (increased 94 fold, p=0.02) and *Pdcd1* (increased 26 fold, p=0.05).

	Gene	p-value	Fold change
1	*Tyk2*	0.002	1201
2	*Il18r1*	0.003	107
3	*Igb*	0.003	351
4	*Cr2*	0.004	445
5	*Ccr6*	0.005	125
6	*Ccr4*	0.007	49
7	*Cox2*	0.008	226
8	*Cd22*	0.010	134
9	*Nmrk1*	0.011	69
10	*Pax6*	0.013	26
11	*Il2*	0.013	35
12	*IgK-C*	0.018	831
13	*Il15ra1*	0.019	22
14	*Ctla4*	0.021	94
15	*Ifng.ET*	0.021	24
16	*Ly6a/e*	0.021	-7
17	*Oas2*	0.023	-10
18	*Cxcr3*	0.025	14
19	*Bid*	0.025	8
20	*Il15ra2*	0.025	90
21	*Blnk*	0.026	219
22	*Jun.1*	0.026	-12
23	*Fos*	0.027	-6
24	*Socs3*	0.028	-8
25	*Ltbr*	0.029	41
26	*Ms4a1*	0.030	39
27	*Itgb1*	0.031	14
28	*H2-Aa*	0.032	6
29	*Cd79a*	0.032	21
30	*Ccr9*	0.036	-21
31	*Prf1*	0.036	-8
32	*Cd74*	0.037	7
33	*Ptpn13*	0.039	8
34	*Cd44.1*	0.039	9
35	*Ox2*	0.040	19
36	*Cd19*	0.042	67
37	*Il7.1*	0.047	18
38	*Cd5.1*	0.048	3
39	*IgL-C1*	0.050	47
40	*Pdcd1.1*	0.051	26
41	*Il23r*	0.057	8
42	*ICOS*	0.058	6
43	*Il1b.ET*	0.059	22

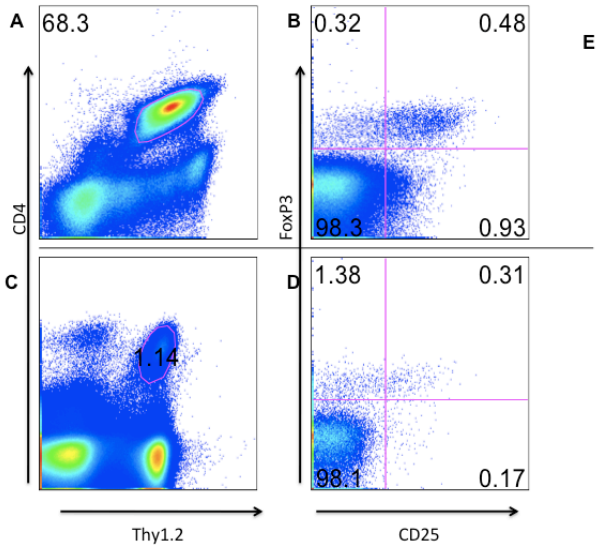
Table 3.6 Gene expression in antigen specific T cells following in. peptide + LPS. Cells from TCR transgenic Marilyn mice were transferred into Thy1.1 recipients that then received i.n. HY^{Ab}*Dby* + LPS. Five days later Thy1.2+ CFSE+ Marilyn cells were sorted, Cells were pooled and RNA was extracted and analysed by quantitative PCR array. Down regulated genes are shown in blue.

3.10 Protein expression of FoxP3 by intracellular staining

The upregulation of FoxP3 5 days after i.n. peptide alone was also assessed by flow cytometric analysis (Figure 3.9). Before transfer 0.48% of the $CD4^+$ population in Marilyn mice expressed $CD25^+$ and $FoxP3^+$. This is much lower than in Marilyn $Rag^{+/-}$ mice where the $CD4^+FoxP3^+$ population is about 5% but higher than expected from $Rag^{-/-}$ Marilyn mice. As these were naïve animals that have not encountered male antigen these cells are naturally occurring Tregs.

After transfer despite depleting $CD8^+$ and $B220^+$ cells with microbeads before flow cytometric analysis, the majority of cells were of recipient origin and there were far fewer $CD4^+$ cells to analyse for FoxP3 expression. The low recovery made interpretation less straightforward.

There was a slight decrease in the percentage of $FoxP3^+CD25^+$ T cells (from 0.48% in WT cell to 0.29% after transfer). This was not expected but does not appear to be significant. However, the percentage of $CD25^-FoxP3^+$ cells was increased from 0.32% to an average of 1.43%. This is in agreement with previous results from this laboratory which showed that tolerance could be transferred by both $CD4^+CD25^+$ and $CD4^+CD25^-$ populations (Chai, 2004).



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	% $CD4^+Thy1.2^+$	% $FoxP3^+CD25^-$	%$FoxP3^+CD25^+$	%$FoxP3^-CD25^+$
WT	68.3	0.32	0.48	0.93
Peptide treated	1.14	1.43 +/- 0.25	0.29 +/- 0.06	0.2 +/- 0.06

Figure 3.9 FoxP3 protein expression in WT Marilyn mice (A and B) and 5 days after transfer into Thy1.1 recipients and treatment with i.n. HY-A^b*Dby* peptide (C and D). (A) Before transfer all CD4+ cells were Thy1.2+ (B) a small percentage of the cells were $CD25^+FoxP3^+$. (C) 5 days after transfer and peptide treatment. Transferred Marilyn T cells ($CD4^+Thy1.2^+$) were only a small percentage of the total cells. $Thy1.2^-$ cells were recipient cells. (D) Gated on $CD4^+Thy1.2^+$ cells, these cells have an increased percentage of FoxP3+ cells and increased percentage of $CD25^-FoxP3^+$ cells. (E) Shows the average FoxP3 expression in $CD4^+Thy1.2^+$ cells after transfer into Thy1.1 recipient and i.n. HY-A^b*Dby* peptide.

3.11 Induction of tolerance after adoptive transfer Marilyn cells

It has been previously shown that i.n. administration of the HY-A^b*Dby* peptide can induce tolerance in female mice to male skin grafts. In the experiments described above, cells from Marilyn mice were transferred into wild type recipients 24h before peptide administration. The transfer of a large number of antigen specific T cells might result in i.n. HY-A^b*Dby* peptide no longer being able to induce tolerance to male skin grafts. I therefore repeated the experiments in which female mice received adoptive transfer of Marilyn T cells and between three and seven doses (of 100μg) of HY-A^b*Dby* peptide. None of these mice rejected their skin grafts (Figure 3.10). There was no difference between 3 and 7 doses indicating that even with the transfer of a large cohort of peptide-specific cells, 3 doses of 100μg peptide is sufficient to induce tolerance.

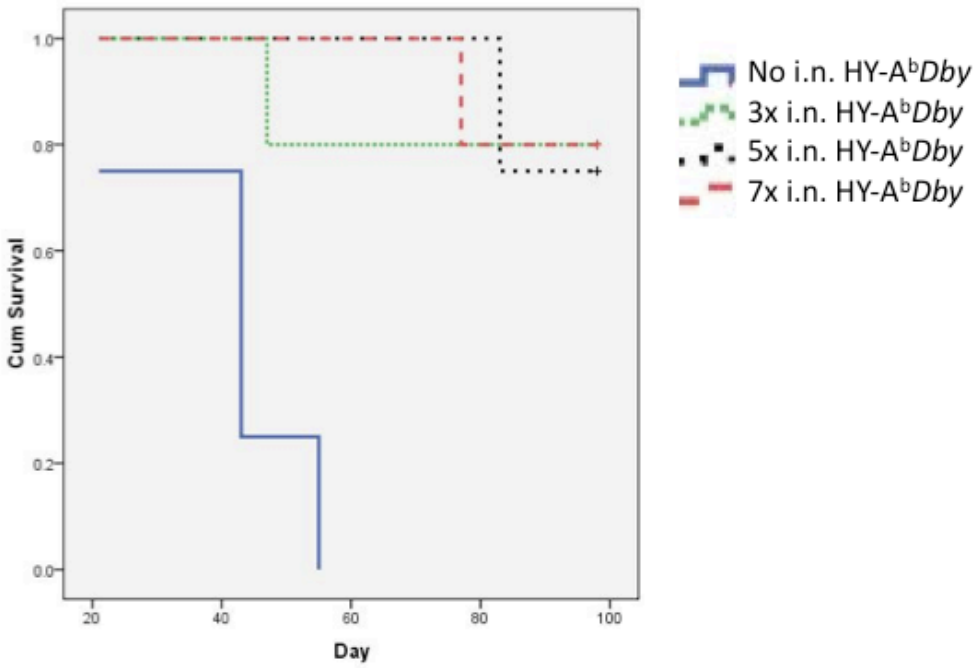


Figure 3.10 i.n. HY-A^b*Dby* peptide can still induce tolerance to male skin grafts after transfer of Marilyn cells. 20x10^6 Marilyn cells were transferred into female recipient mice, which subsequently received either no peptide (4 mice) 3 (5 mice), 5 (4 mice) or 7 (5 mice) doses of 100μg i.n. HY-A^b*Dby* peptide. 10 days after i.n. peptide recipients were then given male skin graft. Grafts were checked every 2-5 days and were scored as rejected when either less than 10% of the graft remained or skin failed to engraft after more than 10 readings.

3.12 Discussion

The aim of the experiments described in this chapter was to analyse gene expression in antigen specific T cells during the tolerance induction phase. It was found that 5 days after i.n. HY-A^b*Dby* antigen specific T cells had divided up to 8 times; this was more than when peptide was given with LPS, but comparatively fewer cells responded to peptide, as seen by the larger proportion of undivided cells in the peptide alone sample (Fig 3.6b cf Fig 3.7b). Following treatment with peptide alone fewer cells responded, but those that did respond went through more divisions than when peptide was administered with LPS. This was unexpected, but could be explained by the gene expression data showing that following peptide alone genes such as *Grp94, Ptpn13* and *Ox2* that protect against apoptosis were up regulated. This protection against apoptosis allowed the relatively few cells expressing these genes to survive and divide more extensively. When peptide was given with LPS, many more cells responded since LPS is a potent adjuvant. However, since these cells did not upregulate the anti-apoptotic genes to the same extent the results could be explained by the fact that there was more limited division by each cell.

I have also carried out experiments looking at earlier time points after i.n. peptide administration and the results (not shown) show that the antigen specific T cells first divide after 36 hours. Furthermore quantitative PCR analysis showed that there were no significant changes in gene expression 24 hours after i.n. peptide. However, by five days after peptide there were many changes in gene expression and these are consistent with the hypothesis that antigen specific T cells have become adaptive regulatory T cells.

3.13 Analysis of gene expression

To facilitate comparison, genes expressed by cells from mice treated with peptide alone, peptide + LPS and by both populations have been grouped according to their location in the cell (Table 3.7). Below they are discussed in relation to generic function

Table 3.7 (following page) Genes significantly changed in antigen specific T cells after treatment with either i.n. HY-A^b*Dby* alone or with LPS. Genes are arranged by cellular location. Blue font indicates the gene was found to be down regulated.

	Peptide alone	Both	Peptide + LPS	
Plasma membrane	lymphocyte-activation gene 3 (*Lag3)* TNF receptor superfamily 13c (*Baffr*)	Ctla4 programmed cell death 1 (*Pdcd1*) interleukin 18 receptor 1 interleukin 15 receptor, alpha chain CCR9 CCR4 CD5 CD44 lymphocyte antigen 6 complex, locus A (*Ly6a/e*)	inducible T-cell co-stimulator (*Icos*) CXCR3 integrin beta 1 (fibronectin receptor beta) (*Itgb*) CCR6 CD19 CD20 (*Ms4a1*) CD22 (Bl-Cam) CD74 CD79A (immunoglobulin-associated alpha) CD79B (Igb)	CD244 natural killer cell receptor 2B4 (*Nmrk*) Lymphotoxin B receptor (*Ltbr*) histocompatibility 2, class II antigen A, alpha (*H2-Aa*) interleukin 23 receptor (Il-*23r*) perforin 1 (pore forming protein) (*Prf*) complement receptor 2 (*Cr2*)
Cytoplasm	Heat Shock protein 90β member 1 (Grp94, *Tra1.1*),	tyrosine kinase 2 (*Tyk2*) NAD(P)H dehydrogenase, quinone 2 (*Ox2*) prostaglandin-endoperoxide synthase 2 (*Ptgs2, Cox2*)	protein tyrosine phosphatase, non-receptor type 13 (*Ptpn13*) B-cell linker (*Blnk*) BH3 interacting domain death agonist (*Bid)*	2'-5' oligoadenylate synthetase 2 (*Oas2*) suppressor of cytokine signalling 3 (*socs3*)
Nucleus	forkhead box P3 (*Foxp3*) nuclear factor, interleukin 3, regulated (*Nfil3*) SFFV proviral integration 1 (*Sfpi1* or *PU.1*)	paired box gene 6 (*Pax 6*) Jun oncogene (*Jun*)		FBJ osteosarcoma oncogene (*Fos*)
Extracellular	interleukin 13 (*Il-13*) CCL3 tachykinin 4 (*Tac4*) matrix metallopeptidase 13 (*Mmp13*) immunoglobulin heavy constant gamma 1 (*IgG1.1*)	interferon gamma (*Ifng*)	interleukin 1 beta (*Il-b*) IL-2 (*Il2*) IL-7(*Il7*)	immunoglobulin kappa chain, constant region (*IgK-C*) immunoglobulin lambda chain, constant region 1 (*IgL-C*)

3.14 Genes expressed following treatment with peptide alone

Plasma membrane

Following treatment with i.n. HY-A^b*Dby* peptide, antigen specific $CD4^+$ T cells upregulated the genes that encode the cell surface proteins LAG3 (gene expression increased 34 fold, p=0.04) and PD1 (gene expression increased 17 fold, p=0.05). LAG3 binds to MHC class II on the surface of APCs and has been shown to inhibit DC activation (Liang et al., 2008). PD1 can also have inhibitory effects on DC activation. PD1 binds to its ligands PDL1 and PDL2 expressed on APCs sending an inhibitory signal to the T cell. In addition, there is evidence from studies using bone marrow derived DCs cultured with PD1-Ig fusion protein that signalling through PD1 could inhibit DC activation and increase IL-10 secretion. This was found to be independent of IDO activation (Keir et al., 2008). In the experiments reported here, following i.n. peptide, the inhibition of dendritic cell maturation would prevent the upregulation of MHC class II, co-stimulatory molecules and secretion of proinflammatory cytokines. DCs also need maturation signals to traffic to the lymph nodes and present the peptide to naïve T cells. If the DC's cannot mature then they will not be able to induce T cell proliferation.

Ctla4 was upregulated after treatment with both peptide alone (increased 27 fold, p=0.01) and peptide + LPS (increased 94 fold, p=0.02). CTLA4 is expressed on activated T cells and binds to CD80 or CD86 on the surface of the APC, this results in inhibition of T cell activation. CTLA4 deficient mice which lack this inhibitory mechanism develop fatal lymphoproliferative disorders (Tivol et al., 1995, Waterhouse et al., 1995). In addition, CTLA4 has been shown to initiate tryptophan catabolism by IDO in B7 expressing DCs (Mellor et al., 2004), and Tregs that express CTLA4 can also induce IDO expression in DCs. As regulatory T cells constitutively express high amounts of CTLA4, it is thought that activation of IDO could be a major mechanism by which Tregs induce tolerance (Fallarino et al., 2003). In this model of i.n. peptide induced graft tolerance LAG3, PD1 and CTLA4 could all act on the DC to prevent maturation and increase IDO activity.

In response to peptide alone the cytokine receptor gene *Il18r* receptor was upregulated (increased 70 fold, p=0.004). This is of interest as IL-18 was first identified as a cytokine that could induce IFNγ production by T cells (Okamura et al., 1995). In the experiments reported here, following i.n. peptide alone, *Ifnγ* was also found to be upregulated. The IL-18 receptor is comprised of two subunits: IL-18 R and an accessory chain, AcPL. Binding of IL-18 to the receptor can activate AP1 and NFκB (Matsumoto et al., 1997). This provides an attractive mechanism by which signalling through the IL-18 receptor could lead to IFNγ gene transcription in these, male specific $CD4^+$ T cells.

After treatment with both peptide alone and peptide + LPS the *Il15rα* gene was up regulated which suggests the cells maybe responding to the T cell growth factor IL-15. In T cells the IL-15 receptor is comprised of IL-2Rβ and the common γ chain. The IL-2Rβ subunit is shared with the IL-2 receptor. Binding of IL-15 activates JAK1 or JAK3 and STAT3 or STAT5 in T cells (Tagaya et al., 1996) and stimulates cell growth.

The gene *Baffr* was also found to be upregulated after peptide alone (expression increased 39 fold, p=0.045). BAFF-R (B cell activating factor receptor) belongs to the TNF receptor superfamily and was first described in B cells as being important for B cell survival and maturation. However, it can also be expressed on a subset of $CD4^+$ T cells after CD3 stimulation (Mackay and Leung, 2006). Work using a cardiac allograft model suggested that binding of BAFF to BAFF-R expressed on $CD4^+$ T cells provides a co-stimulatory signal which stimulates proliferation *in vitro* and *in vivo* (Ye et al., 2004). This paper also showed that BAFF-R was highly expressed on $CD4^+CD25^+$ T cells, although the authors did not investigate the significance of this. Therefore, in some circumstances where antigen presentation is not optimal, BAFF binding to BAFF-R may provide additional survival signals to the $CD4^+$ T cell. In the experiments reported here when peptide was given alone, there would be a lack of co-stimulatory signals and therefore a survival signal such as this could be important in stimulating some proliferation in response to antigen.

In both conditions the chemokine receptor gene *Ccr9* was down regulated (following peptide alone, gene expression decreased 15 fold, p=0.047). Several studies have found that CCR9 is expressed on T cells found in the small intestine (Kunkel et al., 2000, Papadakis et al., 2001, Kunkel and Butcher, 2002). Therefore the downregulation of the gene reported here may prevent these cells migrating to the gut.

In both conditions the chemokine receptor gene *Ccr4* was upregulated (following peptide alone, gene expression increased 29 fold, p=0.023). CCR4 is expressed by memory T cells and is needed for migration into the skin under both inflammatory and steady state conditions. The ligand for CCR4 is TARC which is found on the endothelium. CCR4 has been shown to be important in the migration of Tregs. In a mouse model of Colitis, CCR4 deficient Tregs could not accumulate in the mesenteric lymph node and therefore could not prevent disease (Yuan et al., 2007). The upregulation of CCR4 allows these T cells to migrate through different tissues and accumulate in the lymph nodes. This is crucial for both immunogenic and tolerogenic responses.

The gene *Cd5*, encodes a cell surface molecule present on all T cells and a subset of B cells. In resting cells it is constitutively associated with the protein tyrosine phosphatase, SHP1. Upon TCR stimulation, CD5 cross links with the TCR-CD3 complex and inhibits the resulting increase in intracellular Ca^{2+} (Perez-Villar et al., 1999). It has also been shown that $CD4^{+}CD25^{+}$ Tregs from $CD5^{-/-}$ mice were more effective at suppressing T cell proliferation than WT $CD4^{+}CD25^{+}$cells (Dasu et al., 2008). The authors suggest that this is because CD5 down regulates the signalling events following TCR signalling. Therefore in my experiments following i.n. peptide alone, the increase in the expression of CD5 (increased 4 fold, p=0.03) could cause a reduction in intracellular Ca^{2+} and prevent the T cell from responding. This may be an important mechanism in the induction of tolerance in this model.

Also in both conditions, *Cd44* was upregulated (following peptide alone gene expression increased 7 fold, p=0.02). CD44 has been shown to be involved in a wide range of functions including cell-cell interactions, cell adhesion and

migration (Naor et al., 2008). T cells upregulate CD44 after encounters with cognate antigen, therefore CD44 is frequently used as a marker of antigen experienced T cells. This reinforces the fact that following i.n. peptide alone, small numbers of antigen specific T cells proliferate and upregulate markers such as CD44.

After peptide alone and peptide + LPS the GPI-linked surface protein Ly-6A/E (Sca-1) was down regulated. Following peptide alone gene expression decreased 8 fold, p=0.04). Sca-1 is expressed on resting lymphocytes and downregulated following antigen stimulation (Yang et al., 2005). As with CD44 this confirms that these cells have received a signal through their antigen receptor.

Cytoplasm

Following treatment with peptide alone the gene for Heat Shock Protein 90β, member 1(*Tra1.1* or *Grp94)* was also upregulated (gene expression increased 10 fold, p=0.025). This small chaperone protein is retained in the ER where is involved in the folding and assembly of secretory and membrane proteins. It has also been shown to protect against apoptosis in a mouse lymphoma cell line (McCormick et al., 1997).

Following both peptide alone and peptide +LPS, tyrosine kinase 2 (*Tyk2*) a member of the Janus tyrosine kinase family was upregulated (following peptide alone gene expression increased 685 fold, p=0.002 and after peptide + LPS expression increased 1201 fold, p=0.002) . JAK kinases associate with cytokine receptors at the plasma membrane. The binding of cytokines causes the receptor to dimerise and this activates the JAK kinases. Activated JAKs phosphorylate tyrosine residues on the cytokine receptor. Signal transducer and activator of transcription (STAT) proteins can then bind to the phosphorylated sites. The STAT proteins are then phosphorylated by JAKs and can dimerise. The STATs then leave the receptor and translocate to the nucleus where they activate gene transcription (Shuai and Liu, 2003). There are 4 members of the JAK family, JAK1, JAK2, JAK3 and TYK2. Studies using mice that are deficient

in TYK2 have shown that the TYK2 associates with receptors for IL-12, IFNα and IFNβ (Karaghiosoff et al., 2000). However mutations in the human TYK2 gene give rise to a broad immunodeficient phenotype. Further studies in these patients suggest that TYK2 also associates with IL-23, IL-10 and IL-6 (Watford and O'Shea, 2006). The IL-6 receptor has the GP130 receptor chain, which is also shared among receptors for other cytokines including IL-11, IL-27, IL-31, oncostatin M (OSM), ciliary neurotrophic factor, cardiotrophin 1 and cardiotrophin-like cytokine, so the authors suggest that receptors for these cytokines may also use TYK2 as the JAK signalling protein (Watford and O'Shea, 2006).

Antigen specific $CD4^+$ cells upregulated *Tyk2* in both tolerising and immunising conditions. This suggests that these cells are receiving signals through one or more of these cytokine receptors and using Tyk2 to phosphorylate STAT proteins.

NAD(P)H dehydrogenase quinone 2 (*Ox2*) is a cytosolic enzyme that catalyses the breakdown of quinones which are cytotoxic (Belinsky and Jaiswal, 1993). This gene was found to be upregulated in both conditions (following peptide alone, expression of this gene increased 14 fold, p=0.04. Following peptide + LPS it was 19 fold, p=0.04). and could protect the cells from apoptosis in both conditions.

This seems to fit with the original microarray data which showed that antigen specific $CD4^+$ T cells from mice treated with i.n. peptide alone upregulated many of anti apoptosis genes such as Bcl2. Irrespective of the number of cells transferred, cell recoveries were always smaller from mice treated with peptide alone. This suggests that many cells underwent apoptosis in these conditions. The RNA was extracted from cells that had survived and had divided in response to peptide and these may have been protected from apoptosis by the upregulation of genes such as *Tra1* and *Ox2.*

Prostaglandin-endoperoxide synthase 2 (*Ptgs2*) also known as cyclooxygenase 2 (*Cox2*) was upregulated in both tolerising (expression increased 133 fold, p=0.02) and immunising conditions (gene expression increased 226 fold,

p=0.008). COX-2 is an enzyme that produces prostaglandins and thromboxane from arachdonic acid and has been shown to have a role in the function of human induced Tregs by producing of PGE_2 which can suppress effector T cells (Mahic et al., 2006). It may carry out a similar role in these tolerant antigen specific T cells following i.n. peptide.

Nucleus

Following peptide alone *Foxp3* and *Nfil3* were upregulated (expression of *FoxP3* increased 184 fold, p=0.005 and expression of *Nfil3* increased 4 fold, p=0.04). FoxP3 is crucial for the function of regulatory T cells, and its upregulation here is a very strong indication that i.n. peptide alone induces a population of antigen specific regulatory T cells. The exact function of FoxP3 is not yet known, however, it has been proposed that it may work by either directly inhibiting transcription factors such as NFAT, NF-κB and AP1, or by transcribing an unknown protein that then blocks TCR signals (Campbell and Ziegler, 2007, Josefowicz and Rudensky, 2009).

Nfil3 (also known as E4 binding protein 4 - E4BP4) is a transcription factor that can bind and repress viral promoter sequences. It was also found to be upregulated following i.n. peptide in the preliminary microarray data. The gene was originally cloned from stimulated human T cells (Zhang et al., 1995). In pro-B cells expression of NFIL3/E4BP4 is dependent on IL-3. It is known to be involved in many different cell processes including cell survival, the anti-inflammatory response and circadian mechanisms (Cowell, 2002). The glucocorticoid dexamethasone has been shown to increase the expression of NFIL3/E4BP4. It is thought that NFIL3/E4BP4 may then bind to the promoters of three repressor genes – Cox-2, iNOS and cPLA2 (Wallace et al., 1997). This would fit with these results here as expression of Ptgs2 (Cox-2) was upregulated after both treatments.

The paired box gene transcription factor *Pax6* was upregulated after both i.n. peptide alone and peptide +LPS (following peptide alone gene expression

increased 37 fold, p=0.016). Pax 6 is known to be involved in the development of the eye and brain so the functional relevance of its upregulation here is not clear. *Jun* was downregulated following both peptide alone and peptide +LPS (following peptide alone gene expression decreased 10 fold, p=0.05). Jun is activated by the MAPK kinase pathway and forms the transcription factor AP1 with Fos (Liu et al., 2007). The MAPK pathway can be activated by ligation CD28 on the surface of the T cell (Fathman and Lineberry, 2007). These antigen specific $CD4^+$ T cells may receive signals through their TCR without any co-stimulatory signals. This may lead to a down regulation of Jun as it is not receiving any activation signals through the MAPK kinase pathway.

Sfpi1 (PU.1) was downregulated following i.n. peptide alone (gene expression decreased 14 fold, p=0.019). PU.1 is a member of the Ets transcription factor family and is required for the normal development of many cells of the immune system including T cells. Mutations in the *Sfpi1* gene have been shown to be lethal due to multiple defects in haematopoiesis (DeKoter et al., 2007). It may be down regulated here as part of the normal cell cycle as all the cells that RNA was extracted from were proliferating.

Extracellular

Interestingly *Ifnγ* was also found to be upregulated following both i.n. peptide alone (fold change=68, p=0.007) and i.n. peptide + LPS (fold change=24, p=0.02). This is cytokine usually described as being proinflammatory as it can upregulate antigen processing and presentation in both professional and non professional APCs, affect cellular migration, growth and maturation in a variety of responder cells (Schroder et al., 2004). However, there is also evidence that IFNγ may also have an immunomodulatory effects. For example, mice that are deficient in IFNγ or IFNγR have accelerated EAE, suggesting that IFNγ may limit this disease by suppressing activated $CD4^+$ T cells (Ferber et al., 1996, Chu et al., 2000). It has also been shown that IFNγ was not needed for rejection of MHC

mismatched cardiac grafts as IFNγ deficient mice reject transplants in a similar time frame to WT mice (Saleem et al., 1996, Bishop et al., 2001).

There is also evidence that IFNγ may actually be required for the induction of tolerance to transplanted tissues, this mainly comes from work from Kathryn Wood's group. Most recently they have shown that T cells cultured with immature DCs and IFNγ *ex vivo* became FoxP3^{+}. These cells could then induce tolerance to MHC mismatched skin grafts when transferred into Rag$^{-/-}$ recipient mice. They showed that the culture with IFNγ selected for FoxP3^{+} T cells by enhancing activation induced cell death in the non-Treg population and promoting conversion of naïve CD4^{+} T cells into Tregs. This process was found to be dependent on STAT1 and nitric oxide (Feng et al., 2008a, Feng et al., 2008b).

These processes could also explain a possible mechanism by which tolerance is induced by i.n. peptide. As in the in vitro cell culture experiments described above, IFNγ may promote Treg conversion from naïve T cells and death in non-Treg cells. This would explain why there are fewer cells proliferating after i.n. peptide alone compared to peptide + LPS. It could be that many cells undergo cell death, while the FoxP3^{+} cells survive and proliferate.

It is also of note that IFNγ can induce IDO expression in DCs (Grohmann et al., 2002). The proposed mechanism is via the IFNγR expressed on DCs, which signals through STAT1, and this can then upregulate IDO gene expression (Taylor and Feng, 1991, Hassanain et al., 1993, Babcock and Carlin, 2000). Again this is an another example of how the tolerogeneic antigen specific CD4^{+} T cells could induce tolerance by modifying the antigen presenting DC either at the time of initial tolerance induction or during linked suppression.

After peptide alone *Il-13* was upregulated (*Il*-13 gene expression increased 8 fold, p=0.039). IL-13 has proven roles in resistance to parasitic infections, intracellular organisms and plays a central role in allergic asthma (Wynn, 2003). Of particular interest here is that IL-13 can activate the immunomodulatory enzyme Arginase 1.

Arginase 1 is expressed in macrophages and DCs (Munder et al., 1999). It competes with the enzyme nitric oxide synthase (NOS) for its substrate L-arginase which it converts into ornithine and urea. NOS converts L-arginine into nitric oxide which is proinflammatory. It is thought that the upregulation of arginase I represents a immunomodulatory mechanism as this prevents the synthesis of the proinflammatory mediator (Bronte and Zanovello, 2005). Indeed it has been shown that Arginase 1 is upregulated in DCs conditioned with IL-10 (Nolan et al., 2004). Expression of arginase I can be induced by Th2 cytokines. In smooth muscle cells, it has been shown that IL-13 can upregulate arginase I activity via cAMP and signalling in the JAK/STAT pathway (Wei et al., 2000). In the experiments reported in this thesis IL-13 could be inducing the expression of this enzyme in DCs. This could be another mechanism by which these antigen specific $CD4^+$ T cells modulate the function of DCs.

Following peptide alone the *Ccl3* gene was upregulated (gene expression increased 15 fold, p=0.057). CCL3 (also known as MIP1α) is a chemokine that recruits proinflammatory cells to the site of infection. It is known to promote dendritic cell chemotaxis and transendothelial migration (Menten et al., 2002). Under these tolerising conditions, secretion of CCL3 may encourage DC interactions with regulatory $CD4^+$ T cells that inhibit DC maturation, either (or both) at the time of tolerance induction and during linked suppression.

Following peptide alone Tachykinin 4 (*Tac4*) was found to be upregulated (gene expression increased 11 fold, p=0.056). This gene encodes hemokinin 1 (HK-1) which may have a role in T cell development (Patacchini et al., 2004, Page, 2006). It has not been described in regulatory T cells before so its function here is not known.

These cells also upregulated Matrix metalloproteinase 13 (*Mmp13*) (expression increased 175 fold, p=0.027). Matrix metalloproteinases can break down the extracellular matrix - specifically, Mmp13 cleaves type II collagen (Murphy, 1995, Birkedal-Hansen, 1995, Lijnen and Collen, 1999). It has been suggested that the Mmps may have a role in cleaving adhesion molecules from the cell surface and therefore affecting the migration of cells (Salmi and Jalkanen, 2005). It could be

that by up regulating both these genes after treatment with peptide alone, chemokine molecules are being cleaved from the cell surface, altering the ability of these cells to migrate. However, further work is needed to investigate this.

Finally some immunoglobulin genes appeared to be upregulated in antigen specific $CD4^+$ following peptide alone. *IgG1.1* was found to be significantly upregulated (expression increased 36 fold, p=0.038). However, when this experiment was repeated the results were not found to be consistent (see appendix). It seems unlikely that Ig genes would be expressed in $CD4^+$ T cells and as both the fold change and p value were variable between experiments these genes will not be included in further analysis.

3.15 Genes expressed following treatment with peptide + LPS

Plasma membrane

Overall more genes were altered after i.n. peptide + LPS. This included many proinflammatory genes as discussed below. It also includes the regulatory genes – *Ctla4* (gene expression increased 94 fold, p=0.021) and *Pdcd1* (gene expression increased 26 fold, p=0.051).

CTLA4 is a normally upregulated in activated T cells to prevent any potentially pathogenic lymphoproliferation. As described above mice that are deficient in CTLA4 have fatal lymphoproliferative diseases (Tivol et al., 1995, Waterhouse et al., 1995) demonstrating the importance of this molecule in controlling T cell proliferation. Therefore following peptide +LPS this molecule is part of a negative feedback system that will prevent uncontrolled proliferation.

Similarly, PD1 inhibits TCR signalling and could also prevent uncontrolled proliferation. Mice that are deficient in PD1 develop fatal autoimmune dilated cardiomyopathy (Nishimura et al., 2001). Even though these are immunising conditions it is important to have negative feedback mechanisms to control T cell proliferation.

The *Il15rα* gene was found to be upregulated in both conditions (gene expression increased 22 fold, p=0.021 following i.n. peptide + LPS), suggesting

that in both conditions the cells are responding to the T cell growth factor Il-15. This cytokine may be driving the proliferation seen in both conditions.

In response peptide + LPS the cytokine receptor gene *Il18r* receptor was upregulated (fold change increased 107 fold, p=0.003). As described above IL-18 can induce IFNγ production by T cells (Okamura et al., 1995). IFNγ was also upregulated after peptide + LPS, therefore the same mechanism could be utilised in both immunising and tolerising conditions.

Ccr9 was downregulated following i.n. peptide + LPS (gene expression decreased 21 fold, p=0.036). CCR9 binds CCL25 which recruits T cells to the gut (Svensson et al., 2002). Several studies have found that CCR9 is expressed on T cells found in the small intestine (Kunkel et al., 2000, Papadakis et al., 2001, Kunkel and Butcher, 2002). Therefore the downregulation of the gene reported here may prevent these cells migrating to the gut.

In both conditions the chemokine receptor *Ccr4* was upregulated (gene expression increased 49 fold, p=0.007 following i.n. peptide + LPS). CCR4 is upregulated on T cell that have been activated by DCs in the peripheral lymph nodes. CCR4 expression along with other chemokines receptors allows T cells to migrate into the skin (Bono et al., 2007). In these T cells that have encountered antigen in the presence of LPS – i.e. in a pro-inflammatory environment, the upregulation of CCR4 is going to allow migration and entry to the site of inflammation.

As described above CD5 is a negative regulator of TCR signalling its expression was increased following peptide + LPS (increased 3 fold, p=0.048). In these conditions CD5 may act as a negative feedback molecule to prevent inappropriate immune responses and to control the lymphoproliferation that occurs. Indeed, it has been shown that thymocytes from $CD5^{-/-}$ mice are hyperresponsive to antigen stimulation (Tarakhovsky et al., 1995).

Also in both conditions, *Cd44* was upregulated (following i.n. peptide + LPS gene expression increased 9 fold, p=0.039). $CD44^{low}$ cells are often described as being naïve since T cells upregulate CD44 after encountering antigen. This is

the same in both conditions, i.e. the T cell has recognised peptide and responded by proliferating so it can no longer be considered naïve.

Similarly *Ly6a/e*, a marker of naïve T cells was down regulated (expression decreased 7 fold, p=0.021) showing that these cells have responded to peptide (Yang et al., 2005).

Icos was upregulated in antigen specific $CD4^+$ T cells following peptide + LPS (gene expression increased 6 fold, p=0.058). The binding of ICOS to ICOSL provides a co-stimulatory signal to the T cell that is similar to the binding of B7 molecules to CD28 (Yoshinaga et al., 1999). These co-stimulatory signals are essential for optimal T cell responses and enable T cells to proliferate, produce cytokines and provide help to B cells. ICOS is expressed on activated T cells and resting memory cells (Mages et al., 2000). This suggests that these cells are provided with a strong co-stimulatory signal. It is interesting to note that following peptide + LPS both *Ctla4* and *Icos* were upregulated, in contrast following peptide alone only *Ctla4* was upregulated. CTLA4 provides a negative signal which will balance the stimulatory signals through ICOS following peptide + LPS. But under conditions of peptide alone the balance of signals will be very different as only the negative signal is present. This may explain why fewer of the cells survived following peptide alone.

After peptide + LPS *Cxcr3* was upregulated (increased 14 fold, p=0.025). CXCR3 helps recruit lymphocytes to the lymph nodes and the skin in inflammatory conditions. It is expressed by memory cells and activated T cells (Qin et al., 1998).

These cells under immunising conditions also upregulated the gene for intergrin beta 1(*Itgb*) (gene expression increased 14 fold, p=0.031). Intergrin $\beta_1$can pair with different alpha chains to form receptors for collagen, firbronectin, fibrinogen or laminin. For example, $\alpha_4\beta_1$ (VLA4) is the receptor for VCAM expressed on the endothelial cell wall. Under these conditions *Itgb* was upregulated which would make transendothelial migration possible.

Expression of *Ccr6* was also found to be upregulated following i.n. peptide + LPS (gene expression increased 125 fold, p=0.005). CCR6 was first described as

being expressed on some DC subsets, B cells and memory T cells (Liao et al., 1999). The ligands for CCR6 are CCL20 and β defensin; these are produced at site of inflammation. Mice deficient in CCR6 have altered responses both homeostatic and inflammatory stimulation (Varona et al., 2001). This could fit well with the results of these experiments, following i.n. peptide + LPS, CCR6 may be expressed on memory T cells and involved in the migration to sites of inflammation.

Taken together changes in these three molecules will affect the migratory patterns of these cells. As these changes were not seen following treatment with peptide alone it suggests that peptide treatment may induce tolerance by preventing the migration of antigen specific T cells to the skin graft and/or draining lymph node. This will be further investigated in Chapter 3.

Following peptide + LPS a number of B cell genes were upregulated. This included genes expressed at the cell surface such as *Cd19, Cd20 (Ms4a1), Cd22, Cd74, Cd79*α and β (*Igb*). *Blnk* a gene involved in B cell signalling was also found to be upregulated and some immunoglobulin genes such as *IgK-C* (Immunoglobulin kappa chain, constant region) and *IgL-C1* (lamda chain, constant region).

This suggests that there might be B cell contamination of the $CFSE^+$ $Thy1.2^+$ cells. However, these cells were transferred from transgenic Marilyn mice that do not have any B cells in the periphery and Thy1.2, which was used to sort this population, is only expressed on the surface of T cells. Furthermore, this B cell gene expression pattern was only seen in the mice treated with peptide + LPS. LPS binds to TLR4 which is expressed widely on cells both of the innate and adaptive immune system, including $CD4^+$T cells (Akira and Takeda, 2004). B cells express TLR4 and signalling through this receptor in B cell has been shown to be essential for optimal responses involving IgM and IgG (although it is not needed for IgE responses) (Pasare and Medzhitov, 2005). There are no examples in the literature of T cells upregulating immunoglobulin genes in response to stimulation with LPS, but it maybe a response of these cells to the

particular conditions employed here, since, these are genes which are known to be activated by LPS in other cell types.

Another costimulatory molecule *Cd244* (2B4) (*Nmrk*) was found to be upregulated following peptide + LPS (expression increased 69 fold, p=0.011). This molecule has a proposed role in T-T cell interactions. 2B4 is expressed on NK cells, mast cells and subsets of γδ T cells. Its expression can be induced on $CD8^+$ T cells when stimulated under certain conditions i.e. virally infected $CD8^+$ up regulate 2B4 (Flier et al., 2001). It can bind to CD48 which is broadly expressed on haematopoietic cells and can be upregulated in response to viral infection and by stimulation by IFNαβ and IFNγ. It has been suggested that T cells can co-stimulate each other through binding of 2B4 to CD48 (Assarsson et al., 2005). It could be possible that in these experiments there is T-T interaction but further work would be needed to confirm this.

The gene for Lymphotoxin β receptor was also increased following peptide + LPS (expression of *Ltbr* increased 41 fold, p=0.029). The receptor can bind either lymphotoxin αβ heterodimer or LIGHT (Gommerman and Browning, 2003). Previously the receptor was found to be expressed on non-haematopoetic stromal cells (Murphy et al., 1998, Browning et al., 1997, Browning and French, 2002). As these cells were sorted on Thy1.2 expression it seems unlikely that there would be contaminating stromal cells. It might be that the gene is being transcribed but is not being translated into protein.

Following peptide + LPS the genes *H2-Aa* was upregulated (increased 6 fold, p=0.032). This gene encodes the MHC class II molecules H2-Aα. It isn't clear whether $CD4^+$ T cells are able to process and present antigen. Work done in the late 1970s by Schlossman (Ko et al., 1979) and Kunkel (Evans et al., 1978) demonstrated that human peripheral $CD4^+$ T cells could express MHC class II and the frequency of these cells increased after T cell activation. However, mice do not express MHC class II on their cell surface because their cells cannot transcribe the class II transactivator (C2ta) which is required for MHC class II expression. Transfecting mouse $CD4^+$ T cells with the human form of CIITA allowed MHC class II to be expressed (Chang et al., 1995). Furthermore, in H2B

mice the E molecule is not expressed as EB1 cannot pair with the A alpha chain (Mathis et al., 1983). So although the genes may be upregulated they will not be expressed.

Following peptide + LPS *Il-23r* was upregulated (expression increased 8 fold, p=0.057). IL-23 is part of the IL-12 family and is proinflammatory. Studies in mice that are deficient in the IL-23p19 subunit have shown these mice do not develop EAE or collagen induced arthritis in conditions where controls do (Cua et al., 2003, Murphy et al., 2003). The Il-23R is expressed on human Th17 cells and thought to be important in their differentiation from naïve $CD4^+$ T cells (Annunziato et al., 2008). Binding of IL-23 to its receptor activates tyrosine kinase 2 and STAT1, STAT3, STAT5 and weakly activates STAT6. This can stimulate the secretion of IL-17 and the proliferation of $CD4^+$ memory cells (Yoshimura et al., 2007) . This may indicate that i.n. peptide + LPS can induce the differentiation of proinflammatory Th17 cells, however further work would be need to clarify this.

Perforin (*Pf1*) was found to be significantly down regulated following peptide +LPS treatment (gene expression decreased 8 fold, p=0.036). Perforin is known for its lytic function in $CD8^+$ T cells and NK cells, this is crucial for the immune response. However, recent evidence has also proposed a role for this protein in $CD4^+$ T cells. Perforin deficient mice have hyperproliferative responses and secrete more IL-2. In vitro $CD4^+$ T cells from these mice have an increased calcium flux which increases the activation of NFAT (Bi et al., 2009). The down regulation of this gene in these conditions suggests that this may promote a proliferative response which was seen as more cells divided following peptide + LPS than following peptide alone.

The complement receptor CR2 was also upregulated following peptide + LPS (gene expression increased 445 fold, p=0.004). CR2 is expressed by T cells although it's exact role is not known (Erdei et al., 2009). There have been suggestions that CR2 might increase the adherence of binding to cells that express C3 (Levy et al., 1992). It is not clear why this receptor would be upregulated after i.n. peptide + LPS.

Cytoplasm

After both treatments the genes *Ox2* and *Ptgs2* (or *Cox2*) were upregulated (*Ox2* expression increased 19 fold, p=0.04 and *Cox2* expression increased 226 fold, p=0.008). These genes encode enzymes that are likely to have many roles in cellular function. Following peptide + LPS *Ptpn13* was also upregulated (expression increased 8 fold, p=0.039). PTPN13 and OX2 protect against apoptosis which is relevant following both treatments, and Cox-2 produces prostaglandins and thromboxane from arachdonic acid which have many cellular functions. Therefore it is not surprising that they are upregulated in both conditions.

Tyk2 was also upregulated in both conditions (following i.n. peptide + LPS gene expression increased 1201 fold, p=0.002). As described above tyrosine kinase 2 associates with cytokine receptors at the plasma membrane and signal cytokine binding through the JAK/STAT pathway. Its upregulatation suggests that these cells are receiving signals through cytokine receptors that use TYK2. In particular the IL-23 receptor that was also upregulated in these cells uses TYK2 to signal though the JAK/STAT pathway (Parham et al., 2002).

The enzyme 2'-5' oligoadenylate synthetase 2 (*Oas2*) was found to be significantly down regulated (gene expression decreased 10 fold, p=0.023). This enzyme has roles in shutting down of protein biosynthesis and the induction of apoptosis and regulation of cell growth (Eskildsen et al., 2002, Eskildsen et al., 2003) its being down regulated here may be another example of a pro apoptotic gene being down regulated and thereby allowing these cells to survive.

Suppressor of cytokine signalling 3 (*Socs3*) was also downregulated (gene expression decreased 8 fold, p=0.028). This molecule is induced by cytokine signalling and negatively regulate cytokine signalling through the JAK/STAT pathways (Yoshimura et al., 2007). SOCS3 is known to be induced by TLR signalling and strongly suppresses STAT3 signalling (Yoshimura et al., 2007). However in the experiments reported here SOCS3 was down regulated. This

may promote further signalling through the TLRs and STAT3 which is used by other cytokines signalling through the JAK/STAT pathway.

The gene for the pro-apoptosis molecules Bid was also found to be upregulated (*Bid* expression increased 8 fold, p=0.025). Bid is a member of the Bcl-2 family that is activated following signalling through cell surface death receptors such as Fas (Wang et al., 1996) . It is thought that Bid translocates to the mitochondria where it causes the release of cytochrome C which mediates apoptosis (Luo et al., 1998). Mice that are deficient in Bid have been shown to be protected from apoptosis (Yin et al., 1999). The upregulation of Bid reported here suggests that in immunising conditions some of these antigen specific $CD4^+$ T cells may be undergoing apoptosis.

Nucleus

Following treatment with peptide + LPS and peptide alone *Pax6* was upregulated (gene expression increased 26 fold, p=0.013). As discussed above the function of *Pax6* in T cells is not known.

In these experiments following peptide + LPS both *Fos* and *Jun* were significantly down regulated (*Fos* gene expression decreased 6 fold, p=0.027 and *Jun* gene expression decreased 12 fold, p=0.026). Fos and Jun are both activated by signalling through the MAPK kinase and together form the transcription factor AP1. AP1 binds to many gene promoters and induces the transcription of many genes, including IL-2 (Liu et al., 2007). As both these genes were downregulated it suggests that 5 days after the first dose of peptide cells might be beginning to slow their proliferation and favour a reduction in Th1 type cytokines, therefore by downregulating *Fos* and *Jun.*

Extracellular

Following peptide + LPS, the antigen specific T cells upregulated the gene for the pro-inflammatory cytokine IL-1β (*Il1-b* gene expression increased 22 fold,

p=0.059). There are two forms of IL-1, IL-1α and Il-1β which are encoded by two separate genes. It has been shown that LPS can induce the expression of IL-1β in macrophages but further signals are need for it to be cleaved and secreted (Hsu and Wen, 2002). Its expression here is probably induced by LPS which was given i.n. with the peptide.

Il-2 was also found to be upregulated following peptide + LPS (gene expression increased 35 fold, p=0.013). IL-2 is a crucial growth factor for T cells (Rochman et al., 2009). Similarly, IL-7 was also found to be upregulated (gene expression increased 18 fold, p=0.047). IL-7 is also a lymphocyte survival factor (Mazzucchelli and Durum, 2007, Surh and Sprent, 2008). Both these cytokines promote survival and T cell growth.

3.16 Conclusions

The aim of these experiments was to determine the changes in gene expression that take place in antigen specific T cell during the tolerance induction phase.

Although there was some variability in the gene expression data (see appendix I), this was found to be mainly due to variations in the conditions of the experiments (for example – different set of primers on different plates, different types of master mix being used). In order to take account of this, only genes that were consistently shown to be up or down regulated were included in the final analysis.

When the HY-A^b*Dby* peptide was given i.n. alone, fewer cells were recovered than if the peptide was presented under immunising conditions (i.e. with LPS). Despite being fewer in numbers these cells appeared to go through more divisions than when peptide was given with LPS (Figures 3.6 and 3.7).

Gene expression analysis of the cells recovered after peptide only treatment revealed that FoxP3 was significantly upregulated in these cells and this was confirmed by flow cytometry (Figure 3.9). This demonstrates that i.n. peptide can convert a naïve CD4$^+$ population into a regulatory FoxP3$^+$ population. I found no evidence of an upregulation of CD25, this was not unexpected as not all

regulatory cells are $CD25^+$ and in previous work it was shown that tolerance to male skin grafts could be transferred to other mice by both the $CD25^+$ and $CD25^-$ T cell subsets (Chai et al., 2004, Surh and Sprent, 2008, Mazzucchelli and Durum, 2007).

The genes that were upregulated after i.n. peptide alone can be grouped into 4 categories:

1. Genes typically expressed in by Tregs such as *FoxP3* and *Ctla4*. These genes are known to be expressed by regulatory T cells that prevent effector T cells from responding to antigen.
2. Anti-apoptotic genes such as *Grp94*, *Ptpn13* and *Ox2*. It is known that the protein products of these genes have a role in cell survival. As fewer cells survived following i.n. peptide alone these proteins might be acting to protect the cells that do survive from apoptosis.
3. Genes that encode proteins that inhibit T cell activation such as *Ctla4, Cd5* and *Pdcd1.* These gene products change the balance of signal that the T cell receives, preventing them from differentiating into effector cells.
4. Genes that encode proteins that modify the properties of dendritic cells such as *Pdcd1, Lag3, Ctla4, Ifng* and *Il13*. When transcribed these proteins can modify the function of antigen presenting cells by inducing enzymes such as IDO and L-Arginase which, in turn can prevent T cells from becoming fully activated effector cells.

In order to achieve antigen specific tolerance it is probable that genes in all these four areas work together to alter the balance of signals the T cell receives, and it is a combination of these mechanisms that are needed to induce tolerance.

When peptide was given with LPS, more genes were found to be significantly changed. This included genes that are involved with migration, cell cycle and cytokine signalling. Therefore inducing tolerance by i.n. peptide was not only due to increasing expression of regulatory genes but also preventing or failing to upregulate, a whole host of genes that are transcribed in activated $CD4^+$ T cells.

Chapter 4: Linked suppression phase

4.1 Introduction

Linked suppression is the process by which tolerance to one antigen spreads to other antigens expressed on the same donor tissue. Linked suppression was first described by Waldmann and Cobbold. They demonstrated that CBA mice could be made tolerant to minor mismatched skin grafts from B10.BR mice, by non-depleting anti CD4 and/or CD8 antibodies. After 2 months these same recipient mice would accept another B10.BR skin graft, and reject a skin graft from a third party strain – e.g. CBK (transgenic CBA mice expressing the Kb class I molecule) even if the skin graft was placed in the same graft bed as the B10.BR graft. However 50% of the recipient mice did accept grafts from (B10.BRxCBK)F1 mice. 4 months later the recipients that had accepted the (B10.BRxCBK)F_1 graft would then accept CBK skin grafts. The authors concluded that regulation must happen in the local microenvironment in order for tolerance to spread to other epitopes. However the authors were not clear what it is about the environment that causes linked suppression (reviewed by Waldmann and Cobbold, 1998).

Following i.n. administration of the male HY-A^b*Dby* peptide, B6 female mice become tolerant to syngeneic male skin grafts or male spleen cells, even though these cells express an additional two MHC class I restricted HY epitopes - HY-D^b*Uty* and HY-D^b*Smcy*. Furthermore, i.n. HY-A^b*Dby* can also induce tolerance to male skin grafts in female (CBAxB6)F_1 in this case the syngeneic grafts expressed 1 additional class II and 4 additional class I peptide epitopes (Chai et al., 2004) The mechanism by which tolerance spreads to other peptide epitopes is not known. However, it is very different from the initial induction of tolerance by i.n. peptide where no response of endogenous HY specific CD4 T cells was detectable. Following grafting it was shown that within 8 days $CD4^+$ T cells from the graft draining lymph nodes of tolerant mice were able to secrete IFNγ when

stimulated with peptide *in vitro*. Also, 21 days after skin grafting both the $CD4^+$ and the $CD8^+$ cells secrete IFNγ (Chai et al., 2004). As discussed in chapter 1 - IFNγ can also behave as an immunomodulatory cytokine and thus maybe involved in the mechanism of linked suppression here.

It is thought that linked suppression is only possible if APCs present both the tolerant antigen and the third party antigen at the same time. The evidence for this is the requirement for the antigens to be present on the same allograft (Madsen et al., 1988, Wong et al., 1996) or the same cells *in vitro* (Holán and Mitchison, 1983, Frasca et al., 1997, Marelli-Berg et al., 1997) for tolerance to spread to the third party antigens.

Therefore it has been proposed that the APC mediates linked suppression in two possible ways (Cobbold et al., 2006):

1. The APC becomes tolerogenic so both donor and third party antigens are presented to naïve or effector T cells in a tolerogeneic fashion.
2. The APC allows potential effector cells and regulatory T cells to come together in close proximity. Regulatory T cells can then inhibit effector T cells either by direct contact or cytokine mediated suppression, or indirect competition for cytokine and co-stimulatory ligands.

In order to investigate the mechanism of linked suppression this model of i.n. peptide induced tolerance, gene expression in antigen specific $CD8^+$ and $CD4^+$ T cells in tolerant and rejecting mice after antigen challenge with either male skin graft or transfer of male spleen cells will be examined.

4.2 Gene expression in antigen specific $CD8^+$ T cells during linked suppression

4.3 $CD8^+$ HY-D^bUty tetramer $^+$ cells after skin grafting

To compare gene expression in antigen-specific CD8+ T cells from tolerant and rejecting mice at the time that linked suppression is induced, cells were isolated from spleen, graft draining and non draining LN from female B6 mice shortly after male skin graft emplacement. The tolerised group were given i.n. HY-AbDby 10 days before grafting. CD8+ HY-DbUty tetramer+ T cells were isolated by flow cytometry sorting on day 21 after skin grafting. This time point was chosen because it has been previously shown that more cells secreting IFNγ (therefore tetramer positive) could be detected in the graft draining lymph nodes of peptide treated mice than at earlier time points (Chai et al., 2004) thus optimizing the likelihood of being able to isolate sufficient numbers of cells for RNA preparation. Figure 4.1 (4 individual female mice) shows, that even at this time point only small numbers of $CD8^+$ HY-D^b*Uty* tetramer$^+$ cells could be isolated following i.n. HY-A^b*Dby* peptide and male skin grafting. An average of 3127 $CD8^+$ HY-D^b*Uty* tetramer$^+$ cells were recovered per peptide treated mouse. This presented technical difficulties in isolating a sufficient amount of high quality RNA that would be suitable for the quantitative PCR array. For this reason an alternative method of inducing tolerance to haematopoietic cells was used.

4.4 Tolerance to Haematopoietic cells

As an alternative means of measuring tolerance induction by i.n. administration of HY-A^bDby peptide, intravenous (i.v.) injection of a mixture of male and female splenocytes differentially labelled with CFSE was used (Chai et al., 2004). A 1:1 ratio of male (high CFSE) to female (low CFSE) labelled spleen cells were transferred (i.v.) into female mice. The male cells in this assay will act as both an immunogen and a target. If no peptide treatment was given, male cells were killed thus decreasing the ratio of male to female cells. If the recipients were

given i.n. HY-A^{b}*Dby* peptide, fewer male cells were killed and the ratio of male to female cells remained high (Figure 4.2).

The ratio of male to female cells remained high in 6 of the 8 treated mice, indicating reduced killing. Nevertheless there was some variation in the effect of peptide treatment on the extent to which the male cells were killed in individual mice (Figure 4.2B). Similar findings have been reported earlier using this model (Chai et al., 2004). The reasons for this are not clear. However, all the female recipients, regardless of whether or not they had rejected male spleen cells, were shown to be tolerant of a subsequently placed male skin graft (Chai et al., 2004). The variation may be due to the fact that the in vivo killing assay is much more sensitive as the male antigen is more accessible on the circulating cells. By comparison the skin grafting is less sensitive and the male antigen is probably much less available.

It was found that the recoveries of CD8^{+} HY-D^{b}*Uty* tetramer positive T cells from both tolerant and rejecting mice was greater after the *in vivo* cytotoxicity assay than after skin grafting. On average of 8125 CD8^{+} HY-D^{b}*Uty* tetramer positive T cells per peptide treated mouse (Figure 4.3) were recovered. As the number of cells recovered was larger, and the assay was quicker to perform than the skin grafting experiments, this method was chosen. It was possible to pool cells from several mice and extract good quality RNA for the Q-PCR array.

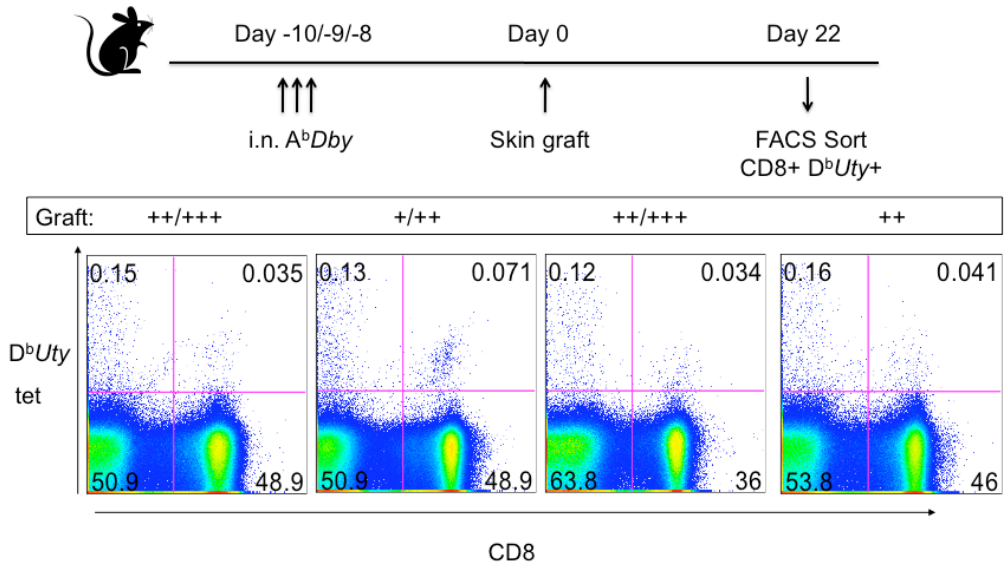


Figure 4.1 CD8$^+$ HY-D^b*Uty* tetramer$^+$ cells from 4 female mice 22 days after male grafting. Female mice were given 3 doses of i.n. A^b*Dby* peptide, and received a male skin graft 10 days later. 22 days after grafting, the grafts were scored from rejected (-) where less than 10% of the graft remained to +++ where the graft shown no signs of rejection. CD8$^+$ HY-D^b*Uty* tetramer$^+$ cells were isolated from pooled spleen and LN cells by flow cytometry sorting. The recovery of cells was low.

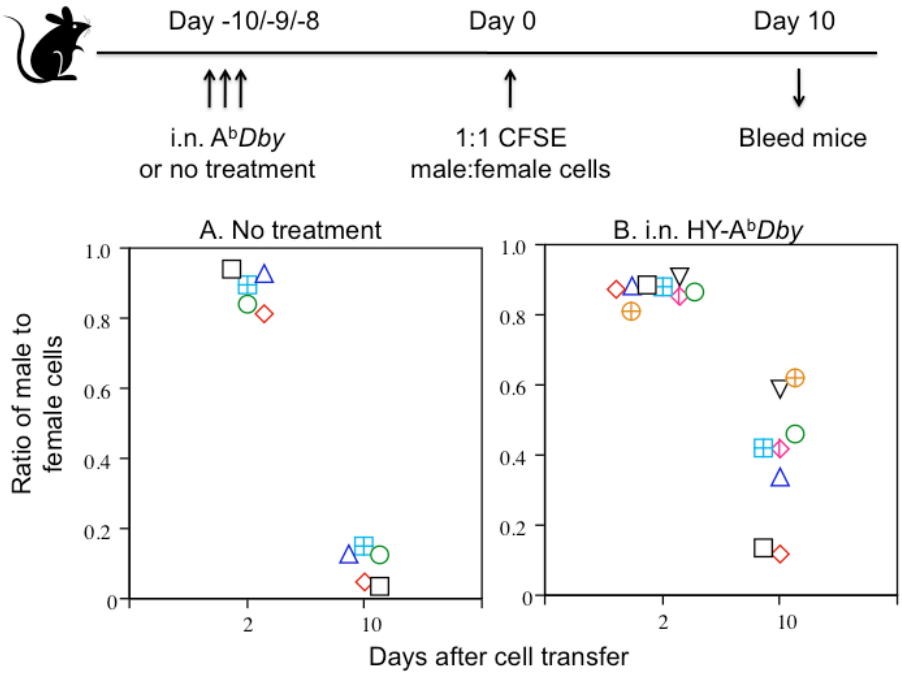


Figure 4.2 i.n. HY-A^b*Dby* can induce tolerance to CFSE labelled male spleen cells. Male spleen cells were labelled with a high concentration of CFSE (5mM) and female spleen cells were labelled with a low concentration (0.5mM) of CFSE. The cells were mixed at a 1:1 ratio and injected i.v. into female mice either after no treatment or 10 days after i.n. HY-A^b*Dby*. 3 and 10 days after CFSE cell transfer, mice were bled. The ratio of male:female cells was calculated from flow cytometric analysis (A) no treatment (5 mice) (B) 3 doses of i.n. HY-A^b*Dby* peptide (8 mice).

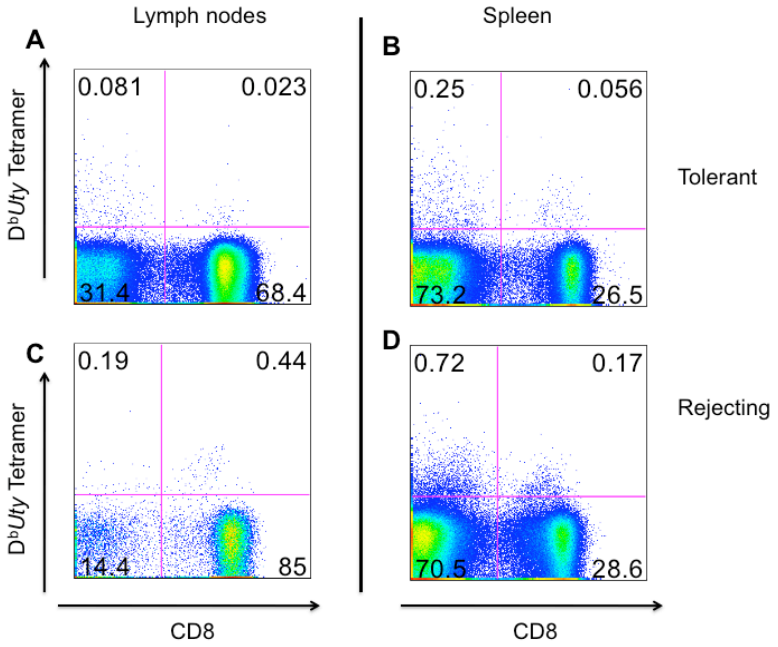


Figure 4.3 CD8$^+$ D^b*Uty*$^+$ cells in spleen and lymph nodes following administration of male cells. Female mice were given i.n. HY-A^b*Dby* peptide and 10 days later given male and female spleen cells differentially labelled with CFSE. Recipient mice were bled to ensure that i.n. HY-A^b*Dby* had induced tolerance to the male spleen cells. Between 10 and 15 days after the transfer of CFSE labelled cells, mice were culled and the spleen and lymph nodes were stained for CD8 and HY-D^b*Uty* tetramer (A) lymph nodes and (B) spleen from tolerant mouse. (C) Lymph nodes and (D) spleen from rejecting mouse. Double positive CD8$^+$ HY-D^b*Uty*$^+$ cells were collected by flow cytometric sorting. In this experiment 5 control mice yielded 73,100 CD8$^+$ HY-D^b*Uty*$^+$ cells and 56,880 CD8$^+$ HY-D^b*Uty*$^+$ cells were recovered from 7 peptide treated mice.

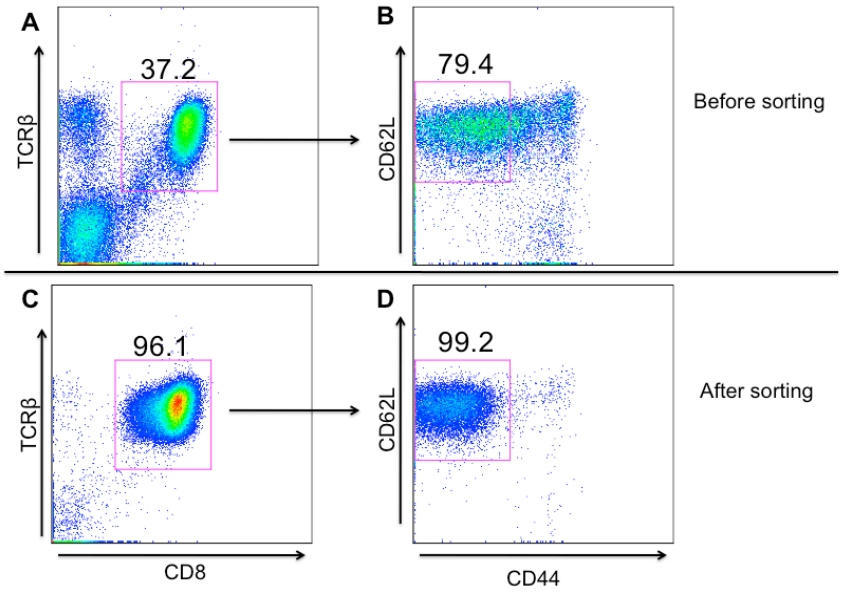


Figure 4.4 Naïve CD8$^+$ T cells. Cells from spleen and lymph nodes of WT B6 mice were stained with CD8, TCRβ, CD44 and CD62L. (A) Before sorting 37% cells were CD8$^+$ TCRβ$^+$ (B) 79% of those cells were CD44^{low}CD62L^{high} (C) After sorting this population was enriched to 96% CD8$^+$ TCRβ$^+$ (D) of those cells 99% were CD44^{low}CD62L^{high}.

4.5 Gene expression in $CD8^+$ T cells from tolerant or rejecting mice

RNA was extracted from naïve sorted $CD8^+$ $TCR\beta^+$ $CD44^{low}$ $CD62L^{high}$ T cells (Figure 4.4) and $CD8^+$ HY-D^b*Uty* $tetramer^+$ T cells from mice that were either tolerant of or had rejected CFSE labelled male spleen cells. The RNA was reverse transcribed and gene expression was analysed by quantitative PCR array. As the number of cells was small, the quantitative PCR method had to be modified; instead of 100ng RNA per array 60ng was used.

In mice that had been given i.n. HY-A^b*Dby* peptide and therefore not rejected CFSE labelled male cells, $CD8^+$ HY-D^b*Uty* $tetramer^+$ cells from these mice showed significant changes in the expression of 16 genes, only two of which (*IL-5* and *Lef1*) were downregulated (Table 4.1). 27 genes were significantly changed in $CD8^+$ HY-D^b*Uty* $tetramer^+$ cells from rejecting mice, including 5 (*Il5, Socs1, Traf1, Sele, Il11ra*) genes that were down regulated (Table 4.2).

There was some overlap in the genes that were significantly changed in both tolerant and rejecting cells. The genes have been arranged by cellular location (Table 4.3).

Rank	Gene	P Value	Fold Change	Ave Ct Naïve	Ave Ct Tolerant
1	*Ccr10*	0.007	1043.74	40.00	33.28
2	*Il5*	0.008	-12.92	33.88	40.00
3	*Plau*	0.014	22.41	37.90	35.25
4	*Pdcd1*	0.014	19.28	33.67	31.79
5	*Ccl4*	0.014	15.92	31.04	29.12
6	*Lag3*	0.020	60.21	33.77	30.40
7	*Ccl5*	0.025	13.00	28.18	26.68
8	*Lef1*	0.027	-5.07	26.40	31.13
9	*Cd36*	0.030	29.85	32.56	30.32
10	*Cdkn1a*	0.031	9.35	32.19	31.28
11	*Klrc1*	0.032	6.62	32.39	31.68
12	*Tnfrsf11a*	0.032	11.90	32.66	31.09
13	*Csf1*	0.035	86.28	37.11	32.81
14	*Prdm1*	0.048	2939.12	32.78	27.15
15	*Ccl3*	0.048	15.32	31.22	29.00
16	*C3*	0.050	18.65	33.88	31.85

Table 4.1. Genes that are significantly changed in $CD8^+$ HY-D^b*Uty*$^+$ T cells from tolerant mice. Female mice were given 3 doses of i.n. HY-A^b*Dby* peptide, and 10 days later given CFSE labelled male and female spleen cells, 10 days later the mice were shown to have not rejected the CFSE labelled male cells. Endogenous $CD8^+$ HY-D^b*Uty*$^+$ cells were isolated by flow cytometric sorting. RNA was extracted from sorted $CD8^+$ HY-D^b*Uty*$^+$ T cells and naïve $CD8^+$ T cells. Gene expression was compared by quantitative PCR array and GPR software. For naïve samples the quantitative PCR array was performed on preparations from three mice on three separate plates. For tolerant samples the array was performed on preparations from at least two pooled mice. Each pool was run once on one plate, four pools were run in total. The average cycle threshold (Ct) is given. The global pattern recognition software calculates a list of significantly changed genes, the P value and the fold change.

Rank	Gene	P-Value	Fold change	Naïve Average Ct	Rejecting Average Ct
1	*IgG1-C2a*	0.0051	37.83	32.86	30.16
2	*Il5*	0.0076	-11.83	33.88	40.00
3	*Pdcd1*	0.0082	28.42	33.67	31.47
4	*Ccl5*	0.0082	31.20	28.18	25.72
5	*Lag3*	0.0097	61.72	33.77	30.00
6	*Gzmb*	0.0115	43.27	31.08	27.81
7	*C3*	0.0130	20.72	33.88	31.79
8	*IgK-C*	0.0136	11.50	25.15	24.09
9	*IgA-C2v1*	0.0160	11.65	27.58	26.63
10	*Ctsb*	0.0164	13.17	28.19	27.01
11	*Itgax*	0.0164	26.77	32.19	29.74
12	*Ifng*	0.0201	25.58	32.17	29.70
13	*Socs1*	0.0206	-6.52	27.98	33.28
14	*Cd36*	0.0219	19.49	32.56	30.94
15	*H2-Eb1*	0.0223	18.91	28.91	26.90
16	*Casp3*	0.0232	7.62	29.86	29.41
17	*Cx3cr1*	0.0233	8.02	32.95	32.40
18	*Tnfrsf9*	0.0236	7.87	31.59	31.19
19	*Ccl4*	0.0246	8.07	31.04	30.46
20	*Fasl*	0.0283	45.00	32.68	30.03
21	*Cxcr3*	0.0312	21.04	31.66	29.26
22	*Itgb1*	0.0323	4.23	27.71	28.16
23	*Traf1*	0.0349	-5.49	28.31	33.30
24	*Tnfrsf11a*	0.0350	8.95	32.66	31.90
25	*Sele*	0.0401	-13.96	31.83	39.07
26	*Il11ra1*	0.0424	-6.67	30.28	35.93
27	*Cd74*	0.0492	9.51	25.05	24.32

Table 4.2. Genes that are significantly changed in $CD8^+$ HY-D^b*Uty*$^+$ T cells from rejecting mice. Female mice were given CFSE labelled male and female spleen cells, 10 days later CFSE labelled male cells were rejected and the endogenous $CD8^+$ HY-D^b*Uty* were isolated by flow cytometric sorting. RNA was extracted from sorted $CD8^+$ HY-D^b*Uty*$^+$ T cells and naïve CD8 T cells and compared by quantitative PCR array. For naïve samples the quantitative PCR array was performed on preparations from three mice on three separate plates. For rejecting samples the array was performed on preparations from at least two pooled mice. Each pool was run once on one plate, three pools were run in total. The average cycle threshold (Ct) is given. The global pattern recognition software calculates the list of significantly changed genes, the P value and the fold change

Table 4.3 (following page) Genes that are significantly either up or downregulated in $CD8^+$ HY-D^b*Uty*$^+$ cells from either rejecting mice or tolerant mice, are shown by their cellular location. Some genes were found to be up or down regulated after both treatments - these are shown in the middle column. Genes that are downregulated are shown in blue.

	Tolerant	Both	Rejecting
Plasma membrane	chemokine (C-C motif) receptor 10 (*Ccr10*) killer cell lectin-like receptor subfamily C, member 1 (*Klrc1*)	programmed cell death 1 (*Pdcd1*) lymphocyte-activation gene 3 (*Lag3*) CD36 antigen (*Cd36*) tumour necrosis factor receptor superfamily, member 11a/Rank (*Tnfrsf11a*)	chemokine (C-X3-C) receptor 1 (*Cxc3cr1*) chemokine (C-X-C motif) receptor 3 (*Cxcr3*) E-Selectin (*Sele*) Integrin alpha X (*Itgax*) Integrin beta 1 (fibronectin receptor beta)/cd29 (*Itgb1*) histocompatibility 2, class II antigen E beta (*H2-Eb1*) tumour necrosis factor receptor superfamily, member 9/4-1bb (*Tnfrsf9*) Fas ligand (TNF superfamily, member 6) (*Fasl*) Interleukin 11 receptor, alpha chain 1 (*Il11ra*)
Cytoplasm			Caspase 3 (*Casp3*) Cathepsin B (*Ctsb*) suppressor of cytokine signalling 1 (*socs1*) Tnf receptor-associated factor 1 (*Traf1*)
Nucleus	lymphoid enhancer binding factor 1 (*Lef1*) PR domain containing 1, with ZNF domain (*Prdm1* or Blimp1) cyclin-dependent kinase inhibitor 1A (P21) (*Cdkn1a*)		
Extracellular	chemokine (C-C motif) ligand 3/MIP1 alpha (*Ccl3*) colony stimulating factor 1 (*Csf1*) plasminogen activator, urokinase (*Plau*)	Interleukin 5 (*Il5*) Chemokine (C-C motif) ligand 4 (Mip1β) (*Ccl4*) Chemokine (C-C motif) ligand 5 (Rantes) (*Ccl5*) complement component 3 (*C3*)	granzyme B/Ctla1 (*Gzmb*) interferon gamma (*Ifng*) IgG1 (*IgG1*) immunoglobulin kappa chain, constant region (*IgK-C*) immunoglobulin heavy chain 2 (serum IgA) (*IgA-C2v1*)

4.6 Discussion: Gene expression in HY peptide specific CD8$^+$ T cells

4.7 Cells from tolerant mice

Plasma membrane

The gene with the greatest fold change in CD8$^+$ HY-D^b*Uty*$^+$ cells from mice made tolerant to male cells by i.n. HY-A^b*Dby* peptide was *Ccr10* (p=0.007 fold change= 1043.7). Expression of this gene was undetectable in naïve cells but high in RNA from tolerant mice, hence the large fold change. CCR10 is expressed by a subset of CD4$^+$ and CD8$^+$ T cells and is involved in homing to the skin and gut. The CCR10 ligands are CCL27, which is expressed in the skin and CCL28 which is expressed in the gut. In order to enter these sites, T cells need to express appropriate co-receptors such as the intergrin: $\alpha 4\beta 7$ for the gut and CCR4 for the skin (Marelli-Berg et al., 2007). There were primer sets for several integrins included on the plates used in these experiments, these genes were found to be expressed but their expression was not significantly different to that of naïve cells. For example, two genes: *Itgax* (p=0.07, fold change = 11.1, average Ct = 30.59) and *Itgb1* (p=0.08 fold change= 8.05 average Ct = 27.17). So these genes are being transcribed but at a similar level to naïve T cells. This is in contrast to the same cells taken from rejecting mice that were found to significantly upregulate *Itgax* (p=0.01, fold change = 26.77) and *Itgb1* (p=0.03, fold change = 4.23). This suggests that the ability of these cells to migrate into peripheral tissues could be impaired in tolerant mice.

The genes *Pdcd1* and *Lag3* were up regulated in tetramer$^+$ CD8$^+$ cells from both tolerant and rejecting mice.

As discussed in chapter 1, *Pdcd1* encodes the cell surface protein Programmed death 1 (PD1) and is expressed by both CD4$^+$ and CD8$^+$ T cells. This cell surface antigen was found to be upregulated on antigen specific CD4$^+$ T cells during the tolerance induction phase. Binding of PD1 to either of its ligands sends a negative regulatory signal to the T cell that prevents it from proliferating (reviewed by Keir et al., 2008). This binding can also send a signal to the DC that prevents maturation and increases secretion of IL-10 (Kuipers et al., 2006).

The inhibition of dendritic cell maturation would effectively decrease the amount of antigen being presented. Any antigen that was presented would be in the absence of co-stimulation and pro-inflammatory cytokines and therefore will not be able to fully activate CD4+ and CD8+ T cells. This may lead to tolerance spreading to other antigens presented at the same time.

In addition, PD1 and LAG3 have been shown to be upregulated on exhausted $CD8^+$ T cells. Following chronic viral infection with LCMV, Wherry *et al.*, found that a number of inhibitory receptors were upregulated on antigen specific $CD8^+$ T cells including PD1 and LAG3 (Wherry et al., 2007). In the current analysis, tolerant $CD8^+$ T cells have seen antigen but have not become cytotoxic effector T cells, and therefore may share some similarities with exhausted $CD8^+$ T cells that are unable to respond to antigen because of chronic stimulation.

Killer-Cell lectin like receptor family C, member 1 (*Klrc1*) was also upregulated on these cells and is a cell surface receptor expressed on NK cells and some CD8+ T cell subsets. It forms a heterodimer at the cell surface with CD94 to form the NKG2 receptor which binds the non-classical MHC molecule Qa-1 in mice. Recently, CD8+ CD94+ cells shown to mediate tolerance in mouse model of ocular-induced tolerance, known as anterior chamber-associated immune deviation (ACAID) (He et al., 2008). It was shown that these CD8+ CD94+ were suppressive and their suppressive activity was associated with an increase in secretion of TGFβ. This suggests that the expression of CD94 in antigen specific $CD8^+$ T cells could mark these cell as being part of a suppressive $CD8^+$ population. Although the gene for TGFβ - *Tgfb* was not found to be significantly upregulated ($p=0.17$ fold change= 1.2) the levels in both naïve and tolerant mice was sufficiently high for TGFβ to have some function in these cells.

$CD8^+$ HY-D^b*Uty*$^+$ cells also upregulated *Cd36* in both tolerising and rejecting conditions. In mice, CD36 is expressed on many cell types including some haematopoietic cells. It has been shown to be differentially regulated on APCs: DC's and macrophages expressing higher surface levels than B cells (Corcoran et al., 2002). Therefore it may have additional roles in antigen processing and presentation. CD36 can bind to multiple ligands involved in many physiological

processes such as cell adhesion, scavenging of apoptotic cells and fatty acid metabolism however, its role in T cells is not known.

Receptor Activator of Nuclear Factor κB (*Tnfrsf11a* or Rank) was also upregulated in both rejecting and tolerant cells. This receptor is expressed on osteoclasts, where it has a role in bone growth and the development of lactating mammary gland during pregnancy. It has been shown that expression of Rank can be induced on activated human peripheral blood T cells by *in vitro* IL-4 and TGFβ stimulation (Anderson et al., 1997). The expression of Rank and RankL on activated T cells and dendritic cells is similar to the structurally related molecules – CD40 and CD40L which are also part of the TNF superfamily of receptors (reviewed by Theill et al., 2002). In a model using mouse keratinocytes, Loser et al., (Loser et al., 2006) have shown that overexpression of RankL on these cells causes functional changes in the DCs in the skin, which lead to an increase in numbers of regulatory T cells in the periphery. Therefore the upregulation of Rank on tolerant antigen specific $CD8^+$ T cells may also have an effect on the presenting DC and cause an increase in the number of regulatory T cells.

Nucleus

In the cell nucleus, lymphoid enhancer binding factor 1 (*Lef1*) was found to be downregulated in tolerant $CD8^+$ T cells. LEF1 is a DNA binding protein, part of WNT signalling pathway that is important in T development and also found in mature T cells. It forms a complex with RUNX1 and CBFβ which can activate MHC class I transcription (Howcroft et al., 2005). It has also been shown that LEF1 expression is downregulated in human $CD8^+$ T cells following *in vivo* antigen stimulation (Willinger et al., 2006). In the current analysis *Lef1* is downregulated which suggests these cells have been stimulated through their TCR and differentiated as their gene expression is different to naïve $CD8^+$ T cells.

Two additional genes that are expressed in the nucleus Prdm1 and Cdkn1a were both upregulated in tolerant CD8+ T cells. *Prdm1* (or BLIMP1) has been

described as a key regulator of T cell homeostasis and activation. Evidence for this has come from reports showing that transcription of the BLIMP gene increases after TCR stimulation and that mice deficient in BLIMP develop fatal colitis (Martins et al., 2006). Furthermore, mutations in the BLIMP gene can lead to a lethal multi-organ inflammatory disease (Kallies et al., 2006) taken together these results suggest that BLIMP has a crucial role in controlling inflammation.

More recent work has shown that BLIMP1 can mediate these effects by directly repressing IL-2 and Fos transcription and this prevents T cell proliferation. Current evidence suggests that IL-2 induces the transcription of *Prdm1* in T cells. BLIMP then prevents the transcription of the IL-2 gene in a classical negative feedback loop (Martins et al., 2008).

The upregulation of *Prdm1* in $CD8^+$ HY-D^b*Uty*$^+$ from tolerant mice is a strong indicator that these cells may not be able to transcribe IL-2 and therefore are less able to proliferate. This may contribute to the mechanisms that prevent the graft being rejected.

Cdkn1a (Cyclin dependent kinase inhibitor) is a cell cycle protein involved in the G1 to S transition phase. CDKN1A acts at this cell cycle checkpoint to prevent cell proliferation, and thus stop the proliferation of cells that have DNA damage (Abukhdeir and Park, 2008). In these $CD8^+$ HY-D^b*Uty*$^+$ cells from tolerant mice, CDKN1A may be another protein that prevents further cellular proliferation in the face of antigen stimulation and therefore prevents graft rejection.

Secreted

The genes for some secreted proteins were also significantly changed.

$CD8^+$ HY-D^b*Uty*$^+$ cells significantly downregulated ($p=0.008$ fold change= -12.92) the gene for the cytokine IL-5 in tolerant and rejecting mice. IL-5 is a B cell growth factor that induces the terminal differentiation of late developing B cells into Ig secreting plasma cells. The downregulation of this gene will reduce B cell activity.

Both tolerant and rejecting $CD8^+$ HY-D^b*Uty*$^+$ cells upregulated the genes for chemokines that can affect T cell migration.

The genes *Ccl4* (p=0.014, fold change= 15.92), *Ccl5* (p=0.025, fold change= 13.0) and *Ccl3* (p=0.048, fold change= 15.32) were significantly upregulated in antigen specific T cells from tolerant mice.

CCL4 (Mip1 beta) and CCL5 (Rantes) bind to CCR5 expressed on T cells – causing them to migrate towards the site of inflammation. CCL4 has been shown to also have some regulatory functions. (Joosten et al., 2007) describe a human $CD8^+$ $LAG3^+$ $CD25^+$ $FoxP3^+$ Treg subset that suppresses T cell proliferation through the secretion of CCL4. CCL5 can induce the expression of integrins which are involved in the adhesion and movement of T cells through the vascular wall reviewed by (Krensky and Ahn, 2007). The upregulation of these cytokines may indicate these antigen specific T cells are attracting other T cell to their location – this may be necessary for both tolerance and rejection responses.

The gene for *Ccl3* was also found to be upregulated only in tolerant T cells. CCL3 can bind CCR1 and CCR5. CCR1 is expressed on DC's and binding may increase DC maturation (Bachmann et al., 2006). As discussed in the introduction, DC maturation is distinct from their ability to induce immunity. Therefore, DC's can mature but if they are not fully licensed they will not be able to elicit a T cell response. Therefore the upregulation of this gene does not necessarily mean tolerance will be broken. Indeed some mature DC that express IDO have been shown to be tolerogenic (Munn et al., 2004).

In addition, Colony stimulating factor 1 (M-CSF, *Csf-1*) was found to be upregulated in tolerant cells. M-CSF is the growth factor for cells of the monocyte-macrophage lineage. It can induce these cells to secrete pro-inflammatory cytokines. It may also have a role in the differentiation of DCs, since these cells also express the CFS1 receptor during differentiation. Furthermore, CSF1 deficient mice have reduced numbers of both plasmacytoid and CD11c high DC subsets (MacDonald et al., 2005).

The upregulation of *Csf-1* suggests that these tolerised $CD8^+$ cells can controlling DC maturation. As these mice are tolerant of the skin graft even though DC's mature they may not be fully licensed to initiate a T cell response.

Plasminogen activator, urokinase (*Plau* or uPA) was found to be upregulated in tolerant cells. It is a serine protease that catalyses the conversion of plasminogen to plasmin. It can also bind to the receptor; uPAR, which is expressed on endothelial cells and haematopoietic cells. uPAR associates with integrins to modulate cell adhesion and migration. uPA and uPAR have been shown to be involved in a number of processes such as cell migration and proliferation (Mondino and Blasi, 2004). Therefore it's upregulation in antigen specific $CD8^+$ T cells from tolerant mice suggests that these cells may have different migratory capacity compared to the same cells from rejecting mice. The migration of antigen specific T cell after skin grafting will be examined in more detail in chapter 3.

The gene for the complement component C3 was also upregulated in both rejecting and tolerising conditions. C3 is central to both the classical and alternative pathways of complement activation. It has been shown that T cells can produce C3 (reviewed by Li et al., 2007, Pantazis et al., 1990). There is some evidence that the active products of C3: C3b and iC3b can bind to CR3 expressed on APCs. This leads to the production and secretion of TGFβ and upregulation of IL-10 RNA in a rat model of tolerance in the anterior chamber of the eye (Sohn et al., 2003). Again this may suggest that the antigen specific $CD8^+$ T cells can alter the function of the antigen presenting DC, in this case by altering the cytokine transcription. The switch to transcribing cytokines that inhibit T cell proliferation could provide a mechanism for linked suppression. Any further antigen presentation by these DCs will be in the presence of these cytokines, which may spread tolerance to other T cell epitopes.

4.8 Cells from rejecting mice

$CD8^+$ HY-D^b*Uty* $tetramer^+$ cells from rejecting mice showed significant changes in the expression of more genes than did the same cells from tolerant mice.

The most highly upregulated was somewhat surprisingly *IgG1-C2a.* Two additional immunoglobulin genes: *IgK-C* and *IgA-C2v1* were also upregulated. I found similar upregulation of a number of B cell genes during the tolerance induction phase (reported in chapter 1) following treatment with i.n. HY-A^b*Dby* peptide+ LPS. Again, the reasons for this are not clear. The conditions here were different to those in the induction phase: no transgenic T cells were transferred, the cells were B cell depleted before being sorted and cells were sorted on the basis of both binding to the tetramer and $CD8^+$ staining. Therefore the B cell contamination is unlikely. There is no evidence in the literature to suggest that T cells can transcribe immunoglobulin genes – therefore it seems that there could be some non-specific amplification of these genes during the quantitative PCR reaction. As the amplification temperatures had to be the same for all the primer sets across the plate – it could be that this was not optimal for these primers and this lead to some non-specific binding and amplification. This could explain why it was seen across different experiments with different conditions.

Considering the next most highly upregulated gene products that are expressed in the plasma membrane, *Pdcd1* and *Lag3* were also found to be upregulated on tetramer positive cells from rejecting mice. These molecules have regulatory functions that control lymphoproliferation. This is important in the control of the immune response leading to rejection of the male graft, preventing it from becoming an overwhelming response.

These molecules have also been shown to be expressed on exhausted T cells after chronic infection (Wherry et al., 2007). These cytotoxic $CD8^+$ cells were in the process of rejected the female skin graft and so may share some similarities with exhausted $CD8^+$ T cells that have also responded to antigen.

Cd36 and *Tnfrsf11a* (or *Rank*) were also upregulated in these cells. CD36 has many roles in physiological processes but its role in T cells is not known. As discussed above, Rank binding to RankL (expressed on DCs) is structurally similar to CD40 binding CD40L. This may provide a co-stimulatory signal to the T cell similar to CD40, which would stimulate $CD8^+$ T cells to reject the graft.

Many genes involved in cell migration such as *Cxc3cr1*, which is a G protein coupled receptor for the proinflammatory CX3C chemokine Fractalkine, were upregulated. Fractalkine has been shown to be both a chemoattractant and an adhesion molecule (Crola Da Silva et al., 2008). These cells also upregulate the gene for CXCR3 which binds CCL21. CXCR3 is involved in T cell recruitment in response to inflammatory stimuli (reviewed by Marelli-Berg et al., 2007). This suggests that these antigen specific $CD8^+$ T cells have the chemokine receptors they need to migrate to the site of inflammation.

Rejecting $CD8^+$ HY-D^b*Uty*$^+$ downregulated the gene for E-Selectin which is normally expressed on the surface of endothelial cells. This gene was found to be expressed in naïve $CD8^+$ T cells but its expression was undetectable in $CD8^+$ HY-D^b*Uty*$^+$ cells. Usually after T cell activation in the skin draining lymph nodes $CD8^+$ T cell upregulate E-selectin ligand (Edele et al., 2008). Therefore the significance of its expression in naïve T cells is unclear. Contamination of the naïve T cell preparation with endothelial cells seems unlikely as naive CD8 T cells were sorted to a high purity on the basis of $CD8^+$ $TCR\beta^+$ $CD44^{low}$ $CD62L^{high}$ expression (Figure 4.4).

The intergrins α_X (*Itgax)* and β_I (*Itgb1)* were also upregulated in rejecting $CD8^+$ HY-D^b*Uty*$^+$ cells. The intergrins are involved in the firm adhesion of rolling lymphocytes to the endothelium. Integrin α_X usually associates with a β_2 chain to form a receptor for fibrinogen. This receptor mediates cell-cell contact during inflammatory responses. Integrin β_I can pair with different α chains to form receptors for collagen, firbronectin, fibrinogen or laminin e.g. $\alpha_4\beta_I$ (VLA 4) is the receptor for Vcam. This suggests that these $CD8^+$ tetramer$^+$ cells can migrate into peripheral tissues, antigen specific migration after skin grafting will be analysed in further detail in Chapter 3.

$CD8^+$ HY-D^b*Uty* tetramer$^+$ cells from rejecting mice were also found to upregulate the MHC class II beta chain gene - *H2-Eb1* ($p=0.02$, fold change=18.91). As discussed in Chapter 1, in H2B mice the E molecule is not expressed as EB1 cannot pair with the A alpha chain (Mathis et al., 1983). So although the genes are upregulated they will not be expressed.

The receptor 4-1BB (*Tnfrsf9*) a member of the TNF receptor family was upregulated in these cells from rejecting mice. It is a receptor for Tnfsf14 (4-1BBL) that is expressed on other T cells and binding has a co-stimulatory effect. This is essential for T cell survival. Experiments from our laboratory have shown that in low responder CBA mice, the only way to consistently elicit a male skin rejection response was to pre-treat female mice with anti-4-1BB at the same time as immunising with LPS treated male cells (Chai et al, unpublished data). Therefore it's upregulation emphasises the importance of this molecule in $CD8^+$ T cell survival and in the rejection of skin grafts.

FasL belongs to the TNF family and was found to be upregulated in rejecting $CD8^+$ $tetramer^+$ T cells (p=0.03, fold change = 45.0). Cytotoxic $CD8^+$ T cells can induce apoptosis in target cells by two methods – FasL binding to Fas and by secreting granules containing perforin and granzyme. Binding of FasL to Fas induces the formation of the Death Inducing Signalling Complex (DISC), this results in DNA fragmentation and apoptosis (Nagata and Golstein, 1995). Therefore the upregulation of this gene confirms the antigen specific $CD8^+$ T cells are mediating rejection of the skin graft.

$CD8^+$ HY-D^b*Uty* $tetramer^+$ cells from rejecting mice downregulated the gene *Il11ra* (p=0.04 Fold Change= -6.67). The IL-11 receptor α chain pairs with the gp130 subunit to form the IL-11 receptor, part of the IL-6 family known to be involved in inflammation. However, IL-6 also has crucial roles in haematopoesis (Heinrich et al., 2003). This gene was expressed in naïve cells (Average Ct= 30.38) rejecting cells albeit at a lower level (Average Ct= 35.93) suggesting it may have another role in the proinflammatory environment of the rejecting skin graft.

Cytoplasm

Caspase 3 was found to be upregulated in these cells (p=0.02, fold change=7.62). This is one of the enzymes involved in the intracellular signalling

cascade that results in apoptosis. Therefore it could be that these cells are also sensitive to apoptosis.

The gene for Cathepsin B (*Ctsb*) was also found to be upregulated in these cells (p=0.016, fold change= 13.17). This endopeptidase it thought to be involved in protecting cytotoxic $CD8^+$ T cells from their own lytic granules. Following exocytosis of granules containing perforin and granzyme B, cathepsin B is expressed on the surface of the degranulating T cell. It is thought cathepsin B can cleave perforin and therefore prevent the granule contents from 'backfiring' and killing the cell that secrete them (Balaji et al., 2002). The upregulation of the gene here is consistent with the function of these cells as antigen specific cytotoxic T cells.

$CD8^+$ HY-D^b*Uty* $tetramer^+$ cells from rejecting mice down regulated Suppressor of cytokine signalling 1 (*Socs1*). The SOCS family of genes act as negative regulators of cytokines that signal through the JAK/STAT pathway, therefore, when they are downregulated it allows increased signalling by these cytokines.

Traf1 was found to be downregulated in these rejecting $CD8^+$ T cells. TRAF1 is an adaptor protein and part of the signal transduction pathway that binds to cytoplasmic domains of TNF receptor family (such as Tnfrsf11a, see above) and links it to different signalling pathways and kinases. Through this action it mediates the activation of NFKB and JNK. As this gene was downregulated compared to naïve T cells it may make these cells less responsive to signalling through TNF receptors.

These rejecting $CD8^+$ HY-D^b*Uty*$^+$ cells also upregulated the genes for IFNγ (p=0.02, fold change= 25.58). As discussed in Chapter 1, although IFNγ can be considered as an immunomodulatory cytokine, it is mostly thought to act in a proinflammatory fashion. IFNγ has many effects on both the innate and adaptive immune response (reviewed by Schroder et al., 2004). Here, it's up regulation could in turn upregulate the class I and class II antigen processing and presentation pathways in other cells and activate the innate immune response.

Most significantly/importantly, Granzyme B was highly upregulated in these cells (p=0.01, fold change=43.27). One of the two methods by which cytotoxic $CD8^+$

T cells can kill target cells in to exocytose granules that contain perforin and granzyme. These can induce apoptosis in the target cell, by cleaving pro-apoptotic proteins in the target cell. However granzyme B is dependent on perforin for delivery to the target cell (reviewed by Trapani and Smyth, 2002). Perforin was also expressed in these cells but was not considered significant. Together with upregulation of FasL on the cell surface and the cytoplasmic enzyme cathepsin B strongly suggests that these cells are cytotoxic. Significantly, none of these genes were found to be upregulated tetramer positive $CD8^+$ T cells in mice made tolerant to male cells by i.n. peptide.

4.9 Conclusions: Gene expression in $CD8^+$ T cells during linked suppression

There were significant differences in the gene expression in antigen specific $CD8^+$ T cells taken from mice rejecting or tolerant to CFSE labelled male cells.

The most striking of these was the upregulation of genes involved in target cell killing. The genes for granzyme B, FasL and cathepsin B were only found to be upregulated in $CD8^+$ from rejecting mice. As expected, these genes were not upregulated in the same cells from tolerant mice. The most straightforward explanation is that in tolerant mice, the $CD8^+$ effector T cells are being controlled by regulatory T cells. The presence of these cells results in incomplete $CD8^+$ T cell activation, as shown here by the different pattern of gene expression following challenge with male antigen.

There were also differences in expression of chemokine receptor genes. $CD8^+$ T cells from tolerant mice upregulated the gene *Ccr10* which allows migration to gut and skin. Whereas $CD8^+$ T cells from rejecting mice upregulate chemokine receptors (*Cxcr3* and *Cx3xcr1*) and integrins (α_x *and* β_1) so they can migrate and enter the site of inflammation. This suggests that the cells from rejecting mice have a greater migratory capacity and can enter more tissues than the equivalent cells from tolerant mice. Again this provides further indications that

tolerance may be maintained by antigen specific T cells with altered migration properties. This will be explored further in chapter 3.

In the $CD8^+$ cells from tolerant mice there was upregulation of some regulatory genes that can affect DC maturation – namely Lag3 and Pd1. Even though these are $CD8^+$ cells, modifying the antigen presenting cell could make it more likely for tolerance to spread to other epitopes presented at the same time. It has been suggested that the APC acts as the link that allows tolerance to spread to other epitopes (Cobbold et al., 2006). The results presented here argue that the $CD4^+$ and $CD8^+$ T cells in tolerant mice act by modifying the maturation of the DC favouring the idea that the role of the DC here is to present antigen in a tolerogeneic fashion rather than simply bringing the regulatory T cells and effector T cell into close proximity to one another.

4.10 Gene expression in antigen specific $CD4^+$ T cells during linked suppression

In order to analyse gene expression in $CD4^+$ T cells, two methods were used to isolate these cells. Firstly, $CD4^+$ T cells were isolated by a cytokine secretion assay; secondly cells from Marilyn mice were transferred into recipient mice that then received i.n. peptide and CFSE labelled male spleen cells. Cell recoveries were very low in both cases.

4.11 IFNγ capture assay

Following male skin graft emplacement, $CD4^+$ T cells from the graft draining lymph node of tolerant mice that had been pre-treated with i.n. HY-A^b*Dby* peptide secrete IFNγ when stimulated in vitro with female cells pulsed with HY-A^b*Dby* peptide 8 and 21 days after receiving the graft (Chai et al., 2004). To isolate HY-specific $CD4^+$ T cells secreting IFNγ in response to the male graft, I planned to use the IFNγ capture assay which uses magnetic beads to isolate cells from culture that are secreting IFNγ. In these experiments female mice would received i.n. HY-A^b*Dby* peptide 10 days prior to a male skin graft. 21 days after the graft, cells from the lymph nodes and spleens of recipient mice would be restimulated *in vitro* with HY-A^b*Dby* peptide. IFNγ secreting $CD4^+$ T cells would be isolated and RNA extracted for quantitative PCR analysis.

Preliminary work was done to evaluate whether the assay would be sensitive enough to be able to detect and isolate the small numbers of cells that secrete IFNγ in response to HY-A^b*Dby* peptide. Figure 4.5 shows the gating strategy used to analyse the results all of these experiments (Figures 4.6 - 4.10). Figure 4.6 shows the results obtained when unimmunised female B6 cells were stimulated *in vitro* with HY-A^b*Dby* peptide. As expected, very few $CD4^+$ cells

secreted IFNγ, indicating that the background level of staining in this assay was approximately 0.2%.

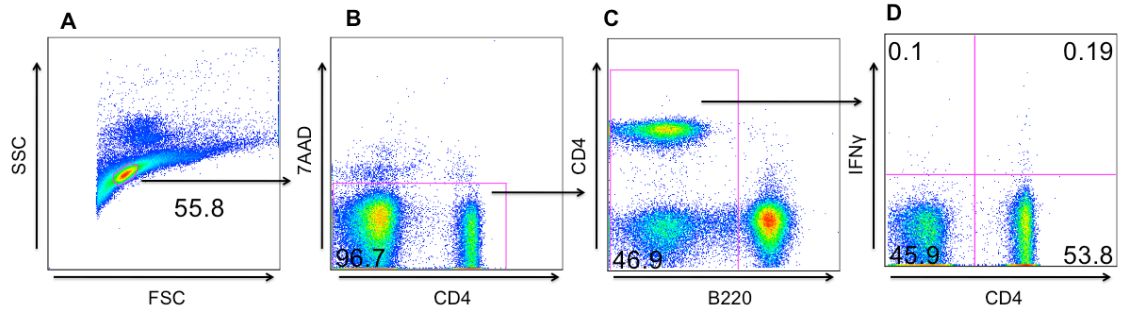


Figure 4.5: Gating strategy for IFNγ capture assay. (A) Cell preparations were gated on lymphocytes (B) 7AAD$^+$ cells (dead cells) were gated out (C) B220$^+$ cells (B cells) were gated out (D) final plot shown CD4 versus IFNγ. This strategy was used is all the experiments shown here.

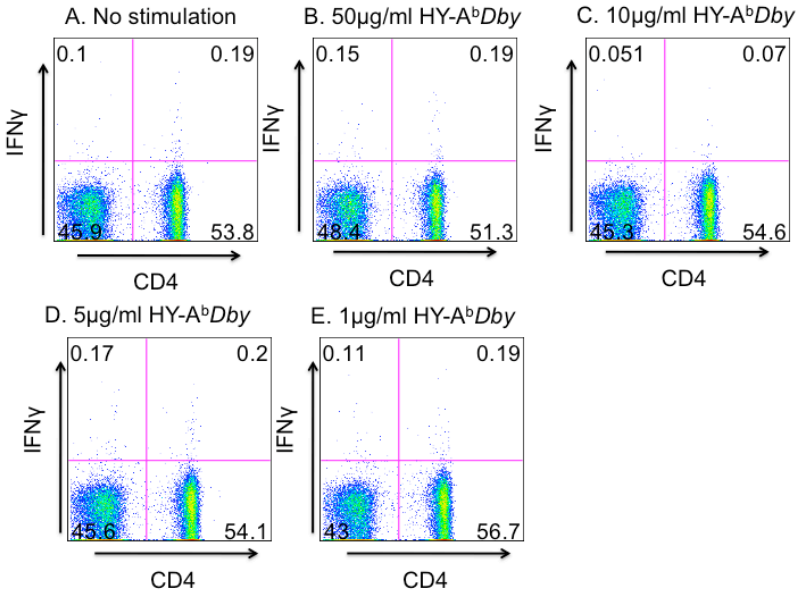


Figure 4.6 Spleen cells from unimmunised B6 female secrete little IFNγ when stimulated with HY-A^b*Dby* peptide for 3h (A) no HY-A^b*Dby* (B) 50µg/ml (C) 10µg/ml (D) 5µg/ml (E) 1µg/ml

.

It was predicted from previous results that the number of $CD4^+$ cells secreting IFNγ 21 days after male skin graft would be small and therefore it was important to test the sensitivity of the assay. In order to do this female WT B6 cells were mixed with different proportions of cells from TCR transgenic Marilyn mice that will secrete IFNγ when stimulated with the HY-A^b*Dby* peptide *in vitro*.

Figure 4.7 shows IFNγ production by B6 and Marilyn cells mixed at different ratios and stimulated with either 5 or 50ug/ml of HY-A^b*Dby* peptide for 6 or 17 hours. When Marilyn cells were stimulated with HY-A^b*Dby* peptide for 17 hours there was a lot of cell death resulting in lower cell recovery. IFNγ detection was greater than background levels when 1:10 Marilyn:WT, especially after 17h when 20% of the cells were $CD4^+IFN\gamma^+$. However at lower ratios of 1:100 or 1:1000 Marilyn:WT, which is around the expected frequency of the antigen specific $CD4^+$ T cells after male skin graft, it was difficult to distinguish $CD4^+IFN\gamma^+$ cells above background levels, even at the higher concentration of peptide and the longer incubation time. Another problem encountered with the IFNγ capture assay, was that the $CD4^-$ population also seem to secrete IFNγ (Figure 4.7). Since the peptide is MHC class II restricted it seems that this must be non-specific IFNγ production or an artefact of the capture assay.

Figure 4.8 shows the separation of IFNγ expressing cells into positive and negative fractions using magnetic beads. Although there were very few $IFN\gamma^+$ cells in the negative fraction, there was a high percentage of $IFN\gamma^-$ cells in the positive fractions, suggesting that it would be better to separate these cells by flow cytometric sorting. The highest percentage (5%) of $CD4^+$ $IFN\gamma^+$ cells were seen from a female B6 mouse that had been immunised with male spleen cells. However, the mixtures of Marilyn and B6 cells only gave a small number of $CD4^+$ $IFN\gamma^+$ cells, fewer than the cells from the B6 spleen incubated with no peptide. These results indicated that the cytokine capture assay was unlikely to be sensitive enough to detect and separate the small numbers of cells that would result from treatment with i.n. HY-A^b*Dby* peptide and male skin graft.

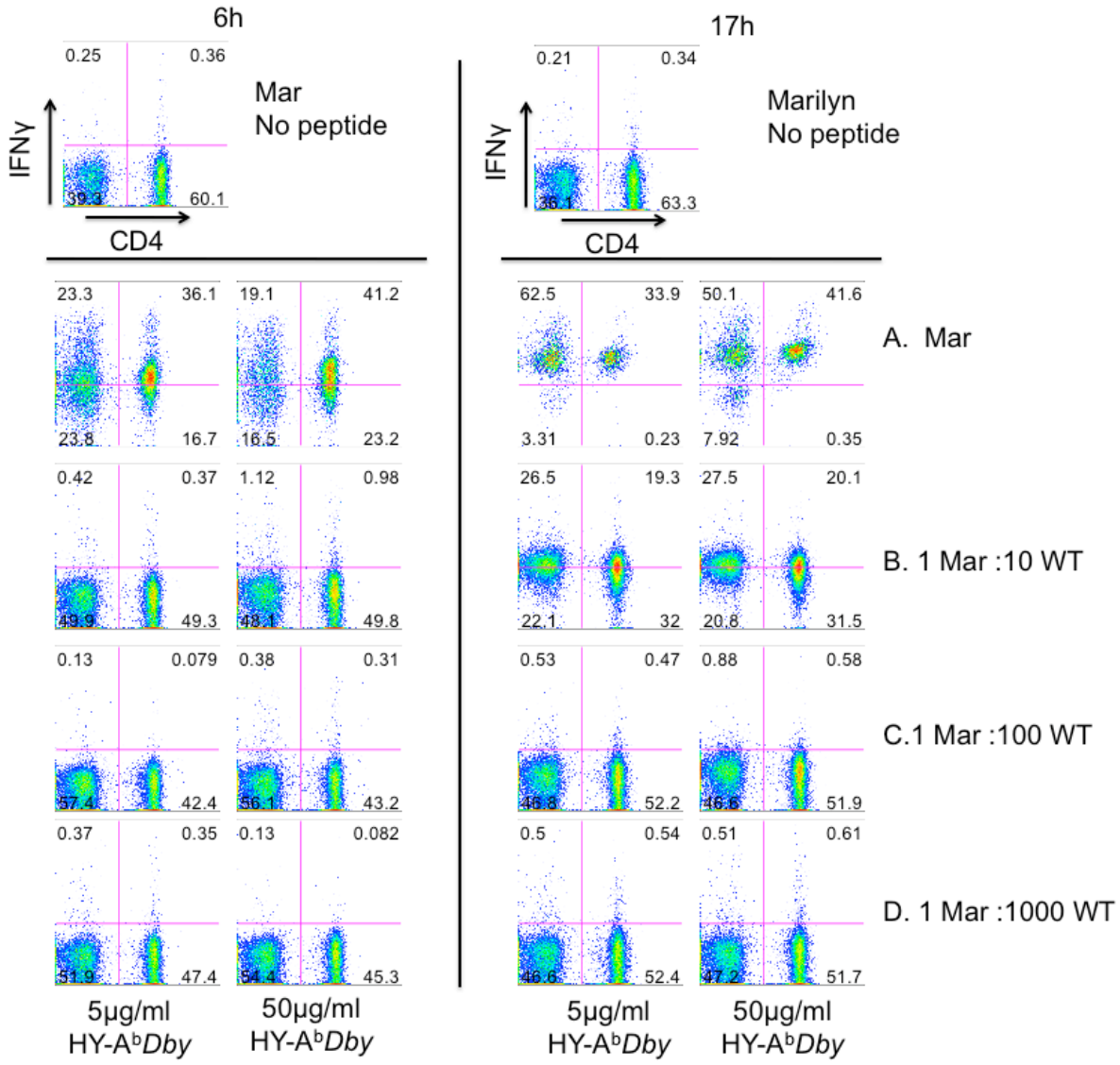


Figure 4.7 Sensitivity of the Cytokine capture assay: IFNγ production by Marilyn cells mixed in different ratios with WT B6 spleen cells. Cells were stimulated with either 5 or 50ug/ml HY-A^b*Dby* peptide for 6 (left panel) or 17 hours (right panel). Mar=Marilyn cells.

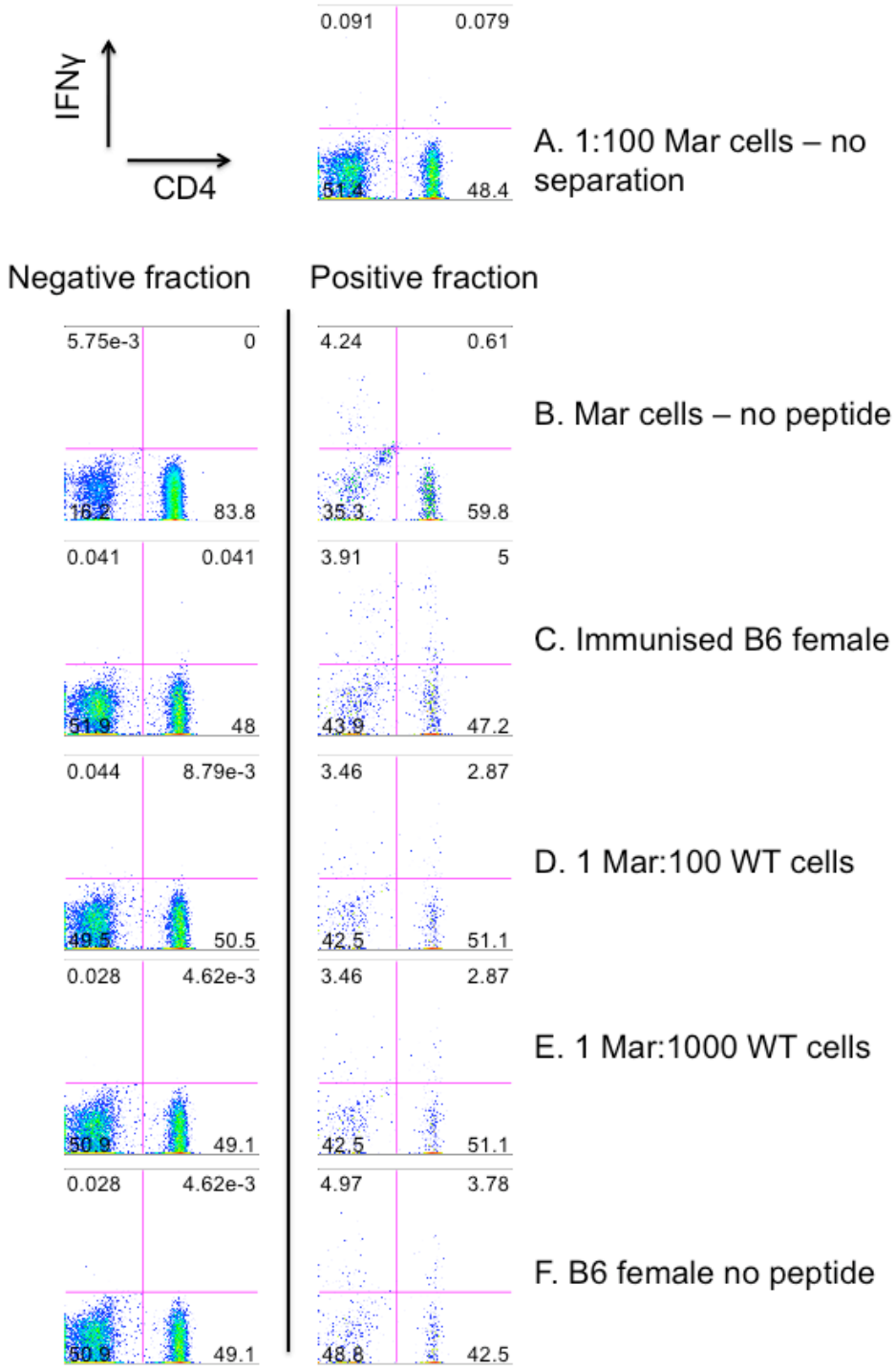


Figure 4.8 WT B6 spleen cells were mixed at different ratios with Marilyn cells and stimulated with 20ug/ml HY-A^{b}*Dby* for 4h. (A) Before separation. Cells were then separated by IFNγ microbeads into negative fraction (left panel) and positive fraction (right panel). (B) Marilyn cells incubated without peptide (C) Cells from B6 female immunised i.p. with male cells 7 days earlier. (D) Mixture of 1 Mar:100 WT cells (E) Mixture of 1 Mar to 1000 WT cells (F) WT B6 spleen incubated without peptide. Mar=Marilyn cells

It has been shown that similar numbers of $CD4^+IFN\gamma^+$ were detected 5 days after i.n. HY-A^b*Dby* peptide +LPS and 21 days after male skin graft (Chai et al., 2004). To further investigate if the IFNγ capture assay would be sensitive enough to detect and isolate these cells, I firstly examined cells from the graft draining LN and spleen of mice 5 days after i.n HY-A^b*Dby* peptide+LPS treatment (Figure 4.9). In the lymph nodes 7% of the cells were $CD4^+IFN\gamma^+$. Although this represents a large percentage, the actual cell number was small, and it was therefore difficult to extract a sufficient quantity of high quality RNA for Q-PCR analysis.

Figure 4.10 compares the yields of unseparated and bead purified IFNγ-secreting cells from lymph nodes and spleen from a WT B6 mouse given i.n. HY-A^b*Dby* peptide+LPS 5 days before. The aim was to determine whether cells should be bead-purified before flow cytometry sorting. Enriching for the $IFN\gamma^+$ population before sorting would mean the flow cytometry sorting was quicker but would also mean that there would be very few cells to set the compensation and adjust the machine, and this could lead to significant loss of cells.

Spleen cells that had not been restimulated with peptide gave a background of 0.17% $CD4^+IFN\gamma^+$ cells after microbead separation. When the cells were restimulated with peptide, the percentage increased to 1.61% $CD4^+IFN\gamma^+$ cells. However, when lymph node cells were restimulated *in vitro* with peptide and separated with microbeads only 0.23% were $CD4^+IFN\gamma^+$ cells, marginally above the background level of staining. In the unseparated samples a low percentage of cells were positive but the actual number of cells is larger. This indicates that it would give better yields to separate the $CD4^+IFN\gamma^+$ cells by flow cytometric sorting and omit the microbead enrichment step.

Following i.n. HY-A^b*Dby* peptide and skin graft or immunisation with male splenocytes only small number of antigen specific $CD4^+$ cells could be detected. Therefore, it was decided that the IFNγ capture assay would not be a suitable method to isolate these cells. There were additional problems with the assay including non-specific $CD4^-$ T cells becoming $IFN\gamma^+$ after *in vitro* stimulation with peptide, although these would have been separated in the flow cytometric

sorting. There were also concerns that the *in vitro* stimulation might alter the gene expression in both the tolerant and rejecting antigen specific T cells. Therefore another approach was tried.

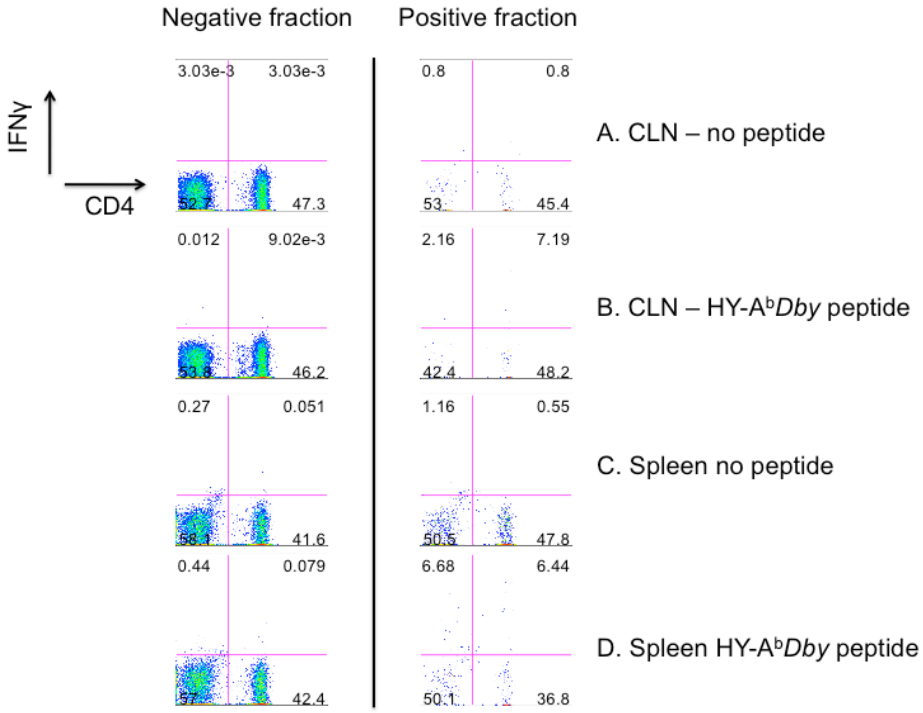


Figure 4.9 CD4$^+$IFNγ$^+$ cells from cervical lymph nodes (CLN) and spleen of a mouse given i.n. HY-A^b*Dby* + LPS. 5 days after i.n. peptide cells from the CLN were incubated with either (A) no peptide or (B) 20ug/ml HY-A^b*Dby* peptide for 4 hours. (C and D) Cells from the spleen were treated in the same way. Cells were then separated into positive (right panel) and negative (left panel) fractions using microbeads.

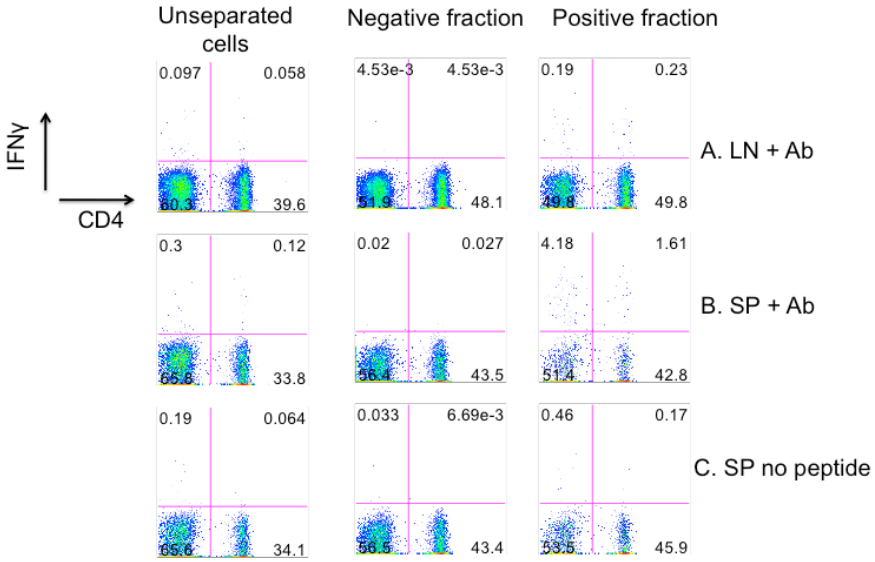


Figure 4.10 B6 mice were given i.n. Ab peptide+LPS and five days later spleen and lymph nodes were taken and restimulated with 20ug/ml HY-A^b*Dby* peptide for 4 hours. (A) Lymph nodes restimulated with HY-A^b*Dby* peptide (B) Spleen restimulated with HY-A^b*Dby* peptide (C) Spleen cells with no peptide restimulation. Unseparated and both the positive and negative fractions after microbead separation are shown.

4.12 Marilyn transfer and immunisation with male cells

Having shown that adoptive transfer of HY-A^{b}*Dby* TCR transgenic T cells prior to i.n. peptide treatment did not compromise tolerance induction to a male skin graft (Fig 3.10) I decided use adoptive transfer of these cells in peptide-tolerised mice and this time challenge with male haematopoietic cells to try to isolate enough cells to enable me to examine gene expression in 'tolerant' CD4+ T cells at the time of induction of linked suppression. In these experiments there was surprisingly no apparent delay in the rejection of CFSE labelled male cells as seen by the change in M:F ratio in peptide-tolerised mice (Figure 4.11). This is in contrast to the results obtained with skin grafting (Fig 3.10) and of using haematopoietic cells as targets following i.n. peptide treatment in the absence of adoptive transfer of Marilyn cells (Fig 4.2). This rapid clearance of male cells may be because the haematopoietic cells are more sensitive targets and following the transfer of large number (120 x10^{6}) of antigen specific Marilyn T the amount i.n. peptide is not sufficient to cause tolerance.

Despite the apparent lack of tolerance shown by survival of the male cells, the *in vivo* expansion of the Marilyn CD4^{+} T cells was very low in the mice given peptide (Figure 4.12B). Again this is in stark contrast to the untreated animals where considerable expansions of the adoptively transferred the Marilyn CD4^{+} cells were seen (Figure 4.12A). After sorting 115,000 Marilyn cells were recovered from 2 mice given no treatment. Only 15,000 Marilyn cells could be recovered from 4 mice given peptide treatment. RNA was extracted from these cell pools, reverse transcribed and gene expression was analysed by quantitative PCR array. There was only enough RNA to run on one plate and this was with a smaller amount (40ng/plate) than in previous experiments and the recommended amount (100ng/plate). The gene expression was compared to that of sorted naïve CD4^{+} T cells. The results of gene expression analysis is shown for tolerant mice in Table 4.3 and in rejecting mice in Table 4.4.

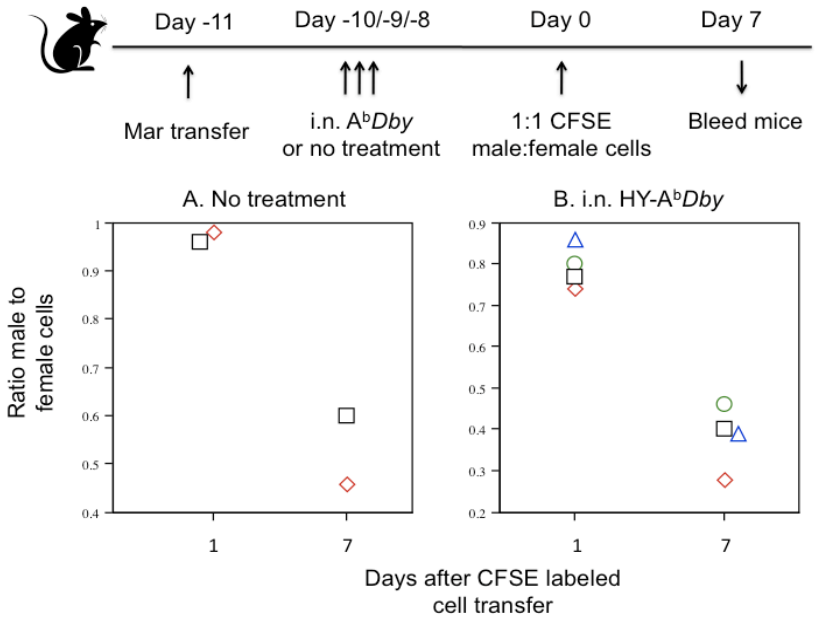


Figure 4.11. Induction of tolerance to haematopoietic cells grafts following adoptive transfer of Marilyn cells. 120x10^6 Marilyn cells were transferred into each female recipient that was then given either (A) no treatment (2 mice) or (B) 3 doses of i.n. A^b*Dby* peptide (4 mice). 10 days after peptide treatment CFSE labelled male and female spleen cells were transferred into these recipient mice. The ratio of male to female cells in the peripheral blood was analysed by flow cytometry 1 day and 7 day after transfer.

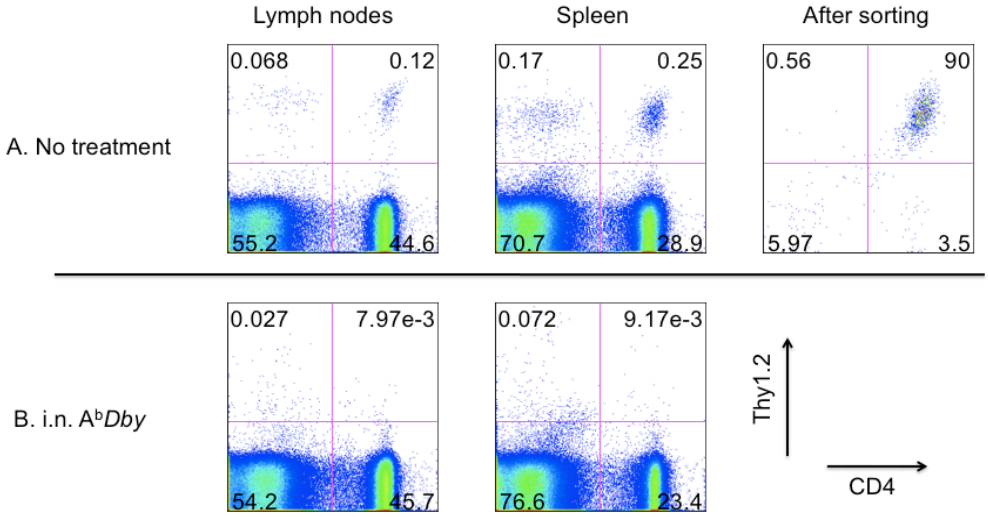


Figure 4.12 Expansion of CD4$^+$Thy1.2$^+$ Marilyn T cells after *in vivo* transfer male cells. Marilyn cells were adoptively transferred to female recipients, which then received (A) no treatment (B) 3x i.n. HY-A^b*Dby*. 10 days after peptide treatment the mice were given CFSE labelled male and female spleen cells. 10 days after CFSE labelled cell transfer, the Marilyn cells were sorted by flow cytometry on the basis of CD4$^+$ Thy1.2$^+$ staining. Care was taken to gate out CFSE positive cells. Examples from the lymph nodes and spleens of two mice are shown. There were too few cells to show post-sorting purity in the mice treated with i.n. HY-A^b*Dby*.

Table 4.4 (Following page) Genes significantly changed in CD4$^+$ Thy1.2$^+$ Marilyn cells after i.n. HY-A^b*Dby* peptide at the time of induction of linked suppression. Thy1.2$^+$ Marilyn cells were transferred into Thy1.1$^+$ female recipient mice. These mice were then given 3x i.n. HY-A^b*Dby* peptide and 10 days later were given CFSE labelled male and female spleen cells. Up to 10 days after the CFSE labelled cells CD4$^+$ Thy1.2$^+$ were isolated by flow cytometric sorting. RNA was extracted and analysed by quantitative PCR array. 93 genes were found to be up or down regulated compared to RNA preparations from naïve CD4$^+$ T cells. Genes that were downregulated are shown in blue

Rank	Gene Name	P-Value	Fold Change	Ave Ct naïve	Tolerant Ct
1	Pax5	0.0008	36792.8	40.00	27.98
2	Cd80	0.0017	1516.9	40.00	32.58
3	IgK-C	0.0022	557.9	36.69	30.74
4	Il17f	0.0023	698.4	39.37	33.10
5	Mmp2	0.0025	476.0	40.00	34.25
6	Klra1	0.0026	428.9	40.00	34.40
7	Ccr10	0.0031	2528.0	39.68	31.40
8	Il23r	0.0041	114.0	40.00	36.32
9	Src	0.0048	71.6	40.00	36.99
10	IgD-C1v1	0.0055	74.7	32.71	29.66
11	Aire	0.0055	226.2	39.76	35.01
12	Cd14	0.0063	142.8	39.72	35.60
13	IgA-C2v1	0.0068	268.7	34.42	29.33
14	Tcra-C	0.0069	-322.1	23.65	35.14
15	Il1rl1	0.0071	102.5	36.47	32.91
16	Cd4	0.0080	-285.1	25.56	36.86
17	Lifr	0.0080	58.9	39.71	36.87
18	Ly6a	0.0081	-1510.8	25.50	39.03
19	Itgal	0.0087	-495.0	27.02	39.12
20	Ncam1	0.0090	41.9	37.30	34.96
21	Ccl11	0.0092	47.3	36.61	34.15
22	Ifngr1	0.0094	-136.5	27.50	37.79
23	Pparg	0.0098	34.1	33.31	31.34
24	Tcf7	0.0102	-289.3	23.88	35.20
25	Fcgr4	0.0102	293.8	33.57	28.31
26	Cd3eap	0.0104	-1.0	31.77	34.98
27	Mmp13	0.0106	38.1	32.58	30.40
28	Tyk2	0.0106	-1.7	29.04	32.96
29	Tollip	0.0109	-115.6	29.52	39.57
30	Il21r	0.0112	-398.1	26.87	38.55
31	B2m	0.0115	-72.3	21.54	30.87
32	Map2k2	0.0118	-2.7	33.17	37.74
33	Casp8	0.0120	-113.7	26.86	36.87
34	Stat1	0.0120	-98.2	24.22	33.98
35	Ifnar1	0.0121	-2.0	28.69	32.83
36	Calr	0.0122	-97.1	26.05	35.82
37	Psmb8	0.0124	-1.2	28.30	31.69
38	Lcp2	0.0124	-15.9	26.31	33.50
39	Cd3e	0.0127	-60.7	24.30	33.42
40	Nfil3	0.0129	3.7	39.31	34.47
41	Ifi35	0.0130	-75.7	25.25	32.74
42	Ccr7	0.0131	-19.6	25.29	34.25
43	Jak1	0.0132	-54.8	25.41	32.81
44	Ptprc	0.0133	-18.3	25.52	32.84
45	Diablo	0.0135	-6.6	31.08	33.58
46	Il4ra	0.0136	-12.5	26.05	32.28
47	Cd28	0.0136	-8.2	28.16	34.19
48	Tgfb1	0.0137	-7.1	14.94	22.59
49	Rn18s	0.0138	-22.0	27.34	32.86
50	Daxx	0.0139	-103.3	29.99	33.11
51	Rel	0.0139	1.0	34.11	30.68
52	Vav1	0.0144	-8.8	27.35	37.68
53	Stat3	0.0144	-151.2	29.35	36.77
54	Mapk1	0.0144	-18.8	30.73	32.56
55	Traf1	0.0148	-7.3	29.27	31.78
56	Tap1	0.0149	-71.7	33.90	37.40
57	Itgam	0.0150	-1.3	26.81	32.90
58	Fyn	0.0150	-7.5	30.00	34.03
59	Cd69	0.0155	-18.6	25.53	32.92
60	Fos	0.0156	-19.0	33.67	36.52
61	Lif	0.0157	1.2	25.64	31.78
62	Prkcd	0.0161	25.3	38.30	34.93
63	Cd9	0.0163	-13.1	29.35	34.63
64	Ly75	0.0164	-4.4	27.62	33.89
65	Mapk14	0.0173	-1.4	27.23	37.31
66	Cd55	0.0176	-125.1	32.06	34.45
67	Il2rg	0.0180	-56.1	29.28	34.30
68	Tfrc	0.0182	-3.8	28.51	34.30
69	Tnfrsf1b	0.0186	-12.5	28.14	34.92
70	Ccr2	0.0196	8.4	39.29	36.19
71	Lat	0.0198	-29.2	32.16	35.97
72	Fas	0.0203	-1.6	31.69	39.02
73	Mapk3	0.0209	-18.3	26.55	32.53
74	Csf3	0.0211	208.8	39.02	37.25
75	H2-Ab1	0.0220	4.1	35.89	32.03
76	Hmgb1	0.0222	-7.7	27.23	31.65
77	Csf1r	0.0228	4.3	36.94	34.85
78	Ccl3	0.0243	6.0	37.04	33.74
79	Itgb2	0.0249	-55.5	30.15	35.85
80	Abl1	0.0253	-6.0	31.11	38.40
81	Tnfrsf13b	0.0254	-18.2	27.09	36.40
82	Il2ra	0.0284	-15.3	29.64	33.64
83	Prf1	0.0288	-2.1	28.70	37.37
84	Tapbp	0.0297	-52.6	32.02	35.81
85	Ly96	0.0304	-1.8	29.20	36.14
86	Irak1	0.0309	-15.0	29.20	38.75
87	Icam1	0.0315	-99.1	27.33	31.23
88	Nfkbia	0.0347	-98.4	29.48	32.29
89	Cxcl10	0.0348	-1.0	28.83	34.55
90	Il1b	0.0384	26.8	39.02	36.87
91	IgL-C1	0.0386	21.6	37.62	31.20
92	Csf3r	0.0449	123.8	36.10	33.51
93	Ciita	0.0462	1.3	36.72	34.60

Rank	Gene Name	P-Value	Fold Change	Ave Ct naïve	Rejecting Ct
1	IgK-C	0.0010	1470.40	36.69	25.08
2	Il21	0.0016	740.19	40.00	29.36
3	Pax5	0.0026	175.41	40.00	31.44
4	Ifnb1	0.0027	155.35	40.00	31.62
5	Tlr2	0.0037	82.73	39.58	32.10
6	Ms4a1	0.0038	72.17	40.00	32.72
7	Cd80	0.0041	63.46	40.00	32.91
8	Cr2	0.0047	49.20	40.00	33.27
9	Tnfrsf13c	0.0053	32.90	39.94	33.81
10	Il17a	0.0054	38.08	40.00	33.64
11	Ccr7	0.0055	-18.77	25.25	28.49
12	Cd40	0.0057	34.90	40.00	33.77
13	Ccbp2	0.0058	20.90	39.77	34.31
14	Oas2	0.0073	-58.89	27.59	32.36
15	Mmp2	0.0074	22.93	40.00	34.38
16	Tal1	0.0078	21.58	40.00	34.46
17	Lep	0.0084	19.24	40.00	34.63
18	Cd14	0.0090	38.56	39.72	33.24
19	Hrh4	0.0091	28.49	39.84	33.87
20	Sele	0.0099	-14.70	33.81	36.64
21	Cd44	0.0104	-84.24	34.09	39.26
22	IgD-C1v1	0.0105	14.79	32.71	27.72
23	Il5	0.0112	13.43	40.00	35.15
24	Cd2ap	0.0113	-7.30	28.17	30.05
25	Ccr10	0.0115	37.47	39.68	33.23
26	Plau	0.0126	18.65	39.40	34.00
27	Entpd1	0.0127	140.82	36.94	28.22
28	Foxp3	0.0128	8.66	32.44	28.25
29	Pecam1	0.0131	-7.62	26.63	28.55
30	IgA-C2v1	0.0137	31.63	34.42	28.17
31	Il6st	0.0142	-10.32	27.76	30.08
32	Stat1	0.0154	-7.13	24.22	26.03
33	Ccl3	0.0157	43.31	33.35	26.52
34	Tnfaip6	0.0158	9.03	40.00	35.72
35	Il23r	0.0161	8.87	40.00	35.75
36	Il4ra	0.0165	-6.65	27.51	29.23
37	Ccr2	0.0165	29.10	33.66	27.47
38	Nfil3	0.0168	13.24	32.71	27.82
39	Stat5b	0.0176	-5.48	28.05	29.49
40	Casp3	0.0180	11.99	31.66	26.92
41	Lag3	0.0183	155.99	33.95	24.94
42	IgL-C1	0.0184	196.80	39.02	29.45
43	Il10	0.0191	581.25	37.10	26.03
44	Aicda	0.0191	7.41	40.00	36.01
45	Ccl2	0.0192	9.25	39.46	35.11
46	Il17f	0.0193	7.03	39.37	35.45
47	Lifr	0.0196	11.59	39.71	34.98
48	Ifit2	0.0199	-9.19	27.54	29.65
49	C1qa	0.0209	-14.98	32.09	34.85
50	Tnfrsf4	0.0223	5.03	32.73	29.33
51	Daxx	0.0227	-5.41	27.50	28.89
52	Lta	0.0229	-4.98	29.83	31.13
53	IgM-C2v1	0.0259	-4.18	25.64	26.69
54	Cd55	0.0273	-6.77	27.23	28.87
55	Bcl2	0.0288	-4.80	29.13	30.33
56	Lef1	0.0295	-4.87	28.14	29.35
57	Il7r	0.0303	-9.93	27.74	29.90
58	Atm	0.0309	-7.79	27.98	29.83
59	Cx3cr1	0.0309	55.32	38.55	30.60
60	Ccl4	0.0334	380.75	36.20	25.30
61	Socs3	0.0345	-5.69	26.90	28.32
62	Slamf1	0.0364	-6.00	28.86	30.33
63	Ly6a	0.0438	-6.73	25.50	27.07
64	Gata3	0.0463	-4.28	29.47	30.50

Table 4.5 Genes significantly changed in $CD4^+$ $Thy1.2^+$ Marilyn cells at the time of graft rejection. $Thy1.2^+$ Marilyn cells were transferred into $Thy1.1^+$ female recipient mice. 10 days later these mice were given CFSE labelled male and female spleen cells. Up to 10 days after the CFSE labelled cells $CD4^+$ $Thy1.2^+$ were isolated by flow cytometric sorting RNA was extracted and analysed by quantitative PCR array. 64 genes were found to be significantly up or down regulated compared to RNA preparations from naïve $CD4^+$ T cells. Genes that were found to be downregulated are shown in blue.

4.13 Discussion: Gene expression in HY peptide specific $CD4^+$ T cells

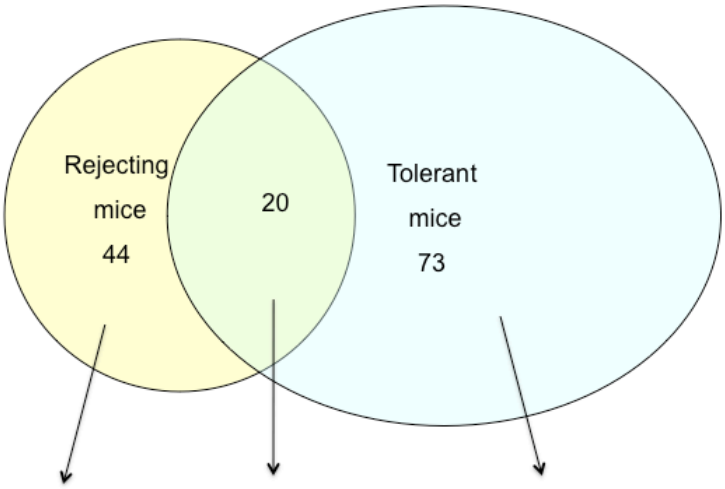
The results of this gene analysis must be interpreted with caution as they do not reach the same standards as the other results described in this thesis. As the cell recoveries were so small only a limited amount of RNA was available to analyse. This meant that instead of running three plates with three different pools of RNA from different animals, as in previous experiments, the RNA from 4 different mice was pooled and run on one plate only. As there was such a small amount the plate was run with only 40ng RNA. This led to two problems:

- The total amount of RNA used was less than half the recommended amount therefore the expression of many genes was undetectable (i.e. they had a Ct of >40 cycles). It may have been that these genes were expressed but because of limiting amounts of recovered RNA, the level was too low to be detected in this particular experiment. Therefore if the genes were expressed in the naïve preparations (Ct of <40) the change in expression was deemed to be significant, giving an artificially large number of genes that were significantly downregulated in the $CD4^+$ T cells from tolerant mice. Therefore genes that were undetectable in the treated group were not included in the results table (but can be seen in appendix II).
- High P values. Because the experiment could not be repeated in triplicate due to the low cell recovery, the variation in the data was artefactually low. This led to an artificially high P value and the number of genes that were found to be significantly changed was also artefactually high. Therefore genes where the difference in Ct value between the naïve and treated samples was less than one were also omitted.

In the tolerant vs naïve experiment these exemptions reduced the number of changed genes from 235 to 93 genes (a full list of 235 genes is given in

appendix II, the edited list of 93 genes is shown in table 4.4). In the rejecting vs naïve group these conditions reduced the number of candidate genes from 75 to 64 genes (a full list of 75 genes is also given in appendix 2, the edited list of 64 genes is shown in table 4.5).

Taking these caveats into account, the data still provide some information on gene expression in these tolerant antigen specific $CD4^+$ T cells after exposure to male antigen, this is summarised in Figure 4.13.



Rejecting		Both	Tolerant	
Aicda	*Il21*	*Ccl3*	*Abl1*	*Jak1*
Atm	*Il5*	*Ccr10*	*Aire*	*Klra1*
Bcl2	*Il6st*	*Ccr2*	*B2m*	*Lat*
C1qa	*Il7r*	*Ccr7*	*Calr*	*Lcp2*
Casp3	*Lag3*	*Cd14*	*Casp8*	*Lif*
Ccbp2	*Lef1*	*Cd55*	*Ccl11*	*Lifr*
Ccl2	*Lep*	*Cd80*	*Cd28*	*Ly75*
Ccl4	*Lifr*	*Daxx*	*Cd3e*	*Ly96*
Cd2ap	*Lta*	*IgA-C2v1*	*Cd3eap*	*Map2k2*
Cd40	*Ms4a1*	*IgD-C1v1*	*Cd4*	*Mapk1*
Cd44	*Oas2*	*IgK-C*	*Cd69*	*Mapk14*
Cr2	*Pecam1*	*IgL-C1*	*Cd9*	*Mapk3*
Cx3cr1	*Plau*	*Il17f*	*Ciita*	*Mmp13*
Entpd1	*Sele*	*Il23r*	*Csf1r*	*Ncam1*
Foxp3	*Slamf1*	*Il4ra*	*Csf3*	*Nfkbia*
Gata3	*Socs3*	*Ly6a*	*Csf3r*	*Pparg*
Hrh4	*Stat5b*	*Mmp2*	*Cxcl10*	*Prf1*
Ifit2	*Tal1*	*Nfil3*	*Diablo*	*Prkcd*
Ifnb1	*Tlr2*	*Pax5*	*Fas*	*Psmb8*
IgM-C2v1	*Tnfaip6*	*Stat1*	*Fcgr4*	*Ptprc*
Il10	*Tnfrsf13c*		*Fos*	*Rel*
Il17a	*Tnfrsf4*		*Fyn*	*Rn18s*
			H2-Ab1	*Src*
			Hmgb1	*Stat3*
			Icam1	*Tap1*
			Ifi35	*Tapbp*
			Ifnar1	*Tcf7*
			Ifngr1	*Tcra-C*
			Il1b	*Tfrc*
			Il1rl1	*Tgfb1*
			Il21r	*Tnfrsf13b*
			Il2ra	*Tnfrsf1b*
			Il2rg	*Tollip*
			Irak1	*Traf1*
			Itgal	*Tyk2*
			Itgam	*Vav1*
			Itgb2	

Figure 4.13 Similarities in gene expression in antigen specific $CD4^+$ T cells during the linked suppression phase. 93 genes were found to be changed in antigen specific $CD4^+$ T cells from mice made tolerant by i.n. HY-A^b*Dby* peptide. 64 genes were found to be changed in the same cells from rejecting mice. 20 of these genes were the same (shown in table). Genes that were downregulated are shown in blue. Genes that were upregulated are shown in black.

Of the 74 genes whose expression was changed in tolerant cells only 19 were upregulated compared to naïve T cells. This is probably still due to the fact that amount of RNA extracted from these cells was low. Even though these genes were detectable in the tolerant samples (i.e. they had a Ct which as less than 40), it was less than in the naïve. For example *Rn18S* had a Ct of 27.34 in naïve sample but only 32.86 in preparations from peptide treated mice.

The genes that were upregulated do not include genes that are typical of regulatory T cells such as *FoxP3*, *Ctla4* and *Pdcd1.* It was also different to the gene expression during the initial tolerance induction phase. For example, the expression of both *FoxP3* and *Lag3* was undetectable in cells taken during the induction of linked suppression. Furthermore, the expression of *Pdcd1, Ctla4* and *IFN*γ was not different to that of naïve $CD4^+$ T cells. This suggests that these cells are no longer actively suppressing the proliferation of effector T cells. Since the $CD4^+$ Marilyn cells did not expand following injection of CFSE-labelled male cells, it seems unlikely that they are indirectly competing with effector T cells for cytokines and co-stimulatory ligands. Therefore these results support the hypothesis that it is the DCs that have previously interacted with the HY-specific $CD4^+$ T cells expressing the regulatory phenotype seen at day 5 that are now presenting antigen in a tolerogeneic fashion to effector and naïve $CD4^+$ and $CD8^+$ T cells and thus inhibiting proliferation. In contrast, *FoxP3* and *Lag3* were found to be upregulated in the rejecting $CD4^+$ T cells following immunization with male spleen cells, suggesting that these cells are expressing regulatory phenotype in order to control the ongoing immune response in the rejecting mice.

Of the small number of genes that were upregulated specifically in tolerant $CD4^+$ T cells there were some genes involved in cell migration for example: *Ccl3*, *Ccr10, Ccr2* and *Ccl11*. This is especially interesting as CCL3 has a role in the maturation of DCs (Trifilo and Lane, 2004). Again this could point to T cells modifying the antigen presentation by DCs to a tolerogeneic phenotype. CCR10 and CCR2 are expressed on activated T cells and are involved in T cell homing. This suggests that these cells have altered migration – this is will be examined further in chapter 3.

4.14 Conclusions: Gene expression in HY specific $CD4^+$ T cells during linked suppression

Due to problems resulting from the very low numbers of antigen specific $CD4^+$ T cells that could be isolated during the linked suppression phase, the analysis of gene expression is not as rigorous as in the previous sections. However, there was no evidence of typically regulatory genes being expressed, and therefore little to suggest that these cells are regulatory. This supports the hypothesis that DCs are the mediators of linked suppression and they present antigen in a tolerogeneic fashion that prevents T cell proliferation. This would mean that donor and third party antigens are presented at the same time by the same DC.

Further work is needed to confirm these findings. This could be done either by repeating the Marilyn cell transfers until a sufficient number of cells are isolated or more recently a class II tetramer for HY-D^b*Uty* has been described (Verginis et al., 2008). This could be used to isolated WT antigen specific $CD4^+$ T cells after transfer of CFSE labelled male and female cells.

Chapter 5: The migration of antigen specific T cells following i.n. peptide and male skin grafting

5.1 Introduction

The gene analysis experiments reported in the two previous chapters have shown that when tolerance is induced by i.n. HY-A^b*Dby* there are changes in the expression of several genes involved in cell migration. For example, during the tolerance induction phase (i.e. before skin grafting) following i.n. HY-A^b*Dby* peptide +LPS, the antigen specific CD4$^+$ T cells upregulated the gene *Cxcr3.* This was not seen when peptide was given alone. Similarly, after skin grafting, during the linked suppression phase, antigen specific CD8$^+$ T cells from rejecting mice upregulated the *Cxcr3* gene. Again this was not seen in the antigen specific CD8$^+$ T cells from tolerant mice: instead these cells upregulated *Ccr10.* After skin grafting, antigen specific CD4$^+$ T cells from tolerant mice upregulated the genes *Ccr10* and *Ccr2*.

This suggests that i.n. HY-A^b*Dby* peptide may lead to changes in the migratory properties of antigen specific T cells that may prevent them migrating to and entering the site of the graft where they could recognise and respond to antigen. This chapter will investigate this by tracking antigen specific cells both *in* and *ex vivo* following tolerance induction or no treatment in female mice following male skin graft.

5.2 CBA mice do not reject male cells or male skin grafts after transfer of CD8$^+$ T cells from C6 TCR transgenic mice

GFP/C6 double transgenic mice were kindly made available for me to use by Professor Julian Dyson. These mice are on a CBA background and their CD8$^+$ T cells are specific for the class I restricted peptide TENSGKDI (HY-K^k*Smcy*) and express GFP. I planned to transfer these T cells into female CBA (H2^k) recipient mice, which would then be given either no treatment or i.n. administration of the H2-E^k restricted peptide REEALHQFRSGRKPI (HY-E^k*Dby*) followed by a male graft. Recipient tissues would be isolated at different time points after grafting and antigen specific CD8$^+$ T cells would be identified by the expression of GFP.

However, it is known that CBA mice are poor responders to HY despite the fact that they can recognise two class I restricted peptides (HY-K^k*Smcy* and HY-D^k*Smcy*) and the MHC class II restricted peptide (HY-E^k*Dby*) described above. This is at least in part because HY-E^k specific CD4$^+$ T cells are sensitive to regulation by CD4$^+$ CD25$^+$ regulatory T cells and therefore do not provide sufficient help for the CD8$^+$ T cell response (Robertson et al., 2007). Initial experiments were done to investigate whether CBA female mice could reject either a male skin graft or male spleen cells following adoptive transfer of transgenic C6 cells. It was thought that transfer of large numbers of C6 cells might boost the response and lead to rejection. C6 cells (10x10^6/mouse) were adoptively transferred into 11 recipient mice. Five of these mice then received a male skin graft. None rejected the male skin graft by day 60 after grafting (data not shown).

The other 6 mice received male and female CFSE labelled cells. 7 days after cell transfer there was little killing of the male cells (Figure 5.1). Even after transfer of large numbers of C6 transgenic cells these mice were unable to reject male skin grafts or consistently reject male haematopoietic cells. Therefore it wasn't possible to use this model to investigate the migration properties of tolerant versus effector HY specific CD8$^+$ T cells.

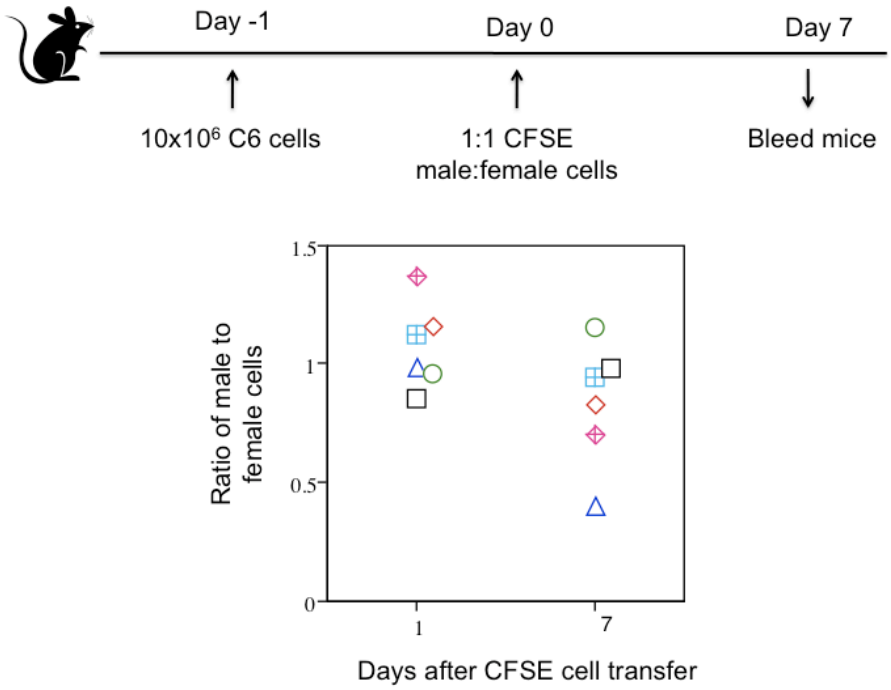


Figure 5.1 Female CBA mice do not consistently reject CFSE labelled male cells after transfer of C6 cells. $10x10^6$ C6 cells were transferred into each recipient, one day a later a mixture of male and female CBA spleen cells labelled with different concentrations of CFSE were transferred. 7 days later the mice were bled and the ratio of male to female (high to low CFSE labelled cells) was determined by flow cytometry Only 3 of the 6 mice partially rejected the CFSE labelled male spleen cells.

5.3 Migration of Matahari T cells following skin grafting

Since the CBA model did not result in consistent male graft rejection, an alternative method was developed using cells from TCR transgenic $Rag^{-/-}$ Matahari mice. The $CD8^+$ T cells from these mice are specific for the HY-D^b*Uty* peptide and are $Thy1.2^+$. These cells were transferred into $Thy1.1^+$ B6 ($H2^b$) mice which are high responders to HY.

Figure 5.2 shows the results of transfer of $0.72x10^6$ $CD8^+V\beta8.3^+$ Matahari cells on day -1 before skin graft. At 22 days after a male or female skin graft, the $Thy1.2^+V\beta8.3^+$ Matahari T cells could not be detected in the graft draining lymph nodes, the spleen or the skin. As discussed in chapter 2, 22 days after male skin graft was chosen as a suitable time point because differences in the number of cells secreting IL-2 and IFNγ from rejecting and tolerant mice were readily detectable at this time (Chai et al., 2004). However, for this experiment 22 days may have been too long after the Matahari transfer to track these cells. In the case of the female graft the cells may have apoptosed since they would not have come into contact with male antigen, and therefore would not have received any survival signals. Following the male graft the cells would be expected to have expanded in response to male antigen and then possibly undergone antigen induced cell death. Alternatively, it may be that too few cells were transferred to detect. Further experiments at earlier time points are needed to confirm if this is the case. Nevertheless, in the case of the male graft, a clear population of endogenous $CD8^+$ cells infiltrating the skin graft can be seen (Figure 5.2.i) but there are no detectable $CD8^+V\beta8.3^+$ Matahari cells (Figure 5.2.i and j).

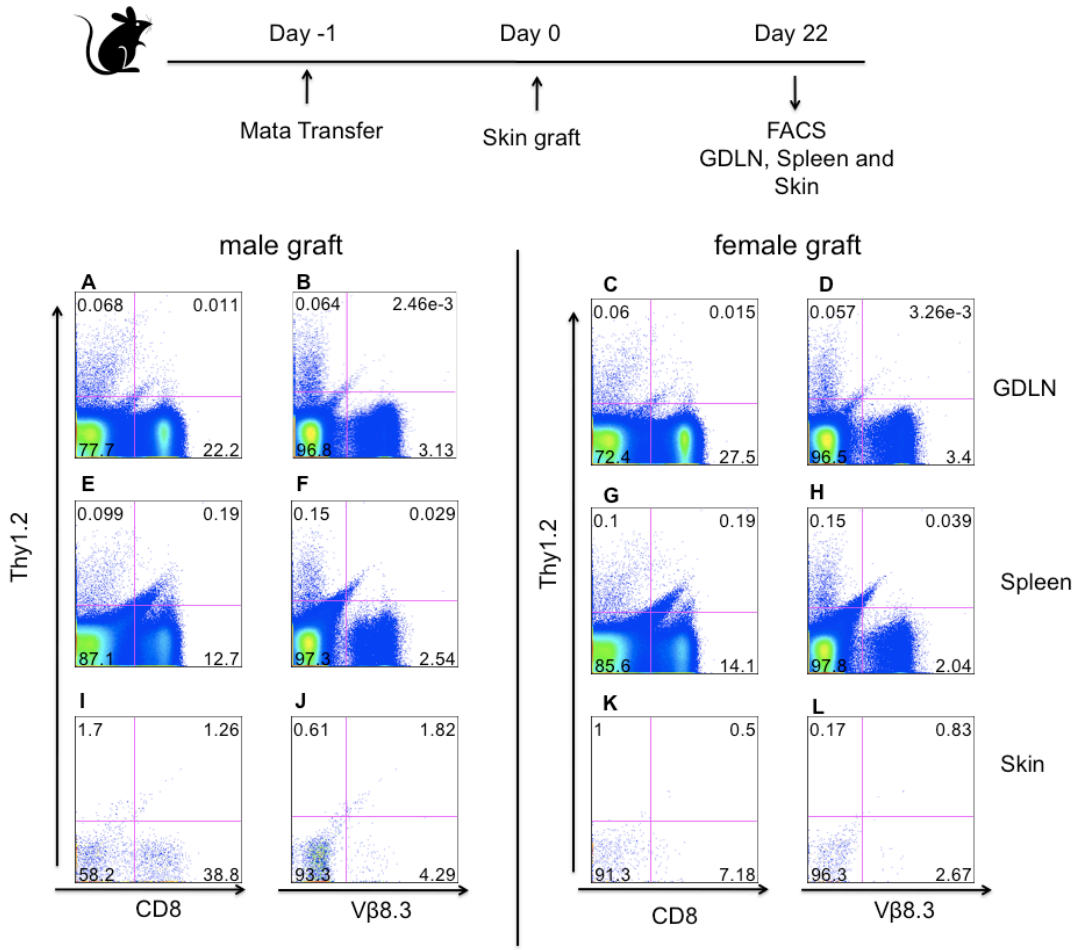


Figure 5.2 22 days after male skin graft, Thy1.2^{+}Vβ8.3^{+} HY-D^{b}*Uty* specific Matahari cells could not be detected in the graft draining lymph nodes, the spleen or the skin. Matahari cells were isolated and pooled from the spleen and mesenteric lymph nodes of 4 Matahari mice, washed and transferred into 4 recipient Thy1.1 mice. Each mouse received approx 100x10^{6} cells. Flow cytometry analysis showed that only 8% of the lymphocytes transferred were CD8^{+}Vβ8.3^{+} this is equivalent to 0.72x10^{6} per mouse. A day later two of the mice received a male skin graft and two of the mice received a female skin graft. At day 22 spleen, skin and LN from these mice were analysed by flow cytometry. Plots are representative of the two mice in each group.

In order to look at the effect of i.n. HY-A^b*Dby* peptide, Matahari cell were then transferred into 4 recipient Thy1.1 mice. A day later one mouse received 3xHY-A^b*Dby* peptide. 10 days later all mice received either a male or female graft. At day 22 the draining and non draining lymph nodes, spleen and the skin were analysed by flow cytometry, plots of CD8$^+$ versus D^b*Uty* tetramer are shown in Figure 5.3 and Figure 5.4.

Again no Thy1.2$^+$ CD8$^+$ cells could be seen in any of the tissues 22 days after grafting by flow cytometry (data not shown). This is probably because it is too long after the transfer of these cells into recipient mice.

Figure 5.3 column A shows the results of tetramer staining from a female Thy1.1 mouse receiving a male skin graft only, with no additional Matahari cells. A small percentage (0.068%) of CD8$^+$ Tetramer$^+$ cells were detected in the graft draining lymph nodes and a similar percentage (0.042%) in the non-graft draining lymph nodes and the spleen (0.067%). CD8$^+$ T cells could be seen to infiltrate into the skin, this represents about 5% of the lymphocytes in the digested skin, of these the percentage of tetramer positive cells infiltrating into the graft was quite high, 0.14%.

Column B shows that when Matahari cells were transferred before a male skin graft the number of CD8$^+$ tetramer$^+$ cells in the graft draining and non-draining lymph nodes and spleen increased to 0.089%, 0.12% and 0.17% respectively. The cells showing this increase in percentage of tetramer positive cells were Thy1.2 negative (data not shown). Thus the transfer of the Matahari cells seems to have boosted the number of endogenous CD8$^+$ HY-D^b*Uty* specific T cells. There was also an increased percentage of both single positive CD8$^+$ T cells (11.1%) and CD8$^+$ HY-D^b*Uty*$^+$ tetramer positive T cells (1.07%) in the skin.

Column C shows that this increased number of antigen specific T cells is not seen when the recipient mice are given a female skin graft. As expected there were very few CD8$^+$ HY-D^b*Uty* specific T cells in the draining (0.012%), non-draining lymph nodes (0.013%) and spleen (0.013%). There was a similar percentage of CD8$^+$ T cells infiltrating the skin, compared with the male graft without Matahari transfer. However, none of these cells was tetramer positive, their presence may just be due to the inflammation caused by surgery.

Column D shows the effect of i.n. HY-A^{b}*Dby* peptide given 10 days before the male skin graft. CD8^{+} HY-D^{b}*Uty* specific T cells were clearly present in the draining lymph node (0.049%) 22 days after the graft. However, very few tetramer positive cells (0.011%) were detected in the non-draining lymph nodes and the spleen (0.014%). The percentages of tetramer positive cells were comparable to those seen in mice receiving a female graft (Column C) rather than those receiving a male graft, particularly with transfer of Matahari cells (see Columns A and B). The level of CD8 T cell infiltration of the skin was 5%, similar no Matahari transfer and female skin graft (Columns A and C) and no CD8^{+} HY-D^{b}*Uty* tetramer positive cells could be detected. Again, the CD8^{+} HY-D^{b}*Uty* specific T cells in these mice were of recipient origin as they were Thy1.2 negative (data not shown).

Tissues taken from the mice in this experiment were frozen and analysed by Microscopy (Figure 5.4).

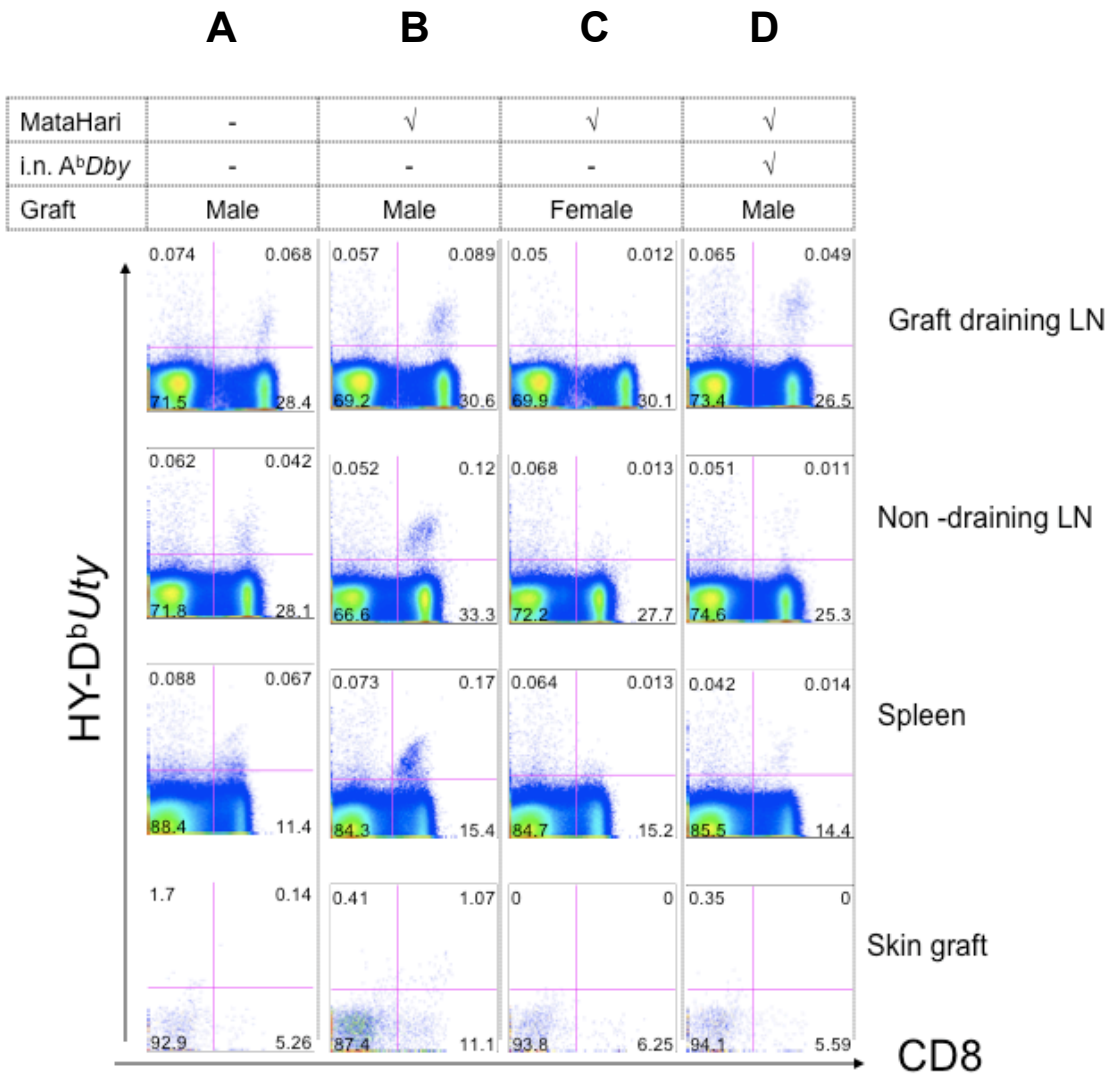


Figure 5.3 Thy1.2^{+} Matahari cells were pooled from the spleen and mesenteric lymph nodes of Matahari mice, washed and transferred into 4 recipient Thy1.1 mice. Each mouse received approx 1x10^{6} CD8^{+}Vβ8.3^{+}cells. A day later one mouse received 3xHY-A^{b}*Dby* peptide. 10 days later all mice received either a male or female graft. At day 22 the draining and non draining lymph nodes, spleen and the skin were analysed by flow cytometry, plots of CD8^{+} versus D^{b}*Uty* tetramer are shown.

Figure 5.4 shows sections of the draining lymph node (DLN), non-draining lymph node (NDLN) and skin stained for CD8 and Thy1.2.

Figure 5.4 A (male skin graft alone) shows some faint $CD8^+$ T cells in the draining lymph nodes (indicated with arrows). In the NDLN and skin the bright green stain is an artefact (indicated with arrow and 'a'). However there are some faintly staining $CD8^+$ T cells.

Figure 5.4 B (Matahari transfer and male skin graft) shows no detectable $CD8^+Thy1.2^+$ T cells in the DLN. There are a few double positive cells in the NDLN and the skin.

Figure 5.4 C (Matahari transfer and female skin graft) has some bright Thy1.2 staining in the DLN and NDLN but this is an artefact as shown by the overlay. There were very few $CD8^+$ T cells infiltrating the skin.

Figure 5.4 D (Matahari transfer, i.n. peptide and male skin graft) the Thy1.2 staining of the DLN is bright but does not seem to overlay with the DAPI and CD8 staining. The NDLN is overexposed but the Thy1.2 staining here clearly does not overlap with the CD8 staining. In the skin no $CD8^+$ cells or $Thy1.2^+$ cells can be seen.

Overall the microscopy supports the flow cytometry data. Fluorescent microscopy is a more sensitive technique than flow cytometry and therefore may have been able to pick up small numbers of Thy1.2 positive cells that were too low to be detected by flow. However, like the flow data, very few Thy1.2 positive cells were found in any of the tissues suggesting that the increase in antigen specific $CD8^+$ T cells after Matahari transfer are recipient cells.

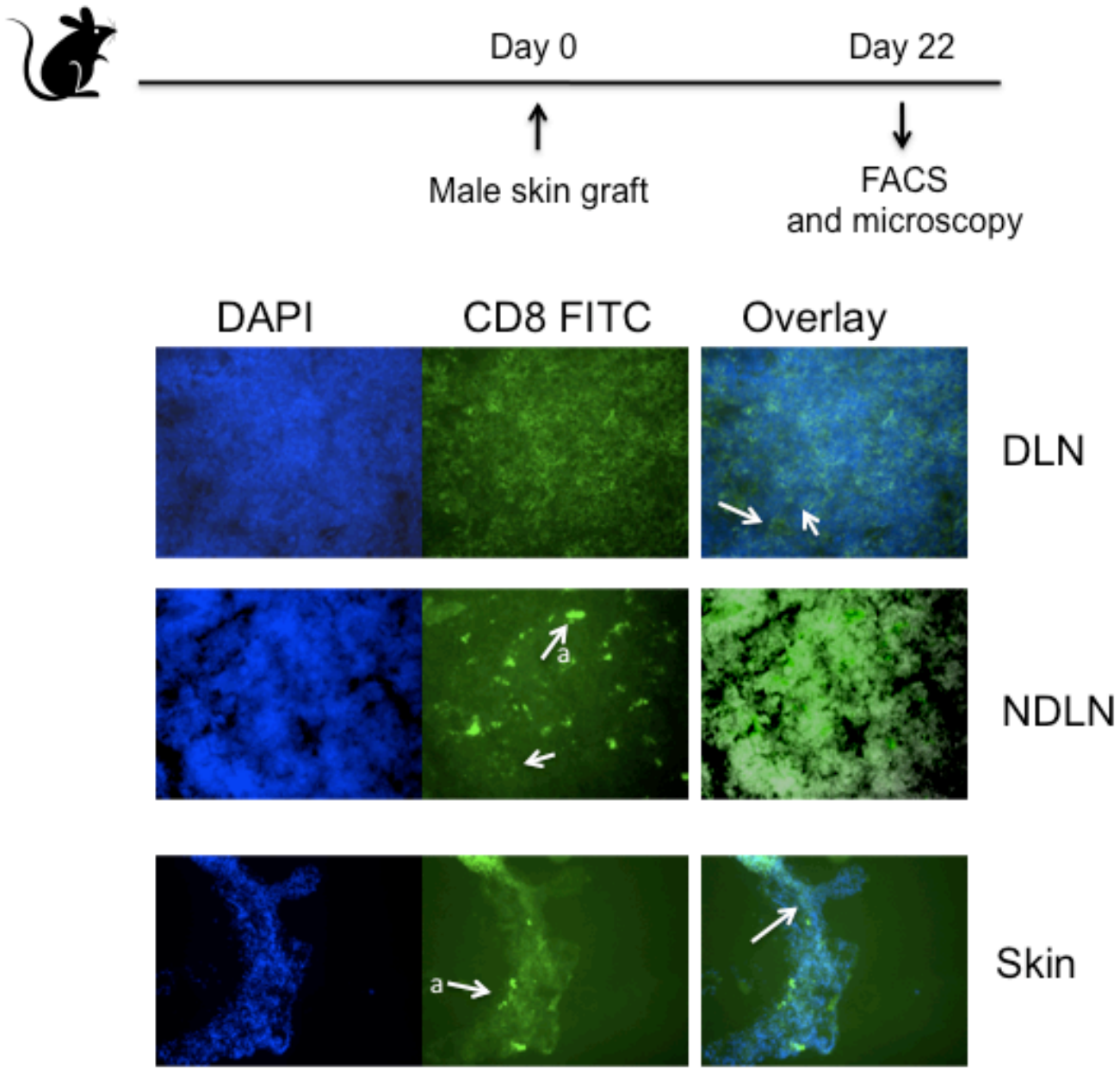


Figure 5.4 Thy1.2^{+} Matahari cells were isolated and pooled from the spleen and mesenteric lymph nodes of Matahari mice, washed and transferred into 4 recipient Thy1.1 mice. Each mouse received approx 1x10^{6} CD8^{+}Vβ8.3^{+} cells. A day later one mouse received 3xHY-A^{b}*Dby* 10 days later all mice received either a male or female graft. At day 22 the draining and non draining lymph nodes and the skin were frozen and microscopy, staining of DAPI, CD8 FITC, Thy1.2 PE (B-D) and overlaps are shown (right panels).

(A) No Matahari cell transfer, no i.n. peptide, male skin graft. Microscopy of lymph nodes x40, skin x20.

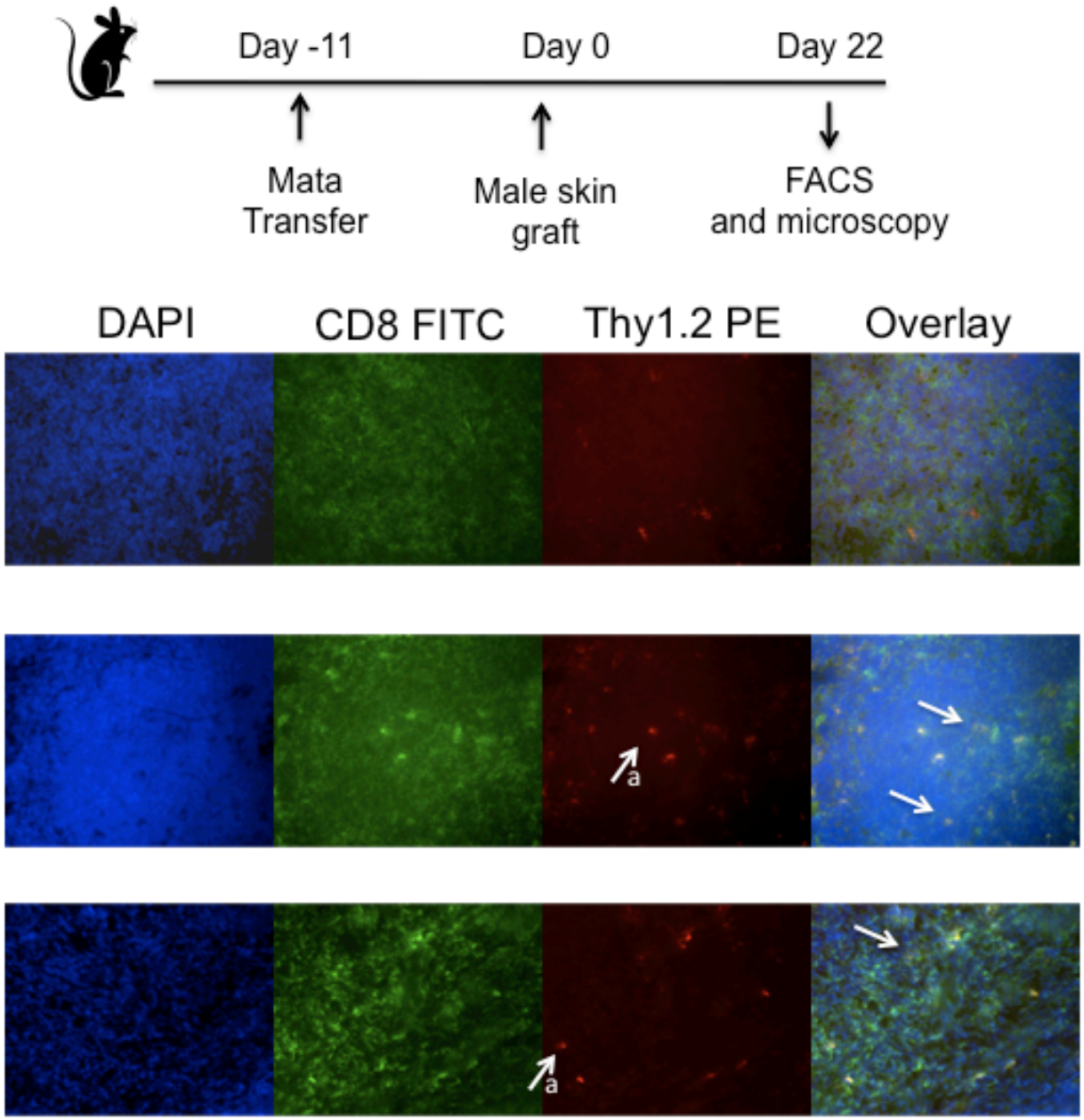


Figure 5.4 B $1x10^6$ $CD8^+V\beta8.3^+$ Matahari cells were transferred – no i.n. HY-A^b*Dby* peptide – male skin graft. Microscopy of Lymph nodes x40, skin x20.

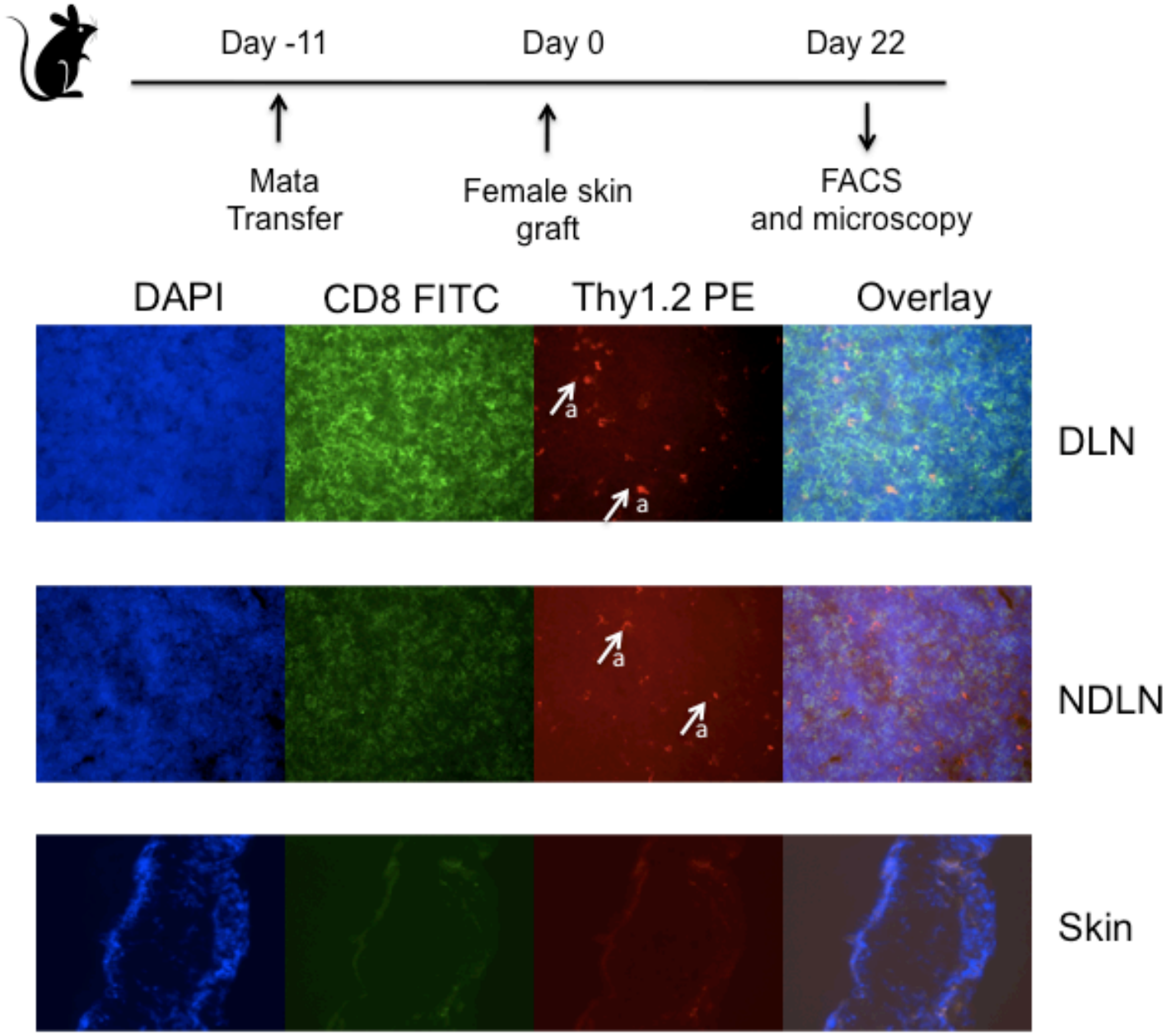


Figure 5.4 C $1x10^6$ $CD8^+V\beta8.3^+$ Matahari cells were transferred, no i.n. peptide, female graft
Microscopy of lymph nodes x40, skin x20

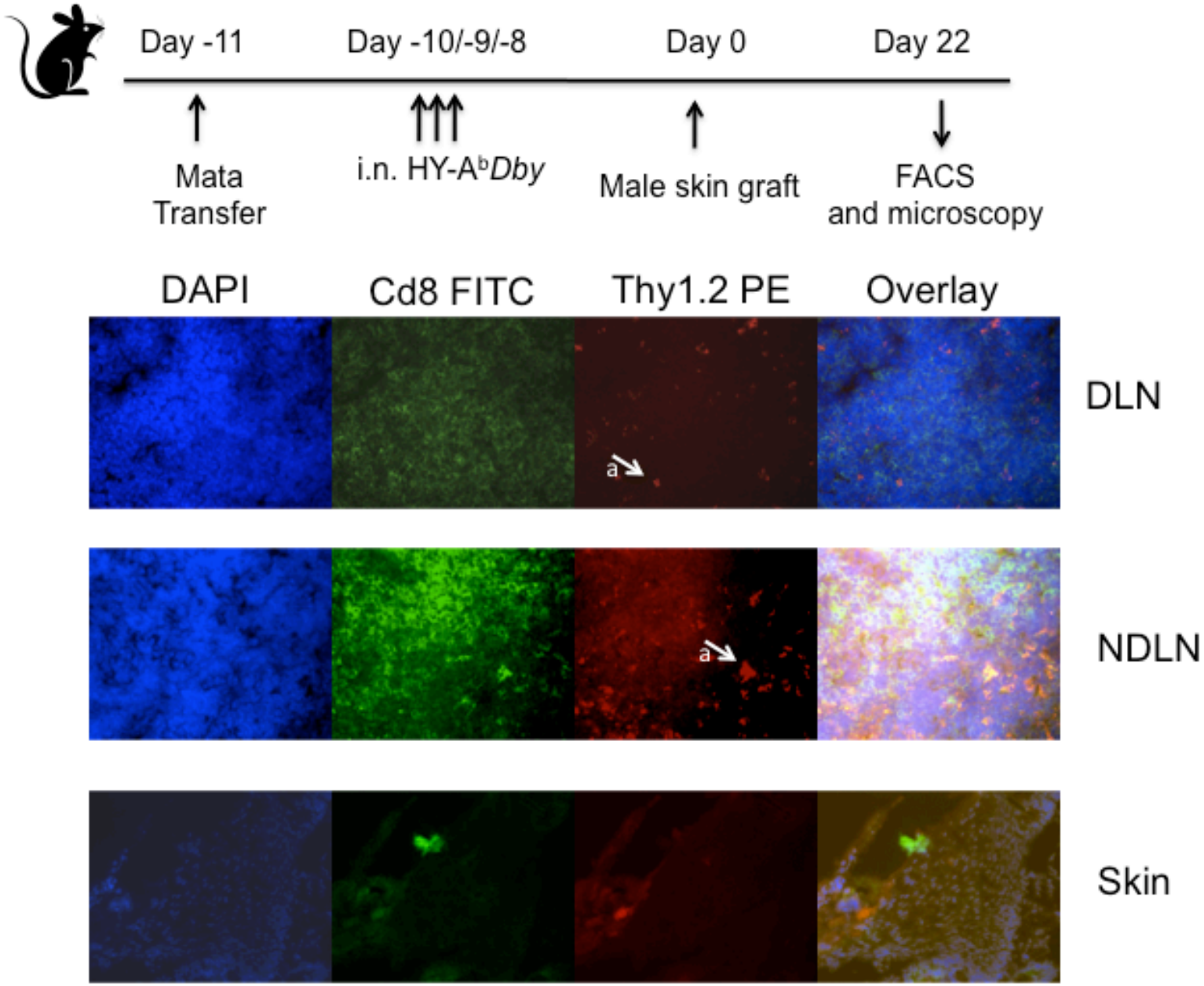


Figure 5.4 D
$1x10^{6}$ $CD8^{+}V\beta8.3^{+}$ Matahari cells were transferred, 3x i.n. HY-A^{b}*Dby* peptide, male skin graft Microscopy of lymph nodes x40 skin x20

5.4 Expression of chemokine receptors on CD8$^+$ HY-D^b*Uty* $^+$ T cells

To further investigate the CD8$^+$ HY-D^b*Uty*$^+$ T cells in the graft draining lymph nodes of mice made tolerant with i.n. HY-A^b*Dby* peptide before male skin grafting, I looked at the expression of chemokine receptor molecules. Using multicolour flow cytometry analysis it was possible to stain cells isolated from the graft draining lymph nodes and spleen for CD8, HY-D^b*Uty* tetramer, CXCR4, CCR7, CXCR3 and LFA-1. These molecules were chosen as they provide information on the trafficking and the possible function of these cells. CXCR4 and CCR7 are involved in constitutive trafficking to the lymph nodes and are expressed by naïve and central memory T cells. CXCR3 allows T cells to migrate in response to inflammation and is expressed by activated effector memory T cells. And the intergrin LFA-1 is constitutively expressed by all T cells but upregulated under inflammatory conditions (Mora and von Andrian, 2006).

In this experiment Matahari cells were adoptively transferred into female recipients in order to boost the number of tetramer positive cells in the draining lymph nodes after skin grafting. The female recipient mice then received either i.n. HY-A^b*Dby* or no treatment. Both groups of mice were given a male skin graft 10 days after the i.n. HY-A^b*Dby*. The lymph nodes and spleens were excised 24 days after skin grafting and pooled for flow cytometry analysis. Figure 5.5 (A) shows the percentage of CD8$^+$ HY-D^b*Uty* tetramer$^+$ cells in the draining and non-draining lymph nodes and the spleen. In all the tissues there were fewer tetramer positive cells in the mice that received i.n. HY-A^b*Dby* peptide. A small percentage of CD8$^+$ HY-D^b*Uty* tetramer$^+$ cells could be seen in the draining lymph node (0.026%). This was not as high as in the previous experiment where CD8$^+$ HY-D^b*Uty*$^+$ cells were only found in the graft draining lymph node, but it was nevertheless higher than the percentage of these cells in the non-draining lymph nodes and the spleen.

Figure 5.5(B) shows histograms of cells gated on the CD8$^+$ HY-D^b*Uty*$^+$ cells 5.5(A). Cells from the same tissue from mice that had received peptide treatment or no treatment are overlaid.

The expression of CXCR4 was very low in the graft draining lymph nodes of both control and peptide treated mice. However, in the non-draining lymph nodes there was an additional population of cells that expressed high levels of CXCR4 in both the peptide treated and control groups. In the spleen the control group showed mostly low-level expression with only a small proportion of the cells expressing CXCR4. In contrast most of the cells from the peptide treated mice expressed high levels of CXCR4.

A different pattern was seen with CCR7 expression. Considering the control mice, in all tissues the expression of CCR7 was quite low and only a small proportion of cells expressed high CCR7 levels. In the peptide treated mice the levels of CCR7 were somewhat higher than in control mice in the non-draining lymph nodes and spleen. Strikingly the levels of CCR7 expression were much higher in the graft draining lymph nodes compared with the controls.

In contrast to the gene expression experiments, CXCR3 expression was similar in all three tissues in both peptide treated and control mice. In the non-draining lymph node and the spleen the control mice expressed slightly higher levels of CXCR3.

LFA-1 expression was higher in control than peptide treated mice in all tissues. Comparing between tissues, the levels of LFA-1 were highest in the graft draining lymph nodes, slightly less in the non-draining lymph nodes and lowest in the spleen. This was the case for both the control and peptide treated mice. In addition, in the draining lymph node the control mice showed a single peak of high expression whereas the peptide treated mice had two peaks. The majority of cells expressed quite high levels of LFA-1 and a small population expressed very high levels of LFA-1.

The histograms shown in this figure are gated on a small number of cells, so appear jagged, but overall the results show that there does seem to be a difference in either the number of cells expressing these chemokine receptors or on their the level of expression.

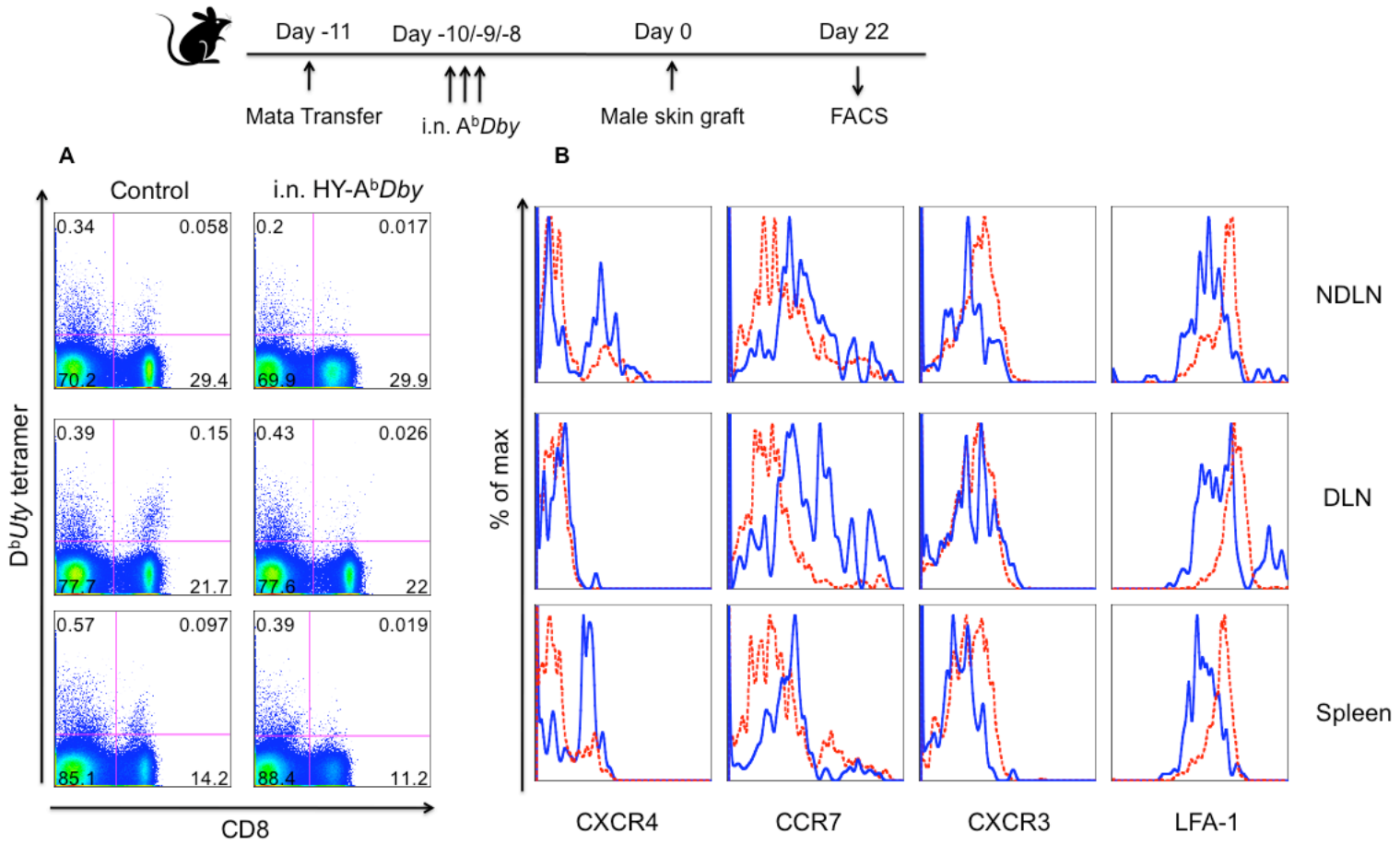


Figure 5.5 Chemokine receptor molecules expressed by CD8$^+$ Tetramer$^+$ cells. Thy1.2$^+$ cells were isolated and pooled from the spleen and mesenteric lymph nodes from 10 Matahari mice, washed and transferred into 8 recipient Thy1.1 mice. Each mouse received approx 14x10^6 Matahari cells. flow cytometry showed 2% of the lymphocytes transferred were CD8$^+$Vβ8.3$^+$ this is equivalent to 2.4x10^4 per mouse. Over the next three days 4 recipient mice received i.n. HY-A^b*Dby*, 10 days later all mice received a male graft. At day 22 the draining and non-draining lymph nodes and the skin harvested, the same tissues from different mice were pooled. Flow cytometry plots of CD8$^+$ versus D^b*Uty* tetramer are shown (left panel) and histograms of CXCR4, CCR7, CXCR3 and LFA-1 are shown on the right. The histograms are gated on CD8$^+$ HY-D^b*Uty* tetramer$^+$ cells. Blue lines from peptide treated mice, red dotted line from control mice.

5.5 *In vitro* migration of CD8$^+$ HY-D^b*Uty* tetramer$^+$ cells

To further examine the *In vitro* migratory capacity of these cells, the experiment was repeated and at day 22 after skin grafting, cells from the graft draining lymph nodes were pooled, counted and following enrichment for CD8$^+$ T cells, 2x10^6 cells were layered onto transwells that had been coated with female endothelial cells. The plates were incubated for 24 hours and the cells were carefully harvested from the upper and lower chamber, stained and analysed by flow cytometry (Figure 5.6).

In all cases the percentage of tetramer positive CD8$^+$ T cells that had migrated into the lower chamber was higher when the cells were from the untreated controls compared with cells from peptide treated mice, even when the number of tetramer positive CD8$^+$ T cells in the upper chamber was similar.

In control mice, as expected, there was a greater number of CD8$^+$ HY-D^b*Uty*$^+$ cells in the graft draining lymph nodes and the spleen in both the upper and the lower chambers compared to peptide treated mice. The overall number (upper and lower chambers) of CD8$^+$ HY-D^b*Uty*$^+$ in the non-draining lymph node was similar in both control and peptide treated mice. However, more CD8$^+$ HY-D^b*Uty*$^+$ cells from the graft draining lymph nodes of control mice migrated from the upper chamber through the endothelial cell layer to the lower chamber compared with the same cells from peptide treated mice.

This migration seen in cells from the graft draining lymph node seems to have been preferential for the CD8$^+$ HY-D^b*Uty*$^+$ cells since the percentage increased by ten fold, from 0.35% in the upper chamber to 3.78% in the lower chamber. In comparison, fewer cells from the graft draining lymph nodes of peptide treated mice migrated to the lower chamber. At the outset, there was a smaller percentage of CD8$^+$ HY-D^b*Uty*$^+$ cells in the upper chamber, 0.28%, and this was enriched to 1.21% in the lower chamber. This represents a fourfold increase in the percentage of antigen specific CD8$^+$ T cells.

There were very few $CD8^+$ HY-D^b*Uty*$^+$ cells in the non-draining lymph nodes of both control and peptide treated mice. Cells from the non-draining lymph nodes of control mice migrated though the endothelial cell layer the lower chamber, and the percentage of $CD8^+$ HY-D^b*Uty*$^+$ cells increased from 0.12% in the upper chamber to 0.97% in the lower chamber, an eightfold increase. The migration of the $CD8^+$ HY-D^b*Uty*$^+$ cells from peptide treated mice resulted in a percentage increase from 0.12% in the upper to 0.34% in the lower chamber. This represented an almost threefold increase and was again lower than the cells from control mice.

Finally, cells from the spleen of untreated control mice tetramer positive $CD8^+$ T cells preferentially migrated to the lower chamber. The percentage of tetramer positive cells increased from 0.23% in the upper chamber to 4.7% in the lower chamber, a twentyfold increase. There were very few tetramer positive T cells in the spleens of peptide treated mice and furthermore very few of these cells migrated to the lower chamber. The percentages increased from 0.056% in the upper chamber to 0.27% in the lower chamber, a fivefold increase.

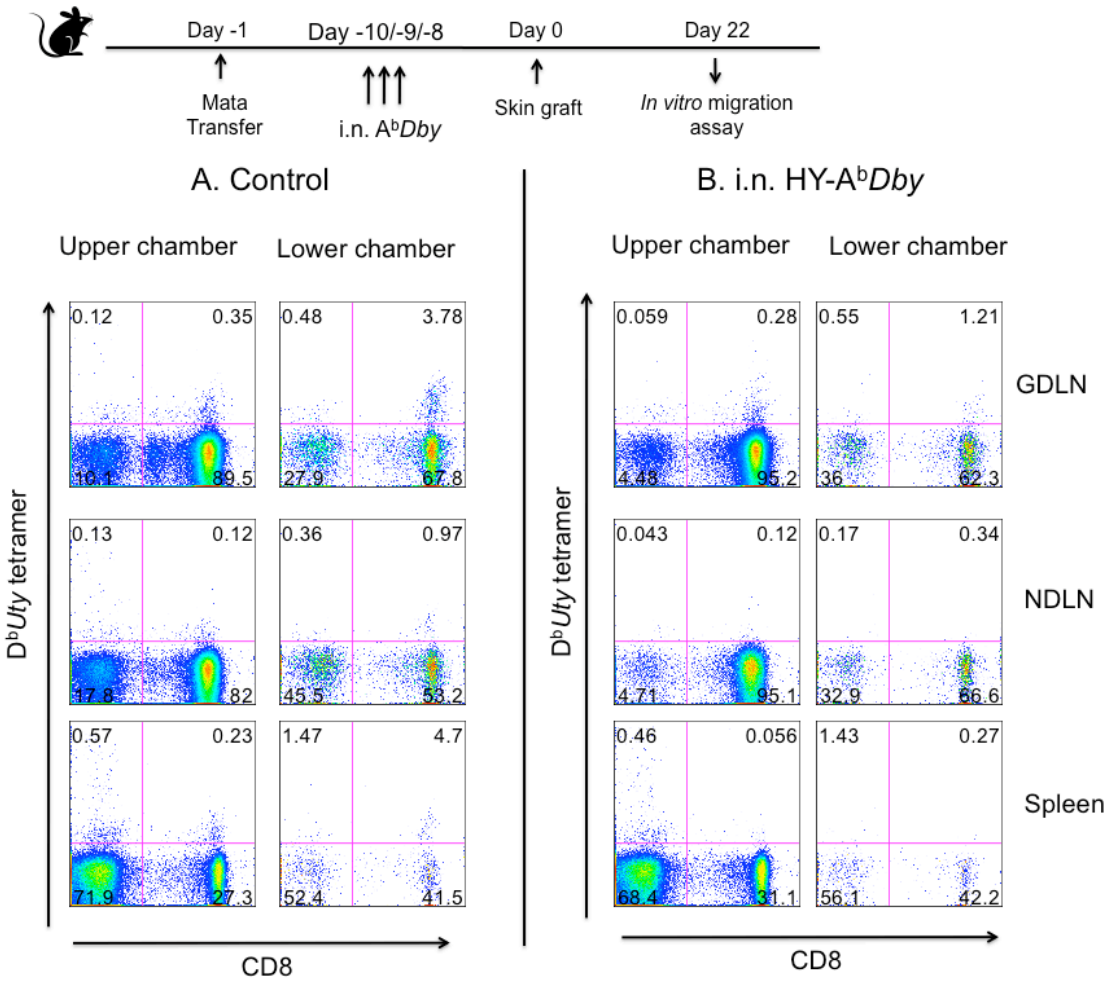


Figure 5.6 *In vitro* migration assay. Approximately 2.4x10^4 CD8$^+$Vβ8.3$^+$ Matahari were transferred into each Thy1.1 recipient. Mice received either i.n HY-A^b*Dby* or no treatment, 10 days later mice were given a male skin graft. 22 days after the graft the lymph nodes and spleen were removed – cells from the same tissues from different mice were pooled. The RBC were lysed, CD4$^+$ T cells and B cells were depleted using dynabeads. 2x10^6 CD8$^+$ enriched cells were added to the upper chamber of each transwell. 24 hours later the upper and lower chambers were carefully harvested, cells were stained and analysed by flow cytometry.

5.6 Do regulatory T cells enter the graft?

Differences in $CD8^+$ T cell migration in the graft draining lymph node have been shown but there is growing evidence in the literature that $CD4^+$ regulatory T cells may enter the graft and regulate both $CD4^+$ and $CD8^+$ T cells directly at the site of the graft (Carvalho-Gaspar et al., 2008). To test this is our model, RNA was extracted from the male skin grafts 24 days after grafting and following Matahari transfer.

Cd4 and *Ifnγ gene* expression was analysed using Sybr green assay and *FoxP3* and *Cd8* gene expression was analysed by Taqman assay. There was considerable variation between individual mice in both the tolerant and rejecting groups and there are therefore no statistically significant differences in gene expression between the two groups (Figure 5.7, Table 5.1).

Nevertheless, considering CD4 expression, the number of $CD4^+$ cells was higher in the peptide treated tolerant group (2.88+/-0.5) compared with control (1.58+/-0.06). The FoxP3 levels were also higher, and although, because of the particularly high variation of expression of this gene the results merely indicate a trend it is suggestive that the $CD4^+$ population might be regulatory.

With respect to CD8 expression, again the trend was for higher expression in the rejecting group, which is expected since, in this model, rejection is mediated primarily by $CD8^+$ T cells. Similarly there is higher expression of IFNγ which is again expected from the results shown in Chapter 2 where the $CD8^+$ T cells from rejecting mice express higher levels of IFNγ.

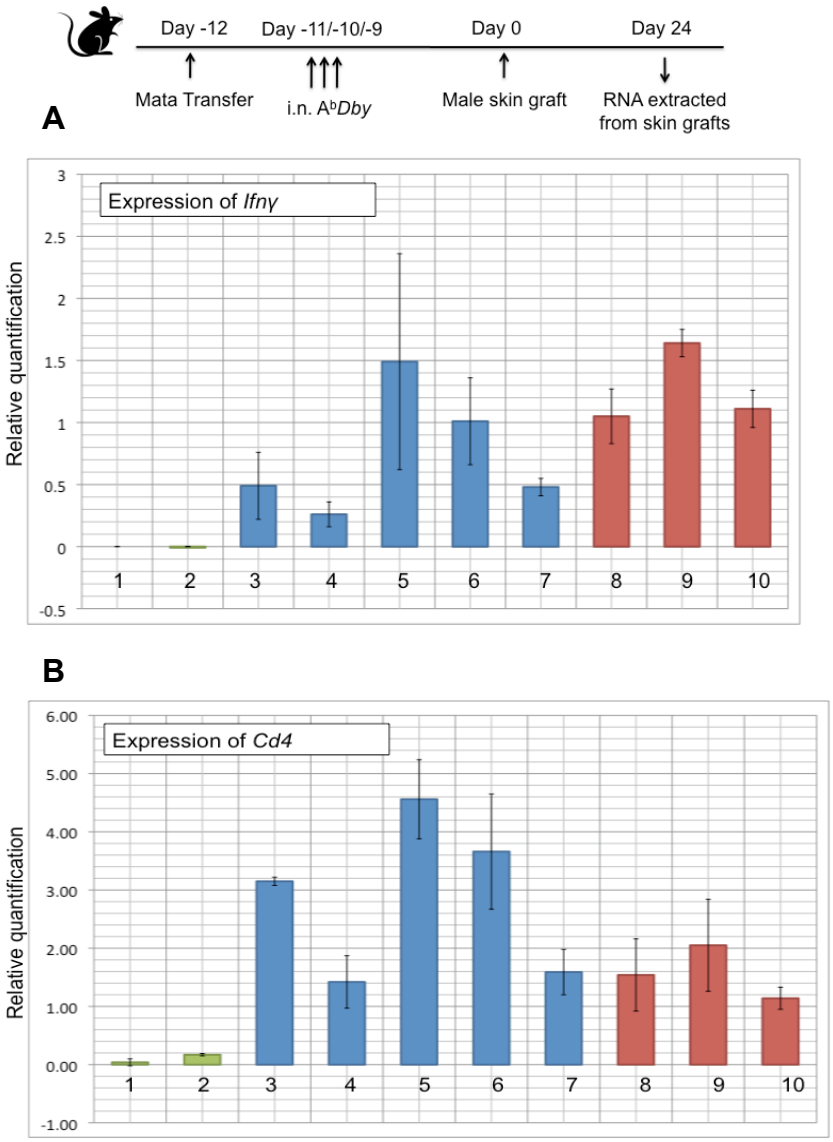
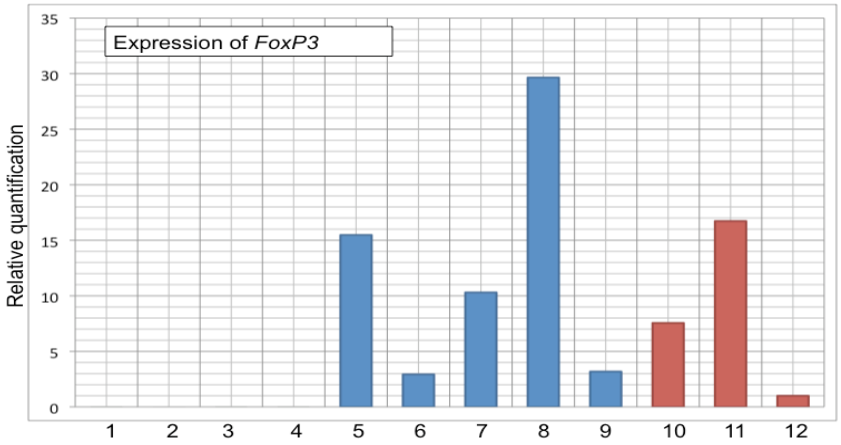


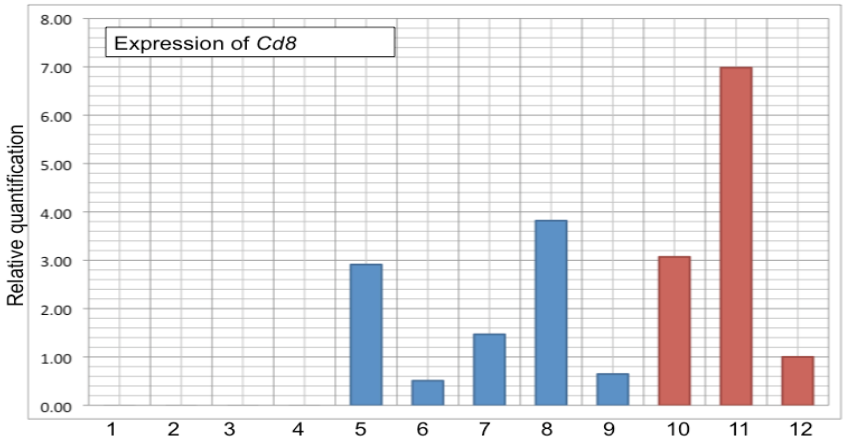
Figure 5.7 Expression of *Ifnγ, Cd4, FoxP3, Cd8* in RNA preparations from male skin grafts 24 days after grafting. Matahari cells were transferred into all Thy1.1 recipients. These mice then either received no treatment or 3x i.n. HY-A^{b}*Dby* followed by a male skin graft. 24 days after grafting skin grafting, the grafts were removed, RNA was extracted, reverse transcribed and gene expression was analysed by Quantitative PCR.

(A) *Ifnγ* and (B) Cd4 gene expression was analysed using Sybr Green assay. Gene expression was normalised to 18S and results are shown relative to the expression in sample 10. Samples 1 and 2 were prepared from the tail skin from WT B6 mice and are shown in green. Samples 3-7 are from peptide treated mice and are shown in blue. Samples 8-10 are from control mice and are shown in red.

C



D



E

	CD4	CD8	FoxP3	IFNγ
WT	0.11 +/- 0.00	0.00+/- 0.00	0.01+/- 0.00	0.00 +/- 0.00
Peptide treated	2.88 +/- 0.5	1.87 +/- 1.45	12.31+/- 11.03	0.75 +/- 0.5
Rejecting	1.58 +/- 0.06	3.68 +/- 3.04	8.43+/-7.9	1.27 +/- 0.06

C) *FoxP3* and (D) *Cd8* gene expression was analysed using a Taqman assay. Results were normalised to 18S and results are shown relative to the gene expression in sample 12. Samples 1-4 were prepared from the tail skin from WT B6 mice and expression of these genes could not be detected in these preparations. Samples 5-9 are from peptide treated mice and are shown in blue. Samples 10-12 are from untreated mice and are shown in red. (E) Table of mean gene expression. The mean value and standard deviations are given for previous 4 charts.

5.7 Discussion: Does i.n. peptide alter the migration of antigen specific T cells?

The results of the gene expression analysis carried out in this project during the tolerance induction and linked suppression phases suggested that HY specific T cells may have altered migratory capacity following i.n. HY-A^{b}*Dby*. In particular, during the tolerance induction phase, HY-specific CD4^{+} upregulated gene *Cxcr3* after i.n. peptide + LPS (Table 3.6) but not after i.n. peptide alone. Following male skin graft male specific CD8^{+} T cells from rejecting mice also upregulated this gene (Table 4.2) whereas the same cell from tolerant mice did not. CXCR3 allows T cells to migrate in response to inflammation and is expressed by activated effector memory T cells, so if it is not upregulated it may mean that in tolerised mice the HY-specific CD8^{+} T cells cannot migrate into the skin and cause rejection.

Furthermore, HY specific CD8^{+} T cells upregulated *Ccr10* after male skin grafting in tolerant mice. CCR10 is expressed by skin homing memory T cells (Jarmin et al., 2000) but can also be involved in homing to the skin draining lymph nodes (Huang et al., 2008). This may enable these antigen specific cells to enter the draining lymph nodes.

To investigate the migration of CD8 T cells after tolerance induction and skin grafting I developed an alternative method of transferring Thy1.2^{+} HY-D^{b}*Uty* peptide specific cells from Matahari mice into Thy1.1 recipient mice. These mice then received i.n HY-A^{b}*Dby* peptide to induce tolerance and 10 days later received a male graft. 22 days after skin grafting it was no longer possible to detect the transferred Thy1.2^{+} cells in the draining lymph nodes, the spleen or the skin graft itself. However, the transfer of large numbers of antigen specific CD8^{+} T cells did boost the endogenous response of the recipient. For this reason the Matahari transfer model was used in further experiments to investigate the migration of antigen specific CD8^{+} T cells in tolerant conditions. It seems likely that this increase in the endogenous response is due to the large number of transferred antigen specific CD8^{+} T cells. This would significantly increase the precursor frequency of HY-specific CD8^{+} T cells that, on encountering male antigen 10 days after transfer could respond by secreting large quantities of cytokines and proinflammatory

mediators that could then recruit and expand the endogenous HY-specific $CD4^+$ and $CD8^+$ T cells. By the time the tissues were isolated (22days after grafting) the transferred population was no longer detectable; these cells having expanded and undergone apoptosis, however, their effects were apparent from the expansion of the endogenous antigen specific T cells .

Following Matahari transfer and a male skin graft, tetramer positive $CD8^+$ T cells were detectable in the graft draining and non-draining lymph nodes, spleen and skin by flow cytometry and some $Thy1.2^+$ Matahari cells could be seen by immunofluorescent staining of the tissues. When tolerance was induced by i.n. HY-A^b*Dby* peptide in these mice the $CD8^+$ tetramer positive cells could be found primarily in the graft draining lymph nodes and few if any detectable in the other tissues. Further investigations using multicolour flow cytometry staining showed that these cells expressed more CCR7 than tetramer positive cells in the draining lymph nodes of rejecting mice. CCR7 is a marker of naïve and central memory T cells and used for homing to the lymph nodes (Förster et al., 1999, Scimone et al., 2004). This suggests there maybe more central memory T cells in the draining lymph nodes of peptide treated mice than rejecting mice. The draining lymph nodes of peptide treated mice had a small population of cells that expressed very high levels of LFA-1. LFA-1 is upregulated in effector T cells suggesting there might be a small population of HY-specific effector T cells that are perhaps being controlled by other cells in the draining lymph node.

There were also differences in $CD8^+$ $tetramer^+$ cells found in the non-draining lymph nodes and spleen. In peptide treated mice, more of these cells expressed the higher level of CXCR4 than in control mice. CXCR4 is a marker of central memory T cells and is involved in homing to the lymph nodes (Okada et al., 2002). In rejecting mice there are more $CXCR3^+$ effector cells, which are involved in migration to sites of inflammation while in peptide treated mice there are central memory $CXCR4^+$ T cells.

To test in vitro migration I used transwells coated with female endothelial cells. When cells from the graft draining lymph nodes from peptide treated mice were added to these wells fewer migrated to the lower chamber compared with cells from control mice. Supporting the idea that these cells

have impaired migration and this might affect their ability to reach the site of the graft.

To further examine the properties of the cells that are present within the skin graft of both tolerant and rejecting skin, the whole graft was digested and gene expression analysed. There was considerable variability in these results, but the trend was that there was more *Cd4* and *FoxP3* expressed in grafts from tolerant mice and more *Cd8* and *IFNγ* expressed in skin that was being rejected. Therefore in this model of tolerance while $CD8^+$ T cells are trapped in the graft draining lymph node, $FoxP3^+$ $CD4^+$ maybe able to migrate into the skin. Further experiments looking at chemokine receptors on $CD4^+$ T cells in the graft draining lymph nodes and the skin are needed to prove this.

Taken together these results suggest that antigen specific $CD8^+$ T cells become trapped in the skin graft draining lymph nodes of tolerant mice and are unable to migrate from to the site of the male graft to carry out their cytotoxic function.

Chapter 6: Conclusions

Post-transplantation antigen specific tolerance is still an elusive goal of clinical transplantation. Currently transplant recipients must take immunosuppressive drugs that prevent them rejecting the graft but also increase the risk of infections (Mueller, 2008) and cancer (Zafar et al., 2008).

In mice, intranasal peptide administration has been used to induce tolerance to the minor antigen, HY, using both mismatched skin grafts and haematopoietic cells, (Chai et al., 2004). It was shown that i.n. administration of the class II restricted peptide, NAGFNSNRANSSRSS, encoded by the *Dby* gene (HY-A^b*Dby*) can induce tolerance to male skin grafts in high responder B6 ($H2^b$) female mice. There appears to be a phenomenon of linked suppression working here as i.n. administration of the single class II peptide is able to induce tolerance not only in B6 recipients that express two additional MHC Class I epitopes but also in (B6xCBA)F_1 mice that express a further class II and two class I restricted HY peptide epitopes, a total of five. The mechanism by which i.n. peptide administration induces tolerance is not clear but tolerance was shown to be transferrable by adoptive transfer of both $CD4^+CD25^+$ and $CD4^+CD25^-$ cell populations into naïve neonatal recipients that received a male skin graft six weeks later. Furthermore it was not dependent on secretion of IL-10 as tolerance could still be induced in IL-$10^{-/-}$ mice (Chai et al., 2004).

Using a model essentially similar to the one that has been developed in our laboratory, Verginis *et al*. have recently also shown that sub-immunogenic quantities of HY-A^b*Dby* peptide can result in transplantation tolerance. In their experimental system the peptide was given via subcutaneous minipump at a dose or either 1 or 10μg over 14 days, much lower than the i.n. dose given in the experiments in this thesis (3 doses of 100μg). In agreement with the findings here, they also showed that HY-A^b*Dby* specific T cells upregulated FoxP3 mRNA. The authors noted an accumulation of $FoxP3^+$ Tregs in the graft draining lymph nodes of peptide-infused WT mice transplanted with male skin (Verginis et al., 2008) but do not comment as to why this might be.

In a T cell receptor transgenic model where the $CD4^+$ T cells recognise a peptide derived from myelin basic protein (MBP), Anderson *et al.* Examined

gene expression in antigen specific T cells following i.n. administration of 10x100μg MBP peptide with 3-4 day intervals between doses. In contrast to the findings reported in this thesis and in the above report by Verginis et al, they found no evidence of FoxP3 being upregulated, instead they found that the transcription factors T-bet, Irf1 and Egr2 were upregulated (Anderson et al., 2006). This is not surprising as previous reports from this group had shown that these MBP Tregs suppress naïve T cells by secretion of IL-10 (Sundstedt et al., 2003). This suggests that in this MBP model, repetitive i.n. treatment results in a $CD4^+$ T cell phenotype that suppresses via different mechanisms to the $FoxP3^+$ Treg population described in this thesis. Suppression in the Anderson et al., model is probably mediated by Tr1 cells that secrete IL-10 and express little or no FoxP3 (Roncarolo et al., 2006).

The aim of this project was to explore the mechanism(s) by which i.n. HY-A^b*Dby* peptide induces tolerance to male grafts. One possibility is that tolerance in this model could be a result of the deletion of antigen specific $CD4^+$ T cells. Without $CD4^+$ T cells to provide sufficient help, then none of the HY epitope specific $CD8^+$ T cells would be able to be primed, to then reject male skin grafts. However, this explanation seems unlikely for several reasons. Firstly the previous work from this laboratory has shown that both $CD4^+$ HY-A^b*Dby* and $CD8^+$ HY-D^b*Uty* could be expanded from long-term tolerant mice (mice that had retained their grafts for greater than 270 days). *In vitro* or *in vivo* re-stimulation with male cells was able to expand these cells although the expansion was smaller than that from mice that had rejected their grafts (Chai et al., 2004). These results clearly argue against deletion of $CD4^+$ T cells resulting in inadequate priming of the HY specific $CD8^+$ T cell population. Secondly, tolerance was found to be transferable; i.v injection of unseparated or $CD8^+$ T cell depleted spleen cells from long term tolerant mice could cause prolonged graft survival in recipient neonatal female mice that received a male skin graft at 6 weeks of age. These results suggest that tolerance is due to regulation rather than depletion.

In order to investigate how i.n. HY-A^b*Dby* peptide induces tolerance to male grafts, cells from TCR transgenic mice specific for HY-A^b*Dby* (Marilyn mice) were labelled with CFSE and transferred into WT B6 female mice that then received a tolerising or immunising dose of peptide and, following the

tolerisation regime, a male graft of skin or haematopoietic cells. Gene expression in the HY-specific cells was analysed during the tolerance induction phase and following immunisation with male cells. Although transferring antigen specific T cells into WT recipients only yielded small numbers of cells in each experiment, it was felt that this model was more physiologically relevant than simply giving the treatment to the transgenic mice alone.

Because of the low cell recovery, and therefore small quantities of recovered RNA, I chose to use real time quantitative PCR analysis with the newly developed Global Pattern Recognition algorithm and primer-coated plates, subsequently Stellarray Plates provided by our collaborators at the Jackson Laboratories in Bar Harbor. However, I did encounter some problems with consistency of gene expression data using this methodology. This was mainly due to having to vary the conditions between experiments. For example, because some of the experiments were carried out in Bar Harbor whilst the rest were carried out in London, it was necessary to use different primer sets and different sources of master mix in different experiments. In addition, as the Q-PCR array is a new technology, during the course of the project it has been going through some development. This resulted in changes in primer sets that are present on the plates. The Q-PCR array has never previously been used on RNA from a sorted T cell population and so the problems I encountered may not have been previously encountered by either the company or by other groups using this technology. In order to minimise the between experiment variation, only genes that were consistently shown to be up or down regulated were included in the final analysis.

Considering the analysis of gene expression in antigen specific $CD4^+$ T cells during the induction phase (i.e. before skin grafting). It was found that these cells proliferated following i.n. peptide and upregulated a smaller number of genes compared to when peptide was given in immunising conditions. One of the genes found to be significantly upregulated following i.n. peptide was FoxP3. This was confirmed by flow cytometry. Since the adoptively transferred population was derived from a $Rag^{-/-}$ background and the transferred cells were CD25 negative this strongly suggests that i.n. peptide administration converts a population of naïve $CD4^+$ T cells into a regulatory

one. This is most likely because a high dose of antigen (3 doses of 100μg) is given and is being taken up and presented by immature DCs over the course of 3 days, in the absence of any other proinflammatory signals. This induces the expression of FoxP3 in antigen specific T cells.

In addition to FoxP3 the antigen specific T cells upregulated genes that protect from apoptosis such as *Grp94, Ptpn13* and *Ox2*. They also upregulated genes that encode proteins that inhibit T cell activation such as *Ctla4, Cd5* and *Pdcd1.* These cells also upregulated a number of genes such as *Pdcd1, Lag3, Ctla4, Ifng* and *Il13* that can modify DC function. For example, back signalling through the PDL1 receptor has been shown to prevent DC maturation (Kuipers et al., 2006). IFNγ can induce IDO expression in DCs (Grohmann et al., 2002). CTLA4 has also been shown to initiate tryptophan catabolism by IDO in B7 expressing DCs (Fallarino et al., 2003) and IL-13 can activate the immunomodulatory enzyme Arginase 1 (Wei et al., 2000). This ability to modulate the APC is of particular interest as it has implications for linked suppression after skin grafting.

If i.n. peptide induces regulatory T cells how do these prevent $CD8^+$ T cells from rejecting male cells or male skin? To answer this question I analysed gene expression by antigen specific HY-D^b*Uty* tetramer positive $CD8^+$ T cells following immunisation with male cells. I also examined gene expression in the $CD4^+$ T cell population at this time point to investigate whether the regulatory phenotype remained following immunisation with male cells.

Not surprisingly, antigen specific $CD8^+$ cells taken from rejecting mice were found to have upregulated genes involved in cell killing such as granzyme B, FasL and cathepsin B. This is consistent with these cells being cytotoxic. These genes were not found in the same cells from tolerant mice suggesting that either regulatory T cells or specialised antigen presentation prevents these cells becoming activated and developing into cytotoxic effector cells.

Antigen specific $CD8^+$ T cells from tolerant mice upregulated the gene *Ccr10*. CCL27 is a CCR10 ligand that has been found at high levels in the skin draining lymph nodes compared to gut draining lymph nodes and spleen (Huang et al., 2008). I therefore suggest that by upregulating *Ccr10* it allows the $CD8^+$ to migrate to the graft draining lymph node.

In contrast the antigen specific $CD8^+$ T cells from rejecting mice upregulated chemokine receptors (*Cxcr3* and *Cx3xcr1*) and integrins (α_x *and* β_1), allowing them to enter peripheral tissues and lymph nodes.

The $CD8^+$ cells from tolerant mice also upregulated *Lag3* and *Pdcd1.* Signalling through these molecules has an inhibitory effect on T cell proliferation, which could prevent these cells differentiating into effector T cells. These molecules are also expressed by exhausted $CD8^+$ T cells after chronic viral infection (Blackburn et al., 2009). My results might therefore suggest that, in these tolerant mice that express the same inhibitory receptors as exhausted T cells, the mechanisms of antigen presentation might also be similar to those in chronic viral infection. Although these markers were also upregulated in $CD8^+$ T cells from rejecting mice, it was in combination with granzyme B, FasL and cathepsin B, which were not were not upregulated in tolerant mice.

Isolating antigen specific $CD4^+$ T cells after immunising with male cells was technically difficult and only very small numbers of cells could be isolated so the results must be interpreted with caution. These cells from tolerant mice did not appear to have upregulated genes that are typical of regulatory T cells such as *FoxP3*, *Ctla4* and *Pdcd1.* In fact, *FoxP3* and *Lag3* were found to be upregulated in the antigen specific $CD4^+$ T cells taken from mice rejecting male cells. Expression of these genes would be expected to be controlling the ongoing immune response in these mice.

The genes *Ccl3, Ccr2* and *Ccl11* were all upregulated in antigen specific $CD4^+$ T cells from tolerant mice. This would allow these cells to home to the lymph node and attract other cells into the local node.

The gene expression in antigen specific $CD4^+$ cells was very different during the tolerance induction phase compared to that following immunisation with male cells. During the tolerance induction phase, the gene expression was consistent with these cells becoming regulatory cells, but there was no evidence for such regulatory cells after challenge with male antigen.

This might be because in these experiments I analysed gene expression in the originally transferred T cell population. During the induction of linked suppression and subsequent long term tolerance recently emigrated HY

specific cells must also become tolerant. Whereas the cells that have been present for 22 days may have become exhausted or inactivated by this time point. It maybe the that it is the initial interaction between the tolerogenic antigen presenting DC and the newly developed HY specific T cells that is causing non-responsiveness rather than the original regulatory T cells (see figure 6.3).

The generation of Tregs from endogenous naïve minor antigen specific T cells has been shown in Herman Walmann's model of transplantation tolerance. In this model new cohorts of naïve T cells either exported from the thymus or introduced experimentally to the tolerant environment also become tolerant (Bemelman et al., 1998, Chen et al., 1996, Qin et al., 1993). Therefore, in the experiments reported here, limiting the gene expression analysis to just the transferred HY-A^b*Dby* specific T cells may have given an incomplete picture of linked suppression. Gene expression in newly developing T cell populations might reveal the upregulation of regulatory genes.

In Chapter 3 I examined the migration of antigen specific $CD8^+$ T cells after male skin grafting. In female mice that were rejecting a male skin graft these cells could be found in the graft draining lymph nodes, the non draining lymph nodes, the spleen and in the skin, albeit in very small numbers. In tolerant mice, induced by i.n. peptide administration, the antigen specific $CD8^+$ T cells could only be found in the graft draining lymph nodes and not in the non-draining lymph nodes, spleen or skin. The expression of CXCR3 and CXCR4 was found to be similar on these antigen specific $CD8^+$ T cells from rejecting and tolerant mice. However, the cells from tolerant mice expressed more CCR7, suggesting there were more cells expressing a central memory phenotype. While antigen specific $CD8^+$ T cells from both mice expressed a high level of LFA-1, tolerant mice had a small population that expressed a very high level of LFA-1^+ cells, suggesting there are a small number of cells that have become activated.

When the antigen specific T cells from the draining lymph node were studied *in vitro* the cells from tolerant mice did not migrate as well as similar cells from rejecting mice.

Together these results suggest that in tolerant mice the antigen specific $CD8^+$ T cells that have migrated to the graft DLN as a result of increased expression of CCR10, cannot then migrate out of the DLN to the site of the male skin graft to mediate rejection. This may be because they do not express the necessary migration molecules to exit the lymph node.

In this thesis I propose that i.n. HY-A^b*Dby* peptide induces tolerance to male skin grafts by inducing antigen specific regulatory $CD4^+$ T cells (Figure 6.1). Work by others in our laboratory has shown that by giving i.n. peptide labelled with CFSE, that as soon as 1 and up to 5 days later the peptide could be found in many peripheral lymph nodes and the spleen (Diane Scott, personal communication). Therefore this antigen presentation is not restricted to the draining lymph nodes or mucosal DCs.

Following i.n. HY-A^b*Dby* antigen specific $CD4^+$ T cells were found to upregulate the genes encoding FoxP3, PD1, CTLA4 and LAG3 (Figure 6.1b). They also upregulated the genes encoding the cytokines IFNγ and IL-13. As described above, the protein products of these genes can modify DCs to make them tolerogenic (Figure 6.1.C). This seems to fit with *in vivo* observations that it takes between 5 and 10 days after i.n. peptide administration for tolerance to develop (Dr J. Chai, personal communication). This suggest that there is some time needed for the tolerogenic DCs to develop.

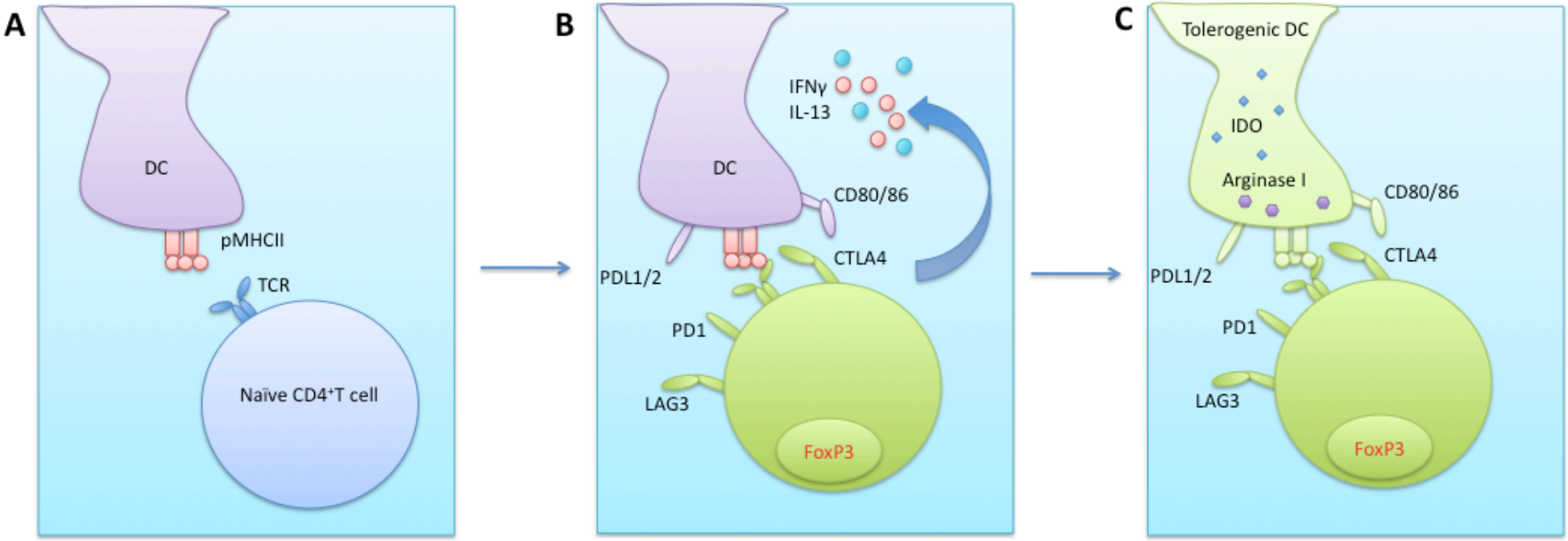


Figure 6.1. Tolerance induction phase. A. Following i.n. HY-A^{b}*Dby* peptide, DC's take up antigen and present it to naïve CD4^{+} T cells. **B.** These T cells were then found to upregulate the genes *FoxP3, Ctla4, Pdcd1, Lag3, Ifnγ* and *Il-13*. CTLA4 and PD1 inhibit T cell activation and CTLA4 can stimulate Tregs to suppress. IFNγ, CTLA4 and IL-13 can all modulate the antigen presenting DC. **C**. The presenting DC to become a tolerogenic DC which expresses IDO and Arginase I (see text for details).

Untreated female $H2^b$ mice will reject a male skin graft. Figure 6.2 shows the interaction between DCs and CTLs in the graft draining lymph node. In these conditions the DC will present both MHC class I and class II restricted peptide in a pro-inflammatory environment. HY-A^b*Uty* specific $CD8^+$ T cells from rejecting mice were found to upregulate genes involved in the cytotoxic killing of cells such as the genes encoding Granzyme B, Cathepsin B, Caspase 3 and FasL. These cells also expressed CXCR3 which allows migration to inflammatory areas. Previous work in this laboratory has shown that after skin grafting HY-A^b*Dby* $CD4^+$ T cells were activated and secreted IL-2 and IFNγ when stimulated with peptide pulsed DCs *in vitro* (Chai et al., 2004). The accumulation of these mechanisms is rejection of the skin graft. It is of note that a number of regulatory genes were upregulated in both antigen specific $CD4^+$ and $CD8^+$ T cells during skin graft rejection. These are considered to be necessary to prevent an uncontrolled immune response.

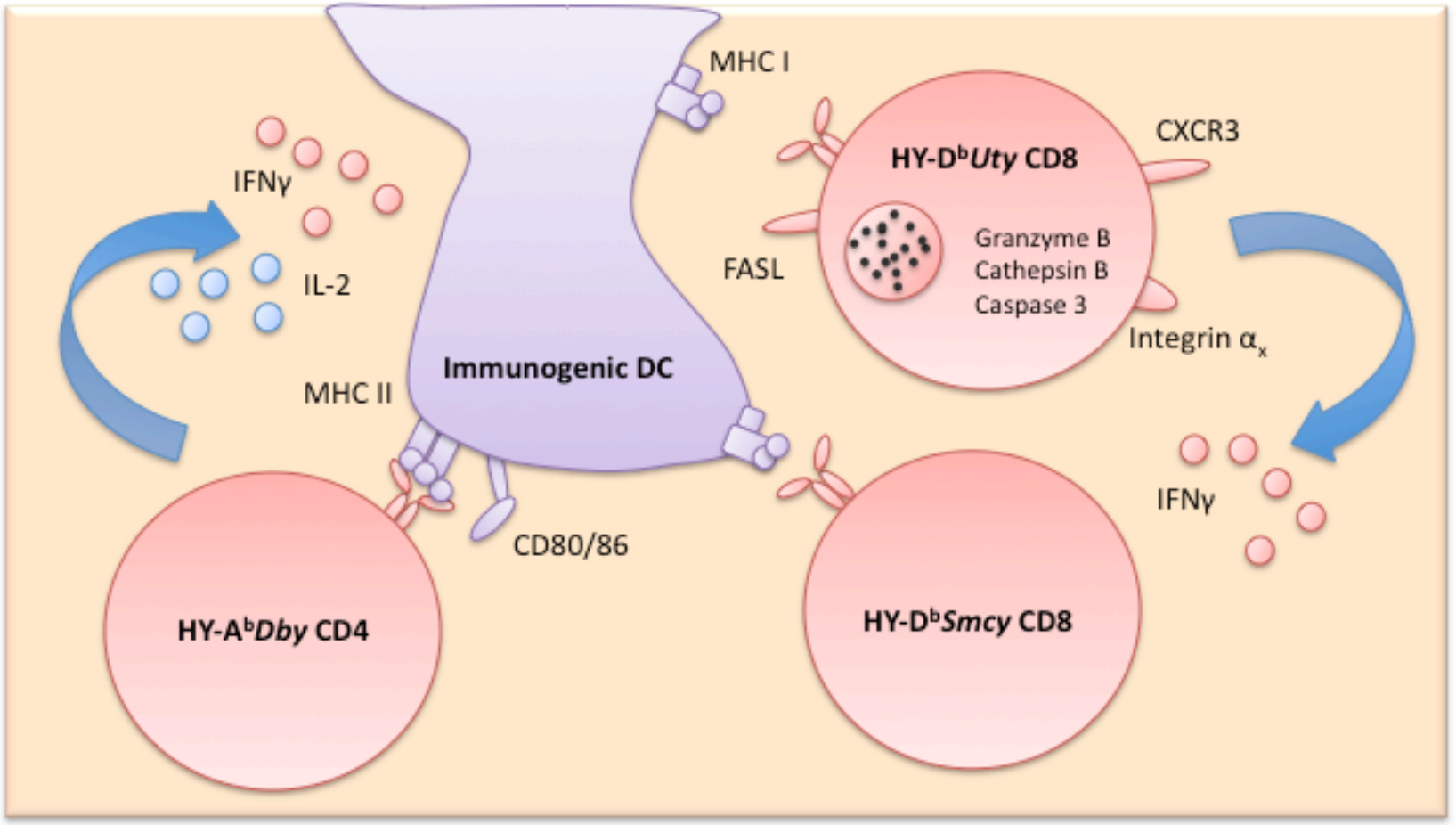


Figure 6.2 Rejection of male skin grafts or cells. In untreated female mice, male cells will be rejected. HY-A^b*Uty* CD8$^+$ cells were shown to upregulate genes for granzyme B, FasL and cathepsin B suggesting these cells become cytotoxic T cells. Previous work has shown that HY-A^b*Dby* CD4$^+$ T cells secrete IFNγ and IL-2.

In mice that have received i.n. peptide and do not reject their male skin grafts there are 2 possible scenarios in the draining lymph node.

- Regulatory T cells in the DLN prevent the proliferation of antigen specific $CD8^+$ T cells. Naturally occurring Tregs are activated in antigen specific fashion but suppress in a non-antigen specific fashion. It is not clear if inducible Tregs act like this. This would mean that the Tregs induced by i.n. peptide prevent the proliferation of both $CD4^+$ and $CD8^+$ T cells in the draining lymph node (Figure 4.3 A).
- Tolerogenic DCs in the DLN actively control $CD4^+$ and $CD8^+$ T cells preventing them from upregulating chemokines they need to exit the lymph node and migrate to the skin graft. As discussed in chapter 2 - this would provide a mechanism for tolerance to spread to other epitopes. The DCs present in the DLN may be made tolerogenic during the induction phase. The continued presence of Tregs in the DLN may provide further signals that either maintain the tolerogenic state or induce more tolerogenic DCs. In turn these DCs could induce more Tregs in a positive feedback loop for which there is some evidence in other models (Yates et al., 2007) (Figure 4.3b).

The results described in this thesis favour the second of these two possibilities.

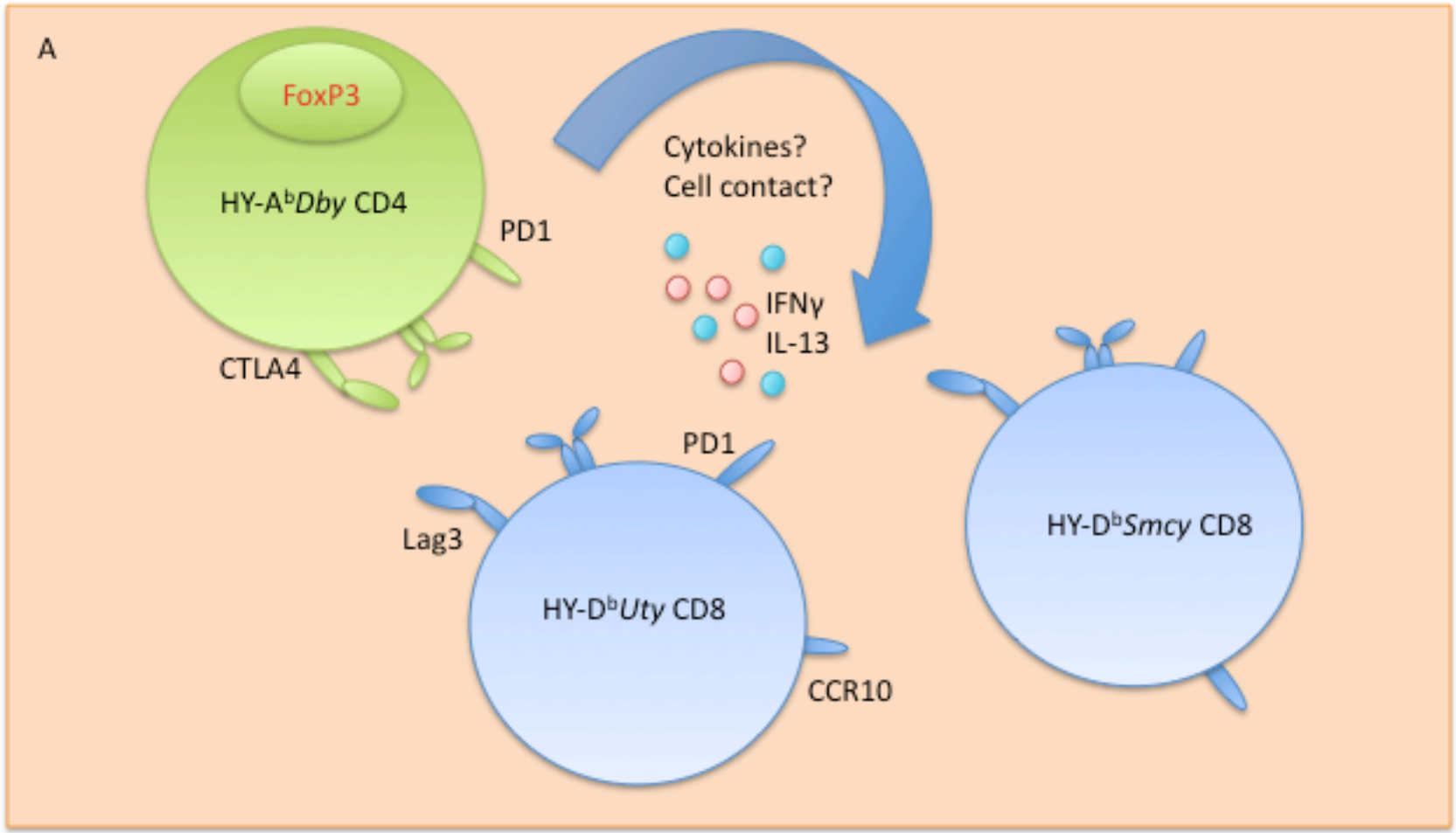


Figure 6.3 A. Linked suppression is mediated by regulatory T cells. In the draining lymph node antigen specific regulatory T cells prevent $CD8^{+}$ T cells from rejecting male cells. Antigen specific $CD8^{+}$ T cells from tolerant mice were found to upregulate the genes for CCR10, LAG3 and PD1.

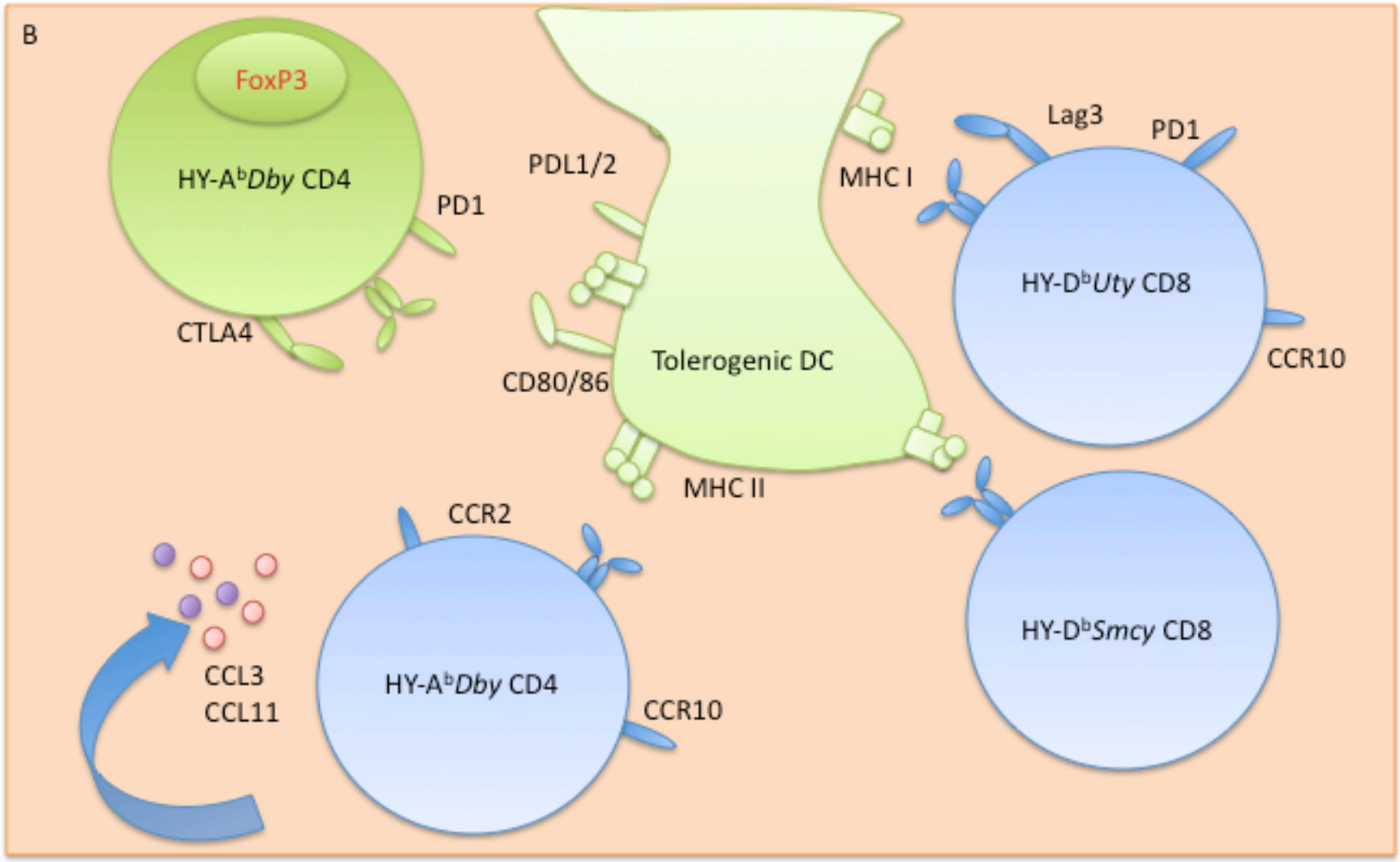


Figure 6.3 B. Linked suppression is mediated by tolerogenic DCs. In the draining lymph node tolerogenic DCs present both MHC class I restricted and MHC class II restricted antigens to $CD8^=$ and $CD4^+$ T cells. Antigen specific $CD4^+$ T cells from tolerant mice were found to upregulate the genes for CCR10, CCR2, CCL3 and CCL11. The presence of Tregs in the DLN could promote the development of more tolerogenic DCs, which in turn could create more Tregs.

6.1 Conclusion

i.n. peptide induces a regulatory population of FoxP3$^+$ CD4$^+$ T cells that can prevent transplantation rejection by modulating dendritic cell function and preventing antigen specific CD8$^+$ T cell from migrating from the graft draining lymph node. An understanding of how tolerance is induced and leads to linked suppression has implications for the induction of tolerance to transplanted organs in the clinic. In the future this may allow for a reduction in immunosuppression following transplantation.

6.2 Further work

The aim of this project was to use a real time PCR array to investigate gene expression in antigen specific T cells. This array has given many candidate genes and the next step would be to look at the protein expression of these genes. In order to look at the mechanism of linked suppression, the gene expression in DCs could be analysed. This would involve extracting the RNA from DC presenting antigen in tolerant and rejecting mice. Although this is possible by laser microdissection, these experiments would be technically demanding.

Furthermore, it is not known to what extent it is possible to extend linked suppression beyond the HY antigens to autosomal minor H antigens. The amino acid sequences of some of these antigens are known and some congenic mice that differ at only one or two loci have been generated for example, 21M (congenic B10.129 – H46^{b}H47^b) and C57BL/10J (H46^{a}H47^a). It may be possible to extend peptide induced tolerance by using both the HY and H46 class II peptides (Sahara and Shastri, 2003) to induce tolerance to either male or female skin grafts in these congenic strains. This will explore extension of antigen specific tolerance to widely expressed autosomal minor H antigens.

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Appendix I

S.1 Gene expression in antigen specific T cells following i.n. peptide alone

The recovery of Marilyn $CD4^+$ T cells 5 days after i.n. peptide alone was consistently low, and the amount of RNA was consequently also low and generally less good quality. Thus RNA was limiting and the experiment could only be done twice. The first analysis comparing naïve and peptide samples was performed by me at the Jackson labs in Bar Harbor. At this time the version of the software gave a score rather than a P value.

The results of this analysis showed that 11 genes were significantly changed, 10 of which were upregulated and 1 (*Ly6a/e*) down-regulated 10 fold (Table S.1). *Foxp3* was ranked as the most significantly changed with a 40 fold increase in expression in the peptide samples (undetectable in the naïve sample). Other significantly upregulated genes were *Ptgs2* (increased 90 fold), *Il18r* (22 fold), *Tyk2* (163 fold), *Ifng* (7 fold), *Lag3* (9 fold) and *Ccr4* (4 fold).

The second experiment was performed on site at Imperial College under slightly different conditions (summarised in Table S.2). The expression of 33 genes was significantly changed, two of which were down-regulated - *Stat6* (–1164.64) and *Sfpi1* (–10.05) and although not statistically significant, *Ly6a/e* was down-regulated again. The significantly upregulated genes included *FoxP3* (266 fold), *Ctla4* (36 fold) and *Pdcd1* (28 fold). The primers for *Ctla4* were not on the plates in the first experiment but were added to new versions used in the second experiment. *Pdcd1* encodes the protein Programmed death 1 (PD1) which is an important inhibitory molecule. These three genes are important for the function of regulatory T cells. Also upregulated were the cytokine genes *Ifng* (95 fold), *Il21* (343 fold), *Il17.1* (32 fold) and the receptors *Il15ra* (128) and *Il18r* (100). Some genes involved in the migration of T cells were also upregulated *Ccr4* (41 fold), *Ccl3* (24 fold) and *Cxcr3* (40 fold).

In total from the two experiments, 38 genes were found to be significantly changed and 31 of these were found to be consistently changed in both the experiments (see Table S.1).

The data from these experiments was reanalysed using the newer version of the software which gives a P value. A summary of this data is given in Chapter 3 (Table 3.5).

	Gene	1. Jax Score	2. IC Score
1	*Baffr.1*		
2	*Ccl3*		
3	*Ccr4*		
4	*Ccr6*		
5	*Cd44.1*		
6	*Cd5.1*		
7	*Cd80.1*		
8	*Ctla4*		
9	*FoxP3*		
10	*Ifng*		
11	*IgG1.1*		
12	*IgG2b*		
13	*Il13*		
14	*Il15ra*		
15	*Il18r1*		
16	*Il21*		
17	*Lag3*		
18	*Ly6a/e*		
19	*Mmp13*		
20	*Mmp1a.ET*		
21	*Nfil3*		
22	*Ox2*		
23	*Pax6*		
24	*Pdcd1.1*		
25	*Ptgs2.ET*		
26	*Ptpn13*		
27	*Sfpi1*		
28	*Stat6*		
29	*Tac4*		
30	*Tra1.1*		
31	*Tyk2*		

Table S.1 Genes that were consistently up or downregulated in both experiments. Cells from TCR transgenic Marilyn mice were transferred into Thy1.1 recipients that then received i.n. HY-A^{b}*Dby*. Five days later Thy1.2^{+} CFSE^{+} Marilyn cells were sorted, Cells were pooled and RNA was extracted and analysed by quantitative PCR array. These experiments gave some variation in the results, the consistent results are shown here and the significance of their up or down regulation. A key to this table is given in Table S.2.

Score	P-Values	Up regulated	Down Regulated
0.8 -1.0	<0.001		
0.6 – 0.79	0.01-0.03		
0.4 – 0.59	0.03 -0.05		
Not significant			
Undetermined			
primers not on plate			

Table S.2 Key for Table S.1 and S.5. Upregulated genes shown in shades of red. Down regulated genes shown in shades of blue depending on the level of significance. Undetermined samples white. Primer set was not present: hatched.

Name	Dates	Location	Master mix	Samples		Other
				Naive	Treated	
1. Jax	Nov 06	Jackson Labs	Jackson Labs	1,2,3	LPS 1,2,3	
					Peptide 1,2,3	
2. IC	Mar 07	IC	AB	1,2,3	LPS 1,2,3	The same primers as the Jax plate except 10 primer sets changed to genes of special interest
					Peptide 1,2,3	
3. Mar	Mar 08	IC	AB	2,4,5	LPS 1,2,3	Custom Stella Array
4. Apr	April 08	IC	AB	1,2,4	LPS 2,3,4	Custom Stella Array

Table S.3: Summary of the different conditions of each quantitative array experiment. Four experiments were done at different times and different locations. RNA preparations from pooled cells from either naïve or treated mice were numbered 1 to 4. Because the recoveries of cells and RNA from mice treated with peptide+LPS was greater, these experiments were repeated four times to test the reproducibility of the IQA/PCR. IC=Imperial College, AB= Applied Biosystems Power Sybr green master mix.

Name	No. of genes changed	No. upregulated	No. Down regulated
		Peptide+LPS	
1. Jax	79	72	7
2. IC	32	27	5
3. Mar	26	18	8
4. Apr	25	19	6
		Peptide alone	
1. Jax	11	10	1
2. IC	33	31	2

Table S.4 Summary of the results of each experiment. Four experiments were done with RNA from mice treated with peptide+LPS and the number of genes significantly changes on each plate varied but the primer sets were also different. As limited amounts of RNA could be recovered from mice treated with peptide alone, there was only enough material for these plates to be run twice.

S.2 Gene expression in antigen specific T cells following i.n. peptide+LPS

Gene expression was analysed in the four experiments and in total 131 genes were found to be significantly changed following i.n. HY-A^{b}*Dby*+LPS compared to naïve samples. However, because the experiments were performed using different primer plates and in two places with different sources of reagents (summarised in Table S.3), the results between experiments were not as consistent as I had expected (see discussion below). Therefore from the list of 131 genes, some were excluded if they were not consistently up or downregulated across the 4 experiments. 54 genes were defined as consistent and are shown in Table S.5. 41 genes were upregulated and 13 genes were downregulated compared to naïve CD4^{+} preparations. Overall more genes were found to be significantly changed compared to when peptide was given alone – although in both conditions the majority of genes were upregulated compared to their expression in naïve Marilyn cells (Table S.4).

	Gene	1. Jax Score	2. IC Score	3 Mar 08 P Value	4 April 08 P Value
1	*B7rp1.1*				
2	*Baffr.1*				
3	*Blnk*				
4	*Bmpr2*				
5	*Casp4*				
5	*Cblb*				
7	*Ccl19*				
8	*Ccr4*				
9	*Ccr6*				
10	*Cd19*				
11	*Cd22*				
12	*Cd44.1*				
13	*Cd48*				
14	*Cd5.1*				
15	*Cd74*				
16	*Cd79a*				
17	*Cox2*				
18	*Ctla4*				
19	*Cxcr3*				
20	*E2a*				
21	*Ebf1*				
22	*Egf*				
23	*Eif2s3y*				
24	*Entpd1*				
25	*FasL*				
26	*Fcgr3*				
27	*Fos*				
28	*H2-Aa*				
29	*H2-Eb1*				
30	*Icos*				
31	*Igb*				
32	*IgK-C*				
33	*IgL-C1*				
34	*IL11ra*				
35	*Il15ra1*				
36	*Il1b*				
37	*Il23r*				
38	*Itgb1*				
39	*Jun.1*				
40	*Kit*				
41	*Ly6a/e*				
42	*Ms4a1*				
43	*Mthfr*				
44	*Nfatc2*				
45	*Nmrk*				
46	*Oas2*				
47	*Ox2*				
48	*Pax6*				
49	*Prf1*				
50	*Ptgs2.ET*				

	Gene	1. Jax Score	2. IC Score	3 Mar 08 P Value	4 April 08 P Value
51	*Ptpn13*				
52	*Sfpi1*				
53	*Socs1*				
54	*Socs3*				

Table S.5 Genes that were consistently up or downregulated in four experiments. Cells from TCR transgenic Marilyn mice were transferred into Thy1.1 recipients that then received i.n. HY^{Ab}*Dby* + LPS. Five days later Thy1.2+ CFSE+ Marilyn cells were sorted, Cells were pooled and RNA was extracted and analysed by quantitative PCR array. These experiments gave some variation in the results, the consistent results are shown here and the significance of their up or down regulation. A key to this table is given in Table S.2. In experiments 1 and 2 the results are given as a score while experiments 3 and 4 the results are given as a P value.

S.3 Reproducibility of the IQA

During the course of this project different quantitative PCR arrays had to be used because the original arrays were no longer available from the Jackson Laboratory. The new arrays were comprised of different primer sets, thus introducing a degree of variation. When experiments were repeated using the same plates and primers, occasionally variable results in both the level of significance and fold change were found indicating that the reproducibility of the IQA was not always perfect. This is partly due to slightly varying conditions between experiments, summarised in Table S.3.

The original experiments were done at the Jackson labs and resulted in 79 genes being significantly changed in the peptide+LPS samples and 11 genes being changed in the peptide alone samples (Table S.4). The plates and primers used in the second experiment were very similar to the first except that 10 primers had been substituted for primers of genes that had been found to be changed in the original microarray data, and therefore were of special interest. However, in this experiment a different master mix was used and this could have had a considerable effect on the results because the calculation of significance for each gene is relative to the expression of all other genes.

To test reproducibility, identical plates that had been custom prepared by BHB to contain all of our genes of interest, were set up using the same master mix and analysed with the newest version of the GPR software (Experiments 3 and 4) Although there was not sufficient RNA to use exactly the same RNA preparations on these plates, overall the results from experiments 3 and 4 were similar thus giving confidence that the methodology was reliable (Table S.4).

Appendix II

S.4 Raw data: Significant genes in CD4$^+$ Thy1.2$^+$ Marilyn cells after i.n. HY-A^b*Dby* peptide during induction of linked suppression.

Table S.6 Genes significantly changes in CD4+ Thy1.2+ Marilyn cells after i.n. HY-AbDby peptide at the time of induction of linked suppression. Thy1.2+ Marilyn cells were transferred into Thy1.1+ female recipient mice. These mice were then given 3x i.n. HY-AbDby peptide and 10 days later were given CFSE labelled male and female spleen cells. Up to 10 days after the CFSE labelled cells CD4+ Thy1.2+ were isolated by flow cytometry sorting. RNA was extracted and analysed by quantitative PCR array. 235 genes were found to be significantly up or down regulated compared to RNA preparations from naïve CD4+ T cells. Genes that were downregulated are shown in blue.

Rank	Gene	P value	Fold change	Ave Ct naïve	Tolerant Ct
1	*Pax5*	0.0008	36792.8	40.0	28.0
2	*Cd80*	0.0017	1516.9	40.0	32.6
3	*Thy1*	0.0021	-8938.1	23.7	40.0
4	*IgK-C*	0.0022	557.9	36.7	30.7
5	*Il17f*	0.0023	698.4	39.4	33.1
6	*Mmp2*	0.0025	476.0	40.0	34.3
7	*Klra1*	0.0026	428.9	40.0	34.4
8	*Ccr10*	0.0031	2528.0	39.7	31.4
9	*Pecam1*	0.0039	-1162.3	26.6	40.0
10	*Nfatc1*	0.0040	-861.8	27.1	40.0
11	*Il23r*	0.0041	114.0	40.0	36.3
12	*Src*	0.0048	71.6	40.0	37.0
13	*IgD-C1v1*	0.0055	74.7	32.7	29.7
14	*Aire*	0.0055	226.2	39.8	35.0
15	*Cd34*	0.0056	21.0	36.3	35.1
16	*Il12a*	0.0056	22.2	33.0	31.8
17	*Ccl19*	0.0056	22.9	34.2	32.8
18	*Notch1*	0.0061	-371.5	28.3	40.0
19	*Cd14*	0.0063	142.8	39.7	35.6
20	*Stat5b*	0.0064	-435.4	28.0	40.0
21	*Socs3*	0.0065	-1043.4	26.9	40.0
22	*Psmb9*	0.0066	-451.0	28.0	40.0
23	*IgA-C2v1*	0.0068	268.7	34.4	29.3
24	*Tcra-C*	0.0069	-322.1	23.7	35.1
25	*Il1rl1*	0.0071	102.5	36.5	32.9
26	*Zap70*	0.0071	-423.1	28.1	40.0
27	*Ptpn6*	0.0075	-644.2	27.6	40.0
28	*Lamp1*	0.0077	11.1	33.9	33.6
29	*Ctsb*	0.0078	-593.1	27.7	40.0
30	*Il6st*	0.0079	-541.3	27.8	40.0

Rank	Gene	P value	Fold change	Ave Ct naïve	Tolerant Ct
31	*Cd4*	0.0080	-285.1	25.6	36.9
32	*Lifr*	0.0080	58.9	39.7	36.9
33	*Ly6a*	0.0081	-1510.8	25.5	39.0
34	*Cfb*	0.0081	6.7	33.2	33.6
35	*Il27ra*	0.0081	-547.0	27.8	40.0
36	*Icosl*	0.0082	5.0	33.0	33.8
37	*C8a*	0.0082	8.5	34.4	34.5
38	*Tgfbr1*	0.0082	5.4	30.2	30.9
39	*Tnfrsf4*	0.0083	2.8	32.7	34.4
40	*Ifit2*	0.0086	-664.5	27.5	40.0
41	*Rbpj*	0.0086	-166.4	29.4	40.0
42	*Itgal*	0.0087	-495.0	27.0	39.1
43	*Bax*	0.0089	-194.2	29.2	40.0
44	*Ncam1*	0.0090	41.9	37.3	35.0
45	*Runx1*	0.0091	2.1	29.4	31.5
46	*Ccl11*	0.0092	47.3	36.6	34.1
47	*Klrd1*	0.0093	1.6	33.0	35.5
48	*Ifngr1*	0.0094	-136.5	27.5	37.8
49	*Cd2*	0.0097	-2062.3	26.0	40.0
50	*Myd88*	0.0097	-180.4	29.3	40.0
51	*Pparg*	0.0098	34.1	33.3	31.3
52	*Cbl*	0.0098	2.6	28.7	30.5
53	*Lta*	0.0102	-129.3	29.8	40.0
54	*Tcf7*	0.0102	-289.3	23.9	35.2
55	*Itgb1*	0.0102	2.1	29.5	31.6
56	*Fcgr4*	0.0102	293.8	33.6	28.3
57	*Oas2*	0.0104	-646.6	27.6	40.0
58	*Cd3eap*	0.0104	-1.0	31.8	35.0
59	*Casp2*	0.0104	-127.3	29.8	40.0
60	*Traf2*	0.0105	-119.3	29.9	40.0

Rank	Gene	P value	Fold change	Ave Ct naïve	Tolerant Ct
61	*Mmp13*	0.0106	38.1	32.6	30.4
62	*Tyk2*	0.0106	-1.7	29.0	33.0
63	*Ikbkg*	0.0106	1.2	29.3	32.2
64	*Bcl6*	0.0108	-74.3	30.6	40.0
65	*Tollip*	0.0109	-115.6	29.5	39.6
66	*Il21r*	0.0112	-398.1	26.9	38.6
67	*Bcl2*	0.0113	-209.5	29.1	40.0
68	*Ncf4*	0.0114	-80.9	30.5	40.0
69	*B2m*	0.0115	-72.3	21.5	30.9
70	*Mapk8*	0.0115	-89.6	30.4	40.0
71	*Atf1*	0.0116	-114.0	30.0	40.0
72	*Map2k2*	0.0118	-2.7	33.2	37.7
73	*Atm*	0.0118	-502.8	28.0	40.0
74	*Tnfrsf18*	0.0120	-66.6	30.8	40.0
75	*Casp8*	0.0120	-113.7	26.9	36.9
76	*Stat1*	0.0120	-98.2	24.2	34.0
77	*Adam17*	0.0121	-47.1	31.3	40.0
78	*Ifnar1*	0.0121	-2.0	28.7	32.8
79	*Bcap31*	0.0121	-223.5	29.1	40.0
80	*Calr*	0.0122	-97.1	26.0	35.8
81	*Irf3*	0.0122	-348.4	28.5	40.0
82	*Psmb8*	0.0124	-1.2	28.3	31.7
83	*Lcp2*	0.0124	-15.9	26.3	33.5
84	*Nfkb1*	0.0125	-417.1	28.2	40.0
85	*Il16*	0.0125	-779.2	27.4	40.0
86	*H2-Aa*	0.0125	3.9	30.2	31.3
87	*Cd79a*	0.0126	3.1	33.1	34.7
88	*Il7r*	0.0127	-612.4	27.7	40.0
89	*Cd3e*	0.0127	-60.7	24.3	33.4
90	*Malt1*	0.0128	-96.7	30.2	40.0
91	*Icos*	0.0128	2.9	29.6	31.2
92	*Cd2ap*	0.0128	-9.2	28.2	34.6
93	*Nfil3*	0.0129	3.7	32.7	33.9
94	*Itga1*	0.0129	180.3	39.3	34.5
95	*Ifi35*	0.0130	-75.7	30.0	39.4
96	*Foxp3*	0.0131	-20.6	32.4	40.0
97	*Ccr7*	0.0131	-19.6	25.3	32.7
98	*Jak1*	0.0132	-54.8	25.3	34.2
99	*Ptprc*	0.0133	-18.3	25.4	32.8
100	*H2-D*	0.0133	-17.5	25.5	32.8
101	*Marco*	0.0133	38.8	35.3	33.0
102	*Nfkb2*	0.0134	-206.9	29.2	40.0
103	*Diablo*	0.0135	-6.6	29.6	35.4
104	*Chuk*	0.0135	1.5	31.1	33.6
105	*Il4ra*	0.0136	-12.5	27.5	34.3
106	*Cd28*	0.0136	-8.2	26.0	32.3
107	*Mthfr*	0.0136	-22.2	32.3	40.0
108	*Socs1*	0.0137	-119.8	30.0	40.0
109	*Tgfb1*	0.0137	-7.1	28.2	34.2
110	*Cblb*	0.0138	-203.9	29.2	40.0
111	*Rn18s*	0.0138	-22.0	14.9	22.6
112	*Ifnar2*	0.0138	-5.0	27.3	32.9
113	*Tcrd-C1*	0.0139	6.9	30.9	31.2
114	*Daxx*	0.0139	-103.3	27.5	37.4
115	*Rel*	0.0139	1.0	30.0	33.1
116	*Sigirr*	0.0141	-110.2	30.0	40.0
117	*Il6*	0.0141	78.6	34.1	30.7

Rank	Gene	P value	Fold change	Ave Ct naïve	Tolerant Ct
118	*Irak2*	0.0142	2.6	31.3	33.0
119	*Vav1*	0.0144	-8.8	27.1	33.4
120	*Stat3*	0.0144	-151.2	27.4	37.7
121	*Mapk1*	0.0144	-18.8	29.4	36.8
122	*Gata3*	0.0147	-173.1	29.5	40.0
123	*Il1rap*	0.0147	2.5	30.7	32.6
124	*Traf1*	0.0148	-7.3	31.3	37.4
125	*Cd74*	0.0148	1.6	29.3	31.8
126	*Tap1*	0.0149	-71.7	27.2	36.5
127	*Itgam*	0.0150	-1.3	33.9	37.4
128	*Fyn*	0.0150	-7.5	26.8	32.9
129	*Mif*	0.0151	-1.8	30.0	34.0
130	*Il12rb1*	0.0152	7.2	30.6	30.7
131	*Ada*	0.0152	-6.4	34.1	40.0
132	*Slamf1*	0.0153	-266.2	28.9	40.0
133	*Cd69*	0.0155	-18.6	26.2	33.6
134	*Fos*	0.0156	-19.0	25.5	32.9
135	*Lif*	0.0157	1.2	33.7	36.5
136	*IgM-C2v1*	0.0159	-7.7	25.6	31.8
137	*Prkcd*	0.0161	25.3	27.8	26.0
138	*Fn1*	0.0162	76.5	38.3	34.9
139	*Cd9*	0.0163	-13.1	29.9	36.8
140	*Ly75*	0.0164	-4.4	29.3	34.6
141	*Lck*	0.0165	-8.5	27.6	33.9
142	*Egf*	0.0171	-2.0	35.9	40.0
143	*Ebf1*	0.0172	16.3	34.4	33.4
144	*Mapk14*	0.0173	-1.4	29.2	32.8
145	*Cd55*	0.0176	-125.1	27.2	37.3
146	*H2-Eb1*	0.0177	1.7	32.1	34.5
147	*Ctsd*	0.0178	-24.2	27.5	35.3
148	*Tnfrsf11a*	0.0179	79.2	33.6	30.0
149	*Il2rg*	0.0180	-56.1	25.4	34.3
150	*Tfrc*	0.0182	-3.8	29.3	34.3
151	*Bcl3*	0.0182	-6.4	28.5	34.3
152	*Ifitm1*	0.0182	57.8	38.4	35.5
153	*Ptpn22*	0.0183	-6.5	27.9	33.8
154	*Syk*	0.0185	24.4	34.4	32.8
155	*Tnfrsf1b*	0.0186	-12.5	29.6	36.4
156	*Nod2*	0.0187	-5.3	34.5	40.0
157	*Lef1*	0.0189	-12.4	28.1	34.9
158	*Cdh5*	0.0189	194.8	38.6	33.3
159	*Cd6*	0.0193	-217.2	28.5	39.3
160	*Ccr2*	0.0196	8.4	33.7	33.6
161	*Sele*	0.0197	-8.2	33.8	40.0
162	*Il4*	0.0197	53.0	39.3	36.2
163	*Lat*	0.0198	-29.2	27.6	35.6
164	*Icam2*	0.0201	-80.2	30.6	40.0
165	*Ebi2*	0.0201	-95.4	30.3	40.0
166	*Fas*	0.0203	-1.6	32.2	36.0
167	*Casp3*	0.0206	-37.0	31.7	40.0
168	*Tnfsf10*	0.0208	-99.2	30.3	40.0
169	*Mapk3*	0.0209	-18.3	31.7	39.0
170	*Card11*	0.0211	-7.5	26.5	32.5
171	*Fasl*	0.0211	-54.8	31.1	40.0
172	*Csf3*	0.0211	208.8	38.5	32.9
173	*Sod1*	0.0214	-343.2	28.7	40.0
174	*IgG2a-*	0.0215	24.2	39.0	37.2

Rank	Gene	P value	Fold change	Ave Ct naïve	Tolerant Ct
	C3v1				
175	*Tnf*	0.0218	-132.2	30.0	40.0
176	*Inpp5d*	0.0220	-337.0	28.7	40.0
177	*H2-Ab1*	0.0220	4.1	31.3	32.3
178	*Ifna1*	0.0220	97.9	35.9	32.0
179	*Hmgb1*	0.0222	-7.7	27.2	33.3
180	*Cxcr4*	0.0225	-2.6	27.2	31.6
181	*Csf1r*	0.0228	4.3	33.6	34.5
182	*Ceacam1*	0.0231	-9.4	33.6	40.0
183	*Entpd1*	0.0233	28.6	36.9	34.8
184	*Ncr1*	0.0237	1.5	33.2	35.7
185	*Ccr4*	0.0241	2.7	34.2	35.7
186	*H2-K*	0.0241	-7.3	26.4	32.4
187	*Rorc*	0.0241	17.8	34.2	32.8
188	*Csf1*	0.0242	-1.4	35.6	39.0
189	*Tnfsf11*	0.0243	29.7	31.7	29.7
190	*Ccl3*	0.0243	6.0	33.4	33.7
191	*Klrc1*	0.0243	55.4	37.0	33.7
192	*Itgb2*	0.0249	-55.5	25.8	34.6
193	*Abl1*	0.0253	-6.0	30.2	35.8
194	*Tnfrsf13b*	0.0254	-18.2	31.1	38.4
195	*Selplg*	0.0255	-80.9	27.1	36.4
196	*Bid*	0.0257	-42.9	31.6	40.0
197	*Itga2b*	0.0258	9.6	33.3	32.8
198	*Birc3*	0.0260	-39.4	27.0	35.3
199	*Cd59a*	0.0263	71.6	38.3	34.7
200	*Gzma*	0.0263	-19.2	32.7	40.0
201	*Il2ra*	0.0284	-15.3	31.8	38.7
202	*Prf1*	0.0288	-2.1	29.6	33.6
203	*C1qa*	0.0297	-29.3	32.1	40.0
204	*Tapbp*	0.0297	-52.6	28.7	37.4
205	*Ly96*	0.0304	-1.8	32.0	35.8
206	*Irak1*	0.0309	-15.0	29.2	36.1
207	*Icam1*	0.0315	-99.1	29.2	38.7

Rank	Gene	P value	Fold change	Ave Ct naïve	Tolerant Ct
208	*Irf1*	0.0316	-2.0	27.3	31.2
209	*Camp*	0.0328	764.7	33.3	23.9
210	*Bcl2l1*	0.0328	-12.5	33.4	40.0
211	*Il11ra1*	0.0330	-7.5	34.0	40.0
212	*Frap1*	0.0336	-64.6	31.0	40.0
213	*Cd40lg*	0.0338	-773.5	27.8	40.0
214	*Cd180*	0.0344	4.0	35.4	36.2
215	*Nfkbia*	0.0347	-98.4	25.7	35.1
216	*Cxcl10*	0.0348	-1.0	29.5	32.3
217	*Nfatc2*	0.0354	-6.8	28.8	34.5
218	*Ptafr*	0.0358	145.3	37.3	32.2
219	*Irf8*	0.0359	-48.8	31.4	40.0
220	*Ltbr*	0.0361	14.4	36.0	34.9
221	*Akt1*	0.0362	-1.6	30.3	34.0
222	*Cd44*	0.0363	-7.9	34.1	40.0
223	*Cd8a*	0.0364	-71.2	31.0	40.0
224	*Il1b*	0.0384	26.8	33.2	30.9
225	*IgL-C1*	0.0386	21.6	39.0	36.9
226	*Il18r1*	0.0387	-78.6	30.9	40.0
227	*Cxcl2*	0.0395	-2.7	35.7	40.0
228	*Nos2*	0.0404	-6.4	34.5	40.0
229	*Casp1*	0.0419	-23.3	32.6	40.0
230	*Cd1d1*	0.0426	-61.1	31.2	40.0
231	*Csf3r*	0.0449	123.8	37.6	31.2
232	*Tlr7*	0.0461	29.3	36.1	33.5
233	*Ciita*	0.0462	1.3	32.7	34.9
234	*H2-Ea*	0.0486	20.4	36.7	34.6
235	*Bad*	0.0496	-10.8	33.7	40.0

S.5 Raw data: Significant genes in CD4$^+$ Thy1.2$^+$ Marilyn cells after i.n. HY-A^b*Dby* peptide during graft rejection.

Table S7 Genes significantly changed in CD4$^+$ Thy1.2$^+$ Marilyn cells at the time of graft rejection. Thy1.2$^+$ Marilyn cells were transferred into Thy1.1$^+$ female recipient mice. 10 days later these mice were given CFSE labelled male and female spleen cells. Up to 10 days after the CFSE labelled cells CD4$^+$ Thy1.2$^+$ were isolated by flow cytometry sorting RNA was extracted and analysed by quantitative PCR array. 75 genes were found to be significantly up or down regulated compared to RNA preparations from naïve CD4$^+$ T cells. Genes that were found to be downregulated are shown in blue.

Rank	Gene	P Value	Fold change	Ave Ct naïve	Rejecting Ct
1	*IgK-C*	0.0010	1470.40	36.7	25.1
2	*Il21*	0.0016	740.19	40.0	29.4
3	*Pax5*	0.0026	175.41	40.0	31.4
4	*Ifnb1*	0.0027	155.35	40.0	31.6
5	*Tlr2*	0.0037	82.73	39.6	32.1
6	*Ms4a1*	0.0038	72.17	40.0	32.7
7	*Cd80*	0.0041	63.46	40.0	32.9
8	*Cr2*	0.0047	49.20	40.0	33.3
9	*Tnfrsf13c*	0.0053	32.90	39.9	33.8
10	*Il17a*	0.0054	38.08	40.0	33.6
11	*Ccr7*	0.0055	-18.77	25.3	28.5
12	*Cd40*	0.0057	34.90	40.0	33.8
13	*Ccbp2*	0.0058	20.90	39.8	34.3
14	*Egf*	0.0071	-36.66	35.9	40.0
15	*Oas2*	0.0073	-58.89	27.6	32.4
16	*Mmp2*	0.0074	22.93	40.0	34.4
17	*Tal1*	0.0078	21.58	40.0	34.5
18	*Lep*	0.0084	19.24	40.0	34.6
19	*Cd14*	0.0090	38.56	39.7	33.2
20	*Hrh4*	0.0091	28.49	39.8	33.9
21	*Sele*	0.0099	-14.70	33.8	36.6
22	*Cd44*	0.0104	-84.24	34.1	39.3
23	*IgD-C1v1*	0.0105	14.79	32.7	27.7
24	*Il5*	0.0112	13.43	40.0	35.1
25	*Cd2ap*	0.0113	-7.30	28.2	30.0
26	*Ccr10*	0.0115	37.47	39.7	33.2
27	*Plau*	0.0126	18.65	39.4	34.0
28	*Entpd1*	0.0127	140.82	36.9	28.2
29	*Foxp3*	0.0128	8.66	32.4	28.2
30	*Pecam1*	0.0131	-7.62	26.6	28.5
31	*IgA-C2v1*	0.0137	31.63	34.4	28.2
32	*Il6st*	0.0142	-10.32	27.8	30.1
33	*Stat1*	0.0154	-7.13	24.2	26.0
34	*Ccl3*	0.0157	43.31	33.4	26.5
35	*Tnfaip6*	0.0158	9.03	40.0	35.7
36	*Il23r*	0.0161	8.87	40.0	35.7
37	*Il4ra*	0.0165	-6.65	27.5	29.2
38	*Ccr2*	0.0165	29.10	33.7	27.5
39	*Nfil3*	0.0168	13.24	32.7	27.8
40	*Stat5b*	0.0176	-5.48	28.0	29.5
41	*Casp3*	0.0180	11.99	31.7	26.9
42	*Lag3*	0.0183	155.99	34.0	24.9
43	*IgL-C1*	0.0184	196.80	39.0	29.4
44	*Il10*	0.0191	581.25	37.1	26.0
45	*Aicda*	0.0191	7.41	40.0	36.0
46	*Ccl2*	0.0192	9.25	39.5	35.1
47	*Il17f*	0.0193	7.03	39.4	35.5
48	*Lifr*	0.0196	11.59	39.7	35.0
49	*Ifit2*	0.0199	-9.19	27.5	29.6
50	*C1qa*	0.0209	-14.98	32.1	34.9
51	*Tnfrsf4*	0.0223	5.03	32.7	29.3
52	*Daxx*	0.0227	-5.41	27.5	28.9
53	*Lta*	0.0229	-4.98	29.8	31.1
54	*Ccl19*	0.0247	-3.76	34.2	35.1
55	*IgM-C2v1*	0.0259	-4.18	25.6	26.7
56	*Cd55*	0.0273	-6.77	27.2	28.9

Rank	Gene	P Value	Fold change	Ave Ct naïve	Rejecting Ct
57	*Ifnar2*	0.0278	-3.91	27.3	28.3
58	*Bcl2*	0.0288	-4.80	29.1	30.3
59	*Lef1*	0.0295	-4.87	28.1	29.4
60	*Il7r*	0.0303	-9.93	27.7	29.9
61	*Atm*	0.0309	-7.79	28.0	29.8
62	*Cx3cr1*	0.0309	55.32	38.5	30.6
63	*Ccl4*	0.0334	380.75	36.2	25.3
64	*Socs3*	0.0345	-5.69	26.9	28.3
65	*Slamf1*	0.0364	-6.00	28.9	30.3
66	*B2m*	0.0396	-3.35	21.5	22.3
67	*Jak1*	0.0427	-2.97	25.3	25.8
68	*Thy1*	0.0430	-3.34	23.7	24.4
69	*Tcf7*	0.0433	-3.67	23.9	24.7
70	*Cd69*	0.0437	-3.46	26.2	27.0
71	*Ly6a*	0.0438	-6.73	25.5	27.1
72	*Casp8*	0.0461	-2.88	26.9	27.4
73	*Gata3*	0.0463	-4.28	29.5	30.5
74	*Myd88*	0.0474	-2.82	29.3	29.8
75	*Cfb*	0.0483	-2.62	33.2	33.5