

AUSTRALIAN NATIONAL UNIVERSITY

LIBRARY

The persons whose signatures appear below have consulted this thesis by **TOSEI MURASE** and are aware that it is available for study only and that no quotations, or substantive information not otherwise available, may be published therefrom without the consent of the author and of

.....

Name (PRINT & Sign)	Date	Name (PRINT & Sign)	Date

Permission is given / ~~not given~~ to the University Librarian or his representative to allow persons other than students or members of staff of the University to consult my thesis only for the purposes of private study and research.

Roles of Notch and NF- κ B Signaling in Allogeneic Responses

By

Tosei Murase

**A thesis submitted for the degree of
Doctor of Philosophy
in the Australian National University**

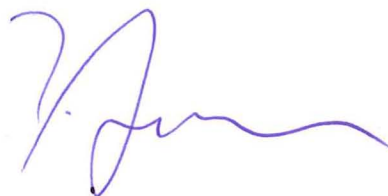
December 2006

No



Statement

The studies presented in this thesis constitute the original work of the author unless otherwise stated in the text. This thesis conforms to the Australian National University guidelines and regulations, and the work contained within has not been submitted for the purpose of obtaining any other degree at this or other universities.



Tosei Murase

December, 2006

List of abbreviations

ADCC	Antibody-Dependent Cytotoxicity
ANU	Australian National University
APC	Antigen Presenting Cell
BCR	B cell Receptor
bp	base pair(s)
CI-MPR	Cation-Independent Mannose-6-Phosphate Receptor
CLP	Common Lymphoid Precursor
CSL	CBF1/RBP-J kappa, Suppressor of Hairless, Lag-1
CTL	Cytotoxic T Lymphocyte
CTLA	Cytotoxic T Lymphocyte Antigen
DC	Dendritic Cell
DII	Delta-Like Ligand
DNA	Deoxyribonucleic Acid
DTH	Delayed Type Hypersensitivity
EGF	Epidermal Growth Factor
FACS	Fluorescence Activated Cell Sorter
Foxp3	Forkhead box p3
GFP	Green Fluorescent Protein
GITR	Glucocorticoid Induced Tumor Necrosis Factor Receptor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HT	High Titer
IC	Intracellular
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IKK	I κ B Kinase
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
i.p	intraperitoneal
i.v	intravenous
Jag	Jagged
JCSMR	John Curtin School of Medical Research
kDa	kilo Dalton
Lfng	Lunatic Fringe
LPS	Lipopolysaccharide
LT	Low Titer
MCP	Monocyte Chemotactic Protein

MFI	Mean Fluorescence Intensity
Mfng	Manic Fringe
MHC	Major Histocompatibility Complex
mRNA	Messenger Ribonucleic Acid
NCR	Notch Cytokine Response
NF- κ B	Nuclear Factor-Kappa B
NIK	NF- κ B-Inducing Kinase
NK	Natural Killer
ODN	Oligodeoxynucleotides
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pDONR	donor vector
PDTC	pyrroclidine dithiocarbamate
PMA/I	phorbol myristate acetate/Ionophore
Rfng	Radical Fringe
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th	T helper
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
UV	Ultraviolet
VCAM	Vascular Cell Adhesion Molecule

Acknowledgement

This work would not have been possible without the assistance, guidance and encouragement of many people. It is, of course, impossible to mention them all here.

I am deeply indebted to Dr. Charmaine Simeonovic and Professor Ian Ramshaw for giving me an opportunity to work on these studies and for taking me through the project with great patience. This work owes much to their incisive criticism and guidance over the years. I would also like to thank Dr. Gerard Hoyne for helping me with his expertise in Notch signaling and I owe greatly to his advice and assistance in completing this work. The members of Dr Simeonovic lab, Ms. Sarah Popp, Mr. Andrew Ziolkowski, Ms. Karla Harris, Ms. Debra Brown, Mr. Peter Hamilton, Ms. Celina Ann-Lynch, and Mr. Darren Newington provided me with tremendous technical support. For their assistance and encouragement, I am extremely grateful. I would like to thank Ms. Anne Prins for processing thousands of histology sections. Mr. David Mann, Ms. Jill Medvesky, Dr. Charani Ransinghe and Dr. Jodie Harrison helped me extensively during the generation and characterization of JAWS II cell constructs. Mr. Geoff Osbourne and Ms. Sabine Gruninger provided me with excellent support in FACS sorting and analyses. Dr. Mark Hulett kindly donated a Phoenix Eco cell line which was an integral component during the construction of JAWS II cells over-expressing Notch-related molecules. Dr. Sudah Rao, Dr. Torsten Juelich and Ms. Elissa Sutcliffe helped me extensively with protein work. I am grateful for their professional guidance and support as well as for being great friends inside and outside school. I also owe a great deal to Dr. Daniel Eichner, Mr. Geoff Sjollema, and Ms. Esther Ng for kindly reading and improving my thesis drafts. Without their friendship and help, I would have been hopelessly handicapped in completing this thesis.

I was extremely lucky to be surrounded by wonderful people in Australia and overseas. I

would like to thank Prof. Keith Williams and his colleagues who introduced me the world of science when I was at high school. I cannot thank enough for the experience and guidance that I was privileged to have at his laboratory and company. I would sincerely thank Michael Lavis for being a great friend since my undergraduate years. I would not have even made to the stage of starting this PhD without his kind support and friendship. I would also like to thank Hiroto Yamauchi for giving me the 'driving energy' through his great friendship throughout. Takashi Terada continuously gave me encouragement and wisdoms from Singapore and Japan and I am grateful that he always gave me support to continue on over tough and rough times. I was extremely lucky to have wonderful house-mates, Tao Kong, Torsten Juelich and Paul D'Arcy who made my time living in Weetangera so bright and colorful. I also owe much to Tien Luu, Allen Cheung, Haruo Nakagawa, Kojiro Kurahashi, Christian von Luebke, Runako Samata, Kyong-He Moon, Youn-Min Park, Steve Jarvis, Aki Asano, Kris Funston, Mark Nolan, Ryoko Tsurumaru, Yoko Yamaguchi, Shiro Armstrong, Tomohiko Satake, Naoya Fujita, Mark Grant, Kanako Nakahiro, Junko Sakata, Joe Marumo, Keisuke and Kasumi Horikawa, Ross and Jayne Garnaut and many others whom I am always privileged to be able to relax and share time with.

My life in Canberra would not have even come about without the generosity of the Drysdale family. Peter, Liz, Ben and Tigger treated me as their own family member throughout 11 years in Canberra and I cannot thank enough for their friendship, care, support and encouragement.

Finally, I would like to thank my family for their love. Your presence and encouragement allowed me to keep going forward. Arigatou.

Abstract

The induction of robust allograft tolerance is the ultimate goal for clinical transplantation. Although studies have identified that dendritic cells (DCs) are important for induction of antigen-specific tolerance, the requirements for generating tolerogenic DCs are yet to be elucidated. Recently, it has been demonstrated the modulation of two signaling pathways, Notch and nuclear factor κ B (NF- κ B) can render DCs tolerogenic. The studies documented here examine (1) whether immature DCs over-expressing Notch-related molecules (Jagged-1, Delta-like-1 (Dl1-1), Lunatic Fringe (Lfng), and Manic Fringe (Mfng)) act as immunoregulatory DCs and promote allograft survival; and (2) whether DCs deficient in NF- κ B signaling inhibit the alloreactive T cell response and promote allograft survival.

The immature DC cell line (JAWS II cells (H-2^b)) was retrovirally transduced to over-express murine (m)Jagged-1, mDl1-1, mLfng, or mMfng. JAWS II cells over-expressing Notch related molecules remained immature following transduction, however, these cells were unable to modulate an alloreactive T cell (H-2^k) response *in vitro*. Pretreatment of allogeneic CBA/H mice (H-2^k) with transduced JAWS II cells failed to promote C57BL/6 (H-2^b) thyroid allograft survival. Cellular transplantation of JAWS II cells over-expressing Notch related molecules were also acutely rejected in CBA/H mice suggesting lack of immunomodulation by genetically engineered JAWS II cells *in vivo*. In addition, cellular grafts of JAWS II cells to H-2-compatible mice (H-2^b) were chronically rejected, indicating that JAWS II cells and C57BL/6 mice differ at one or more minor histocompatibility loci.

The NF- κ B inhibitor, BAY11-7082 (BAY), and cRel inhibitor, Pentoxifylline (Ptx), were used to prevent NF- κ B signaling in C57BL/6 (H-2^b) splenocytes and bone marrow-derived DCs (BMDCs). BAY treatment abrogated the capacity of splenocytes (and to a lesser extent

BMDCs) to stimulate allogeneic (H-2^k) T cells. This effect correlated with reduced expression of costimulatory molecules and major histocompatibility complex (MHC) Class II molecules on the treated splenocyte and BMDC population. It was also shown that BAY-treated splenocytes did not produce inflammatory cytokines including interferon (IFN)- γ , tumor necrosis factor (TNF)- α , IL-2 and IL-4, and produced less IL-5 compared to untreated splenocytes. Although allogeneic T cells did not proliferate in response to BAY-treated splenocytes, subsequent T cell proliferation in response to a secondary stimulus was observed *in vitro*. T cells previously exposed to BAY-treated splenocytes also failed to inhibit naïve T cell proliferation indicating lack of regulatory T cell differentiation.

Based on the finding that BAY treatment inhibited the capacity of APCs to induce alloreactive T cell proliferation *in vitro*, we examined whether BAY treatment of thyroid tissue or adult islets prior to transplantation inhibited the immunostimulatory capacity of donor passenger leukocytes to prime recipient T cells. Allografts precultured with BAY were rejected with similar kinetics to control cultured allografts, indicating that *in vitro* exposure to BAY was not sufficient to prevent alloreactive T cell activation by donor passenger leukocytes. However, CBA/H (H-2^k) mice which received BAY-treated splenocytes or BAY-treated BMDCs intravenously prior to implantation of C57BL/6 (H-2^b) thyroid tissue demonstrated prolonged allograft survival. This finding indicates that an *in vivo* environment provides additional signal(s) which are absent in the *in vitro* system and which are necessary for modulating alloresponses. The mechanism by which BAY splenocytes and BAY BMDCs prolong allograft survival requires further investigation.

Although the potential for Notch signaling to promote alloantigen-specific tolerance remains unresolved, these studies suggest that inhibition of NF- κ B signaling in DCs represent a potential approach for promoting allograft survival.

Table of contents

Statement.....	ii
List of abbreviations	iii
Acknowledgement.....	v
Abstract	vii
Table of contents	ix
Chapter 1	1
1.1 Introduction	2
1.2 Immunobiology of transplantation	2
1.2.1 T cells in transplantation	2
1.2.2 Priming of alloreactive T cells.....	9
1.3 Current immunotherapeutic strategies	15
1.4 Tolerance induction	20
1.4.1 DCs and tolerance induction	20
1.4.2 DC tolerance and transplantation.....	24
1.4.3 Regulatory T cells and tolerance	26
1.5 Notch signaling pathway	30
1.5.1 Structure of Notch receptors	31
1.5.2 Notch signaling pathway and its modulation	34
1.5.3 Immune regulation by Notch signaling	38
1.5.4 Notch Signaling pathway and tolerance Induction.....	42
1.6 NF- κ B transcription family	43
1.6.1 NF- κ B signaling pathways	45
1.6.2 NF- κ B and the immune system	49
1.6.3 NF- κ B and dendritic cell function.....	53
1.6.4 NF- κ B and tolerance induction	55
1.6.5 NF- κ B and allotransplantation	56
1.7 Scope of thesis	59
Chapter 2	61
2.1 Mice and reagents	62
2.1.1 Animals.....	62
2.1.2 Media and buffers	62
2.1.3 Cell lines	62
2.2 Molecular techniques	63
2.2.1 RNA extraction	63
2.2.2 Reverse transcription of mRNA to cDNA	63

2.2.3	Semi-quantitative RT-PCR analysis	64
2.2.4	Polymerase chain reaction for Gateway™ cloning technology	65
2.2.5	Sequencing of pKMV constructs with Notch-related genes.....	65
2.2.6	Restriction digest	66
2.3	<i>In Vitro</i> techniques	66
2.3.1	Production of retrovirus and retroviral transduction of JAWS II cells	66
2.3.2	Splenocyte preparation	70
2.3.3	Lymph node cell preparation	71
2.3.4	Isolation of bone marrow-derived DCs	71
2.3.5	Flow cytometry analysis.....	71
2.3.6	Mixed lymphocyte reaction (MLR).....	73
2.3.7	Nuclear extraction	74
2.3.8	Western blot.....	75
2.4	<i>In Vivo</i> techniques.....	76
2.4.1	Thyroid and adult islet isolation and transplantation.....	76
2.4.2	Cellular transplantation.....	79
2.4.3	Harvest of grafts	80
2.4.4	Histological analysis	80
2.4.5	Morphometric analysis of histological sections of transplants	80
Chapter 3	81
3.1	Introduction	82
3.2	Construction of pKMV-Δ Gateway™ destination vector	85
3.3	Construction of pDONR of Notch related genes.....	90
3.4	Construction of pKMV vector carrying Notch-related genes.....	91
3.5	Construction of JAWS II cells over-expressing Notch related molecules	91
3.6	mJagged-1, mLfng, mMfng and Delta-III1 expression.....	98
3.7	Surface marker profiles of JAWS II cells.....	101
3.8	Functional studies of JAWS II cells transduced with Notch related molecules	106
3.9	Discussion	111
Chapter 4	115
4.1	Introduction	116
4.2	Thyroid allotransplantation in mice receiving a single treatment of JAWS cells over-expressing Notch related molecules (LT).....	117
4.3	Thyroid allotransplantation in mice receiving a single treatment of JAWS cells over-expressing Notch related molecules (HT).....	122
4.4	Thyroid allotransplantation in mice receiving multiple treatment of JAWS-II cells over-expressing Notch-related molecules (HT)	129
4.5	Immunogenicity of JAWS II cells in MHC-compatible and MHC-incompatible mice	139

4.6 Discussion	142
Chapter 5	149
5.1 Introduction	150
5.2 Confirmation of NF- κ B signaling inhibition by BAY11-7082 and pentoxifylline treatment.....	152
5.3 Inhibition of immunostimulatory property of splenocytes with BAY treatment.....	153
5.4 Effects of BAY treatment in cytokine production	158
5.5 Effects of BAY treatment on splenocyte proliferation	160
5.6 Effects of BAY in splenocytes at different stages of activation.....	163
5.7 Effects of Pentoxifylline treatment on splenocytes	166
5.8 Effects of BAY on BMDCs.....	168
5.9 Characterization of T cells exposed to BAY splenocytes	172
5.10 Proportion of CD4 ⁺ and CD8 ⁺ T cells in primary and secondary MLR.....	176
5.11 Discussion	178
Chapter 6	186
6.1 Introduction	187
6.2 Identification of the optimal dose of BAY for thyroid tissue.....	188
6.3 Assessment of survival of BAY-cultured thyroid allografts.....	193
6.4 cRel ^{-/-} thyroid allotransplantation	200
6.5 Effect of culturing adult islets with BAY11-7082 prior to allotransplantation	203
6.6 Thyroid allotransplantation to recipient mice preconditioned with BAY-treated donor splenocyte or BMDC.....	206
6.7 Discussion	212
Chapter 7	217
7.1 General discussion	218
Chapter 8	226
8.1 Bibliography.....	227
Appendix.....	261
Appendix 1 Media and Buffers.....	262
Appendix 2 Primers used for PCR reactions and their conditions	264
Appendix 3 Primers used for PCR amplification of Dll-1 with attB sites on 5' and 3' ends and their reaction conditons.....	265
Appendix 4 Primers used for Sequencing.....	266
Appendix 5 Sequencing data	266