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Roles of Notch and NF-κB Signaling in Allogeneic Responses

By

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Statement

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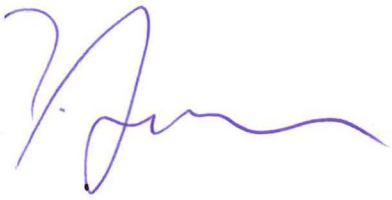

Tosei Murase

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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
Dll	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	IκB 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	Lunativc 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-κB	Nuclear Factor-Kappa B
NIK	NF-κB-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

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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.

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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 κB (NF-κB) can render DCs tolerogenic. The studies documented here examine (1) whether immature DCs over-expressing Notch-related molecules (Jagged-1, Delta-like-1 (Dl1-l), Lunatic Fringe (Lfng), and Manic Fringe (Mfng)) act as immunoregulatory DCs and promote allograft survival; and (2) whether DCs deficient in NF-κB signaling inhibit the alloreactive T cell response and promote allograft survival.

The immature DC cell line (JAWS II cells ($H\text{-}2^b$)) was retrovirally transduced to over-express murine (m)Jagged-1, mDll-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 ($H\text{-}2^k$) response *in vitro*. Pretreatment of allogeneic CBA/H mice ($H\text{-}2^k$) with transduced JAWS II cells failed to promote C57BL/6 ($H\text{-}2^b$) 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 ($H\text{-}2^b$) were chronically rejected, indicating that JAWS II cells and C57BL/6 mice differ at one or more minor histocompatibility loci.

The NF-κB inhibitor, BAY11-7082 (BAY), and cRel inhibitor, Pentoxifylline (Ptx), were used to prevent NF-κB signaling in C57BL/6 ($H\text{-}2^b$) 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-κB signaling in DCs represent a potential approach for promoting allograft survival.

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Chapter 1

Introduction

1.1 Introduction

During the past several decades, there has been tremendous progress in understanding allograft rejection and inducing transplant tolerance. It is understood that while the innate immune system can contribute to the rejection process, the adaptive immune response plays the dominant role in both acute and chronic allograft rejection (Bolton et al., 1989; Heeger, 2003). The major obstacle in successful allotransplantation is the histocompatibility disparity between the donor and recipient. The requirement of alloreactive T cells and antigen presenting cells (APCs) in allograft rejection is well documented (Heeger, 2003; Lafferty et al., 1983; Simeonovic et al., 1996). Much progress has been made in elucidating the mechanism by which these mediators cause graft destruction. Immunosuppressive regimes, including costimulatory blockade with or without donor-specific transfusion, have been a major development in controlling the anti-graft immune response (Parker et al., 1995). Furthermore, although immunosuppressive drugs can prevent graft rejection, the treatment dampens the body's general immune capacity. The major focus in transplantation studies is, therefore, the induction of alloantigen specific unresponsiveness in recipients or allo-specific tolerance. Recent investigations have demonstrated that dendritic cells (DCs) are capable of mediating immune tolerance to specific antigens via induction of protective or regulatory T cell immune responses (Jonuleit et al., 2001; Mondino et al., 1996). There is accumulating evidence that modulation of Notch signaling pathway can potentially induce antigen-specific tolerance (Osborne and Miele, 1999). It is also evident that the nuclear factor κB (NF-κB) pathway is a major player in activation of T cell-mediated immunity (Caamano and Hunter, 2002; Viatour et al., 2005).

1.2 Immunobiology of transplantation

1.2.1 T cells in transplantation

Allograft rejection is essentially mediated by T cells (Auchincloss and Sultan, 1996; Gill et

al., 1996; Yamamoto et al., 1990). The critical role of T cells in allograft rejection is attributed to their capacity to respond to allogeneic MHC molecules much greater than to nominal foreign antigen (Lechler et al., 1990). Alloreactive T cells represent the naïve T cell population that recognizes allogeneic MHC antigens in the context of self-MHC with high specificity (Auchincloss and Sultan, 1996; Matesic et al., 1998; Warrens et al., 1994). Because of the similarity between allogeneic and self MHC molecules, alloreactive T cells are stimulated by MHC antigens without requirement of processing and presentation as peptides (Auchincloss and Sultan, 1996; Warrens et al., 1994). The critical requirement of T cells in allograft rejection was demonstrated by Miller who showed an absence of allograft rejection in thyrectomized rats or mice lacking T cells (Miller, 1963). Similarly, studies have shown that skin and cardiac allografts were not rejected in the absence of T cells (Hall et al., 1978; Rosenberg and Singer, 1992). Other studies demonstrated that reconstitution of T cells alone can induce acute rejection in immunodeficient mice in a skin allograft model (Dallman et al., 1982; Loveland et al., 1981). Furthermore, Pearson *et al.* showed an induction of alloreactive T cell tolerance alone could protect allografts long-term (Pearson et al., 1992). These studies collectively indicate that T cells are the principal effector cells that need to be controlled in order to prevent the allograft rejection.

Although allograft rejection process is primarily mediated by T cells, immune cells involved in allograft rejection depend on whether it is an organ allograft (e.g. heart, lungs, and kidneys) or cellular allograft (e.g. pancreatic islets). Since organ allografts are revascularized by donor-derived endothelial cells, they are predisposed to hyperacute rejection mediated by pre-existing antibodies against these endothelial cells (reviewed in (Snanoudj et al., 2005)). B cell response as well as T cell response is therefore an important component in rejection of vascularized organ allografts. In contrast, cellular allografts become revascularized by host vascular endothelium therefore they are not subjected to hyperacute rejection mediated by

the antibodies. Thus, cellular allograft rejection is dominated by anti-allograft T cell responses.

1.2.1.1 $CD8^+$ T cells

The contribution of different T cell subsets to rejection depends on the type of tissue transplanted and the donor/recipient mouse strains used. Cellular allograft rejection is predominantly a Th1 mediated immune response and $CD8^+$ T cells have been strongly associated with graft rejection (Simeonovic et al., 1999; Yamamoto et al., 1990). While High-oxygen-culture of allogeneic thyroids and islets results in a permanent allograft survival, these grafts can be rejected by host immunization with donor-type spleen cells (Bowen et al., 1981; Lafferty et al., 1983). Subsequent experiments revealed that this induced rejection was mediated by $CD8^+$ T cells (Prowse et al., 1983; Warren et al., 1986). Anti-CD8 blockade and adoptive transfer studies have also shown that $CD8^+$ T cells are the primary effector cells in skin and pancreatic islet allograft destruction (Jones et al., 2001; Simeonovic et al., 1996; Simeonovic et al., 1999; Yamamoto et al., 1990). Consistently, MHC Class I-deficient islet allografts are permanently accepted, further indicating the central role of $CD8^+$ T cells in rejection of cellular grafts (Markmann et al., 1992). Although $CD8^+$ T cells are known to be the main effector population in an anti-allograft response, the precise mechanisms by which these cells cause graft destruction is not clearly understood. The perforin/granzyme pathway and Fas/FasL-mediated apoptosis are the two main pathways by which cytotoxic $CD8^+$ T cells mediate target cell lysis (Ahmed et al., 1997). There is evidence showing contributions of these apoptotic pathways to transplant rejection in a range of species (Kown et al., 2000; Narula et al., 2001; Shim et al., 2002). Several studies have demonstrated prolonged allograft survival in mice and humans by inhibiting these apoptotic pathways (Kagi et al., 1994; Krupnick et al., 2002; Wever et al., 1998). Motyka *et al.* reported that the cation-independent mannose-6-phosphate receptor

(CI-MPR)-mediated apoptosis represent another pathway that can be employed by $CD8^+$ T cells to destroy allografts (Motyka et al., 2000). It was shown that CI-MPR can also facilitate granzyme-B mediated apoptosis and the degree of murine islet allograft rejection was significantly reduced in CI-MPR-deficient recipients (Motyka et al., 2000). However, perforin-deficient and Fas-deficient mice showed a lack of perforin and Fas alone or together did not prevent allograft rejection (Ahmed et al., 1997; Schulz et al., 1995). These studies indicate perforin and Fas pathways alone are not the primary effector mechanism for allograft rejection.

The rejection of skin and fetal proislet allografts correlates with a Th1 cytokines profile including IL-2, IFN-γ, IL-12 and IL-3 (Matesic et al., 1998; Simeonovic et al., 1999). Studies revealed enhanced expression of IL-2 and IFN-γ during pro-islets and adult islet allograft rejection (O'Connell et al., 1993; Simeonovic et al., 1996). Likewise, pretreatment of adult islet with IFN-γ has been shown to evade cyclosporine A induced protection of allografts in rat (Hao et al., 1990) indicating cytokines as important mediator of allograft rejection. Nevertheless, cardiac allografts in IFN-γ knockout mice demonstrated that rejection was not prevented in the absence of IFN-γ (Saleem et al., 1996). IL-2 knockout mice were also shown to be able to reject islet allografts *in vivo* with CD4+ and CD8+ T cell infiltration into the graft, granzyme B activity, and enhanced expression of cytokines including IFN-γ, IL-4, IL-7 and IL-10 (Steiger et al., 1995). Furthermore, skewing the cytokine profile from Th-l type to Th-2 type immune response using IFN-γ or IL-2 deficient mice (Dai et al., 1998; Nicolls et al., 2002; Simeonovic et al., 1999) failed to promote allograft survival. Consistently, there are several reports that Th-2 type cytokines such as IL-4 and IL-10, have been observed in graft rejecting mice indicating the participation of Th-2 type cytokines can occur during the rejection of some allografts (Langer et al., 2004). In addition, it has been suggested that other immune cells such as eosinophils or neutrophills

could contribute to in the destruction of the graft in such settings (Almirall et al., 1993; Le Moine et al., 1999; Le Moine et al., 1999; Surquin et al., 2005). Together although cytokines are critical in directing immune responses, these studies suggest that the polarization of the cytokine response may contribute but is not essential for allograft rejection.

1.2.1.2 $CD4^+$ T cells

$CD4^+$ T cells can also play influential roles in mediating graft rejection. It was shown that depleting anti-CD4 monoclonal antibody administration to cardiac allograft recipients can allow permanent graft survival in mice (Jaques et al., 1998; Krieger et al., 1996). In accordance, prolonged cardiac allograft survival was observed in CD4-knockout mice (Youssef et al., 2004). In acute phase, $CD4^+$ T cells can produce Th1 proinflammatory cytokines and provide helper signals to enhance the activation of $CD8^+$ T cells and they can also initiate the production of alloantibodies (Krieger et al., 1996). $CD4^+$ T cells are therefore considered to play an important role in organ allograft rejection. In cellular allografts, however, $CD4^+$ T cells have been considered to play a less prominent role during rejection. Cellular allografts including hand-picked adult islet allografts do not express MHC Class II molecules prior to acute rejection (Markmann et al., 1987) and expression of MHC Class II molecules is evident only after the islets have been damaged (Warren et al., 1992). Since donor MHC Class II molecules are absent for recipient $CD4^+$ T cells to recognize, $CD4^+$ T cell may play a secondary effector role. Simeonovic *et al.* demonstrated that anti-CD4 monoclonal antibody treatment only resulted in a transient restoration of adult islet allograft survival while anti-CD8 monoclonal antibody treatment protected the graft indefinitely (Simeonovic et al., 1996). Observation that $CD4^+$ T cells alone were not sufficient for islet allograft rejection where $CD8^+$ T cell were also required, confirms that $CD4^+$ T cell are not primary effector cell population (Wolf et al., 1995). Although $CD4^+$ T cells are not primarily involved in cellular allograft rejections, there are studies indicating some role of $CD4^+$ T

cells in allograft rejection. Depleting anti-CD4 monoclonal antibody treatment was shown to be effective in inducing prolonged survival of murine adult islet allografts (Alters et al., 1993). Coloumbe *et al.* also demonstrated that the degree of islet allograft rejection was maximal when both $CD4^+$ T cells and $CD8^+$ T cells were present although $CD8^+$ T cells alone could induce rejection (Coulombe et al., 1999). Collectively, these studies indicated that $CD4^+$ T cell does not act as the primary effector cells but play a role to exacerbate cellular allograft rejection. It has been shown that indirectly primed $CD4^+$ T cells produce IL-2 and IFN-γ (Benichou et al., 1999). Via production of these cytokines and cell contact dependent helper signals (such as CD40), $CD4^+$ T cells can amplify cytotoxic $CD8^+$ T cell activity leading to increased graft damage (Lovegrove et al., 2001; Pettigrew et al., 1998; Schoenberger et al., 1998).

$CD4^+$ Th1 cells can also directly damage allografts via delayed type hypersensitivity (DTH). DTH has been considered as another contributing factor for allograft rejection (Rocha et al., 2003). DTH is facilitated by $CD4^+$ T cells secreting proinflammatory cytokines, including, IFN-γ and TNF-α (Rocha et al., 2003). These cytokines mediate graft destruction by activating monocytes and macrophages, which produce proteolytic enzymes, nitric oxide, and other soluble factors to cause damage to the graft (Le Moine et al., 1999; Mogayzel et al., 2001; Wyburn et al., 2005). The activation of these cells also leads to further amplification of cytokines and chemokines. The contribution of DTH in allograft rejection has been demonstrated in a number of studies (Dalloul et al., 1996; Saleem et al., 1996; Vella et al., 1997). In a murine skin allograft model, adoptive transfer of $CD4^+$ T cells from $CD8^+$ T cell deficient mice could induce allograft rejection in the absence of a CTL response indicating that DTH alone could mediate graft rejection (Dalloul et al., 1996). Furthermore, Valujskakh *et al.* also showed the adoptive transfer of allo-antigen specific Th1 cells could induce skin allograft rejection via DTH (Valujskikh et al., 1998).

While it is still controversial, several studies suggest that direct priming of $CD4^+$ T cells by donor APCs is important in priming alloreactive $CD8^+$ T cells. It was shown that cross-priming of some alloreactive $CD8^+$ T cells requires helper signals derived from $CD4^+$ T cells, such as IL-2 and CD40 (Bennett et al., 1997; Schoenberger et al., 1998). These $CD4^+$ T cells can only prime $CD8^+$ T cells when both populations recognize the same DC (Bennett et al., 1997). These studies collectively suggest that activation of DCs by $CD4^+$ T cells via CD40-CD40L ligation is important for subsequent priming of $CD8^+$ T cells that interact with the same DC (Bennett et al., 1997; Schoenberger et al., 1998).

During the last decade, $CD4^+$ T cells, in particular, $CD4^+$ $CD25^+$ regulatory T cells have emerged as a critical population required for induction of transplant tolerance (discussed further in Section 1.4.3) (Feng and Wood, 2004; Gill et al., 1996; Rossini et al., 1999; Sanchez-Fueyo et al., 2002; Taylor et al., 2001). A number of studies have demonstrated that $CD4^+$ T cells in allograft tolerant mice possess a capacity to suppress syngeneic alloreactive $CD8^+$ and $CD4^+$ T cell responses (Coulombe et al., 1999; Rossini et al., 1999; Sanchez-Fueyo et al., 2002; Taylor et al., 2001). Rossini *et al.* showed that islet allograft tolerance induced by donor-specific transfusion in combination with anti-CD154 treatment requires presence of $CD4^+$ T cells, IFN-γ and CTLA-4 (Markees et al., 1998; Rossini et al., 1999). Consistently, high-oxygen-culture induced islet allograft tolerance has shown to be $CD4^+$ T cell-dependent (Coulombe et al., 1999). This study also showed that these tolerant states to islet allograft can be adoptively transferred to naïve mice by $CD4^+$ but not $CD8^+$ T cells harvested from tolerant mice (Coulombe et al., 1999). In this process, peripheral $CD4^+CD25^+$ T cells have been identified to be an important population for tolerance maintenance (Gill et al., 1996; Sanchez-Fueyo et al., 2002). Taken together these studies indicate an active involvement of $CD4^+$ T cells (regulatory T cells) in induction and

maintenance of allograft tolerance.

1.2.2 Priming of alloreactive T cells

1.2.2.1 Priming of T cells via direct pathway

Recipient T cells can be primed by APCs in two ways; by donor-derived (via direct presentation) and recipient-derived (via indirect presentation) APCs [Figure 1.1] (Gould and Auchincloss, 1999; Heeger, 2003). The direct pathway is a process in which donor-derived APCs carried within the graft (passenger leukocytes) primes recipient-derived alloreactive T cells. It is considered that the direct pathway is particularly important during the acute rejection of MHC-disparate cellular allografts.

Lafferty *et al.* showed that alloantigens are presented to alloreactive T cells by passenger leukocytes, consisting of APCs carried within the graft (Lafferty et al., 1975; Lafferty et al., 1983; Simeonovic et al., 1984). These studies showed removal of these passenger leukocytes from thyroid and islet allografts by a high oxygen culture indefinitely prolonged the survival of these allografts (Lafferty et al., 1975; Simeonovic et al., 1984). Subsequent study showed that allograft rejection was restored when these recipient mice received donor type splenocytes (Lafferty, 1980). Passenger leukocytes express major histocompatibility complex (MHC) class I and II alloantigens together with costimulatory molecules and are thus capable of providing the two necessary signals for activation of alloreactive T cells. These studies established the importance of direct pathway in cellular allograft rejection. Following Lafferty's report, the importance of donor derived APCs but not the recipient APCs was further highlighted by Gould and Auchincloss (Gould and Auchincloss, 1999; Lafferty et al., 1983). Using mixed lymphocyte reactions, they were able to demonstrate that alloreactive T cell proliferation was not affected by the removal of recipient APCs whereas it was significantly reduced by the removal of donor APCs (Gould and Auchincloss, 1999). Recent

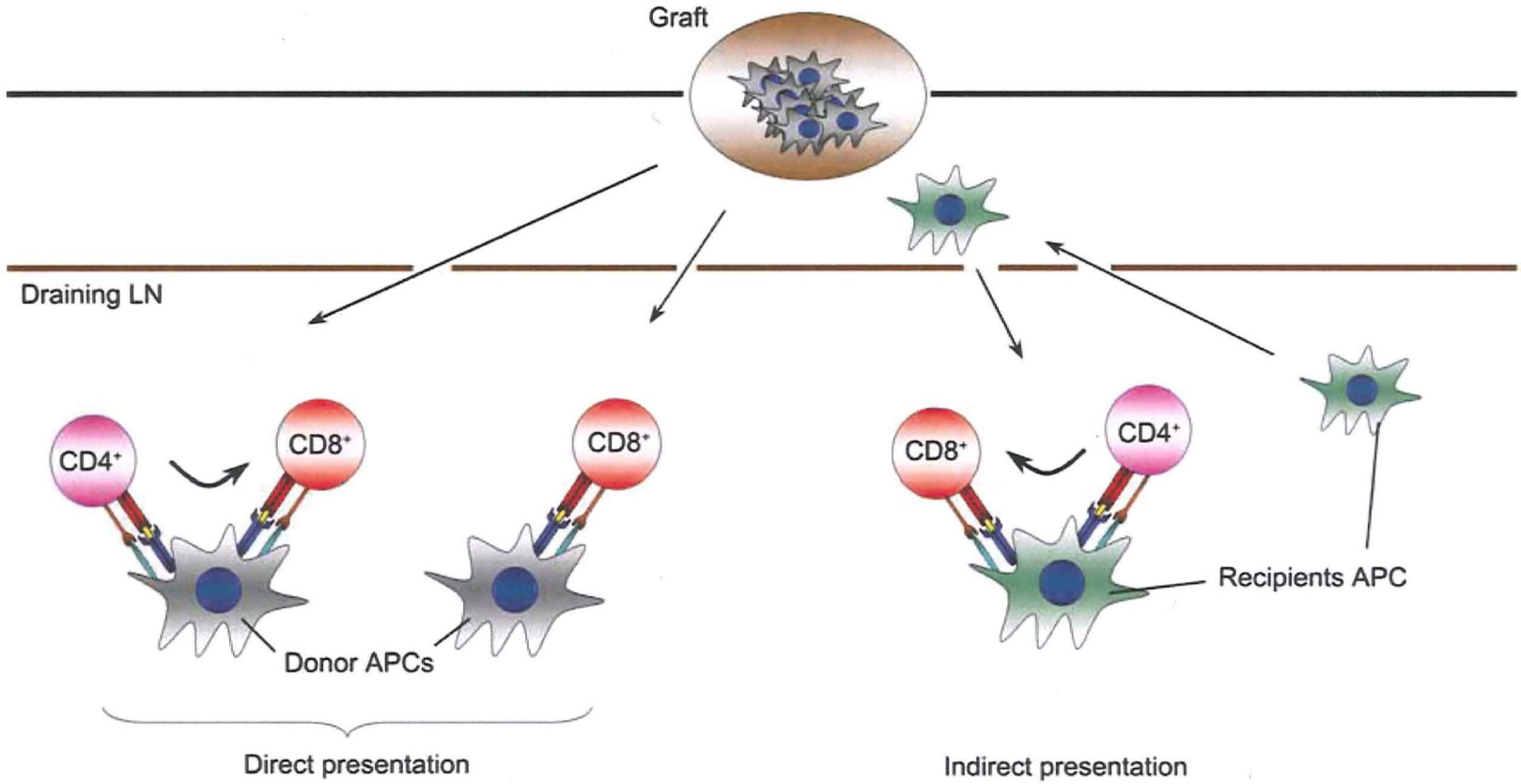

Graft
Draining LN
CD4+
CD8+
CD8+
Donor APCs
Direct presentation
CD8+
CD4+
Recipients APC
Indirect presentation

Figure 1-1: Schematic diagram of the direct and indirect presentation of allopeptides. In the direct pathway, T cells recognize intact MHC molecules on donor APCs. The donor APCs presenting alloantigen together with costimulatory molecules can activate alloreactive $CD8^+$ and $CD4^+$ T cells. In the indirect pathway, T cells recognize processed alloantigen by recipient APCs. The alloantigen can be presented to $CD4^+$ T cells and also to $CD8^+$ T cells by cross presentation.

studies detected donor APCs in recipient lymphoid tissues post-transplant and donor APCs migrating into T cell rich areas of the draining lymph nodes where they may potentially prime alloreactive T cells (Larsen et al., 1990; Saiki et al., 2001). Subsequent experiments demonstrated that these secondary lymphoid organs are critical in allograft rejection presumably due to the initial priming of T cells (Lakkis et al., 2000). These experiments demonstrated that removal of secondary lymphoid organs prevented the graft rejection; however, survival was not due to tolerance but rather T cell ignorance against the graft (Lakkis et al., 2000).

Allogeneic (donor-type) APCs express their own set of MHC molecules capable of priming recipient alloreactive T cells through the direct allorecognition pathway. Approximately 10 percent of self-restricted T cell clones bear T cell receptors (TCR) capable of recognizing allogeneic MHC/peptide complexes (Ben-Nun et al., 1983). The frequencies of T cell precursor recognizing allogeneic MHC/peptide was reported to be a 10- to 100-fold higher than to nominal foreign antigens (Shoskes and Wood, 1994). These cross-reactive T cells can recognize any peptide present in allogeneic MHC molecules (Smith and Lutz, 1997). Crystal structure analysis has shown that interaction of cross-reactive TCRs and allogeneic MHC is achieved in a manner similar to the interaction between self-MHC molecules (Reiser et al., 2000). The 'plasticity' of T cells specific for syngeneic MHC/peptide has been demonstrated by cross-reactivity with allogeneic MHC (Ben-Nun et al., 1983). In addition to allogeneic cross-reactivity, MHC/minor antigenic peptides can interact in a similar manner. For example, the rejection of MHC-compatible male tissues by female recipient mice is due to anti-Y minor alloantigen response (Scott et al., 1997).

The exact duration for which donor APCs are present in the recipient is unclear but it has been suggested that the survival of donor allogeneic APCs in the recipient is limited (Saiki et

al., 2001). It is therefore considered that direct priming is important for acute allograft rejection. Consistent with this hypothesis, a recent study reported that direct alloreactivity was not detectable in the peripheral blood of renal allograft recipients with chronic allograft dysfunction several years after transplantation (Baker et al., 2001).

1.2.2.2 Priming of T cells via indirect pathway

The indirect pathway is a process in which donor-derived antigens are taken up by recipient-derived APCs and presented to recipient T cells. This pathway allows recipient T cells to respond to graft-derived alloantigen in the context of syngeneic MHC complex i.e. host MHC/allo-peptide complex. Indirect presentation could occur in a number of ways. In the first scenario, donor DCs migrate to the recipient's secondary lymphoid organs where they are endocytozed by recipient APCs, which present antigens to syngeneic host T cells (Benichou et al., 1992). Secondly, donor alloantigens/cellular debris from the graft could reach the recipient's secondary lymphoid organ through the bloodstream then become processed by recipient APCs (Demaria and Bushkin, 2000). Finally, recipient APCs could enter the graft where they endocytoze alloantigen then migrate to the recipient's secondary lymphoid organ and prime recipient's T cells (Denton et al., 1999). Accumulating evidence suggests that the indirect pathway plays a very prominent role in the chronic phase of graft rejection of organ allografts (Baker et al., 2001; Liu et al., 1996; Vella et al., 1997). In cellular allografts, a long term survival of high oxygen cultured thyroid and adult islet allograft (Lafferty et al., 1975; Lafferty et al., 1983) argues that indirect processing of MHC alloantigen is less prominent in neovascularized allograft rejection.

In acute rejection, the frequency of directly primed T cells is higher than those indirectly primed (Benichou et al., 1999; Liu et al., 1993). This observation, together with the strength of T cell stimulation by the direct pathway in comparison to the indirect pathway, seemed to

suggest that the indirect pathway may contribute less to the induction of acute allograft rejection (Auchincloss and Sultan, 1996). However, the contribution of the indirect pathway in allograft rejection has been demonstrated by studies showing accelerated skin and kidney allograft rejection in recipient previously immunized with peptides of donor MHC antigens (Benham et al., 1995; Fangmann et al., 1992). Studies have shown that the source of peptides presented via the indirect pathway is frequently donor MHC derived (Benichou et al., 1994; Benichou et al., 1992; Gould and Auchincloss, 1999; Watschinger et al., 1994). In organ allografts, the indirect responses could play an influential role in graft rejection. The indirect pathway alone has been shown to be capable of inducing graft destruction in a skin allograft model (Vella et al., 1997). Auchincloss *et al.* showed that indirect response could initiate skin allograft rejection by using MHC Class II deficient mice as the graft donor. Since these skin grafts could not directly prime recipient $CD4^+$ T cells, the allograft rejection suggested that indirectly primed T cells could induce allograft rejection (Auchincloss et al., 1993). The prominent role of indirect priming in cardiac allograft rejection has also been demonstrated (Reed et al., 1996). In contrast to organ allografts, the indirect pathway is considered to play a less prominent role in cellular allograft rejection (Nicolls et al., 2001). Although, the direct presentation of alloantigen is the predominant pathway in cellular allograft rejection, the contribution of indirect pathway in cellular graft rejection has also been reported. Indirect $CD4^+$ T cell-dependent reactivity was found to contribute to islet allograft rejection and this recognition process required donor-derived APCs as the source of antigen (Nicolls et al., 2001). This finding indicated that donor-derived APCs can also trigger indirect form of anti-allograft immune response leading to rejection.

It has been reported that $CD4^+$ T cells recognize allogeneic MHC Class I peptides exclusively via the indirect pathway (Pettigrew et al., 1998). The molecular mechanism by which the indirect pathway mediates graft destruction is unclear. However, it has been

suggested that indirect antigen presentation by recipient DCs could be responsible (Rifle and Mousson, 2002). Indirectly primed $CD4^+$ T cells can migrate into the graft and encounter host APCs that have entered the graft and processed alloantigen in the context of MHC Class II. This promotes bystander damage by non-specific inflammatory responses (Heeger, 2003). In addition, indirectly primed $CD4^+$ T cells have also been associated with graft fibrosis and vasculopathy (Pietra et al., 2000). Cross-priming may also facilitate the processing of alloantigen to MHC Class I, thereby priming host $CD8^+$ T cells (Heath and Carbone, 2001).

Interestingly, indirect priming has been associated with allograft tolerance. Oral administration of allogeneic MHC antigens to recipient prior to allogeneic skin graft was also reported to promote T cell unresponsiveness to the allografts (Sayegh et al., 1992). Since the mechanism would involve these alloantigens to be processed by recipient APCs, the indirect pathway was considered to be responsible for this phenomenon. Similarly, the effectiveness of donor-specific transfusion of donor-type splenocytes in protecting both organ and cellular allografts (Kataoka et al., 2002; Rossini et al., 1999; Shoskes and Wood, 1994) also supports the role of the indirect presentation pathway in induction of transplant tolerance. Yamada *et al.* employed a model lacking the indirect pathway and demonstrated that costimulatory blockade is not effective in the absence of indirect processing of alloantigen (Yamada et al., 2001). This study employed MHC Class II deficient mice, which are incapable of mounting an indirect allogeneic response, as skin and cardiac allograft recipients; and observed these recipient mice were resistant to costimulatory blockade-mediated allograft tolerance (Yamada et al., 2001). These studies collectively suggested that indirect priming may be necessary for the maintenance of transplant tolerance.

1.3 Current immunotherapeutic strategies

Immunological tolerance can be defined as the state in which the immune system, in the

absence of ongoing exogenous immunosuppression, does not mount an immunological response against specific foreign antigens, while responses to other antigens are preserved (Sanchez-Fueyo et al., 2002). Induction of allograft tolerance is considered to be the ultimate goal in transplantation. Since allograft rejection involves a broad range of immune cells, controlling the anti-graft response has been a tremendous challenge clinically. Although heavy immunosuppression prevents grafts from being destroyed by the recipient immune system, it also predisposes recipients to undesired side effects and opportunistic infections, including pulmonary infections (Duncan and Wilkes, 2005). Progress in understanding transplantation immunology has led to a remarkable improvement in designing therapeutic drugs, however, the consistent establishment of alloantigen-specific tolerance still remains an elusive goal.

Current therapeutic strategies employ immunosuppressive agents, such as corticosteroids, calcineurin inhibitors (e.g. cyclosporine A and Tacrolimus), antimetabolites (e.g. azathioprine and mycophenolate Mofetil), rapamycin inhibitors, and monoclonal antibodies to control mainly T and B cell activities with additional administration of anti-fungal/bacterial pharmaceuticals to control secondary infections (Duncan and Wilkes, 2005). These immunosuppressive agents mainly target two components involved in T cell activation; costimulatory molecules and inflammatory cytokines. The molecules/pathways downregulated/inhibited by these agents include transcription factors (nuclear factor of activated T cells (NFAT), nuclear factor-κB (NF-κB) and activator protein (AP)-1), cytokines (IL-2, IFN-γ, TNF-α, IL-1, -3, -6, -8, and -10), costimulatory molecule interactions (CD28/B7 and CD40/CD154), and MHC molecules (reviewed in (Duncan and Wilkes, 2005) and (Shapiro et al., 2003)). A major problem with these agents is that the immunosuppressive effects are not alloantigen-specific and recipients are predisposed to high risks of opportunistic infections. Another problem with current immunosuppression is that

therapeutic agents not only block effector T cell activities but they could potentially inhibit the development of protective or regulatory T cells. It has been shown that IL-2 as well as costimulatory signaling are important for development and survival of regulatory T cells which are critical for the maintenance of peripheral tolerance (Fontenot et al., 2005) (Section 1.4.3). IL-2 promotes regulatory T cell differentiation via the STAT5 pathway and IL-2 deficiency results in a significant reduction in the number of regulatory T cells in the periphery (Antov et al., 2003; Burchill et al., 2003; Malek et al., 2002; Papiernik et al., 1998). Development of severe systemic autoimmune disease in IL-2 deficient mice further confirms the role of IL-2 in immune regulation (Klebb et al., 1996; Sadlack et al., 1994). These studies collectively suggest that while these therapeutic approaches ameliorate effector cell-mediated graft damage, the current immunotherapy may also have detrimental effects on the development and maintenance of tolerogenic T cells.

High-oxygen culture can promote indefinite survival of cellular allografts by elimination of the passenger leukocytes in rodents (Lafferty et al., 1975; Simeonovic et al., 1984), however, its application to the clinical setting has been hampered by problems in optimizing culture conditions to facilitate human islet survival (Benhamou et al., 1998). It has been shown that fetal β cells were rapid destroyed under 95% O_2 condition and other culture condition were also unable to sustain islet viability (Benhamou et al., 1998; Mandel et al., 1982). Although *in vitro* studies of some islet culture conditions have reported abrogation of allogeneic stimulation to the cultured islets, in accordance with rodent studies (Benhamou et al., 1998), an optimal condition to preserve islet viability is yet to be achieved.

A primary target of current therapeutic strategies is donor-derived costimulatory activity. This strategy is based on observation that complete T cell activation requires TCR-MHC/peptide and costimulatory signal and antigen stimulation of T cells in the

absence of costimulatory molecules leads to T cell death or anergy (Chen and Nabavi, 1994; Kosmaczewska et al., 2001; Najafian and Sayegh, 2000). There are a number of costimulatory molecule interaction whose inhibition can potentially be beneficial for transplantation including CD28/B7 and CD40/CD156(CD40L) (reviewed in (Rothstein and Sayegh, 2003)). CD28/B7 interaction provides T cell survival signals, and promotes Th1 and Th2 cell differentiation (Alegre et al., 2001). Studies using CD28-deficient mice revealed prolonged survival of islets and skin allografts, indicating the efficacy of targeting CD28/B7 signaling (Mandelbrot et al., 1999; Szot et al., 2000). One strategy being exploited to target this CD28/B7 (CD80 and CD86) interaction is the utilization of cytotoxic T lymphocyte antigen (CTLA-4) homologues. CTLA-4 competitively binds to B7 molecules and delivers negative signals resulting in inhibition of T cell activation (Alegre et al., 2001; Linsley et al., 1991). One study showed that CTLA-4-Ig, a soluble fusion protein that binds to B7 molecules with high affinity, treatment inhibited 50% of alloreactive T cells and significantly reduced Th1 cytokine production *in vitro* (Judge et al., 1996). Steurer *et al.* showed that pretreatment of *ex vivo* islet allografts with CTLA-4-Ig prior to transplantation inhibited allogeneic T cell response *in vitro* and induced allograft tolerance *in vivo* (Steurer et al., 1995). Co-transplantation of host-syngeneic myoblast transduced with CTLA4-Ig cDNA has also been shown to prolong islet allograft survival (Chahine et al., 1995). Furthermore, pretreatment of recipients with donor-derived DCs over-expressing CTLA-4-Ig was shown to prolong islet allograft survival *in vivo* (O'Rourke et al., 2000). These studies indicated that blockade of B7 costimulatory molecule signaling to recipient T cells may be beneficial in preventing cellular allograft rejection. Recent studies of non-human primate islet allografts reported CTLA4-Ig treatment prolonged islet allograft survival in non-human primate models (Adams et al., 2002; Levisetti et al., 1997), indicating positive effects of CTLA4 blockade approach.

Another costimulatory molecule targeted in preventing allograft rejection is the CD40/CD154 interaction (Benhamou et al., 1998). CD40 is a costimulatory molecule important for the activation and maturation of DCs (Blair et al., 2000; van Essen et al., 1995). CD40 ligation initially provides a costimulatory signal to DC which augments their ability to prime T cells by upregulating the expression of B7 molecules (Fairchild and Waldmann, 2000; Heath and Carbone, 2001). Thus CD40/CD154 signaling can also be targeted for preventing the activation of alloreactive T cells and allograft rejection. A number of cellular and organ allograft studies in rodents and primates using anti-CD154 antibody treatment to inhibit costimulation, support the efficacy of this strategy (Kirk et al., 1997; Larsen et al., 1996; Larsen et al., 1996; Parker et al., 1995). The efficacy of blocking CD40/CD154 interaction by treating recipient mice with monoclonal Ab was first demonstrated by Parker *et al.* (Parker et al., 1995). The study demonstrated that administration of anti-CD154 antibody together with allogeneic lymphocytes resulted in indefinite survival of islet allografts (Parker et al., 1995). Donor specific transfusion (DST) together with anti-CD154 antibody treatment was also shown to induce alloantigen-specific tolerance to islet allografts by inducing deletion of alloreactive $CD8^+$ T cells (Iwakoshi et al., 2000; Zheng et al., 1999). Abrogation of tolerance in DST/anti-CD154 mAb treated mice by anti-CTLA antibody administration indicated that such allograft tolerance was also CTLA4-dependent (Iwakoshi et al., 2000; Zheng et al., 1999). Anti-CD154 mAb treatment also resulted in a long-term survival of islet allografts in a non-human primate model further suggesting the beneficial effects of this strategy (Kenyon et al., 1999).

Recently, a more novel costimulatory pathway, inducible costimulatory (ICOS)/B7RP-1 (Nakamura et al., 2003), also show some potential as therapeutic targets. ICOS is expressed preferentially on activate T cells and binds to costimulatory molecule B7RP-1 expressed on APCs (Yoshinaga et al., 1999). Studies have detected graft-rejection-associated upregulation

of ICOS leading to expansion of alloreactive $CD8^+$ T cells in islet allograft *in vivo*, and blockade of ICOS/B7BP-1 interaction by anti-ICOS mAb significantly improved islet allograft survival (Nakamura et al., 2003). Consistently, Nanji *et al.* demonstrated a $CD4^+CD25^+$ regulatory T cell-mediated prolongation of islet allograft survival by treating recipients with anti-ICOS mAb with or without CD154 mAb or CTLA4-Ig (Nanji et al., 2004; Nanji et al., 2006). In a skin allograft model, deletion of $CD4^+CD25^+$ T cells in recipient mice abrogated allospecific tolerization via CD40/CD154 or CD28/CTLA4 blockade (Jarvinen et al., 2003; Taylor et al., 2001). Despite a difference in skin and cellular allograft rejection mechanism, these reports indicated that costimulatory blockade-induced tolerance is likely mediated by $CD4^+CD25^+$ regulatory T cells.

1.4 Tolerance induction

1.4.1 DCs and tolerance induction

The capacity of DCs to play a dual role in induction and regulation of immune responses has attracted much attention. Studies have shown that while mature DCs induce antigen specific immunity, immature, non-activated DCs can induce tolerance(Banchereau et al., 2003). Their tolerance-inducing properties are currently being explored as new anti-graft rejection strategies (Fairchild and Waldmann, 2000).

DCs have been shown to delete self-reactive T cells in the thymus during central tolerance induction (Brocker et al., 1997). Recent studies have shown that DCs are also critical regulators of peripheral tolerance (Banchereau et al., 2000; Banchereau et al., 2003; Barratt-Boyes and Thomson, 2005; Hackstein et al., 2001; Jonuleit et al., 2001; Steinbrink et al., 1997). It has been speculated that maintenance of peripheral tolerance requires the presentation of exogenous self-antigens to T cells (Lutz and Schuler, 2002). In agreement with this hypothesis, T cell tolerance has been shown to require the presentation of

cell-derived self-antigens by APCs and DCs have been identified as a crucial component in establishing peripheral tolerance (Adler et al., 1998; Kurts et al., 1997). It has also been shown that some populations of rat intestinal DCs with immature phenotype constitutively transport apoptotic epithelial cells to the T cell areas of secondary lymphoid organs when infection is not present (Huang et al., 2000). The author argues that this constant surveillance of immature DCs is important for the maintenance of peripheral tolerance (Huang et al., 2000).

Although it is still controversial, the maturation status of DCs is thought to be an important determinant for their capacity to induce peripheral tolerance. It is well established that fully mature DCs induce immunity by presenting antigen in the context of inflammatory signals. Mature DCs prime effector T cells by expressing high levels of MHC molecules and costimulatory molecules (e.g. B7 molecules and CD40) as well as producing inflammatory cytokines (Banchereau et al., 2000; Jorgensen et al., 2002). In contrast, immature DCs express low levels of MHC molecules and no costimulatory molecules (Banchereau et al., 2000; Lutz and Schuler, 2002). Studies have shown that antigen presentation in the absence of costimulation induces T cell anergy and potentially induce $CD8^+$ T cell tolerance (Lutz and Schuler, 2002; Schwartz et al., 1989). The importance of immature DCs in tolerance induction was further highlighted by studies demonstrating peripheral tolerization of $CD4^+$ and $CD8^+$ T cells were induced by immature DCs *in vivo* (Bonifaz et al., 2002; Hawiger et al., 2001). Monocyte-derived immature DCs have been shown capable of inducing antigen-specific inhibition of $CD8^+$ T cell activity when pulsed with influenza matrix peptide and keyhole limpet hemocyanin (Dhodapkar et al., 2001). In this experiment, influenza matrix peptide-specific-IL-10 producing T cells were generated with reduced IFN-γ production (Dhodapkar et al., 2001). Furthermore, a study using an experimental autoimmune uveoretinitis model demonstrated that immature, but not mature, peptide-loaded

bone marrow-derived DCs were shown to be capable of inducing tolerance via increased production of IL-10 and IL-5 and decreased levels of IFN-γ and IL-2 (Jiang et al., 2003). Although these studies suggest immature but not mature DCs mediate tolerance induction, there are also studies demonstrating mature DCs can also facilitate tolerance induction under certain conditions. In a steady state, DCs continually endocytose self-antigens such as apoptotic cells and acquire some properties of mature DCs (Lutz and Schuler, 2002). Recent studies have also reported that while immature DCs induce $CD8^+$ T cell ignorance, mature DCs can induce $CD8^+$ T cell tolerance by promoting transient cell proliferation followed by apoptosis *in vitro* (Albert et al., 2001). Akbari *et al.* demonstrated IL-10-producing pulmonary DCs pulsed with OVA-peptide were also capable of inducing tolerance by inducing $CD4^+$ regulatory T cell differentiation and these DCs exhibited a mature phenotype when reaching draining LNs (Akbari et al., 2001). Although further investigation is needed in order to verify whether the maturation status influences tolerogenic properties of DCs, above studies collectively indicate that DCs may have the potential to induce antigen-specific tolerance to allografts.

Major strategies currently employed to selectively enhance the tolerogenic properties of DCs include the use of immature DCs (or DCs chemically arrested before maturation), and use of genetically engineered DCs expressing immunomodulatory or immunosuppressive molecules(Hackstein et al., 2001). Studies demonstrated that immature DCs generated with low doses of GM-CSF in the absence of IL-4 were maturation-resistant and they were capable inducing alloantigen-specific T cell unresponsiveness *in vitro* and *in vivo* (Lutz et al., 2000). Jonuleit *et al.* showed that repetitive *in vitro* stimulation of allogeneic human T cells with immature monocyte-derived DCs, leads to the generation of non-proliferating IL-10 producing regulatory T cells (Jonuleit et al., 2000). These regulatory T cells exert immunomodulatory activity in a contact-dependent, antigen-non-specific manner (Jonuleit et

al., 2000). Treatment of immature DCs with IL-10 *in vitro* has also been described effective in induction of tolerogenic DCs (Steinbrink et al., 1999; Steinbrink et al., 1997; Yang and Lattime, 2003). Studies revealed that IL-10 treatment of immature DCs can results in generating immunoregulatory DCs that are capable of inhibiting $CD4^+$ and $CD8^+$ T cell responses in an antigen-specific manner (Steinbrink et al., 1999; Steinbrink et al., 1997; Yang and Lattime, 2003). DCs treated with another immunomodulatory cytokine, TGF-β, were also reported to induce tolerance by promoting $CD8^+$ regulatory T cell differentiation in experimental autoimmune encephalomyelitis model (Faunce et al., 2004). Treatments of DCs with pharmaceuticals such as vitamin D3 metabolite $1\alpha,25\text{-}(OH)_2D_3$, *N*-acethyl-L-cysteine, and common immunosuppressive drugs have demonstrated to arrest DCs at immature stage, and these DCs can induce T cell anergy or regulatory T cell differentiation (Piemonti et al., 1999; Piemonti et al., 2000; Verhasselt et al., 1999). Another apporach of genetically modifying DCs to express several immunoregulatory molecules, including IL-10, TGF-β and CTLA-4Ig also generated tolerogenic DCs (Takayama et al., 2000; Takayama et al., 1998; Tomasoni et al., 2005). Takayama *et al.* demonstrated that retrovirally transduced DCs over-expressing IL-10 exhibited a immature DC phenotype with low levels of MHC class II and costimulatory molecule expression (Takayama et al., 1998). These $IL\text{-}10^{+/+}$ DCs promoted alloantigen-specific T cell hyporesonsiveness *in vitro* (Takayama et al., 1998). Similarly, retroviral transduction of myeloid DCs with CTLA4-Ig reduced their capacity to prime alloreactive T cells and this gene delivery turned DCs to become capable of inducing alloantigen-specific T cell anergy *in vitro* (Takayama et al., 2000). Recently, induction of antigen-specific tolerance by DCs lacking NF-κB signaling, which is required for DC maturation, was demonstrated *in vivo* (Tomasoni et al., 2005). It was shown that DCs, carrying adenoviral vector encoding for a defective form of IkappaB kinase(IKK)2, were inhibited from maturation, and $IKK2^{-/-}$ DCs promoted $CD4^+$ regulatory T cells-mediated tolerance (Tomasoni et al., 2005). Evidence from the above studies suggests DCs are indeed

capable of mediating tolerance with antigen-specificity.

Despite these observations that DCs can induce antigen-specific tolerance, precise mechanisms of tolerogenic DCs generation and the mechanisms of antigen-specific tolerance mediated by tolerogenic DCs are not fully understood. Recent development indicated that IL-10 and TGF-β may play important roles in DC-mediated tolerance (Steinbrink et al., 1997; Yamaguchi et al., 1997). IL-10 or TGF-β pretreatment of DCs effectively prevent immature DCs from maturation (reviewed in (Jonuleit et al., 2001; Mahnke et al., 2002; Wallet et al., 2005)). IL-10 or TGF-β treated DCs are shown to be kept immature by failing to upregulate costimulatory molecules such as CD40, CD80 and CD86, and secrete inflammatory cytokines such as IL-1β, IL-6, IL-12 and TNF-α (Jonuleit et al., 2001; Mahnke et al., 2002; Wallet et al., 2005). It has also been shown that IL-10 can also mediate induction of tolerogenicity in DC by preventing maturation via inhibition of NF-κB activation (Allavena et al., 1998; Rescigno et al., 1998). Low levels of costimulatory molecules and absence of inflammatory cytokines are therefore considered important characteristics of tolerogenic DCs. A recent study reported that the important contribution of IL-10 in generating tolerogenic DCs also depends on induction of inhibitory immunoglobulin-like transcript 3 (ILT3) and ILT-4 on DCs (Manavalan et al., 2003). This study demonstrated that treatment of monoclonal antibodies for ILT-3 and ILT4 completed blocked T cell anergy induced by IL-10 treated DCs (Manavalan et al., 2003). These studies collectively indicated IL-10 render DC tolerogenic by inhibiting maturation and upregulating ILT3 and ILT4 expression. Additional signals required to license tolerogenicity remains to be elucidated.

1.4.2 DC tolerance and transplantation

Because DCs can demonstrate tolerogenic properties, attempts to prevent allograft rejection using DCs have been actively exploited in a number of allograft models (reviewed in

(Barratt-Boyes and Thomson, 2005)). It was reported that administration of maturation-resistant-immature DCs prolonged cardiac allograft survival by inducing alloreactive T cell hyporesponsiveness *in vivo* (Lutz et al., 2000). These DCs were found to be resistant to LPS and cytokine activation, and exhibited a low costimulatory molecule expression profile even after stimulation (Lutz et al., 2000). Taner *et al.* also demonstrated that pre-transplant infusion of recipient-derived immature DCs pulsed with alloantigen significantly improved cardiac allograft survival in rat (Taner et al., 2005). Analysis revealed that donor-antigen specific T cell hyporesponsiveness via downregulation of IL-2 and IFN-γ attributed to the prolongation of allograft survival (Taner et al., 2005). $CD4^+$ regulatory T cell-dependent prolongation of renal allografts by administration of NF-κB-signaling-deficient immature DCs (Tomasoni et al., 2005) further suggested implications of immature DCs for allograft tolerance induction. Allograft protection using DCs expressing immunomodulatory molecules have also been documented. *In vitro* studies showed that allogeneic DCs expressing CTLA4-Ig can inhibit production of IFN-γ by alloreactive T cells and enhance production of IL-4 and IL-10 (Takayama et al., 2000). Takayama *et al.* demonstrated that retrovirally transduced CTLA4-Ig expressing DCs can impair the proliferation and effector functions of $CD8^+$ T cells in alloantigen-specific manner *in vitro* (Takayama et al., 2000). In accordance, pretreatment of recipient mice with DCs expressing CTLA4-Ig prolonged murine islet allograft survival *in vivo* (O'Rourke et al., 2000). Although this study did not examine whether the prolongation was due to regulatory T cell differentiation or alloreactive T cell anergy, CTLA4-Ig expressing DCs expressed reduced level of CD80 and these DCs were unable to induce allogeneic T cell proliferation *in vitro* (O'Rourke et al., 2000). A study of renal allografts also showed that administration of recipients with DCs over-expressing IL-10 and TGF-β moderately prolonged graft survival (Gorczynski et al., 2000). Despite the above studies demonstrating capacity of DCs to induce allospecific tolerance presented potential strategies target to promote tolerance to allografts,

consistent and permanent graft acceptance with DC administration alone is yet to be achieved.

1.4.3 Regulatory T cells and tolerance

1.4.3.1 Regulatory T cell mediated T cell suppression

Over the last decade, researchers have identified immunoregulatory activities in a number of T cell subpopulations, including $CD4^+$ T cells (Taylor et al., 2001), $CD8^+$ T cells (Zhou et al., 2001), and NK-T cells (Zeng et al., 1999). Among these T cells, $CD4^+$ T cell populations expressing the α-subunit of the IL-2 receptor, CD25, are described to have potent immunoregulatory function. Naturally occurring $CD4^+CD25^+$ regulatory T cells constitute up to 10% of the T cell repertoire in the periphery (Feng and Wood, 2004). These T cells are anergic to stimulation through the T cell receptor, and capable of suppressing the function of other T cells present in the same microenvironment (Feng and Wood, 2004).

CD25 is the α-chain of the IL-2 receptor and it is used as a surface marker to distinguish regulatory T cells (Malek and Bayer, 2004). While $CD25^+$ T cells can represent both regulatory T cells and activated effector T cells, there are differences in the level of expression of CD25 by both these cell types. Regulatory T cells show high and sustained expression levels of CD25, whereas activated effector T cells express lower levels of CD25 only transiently following activation (Kuniyasu et al., 2000; Wood and Sakaguchi, 2003). Studies have identified other surface markers such as CD45RB, CTLA-4, glucocorticoid induced tumor necrosis factor receptor family related gene (GITR), CD102, CD122 and CD62 on regulatory T cells (Davies et al., 1999; Ermann et al., 2004; Lehmann et al., 2002; McHugh et al., 2002; Shimizu et al., 2002; Takahashi et al., 2000; Zelenika et al., 2002). While these markers have also been reported to be expressed on the $CD4^+CD25^+$ regulatory T cell population, they are unlikely to be regulatory T cell specific. Recently, Hori *et al.*

identified high expression of a transcription factor, Forkhead box p3 (Foxp3), in regulatory T cells (Hori et al., 2003). They also showed that Foxp3 expression is specific to $CD4^+CD25^+$ T cells and cannot be induced in naïve T cells following activation (Hori et al., 2003). It was also demonstrated that T cell populations expressing Foxp3 proliferated poorly and produced IL-2 and IL-10 upon TCR stimulation (Yagi et al., 2004). Foxp3-deficient mice have also been shown to generate few $CD4^+CD25^+$ regulatory T cells (Yagi et al., 2004). Although Foxp3 is a most specific intracellular marker for regulatory T cells, the precise molecular mechanism by which Foxp3 is expressed is not known.

$CD4^+CD25^+$ regulatory T cells are very potent suppressors of immunogenic T cells and recent studies have shown $CD4^+CD25^+$ regulatory T cells can induce linked-suppression (Cederbom et al., 2000; Dieckmann et al., 2002; Hussain and Paterson, 2004; O'Garra et al., 2004). Regulatory T cell effects include inhibition of proinflammatory cytokine production, production of immunosuppressive cytokines, downregulation of expression of costimulatory molecules and adhesion molecules, inhibition of T cell proliferation, induction of T cell anergy, induction of apoptosis in effector T cells, and conversion of effector T cells into a regulatory phenotype (Cederbom et al., 2000; Dieckmann et al., 2002; Hussain and Paterson, 2004; O'Garra et al., 2004).

IL-2 deficiency in mice results in the absence of $CD4^+CD25^+$ regulatory T cells in the thymus and the periphery, indicating the importance of IL-2 in the development and the maintenance of $CD4^+CD25^+$ regulatoryT cells (de la Rosa et al., 2004; Papiernik et al., 1998). This finding was also consistent with the report that cytokines, such as IL-2 and IFN-γ, were necessary for long term allograft survival (reviewed in (Wood and Sakaguchi, 2003)). IL-10 has been shown to possess a potent anti-inflammatory function limiting excessive inflammatory responses (Trinchieri, 2004). Blockade of IL-10 by anti-IL-10 or anti-IL-10

receptor antibody results in abrogation of skin-allograft acceptance, mediated by regulatory T cells (Hara et al., 2001; Kingsley et al., 2002). These studies further showed that IL-10 plays an important role in the induction and the maintenance of tolerance mediated by regulatory T cells (Hara et al., 2001; Kingsley et al., 2002). Inhibition of TGF-β has also been shown to block the induction of T cell unresponsiveness to alloantigens (Josien et al., 1998). It was also demonstrated that naturally occurring $CD4^+CD25^+$ T cells express surface-bound latent TGF-β (Nakamura et al., 2001). Regulatory T cells have been proposed to mediate effector T cell suppression in a cell-contact dependent manner through surface-bound TGF-β (Nakamura et al., 2001). Furthermore, It has been reported that levels of TGF-β signaling correlated with the expression of Foxp3 (Schramm et al., 2004). CTLA-4 is important in the suppression of activated T cells and is associated with $CD4^+CD25^+$ regulatory T cells (O'Rourke et al., 2000; Walunas et al., 1994). $CD4^+CD25^+$ regulatory T cells have been shown to be the only subset of T cell that expresses CTLA-4 constitutively (Annunziato et al., 2002; Jonuleit et al., 2001; Jonuleit et al., 2001; Levings et al., 2001). The loss of CTLA-4 function results in the incapacitation of regulatory T cell mediated alloantigen-specific T cell suppression (Kingsley et al., 2002; Sanchez-Fueyo et al., 2002). An *in vitro* study has also shown that regulatory T cells can downregulate the expression of the costimulatory molecules CD80 and CD86 on APCs, rendering them less able to activate T cells (Cederbom et al., 2000). Furthermore, human regulatory T cells have been shown to inhibit the expression of MHC and costimulatory molecules on immature DCs and induce apoptosis in mature DCs (Frasca et al., 2002). The above studies collectively suggest that regulatory T cell mediated tolerance is the dominant mechanism in induction and maintenance of the peripheral tolerance.

1.4.3.2 $CD4^+CD25^+$ T cells in transplantation

The relevance of regulatory T cells in transplantation has been recognized in a number of

studies. These observations indicate the potency of regulatory T cells to inhibit alloreactive T cell activity was demonstrated by an experiment showing that $CD4^+CD25^+$ T cells, generated via costimulation blockade *in vivo*, are capable of suppressing T cell response to alloantigen by a 100-fold excess of donor alloantigen specific $CD8^+$ T cells *in vitro* (Lin et al., 2002). Donor Class II MHC-specific $CD4^+CD25^+$ T cells have been found in the draining lymphoid organs of recipient with long-term graft survival (Graca et al., 2002; Hara et al., 2001; Kingsley et al., 2002). It can be speculated that $CD4^+CD25^+$ T cells in the draining lymphoid tissue can inhibit the activation of alloreactive T cells, while $CD4^+CD25^+$ T cells at the graft site can suppress alloreactive T cells which have escaped immunoregulation at the draining lymphoid organs. Interestingly, it has been shown that repeated antigenic exposure in the periphery is sufficient to generate these regulatory T cells (Taams et al., 2002) and the presence of the graft itself is critical for the maintenance of allotolerance *in vivo* (Hamano et al., 1996).

Studies indicate that the generation and maintenance of alloantigen-specific $CD4^+CD25^+$ regulatory T cells may be the key for achieving the ultimate goal, alloantigen-specific tolerance (Hall et al., 1990; Hara et al., 2001; Hoffmann et al., 2002; Kingsley et al., 2002). It was also shown that recipient $CD4^+CD25^+$ T cells play a potent role in the induction and maintenance of alloantigen specific tolerance (Hara et al., 2001; Sanchez-Fueyo et al., 2002). Hall *et al.* demonstrated that cardiac allograft tolerance can be adoptively transferred by $CD4^+CD25^+$ T cells isolated from tolerant mice (Hall et al., 1990). Sanches-Fueyo *et al.* also demonstrated that islet allograft tolerance can be transferred to naïve isogeneic mice by adoptive transfer of $CD4^+CD25^+$ T cells (Sanchez-Fueyo et al., 2002). Such tolerance has been shown to be dependent on $CD4^+CD25^+$ T cells which weakly proliferate in response to donor cells and suppress both naïve $CD4^+$ and $CD8^+$ T cells from responding to donor cells but not to third part cells (Sanchez-Fueyo et al., 2002). These studies strongly suggest the

critical role of $CD4^+CD25^+$ cells in induction of allograft tolerance. $CD4^+CD25^+$ T cells are also present in human peripheral blood, indicating that such cell populations could also function in establishing transplantation tolerance in humans (Jonuleit et al., 2001; Levings et al., 2001).

Whether allopeptide presentation to $CD4^+CD25^+$ T cells is achieved via the direct or indirect pathway is not known. Some studies have suggested that allopeptide recognition by $CD4^+CD25^+$ regulatory T cells requires indirect presentation (Wise et al., 1998; Yamada et al., 2001). It was shown that recipient MHC Class II expression (indicating antigen presentation through indirect pathway) is necessary in order to achieve long-term survival of cardiac allografts (Yamada et al., 2001). Thus, it can be speculated that the indirect pathway is involved in this maintenance of graft protection. Furthermore, Wise et al. showed that linked suppression can operate through indirect recognition in a skin allograft model (Wise et al., 1998). However, the direct pathway could well be involved in alloantigen recognition by $CD4^+CD25^+$ T cells in the early post-transplant period as alloreactive T cells may potentially recognize alloantigen presented by donor APCs.

Recent developments have indicated that several signaling pathways are involved in controlling regulatory T cell development, including the Notch and NF-κB signaling pathways (Bettelli et al., 2005; Hoyne et al., 1999; Iruretagoyena et al., 2006; Ng et al., 2001; Wong et al., 2003). Manipulation of these pathways may therefore promote alloantigen-specific tolerance after transplantation via generation of regulatory T cells.

1.5 Notch signaling pathway

Notch represents a large single transmembrane receptor family expressed on many types of cells, including neural cells (Sakamoto et al., 2002) and hematopoietic cells (Bash et al.,

1999; Moloney et al., 2000). Notch1 was originally isolated as a gene that is rearranged by translocation in human acute T cell lymphoblastic leukemia/lymphoma (Carlesso et al., 1999). In this disease, Notch is constitutively active and produces T cell neoplasm in mice (Carlesso et al., 1999). To date four families of Notch receptors have been identified; Notch1, Notch 2, Notch 3 and Notch 4 (MacDonald et al., 2001). Among these, Notch 1-3 are expressed on hematopoietic and myeloid progenitors, and Notch1 is expressed in splenic cells and peripheral blood lymphocytes (Bash et al., 1999; Bigas et al., 1998; Hoyne et al., 2000; MacDonald et al., 2001). Notch 4 expression is restricted to endothelial cells (Bigas et al., 1998).

Recent studies have demonstrated that Notch is not a single functional gene. Rather, expression of Notch in different cell types at different stages of development may act as a 'switch' in multiple developmental events. Thus, stimulation of Notch activity *in vivo* is likely to be time-dependent and cell type-specific, allowing cells to receive signals and adopt alternate differential fates at appropriate times (Milner et al., 1996). Notch pathways are initiated by direct receptor/ligand interactions between neighboring cells. Notch activation has been reported to mediate a wide range of cell-fate determination processes, including neural cell development (Sakamoto et al., 2002), embryogenesis (Hoyne et al., 2000), common lymphocytes precursor development into T/B cells (MacDonald et al., 2001), $\alpha\beta/\gamma\delta$ lineage differentiation of T cells (Bigas et al., 1998; MacDonald et al., 2001), and $CD4^+/CD8^+$ T cell lineage differentiation (Bigas et al., 1998; Hoyne et al., 2000; MacDonald et al., 2001).

1.5.1 Structure of Notch receptors

Notch proteins are synthesized from single polypeptide precursors which are proteolytically processed to form a heterodimer (Osborne and Miele, 1999). Notch receptors have two

domains; extracellular and intracellular (Osborne and Miele, 1999). Recent studies have demonstrated that a truncated Notch molecule lacking its extracellular domain behaves as a constitutively activated form, indicating that its intracellular domain plays a central role in facilitating the Notch signaling pathway (Bigas et al., 1998). It has been shown that ligand binding to Notch results in proteolytic cleaving of the intracellular domain which activates the Notch signaling pathway within the expressing cell (Milner and Bigas, 1999).

The extracellular domain of Notch consists of 29 to 36 copies of epidermal growth factor (EGF) repeats and Lin-12/N repeats [Figure1.2] (Fleming, 1998; Sakamoto et al., 2002). It has been shown that the 11^{th} and 12^{th} repeat regions are required for binding of the Notch ligands, Delta-like and Serrate/Jagged (Rebay et al., 1991; Sakamoto et al., 2002). Recent studies showed that the EGF repeat region is also the target of Fringe-mediated glycosylation which results in a modulation of the nature of Notch activation (Moloney et al., 2000). Lin-12/N-repeats have been shown to interact with furin-like convertase or transmembrane metalloprotease kuzbanian, functioning as a receptor involved in physical cell-cell interactions of Notch receptors and their ligands (Bigas et al., 1998).

The intracellular (IC) domain consists of 4 major components: 6CDC10/SW161/ankyrin repeats (Bigas et al., 1998; Sakamoto et al., 2002), Notch cytokine response (NCR) region [Figure 1.2] (Bigas et al., 1998), 1 or 2 opa region (Sakamoto et al., 2002), and PEST domain (Sakamoto et al., 2002). The ankyrin repeat region is the most highly conserved portion of the Notch molecule and is thought to be responsible for putative nuclear localization signals and intracellular signal transduction (Bigas et al., 1998). The ankyrin repeat regions are shown to play an important role in interacting with downstream transcription factors, such as CBF1, Suppressor of Hairless, Lag-1 (CSL) (Barrick and Kopan, 2006; Le Gall and Giniger, 2004). The lack of ankyrin repeat region results in a

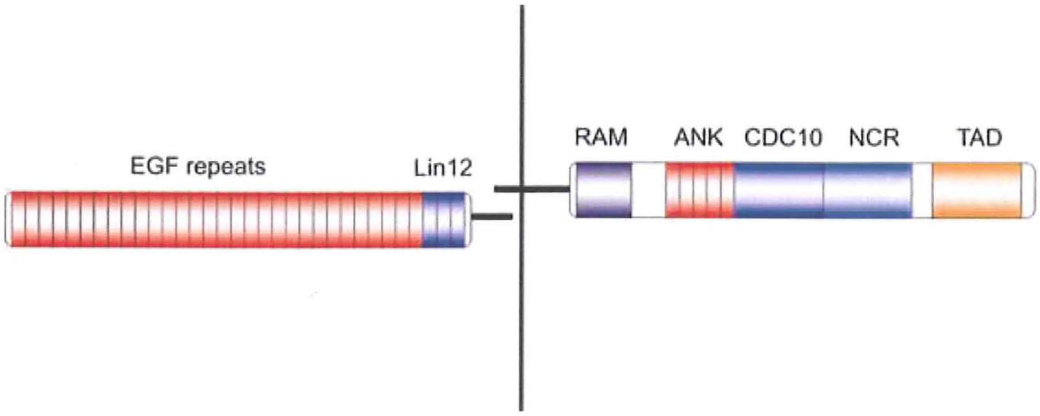


Figure 1-2: Schematic diagram of Notch receptor

The extracellular domain of Notch contains EGF repeats and LNR12 region. The intracellular domain contains RAM23 domain, ankyrin (ANK) repeats, cdc10, NCR regions and PEST domain. Notch 1 and 2 contains N-terminal transactivation (TAD) domains.

(Adapted from (Bigas et al., 1998))

dominant negative form of Notch (Fleming, 1998), indicating the importance of this region in facilitating effective Notch signaling. The NCR region is located immediately adjacent to C-terminal of the CDC10 domain (Bigas et al., 1998). A recent experiment has demonstrated that the NCR region may interact with cytokine signaling pathway components upon cytokine induction and the cytokine specificity of Notch1 and Notch2 can be transferred with the NCR region (Bigas et al., 1998). Thus it was speculated that NCR region is important in regulating cytokine production via the Notch signaling pathway. It was also suggested that the NCR region may influence the subcellular trafficking of the IC domain thereby having effects on the functional activities of Notch (Bigas et al., 1998). PEST domain is required to maintain protein stability by preventing hyperphosyphorylation of IC domain (Fryer et al., 2004).

1.5.2 Notch signaling pathway and its modulation

The Notch signaling pathway is activated by a Notch ligand binding to the Notch receptor [Figure 1.3] (Sakamoto et al., 2002). This activation leads to processing of the IC domain where it is proteolytically cleaved at the transmembrane domain by γ-secretase (MacDonald et al., 2001). This cleaving event requires presenilin-1 and occurs within or near the membrane (Osborne and Miele, 1999). The IC domain is then translocated into the nucleus where it undergoes heterodimerization (MacDonald et al., 2001; Sakamoto et al., 2002). This form of IC domain binds to the transcription factor CSL (Izon et al., 2002; MacDonald et al., 2001). In its inactive state, CSL is bound to corepressors, such as N-CoR/SMRT and CIR (Izon et al., 2002; MacDonald et al., 2001). Upon binding with the IC domain, CSL displaces the corepressors and recruits coactivators, such as mastermind KIP, PCAF and GCN5 (Allman et al., 2001; Bash et al., 1999; Sakamoto et al., 2002). The formation of this IC/CSL/coactivator complex triggers the transcription of Notch signaling target genes, including HES-1, HES-5, and CD23 (Allman et al., 2001; Bash et al., 1999; Le Gall and

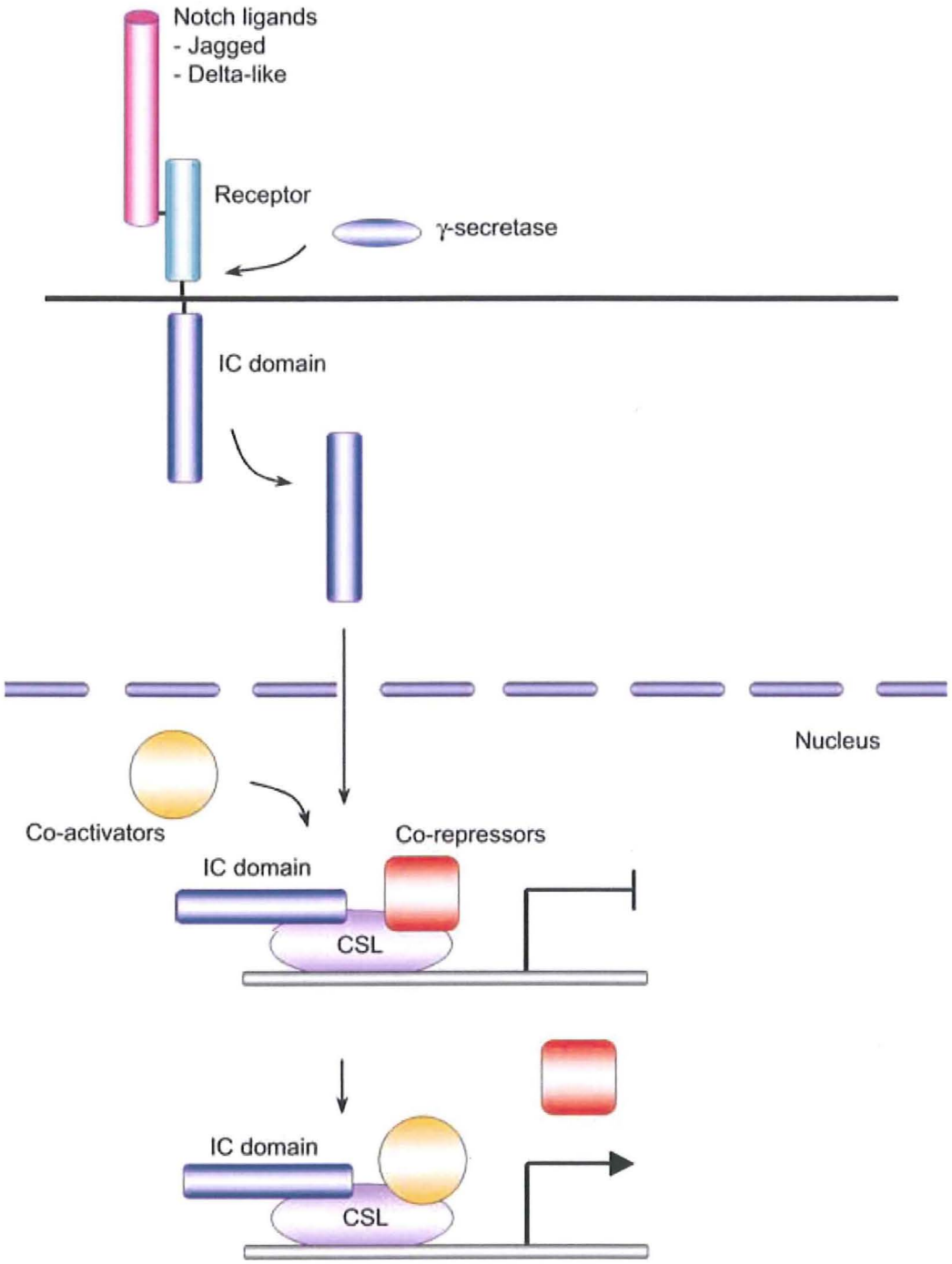

Notch ligands
- Jagged
- Delta-like
Receptor
γ-secretase
IC domain
Nucleus
Co-activators
Co-repressors
IC domain
CSL
IC domain
CSL

Figure 1-3: Schematic diagram of the Notch signaling pathway

Interaction of Notch receptors with ligands leads to proteolytic cleavage of extracellular domain by γ-secretase. The cleaved IC domain translocates into the nucleus from heterodimers. In the nucleus, they recruit co-activators and replaces co-repressors bound to CSL complex. This dissociation of co-repressors and binding of IC domain together with co-activators triggers transcriptional activation of the target genes.

Giniger, 2004; MacDonald et al., 2001; Sakamoto et al., 2002). Activation of these target genes via Notch signaling has been shown to influence a morphogenesis and cellular differentiation in the immune system as described in Section 1.5.3.

There are modulators of Notch signaling pathway such as Fringe molecules which have been shown to regulate the ligand binding specificity of Notch receptors (Hicks et al., 2000; Moloney et al., 2000; Pear and Radtke, 2003). There are three Fringe molecules identified to date, Lunatic (Lfng), Radical (Rfng), and Manic Fringe (Mfng). The mode in which Fringe proteins alter the Notch pathway is still not clearly understood. Recent studies have identified that Fringe proteins are glycosyltransferases localized in the Golgi apparatus, and are capable of modifying Notch receptors (Hicks et al., 2000; Moloney et al., 2000). Koch *et al.* demonstrated that ectopic expression of Lfng in the thymus potently inhibit Notch-1 signaling (Koch et al., 2001). Studies also showed that Fringe molecules have capacity to alter the sensitivity of Notch ligand binding specificity (Izon et al., 2002). Recent drosophila studies have consistently shown that Lfng interacts with both Delta and Serrate *in vitro* (Koch et al., 2001). In developing fly wings, Fringe proteins, such as Lfng and Mfng, have been shown to preferentially activate Notch by the Delta-pathway and inhibit Notch activation by Serrate (Koch et al., 2001; Panin et al., 1997). It has been shown that Mfng suppresses binding of Jagged-1 to Notch twice as potently as Lfng, directing Delta-mediated Notch activation (Shimizu et al., 2001). Recent study demonstrated that glycosylation of EGF-12 region of the Notch receptor by Fringe is responsible for the modulation of ligand-binding specificity (Lei et al., 2003). In addition to Fringe molecules, Deltex has been identified a modulator of Notch (Gorman and Girton, 1992; Izon et al., 2002; Xu and Artavanis-Tsakonas, 1990). Mammalian Deltex is known to bind to the ankyrin repeats and C-terminal of the RING finger domains of Notch-1 (Izon et al., 2002). While Drosophila study identified Deltex as a positive regulator of the Notch pathway (Matsuno et al., 1995),

human homologue, Deltex-1, has been shown to antagonize Notch-1 function (Sestan et al., 1999). Although further investigation is required to determine the role of Deltex in regulating the Notch pathway, negative modulation by Deltex is further supported by antagonizing effects of Deltex-1 in Notch-1 mediated BM cell development (Izon et al., 2002).

1.5.3 Immune regulation by Notch signaling

1.5.3.1 Effects of Notch signaling in cellular kinetics

Studies have revealed that Notch-1 plays a role in regulating cell cycle kinetics and differentiation processes in a number of cells types (Bigas et al., 1998; Milner et al., 1996). There is evidence that Notch can inhibit differentiation/proliferation of certain cell types while enhancing those processes in other cell types (MacDonald et al., 2001; Milner and Bigas, 1999; Pear and Radtke, 2003). It has been observed that Jagged-2-induced Notch activation delays the proliferation of human myeloid $CD34^+$ hematopoietic precursors and monocytic differentiation in human HL-60 cells, in the absence of growth-promoting cytokines (Carlesso et al., 1999). Notch activation by Jagged-1 in marrow stromal cells has also been shown to inhibit granulocyte differentiation in the murine 32D cell line in response to granulocyte colony-stimulating factor (G-CSF), promoting the expansion of undifferentiated cells (Li et al., 1998; Milner et al., 1996). These studies collectively suggest that Notch is most likely involved in the maintenance of hematopoiesis.

1.5.3.2 T/B cell lineage differentiation

Recent studies have revealed that the expression of Notch-1 is an essential factor in determining T and B cell lineage differentiation from common lymphoid precursors (Radtke et al., 1999). It has been shown that bone marrow progenitors lacking Notch-1 become B cells when they enter the thymus (Wilson et al., 2000). Another study showed that blocking RBP-J activity, which is important in the signaling pathways of all the Notch receptor family,

results in B cell development in the thymus (Han et al., 2002). MacDonald *et al.* have also shown the importance of Notch signaling in common lymphoid precursor (CLP) development in the thymus and gastrointestinal tract (MacDonald et al., 2001). They demonstrated that CLP differentiated into T cells in the presence of thymic stromal cells expressing Jagged-1, while CLP receiving no Notch signals became B cells (MacDonald et al., 2001). While Notch-1 appears to be expressed on T cells, Notch-2 has been shown to be preferentially expressed on B cells (Saito et al., 2003). This may indicate Notch-2 promotes B cell differentiation. It has also been reported that enhanced Notch-2 expression promote differentiation of BM stromal cells into myeloid precursors, instead of lymphoid precursors (Milner and Bigas, 1999). In addition, Notch-1-induced T cell lineage differentiation was shown to be antagonized by Deltex-1 (Izon et al., 2002). Study has shown that Deltex-1 expressing BM cells preferentially developed into B cells (Izon et al., 2002), indicating Deltex-1 plays a role in inhibiting T cell lineage commitment.

Consistent with these findings, a study allowing inducible inactivation of Notch1 by employing Cre/*loxP* technology, showed an accumulation of B cells in the thymus, completely inhibiting T cell differentiation (Radtke et al., 1999; Wolfer et al., 2001). It has been reported that the ligation of Notch 1 or Delta-4 in bone marrow precursors leads to T cell development while blocking B cell development(Dorsch et al., 2002; Pui et al., 1999; Yan et al., 2001). HES is one of the target genes of Notch (Jarriault et al., 1998; Kim and Siu, 1998). HES-1 has been shown to inhibit transcription of bHLH genes that specify the differentiation of CLP into B cells (Guidos, 2002). It has been repeatedly demonstrated that HES-1 and HES-5 block B cell development (Kawamata et al., 2002). Thus HES-1 could be a factor preventing B cell lineage development and promoting T cell lineage commitment. Consistently, Notch-1 signaling is considered capable of inhibiting apoptosis in committed T cell progenitors, while being capable of inducing apoptosis in B cells (Jehn et al., 1999).

Jehn *et al.* showed Notch-1 and the nuclear receptor protein Nur77 interactions provide T cell protection against TCR mediated apoptosis (Jehn et al., 1999). These studies suggest that Notch signaling is critically required for driving T cell differentiation from CLP.

B cell lineage development from CLP requires genes such as EBF, PU.1 and E2A, which encode bHLH transcription factors including recombinase activating genes (RAG)-1 and RAG-2, Igα, Igβ, λ5, VpreB, Igμ, and Pax-5 (Guidos, 2002; Koch et al., 2001). Recent investigations have shown that Notch-1 activation preferentially induces T cell lineage development rather than B cell lineage by interfering with this cascade (Pui et al., 1999). Notch-1 activation was reported to block bHLH transcription factor E2A and B cell specific genes and prevented early B cell lymphopoiesis while ectopically inducing thymic-independent $CD4^+CD8^+$ T cells in the bone marrow transplantation studies (Pui et al., 1999). However, the mechanism by which Notch-1 inhibit bHLH genes is not clearly understood.

1.5.3.3 Notch in αβ/γδ-TCR lineage differentiation

T cell progenitors entering the thymus commit to either αβ- or γδ-T cell lineages. The involvement of Notch signaling in this fate determination is not clearly understood. Washburn *et al.* suggested that Notch1 signals promote the αβ-lineage differentiation over the γδ-lineage (Washburn et al., 1997). However, conflicting data suggest that Notch1 signaling does not promote γδ-lineage differentiation, but instead may play a role in αβ-lineage formation (Wolfer et al., 2002). Further studies are needed to elucidate the role of Notch signaling in the γδ-TCR lineage differentiation of T cells.

1.5.3.4 Notch in CD4/CD8 lineage differentiation

Robey *et al.* reported that constitutive expression of activated Notch in developing

thymocytes promoted $CD8^+$ T cell differentiation over $CD4^+$ T cell differentiation in transgenic mice (Robey et al., 1996). This result suggests that Notch acts on T cell precursors at the $CD4^+CD8^+$ stage and enhances $CD8^+$ T cell differentiation but not $CD4^+$ T cell development. Furthermore, it was shown that upregulated Notch signaling directs thymocytes already bearing MHC Class II compatible TCRs (which normally become $CD4^+$ T cells) to become $CD8^+$ T cells instead (Robey, 1999). Interestingly, activated Notch allows the development of the $CD8^+$ T cell lineage in the absence of MHC Class I or II proteins, but not in the absence of both MHC Class I and II (Robey et al., 1996). This indicates that Notch signaling is capable of overriding the precursor cells bearing MHC Class II, forcing them to differentiate into the $CD8^+$ T cell lineage. Thus, although the overall population of $CD4^+CD8^+$ T cell precursors remains unaffected, the proportion of $CD4^+$ and $CD8^+$ T cells generated in the thymus is altered (Robey, 1999). It was also demonstrated that activated Notch 2 potentiates CD8 lineage maturation (Witt et al., 2003).

The mode in which Notch induces preferential development of the $CD8^+$ T cell lineage over the $CD4^+$ lineage is not clearly understood. However, one study suggested that one of the downstream target genes in the Notch signaling pathway, HES-1, could be at least partially responsible for the preferential $CD8^+$ lineage differentiation (Kim and Siu, 1998)

Whether Notch expression actually influences T cell differentiation into $CD4^+$ or $CD8^+$ fates still remains controversial. Wolfer *et al.* have challenged previous findings by showing normal thymocyte development in $Notch^{lax/lax}$ $CD4^-$Cre mice, whose Notch is inactivated in a tissue-specific manner (Wolfer et al., 2001). They also reported that the lack of Notch-1 expression did not alter the proportion of $CD4^+$ and $CD8^+$ thymocytes nor the maturation or survival of mature $CD4^+$ and $CD8^+$ T cells (Wolfer et al., 2001). Further investigation is required to clarify the contribution of Notch signaling in $CD4^+/CD8^+$ T cell lineage

differentiation.

1.5.4 Notch Signaling pathway and tolerance Induction

It has been suggested that Jagged-1-Notch interaction interferes with the progression of T cells to a Th1 phenotype and drives the formation of regulatory T cells at this point (Yvon et al., 2003). Recently *in vitro* stimulation of $CD45RA^+$ naïve T cells by Jagged-1 induced proliferation of $CD4^+$ T cells displaying a regulatory phenotype (Yvon et al., 2003). This regulatory T cell population had lower IL-2, IL-5 and IFN-γ, higher TGF-β production, upregulated expression of Deltex and induced alloantigen-specific hyporesponsiveness (Yvon et al., 2003). Ng *et al.* showed that $CD4^+CD25^+$ regulatory T cells express high levels of Deltex and the magnitude of Deltex and Notch4 expression is highly upregulated following anti-CD3 and anti-CD28 stimulation (Ng et al., 2001). Anastasi et al. showed that transgenic mice which constitutively express Notch-3 are resistant to streptozotocin-induced diabetes and increased number of $CD4^+CD25^+$ regulatory T cells correlated with the resistance to diabetes (Anastasi et al., 2003). Furthermore, injection of Jagged-1 transfected DCs pulsed with a house dust mite antigenic peptide, rendered mice profoundly tolerant to subsequent challenges with intact house dust mite protein (Hoyne et al., 1999). In support, another study has shown that over-expression of Jagged-1 on B cells resulted in the induction of antigen-specific regulatory T cells (Vigouroux et al., 2003). These studies collectively strongly suggest that Notch signaling is involved in regulatory T cell differentiation.

Past studies investigating the potential roles of the Notch signaling to induce allograft tolerance are limited. Recently, pretreatment of recipient mice L-cells overexpressing the Notch ligand, Delta-like-1, resulted in prolonged survival of cardiac allografts due to expansion of alloantigen-specific $CD8^+$ T cells with regulatory function (Wong et al., 2003). This study indicates that Notch signaling has the potential to induce tolerance via the

induction of $CD4^+CD25^+$ regulatory T cell differentiation. However, the impact of Notch signaling in the generation of immunoregulatory DCs and alloantigen-specific tolerance has not been investigated.

1.6 NF-κB transcription family

The transcription family, NF-κB, is expressed in virtually all cell types and involved in the regulation of over 200 genes (Pahl, 1999; Xu et al., 2003), including critical factors involved in immune response. NF-κB family consists of five members; NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel [Figure 1-4] (Liou and Hsia, 2003). NF-κB family members can be distinguished on the basis of their Rel-homology domain, which is responsible for sequence-specific DNA binding, dimerization, and interaction with inhibitory proteins, IκB (Caamano and Hunter, 2002; Viatour et al., 2005). These members form either homo or heterodimers and the balance between homo- or heterodimers formed determines the level of transcriptional activity (Caamano and Hunter, 2002). NF-κB1, NF-κB2, and RelA are expressed in all cell types and they are expressed in thymus at the highest level (Liou and Hsia, 2003). In contrast, RelB and cRel are exclusively expressed in lymphoid tissues; cRel is found in hematopoietic cells and RelB in spleen and thymus (Liou and Hsia, 2003). NF-κB can play a critical role in promoting immunity during infections. For example, NF-κB is involved in expression of genes important for recruiting inflammatory cells to the site of infection and their effector functions e.g. adhesion molecules [intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1)], cytokines [such as IL-1, IL-2, IL-6, IL-12, TNF-α, IFN-γ] and chemokines [monocyte chemotactic protein-1 (MCP-1) MIP-1α, RANTES and IL-8](Elewaut et al., 1999; Hoffmann et al., 2002; Rhodus et al., 2005; Vila-del Sol and Fresno, 2005). NF-κB is also strongly associated with immune cell proliferation and anti-apoptotic gene expression (Caamano et al., 1998; Kahn-Perles et al., 1997; O'Keeffe et al., 2005). The NF-κB target genes also include IκB genes whose

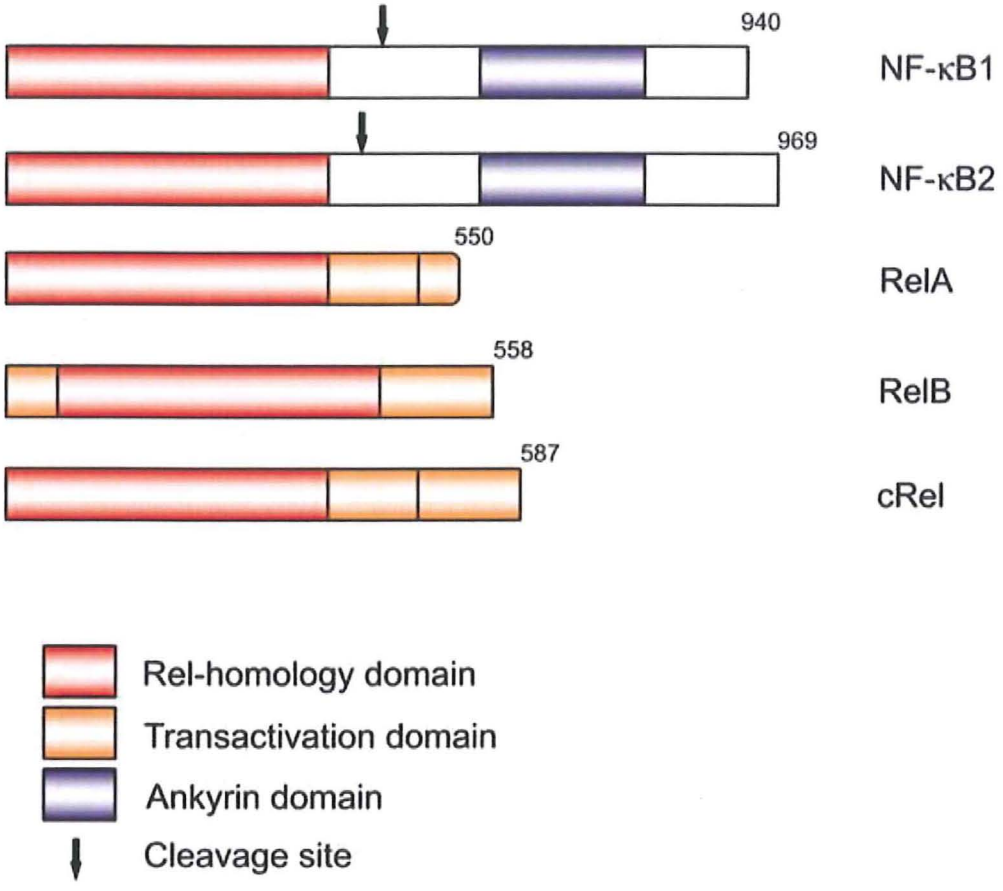


Figure 1-4: Schematic diagram of NF-κB proteins

Members of NF-κB proteins are shown. The NF-κB family members can be distinguished on the basis of their Rel-homology domains, which is important for dimerization and interaction with inhibitory proteins, IκB. The arrow points to the proteolytic cleavage sites of NF-κB1 (p100/p52) and NF-κB2 (p105/p50). The number of amino acids in each member is shown on the right.

(Adapted from (Siebenlist et al., 2005))

transcriptional activation leads to downregulation of NF-κB in an autocrine manner (Brown et al., 1993; Caamano and Hunter, 2002; Sun et al., 1993).

1.6.1 NF-κB signaling pathways

In the resting state, NF-κB is kept in an inactive form by inhibitory IκB proteins (Viatour et al., 2005). IκB proteins are NF-κB specific inhibitors and they bind to the Rel-homolog domain of NF-κB, preventing their translocation into the nucleus (Ghosh et al., 1998). The phosphorylation followed by subsequent protease-dependent degradation of IκB protein has been recognized as an essential step for NF-κB activation (Bonizzi and Karin, 2004). Dissociation of IκB allows NF-κB to translocate into the nucleus where they bind to the κB motif in promoter regions of target genes and regulate their transcriptional activities (Caamano and Hunter, 2002). A number of extracellular stimuli induce NF-κB activation and there are 2 main NF-κB activation pathways; classical and alternative pathways [Figure 1.5] (Bonizzi and Karin, 2004). The molecular pathway to activate NF-κB is stimulus/receptor-specific and depends on whether p105 or p100 precursor is processed. The first pathway (classical pathway) involves processing of p105 precursors (Bonizzi and Karin, 2004). This pathway is activated in response to proinflammatory cytokines such as TNF-α and IL-1β, viruses, bacterial products, toll-like receptor (TLR) signaling, and antigen receptor binding (Bonizzi and Karin, 2004; Caamano and Hunter, 2002; Medzhitov, 2001). Signals through the TNF-receptor recruit adaptor molecules, TNF-receptor associated factor (TRAF)-2 or PKCθ (which subsequently activates CARMA1/MALT1/Bcl10) to the cytoplasmic membrane and subsequently recruits and activates the IκB-kinase (IKK) complex (Devin et al., 2000; Hsu et al., 1995; Lucas et al., 2004). The IKK complex consists of a number of subunits, including IKKα, IKKβ, and IKKγ, and the activation of the IKK complex leads to phosphorylation and ubiquitylation of IκB (Devin et al., 2000; Ghosh and Karin, 2002; Ghosh et al., 1998). Phosphorylation of IκB results in IκB degradation followed

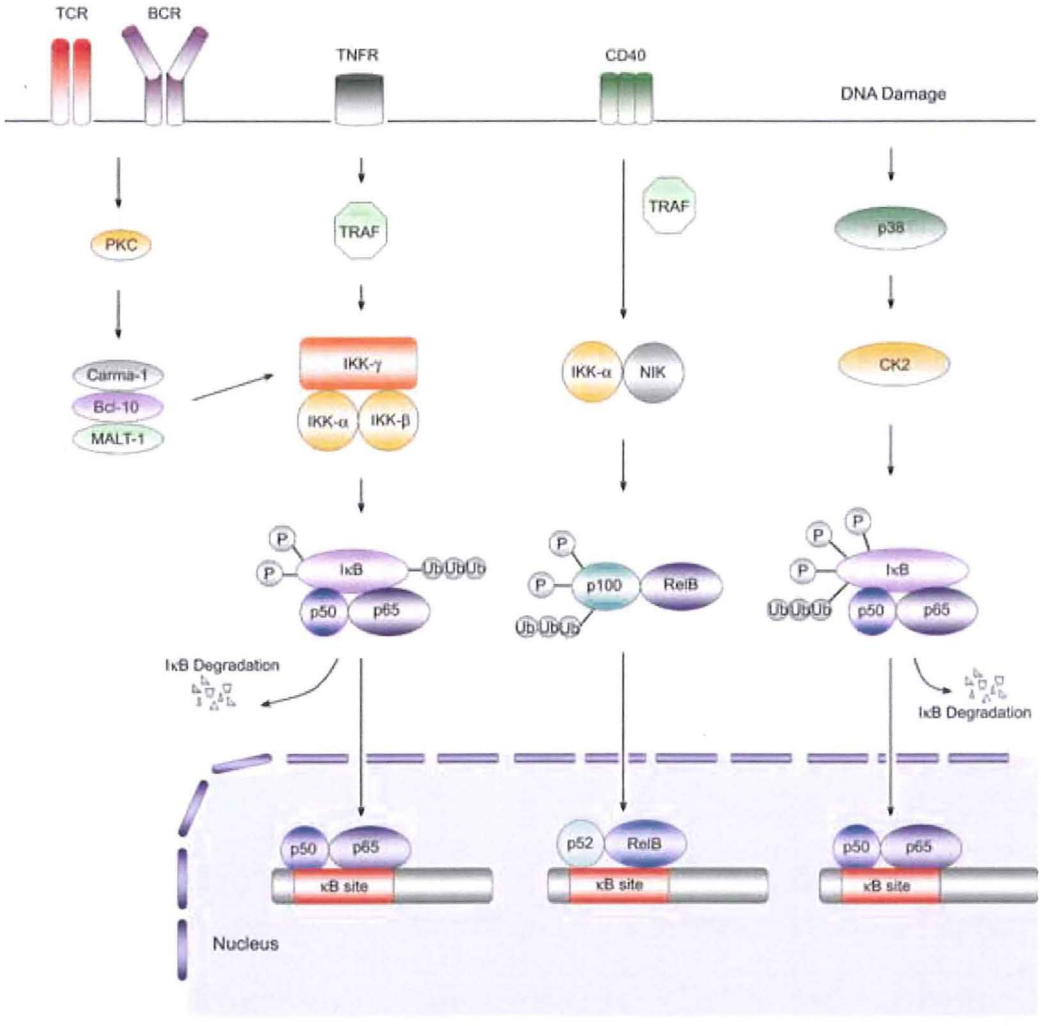

TCR
BCR
TNFR
CD40
DNA Damage
PKC
TRAF
TRAF
p38
Carma-1
Bcl-10
MALT-1
IKK-γ
IKK-α
IKK-β
IKK-α
NIK
CK2
P
IκB
Ub Ub Ub
p50
p65
P
p100
RelB
Ub Ub Ub
P
IκB
Ub Ub Ub
p50
p65
IκB Degradation
IκB Degradation
p50
p65
κB site
p52
RelB
κB site
p50
p65
κB site
Nucleus

Figure 1-5: Schematic diagram of NF-κB pathway

NF-κB pathway can be divided into 3 pathways; classical, alternative and atypical.

The classical pathway can be activated by TCR/BCR receptor or TNFR/TLR/IL-1R binding. The TCR/BCR receptor binding activates PKC which in tern activates the IKK complex via Carma1/Bcl-10/MALT1 complex activation. TNFR binding activates TRAF which activates the IKK complex. The alternative pathway is activated by ligation of CD40L or LTβ which results in activated of TRAF. In this pathway, NIK/IKK complex is activated which facilitates processing of p100. The atypical pathway is IKK independent pathway and this pathway is activated by direct DNA damage such as UV light. The atypical pathway relies on sequential p38 and CK2 activation to phosyphorylate IKK. The dissociation of IKK complex from NF-κB family members results in the nuclear translocation where they bind to the κB sites of the target genes to activate transcription.

(adapted from (Siebenlist et al., 2005))

by NF-κB nuclear translocation (Yamaoka et al., 1998). This pathway leads to the transcription of a broad range of soluble inflammatory mediators, genes associated with apoptosis, macrophage, B and T cell functional genes (Caamano and Hunter, 2002; Dieckhoff et al., 2005; Gerondakis et al., 1996; Liou et al., 1999; Lucas et al., 2004; Weih et al., 1995). The activation of T cells via TCR signaling requires this route of NF-κB activation. Recent studies employing knock out mice demonstrated the essential roles of PKCθ interacting with Bcl10/CARMA1/MALT1 units in activating IKK complex (Lucas et al., 2004; Sun et al., 2000).

The second pathway (alternative pathway) is triggered by exogenous cytokine stimuli, such as lymphotoxin β, B cell activating factor (BAFF), CD40L and viral proteins (Atkinson et al., 2003; Bonizzi and Karin, 2004). This NF-κB pathway involves TRAF molecules recruited to the cellular membrane and NF-κB-inducing kinase (NIK) (Xiao et al., 2001). NIK activates IKK heterodimer which does not contain IKKγ and this form of IKK acts on p100, another member of IκB inhibitor protein, by ubiquitinating and cleaving p100 to produce p52 (Xiao et al., 2001). The p52 forms heterodimer with RelB and translocate into the nucleus (Bonizzi and Karin, 2004).

The NF-κB pathway can also be activated without IKK complex adding further complexity to NF-κB activation process [Figure 1.4] (Tergaonkar et al., 2003). This pathway (atypical pathway) is activated when the cell is exposed to external stress such as oxidative stress and DNA damage by UV (Kato et al., 2003; Tergaonkar et al., 2003). These stimuli induce protease-mediated IκB dissociation by p38-activated casein kinase 2 (CK2), resulting in nuclear translocation of p50p65 heterodimers (Kato et al., 2003).

1.6.2 NF-κB and the immune system

NF-κB plays central roles in development and maintenance of immunity (Caamano and Hunter, 2002; Lucas et al., 2004; Siebenlist et al., 2005). During development, deficiencies in NF-κB components result in a number of defects in immune architecture. While $cRel^{-/-}$ mice have been shown to develop normally and acquire a structurally normal immune system (Gerondakis et al., 1996; Liou et al., 1999), transgenic mice lacking RelB develop normally but suffer from abnormal lymphocyte and macrophage distribution in the spleen as well as defects in germinal center formation (Poljak et al., 1999; Weih et al., 2001). NF-κB2 is also required for normal formation of germinal centers and $NF\text{-}\kappa B2^{-/-}$ mice exhibited severe deficiency of B cells in their lymph nodes and spleen (Caamano et al., 1998; Franzoso et al., 1998; Weih et al., 1995). This disruption could be due to immune cells dying from excessive apoptosis in these mice (Hirotani et al., 2005). In fact, NF-κB activation has been associated with inhibition of apoptosis via activation of anti-apoptotic genes, including TRAF1, TRAF2, cIAP-1, cIAP-2, IEX-1L, Bcl-x_L, Bfl-1/A1 (Beg et al., 1995; Grumont et al., 1999; Khoshnan et al., 2000; Van Antwerp et al., 1996; Wang et al., 1998; Wu et al., 1998). It has been shown that macrophages from mice lacking IKKβ , thus lacking NF-κB signaling, become very sensitive to LPS-induced apoptosis (Hsu et al., 2004). The anti-apoptotic role of NF-κB has also been reported in T and B cells pre- and post-activation (Bonizzi and Karin, 2004; Ferreira et al., 1999; Siebenlist et al., 2005). These studies collectively indicate the important role of NF-κB signaling in maintaining architecture of immune organs and promoting survival of leukocytes. This survival signal may contribute to sustaining the immune response during the process of pathogen clearance.

NF-κB defects result in dysregulation of not only survival but also effector functions of innate and adaptive immune cells. NF-κB is a critical factor orchestrating the levels of soluble factors, cytokine and chemokines, which significantly influence the recruitment and

activation of immunity. Cytokines (such as IL-1, IL-2, IL-6, IL-12, IFN-γ, TNF-α, LTα, LTβ and GM-CSF), and chemokines (such as IL-8, MIP-1α, MCP1, RANTES, and eotaxin), as well as adhesion molecules (such as ICAM, VCAM, and E-selectin) have been reported to be under transcriptional control of NF-κB (Finn et al., 2001; Ghosh and Karin, 2002; Grigoriadis et al., 1996; Hoffmann et al., 2002; Mason et al., 2002). It was shown that mast cells produce IL-9 in NF-κB dependent manner (Stassen et al., 2001). Several studies have shown that NK cell production of IFN-γ is regulated in an NF-κB classical pathway-dependent manner (Caamano et al., 1998; Garcia et al., 1999; Hunter et al., 1997; Nandi et al., 1994). Caamano *et al.* employed RelB deficient mice and identified RelB as one of regulating factor in this process (Caamano et al., 1998). They showed that lack of RelB results in a defect in the ability of NK cells to produce IFN-γ in response to immunological stimuli (Caamano et al., 1998). Macrophages from $RelB^{-/-}$ mice also display abnormal cytokine production such as over-production of IL-1 and impaired production of TNF-α (Caamano et al., 1998). cRel also plays a role in controlling the levels of TNF-α, IL-6, IL-12 and GM-CSF in macrophages (Caamano et al., 1999; Grigoriadis et al., 1996; Mason et al., 2002). While these soluble mediators are involved in amplification of effector functions in innate immune cells, their production of cytokines such as IFN-γ, TNF-α, IL-12, and GM-CSF, could also direct development of adaptive immunity involving T cell, B cells and DCs.

It is well established that the NF-κB pathway is required for T and B cell development. Studies have shown that lack of NF-κB results in reduction of both T and B cells numbers with increased levels of apoptosis (Boothby et al., 1997; Caamano et al., 1998; Esslinger et al., 1997; Ferreira et al., 1999; Senftleben et al., 2001). Transgenic mouse studies revealed that mice deficient in NF-κB exhibits a significant reduction in B cells and $CD8^+$ T cells in the periphery (Caamano et al., 1998; Esslinger et al., 1997). Furthermore, NF-κB activities

are also associated with effector functions of T and B cells. It was shown that mice deficient in Bcl-10 (which is important for TCR/BCR mediated NF-κB activation; see Figure 1.4) were severely immunocompromized and T and B cells from these mice were unable to proliferate in response to receptor or PMA/ionomycin-induced activation (Ruland et al., 2001).

While NF-κB signaling protects B cells from apoptosis, NF-κB allows B cells to mature upon antigen-stimulation (Franzoso et al., 1998). Franzono *et al.* reported that B cells in NF-κB2$^{-/-}$ mice were not able to mature and displayed impaired IgA and IgG antibody production in response to antigen-challenge (Franzoso et al., 1998). Similarly, the requirement of RelA and RelB in IgA and IgG antibody production has been documented (Doi et al., 1997; Li et al., 2003). These studies illustrate that NF-κB are regulators of antibody production in B cells. Attenuation of B cell response by NF-κB deficiency is known to render mice to become more susceptible to infections, for example, against gram-positive bacterial infections due to a compromised B cell response to LPS (Sha et al., 1995).

As described in the last section, NF-κB dependent innate production of cytokines, such as IFN-γ, TNF-α, IL-6 and IL-12 conditions the microenvironment to activate T cell responses (Caamano et al., 1999; de Haij et al., 2005; Hirotani et al., 2005; Mason et al., 2002). The absolute requirement of NF-κB in T cell mediated immunity is well established. It has been shown that the inhibition of NF-κB in T cells results in a significant reduction in T cell proliferation (Boothby et al., 1997; Ferreira et al., 1999; Mora et al., 2001; Ruland et al., 2001). Studies by Ishikawa *et al.* and Carrasco *et al.* determined that NF-κB1 and cRel are the major factors associated with T cell proliferation (Carrasco et al., 1998; Ishikawa et al., 1998). TCR mediated activation of T cells relies on the NF-κB pathway for subsequent transcriptional activation of inflammatory cytokines (Siebenlist et al., 2005). It is now clear

that TCR signaling activates PKC-θ which interacts with BcL-10/Carma1/MALT-1 complex, leading to NF-κB pathway activation (Sun et al., 2000). Mora *et al.* showed that failure of T cells to proliferate in NF-κB-deficient mice is, at least partially, due to defects in STAT5α activation by the NF-κB pathway (Mora et al., 2001). The transcription factor STAT5α is one of the NF-κB target genes and is required for T cell proliferation by controlling the production of IL-2 and IL-4 (Mora et al., 2001). T cells which have IκB resistance to degradation were not able to activate STAT5α, and could not proliferate in response to immunization and lacked IL-2 production (Mora et al., 2001). Bcl-10 deficient mice exhibited lack of IL-2 production and CD25 (IL-2 receptor) expression in the T cells which were incapable of proliferating (Jain et al., 1995; Ruland et al., 2001). Furthermore, costimulatory signals, such as B7-CD28, have been shown to induce IL-2 and Bcl-x_L in T cells via cRel nuclear translocation enabling activated T cells to survive and amplify the immune response (Boise et al., 1995; Gerondakis et al., 1996; Kahn-Perles et al., 1997). The findings that cRel is important for the induction of IL-3, IFN-γ, and GM-CSF production by T cells further confirms the role of NF-κB pathway in the amplification of T cell mediated responses (Gerondakis et al., 1996). In addition, The association between NF-κB and DTH responses further suggested a contribution of the NF-κB pathway to T cell mediated immunity (Caamano and Hunter, 2002).

It has been proposed that the NF-κB pathway is also involved in Th1/Th2 lineage differentiation i.e. different NF-κB components direct T cell towards either Th1 or Th2 fates. Lederer *et al.* reported that while resting Th1 and Th2 cells both express comparable levels of RelA and cRel, Th1 cells upregulated RelA expression in response to TCR stimulation whereas Th2 cells could not (Lederer et al., 1996). An experimental model of autoimmune encephalomyelitis has shown that $cRel^{-/-}$ mice were completely defective in generating Th1 response and these mice completely lacked IFN-γ production (Hilliard et al., 2002). Another

report demonstrating the requirement of cRel for IFN-γ production is consistent with this finding (Dieckhoff et al., 2005). Moreover, cRel and RelA are required for optimal production of IL-4, a Th2 type cytokine (Casolaro et al., 1995; Li-Weber et al., 1998). Collectively, cRel could be a key molecule in controlling Th1 response. On the other hand, NF-κB1 is considered as a regulator of Th2 response. NF-κB1$^{-/-}$ mouse studies revealed that Th2 responses were abrogated while Th1 response remained unaffected (Das et al., 2001). They also demonstrated that these mice lacked IL-4 production due to a blockade of Gata3-mediated Th2 cell differentiation (Das et al., 2001). Interestingly, it was demonstrated that cRel does not regulate the activity of T-bet (Th1 specific transcription factor); instead, cRel acts downstream of T-bet (Hilliard et al., 2002). This may suggest that while the NF-κB pathway is a key component of T cell lineage differentiation, differential NF-κB family expression is a result of Th1/Th2 specific transcription factor activation and does not initiate Th1/Th2 lineage differentiation first hand. It could also be speculated that there may be simultaneous involvement of other transcription factors directing Th1/Th2 lineage differentiation together with the NF-κB pathway.

1.6.3 NF-κB and dendritic cell function

Like other immune cells, DCs also require NF-κB signaling for development (Burkly et al., 1995; Wu et al., 1998). A number of studies have indicated that RelB deficiency results in lack of DC subsets including CD8α^{+} DCs and thymic DCs (Burkly et al., 1995; Wu et al., 1998). Recently, Cejas *et al.* reported that RelB deficient mice were unable to activate PKCβ, which is involved in cell growth and division, and they argued that this could account for the problems with DC development (Cejas et al., 2005). TLR-9-mediated activation of NF-κB in DCs has been found to promote DC survival and the limited survival of NF-κB1$^{-/-}$cRel$^{-/-}$ DCs was due to failure to upregulate the anti-apoptotic gene, Bcl-x_L, (O'Keeffe et al., 2005).

NF-κB activation in DCs is required for priming effector T cells (Boffa et al., 2003; Rescigno et al., 1998). Studies have also revealed that NF-κB is involved in activation of DCs and their production of inflammatory cytokines thus enabling DCs to stimulate T cells (Caamano and Hunter, 2002). Correlations between NF-κB expression (p50, RelA, RelB and cRel) and DC activation and severe impairment of T cell priming capacity of DCs in NF-κB deficient mice strongly suggest the absolute requirement of NF-κB in DCs to promote T cell responses (Baltathakis et al., 2001; Clark et al., 1999; Hofer et al., 2001; Neumann et al., 2000). Calder *et al.* demonstrated that over-expression of IκB in DCs significantly attenuated their capacity to stimulate T cell proliferation; production of IFN-γ, IL-4 and IL-10 was reduced upon stimulation of these DCs with tetanus toxoid peptide (Calder et al., 2003). $cRel^{-/-}$ DCs lack IL-12p35 transcription in response to LPS stimulation and IL-12p70 production was absent in these DCs (Grumont et al., 2001; O'Keeffe et al., 2005). Furthermore, $cRel^{-/-}$ DCs have less capacity to produce IFN-γ and IL-4 (Boffa et al., 2003). Production of other inflammatory cytokines such as IL-6 and GM-CSF by DCs is also regulated in a NF-κB-dependent manner, further underlying its importance in T cell priming (Cruz et al., 2001; Mann et al., 2002). T cells exposed to $cRel^{-/-}$ DCs proliferate poorly compared to those that encountered wild type (WT) DCs and the weaker T cell priming resulted much less IL-2 production (Boffa et al., 2003). Analyses of these NF-κB deficient DCs revealed that the NF-κB pathway is critical for upregulation of costimulatory molecules and inflammatory cytokine production by DCs (Kopp and Medzhitov, 1999; Mintern et al., 2002; Speirs et al., 2004). Expression of MHC Class II and costimulatory molecules, such as CD40, CD80 and CD86, have been shown to be under NF-κB transcriptional control (Kopp and Medzhitov, 1999; Mintern et al., 2002; Speirs et al., 2004). Blockade of NF-κB signaling renders DCs incapable of upregulating the expression of these costimulatory molecule expression and they have significantly reduced capacity to prime T cells upon stimulation (Mintern et al., 2002; Morelli et al., 2000).

A study by Andreakos *et al.* revealed that the classical NF-κB pathway involving IKK2, but not the alternative pathway involving NIK, is important for DC activation induced by CD40L or contact with allogeneic T cells, but not by LPS (Andreakos et al., 2003). Thomas *et al.* reported that DC maturation in response to LPS requires the alternative pathway (Thomas et al., 2005). These studies collectively suggest that different routes of NF-κB activation induced by different immunological stimuli allow DCs to mature independently. Despite these studies, factors responsible for the choice of NF-κB activation pathway by DCs following different stimuli and the regulatory mechanism of DC-T cell interaction is still poorly understood.

1.6.4 NF-κB and tolerance induction

The regulatory roles of NF-κB in costimulatory molecule expression and cytokine production in DCs identified the NF-κB as a potential target for the induction of tolerogenic DCs. There is accumulating evidence that NF-κB signaling blockade can induce tolerogenic DCs which, in some cases, can induce regulatory T cell differentiation (Iruretagoyena et al., 2006; Martin et al., 2003; Nouri-Shirazi and Guinet, 2002; Tan et al., 2005). It has been shown that treatment of DCs with the NF-κB inhibitor, N-acetyl-L-cystein, (NAC) prevented DCs from maturing and these DCs promoted antigen-specific alloreactive $CD4^+$ T cell anergy (Nouri-Shirazi and Guinet, 2002). Another study using DCs treated with vitamin C and E (which can inhibit NF-κB signaling) demonstrated alloantigen-specific T cell anergy (Tan et al., 2005); these anergic T cells could suppress naïve T cell proliferation in a contact-dependent manner (Tan et al., 2005). The differentiation of regulatory T cells by DCs lacking NF-κB signaling has also been reported. Treatment of NF-κB inhibitors andrographolide or rosiglitazone, were shown to block costimulatory molecule upregulation and cytokine production in DCs after LPS-activation and the administration of these DCs *in*

vivo resulted in a decreased severity of experimental autoimmune encephalomyelitis (EAE) (Iruretagoyena et al., 2006). The authors found upregulation of Foxp3 in splenocytes harvested from the DC-treated mice and speculated that enhanced regulatory T cell differentiation in these mice, resulted in reduced EAE severity (Iruretagoyena et al., 2006). Martin *et al.* demonstrated that $RelB^{-/-}$ DCs, which lack CD40 expression and reduced MHC class I and II expression, induced antigen-specific tolerance via generation of $CD4^+$ regulatory T cells (Martin et al., 2003). They showed that $RelB^{-/-}$ DCs pulsed with keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) induced antigen-specific unresponsiveness in the $Rel^{-/-}$ DC-treated mice via the generation of IL-10-producing $CD4^+$ regulatory T cells (Martin et al., 2003). These DCs were also capable of preventing the expansion of previously primed T cells (Martin et al., 2003). Although evidence for NF-κB blockade in the generation of tolerogenic DCs and antigen-specific tolerance is still limited, there may be potential for this approach in establishing transplant tolerance.

1.6.5 NF-κB and allotransplantation

Studies have shown a correlation between NF-κB activation and graft rejection. Elevated levels of NF-κB gene expression have been reported in rejecting cardiac and liver allografts and graft- infiltrating cells (Bierhaus et al., 2001; Csizmadia et al., 2001; Lee et al., 2001; Xu et al., 2003). One mechanism by which NF-κB can facilitate graft rejection is through inducing the production of nitric oxide via activation of inducible nitric oxide synthase (iNOS) gene expression (Cooper et al., 1998; Cruz et al., 2001). A study by Cooper *et al.* showed decreased incidence of rat cardiac allograft rejection by administration of pyrroclidine dithiocarbamate (PDTC), an NF-κB inhibitor (Cooper et al., 1998). They demonstrated that reduction of iNOS (NO production) by NF-κB inhibition correlated with the graft protection (Cooper et al., 1998). As described in Section 1.2, T cells are the primary effector cells during allograft rejection. Several studies have shown beneficial effects of

NF-κB modulation in controlling T cell-mediated allograft rejection. Finn *et al.* reported that cardiac allograft tolerance can be induced using transgenic mice lacking NF-κB activity in their T cells as allograft recipients (Finn et al., 2001; Zhou et al., 2003). These mice showed reduced expression of inflammatory cytokines, such as IL-6 and IL-1β, and chemokines, such as MIP-1α, -1β, -2, and MCP-1, early post-transplant (Finn et al., 2001). A later study showed that this graft acceptance was not due to the generation of $CD4^+CD25^+$ regulatory T cells and that tolerance could not be transferred to fresh NF-κB-competent T cells (Zhou et al., 2005). Instead graft survival was attributed to enhanced apoptosis of alloreactive T cells due to a defect in the anti-apoptotic expression of BcL-x_L (Zhou et al., 2005).

Current immunosuppressive drugs e.g. cyclosporine, deoxyspergualin (DSG), and glucocorticoids, have been shown to suppress the immune response by inhibiting NF-κB activation (Auphan et al., 1995; Lee et al., 1999; Scheinman et al., 1995). Contreras *et al.* showed that a brief course of DSG treatment can lead to prolonged kidney allograft acceptance (Contreras et al., 1998). They reported that graft protection was due to inhibition of IL-12 mediated IFN-γ production through NF-κB blockade as well as skewing of the immune response to a Th2 type (Contreras et al., 1998). Lee *et al.* successfully generated immature BMDCs which are capable of inducing alloantigen-specific T cell hyporesponsiveness by cyclosporine administration *in vitro* (Lee et al., 1999). A recent study showed that $cRel^{-/-}$ and NF-κ$B1^{-/-}$ mice are resistant to low-dose streptozotocin-induced diabetes (Lamhamedi-Cherradi et al., 2003; Mabley et al., 2002), possibly due to defects in cytokine production (reduction of IL-12 and TNF-α) by DCs and macrophages. In addition, accelerated apoptosis of granulocytes and macrophages in NF-κ$B1^{-/-}$ mice and of DCs in $cRel^{-/-}$ mice may have contributed (Lamhamedi-Cherradi et al., 2003).

There is evidence that the inhibition of the NF-κB pathway in DCs could promote allograft protection via development of tolerogenic DCs (Bonham et al., 2002; Giannoukakis et al., 2000; Ohmori et al., 2005; Xu et al., 2004). A number of studies have reported that administration of donor-type DCs pretreated with NF-κB inhibitors prolongs allograft survival (Bonham et al., 2002; Giannoukakis et al., 2000; Ohmori et al., 2005; Saemann et al., 2004; Xu et al., 2004). Xu *et al.* showed that DCs treated with NF-κB decoy oligodeoxynucleotides (ODN) were resistant to IL-4- or LPS- induced activation and these DCs failed to prime alloreactive T cells (Xu et al., 2004). *In vitro* administration of these DCs prolonged liver allograft survival and resulted in an increased incidence of apoptosis in graft infiltrating cells (Xu et al., 2004). Similarly, NF-κB decoy ODN-treated DCs have been shown to prolong cardiac allograft survival (Xu et al., 2004). It was also shown that a combination of NF-κB decoy ODN treatment and CTLA4-Ig expression on BMDCs can potentiate alloreactive T cell apoptosis and enhanced IL-10 expression resulting in a prolonged cardiac allograft survival (Bonham et al., 2002).

Recently, Min *et al.* showed that NF-κB inhibition in DC can lead to generation and expansion of regulatory T cells in transplantation settings (Min et al., 2003). Treatment of DCs with the NF-κB inhibitor, LF15-0195, together with anti-CD45RB antibody rendered the DCs tolerogenic and capable of generating $CD4^+CD25^+$ regulatory T cells (Min et al., 2003). Treatment of cardiac allograft recipients with these DCs resulted in alloantigen-specific tolerance and a significant increase in the number of $CD4^+CD25^+$ T cells in the spleen (Min et al., 2003). Furthermore, these regulatory T cells were able to drive the generation of tolerogenic DCs from DC progenitors *in vitro* (Min et al., 2003).

Studies on the modulation of NF-κB pathway in inducing tolerance to cellular allografts are still limited, and no reports demonstrating tolerance to islet allografts by manipulating

NF-κB pathway have been documented. Nevertheless, Kutlu *et al.* demonstrated that human pancreatic β-cells produce a chemokine, MCP-1, in a NF-κB signaling-dependent manner during islet destruction by insulitis (Kutlu et al., 2003). This study, together with the significance of the NF-κB pathway-mediated production of inflammatory cytokines (e.g. IFN-γ, IL-2, and TNF-α)/chemokines during rejection of cellular allografts, suggests a potential benefit of inhibiting of NF-κB to prevent islet allograft rejection.

Collectively, these studies strongly suggest NF-κB signaling can act as a potential target for preventing allograft rejection. Further research is required to ascertain how intervention of the NF-κB pathway can be optimized to promote allograft tolerance.

1.7 Scope of thesis

The aim of this thesis was to investigate whether modulation of Notch and NF-κB signaling in dendritic cells could lead to induction of allograft protection.

The rejection of neovascularized allografts is primarily mediated by T cells and the control of the alloreactive T cell response is a requisite for successful transplantation. The induction of regulatory T cell differentiation is emerging as an effective means to induce alloantigen-specific tolerance. Previous demonstration that modulation of Notch signaling in DCs could promote T cell non-responsiveness suggested a potential role for Notch signaling in the induction of allograft tolerance (Hoyne et al., 1999). It was hypothesized that presentation of allogeneic MHC together with enhanced expression of Notch-related molecules on immature donor-type DCs (using retroviral vectors) may induce differentiation of allo-antigen specific regulatory T cells in the recipient.

The second half of this thesis focuses on the use of an NF-κB inhibitor, Bay11-7082, to

modulate the immunostimulatory capacity of donor-derived passenger leukocytes. Passenger leukocytes carried in the transplant provide both signals (alloantigen and costimulation) required for the activation of alloreactive T cells in the host. The essential roles of NF-κB in DC maturation and in T cell priming by DCs are also well documented. It was hypothesized that blockade of NF-κB activation in passenger leukocytes would inhibit activation of the allograft-specific T cell response and facilitate allograft survival.

Chapter 2

Materials and Methods

2.1 Mice and reagents

2.1.1 Animals

C57BL/6J (H-2^b), CBA/H (H-2^k), and cRel$^{-/-}$ (C57BL/6J background) male mice were obtained from specific pathogen-free facilities at the John Curtin School of Medical Research (JCSMR) at The Australian National University (ANU) and housed in approved containment facilities. Where necessary, mice were purchased from the Animal Resources Center (ARC), Perth, Western Australia. Mice were treated according to ANU animal welfare guidelines. Mice at 7-12 weeks of age were used throughout these studies.

2.1.2 Media and buffers

Media and buffers used are shown in Appendix 1.

2.1.3 Cell lines

The following cell lines were used in these studies;

Phoenix Eco cell line (obtained from Dr. Mark Hulett, JCSMR.) is a retrovirus producer cell line prepared from 293 T cells, a human embryonic kidney cell line. Phoenix cells were maintained at 37°C in 5% CO_2 95% air but they were cultured at 32°C in 5% CO_2 95% air during transfection. JAWS II cells (ATCC accession no. CRL-11904), a C57BL/6-derived immature dendritic cell line (obtained from Dr. Joanne Banyer, JCSMR), were cultured in GM-CSF supplemented JAWS II medium (See Appendix 1) at 37°C in 5% CO_2 95% air. To induce activation, JAWS II cells were incubated with IL-4 (10ng/ml) (Cytolab, Israel), IFN-γ (10ng/ml) (Cytolab), TNF-α (10ng/ml) (Cytolab), and LPS (1μg/ml) (Calbiochem, San Diego, CA) for 48 hours at 5%CO_2 95% air. EL-4 T cell line was obtained from Dr. Sudah Rao and were cultured in supplemented F15 medium (See appendix 1). For activation of EL-4 T cells, cells were activated with PMA (20ng/ml) (Borhringer Mannheim, Germany)/calcium ionophore (1μM) (Sigma-Aldrich, Cat:23187) for 4 hours.

2.2 Molecular techniques

2.2.1 RNA extraction

Control and transduced JAWS II cells were frozen in liquid nitrogen and stored at -70°C until RNA extraction. Total RNA was isolated from control and transduced JAWS II cell using RNAzol B (Tel-Test Inc. Friendswood, Texas) as described by the manufacturer. In brief, 2ml of RNAzol B was added to 10^7 JAWS II cells. To the cell suspension, 200µl chloroform was added and vigorously shaken for 15 secs. The samples were stored on ice (4°C) for 5 mins followed by centrifugation at 12,000g for 15 mins. The aqueous (upper) phase was transferred to a fresh tube, an equal volume of isopropanol (Asia Pacific Specialty Chemicals Ltd, NSW, Australia) was added and the mixture was incubated at 4°C for 15 mins. The mixture was span at 12,000g at 4°C for 15 mins then the supernatant was removed and RNA pellet was washed once with 75% ethanol (Sigma) by vortexing and then centrifuged at 7500g at 4°C for 8 mins. The supernatant was removed and the RNA pellet was suspended in appropriate amount of diethylpyrocarbonate (DEPC)-treated RNase free water and kept at -70°C. The purity of RNA obtained was checked by calculating A260/A280 ratio using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) (the A260/A280 ratio was to be >1.8).

2.2.2 Reverse transcription of mRNA to cDNA

Each RNA sample was diluted to a concentration of 1µg RNA/10µl DEPC water. 30µl RNA solution was incubated at 65°C for 5 mins, and then place on ice for 3 mins. 30µl of the reverse transcription mix (each reaction mix is composed of 5×RT buffer, 2.5µl dNTP's (10mM of each dATP, dCTP, dGTP, dTTP), 1.0µl oligo $dT_{12\text{-}18}$ primers, 1µl RNAsin (40u/µl), 1 µl reverse transcriptase (200u/µl), 2µl 20mM DTT; all supplied by Promega, Wisconsin, WI) was added. The mix was incubated at 37 °C for 60 mins followed by 65 °C for 10 mins to inactivate the reverse transcriptase. The cDNA samples were then stored at -20°C until

PCR analysis.

2.2.3 Semi-quantitative RT-PCR analysis

Semi-quantitative polymerase chain reaction (PCR) was performed to determine the relative expression of mRNA for various Notch-related molecules. 1μg cDNA (in a volume of 20μl) was amplified in a 50μl reaction volume containing 0.2mM dNTPs (Promega), 1-3mM $MgCl_2$ (Invitrogen Australia Pty Ltd, Victoria, Australia), 0.4-1μM forward and reverse primers, 1.4-2.75U Taq polymerase (Fisher Biotech), and reaction buffer (supplied with the enzyme). 10μl aliquots of the reaction mix were sampled at 5-cycle intervals from 25 to 35 cycles. Standard reaction parameters were 94°C 3-5 mins, 94°C 30s-1 min 55-68°C 10-30s, 72°C 30s-1 min. Primers for the house-keeping gene GAPDH were used as controls to indicate that there were similar levels of amplifiable cDNA in samples. Primer sequences for GFP, mMfng, mLfng, mJagged-1, and mDll-1 are as described in Appendix 2.

PCR products were size-fractioned by electrophoresis through 0.8-1.2% agarose gels; the gels were stained in ethidium bromide solution (2μg/ml) for 10-20 mins and photographed under UV illumination. The sizes of the PCR products were verified against a marker of known standards (1kB Plus DNA ladder (Gibco BRL)). Gels were photographed using SynGene BioImaging System Gene Genius and GeneSnap software (Syngene, Frederick, MD) under UV light for the preparation of digital images. The band intensities were measured using Image Gauge version 3.3 (Fuji Photofilm Co. Ltd, Japan) and exported to Microsoft Excel (Microsoft Co, WA). The gene expression was standardized with GAPDH expression and relative expression levels were calculated setting the gene expression in untransduced JAWS II cells as 1.

2.2.4 Polymerase chain reaction for Gateway™ cloning technology

The genes were PCR amplified with primers encoding the gene with *att*B flanking regions and the amplified gene/*att*B were used for constructing pDONR carrying the gene of interest. PCR was performed with 100ng template DNA, 50pM primers (Appendix 3), 5μl 10×Buffer (Supplied with the enzyme), 0.75μl 20mM dNTPs, 1μl 50mM $MgSO_4$(Invitrogen), 1μl Platinum® Pfx DNA polymerase (2.5units) (Invitrogen) in a total volume of 50μl. The amplification condition were: 94°C 2 mins followed by 35 cycles of 94°C and 15 secs and 68°C 3 mins. The PCR products were checked by electrophoresis as described in Section 2.2.3.

2.2.5 Sequencing of pKMV constructs with Notch-related genes

pKMV plasmid containing Notch-related genes were constructed using Gateway Technology. In brief, the Gateway Reading Frame Cassette, RfA, was inserted into pKMV-Δ plasmid (See Section 3.2 in Chapter 3). Notch-related genes flanked by *att*B sequence on 3' and 5' ends were cloned into pKMV-RfA via BP reaction followed by LR reactions (See Section 3.3-3.4 in Chapter 3). Full-length sequencing of the cloned gene within pKMV-plasmid was performed using multiple primers which were designed to cover the inserted gene (See Appendix 4). Reaction mix consisting of 200ng template, 3.2pmol sequencing primer, 2μl Ready Reaction Premix in BigDye® (Biomolecular Resource Facility (BRF, JCSMR) was made up to 20μl with MilliQ water. Sequencing cycles were: 94°C for 5 mins, followed by 30 cycles of 96°C 10 secs, 50°C 5 secs, 60°C 4 mins. Unincorporated dye terminates were removed by treating with Big DyeEx Spin Kit (QIAGEN, CA) according to the manufacturer's instruction and sequences were analyzed by ABI 3730 Capillary Genetic Analyzer by staff at BRF, JCSMR. Sequence data was visualized with Chromas software

(Technelysium, Queensland, Australia) and aligned to reference sequences on nucleotide-nucleotide BLAST (NCBI, http://www.ncbi.nlm.nih.gov/blast/).

2.2.6 Restriction digest

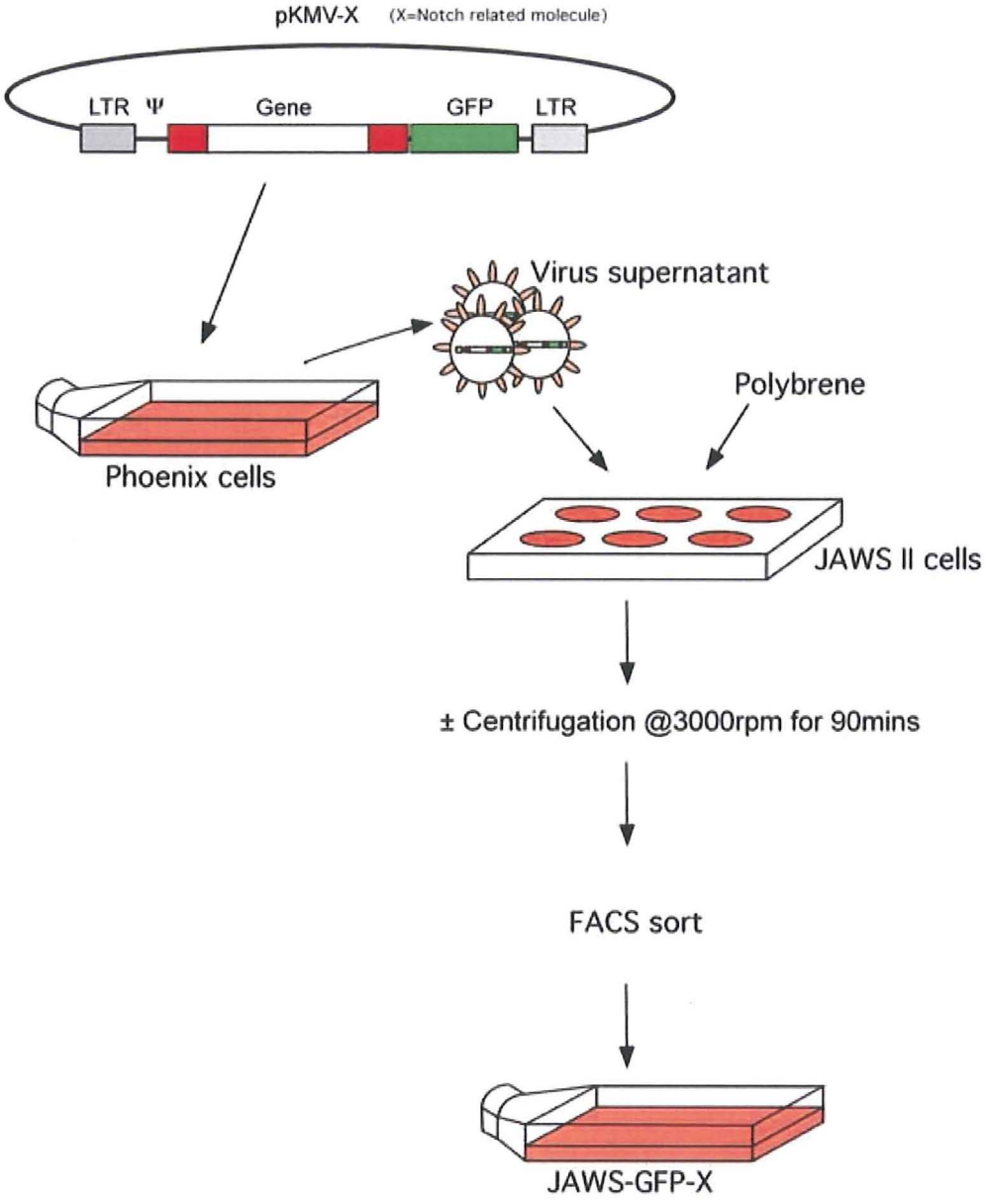
Restriction digests were performed to check the presence of inserted genes in pKMV-Carrying the Notch-related genes. Restriction digests were conducted using 200ng sample DNA, 2.5µl of 10X Buffer, 0.1mg/ml BSA, and 1µl of the appropriate enzymes; NcoI and EcoRI for pKMV-GFP-mMfng; EcoRI and BamHI for pKMV-GFP-mLfng; BglII for pKMV-GFP-mJag-1; EcoRI and SalI for pKMV-GFP-Delta-l1 (all from New England BioLabs, Beverley, USA) in a final volume of 25µl. Reactions were incubated at 37°C for 1 hour, then run on 1.0-1.2% agarose gel for visualization of products.

2.3 *In Vitro* techniques

2.3.1 Production of retrovirus and retroviral transduction of JAWS II cells

2.3.1.1 Low titer retrovirus-transduced JAWS II cells

For production of retroviral constructs, pKMV constructs containing a single Notch-related molecule and GFP was transfected into Phoenix Ecotropic packaging cells (developed by Dr Gary Nolan , Stanford University, Stanford, UC, and provided by Dr Mark Hulett, JCSMR) using calcium phosphate method (www.stanford.edu/group/nolan/protocols/pro_helper_dep.html) (Figure 2-1). In brief, 8µl 2M chloroquine (Sigma) was added to Phoenix cells (at approximately 60% confluence) in T75 flasks (NUNC, Roskilde, Denmark) containing Phoenix medium (See Appendix 1) and incubated for 5 mins at 37°C to increase transfection efficiency and to inhibit degradation of DNA absorbed by the cells(Luthman and Magnusson, 1983). 40µg retroviral DNA (pKMV-GFP-Δ, pKMV-GFP-mJag-1, pKMV-GFP-mLfng and pKMV-GFP-mMfng) were


pKMV-X
(X=Notch related molecule)
LTR
Ψ
Gene
GFP
LTR
Virus supernatant
Polybrene
Phoenix cells
JAWS II cells
± Centrifugation @3000rpm for 90mins
FACS sort
JAWS-GFP-X

mixed with 155µl 2M $CaCl_2$ and 1250µl 2x HBS buffer (pH7.05) and bubbled vigorously for 30 secs to ensure formation of good viral particles. The DNA/$CaCl_2$/HBS mix was added to Phoenix cells and incubated at 32°C in 5%CO_2 95% air. Medium was replaced after 24 hours and the cell culture was incubated for a further 24 hours. The culture supernatant containing retrovirus was then collected. For pKMV-retroviral transduction of JAWS II cells, 1.3ml HIFCS, 2.6µl mGM-CSF (5mg/ml)(PeproTech Inc.), 27.3µl hexadimethrine bromide (polybrene) (5mg/ml) (Sigma) (which is positively charged molecule that binds to cell surfaces and neutralizes surface charge enhance transduction efficiencies by reducing the repulsion between virus and cells) and the viral supernatant was added to JAWS II cells (at 70 % confluence) in 13ml JAWS II medium (Section 2.1.2) and incubated at 37°C 5%CO_2 95% for 48 hours. JAWS II cells expressing GFP was recovered by FACS sort using FACSVantage SE (BD Pharmingen, San Jose, CA) and FACSDiVa Option (Becton Dickinson). Sorted cells were allowed to propagate for approximately 1 week then FACS sorted as described above. This procedure was repeated until ≥80% of total population is GFP-positive.

2.3.1.2 High titer retrovirus-transduced JAWS II cells

Phoenix cell cultures with pKMV constructs were set up as described above (see Section 2.3.1.1). After replacement of Chiloquin-containing medium, virus culture supernatant was collected every 12 hours and snap-frozen in liquid nitrogen. Virus supernatant was stored at -70°C for subsequent transduction. Transfection efficiency was routinely tested by measuring % GFP-positive Phoenix cells using BD FACSCalibur (BD pharmingen; with a single argon laser). Events were collected for a gated live population, and analyzed using CellQuest software version 2.0 (BD Biosciences) to indirectly ascertain a measure of viral titer (>50% GFP-positive cells). JAWS II cells were transduced using centrifugal enhancement method as described in Bahnson et. al(Bahnson et al., 1995). In brief, JAWS II

cells were plated in 6 well-plates ($6x10^5$ cells per well) (Nunc, Cat:140676). 3µl polybrene (5mg/ml) and 3ml virus supernatant was added to each well and the plate was centrifuged at 1300g for 90 mins at 37°C. The virus supernatant was replaced with fresh medium and the cells were incubated at 37°C for 24 hours. JAWS II cells were transduced again using the protocol described above then allowed to propagate. JAWS II cells expressing GFP was recovered by FACS sort. Transduced JAWS II cells were sorted until >80% of the total population becomes GFP-positive.

2.3.2 Splenocyte preparation

Spleens were dissected from C57BL/6 mice and mashed through a fine sieve (BD pharmingen Cat: 352360) into approximately 15ml complete MLC medium. Cell debris was removed by leaving the suspension for 5 mins on ice and the supernatant was then removed. Red blood cells were lysed with approximately 5 ml red cell lysis buffer (Section 2.1.2) per 2×10^7 cells by incubating on ice for 5 mins. Cells were washed by resuspending in MLC medium and centrifuged at 200g at 37°C for 5 mins then resuspended in MLC medium (Section 2.1.2). To induce activation, splenocytes were incubated in MLC medium containing IL-4 (10ng/ml) (Cytolab, Israel), IFN-γ (10ng/ml) (Cytolab), TNF-α (10ng/ml) (Cytolab), and LPS (1µg/ml) (Calbiochem, San Diego, CA) for 48 hours at 5%CO_2 95% air. Splenocytes were then washed with MLC medium and used for further experiments. For experiments with Bay11-7082, splenocytes were incubated for 1 hour with 0.5-20µM Bay11-7082 (Sapphire Bioscience, NSW, Australia) in 5%CO_2 95% air at 37°C. Cells were then washed with MLC medium three times before use. For experiments with pentoxifylline (Sigma), splenocytes were resuspended in 2.5-20mg/ml pentoxifylline in MLC medium and incubated for 1 hour in 5%CO_2 95% air at 37°C. The cells were washed by resuspending in MLC medium 3 times.

2.3.3 Lymph node cell preparation

Lymph nodes (superficial cervical nodes, mediastinal nodes, axillary nodes and inguinal nodes) were dissected from CBA/H and C57BL/6J mice. The lymph nodes (LN) mashed through a fine sieve (BD Pharmingen) into approximately 15ml MLC medium. The supernatant was centrifuged at 200g at 37°C for 5 mins and resuspended in MLC medium to make 3×10^6 cells/ml.

2.3.4 Isolation of bone marrow-derived DCs

Bone marrow cells were flushed from the femur and tibia of C57BL/6 mice with BMDC flush medium (Section 2.1.2). Cells were resuspended in BMDC culture medium (Section 2.1.2) and 2×10^6 cells/6ml/well were cultured in 6-well plates (Nunc) at 37°C in 5%CO_2 95% air. The medium was changed on day 3 with removal of non-adherent cells. Semi-adherent cells were collected on sixth day of culture by gentle aspiration using a pasture pipette and 1×10^6 cells/6ml culture medium were cultured in the 6 well plates for a further 24 hours. For BMDC activation IL-4 (10ng/ml) (Cytolab), IFN-γ (10ng/ml) (Cytolab), TNF-α (10ng/ml) (Cytolab), and LPS (100ng/ml) (Calbiochem) were added and cells were collected and rinsed in fresh culture medium 48 hours post-activation (Jorgensen et al., 2002). For Bay11-7082 experiments BMDCs were incubated in the presence of 5μM Bay11-7082 as described in Section 2.3.2 for 1 hour prior- or post-activation with cytokines/LPS and cells were washed three times before use.

2.3.5 Flow cytometry analysis

Antibodies used for flow cytometry were as follows:

anti-CD40:PE, anti-CD80:PE, anti-CD86:PE, anti-CD69:PE, anti-IA^b:Biotin, anti-H-$2K^b$:Biotin, anti-CD4:PE, anti-CD8α:FITC, anti-CD16/CD32 purified hamster IgG, purified rat IgG, (all from BD pharmingen), anti-Dec205:Biotin (Cedarlane, Ontario,

Canada), anti-CD11c:PE Cy5.5 (Caltag, Burlingame, CA), goat anti-mJagged-1, (R&D Systems, Minneapolis, MN), goat anti-Delta (Santa Cruz Biotechnology, CA), streptavidin PE (Serotec, Oxford, UK), and swine anti-goat:PE (Caltag).

5×10^5 cells were plated in U-shaped 96-well plates (Linbro/Titertek Flow Laboratories, McLean. VA) and spun at 200g for 5 mins at 4°C to pellet the cells. The plates were flicked to remove the medium then 26μl Fc block (1:100 anti-Fc receptor antibody, 1:10 anti-rat IgG antibody, and 1:10 anti-hamster IgG antibody) was added to each well to prevent non-specific binding of antibodies. Plates were then left on ice for 20 mins. The cells were washed with 200μl FACS buffer (Section 2.1.2) in each well and centrifuged at 200g for 5 mins at 4°C. Plates were flicked to remove the buffer/unbound antibody; and primary antibodies at 1:100 dilution were added. After incubation for 1 hour at 4°C, the cells were rinsed as above. For biotinylated antibody-bound cells, the streptavidin:PE at 1:25 dilution was added and left on ice for 30 mins while other antibody-bound cells received FACS buffer only. The cells were washed by adding 200μl FACS buffer and resuspended in 200μl PBS with filtration. Antibody-labeled cells were analyzed on BD FACSCalibur, four-color, dual argon laser system, gated on the live population, and analyzed using CellQuest software version 2.0 (BD Biosciences).

For measuring cytokine levels of IFN-γ, TNF-α, IL-2, IL-4 and IL-5 contained in MLR supernant, Cytometric Bead Array Kit (BD Pharmingen; Cat no.: 551287) was performed following manufacturer's mannual. In brief, MLR of CBA/H LN cells and C57BL/6 splenocytes±10μM BAY treatment was carried out (stimulator:responder ratio of 1:1) (Section 2.3.6.1). MLR supernatant from 10-12 wells of identical responder/stimulator population was collected and pooled into a single 1.5ml microfuge tube. 50μl MLR supernatant was added to a 1.5ml microfuge tube containing 50μl mixed Capture Beads and

50µl Mouse Th1/Th2 Cytokine PE Detection Reagent(BD Pharmingen; Cat no.: 551287). The mixture was incubated at RT for 2 hours pretected from direct exposure to light and centrifuged at 200g for 2 minutes. Supernatant was removed and the pellet was resuspended in 300µl Washing buffer (BD Pharmingen; Cat no.: 551287). Cytokine levels in each sample were then measured by flow cytometry.

2.3.6 Mixed lymphocyte reaction (MLR)

2.3.6.1 Primary and secondary MLRs

C57BL/6 BMDCs or splenocytes (See Section 2.3.2) were irradiated with 3000 rads using Cesium radiation source (CSIRO Entomology, ACT, Australia) and used as stimulator populations. Stimulator population was irradiated in all MLR experiments except for the experiment comparing the effect of BAY-treatment on splenocyte proliferation (Section 5.5) where irradiated (3000 rads)/non-irradiated splenocytes were used as stimulator population. Stimulator populations (JAWS II cells, mature, immature BMDC and splenocytes) with/without Bay11-7082 or pentoxifylline (Sigma) treatment in 100µl MLC medium were plated in 96-well U-bottom plates (Linbro/Titertek, Cat: 76-013-05) at stimulator:responder ratios of 1:1-1:512. $3x10^5$ C57BL/6 or CBA/H LN responder cells (see Section 2.3.3) in 100µl MLC medium were added to stimulator cell populations and plates were incubated in 5%CO_2 95% air at 37°C. At the end of the primary culture, all viable cells cells were considered as T cells. The cells from the primary culture were harvested and $3x10^5$ viable cells were restimulated with fresh C57BL/6 splenocytes using the same stimulator:responder ratios. For examining the suppressive effects of those LN cells, $3x10^5$ naïve CBA/H LN cells were added to the reaction mix together with $3x10^5$ T cells from the primary culture. After 72 hours of co-culture, T cell proliferation was assessed by pulsing the cells with 1µCi of [^{3}H] thymidine (Amersham, Piscataway, NJ) per well. 18-19 hours after the pulse, the cells were harvested onto glass-fiber filter (Packard Bioscience Company, Mariden, CT) using an

automated 96-well harvester (Packard Instruments, Meridien, CT) and 20µl scintillating liquid (Microscint 0, Perkin Elmer Life Sciences) was added to each well. The plates were sealed with microplate press-on adhesive sealing film (Perkin Elmer Life Sciences, Boston, MA) and [^{3}H]thymidine incorporation was determined by liquid scintillation spectroscopy on a TopCount NXT (Packard Instruments). The responses are reported as the mean cpm±SE for triplicate wells.

2.3.6.2 Characterization of MLR stimulator cells

Cells were harvested from MLR culture on day 3. The harvested cells were labeled with antibodies (anti-CD40:PE, anti-CD80:PE, anti-CD86:PE, anti-CD69:PE, anti-I-A^b:Biotin, and anti-H-2K^b:BIOT) as described in Section 2.3.5. H-2K^b positive cells were gated as the stimulator population and surface activation markers were analyzed as described in Section 2.3.5.

2.3.6.3 CD4/CD8 characterization of responding cells

T cells from MLR were harvested on day 4. Cells were stained with anti-CD4:PE and anti-CD8α:FITC antibodies and analyzed as described in Section 2.3.5.

2.3.6.4 ConA stimulation of T cells

The cells from primary MLR were harvested on day 4 and 5×10^5 cells were plated in a U-bottom 96-well plates (Linbro/Titertek, Cat: 76-013-05) containing 200µl MLC medium with 0.5µg/ml Concanavalin A (ConA) (Sigma). The plates were cultured in 5%CO_2 95% air at 37°C. Cell proliferation of these cells was measured as described in Section 2.3.6.1.

2.3.7 Nuclear extraction

Cytoplasmic and nuclear proteins were extracted from Bay11-7082 or pentoxifylline treated splenocytes and pentoxifylline treated EL-4 T cells (See Section 2.3.2). 10^8 cells were rinsed

with PBS and centrifuged at 960g for 3 mins at 4°C. The cell pellet was resuspended in 2ml Buffer A (see Appendix1) using cut-tips (to prevent damaging the nucleus) and left on ice for 5 mins. The cells were pelleted by centrifugation at 960g for 3 mins at 4°C and the supernatant containing the cytoplasmic content was harvested. The pellet (nucleus) was rinsed with 1.5ml Buffer A-NP-40 (see Appendix 1) using a cut tip then centrifuged at 960g for 3 mins at 4°C. The supernatant was discarded. 50µl Buffer C (see Appendix 1) was added and the nucleus was allowed to be lysed on ice for 15 mins on a shaker. The mixture was centrifuged at 11400g for 5 mins at 4°C and the supernatant containing the nuclear extract was harvested. The protein concentrations of cytoplasmic and nuclear extracts were measured by Bradford assay. A standard curve was made with 1-10µg of BSA (New England) in 20% Bradford dye (BIO-RAD Hercules, CA)/MilliQ H_2O using Spectrophotometer (BIO-RAD). 5µl of the cytoplasmic and nuclear extract samples were added into 20% Bradford dye/MilliQ H_2O and the concentration of the protein extracts were measured in the spectrophotometer at 570nm.

2.3.8 Western blot

The cRel protein level in cytoplasmic and nuclear extracts of Bay11-7082 or pentoxifylline treated splenocytes were measured by western blot. Nuclear and cytoplasmic proteins were denatured at 95°C for 10 mins. The samples were then electrophoresed in polyacrylamide gel (10% Longlife Gels, Life Therapeutics, NSW, Australia) with 14.3-220kDa Rainbow ladder (Amersham) in the running buffer. The gel was gently rinsed with tap water to remove SDS and the proteins were then transferred onto nitrocellulose membrane (Osmonics, Minnetonka, MN) in the transfer buffer (Section 2.1.2) at 80volts for 2 hours at 4°C. The membrane was then incubated in PBS 5% milk (Boneland Dairies Pty, VIC, Australia) at room temperature for 1 hour. The membrane was then incubated in Rabbit anti-cRel antibody, anti-RelB (all from Santa Cruz) in milk (1:250) at 4°C overnight. The membrane was then washed in

PBS/0.003% (v/v) Tween®20 (Sigma) for 40 mins. HRP-conjugated anti-rabbit IgG (1:2000) (Calbiochem) in PBS/5% milk was added to the membrane and washed for 2 hours. The membrane was rinsed with PBS 3 times, then 1ml of ECL mix (detection reagent 1 and detection reagent 2)(both from Amersham Cat:RPN2106) was poured onto the membrane and left for 2 mins at room temperature. The membrane was placed onto transparencies and the image was developed in Kodak X-OMAT 1000 processor (Kodak Pty Ltd. Australia) with the exposure time of 5-20 mins.

2.4 *In Vivo* techniques

2.4.1 Thyroid and adult islet isolation and transplantation

CBA/H mice were treated with untransduced or transduced JAWS II cells for Notch studies. JAWS-GFP, JAWS-GFP-mJag-1, JAWS-GFP-mLfng-, JAWS-GFP-mMfng, JAWS-GFP-mDll-1-, or untransduced JAWS II cells ($4x10^5$ per 0.2ml PBS) were intravenously (i.v.) injected into recipient mice at day 14, 7, before transplantation or on the day of transplant (day 0). For NF-κB experiments, CBA/H mice were i.v. injected with 10μM Bay11-7082 treated splenocytes or BMDC (10^6 per 0.2ml PBS) 7 days prior to transplantation.

2.4.1.1 Thyroid isolation

Thyroids from C57BL/6, CBA/H and $cRel^{-/-}$ mice (8-13 week old male donors) were harvested. Mice were sacrificed by CO_2 asphyxiation. Throat area was exposed by shaving the fur and an incision was made using a No.11 surgical blade (Swann Morton Ltd, Sheffield, UK). Throat area was exposed by using forceps. Using fine forceps, lateral muscles were separated and the trachea was exposed. Left and Right thyroid lobes were dissected using picking with fine forceps. The dissected thyroid lobes were placed in 20mM Hepes/Hanks for transplantation. For NF-κB experiments, isolated thyroid tissue was cultured with or

without BAY (0-1mM) at 5%CO_2 95% air or 95% O_2 5% CO_2 gas phases for 12 or 24 hours.

2.4.1.2 Thyroid transplantation

Recipient mice (C57BL/6 or CBA/H 8-13 week old male recipients) were anaesthetized with avertin (0.010-0.012mg/g body weight i.p.) injection. The depth of anaesthesia was determined by reflex response on hind foot pad, and if required, isoflurane (Abbott Australasia Pty Ltd, Kurnell, NSW, Australia) was used as an inhalation anaesthetic to maintain anaesthesia. The left flank of recipient mice was shaved and sterilized with 80% ethanol. The abdominal cavity was exposed by an incision over the left kidney. Cotton buds (Johnson and Johnson, St.Leonards, NSW, Australia) soaked with 20mM HEPES/Hanks was used to keep the kidney exposed during the operation. The exposed kidney was kept moist by constant swabbing with 20mM HEPES/Hanks solution.

A thyroid was transplanted beneath the left kidney capsule of mice treated with JAWS II cell (GFP-, GFP-mJag-1-, GFP-mLfng-, GFP-mMfng, GFP-Dll-1-, or untransduced), Bay11-7082-treated splenocytes, and Bay11-7082-treated BMDC. In brief, a small incision in the kidney capsule was made with 26-gauge needle (Terumo® Needle, Terumo Medical Corp, Elkton, MD).The capsule and the kidney were gently separated using a blunted/rounded, Luer-Lock needle (26G; 2R2, Switzerland) then a thyroid was positioned under the capsule. After the kidney was repositioned in the peritoneal cavity, the wound was closed using surgical autoclips (9 mm; Clay Adams®, Becton Dickinson Primary Care Diagnostics, Sparks MD). The mouse was then allowed to recover from anaesthetics under an incandescent lamp.

2.4.1.3 Isolation of adult mouse islets

Adult islets cultured with Bay11-7082 were transplanted into isogeneic or allogeneic

recipients. Adult mouse islets were isolated from C57BL/6J mice (7-12 week old male donors). Avertin (0.010-0.012mg/g body weight) was injected i.p. to anesthetize the mice and the peritoneum was exposed. An incision was made across the rib cage and the sternum was removed. Sterilized gauze (Multigate Medical Products Pty, NSW, Australia) soaked in medium A (see Appendix 1) was placed over the thorax and the liver lobes were lifted back over the thorax where they were held in place with the gauze. The distal end of the bile duct was clamped with artery forceps to prevent collagenase entering the intestine. A small hole was made in the common bile duct using micro-scissors and the pancreas was inflated by injecting of 3ml collagenase P (Roche Diagnostics GmbH, Mannheim, Germany, Cat: 11914520) in medium A (Section 2.1.2) using a 30 gauge needle (BD Bioscience bent at 45°) attached to a 3ml syringe (Terumo Medical Corp, Elkton, MD). The artery forceps were then unclamped and the pancreas was transferred into a siliconized vial containing 1ml 2.5mg/ml collagenase P in medium A. The tissue was digested at 37°C for 15 mins. The vial was then hand shaken for 30 secs and washed with approximately 20 ml medium A for 5 mins twice followed by 2 washes with medium B (Section 2.1.2) for 5 mins each. Finally the tissue was placed in approximately 20ml of medium A.

Individual islets were hand-picked under a stereo microscope (detail) using a siliconized drawn-out Pasteur pipette to separate the islets from acinar tissues. Picked islets (approximately 400 islets from 4 donor mice) were placed in a petri dish containing approximately 30ml medium A. Islets were picked again and 50 islets were transferred into each well of a U-bottom 96-well culture plate (Linbro/Titertek, Cat: 76-242-05) using custom-made siliconized glass tubes fitted to each well. Islets were cultured in a 6-well plate (Greiner Labortechnic, Frickenhausen, Germany, Cat: 657175) each well containing 2ml of supplemented RPMI medium (Section 2.1.2) with/without appropriate concentrations of Bay11-7082 for 3 hours in 10% CO_2 90% air prior to transplantation.

recipients. Adult mouse islets were isolated from C57BL/6J mice (7-12 week old male donors). Avertin (0.010-0.012mg/g body weight) was injected i.p. to anesthetize the mice and the peritoneum was exposed. An incision was made across the rib cage and the sternum was removed. Sterilized gauze (Multigate Medical Products Pty, NSW, Australia) soaked in medium A (see Appendix 1) was placed over the thorax and the liver lobes were lifted back over the thorax where they were held in place with the gauze. The distal end of the bile duct was clamped with artery forceps to prevent collagenase entering the intestine. A small hole was made in the common bile duct using micro-scissors and the pancreas was inflated by injecting of 3ml collagenase P (Roche Diagnostics GmbH, Mannheim, Germany, Cat: 11914520) in medium A (Section 2.1.2) using a 30 gauge needle (BD Bioscience bent at 45°) attached to a 3ml syringe (Terumo Medical Corp, Elkton, MD). The artery forceps were then unclamped and the pancreas was transferred into a siliconized vial containing 1ml 2.5mg/ml collagenase P in medium A. The tissue was digested at 37°C for 15 mins. The vial was then hand shaken for 30 secs and washed with approximately 20 ml medium A for 5 mins twice followed by 2 washes with medium B (Section 2.1.2) for 5 mins each. Finally the tissue was placed in approximately 20ml of medium A.

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2.4.1.4 Adult islet transplantation

The cultured islets (100 islet equivalent) were transferred into each well of U-bottom 96-well plate (Linbro/Titertek). Culture medium in the well was replaced with 5μl of the recipient mouse blood, which was obtained prior to transplantation from the mouse's tail vein using micro-capillaries (10μl; Drummond Scientific Company, Bromall, PA). The blood was allowed to clot for 10 mins at room temperature. Two blood clots containing islets were transplanted under the kidney capsule of each recipient mouse as described in Section 2.4.1.2 except a micro-capillary tube was used to insert the islets under the kidney capsule.

2.4.2 Cellular transplantation

The untransduced and transduced (GFP-, GFP-mJag-1-, GFP-mLfng-, GFP-mMfng-, and GFP-mDelta-l1-) JAWS II cells ($H\text{-}2^b$) in recipient blood clots were transplanted into C57BL/6J ($H\text{-}2^b$) or CBA/H ($H\text{-}2^k$) mice to examine the survival of these cells in MHC-compatible and MHC-incompatible mice. GFP-, mJag-1-, mLfng-, mMfng-, mDelta-l1-, and control JAWS II cells were transplanted into C57BL/6 (isogeneic) or CBA/H (allogeneic) mice.

5×10^6 cells in culture medium were transferred into each well of U-bottom 96-well plate (Linbro/Titertek, Cat: 76-242-05) and centrifuged at 200g for 5 mins. Culture medium in the well was removed and replaced with 5μl of the recipient mouse blood (See Section 2.4.1.4), prior to transplantation. Two blood clots containing JAWS II cells (5×10^6 cells/clot) were transplanted under the kidney capsule of each recipient mouse as described in Section 2.4.1.4.

2.4.3 Harvest of grafts

For studies of the kinetics of allograft rejection, grafts were harvested on days 7 and 14 post-transplantation. The kidneys carrying the grafts were removed from mice using a pair of scissors and curved forceps. Graft sites were cut into two pieces, using a scalpel (No.11, carbon steel sterile R surgical blades, Swann-Morton®, Sheffield, England), curved forceps, and the aid of a stereo- microscope. The grafted tissue was transferred into 10% neutral buffered formalin (Microscopy and Cytometry Resource Facility, JCSMR) for at least 2 days before further processing.

2.4.4 Histological analysis

Tissue specimens of the animals sacrificed on the Day 7 and Day 14 were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were cut at 80μm intervals by Microtome1512 (Leitz Wetzlar, Germany) and stained with hematoxylin and eosin (H+E). Preparation of slides and staining was done by Ms. Anne Prins (JCSMR).

2.4.5 Morphometric analysis of histological sections of transplants

Morphometry was performed to quantitate surviving donor tissue at the graft site. Digital photographs of 4 representative sections of histological slides were taken with an Olympus IX2-UCB digital camera (Olympus Co., Tokyo, Japan) and the total areas of intact grafts (mm^2) were measured using Scion Image 4.0.2 (Scion Corporation, USA) and Adobe Photoshop version 7.0 (Adobe Systems Inc., San Jose, CA). Survival of graft was represented as the percentage of intact follicles over the total area of the graft;

$$\text{Graft survival}\,(\%) = \frac{\text{Area of intact follicles}}{\text{Total graft area}} \times 100$$

Chapter 3

Construction and *in vitro* Chacterization of JAWS II Cells Overexpressing Notch-Related Molecules

3.1 Introduction

Allograft rejection is predominantly T cell dependent (Heeger, 2003; Rothstein and Sayegh, 2003; Wood and Sakaguchi, 2003; Yamamoto et al., 1990). $CD8^+$ T cells are considered as the primary effector population during the rejection although the efficiency of the anti-graft response is maximal when $CD4^+$ T cells are also present (Heeger, 2003; Jones et al., 2001; Yamamoto et al., 1990). Studies have shown that the depletion of T cell activities in recipients completely abrogate graft rejection indicating the essential role played by T cells during process of allograft rejection (Hall et al., 1978). Controlling T cell immunity against an allograft is, therefore, an absolute requirement to prevent allograft rejection. Current therapeutic approaches using immunosuppression have shown to be effective in protecting grafts, however, induction of antigen-specific allograft tolerance is yet to be achieved (Duncan and Wilkes, 2005). Recent studies have demonstrated that Notch signaling could suppress T cell effector functions in an antigen-specific manner via promoting regulatory T cell differentiation (Vigouroux et al., 2003; Wong et al., 2003) suggesting a potential therapeutic target to induce alloantigen-specific tolerance.

Notch receptor family belongs to the EGF-1-like homeotic gene family (Baldi et al., 2004). There are 4 family members of Notch receptors (Notch1-4) and 2 family members of Notch ligands (Jagged and Delta-like) identified in mammals (MacDonald et al., 2001). These Notch receptors and ligands are expressed on a number of cell types including lymphoid tissues and hematopoietic cells (Baldi et al., 2004; Bash et al., 1999; Hoyne et al., 2000; Kim and Siu, 1998; Sakamoto et al., 2002). A study examining the relative expression of Notch-1 has revealed that lymphocytes in peripheral lymphoid tissues showed the most abundant Notch-1 expression suggesting the importance of Notch signaling in the immune system (Baldi et al., 2004). The Notch signaling pathway has been shown to be involved in controlling differentiation of cells towards specific cell fates, and this is also a case for

differentiation and proliferation of immune cells (Bigas et al., 1998; Hoyne et al., 2000; MacDonald et al., 2001; Ohishi et al., 2001). It is now well accepted that the Notch signaling is a critical regulator of T versus B cell differentiation from CLP (MacDonald et al., 2001; Pui et al., 1999; Radtke et al., 1999).

Notch receptors consist of extracellular and intracellular domains. Characterization of the extracellular domain revealed the EGF-repeat region acting as the Notch signaling inhibitory domain, and the Lin-12/N-repeat region is important for receptor-ligand binding (Bigas et al., 1998; Moloney et al., 2000). Notch signaling transduction is initiated by binding of the Jagged or Delta-like ligands (Hoyne et al., 2000). Upon binding of ligands to the Notch receptor, presenilin dependent, γ-secretase-mediated cleavage occurs at or in the proximity of transmembrane region releasing IC domain (Brou et al., 2000; De Strooper et al., 1999). The released IC domain undergoes heterodimerization and translocates into the nucleus where it binds to CSL complex thereby activating transcription of the target genes, such as HES-1, HES-5, and CD23 (Allman et al., 2001; Bash et al., 1999; Le Gall and Giniger, 2004; MacDonald et al., 2001; Sakamoto et al., 2002).

While the magnitude of receptor or ligand expression regulates the intensity of the Notch signaling, Fringe molecules (Lfng, Mfng and Rfng) have been shown to modify the nature of the Notch signaling as well (von Boehmer, 2005). The Fringe proteins are glycotransferases and they glycosylate Notch receptors in the Golgi apparatus (Carlesso et al., 1999; Moloney et al., 2000; von Boehmer, 2005). The glycosylation of Notch receptors by Fringe proteins has been shown to influence the ligand binding specificities of the receptor (Izon et al., 2002; Moloney et al., 2000). A number of studies have demonstrated the receptor-modification by Fringe proteins promotes Notch activation via preferential binding of Delta-like ligand over Jagged (Koch et al., 2001; Panin et al., 1997; Shimizu et al., 2001). Recent findings that

Notch receptors, ligands and Fringe molecules are expressed in T cells and DCs and accumulating evidence showing the participation of the Notch signaling in development of DCs and DC-mediated tolerance induction suggests implications of the Notch signaling in inducing transplantation tolerance (Cheng et al., 2003; Dontje et al., 2006; Hoyne et al., 1999; Hoyne et al., 2000; Olivier et al., 2006).

Over that last decade, studies revealed that DCs are the key group of leukocytes in induction of regulatory T cell-mediated tolerance (Banchereau et al., 2003; Fairchild and Waldmann, 2000; Heath and Carbone, 2001; Jonuleit et al., 2001). While DCs play central roles in activating allospecific T cells by presenting allopeptides together with costimulatory signals, immature DCs, in particular, are thought to play a critical role in tolerance induction in a number of experimental models (Dhodapkar et al., 2001; Jonuleit et al., 2001; O'Rourke et al., 2000). For example, it has been demonstrated that immature-monocyte derived DCs were able to generate regulatory T cells that produce IL-10 thereby inhibiting $CD8^+$ T cell activities (Dhodapkar et al., 2001). The regulatory roles of immature DCs have also been exploited in transplantation. It was demonstrated a pretreatment of donor-type $CD8\alpha^+$ DCs in recipient mice resulted in the prolonged survival of vascularized heart allografts (O'Connell et al., 2002). Several studies have demonstrated that pretreatment of recipients with donor-type DCs expressing CTLA-4 resulted in donor-specific T cell unresponsiveness *in vitro* (Gorczynski et al., 2000; Takayama et al., 2000; Takayama et al., 1998) and *in vivo* (O'Rourke et al., 2000). Furthermore, administration of DCs overexpressing IL-10 and TGF-β together with OX-2 is reported to be able to improve renal allograft survival (Gorczynski et al., 2000). Although strategies employing DCs appear to be successful in several experimental models, the mechanism by which DCs can induce antigen-specific tolerance is yet to be understood.

Recently, Notch signaling has been shown to be capable of modulating the immune response and, in some reports, induce antigen-specific tolerance. It was shown that $CD4^+CD25^+$ T cells express high levels of Deltex and the magnitude of Deltex and Notch4 expression were highly upregulated following anti-CD3 an anti-CD28 stimulation (Ng et al., 2001). Another study has shown that over-expression of Jagged-1 on B cells, which can act as APCs, resulted in the induction of antigen-specific regulatory T cells (Vigouroux et al., 2003). This study suggests Notch receptor/ligand interactions between APCs and T cells may drive T cells towards a regulatory phenotype. Consistently, it has been demonstrated that injection of Jagged-1 transfected DCs pulsed with a house dust mite antigeneic peptide, rendered mice profoundly tolerant to subsequent challenges with intact house dust mite protein (Hoyne et al., 1999). These studies indicate that Notch signaling has the potential to induce tolerance via the induction of $CD4^+CD25^+$ regulatory T cell differentiation. However, the mechanism by which DC-mediated Notch signaling can generate tolerance is poorly understood.

We hypothesized that donor-type immature DCs over-expressing Notch related molecules may facilitate suppression of alloimmune responses in an antigen-specific manner, possibly through the induction of regulatory T cells. In this chapter, the aim was to construct a monocyte-derived immature DC line (JAWS II cells) overexpressing Notch related molecules and to evaluate whether modulation of Notch signaling alters the capacity of JAWS II cells to stimulate allogeneic T cells *in vitro*.

3.2 Construction of pKMV-Δ Gateway™ destination vector

Retroviral-mediated gene transfer is used extensively as a tool to integrate a gene of interest into cells that are undergoing cell division and to achieve stable transfer and gene expression in those cells (Cornetta and Anderson, 1989; Takayama et al., 1998). The retroviral vector, pKMV-Δ (Figure 3-1), was chosen for transfer of Notch-related genes. The pKMV-Δ plasmid

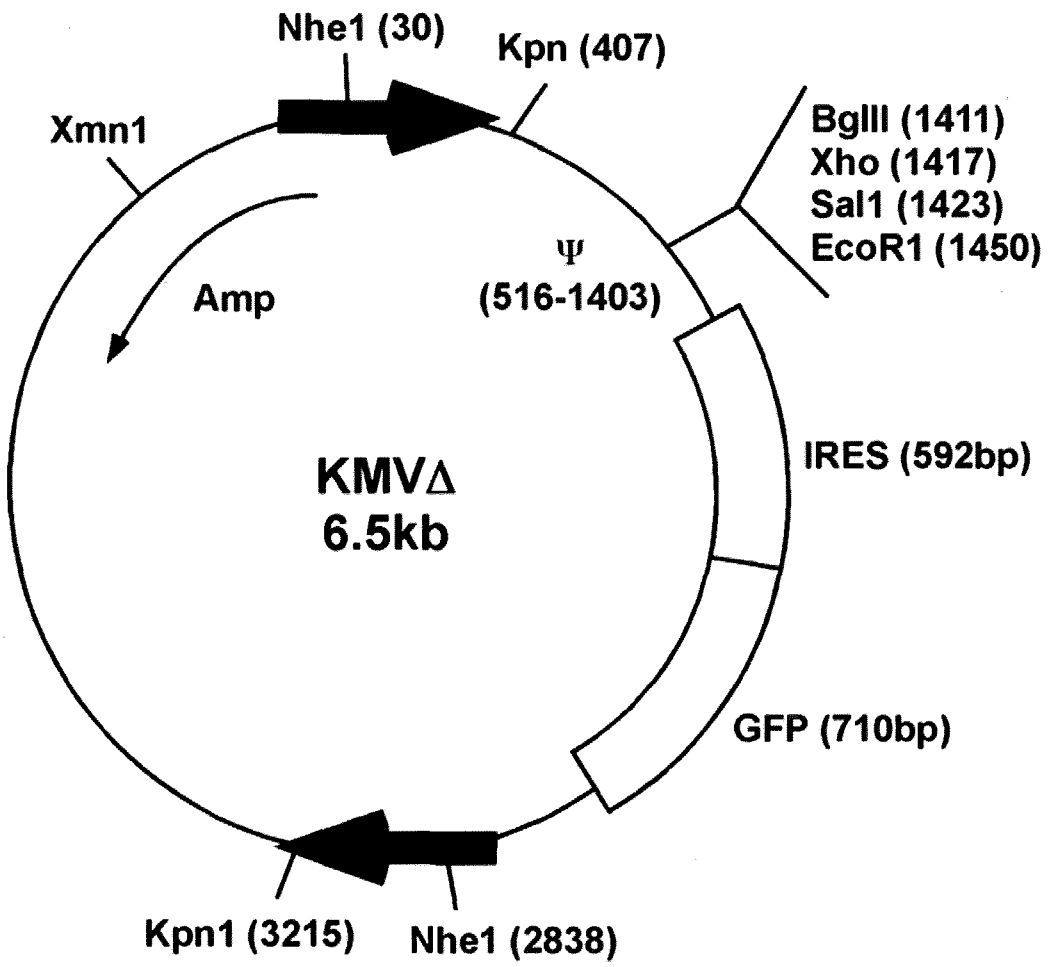


Figure 3-1: **Gene map of pKMV-Δ plasmid.**

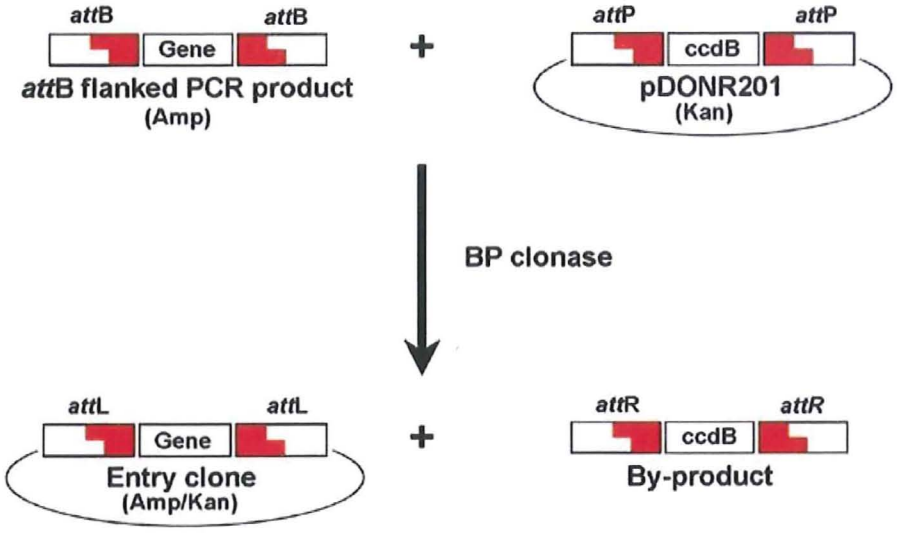
Gateway cassette RfA was inserted into the multiple cloning site (BglII site) of the pKMV-Δ plasmid as described in Section 3.2.

was obtained from Professor Ian Ramshaw (Division of immunology and Genetics, JCSMR). The Gateway cloning technology™ (Invitrogen) was employed to insert Notch-related genes into pKMV. The Gateway Technology employs a bacteriophage lambda site-specific recombination system. This recombination occurs at *att* sites. The recombination is catalyzed by clonase which is a *E.coli* encoded recombinant protein. In this recombinant reaction, switching occurs between DNA molecules flanked at *att* sites and *att* sites of Gateway vectors. *att* sites do not contain stop codon and *att* sites are also modified to minimize the formation of secondary structure. This commercially available technology allows the transfer of different genes into the destination vector in an identical manner.

The process of constructing recombinant vectors consists of two main steps; BP reaction (construction of entry clone) and LR reaction (construction of expression clone) [Figure 3-2]. In the BP reaction, PCR-product *att*B flanking region is inserted into a donor vector (pDONR) carrying *att*P flanking region using BP clonase (which catalyzes the crossing over of heterologous DNA sequences flanked by *att*) [Figure 3-2]. BP reaction generates an entry clone with a gene of interest with *att*L flanking regions LR reaction facilitates *att*L substrate in the entry clone to exchange with *att*R substrate in a destination vector. Resulting clone contains the gene of interest with *att*B flanking region [Figure 3-2] and the clone can be readily used for transfection.

Firstly, the Gateway Reading Frame Cassette, RfA, was cloned into pKMV-Δ at the multiple cloning site as described by the manufacturer (Invitrogen). In brief, pKMV-Δ plasmid was linearized with the restriction enzyme *Bgl-II* (New England BioLabs). The overhangs on both 5' and 3' ends were endfilled with T4 DNA polymerase (Invitrogen) then the 5' end was dephosphorylated using calf intestinal alkaline phosphatase (Invitrogen) following the manufacturer's instructions. The blunt-ended plasmid was purified by the addition of 1:1

BP reaction



LR reaction

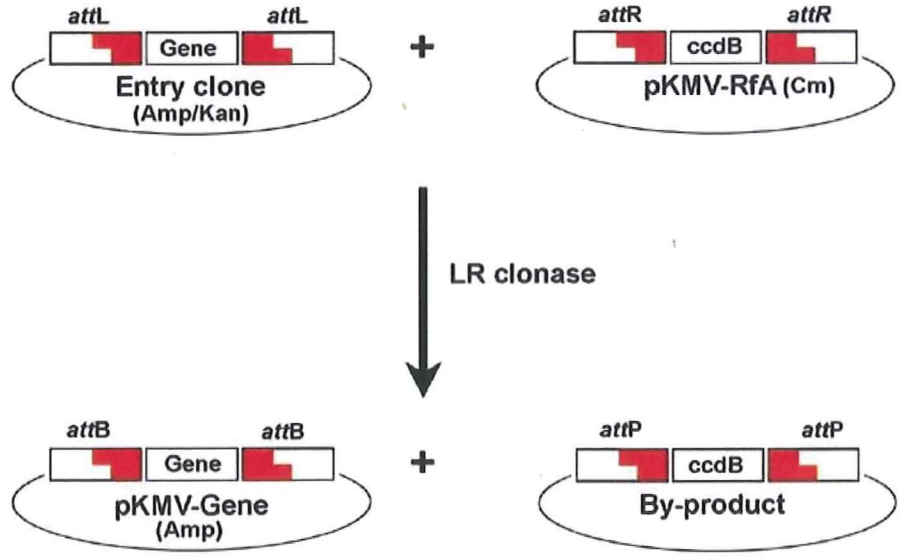


Figure 3-2: Schematic diagram of Gateway™ cloning technology (adapted from the manufacturer's manual)

BP reaction involves the insertion of attB flanked genes of interest (mJag-1, mLfng, mMfng, mDelta-l1) into pDONR201. LR reaction facilitates the transfer of the genes from pDONR into pKMV containing attL sites, creating the final plasmids of pKMV-mJag-1, pKMV-mLfng, pKMV-mMfng, and pKMV-mDelta-l1.

phenol/chloroform and the upper layer free of proteins was collected. DNA was precipitated with ethanol/sodium acetate (NaOAc; pH5.2) and resuspended in TE buffer. The Gateway Cassette RfA was ligated into pKMV-Δ using T4 DNA ligase and the presence of the Gateway cassette was confirmed by 1% agarose electrophoresis. The pKMV-RfA vector was transformed into DB3.1 Competent Cells (Invitrogen) by heat shock and selected for chloramphenicol (Cm). Colonies were hand picked with sterile toothpick and cultured in Luria Broth/Cm. pKMV-RfA was extracted from these cells using QIAprep Spin Miniprep Kit. Since the Gateway Cassette RfA is blunt-ended, the orientation of the insert was checked by restriction digest with *EcoRI* followed by electrophoresis. 7 out of 9 colonies showed the correct orientation and these colonies were used for recombination with entry clones.

3.3 Construction of pDONR of Notch related genes

The pIRES2-EGFP-Delta-l1 plasmid (pIRES2-EGFP from BD Biosciences) was obtained from Professor Margaret Dallman (Department of Biology, Imperial College, London). Delta-l1 plasmid was used as a PCR-template and amplified with primers with *att*B sequence. PCR products were purified from a gel as recommended in Gateway Cloning Technology manual using High Pure PCR Production Purification Kit (Roche Diagnostic Corporation, Indianapolis, USA). The PCR product was then cloned into a donor vector pDONR201 via BP reaction. The BP reaction facilitates transfer of Delta-*att*B PCR product into an attP-containing pDONR201 (carrying a kanamycin resistant gene) resulting in an entry clone containing the Delta-l1 sequence and a byproduct. BP reaction product was transformed into electroporation-competent DH10B *E. coli* cells (Invitrogen) by electroporation (2.5kV for 0.5 secs) and kanamycin resistant colonies were selected. A single colony was picked and DNA was extracted with mini-prep DNA (QIAGEN). The other entry clones (mJagged1, mLfng, and mMfng) used in this thesis were constructed by David Mann and Jodie Harrison

in Professor Ramshaw's laboratory (JCSMR).

3.4 Construction of pKMV vector carrying Notch-related genes

Entry clones containing the gene of interest were then transferred to the pKMV-destination vector via LR reaction. The LR reaction is catalyzed by LR clonase which facilitates the homologous recombination of attL substrate (of the entry clone; pmJag-1, pmLfng, pmMfng, and pmDelta-l1) and attR substrate (of the destination vector; pKMV-attR). 300ng of both entry and destination clones were mixed for the LR reaction. The reaction product was then electroporated into DH10B *E. coli*, which were then selected for ampecillin resistance. DNA was extracted with mini-prep and the presence of the genes of interest was confirmed by restriction digest (NcoI and EcoRI for pKMV-GFP-mMfng; EcoRI and BamHI for pKMV-GFP-mLfng; BglII for pKMV-GFP-mJag-1; EcoRI and SalI for pKMV-GFP-Delta-l1) [See Figure 3-3 (a)- (d)]. Sequencing of all Notch-related genes in respective pKMV constructs were performed using BigDye v3.1 [See Appendix]. The sequences of the constructs were found to be the same as the NCBI nucleotide data base of the genes in question (http://130.14.29.110/BLAST/index.shtml).

3.5 Construction of JAWS II cells over-expressing Notch related molecules

As pKMV constructs lack virus-packaging proteins, gag-pol-env, a virus-producing cell line, Phoenix cells was used for the production of infectious virus [Figure 3-4]. After Phoenix cells were incubated with chloroquine for 5 minutes, pKMV-Δ, pKMV-GFP-mJag-1, pKMV-GFP-mLfng, and pKMV-GFP-mMfng were transfected into Phoenix cells using calcium phosphate (www.stanford.edu/group/nolan/protocols/pro_helper_dep.html) as described in Section 2.3.1. After incubation for 24 hours, medium was replaced to remove chlorquine and cells were incubated for a further 24 hours. Successful transfection was

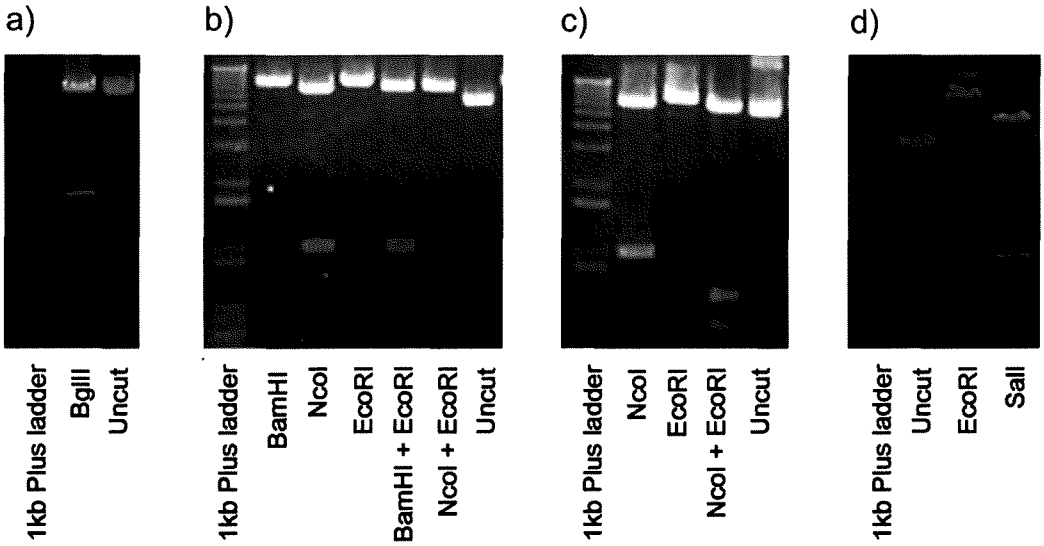


Figure 3-3: Confirmation of mJag-1, mLfng, mMfng and mDll-1 in pKMV-GFP-Δ after LR reaction by restriction digest

Presence of inserted genes was checked by restriction digest as described in Section 2.2.6. a) pKMV-GFP-mJag-1 was digested with BglII yielding 2.2kb + 8.2kb bands; b) pKMV-GFP-mLfng was digested with BamHI, NcoI and EcoRI yielding 950bp band for BamHI+EcoRI and 600bp and 400bp bands for NcoI+EcoRI; c) pKMV-GFP-mMfng was digested with EcoRI and NcoI yielding 600bp and 400bp bands and d) pKMV-GFP-Dll-1 was digested with EcoRI and SalI yielding 2.3kbp band.

confirmed by GFP expression in Phoenix cells (>50% of total population being GFP-positive). The virus supernatant was then harvested and used for transduction of JAWS II cells. JAWS II cells were then transduced with the virus supernatant in the presence of polybrene (Sigma) to enhance transduction efficiency [Figure 2-1]. GFP-positive JAWS II cells were obtained after approximately 1 week incubation (referred to as low-titer-virus-transduced (LT) JAWS II cells). The first transduction yielded 4-11% of total cells GFP-positive (approximately 11% for GFP-JAWS II cells; 13% for mJag-1-GFP-JAWS II cells; 4% for mLfng-GFP-JAWS II cells) [Table 3-1][Figure 3-4 (a), (c), (e) and (g)]. JAWS II cells were then positively selected for GFP by successive FACS sorting over a period of 2-3 weeks until > 80% of the population was GFP-positive.

Subsequently, a centrifugal enhancement method using high-titer virus was adopted and another set of JAWS II cells over-expressing Notch related molecules was constructed (referred to as high-titer-virus-transduced (HT) JAWS II cells) as described in Section 2.3.1. More efficient transduction was observed using this method. With centrifugal enhancement, the initial transduction efficiency increased up to 6-fold more than the non-centrifugal method (approximately 44.7% for GFP-JAWS II cells, 35.6% for mJag-1-GFP-JAWS II cells, and 26.7% for mLfng-GFP-JAWS II cells) [Table 3-1] [Figure 3-4 (b), (d), (f) and (h)]. These cells were also successively FACS-sorted for GFP expression to increase the purity to >80% GFP-positive cells in the total population. As a preliminary study, C57BL/6 primary BMDCs were transduced with pKMV-GFP-mDll-1 (HT) retrovirus by the centrifugal enhancement method [Figure 3-5 (b)]. Retroviral transduction yielded 2.9% of total cells GFP-positive, however, sufficient number of GFP-positive BMDCs could not be obtained for further experiments due to a low transduction efficiency.

The proportion of GFP-positive cells declined over time; this was most likely due to loss of

Method of Transduction	Percentages of GFP-positive cells in the first FACS sort				
	JAWS GFP	JAWS-GFP -mJag-1	JAWS-GFP-mLfng	JAWS-GFP-mMfng	JAWS-GFP-mDelta-l1
Non-centrifuga l	11.0	13.0	10.1	4.0	---
Centrifugal	44.7	35.6	26.7	---	11.0

Table 3-1: Transduction efficiencies of HT and LT pKMV-transduced JAWS II cells. JAWS II cells were transduced with LT pKMV-Δ, pKMV-GFP-mJag1, pKMV-GFP-mLfng or transduced with HT pKMV-Δ, pKMV-GFP-mJag1, pKMV-GFP-mLfng and pKMV-GFP-mDll-1 with centrifugal enhancement method. GFP-positive cells were selected by FACS sorting and their initial transduction efficiencies were compared.

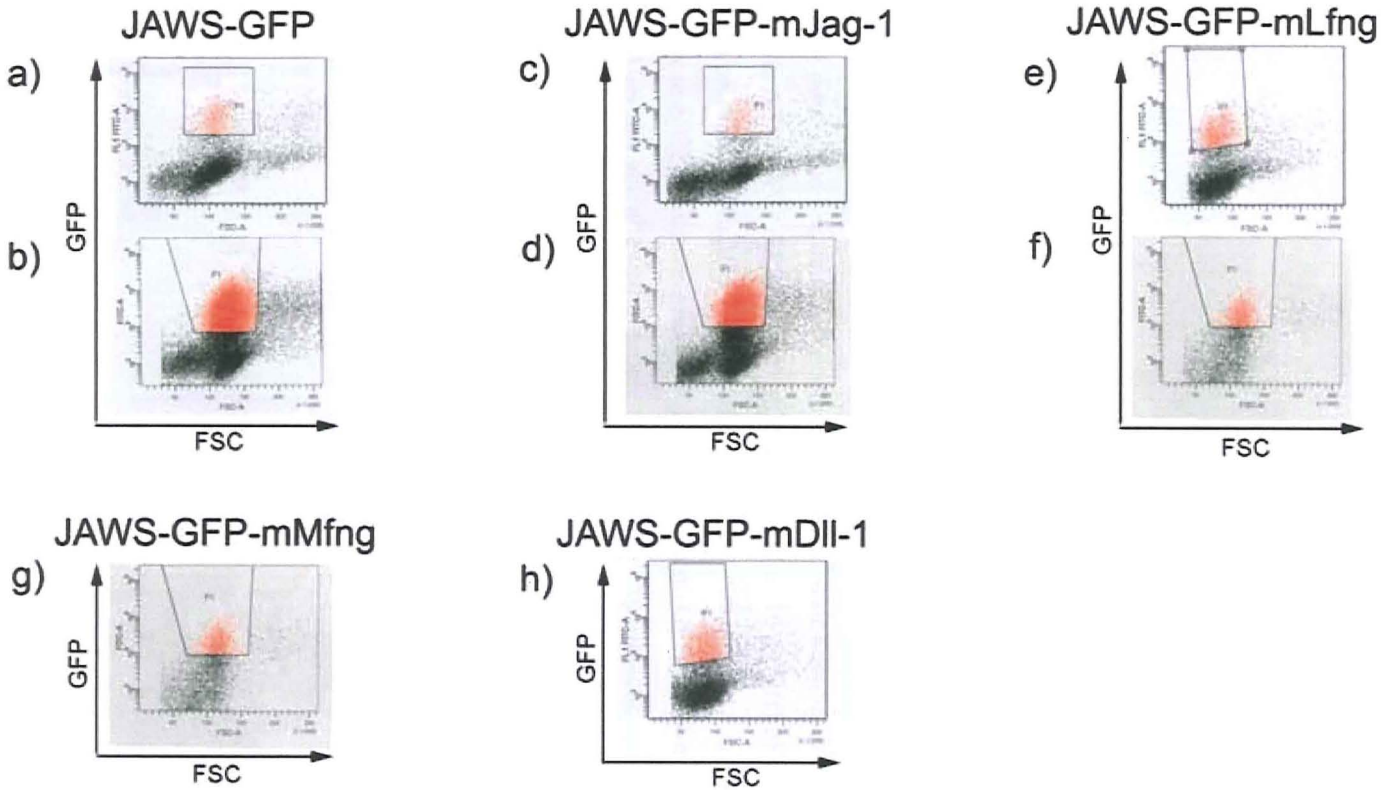

JAWS-GFP
a)
b)
GFP
FSC
JAWS-GFP-mJag-1
c)
d)
GFP
FSC
JAWS-GFP-mLfng
e)
f)
GFP
FSC
JAWS-GFP-mMfng
g)
GFP
FSC
JAWS-GFP-mDll-1
h)
GFP
FSC

Figure 3-4: Transduction efficiency of JAWS cell transduced with pKMV-retrovirus containing Notch-related gene with and without centrifugal enhancement.

JAWS II cells were transduced with (a) pKMV-GFP (LT), (c) pKMV-GFP-mJag-1 (LT), (e) pKMV-GFP-mLfng (LT), and (g) pKMV-GFP-mMfng (LT) in a presence of chloroquine. 4-13% of total population became GFP-positive after the 1st transduction. JAWS II cells were transduced with (b) pKMV-GFP (HT), (d) pKMV-GFP-mJag-1 (HT), (f) pKMV-GFP-mLfng (HT), and (h) pKMV-GFP-mDll-1 (HT) by the centrifugal enhancement method. 11-44% of total cells became GFP-positive after the 1st transduction. GFP-positive cells were then selected by FACS sort and allowed to propagate for further experiments.

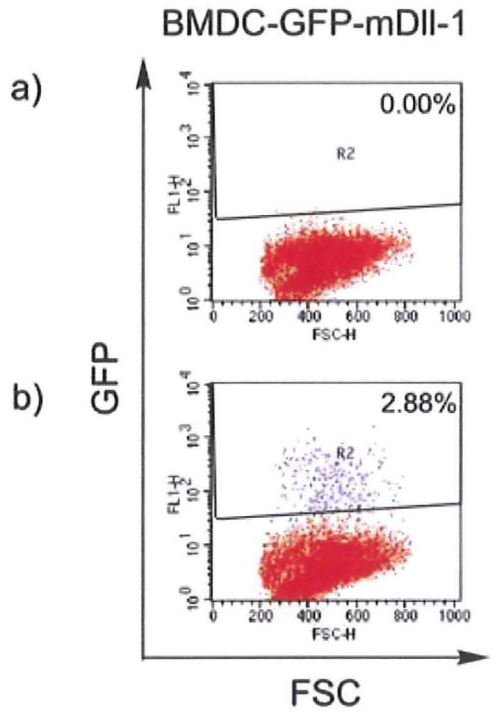


Figure 3-5: Transduction efficiency of BMDCs transduced with pKMV-GFP-mDll-1 with centrifugal enhancement.

(a) untransduced BMDCs were GFP-negative. (b) BMDCs were transduced with pKMV-GFP-mDll-1 (HT) by the centrifugal enhancement method and 2.88% of total cells become GFP-positive after 1st transduction.

the inserted GFP gene and/or overgrowth of the GFP-negative population also present in the cell population after purification. GFP expression levels in the transduced JAWS II cells were monitored and GFP-positive cells were maintained above 80% of the total population by repeated sorting. Interestingly, JAWS II cells transduced with high-titer retrovirus showed much more stable expression of GFP compared with those transduced with low-titer virus. The majority of JAWS II cells transduced with high-titer pKMV-GFP-Δ, pKMV-GFP-mJag1, pKMV-GFP-mLfng, and pKMV-GFP-mDelta-ll1 showed sustained GFP expression over 20 days [Figure 3-6]. On the other hand, over 50% of JAWS II cells transduced with pKMV-GFP-mJag1 (LT) and pKMV-GFP-mLfng (LT) lost GFP expression by day 20 post-transduction [Figure 3-6]. These results indicate that JAWS II cells transduced with high-titer viruses using centrifugal enhancement showed higher transduction efficiency and more successful and stable incorporation of the gene of interest while the gene insertion via LT virus transduction was more transient.

3.6 mJagged-1, mLfng, mMfng and Delta-ll1 expression

To confirm the over-expression of Notch-related genes in JAWS II cells, gene expression was analyzed by semi-quantitative RT-PCR. It is of note that all genes resolved as a single band after electrophoresis thus showing the absence of genomic DNA. Band intensity was standardized with GAPDH and the band intensity was compared between cDNA of untransduced JAWS II cells and transduced JAWS II cells.

Notch related molecules, mJagged-1, mLfng and mMfng were expressed in untransduced JAWS II cells [Figure 3-7 (a)- (c)]. Following transduction with low-titer virus without centrifugal enhancement, there was a 31-fold increase in mJag-1 expression compared to untransduced cells [Figure 3-7 (a)]. Similarly, mLfng and mMfng gene expression was upregulated 10- and 11- fold in mLfng-GFP-JAWS II and mMfng-GFP-JAWS II respectively

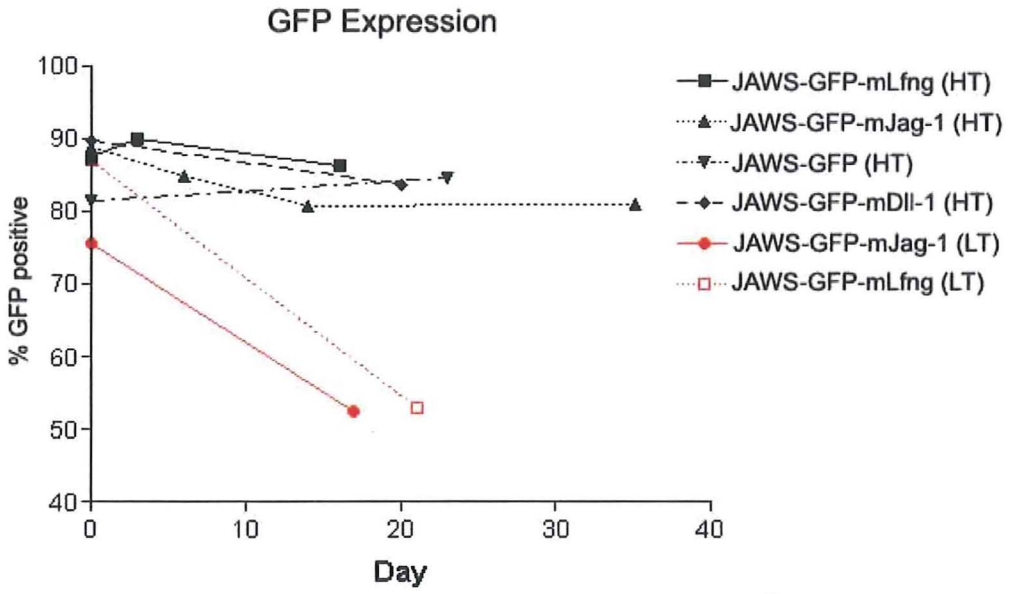


Figure 3-6: Stability of Notch-related genes in JAWS II cells

JAWS II cells were transduced with pKMV-Δ, pKMV-GFP-mJag1, pKMV-GFP-mLfng, pKMV-GFP-mMfng, and pKMV-GFP-mDll-1. Percentages of GFP-positive populations from JAWS-GFP (HT), JAWS-GFP-mJag-1 (LT and HT), JAWS-GFP-mLfng (LT and HT) and JAWS-GFP-mDll-1 constructs were measured by single laser flow cytometry. % GFP-positive cells over the total population were monitored ≥2 weeks post-transduction.

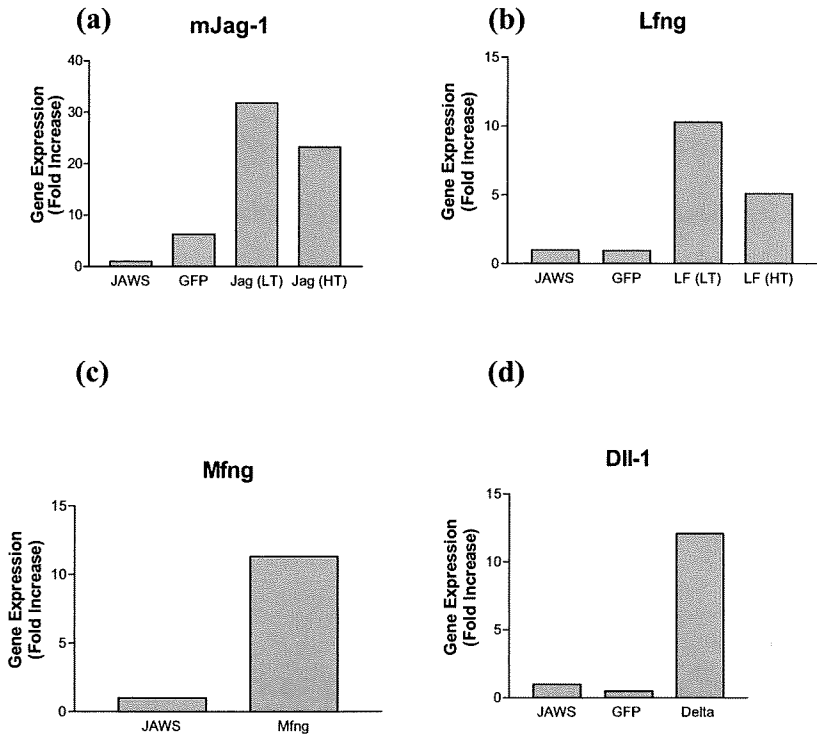


Figure 3-7: Characterization of Notch-related molecule transcripts in pKMV-retrovirus-transduced JAWS II cells by RT-PCR analysis.

RNA from JAWS-GFP-mJag1, JAWS-GFP-mLfng, JAWS-GFP-mMfng, and JAWS-GFP-mDll-1 was purified. cDNA was synthesized followed by amplifications with the standard PCR techniques as described in Section 2.2.3. Band intensities of mJag-1 (a), mLfng (b), mMfng (c) and mDelta-l1 (d) were analyzed by Image Gauge software (Fuji Film). The expression level of each Notch related molecules in retrovirus-transduced JAWS II cells was standardized against corresponding GAPDH levels.

[Figure 3-7 (b) and (c)]. JAWS II cells, transduced with high-titer viruses, a 23-and 5-fold increase in levels of mJag-1, and mLfng gene expression respectively compared to control JAWS II cells [Figure 3-7 (a) and (b)]. However, the magnitude of Notch-related gene expression in high-titer virus transduced JAWS II was notably lower than those in low-titer virus transduced JAWS II cells.

The expression of Delta-ll1 was also examined in untransduced and pKMV-GFP-mDelta-ll1 transduced JAWS II cells. No expression of Delta-ll1 in untransduced JAWS II was observed [Figure 3-7 (d)]. To demonstrate the lack of Delta-l1 expression was not due to poor quality cDNA, RT-PCR using primers for the house-keeping gene, GAPDH, was performed [Figure 3-7 (d)]. pKMV-mDelta-1 transduced JAWS II cells, however, showed a single band at 1650bp, indicating the transduction resulted in induction of mDelta-l1 gene expression [Figure 3-7 (d)].

3.7 Surface marker profiles of JAWS II cells

Since retroviral gene transduction resulted in enhanced expression of the inserted genes, it was necessary to confirm that transduction did not result in intrinsic activation of the JAWS II cells. 4-color FACS analysis was therefore performed using antibodies against DC activation markers (CD40, CD80, CD86, CD69, MHC Class I and II) and DC-specific markers (CD11b, CD11c and CD205) to ascertain the phenotypes of JAWS II cells pre- and post transduction [Table 3-2].Although there was slight upregulation was observed in CD86 and MHC Class II expresion, pKMV-GFP, pKMV-GFP-mJag-1, pKMV-GFP-mLfng, pKMV-GFP-Mfng and pKMV-GFP-mDll-1 transduced JAWS II cells did not show major alterations in overall expression of surface markers compared to untransduced JAWS II cells. The similar phenotypes between untransduced and the transduced JAWS II cell populations indicate that transduction did not intrinsically activated JAWS II cells.

Cell surface marker	Untransduced	JAWS-GFP		JAWS-GFP-mJag-1		JAWS-GFP-mLfng		JAWS-GFP-mMfng	JAWS-GFP-mDll-1	Activated JAWS II
		LT	HT	LT	HT	LT	HT			
CD40	-	-	-	-	-	-	-	-	-	+++
CD80	+++	+++	+++	+++	+++	+++	+++	+++	+++	++++
CD86	+	++	++	++	++	++	++	++	++	++++
CD69	-	-	-	-	-	-	-	-	-	-
MHC Class I	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
MHC Class II	-	±	±	±	±	±	±	±	+	+
CD205	+	+	+	+	+	+	+	+	+	+
CD11b	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
CD11c	+	+	+	+	+	+	+	+	+	+
Jag-1	-	-	-	+	+	-	-	-	-	-
Delta	-	-	-	-	-	-	-	-	-	-

Table 3-2: Phenotypic analysis of JAWS II cells and pKMV transduced JAWS II cells. Expression of DC markers (Dec205, CD11b and CD11c), activation markers, (CD40, CD80, CD86, CD69 MHC Class I and II), mJag-1 and mDelta-l1 on pKMV-GFP, pKMV-GFP-mJag-1, pKMV-GFP-mLfng, pKMV-GFP-mMfng, pKMV-GFP-mDll-1 transduced, untransduced and LPS/cytokine activated JAWS II cells was analyzed by FACS. The degree of surface marker expression was determined by MFI shift between unstained and antibody stained populations. Experiments were repeated 3 times and representative phenotypes are shown.

++++ denotes very strong expression (more than 1 log increase in MFI compared to then unstained control)

+++ denotes strong expression (approximately 1 log increase in MFI compared to the unstained control)

++ denotes moderate expression (approximately 0.5 log increase in MFI compared to the unstained control)

+ denotes weak expression (less than 0.5 log increase in MFI compared to the unstained control)

± denotes slight expression (approximately 0.1 log increase in MFI compared to the unstained control

- denotes no expression (no MFI shift from the unstained control).

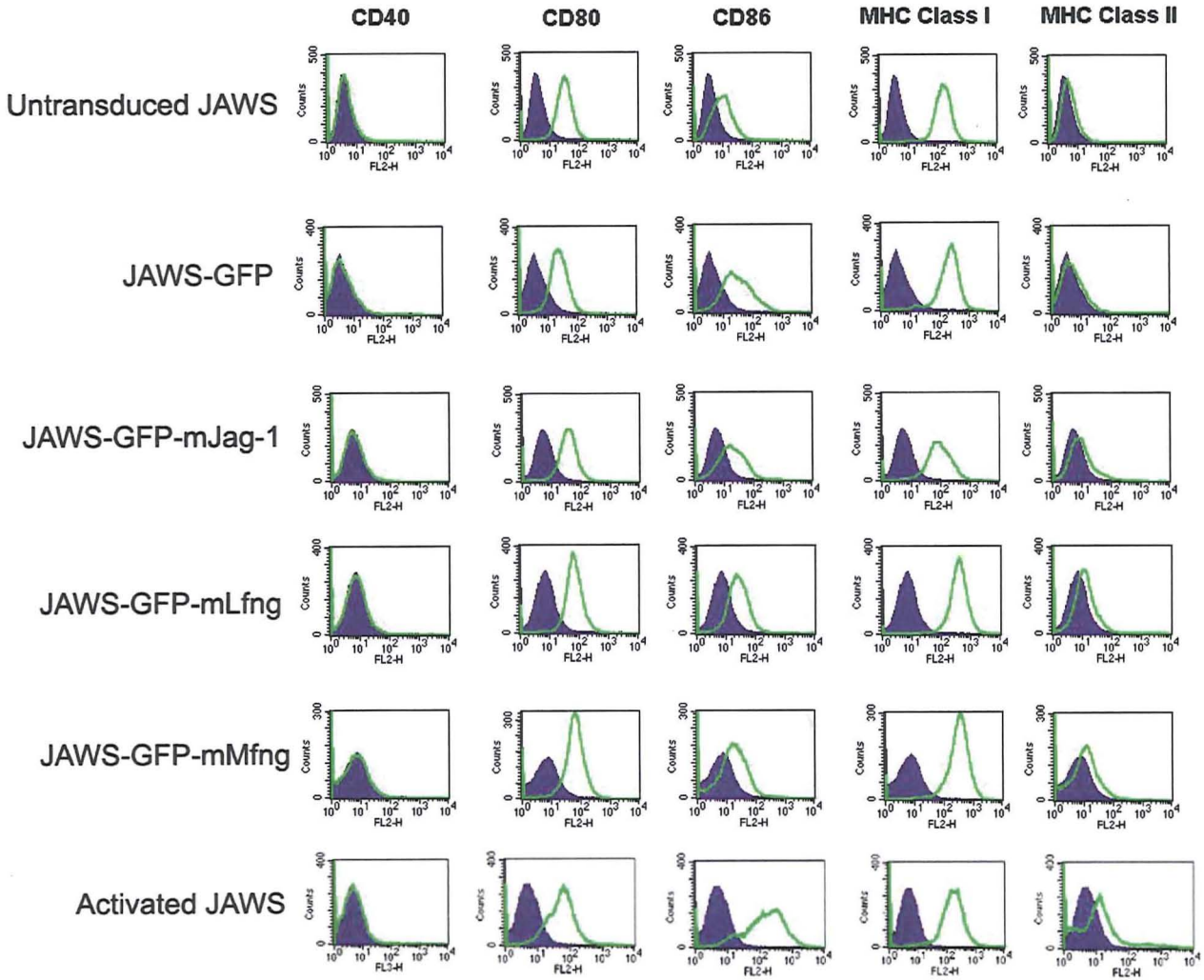

CD40
CD80
CD86
MHC Class I
MHC Class II
Untransduced JAWS
JAWS-GFP
JAWS-GFP-mJag-1
JAWS-GFP-mLfng
JAWS-GFP-mMfng
Activated JAWS
Counts
FL2-H

Figure 3-8: Phenotypic analysis of JAWS II cells and LT pKMV transduced JAWS II cells.

Expression of activation markers, CD40, CD80, CD86, MHC Class I and II on pKMV-Δ, pKMV-GFP-mJag-1, pKMV-GFP-mLfng, pKMV-GFP-mMfng transduced, untransduced, and LPS/cytokine activated JAWS II cells was analyzed by FACS. Closed histograms represent unstained population and open histograms represent population stained with the antibodies. Experiments were repeated 3 times and representative phenotypes are shown.

While untransduced JAWS II cells showed strong MHC Class I surface expression and moderate CD80 and CD86 expression, low MHC Class II expression and no CD40 expression confirmed an immature DC phenotype [Figure 3-8]. When JAWS II cells were activated with cytokines (IFN-γ, TNF-α, IL-4) and LPS stimulation, activation markers CD40, CD80, CD86 and MHC II were all upregulated on stimulated JAWS II cells compared to unstimulated JAWS II cells.

Transduction with low titer pKMV-GFP-Δ, pKMV-GFP-mJag-1, pKMV-GFP-mLfng and pKMV-GFP-mMfng did not alter the expression of CD40, CD80, and MHC Class I [Figure 3-8]. Expression of CD69, CD11c, CD11b were also not affected [Table 3-2]. Transduced JAWS II cells showed slight upregulation in CD86 and MHC Class II surface expression; however, relative to activated JAWS II cells, the low expression levels of these genes and lack of CD40 expression collectively indicate that the transduced JAWS II cells remained immature [Table 3-2]. When the phenotype of JAWS II cells transduced with high-titer pKMV, pKMV-mJag-1, pKMV-mLfng and pKMV-mDelta-l1were examined, no alteration in the surface expression of these activation markers compared to unstimulated JAWS II cells were observed in the transduced cell populations [Figure3-9]. These results indicate that transduction with both LT- and HT-pKMV carrying Notch-related genes did not affect the immature phenotype of JAWS II cells.

3.8 Functional studies of JAWS II cells transduced with Notch related molecules

Previously, it has been shown that over-expression of Notch ligand in APCs can alter the nature of T cell proliferation and in particular could lead to the generation of regulatory T cells (Hoyne et al., 2000). Therefore, JAWS II cells were examined for their ability to prime allogeneic T cells. The capacity of JAWS II cells (C57BL/6 derived; H-2^b) and JAWS II cells

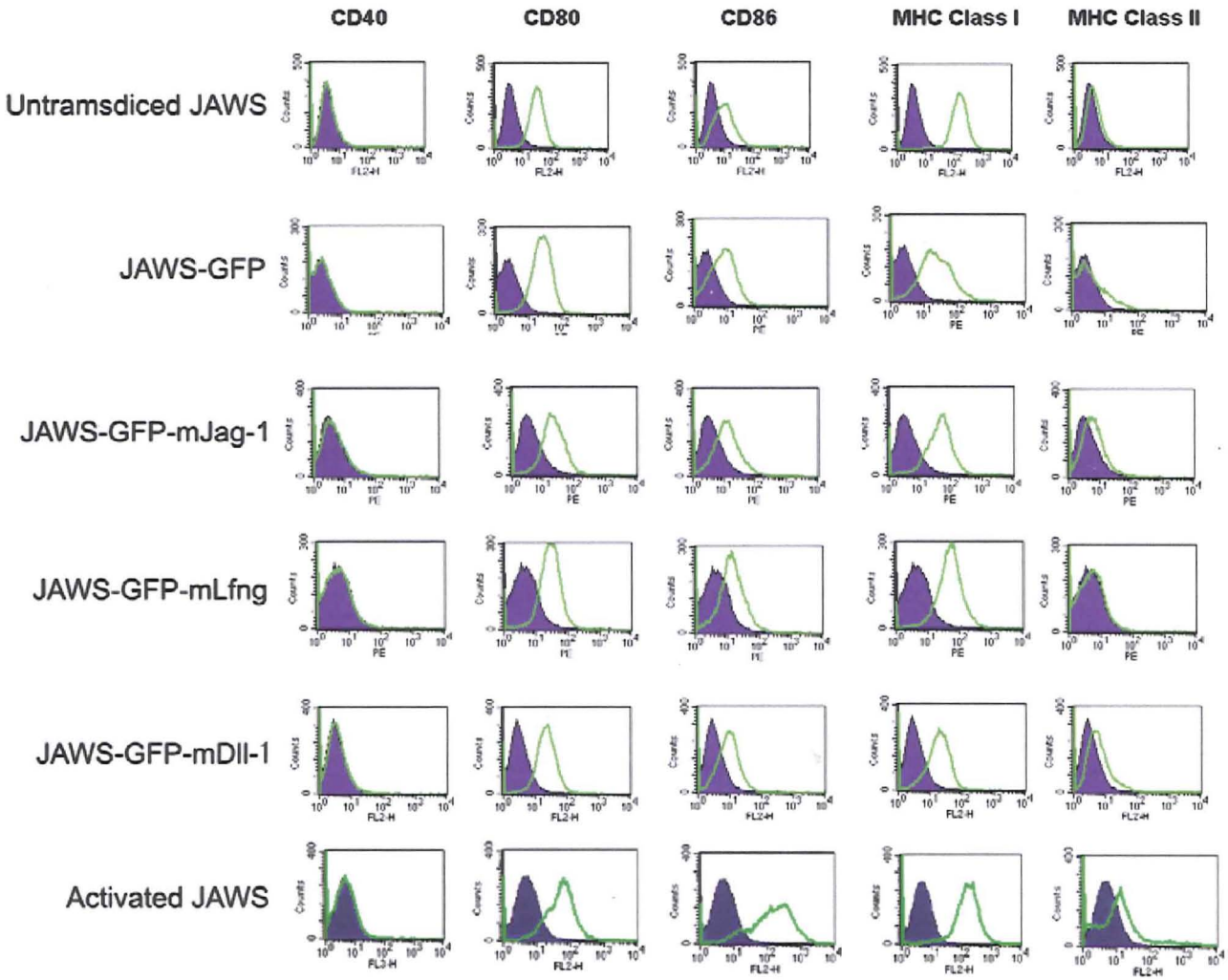

CD40
CD80
CD86
MHC Class I
MHC Class II
Untramsdiced JAWS
JAWS-GFP
JAWS-GFP-mJag-1
JAWS-GFP-mLfng
JAWS-GFP-mDll-1
Activated JAWS

Figure 3-9: Representative phenotypes of JAWS II cells and HT pKMV transduced JAWS II cells.

Expression of activation markers, CD40, CD80, CD86, MHC Class I and II on pKMV-Δ, pKMV-GFP-mJag-1, pKMV-GFP-mLfng, pKMV-GFP-mDll-1 transduced, untransduced and LPS/cytokine activated JAWS II cells was analyzed by FACS. Closed histograms represent unstained population and open histograms represent population stained with the antibodies. Experiments were repeated 3 times and representative phenotypes are shown.

over-expressing Notch related molecules to stimulate allogeneic T cells (from CBA/H mice; H-2^k) was tested in a mixed lymphocyte reactions.

C57BL/6 splenocytes were able to prime allogeneic CBA/H T cells (3[H] thymidine incorporation in response to C57BL/6 splenocytes was 27581±2577cpm at 1:1 stimulator/responder ratio) [Figure 3-10 (a)]. Similarly unstimulated JAWS II cells were readily able to stimulate allogeneic CBA/H T cells at a moderate level with C57BL/6 splenocytes (3[H] thymidine incorporation in response to JAWS II cells was 11347±696cpm at 1:64 stimulator/responder ratio [Figure 3-10 (b)]. However, when JAWS II cells were activated with cytokines and LPS, their T cell priming activity was significantly enhanced ($p=0.02$) compared to unstimulated JAWS II cells (3[H] thymidine incorporation in response to activated JAWS II cells was 17258±1402cpm) yet still significantly less than that of splenocytes ($p=0.01$) [Figure 3-10 (b)]. Unstimulated JAWS-GFP, JAWS-GFP-mJag-1, JAWS-GFP-mLfng, and JAWS-GFP-mDll-1 were also able to prime allogeneic T cells in a similar manner to untransduced JAWS II cells (response to JAWS-GFP was 11233±755cpm; JAWS-GFP-mJag1 was 12295±1403cpm; JAWS-GFP-mLfng was 12048±1113cpm; JAWS-GFP-Dll-1 was 9659±2447; $p>0.05$)[Figure 3-10 (b)- (f)]. Significantly elevated T cell proliferation was also observed when allogeneic T cells were mixed with activated JAWS-GFP, activated JAWS-GFP-mJag1, activated JAWS-GFP-mLfng and activated JAWS-GFP-mDll-1 (response to activated JAWS-GFP was 14805±932cpm; activated JAWS-GFP-mJag1 was 16419±2718cpm; activated JAWS-GFP-mLfng was 19224±1995cpm; activated JAWS-GFP-Dll-1 was 21575±4663) as compared to the unstimulated transduced JAWS II cell population ($p<0.05$) [Figure 3-10 (b)- (f)]. These results indicate that JAWS II cells, which are regarded as an immature DC line, were able to stimulate allogeneic T cells and the response was further enhanced when the cells were activated with cytokines and LPS and underwent maturation. These results also show that

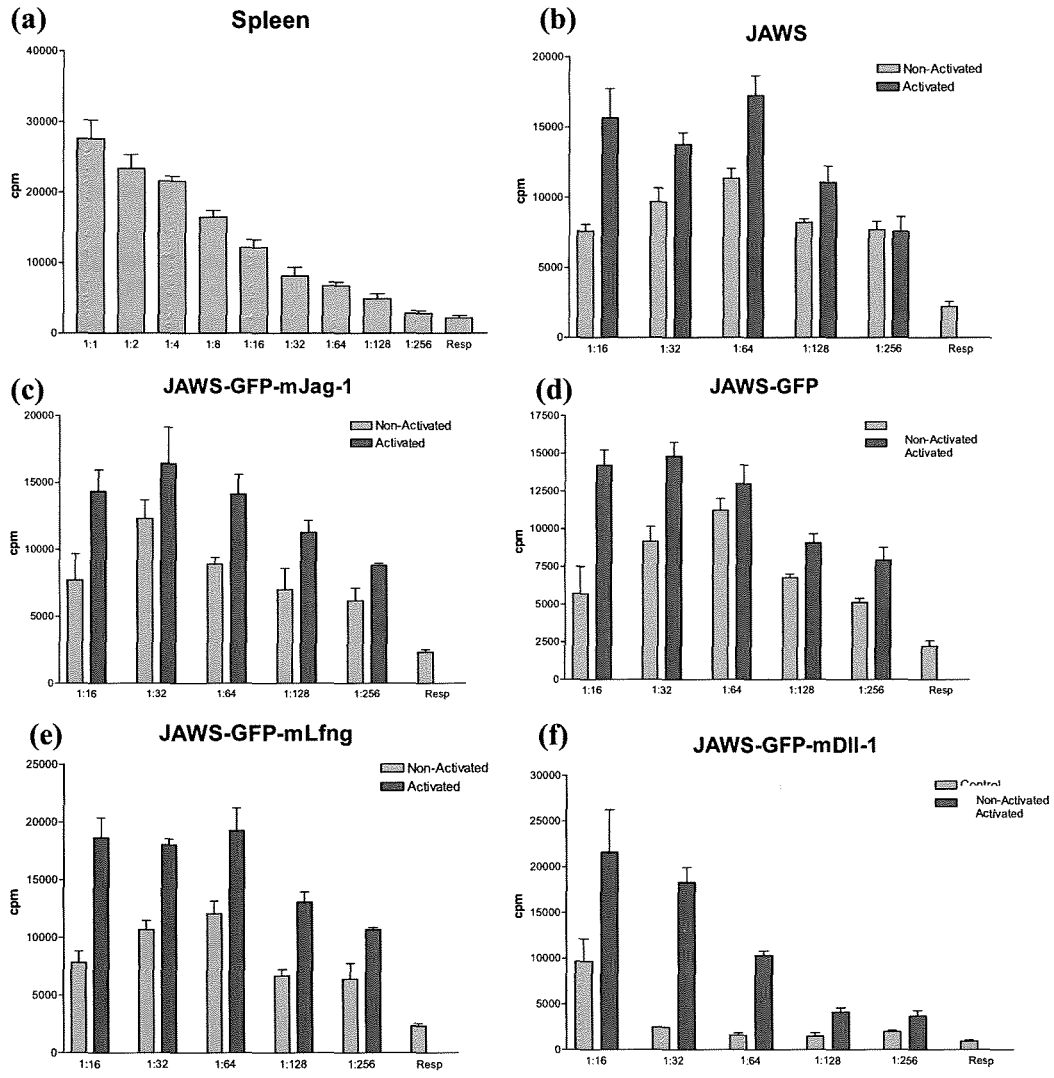


Figure 3-10: Characterization of capacity of unstimulated and activated JAWS II cells over-expressing Notch-related molecules to stimulate allogeneic T cells

CBA/H T cell proliferation was measured in response to (a) C57BL/6 splenocytes, (b) untransduced JAWS, (c) JAWS-GFP, (d)JAWS-GFP-mJag1, (e) JAWS-GFP-mLfng and (f) JAWS-GFP-mDll-1 on Day 4. Mean±SEM cpm from groups of 3 samples tested individually are shown.

modulation in Notch signaling did not markedly affect the T cell immunostimulatory capacity of immature DCs, at least, under these experimental conditions *in vitro*.

3.9 Discussion

This chapter describes the construction of an immature DC cell line, JAWS II cells, over-expressing Notch related molecules, mJag-1, mLfng, mMfng and mDelta-ll1. The levels of transduced gene expression confirmed retroviral gene transduction. The phenotype characterization of JAWS II cell populations suggested that the JAWS II cells had remained immature post-transduction.

Retroviral delivery of genes is widely used to deliver exogenous genes into cells to be used for experimental and therapeutic purposes (Cornetta and Anderson, 1989; Takayama et al., 1998). It is important to achieve long-term expression of inserted genes in order to assess the biological effectiveness of the gene of interest; however, retroviral transduction efficiency is generally low (Takayama et al., 1998). Results presented in this chapter describe the transduction of JAWS II cells with LT virus resulting in over-expression of Notch-related molecules but unstable gene transfer (half-life was approximately 20 days)[Figure 3.6]. Using a centrifugal enhancement method of transduction, HT virus was found to improve both the transduction efficiency (with all pKMV constructs) [Table 3-1, Figure 3-4 and 3-5] as well as the stability of the inserted genes; long term GFP-expression >40 days was achieved [Figure 3-6]. This finding was consistent with previous studies showing a 4- to 5-fold increase in transduction efficiency after centrifugal enhancement (Bahnson et al., 1995; Takiyama et al., 1998). The HT virus was HT because it was harvested from the supernatant of Phoenix cells when they were strongly GFP-positive. The HT virus is independent of the centrifugal enhancement method of transduction. The more stable transduction of JAWS II cells using HT-virus is most like a combination of HT virus and the

method of transduction. In contrast to JAWS II cells, transduction of primary BMDCs with HT virus with centrifugal enhancement showed poor transduction efficiency and did not yield a sufficient number of GFP-positive DCs for further experimentation. Since retroviruses only transduce dividing cells, it is possible that low transduction efficiency was due to a lower rate of cell division in BMDCs compared to JAWS II cells.

Semi-quantitative RT-PCR analysis confirmed that gene expression of Jag-1, Lfng, and mMfng were enhanced and Delta-l1 expression was induced in JAWS II cells following transduction with LT and HT viruses. The notable differences in the magnitude of Notch-related gene expression between LT- and HT- retrovirus-transduced JAWS II cell populations (differs up to 6-fold) may be due to different preparation method. Izon *et al* showed that Notch-1 signaling can arrest thymocyte development in a dose dependent manner (Izon et al., 2001). They demonstrated that there was an inverse relationship between T cell differentiation and the strength of Notch-1 signaling. In our system, LT virus transduced JAWS II cells exhibited greater expression of Notch related genes compared to HT virus transduced JAWS exhibit a lesser degree of over-expression. These two sets of JAWS II cell constructs could be utilized to assess whether the strength of Notch signaling could lead to different levels of allograft protection *in vivo* in the following chapter.

FACS analysis confirmed that there was little difference in phenotype between untransduced and pKMV (both LT and HT) transduced JAWS II populations [Figure 3-8]. Based on their profiles of expressed costimulatory molecules, JAWS II cells remained immature even after transduction. *In vitro* studies showed that JAWS II cells were able to stimulate allogeneic T cells and, as expected, the T cell-priming capacity was significantly enhanced following JAWS II cell activation with cytokines and LPS. A lack of MHC class II on unstimulated JAWS II and relatively low MHC class II expression as well as upregulated expression of

costimulatory molecules on cytokine/LPS stimulated JAWS II cells were consistent with a previous report (Jorgensen et al., 2002). Likewise, JAWS II cells were able to prime allogeneic T cells and their capacity to stimulate T cells was enhanced when JAWS II cells were activated (Haase et al., 2004; Jorgensen et al., 2002).

When the immunostimulatory capacity of JAWS II cells over-expressing Notch related molecules were tested, little difference was seen between untransduced and transduced JAWS II cells. The data from MLRs presented in this chapter indicates that the modulation of Notch signaling during activation of DC cell line does not have gross biological effects *in vitro*. In contrast, to previous reports showed the induction of tolerogenic T cells by over-expressing of Notch-ligands, Jagged-1 or Delta-l1 on stimulating cells (Hoyne et al., 1999; Wong et al., 2003). These reported studies employed DCs enriched for primary APC populations and L-cells to over-express the Notch ligands and the differences in experimental systems could account for the different outcome observed in this study. The difference in allogeneic T cell proliferation could also be due to the fact that JAWS II cells are not physiologically identical to primary DCs (Jorgensen et al., 2002). Previous study demonstrated differential expression of Dll or Jag-1 on APCs can skew T cell response towards Th1 or Th2 type respectively (Amsen et al., 2004). Whether there was a polarization of T cell response into Th1 or Th2 type in the proliferating T cells in response to JAWS II cells over-expressing Notch related molecules was not experimented due to time constraints.

In the work described in this chapter, JAWS II cells (immature DC cell line) over-expressing Notch pathway components, mJag-1, mLfng, mMfng, and mDelta-l1 were constructed. It was shown that two transduction methods and differences in virus titer resulted in JAWS II cells with different transduction efficiencies and stability of gene transfer; nevertheless an immature DC phenotype was obtained. Functional assays showed that, at least *in vitro*,

over-expression did not alter the nature of allogeneic T cell priming activities of JAWS II cells.

As *in vitro* experimental model cannot replicate fully the normal physiological environment, the effect of Notch-related molecule overexpression needed to be assessed *in vivo*. Chapter 4 will examine whether modulation of Notch signaling by pretreatment of recipient mice with pKMV-transduced JAWS II cells can prolong allograft survival, and whether different duration and magnitudes of Notch related gene expression in JAWS II cells affect the graft survival *in vivo*.

Chapter 4

Effects of Administration of JAWS II Cells Over-expressing Notch-related Molecules in Allotransplantation

4.1 Introduction

A number of studies have demonstrated that the over-expression of Notch-related molecules can lead to the generation of regulatory T cells, suggesting the involvement of Notch signaling during tolerance induction (Hoyne et al., 1999; Vigouroux et al., 2003; Wong et al., 2003). Hoyne *et al.* showed that the over-expression of Jagged-1 on APCs pulsed with house dust mite protein induce antigen specific tolerance via generation of regulatory T cells (Hoyne et al., 1999). Antigen-specific tolerance was also induced using an Epstein-Barr-virus (EBV)-positive lymphoblastoid B cell line (LCL) over-expressing Jagged-1 as APCs (Vigouroux et al., 2003). These cells were shown to inhibit cytotoxic T cell proliferation and effector function in antigen-specific manner via increased production of IL-10 (Vigouroux et al., 2003). The observed tolerance was attributed to the activities of $CD4^+CD25^+$ and $CD8^+CD25^-$ tolerogenic T cells, acting in a cell-to-cell contact-dependent manner (Vigouroux et al., 2003). A parallel study showed that this EBV-LCL experimental system can also promote alloantigen-specific regulatory T cell differentiation by employing allogeneic EBV-LCL cells *in vitro*. These findings suggested that manipulation of the Notch signaling pathway in transplantation settings could facilitate graft survival (Yvon et al., 2003). Wong *et al.* reported that modulation of Notch signaling can be used to protect cardiac allografts *in vivo*. It was shown that administration of L-cells over-expressing Delta-1 (Dll-1) in recipient mice resulted in generation of $CD8^+$ regulatory T cells leading to prolonged allograft survival (Wong et al., 2003). Collectively, these studies suggest that the enhanced Notch signaling in APCs can potentially alter immune response in favor of allograft protection. However, the mechanism(s) by which Notch signaling can be used to induce alloantigen-specific tolerance is yet to be identified.

Chapter 3 examined modification of Notch-related molecule expression on JAWS II cells. *In vitro* results indicated no suppression of alloreactive T cells mediated by JAWS II cells

over-expressing Notch-related molecules. Despite these results, it is possible that additional signals or interactions were required to induce allospecific T cell unresponsiveness. While draining lymph nodes (LNs) are required for mounting allospecific T cell responses (Lakkis et al., 2000), there are several reports showing the requirement of lymphoid organs in the induction of allospecific tolerance as well (Bai et al., 2002; Garrod et al., 2006; Ochando et al., 2005). Transportation of apoptotic cells to secondary lymphoid organs by immature intestinal DCs has correlated with the induction of tolerance to self-antigens (Huang et al., 2000). Garrod *et al.* demonstrated that migration of immature viral IL-10-transduced DCs to LNs of recipients is critical for inducing prolonged survival of cardiac allografts. In addition, recent reports showed that LNs, but not other anatomic sites, facilitate the expansion of $Foxp3^+$ regulatory T cells during the induction of tolerance to alloantigens (Ochando et al., 2005). Collectively these findings emphasize the importance of secondary lymphoid organs for promoting tolerance. These studies led us to examine the effects of transduced JAWS II cells (prepared in Chapter 3) in allotransplantation settings *in vivo*.

In this chapter, the aim was to investigate whether the over-expression of Notch related molecules on donor-type immature DCs suppress recipient alloresponses *in vivo*. Previously constructed pKMV-transduced JAWS II cells ($H\text{-}2^b$) were administered to CBA/H ($H\text{-}2^k$) recipients prior to C57BL/6 ($H\text{-}2^b$) thyroid transplantation. The effect of JAWS II cell pretreatment of recipient mice on allograft survival was examined.

4.2 Thyroid allotransplantation in mice receiving a single treatment of JAWS cells over-expressing Notch related molecules (LT)

Over-expression of Notch ligands on APCs has been shown to promote the differentiation of alloantigen-specific regulatory T cells (Vigouroux et al., 2003; Wong et al., 2003). Pretreatment of allograft recipients with these APCs resulted in prolonged survivals of the

grafts (Vigouroux et al., 2003; Wong et al., 2003). On the bases of such previous reports, this study examined the effects of pretreating allogeneic recipient mice (CBA/H, H-2^k) with JAWS cells over-expressing the Notch-related molecules (constructed in Section 3.6) prior to the allotransplantation of C57BL/6J (H-2^b) thyroid tissue. JAWS-GFP-mJag-1 (LT), JAWS-GFP-mLfng (LT), JAWS-GFP-mMfng (LT) cells were injected i.v. into CBA/H recipient mice (4×10^5/mouse) 14 days prior to C57BL/6 thyroid transplantation. JAWS-GFP (LT) and untransduced JAWS II cells (4×10^5/mouse) were used and control cells and CBA/H thyroid was transplanted as isograft controls. Thyroid allografts and isografts were harvested on day 7 and day 14 post-transplant. Histological examination of grafts harvested from PBS-treated, untransduced JAWS and JAWS-GFP (LT)-treated mice showed signs of acute graft rejection including cellular infiltration, hemorrhage, swelling of the graft site, loss of thyroid follicles and scarring [Figure 4-1(a)-(c)]. Grafts from JAWS-GFP-mJag-1 (LT), JAWS-GFP-mLfng (LT), JAWS-GFP-mMfng (LT)-treated mice also showed milder cellular infiltration but the magnitudes of intact follicle loss were similar to those observed in PBS-, untransduced JAWS, and JAWS-GFP (LT) controls [Figure 4-1(d)-(f)]. Isografts contained a mass of full intact follicles without any signs of graft destruction [Figure 4-1(g)].

Morphometric analysis revealed 2.7-fold reduction in the % intact follicles in the day 7 graft harvested from PBS-treated mice compared to that of intact isografts (15.20±0.50% intact follicles in PBS-treated allografts compared to 41.09±3.14% in isograft controls) [Figure 4-2]. Treatment with untransduced JAWS or JAWS-GFP (LT) cells did not significantly improve graft survival compared to PBS grafts ($p>0.05$) (% intact follicles was 14.60±1.35% and 11.96±1.30% respectively) [Figure4-3]. Similarly, the pretreatment of recipients with JAWS-GFP-mJag-1 (LT), JAWS-GFP-mLfng (LT), and JAWS-GFP-mMfng (LT) did not improve the allograft survival compared to PBS-treated mice or mice treated with untransduced JAWS, or JAWS-GFP (LT) (% intact follicles was 14.65±0.79%, 13.64±1.01%

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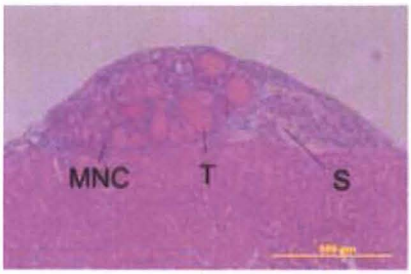

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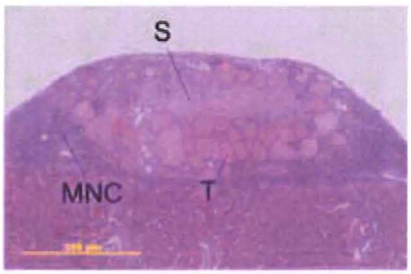

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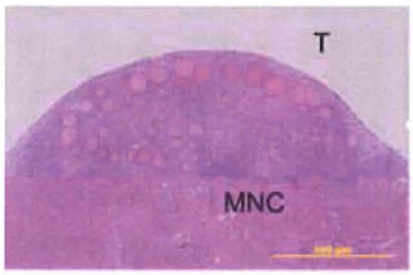

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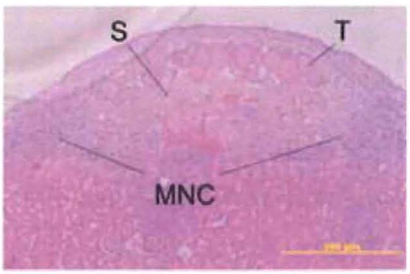

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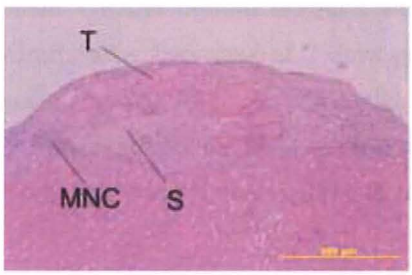

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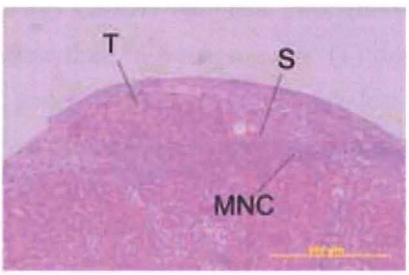

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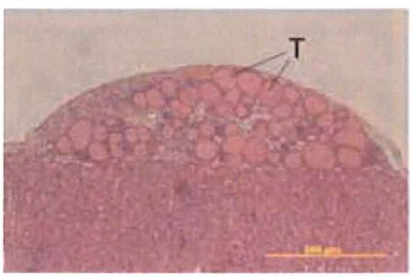

Figure 4-1: Histological appearance of representative day 7 thyroid allografts from mice receiving a single treatment of JAWS II cells over expressing Notch related molecules (LT)

C57BL/6 thyroid allografts were transplanted to (a) PBS-treated, (b) untransduced JAWS-treated, (c) JAWS-GFP (LT)-treated, (d) JAWS-GFP-mJag-1 (LT)-treated, (e) JAWS-GFP-mLfng (LT) treated or (f) JAWS-GFP-mMfng (LT)-treated CBA/H recipient mice. CBA/H thyroid isografts were transplanted to (g) CBA/H recipient mice (n=6-13). Grafts were harvested at 7 days post-transplant as described in Section 2.4.4. (T) depicts intact thyroid follicles, (S) depicts scarred tissues, and (MNC) depicts mononuclear cell infiltrates. Experiments were repeated twice and a representative Histological section of each group is shown. Hematoxylin and eosin, ×10.

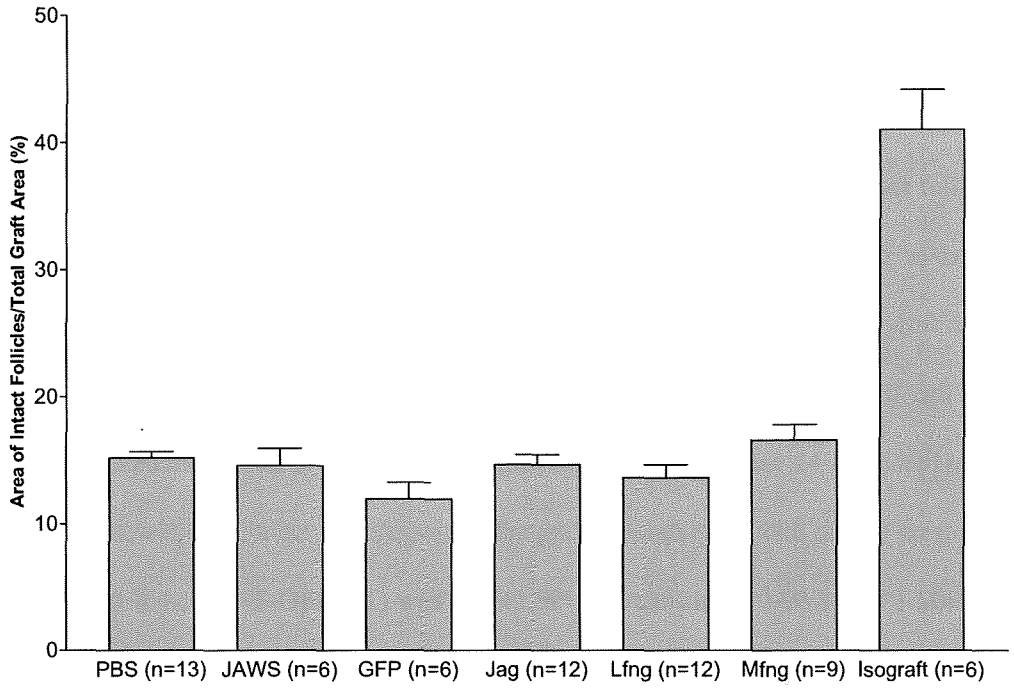

Figure 4-2: Morphometric analysis of C57BL/6 thyroid allografts and CBA/H thyroid isografts at 7 days post-transplant to CBA/H recipients which received a single treatment of JAWS II cells over-expressing Notch related molecules.

CBA/H recipient mice were pretreated with 4×10^5 untransduced JAWS II, JAWS-GFP (LT), JAWS-GFP-mJag-1 (LT), JAWS-GFP-mLfng (LT), or JAWS-GFP-mMfng (LT) cells i.v. 14 days prior to thyroid transplantation. C57BL/6 thyroids were transplanted beneath the kidney capsule on day 0 and the grafts were harvested at 7 days post-transplant. % intact follicles of the total graft area was calculated as described in Section 2.4.5. Data represent mean±SEM.

and 16.61±1.23% for JAWS-GFP-mJag-1 (LT), JAWS-GFP-mLfng (LT) and JAWS-GFP-mMfng (LT), respectively) [Figure 4-2].

By day14, thyroid allografts following all treatments were extensively damaged with few intact thyroid follicles, cellular infiltrates and some scarring [Figure 4-3(a)-(f)]; in contrast isografts remained intact [Figure 4-3(g)]. Morphometric analysis of allografts at day 14 post-transplant confirmed extensive graft rejection [Figure 4-4]. The % of intact follicles in isografts at day 14 was 47.15±9.55% [Figure 4-4] and was similar to isografts at day 7 (% intact follicles of day 7 isografts was 41.09±3.14%) [Figure 4-2], indicating no graft damage. Grafts harvested from PBS-treated, GFP-JAWS (LT) and untransduced JAWS cell treated mice exhibited more extensive destruction of thyroid allografts at day 14 compared to day 7 ($p<0.05$) showing few intact follicles within the grafts (% intact follicles of PBS-treated mice was 2.15±0.76%; JAWS-GFP (LT) was 3.91±1.52%; untransduced JAWS was 1.56±0.96%) [Figure 4-4]. No statistically significant improvement ($p>0.05$) in allograft survival was achieved following JAWS-GFP-mJag-1 (LT), JAWS-GFP-mLfng (LT), and JAWS-GFP-mMfng treatment, in comparison to grafts from control mice (% intact follicles in the graft of JAWS-GFP-mJag-1 was 3.71±0.10%; JAWS-GFP-mLfng was 7.62±2.28%; JAWS-GFP-mMfng was 6.47±1.67%) [Figure 4-4].

These results indicate that a single i.v. treatment of allograft recipients with JAWS cells over-expressing the Notch-related molecules (LT) did not alter the kinetic of thyroid allograft destruction and hence did not provide graft-protection *in vivo*.

4.3 Thyroid allotransplantation in mice receiving a single treatment of JAWS cells over-expressing Notch related molecules (HT)

LT pKMV-retrovirus transduced JAWS cells demonstrated loss of GFP expression (i.e.

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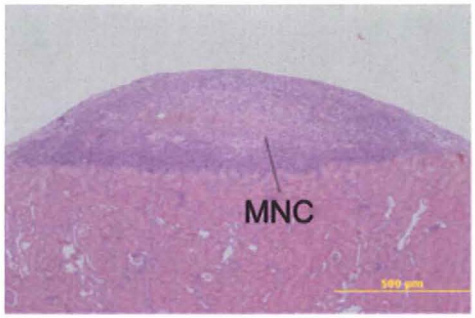

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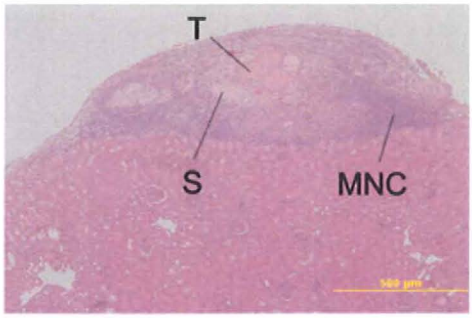

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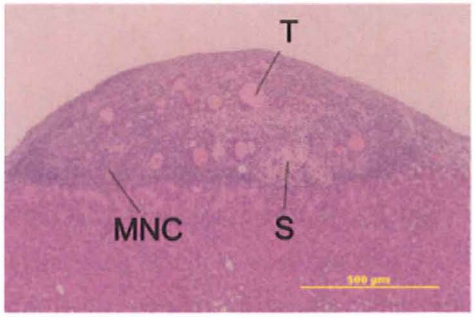

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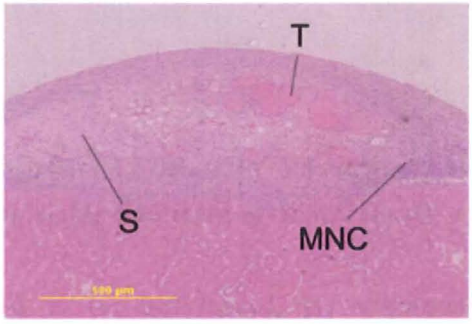

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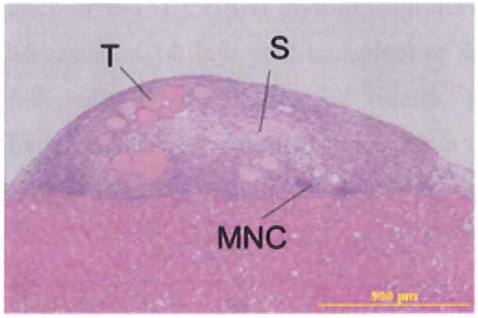

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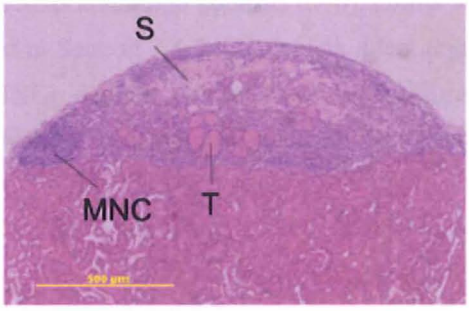

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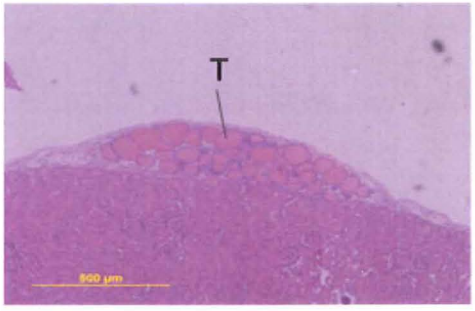

Figure 4-3: Histological appearance of representative C57BL/6 thyroid allografts at 14 days post-transplant to CBA/H mice receiving single treatment of JAWS II cells over expressing Notch related molecules.

C57BL/6 thyroid allografts were transplanted to (a) PBS-treated, (b) untransduced JAWS-treated, (c) JAWS-GFP (LT)-treated, (d) JAWS-GFP-mJag-1 (LT)-treated, (e) JAWS-GFP-mLfng (LT) treated or (f) JAWS-GFP-mMfng (LT)-treated CBA/H recipient mice (n=5-13). CBA/H thyroid isografts (g) served as additional controls (n=6). Grafts were harvested at 14 days post-transplant as described in Section 2.4.4. (T) depicts intact thyroid follicles, (S) depicts scarred tissues, and (MNC) depicts mononuclear cell infiltrates. Experiments were repeated twice and a representative Histological section of each group is shown. Hematoxylin and eosin, ×10.

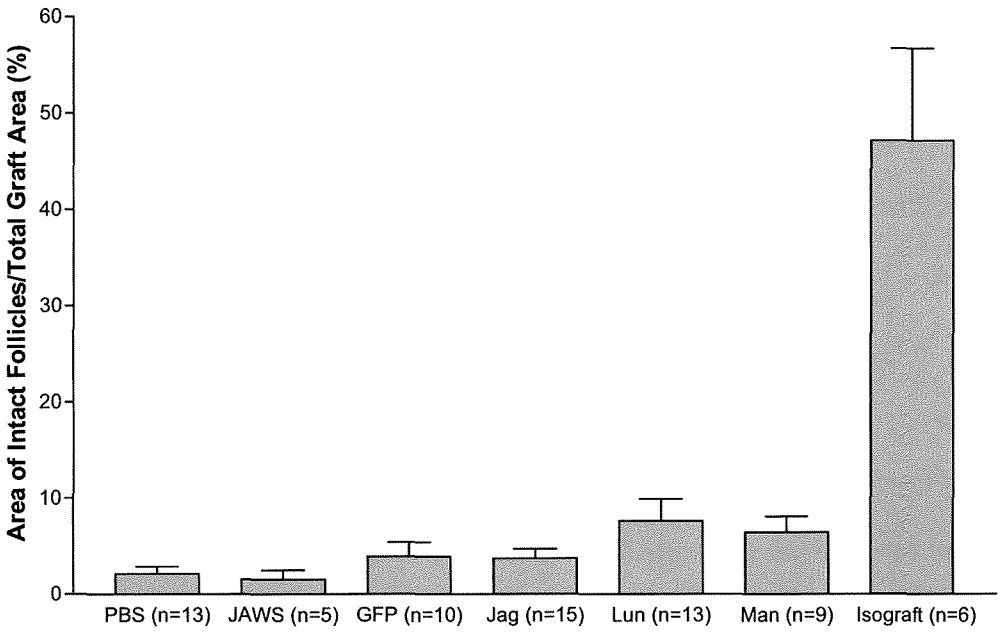

Figure 4-4: Morphometric analysis of C57BL/6 thyroid allografts at 14 days post-transplant to CBA/H mice receiving a single treatment of JAWS II cells over-expressing Notch related molecules (LT).

CBA/H recipient mice were pretreated with 4×10^5 untransduced JAWS II, JAWS-GFP (LT), JAWS-GFP-mJag-1 (LT), JAWS-GFP-mLfng (LT), or JAWS-GFP-mMfng (LT) cells i.v. 14 days prior to thyroid transplantation. C57BL/6 thyroid was transplanted beneath the kidney capsule and the grafts were harvested at 14 days post-transplant. % intact follicles of total graft area was calculated as described in Section 2.4.5. Data represent mean±SEM.

reporter gene) in approximately 50% of total population by 14 days post-transduction while HT pKMV-retrovirus-transduced JAWS cells exhibited much more stable GFP expression (Section 3.5). Thus, the failure of JAWS II cells transduced with Notch-related genes (Section 4.2) to facilitate allotransplantation in treated mice may have been due to loss of the gene of interest from the JAWS cells *in vivo* and/or the rejection of JAWS II cells themselves (H-2 compatible and possible minor non-H-2 incompatibilities). Persistence of the inserted gene following transduction of JAWS II cells may therefore improve the survival of the JAWS II cells as well as the thyroid allografts. Mice were therefore treated with a single injection of JAWS-GFP-mLfng (HT) cells to test whether the administration of JAWS cells stably expressing Lfng would result in prolongation of allograft survival.

4×10^5 JAWS-GFP-mLfng (HT) cells were injected i.v. into CBA/H ($H\text{-}2^k$) recipient mice 14 days prior to receiving C57BL/6 ($H\text{-}2^b$) thyroid grafts. The grafts were harvested on day 7 and day14 post-transplant. The grafts from JAW-GFP-mLfng (HT) treated mice at 7 days post-transplant showed graft rejection including heavy cellular infiltration, hemorrhage, and loss of intact follicles in a similar manner to allografts from mice treated with PBS or untransduced JAWS cells [Figure 4-5 (a)-(c)]. The histological appearance of the grafts was also similar to grafts at 7 days post-transplant to mice pretreated with JAWS-GFP-mLfng (LT) cells [Figure 4-1 (e)].

Morphometric analyses revealed that the tempo of graft destruction in JAWS-GFP-mLfng-treated mice was not significantly different from PBS- and untransduced JAWS II cell treated mice on day 7 (% intact follicles in grafts of PBS-treated mice was 15.20±0.50%: untransduced JAWS II was 15.597±1.35%: JAWS-GFP-mLfng (HT) was 13.84±2.83%) [Figure 4-6]. Histological analyses of grafts harvested at 14 days post-transplant to JAWS-GFP-mLfng (HT) mice also did not show an improvement in the

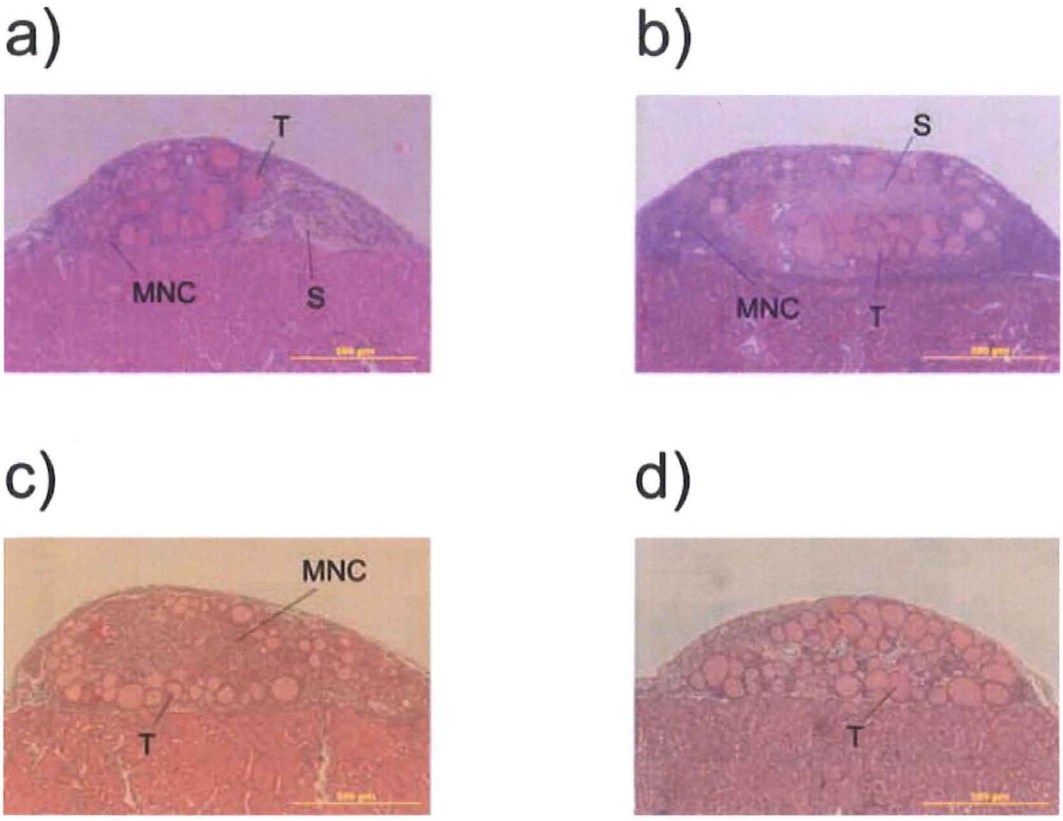

Figure 4-5: Histological appearance of representative C57BL/6 thyroid allografts at 7 days post-transplant to CBA/H mice receiving single treatment of JAWS-GFP-mLfng (HT)

C57BL/6 thyroid allografts were transplanted to (a) PBS-treated, (b) untransduced JAWS-treated, or (c) JAWS-GFP-mLfng (HT) treated CBA/H recipient mice (n=5-13). CBA/H thyroid isografts were transplanted to (d) CBA/H recipient mice (n=6). Grafts were harvested at 7 days post-transplant as described in Section 2.4.4. (T) depicts intact thyroid follicles, (S) depicts scarred tissues, and (MNC) depicts mononuclear cell infiltrates. Experiments were repeated twice and a representative Histological section of each group is shown. Hematoxylin and eosin, ×10.

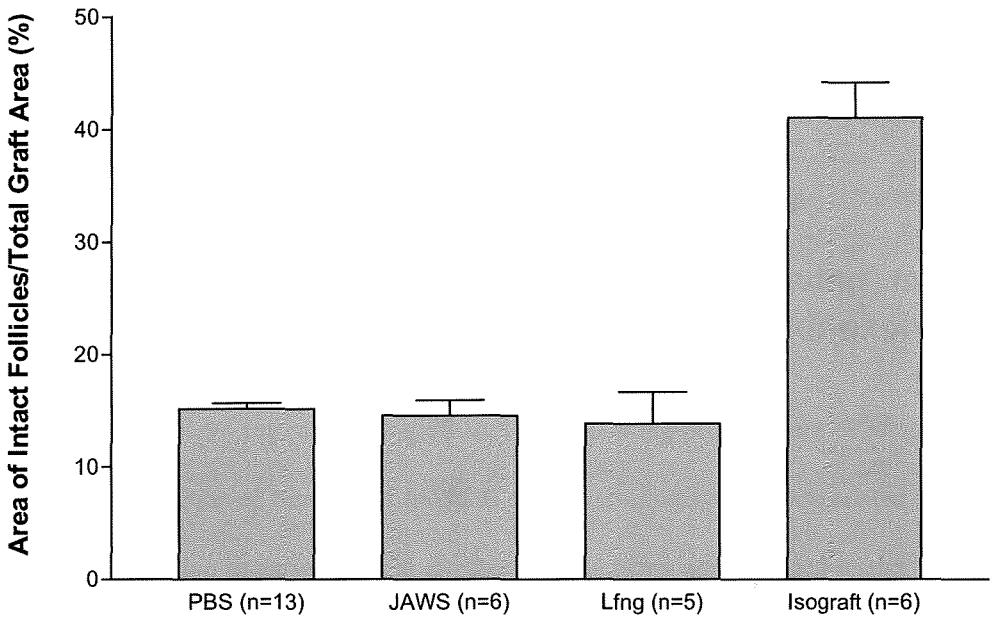

Figure 4-6: Morphometric analysis of C57BL/6 thyroid allografts at 7 days post-transplant to CBA/H mice receiving single treatment of JAWS II cells over-expressing mLfng (HT).

CBA/H recipient mice were pretreated with 4×10^5 untransduced JAWS II, or JAWS-GFP-mLfng (HT) cells i.v. 14 days prior to thyroid transplantation. C57BL/6 thyroids were transplanted beneath the kidney capsule and the grafts were harvested at 7 days post-transplant. % intact follicles of total graft area was calculated as described in Section 2.4.5. Data represent mean±SEM.

allograft survival, compared to control allografts from PBS- and untransduced JAWS II cell treated mice [Figure 4-7]. Morphometric analyses also showed no significant difference between allograft survival in PBS-, untransduced JAWS, JAWS-GFP-mLfng (HT)- treated animals [% intact follicles in PBS-treated mice was 2.40±0.73%; untransduced JAWS mice was 1.25±0.44%; JAWS-GFP-mLfng (HT) was 3.57±1.09%] [Figure 4-8]. These results indicate the lack of allograft protection following treatment of JAWS II cells over-expressing Notch-related molecules was unlikely to be due to the loss of the inserted genes from the JAWS cells following *in vivo* administration.

4.4 Thyroid allotransplantation in mice receiving multiple treatment of JAWS-II cells over-expressing Notch-related molecules (HT)

The failure to achieve improved allograft survival following pretreatment of recipient mice with a single injection of transduced JAWS II cells over-expressing Notch related molecules could have been due to an inadequate number of cells being administered to the recipients. Some reports argue that the relative proportion of regulatory T cells and effector T cells is a critical factor determining the outcome of the immune response (reviewed in (Lohr et al., 2006)). To eliminate this possibility, i.v. injections of 4×10^5 JAWS-GFP (HT), JAWS-GFP-mJag-1 (HT), JAWS-mLfng (HT), JAWS-GFP-Dll-1 (HT) or untransduced JAWS cells were performed on 14 days and 7 days before transplantation and also on the day of transplantation. The grafts were harvested on day 7 and day 14 to examine survival.

Histological sections of grafts at 7 days post-transplant showed similar features of graft rejection in all treated recipient mice. PBS-, untransduced JAWS- and JAWS-GFP (HT) treated control mice showed cellular infiltrates at the graft site, hemorrhage and loss of intact follicles, indicating rejection [Figure 4-9 (a)-(c)]. Treatment with JAW-GFP-mJag-1 (HT), JAWS-GFP-mLfng (HT), and JAWS-GFP-mDll-1 (HT) did not promote graft survival and

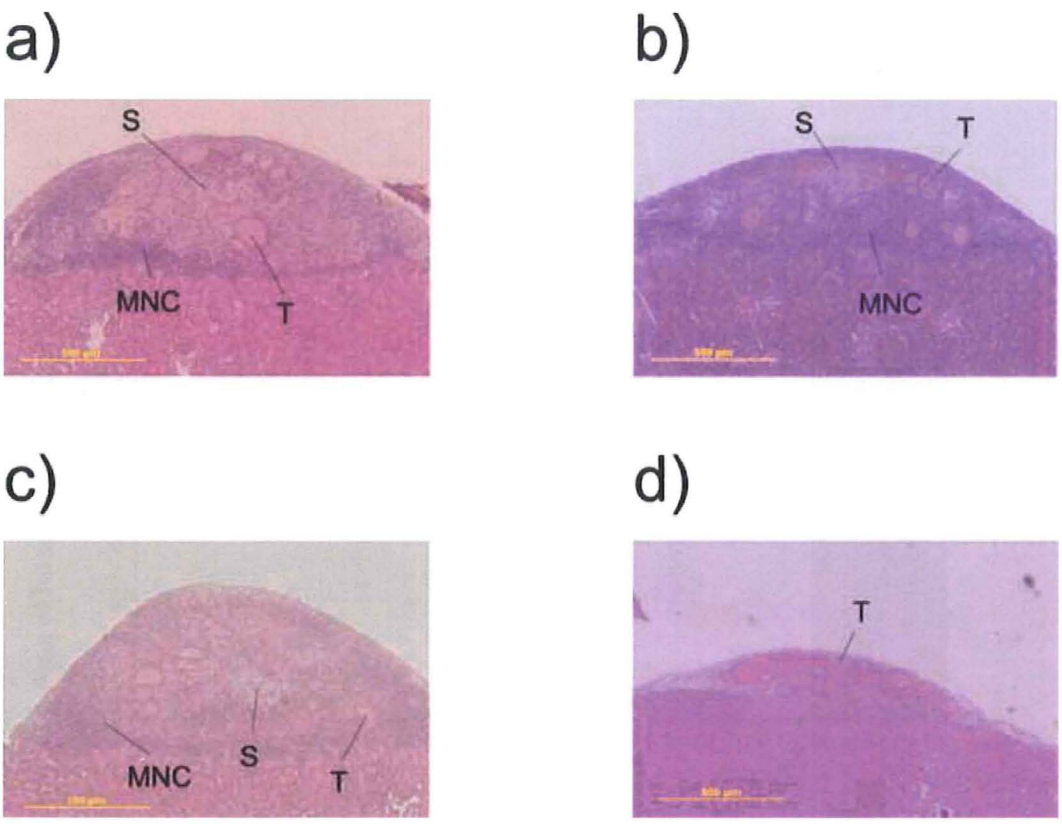

Figure 4-7: Histological appearance of representative C57BL/6 thyroid allografts at 14 days post-transplant to CBA/H mice receiving a single treatment of JAWS II cells-GFP-mLfng (HT)

C57BL/6 thyroid allografts were transplanted to (a) PBS-treated, (b) untransduced JAWS-treated, or (c) JAWS-GFP-mLfng (HT) CBA/H recipient mice (n=5-6). CBA/H thyroid isografts were transplanted to (g) CBA/H recipient mice (n=6). Grafts were harvested at 14 days post-transplant as described in Section 2.4.4. (T) depicts intact thyroid follicles, (S) depicts scarred tissues, and (MNC) depicts mononuclear cell infiltrates. Experiments were repeated twice and a representative Histological section of each group is shown. Hematoxylin and eosin, ×10.

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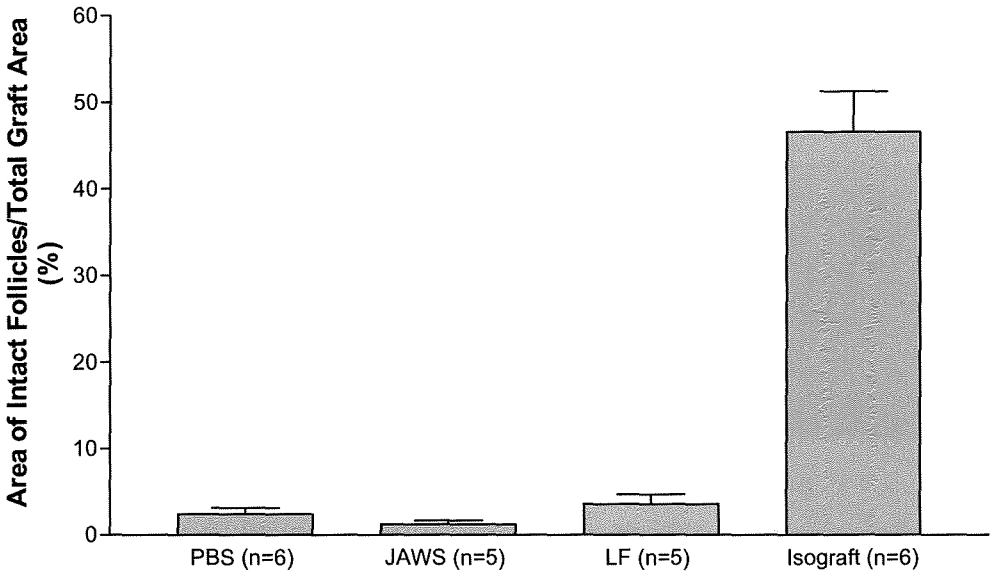
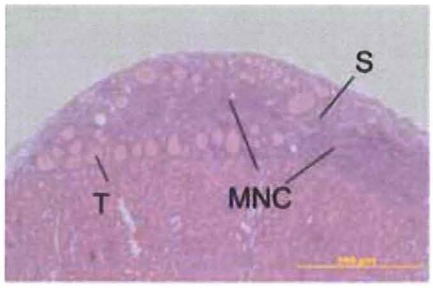


Figure 4-8: Morphometric analysis of C57BL/6 thyroid allografts at 14 days post-transplant to CBA/H mice receiving a single treatment of JAWS II cells over-expressing mLfng (HT).

CBA/H recipient mice were pretreated with 4×10^5 untransduced JAWS II, or JAWS-GFP-mLfng (HT) cells i.v. 14 days prior to thyroid transplantation. C57BL/6 thyroid was transplanted underneath the kidney capsule and the grafts were harvested at 14 days post-transplant. % intact follicles of total graft area was calculated as described in Section 2.4.5. Data represent mean±SEM.

a)



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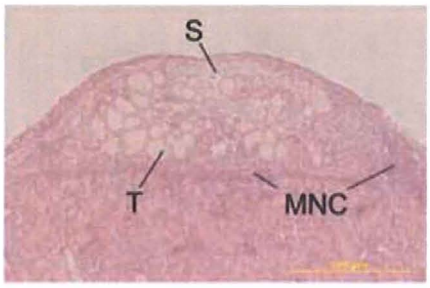

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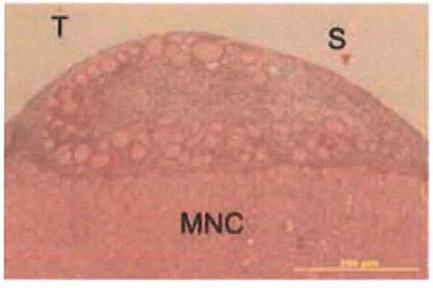

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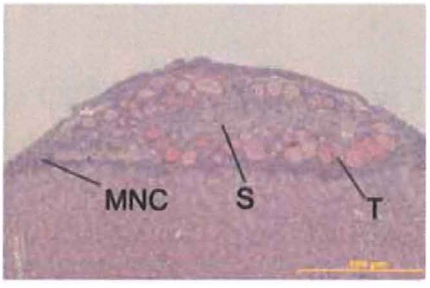

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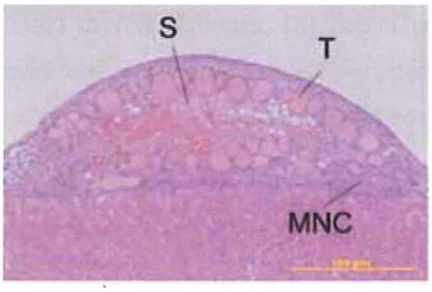

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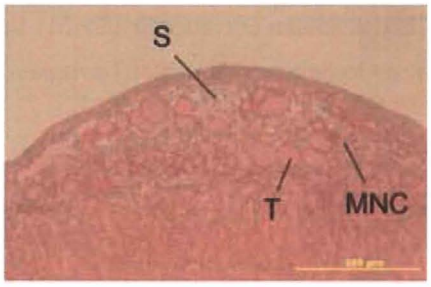

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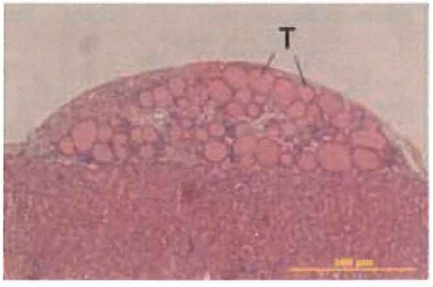


Figure 4-9: Histological appearance of representative C57BL/6 thyroid allografts at 7 days post-transplant to CBA/H mice receiving multiple treatments with JAWS II cells over expressing Notch related molecules (HT)

C57BL/6 thyroid allografts were transplanted to (a) PBS-treated, (b) untransduced JAWS-treated, (c) JAWS-GFP (HT)-treated (d) JAWS-GFP-mJag-1 (HT)-treated, (e) JAWS-GFP-mLfng (HT)-treated or (f) JAWS-GFP-mDll-1 (HT)-treated CBA/H recipient mice (n=4-9). CBA/H thyroid isografts were transplanted to (g) CBA/H recipient mice (n=6). Grafts were harvested at 7 days post-transplant as described in Section 2.4.4. (T) depicts intact thyroid follicles, (S) depicts scarred tissues, and (MNC) depicts mononuclear cell infiltrates. Experiments were repeated twice and a representative Histological section of each group is shown. Hematoxylin and eosin, ×10.

the histological appearance of the allografts resembled those observed in control mice treated with PBS, untransduced JAWS and JAWS-GFP (HT) cells [Figure 4-9(d)-(f)]. Morphometric analysis of grafts of PBS-, untransduced JAWS- and JAWS-GFP (HT) treated mice confirmed the comparable graft damage with approximately 2.1-2.7-fold reduction in the area of intact follicles compared to corresponding isografts (% intact follicles in PBS-treated mice was 18.17±1.98%; untransduced JAWS treated mice was 19.34±2.07%; JAWS-GFP (HT) treated mice was 15.29±2.46%; isograft was 41.09±3.15%) [Figure 4-10]. Similarly, a 1.9-2.5-fold reduction in survival follicles was obtained in the allografts from JAWS-GFP-mJag-1 (HT), JAWS-GFP-mLfng (HT), and JAWS-GFP-mDll-1 (HT)-treated mice (% intact follicles in the graft of JAWS-GFP-mJag-1(HT) was 21.19±1.97%; JAWS-GFP-mLfng (HT) was 21.90±2.20%; JAWS-GFP-mDll-1 (HT) was 16.49±1.86%) compared to day 7 isografts [Figure 4-10]. Statistical analyses revealed there were no significant differences ($p>0.05$) in % intact follicles from treated and control mice.

Histological examination of the grafts harvested from PBS-, untransduced JAWS, JAWS-GFP (HT) revealed extensive damage in the thyroid graft integrity with heavy cellular infiltration [Figure 4-11(a)-(c)]. JAWS-GFP-mJag-1 (HT), JAWS-GFP-mLfng (HT) and JAWS-GFP-Dll-1 (HT) treatment did not result in major histological changes, compared to control treatment [Figure 4-11(a)-(f)]. Morphometric analysis of these histological sections also showed that there was no significant improvement in the % intact follicles at the graft site at 2 weeks after treatments with JAWS-GFP (HT) and untransduced JAWS cells, compared to PBS-treated grafts (% intact follicles in the grafts of PBS-treated mice was 2.95±1.16%; JAWS-GFP (HT) was 7.13±2.12%; untransduced JAWS II cell was 1.14±0.75%) [Figure 4-12]. JAWS-GFP-mJag-1 (HT), JAWS-GFP-mLfng (HT) and JAWS-GFP-Dll-1 (HT) administration also failed to prolong allograft survival (% intact follicles in JAWS-GFP-mJag-1 (HT) was 2.34±1.40%; JAWS-GFP-mLfng (HT) was

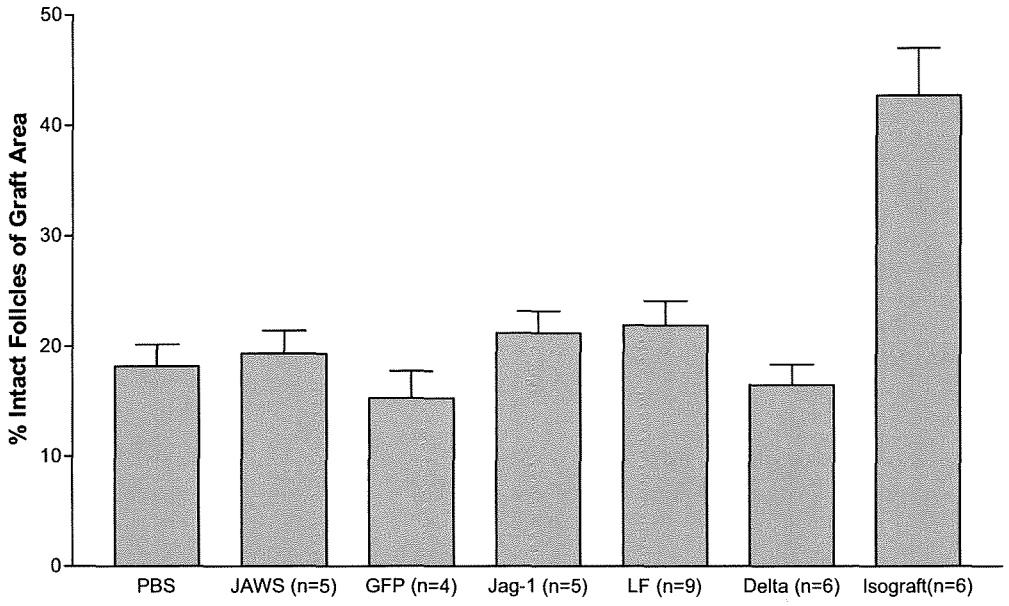
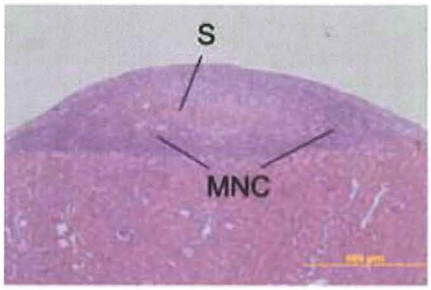


Figure 4-10: Morphometric analysis of C57BL/6 thyroid allografts 7 days post-transplant to CBA/H mice receiving multiple treatments with JAWS II cells over-expressing Notch related molecules (HT).

CBA/H recipient mice were pretreated with 4×10^5 untransduced JAWS II, JAWS-GFP (HT), JAWS-GFP-mJag-1 (HT), JAWS-GFP-mLfng (HT), or JAWS-GFP-mDll-1 (HT) cells i.v. 14, 7 and 0 days prior to thyroid transplantation. C57BL/6 thyroid was transplanted into these mice and the grafts were harvested at 7 days post-transplant. % intact follicles of total graft area was calculated as described in Section 2.4.5. Data represent mean±SEM.

a)



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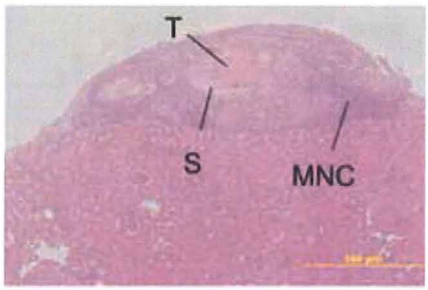

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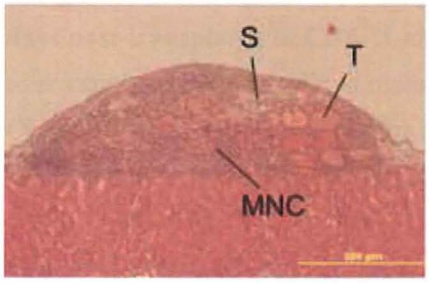

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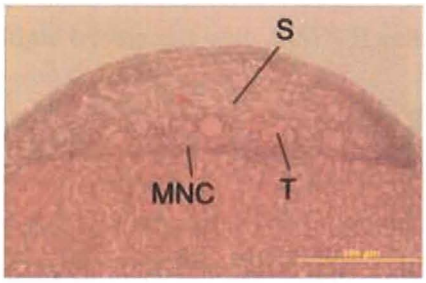

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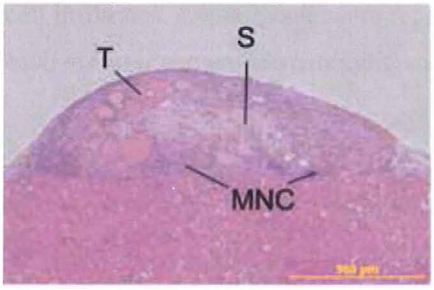

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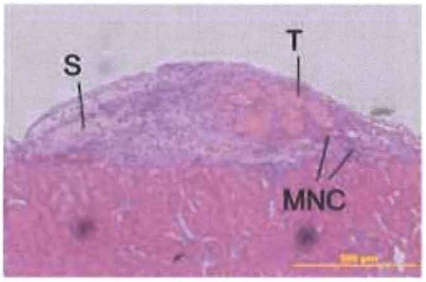

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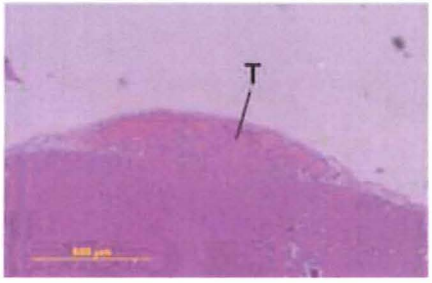


Figure 4-11: Histological appearance of representative C57BL/6 thyroid allografts at 14 days post-transplants to CBA/H mice receiving multiple treatments with JAWS II cells over expressing Notch related molecules (HT)

C57BL/6 thyroid allografts were transplanted to (a) PBS-treated, (b) untransduced JAWS-treated, (c) JAWS-GFP (HT)-treated (d) JAWS-GFP-mJag-1 (HT)-treated, (e) JAWS-GFP-mLfng (HT)-treated or (f) JAWS-GFP-mDll-1 (HT)-treated CBA/H recipient mice (n=5-9). CBA/H thyroid isografts were transplanted to (g) CBA/H recipient mice (n=6). Grafts were harvested at 14 days post-transplant as described in Section 2.4.4.Experiments were repeated twice and a representative Histological section of each group is shown. (T) depicts intact thyroid follicles, (S) depicts scarred tissues, and (MNC) depicts mononuclear cell infiltrates. Experiments were repeated twice and a representative Histological section of each group is shown. Hematoxylin and eosin, ×10.

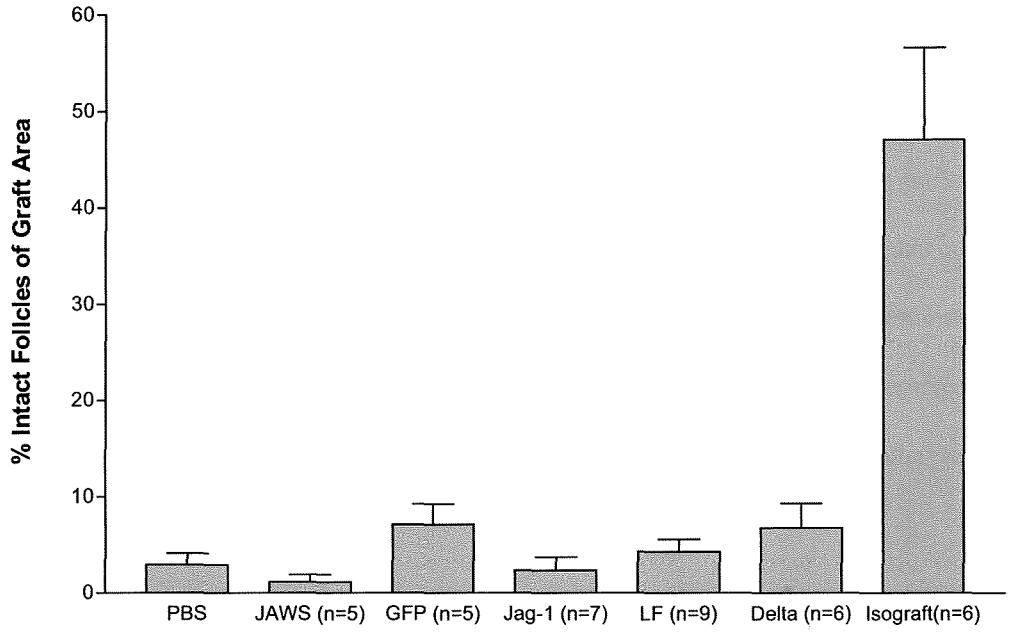


Figure 4-12: Morphometric analysis of C57BL/6 thyroid allografts at 14 days post-transplant to CBA/H mice receiving multiple treatments with JAWS II cells over-expressing Notch related molecules (HT).

CBA/H recipient mice were pretreated with 4×10^5 untransduced JAWS II, JAWS-GFP (HT), JAWS-GFP-mJag-1 (HT), JAWS-GFP-mLfng (HT), or JAWS-GFP-mDll-1 (HT) cells i.v. 14, 7 and 0 days prior to thyroid transplantation. C57BL/6 thyroid was transplanted into these mice and the grafts were harvested at 14 days post-transplant. % intact follicles over total graft area was calculated as described in Section 2.4.5. Data represent mean±SEM.

5.29±1.29%; JAWS-GFP-mDll-1 (HT) was 6.78±2.53%) [Figure 4-12].

These results indicate that the pretreatment of mice with increased number of JAWS II cells over-expressing mJag-1, mLfng and mDll-1 does not have allograft protecting effects.

4.5 Immunogenicity of JAWS II cells in MHC-compatible and MHC-incompatible mice

Results obtained in the previous sections of this chapter demonstrated that the administration of JAWS II cells (H-2^b) over-expressing the Notch-related molecules does not prolong the survival of H-2^b thyroid allografts (Section 4.2-4.4). A possible explanation for this outcome is that the JAWS II cells (H-2^b) were rejected in CBA/H (H-2^k) following i.v. administration. Thus the immunogenicity of JAWS II cells and transduced JAWS II cells over-expressing Notch related molecules was examined by implanting the JAWS cells as cellular grafts in CBA/H mice.

Histological analysis of cellular grafts of untransduced JAWS II cells in CBA/H recipient showed acute destruction of the JAWS II cells by day 7 [Figure 4-13(a)]. Heavy infiltration of lymphocytes was observed in the graft and graft/kidney interface indicating that an immune response had been generated against allogeneic JAWS II cells [Figure 4-13(a)]. Similar results were obtained for cellular grafts of JAWS-GFP (LT), JAWS-GFP-mLfng (LT), and JAWS-GFP-mMfng (LT) cells, demonstrating their limited survival in CBA/H mice [Figure 4-13 (b)-(d)]. When JAWS II (H-2^b) cells were transplanted to H-2-compatible C57BL/6 (H-2^b) mice, these cellular grafts underwent chronic rejection [Figure 4-14(a)]. Cellular grafts of JAWS-GFP (LT), JAWS-GFP-mLfng(LT), and JAWS-GFP-mMfng (LT) cells in C57BL/6 recipient mice exhibited similar histopathology on day 7 [Figure 4-14(b)-(d)]. These findings suggest that H-2 compatible JAWS II cells are not isogeneic to

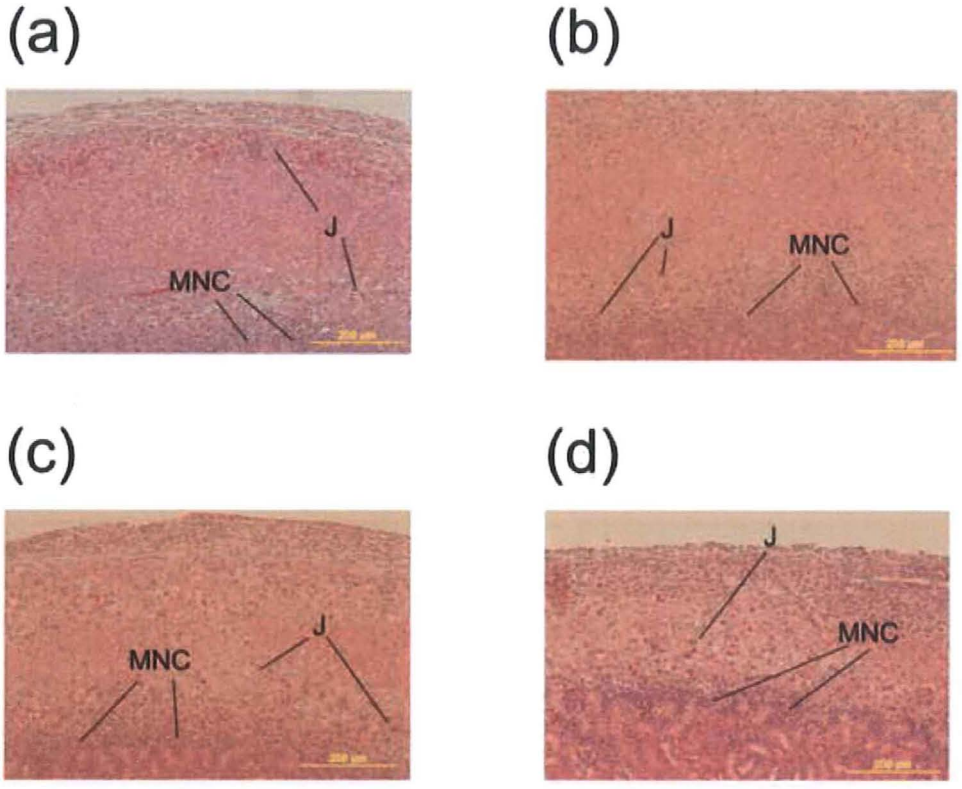


Figure 4-13: Histological appearance of representative cellular transplants of JAWS II (H-2^b) cells in MHC-disparate allogeneic recipients at 7 days post-transplant

1×10^7 (a) untransduced JAWS, (b) JAWS-GFP (LT), (c) JAWS-GFP-Lfng (LT) and (d) JAWS-GFP-Mfng (LT) cells were transplanted in recipient strain blood clots beneath the kidney capsule of CBA/H mice. (J) depicts JSWS II cells and (MNC) depicts mononuclear cell infiltrates. Grafts were harvested at 7 days post-transplant and graft sections as described in Section 2.4.4. Hematoxylin and eosin, ×20.

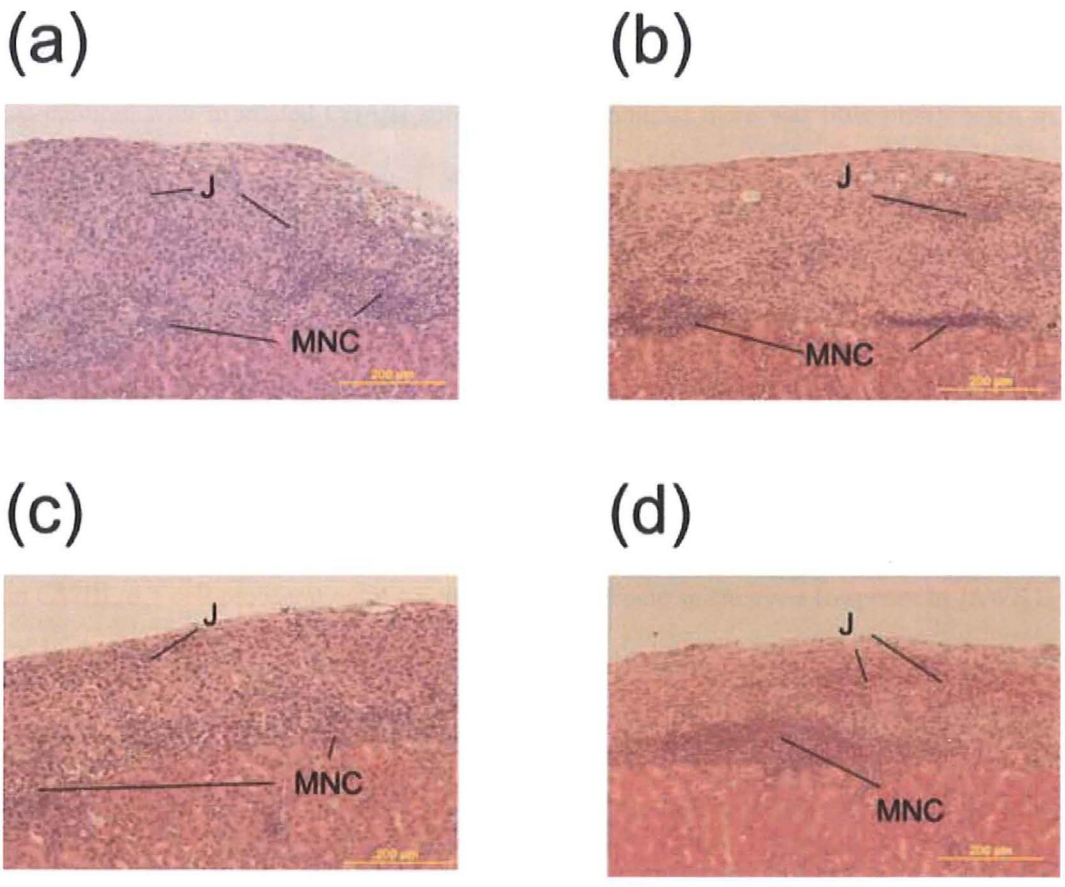


Figure 4-14: Histological appearance of representative JAWS II (H-2^b) cellular transplants in MHC-compatible recipient mice at 7 days post-transplant

1×10^7 (a) untransduced JAWS (H-2^b), (b) JAWS-GFP (LT), (c) JAWS-GFP-Lfng (LT) and (d) JAWS-GFP-Mfng (LT) cells were transplanted beneath the kidney capsule of C57BL/6 (H-2^b) mice in recipient blood clot. (T) depicts intact thyroid follicles, (S) depicts scarred tissues, and (MNC) depicts mononuclear cell infiltrates. Grafts were harvested at 7 days post-transplant as described in Section 2.4.4. Hematoxylin and eosin, ×20.

C57BL/6 cells, and probably express minor histocompatibility alloantigens.

To confirm that JAWS II cells are not isogeneic to C57BL/6 cells, MLR was performed between untransduced JAWS II cells and T cells harvested from C57BL/6 mice. As positive controls for the assay, C57BL/6 (H-2^b) T cells proliferated very strongly when they were co-cultured with irradiated CBA/H splenocytes; in contrast there was little proliferation of C57BL/6 LN cells to irradiated isogeneic C57BL/6 splenocytes (3[H] thymidine incorporation in response to CBA/H splenocytes was 12,625±1005 cpm at 1:1 stimulator/responder ratio; response to C57BL/6 splenocytes was 2219±403cpm; p=0.03) [Figure 4-15]. Although the magnitude of C57BL/6 T cell proliferation in response to irradiated untransduced JAWS II cells was significantly reduced compared to the response to allogeneic splenocytes, untransduced JAWS II cells were able to induce a 2.2-fold increase in C57BL/6 T cell proliferation in comparison to isogeneic splenocytes (response to JAWS II cells was 4895±451cpm; p=0.01) [Figure 4-15]. This finding of proliferative response of MHC-compatible C57BL/6 T cells to JAWS cells is consistent with the rejection of JAWS II cellular grafts in C57BL/6 recipient mice [Figure 4-14]. These experiments suggest that the failure of JAWS II cells over-expressing the Notch signaling components to modulate the immune response to C57BL/6J thyroid allografts could be due to the genetic incompatibility between JAWS II cells and the grafted tissue.

4.6 Discussion

Previous studies have strongly linked DCs to the expansion of antigen-specific regulatory T cells capable of suppressing alloreactivity *in vitro* and *in vivo*, and have implicated tolerogenic DCs in inducing transplantation tolerance (reviewed in (Barratt-Boyes and Thomson, 2005; Lu and Thomson, 2002; Yamazaki et al., 2006)). However, the essential signals provided by DCs to promote transplantation tolerance facilitated by regulatory T cells

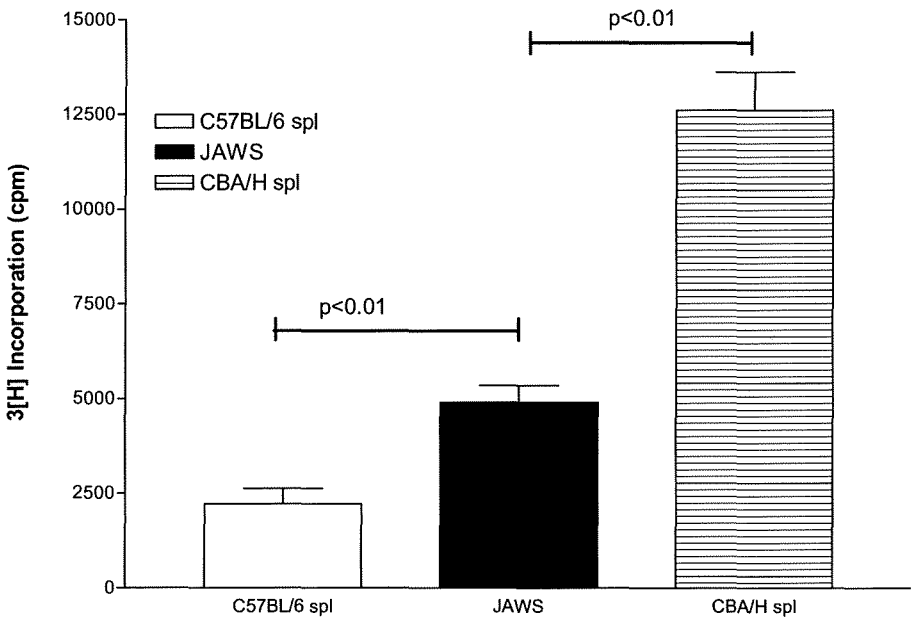


Figure 4-15: Proliferation of MHC-incompatible (CBA/H) and MHC-compatible (C57BL/6) T cells to irradiated JAWS II cells.

C57BL/6 T cell proliferation was measured in response to C57BL/6 splenocytes (blank bar), untransduced JAWS (solid bar), and CBA/H splenocytes (stripe bar) on Day 4. Mean±SEM cpm of triplet cultures are shown.

are ill-defined. In this chapter, the administration of transduced JAWS II cells (a cell line of immature DCs) over-expressing Notch ligands Jag-1 and Dll-1 or Notch modulators Lfng or Mfng, to allograft recipient mice was unable to modulate the anti-allograft rejection response and did not promote C57BL/6 thyroid allograft survival. Allografts harvested from mice treated with JAWS II cells over-expressing Notch related genes exhibited signs of acute allograft rejection including extensive mononuclear cell infiltration into the graft, hemorrhage, and loss of intact thyroid follicles. Such grafts resembled the histological appearance of control grafts from PBS-treated mice. This finding was in contrast to a previous report that pretreatment of allograft recipient mice with L-cells over-expressing Dll-1 prolonged cardiac allograft survival (Wong et al., 2003). It was shown that this effect was due to alloantigen unresponsiveness mediated by the differentiation of $CD8^+$ regulatory T cells with reduced IFN-γ and IL-2 production and enhanced IL-10 production (Wong et al., 2003). Despite the Dll-$1^{+/+}$L cells and JAWS-GFP-mDll-1 cells sharing a similar MHC Class II negative and Dll-$1^{+/+}$ phenotype, no regulatory function of JAWS-GFP-mDll-1 cells in allograft recipients was observed. Acute rejection of JAWS cellular transplants in allogeneic recipient mice indicate a limited survival of JAWS II cells following systemic administration (Figure 4-13). Although limited survival of JAWS II cells over-expressing Notch related molecules may contributed to the failure to promote thyroid allograft protection, no difference in the kinetics of rejection between cellular allografts of untransduced and retrovirally transduced-JAWS II cells indicate over-expression of Notch related molecules did not alter the immunogenicity of transduced JAWS II cells *in vivo*. This observation was consistent with a lack of immunomodulation by JAWS II cells over-expressing Notch related molecules *in vitro* described in Section 3.8. In addition, chronic rejection of cellular transplant of JAWS II ($H\text{-}2^b$) to H-2 compatible C57BL/6 ($H\text{-}2^b$) mice indicate a minor (non-MHC) histocompatibility disparity between C57BL/6 and JAWS II cells (as shown in Figure 4-14). Proliferation of C57BL/6 T cells in response to JAWS II cells confirms this

finding (Figure 4-15). It has been shown that minor histocompatibility differences can also elicit allograft rejections (Simeonovic et al., 1985; Suzuki et al., 1988). Simeonovic et al. demonstrated that BALB/c (H-2^d) pancreatic allografts were acutely rejected in DBA/2 (H-2^d) recipient mice due to histoincompatible minor (non-MHC) antigens (Simeonovic et al., 1985). It is possible that the disparity between minor histocompatibility of JAWS II cells and C57BL/6 mice was generated during the transformation of JAWS II cells from C57BL/6 mice.

The unchanged kinetics of thyroid allograft rejection following the treatment of recipients with single injection of HT virus transduced-JAWS II cells (which demonstrated more stable expression of the gene-of-interest than LT-transduced JAWS II cells as demonstrated in Figures 3-7 in Section 3.5) could not be attributed to the loss of the gene of interest during the tolerizing period in recipient mice. Similarly, it was demonstrated that the administration of different doses of JAWS II cells over-expressing Notch related molecules did not improve allograft survival. This finding suggested that it was unlikely that the lack of allograft protection was due to the administration of insufficient numbers of JAWS II cells.

It has been previously shown that variation in the strength of Notch signaling can result in different outcomes for cell differentiation (Izon et al., 2001). During the process of thymocyte development, it was shown that double positive thymocytes which received strong Notch signaling were less likely to become CD4 single positive T cells than those which received weak signals (Izon et al., 2001). This study demonstrates the importance of the magnitude of Notch signaling in determining the final phenotype following differentiation. As demonstrated in Section 3.6, LT virus-transduced JAWS II cells over-expressed Jag-1 and Lfng 1.5- to 2-fold stronger than JAWS II cells transduced with HT retrovirus. Although there was a difference in the stability of gene incorporation between LT

and HT retrovirus transduced JAWS II cells, the strength of the signal received by responsive recipient T cells is unknown. Indeed weak signaling by transduced JAWS II cells may account for their failure to induce allograft tolerance.

In order to confirm that JAWS II cells over-expressing Notch related molecules have different functional properties from tolerogenic APCS previously demonstrated, it would have been useful to assess the cytokine production profiles of T cells which interacted with the JAWS II cells population. It has been shown that interaction between Dll-1^{+}L-cell and T cells promoted differentiation of CD8^{+} regulatory T cells whose IFN-γ and IL-2 production were significantly reduced while IL-10 production was enhanced (Wong et al., 2003). Similarly, Notch signaling-dependent regulatory T cell differentiation results in decreased production of proinflammatory cytokines, (such as IL-2, IL-5, and INF-γ) and enhanced production of immunomodulatory cytokines (such as TGF-β and IL-10) (Anastasi et al., 2003; Yvon et al., 2003).

The similar kinetics of allograft rejection in recipients treated with PBS and JAWS constructs raised the question of whether effective Notch signaling between transduced JAWS II cells and recipient T cells occurred *in vivo*. It was suggested that the Notch signaling can only occur in *trans*-manner, where responding T cells express only Notch receptors with no ligand expression [Dr. Gerard Hoyne, JCSMR, personal communication; manuscript submitted]. Otherwise, if the T cells constitutively express the Notch ligands together with Notch receptors, *cis*-inhibition of the Notch signaling can occur to cancel Notch signal transduction within the T cells. Construction of allogeneic T cells containing a reporter gene within the promoter region of Notch target gene, such as HES-1 promoter gene, and the examination of reporter gene expression upon co-culture with JAWS II cells over-expressing Notch related molecules may be required to establish an optimal

Notch-receptor (on T cells)/ligand (on JAWS cell) interaction in this experimental model.

Since the JAWS II immature DC cell line does not represent a naturally occurring DC population, experimental models employing primary DCs, such as BMDC or *ex vivo* splenic DCs, are alternative models to the JAWS II cell line for assessing the effects of modulating Notch signaling. Use of primary DCs would ensure that donor transplant tissue and DCs were isogeneic. However, preliminary studies showed that primary bone marrow-derived immature DCs (immature BMDCs) were less efficiently transduced with pKMV retrovirus resulting in low yields of transduced cells over-expressing Notch components for *in vivo* experimentation (Section 3.5 in Chapter 3).

Recent developments in DC research have indicated that the tolerogenic properties of monocyte-derived DCs are still not conclusive (Barratt-Boyes and Thomson, 2005; Quah and O'Neill, 2005; Randolph et al., 1998). Ip *et al.* have shown that immature monocyte-derived DCs which internalized apoptotic cells do not mature and instead, produce a significantly reduced level of IL-12 in response to LPS stimulation *in vitro* (Ip and Lau, 2004). It was also shown that monocyte-derived DCs promoted IL-10-producing regulatory T cells when primed with two species of lactobacilli *in vitro*, suggesting potential use of monocyte-derived DCs for tolerance induction. However, separate studies showing that monocyte-derived DCs develop under inflammatory conditions (Quah and O'Neill, 2005; Randolph et al., 1998) argue against a dedicated tolerogenic role for immature DCs. The results presented in this chapter suggest that over-expression of Notch related molecules did not drive immature DCs to acquire tolerogenic properties. A number of recent studies have demonstrated the capacity of plasmacytoid DCs or myeloid DCs to induce tolerance by promoting regulatory T cell differentiation (Barratt-Boyes and Thomson, 2005; Jonuleit et al., 2000; Kuwana, 2002; Levings et al., 2005; Steinbrink et al., 1997). It is speculated here that

in vivo use of allogeneic myeloid or plasmacytoid DCs over-expressing Notch-related molecules may favor the efficient induction of allograft tolerance.

This chapter demonstrated that the administration of JAWS II cells over-expressing Notch related molecules, mJag-1, mLfng, mMfng, and mDll-1 to transplant recipient mice does not promote allograft survival. It was shown that neither LT nor HT retrovirus-transduced JAWS II cells nor increased numbers of transduced JAWS III cells significantly altered the kinetics of allograft rejection. Cellular transplantation of JAWS II cells and in *vitro* MLR studies showed that JAWS II cells were not isogeneic to C57BL/6 mice, thereby identifying minor histocompatibility differences between JAWS II cells and the donor C57BL/6 thyroid tissues. This finding indicated that the JAWS II cells may not represent a suitable cell population for testing the capacity of immature DCs presenting donor-antigen and over-expressing Notch related molecules, to induce allograft tolerance.

Results described in this chapter indicated that the Notch signaling pathway did not modulate the allogeneic immune response, however, accumulating evidence suggests that another signaling pathway, the NF-κB signaling pathway, can to regulate DC function and their acquisition of a tolerogenic phenotype (Martin et al., 2003; Nouri-Shirazi and Guinet, 2002; Tan et al., 2005). In chapter 5, the potential for the NF-κB signaling pathway to induce allograft tolerance was investigated.

Chapter 5

Role of NF-κB signaling in stimulating allogeneic responses *in vitro*

5.1 Introduction

NF-κB is a transcription family consisting of 5 members, p50, p52, RelA, RelB and cRel (Liou and Hsia, 2003; Pahl, 1999). These proteins are expressed in virtually all cell types, and participate in the regulation of over 200 genes (Pahl, 1999; Xu et al., 2003). A number of studies have demonstrated that defects in NF-κB signaling predisposed animals to viral, bacterial and parasitic infections indicating a critical requirement of NF-κB signaling in immune functions (Caamano and Hunter, 2002; Grigoriadis et al., 1996; Sha et al., 1995). The absolute requirement for NF-κB signaling in T cell proliferation and effector functions has been well documented (Caamano and Hunter, 2002; Ferreira et al., 1999; Gerondakis et al., 1996; Mora et al., 2001). Similarly, DC maturation and their T cell priming capacity has been reported to rely on NF-κB activation (Kopp and Medzhitov, 1999; Mintern et al., 2002; Rescigno et al., 1998; Speirs et al., 2004). Studies have shown that participation of the NF-κB pathway in such broad aspects of adaptive immunity is attributed to its involvement in regulating costimulatory molecule expression (such as CD80, CD86, and CD40), cytokine production (such as IL-1, IL-4, IL-5, IL-6, IL-8, IL-12, IL-18, INF-γ, TNF-α, and GM-CSF), chemokine production (such as MIP-1α, MCP-1, and RANTES) and adhesion molecule expression (such as VCAM-1, ICAM-1, and E-selectin) (Gerondakis et al., 1996; Ghosh et al., 1998; Grigoriadis et al., 1996; Mason et al., 2002; Pahl, 1999). Modulation of the NF-κB pathway therefore represents a potential approach for promoting allograft protection.

Recent investigations have revealed that regulating NF-κB signaling is also an important step for regulatory T cell development. Bettelli *et al.* demonstrated that Foxp3-mediated inhibition of IL-2, IL-4 and IFN-γ production in Th cells is mediated by a direct interaction of Foxp3 with an NF-κB protein (Bettelli et al., 2005). This study showed that the Foxp3 physically interacted with an NF-κB protein and inhibited transcriptional activities of NF-κB thereby preventing inflammatory cytokine production (Bettelli et al., 2005). Since Foxp3 is

associated with regulatory T cell differentiation (Fontenot et al., 2003; Hori et al., 2003), it may be necessary to inhibit the NF-κB pathway in order to induce regulatory T cell differentiation. Recently, NF-κB inhibition has also been shown to be useful in generating tolerogenic DCs. Martin *et al.* reported that DCs lacking RelB activity did not express CD40 upon antigen-stimulation and these DCs were able to inhibit T cell proliferation in an antigen-specific manner (Martin et al., 2003). They showed that this antigen-specific tolerance was due to the induction of IL-10-producing regulatory T cells by DCs lacking RelB function (Martin et al., 2003).

Technologies to allow manipulation of the NF-κB pathway in DCs have provided some promising outcomes for allotransplantation as well. For example, treatment of DCs with an NF-κB inhibitor, NF-κB decoy ODN, induced antigen-specific T cell unresponsiveness in several studies (Giannoukakis et al., 2000; Xu et al., 2004; Xu et al., 2004). It was shown that NF-κB decoy ODN-treatment of DCs prevented the production of inflammatory cytokines, such as IFN-γ, TNF-α, IL-10 and IL-12 (Giannoukakis et al., 2000; Xu et al., 2004). NF-κB ODN-treated DCs also lacked NO production as well as upregulation of costimulatory molecule expression upon IL-4 or LPS stimulation (Giannoukakis et al., 2000; Xu et al., 2004). These studies also demonstrated that donor type DCs treated with this inhibitor could induce alloantigen specific hyporesponsiveness *in vitro* and were capable of prolonging allograft survival when administered to recipient mice prior to transplantation (Giannoukakis et al., 2000; Xu et al., 2004; Xu et al., 2004). Similarly, inhibition of NF-κB signaling in human DCs by treatment with an NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC), also prevented DC maturation by arresting HLA and costimulatory molecule upregulation (Saemann et al., 2004). The finding that PDTC-treated DCs could induce alloantigen-specific hyporesponsiveness *in vitro* (Saemann et al., 2004), further highlighted the potential use of this NF-κB inhibitor in transplantation settings. Tomasoni *et al.*

succeeded in prolonging kidney allograft survival by treating recipients with donor type DCs, which carried a dominant negative form of IKK2 (which is important in activating the NF-κB pathway) (Tomasoni et al., 2005). This fining was attributed to the development of recipient $CD4^+$ regulatory T cells capable of suppressing naïve T cell proliferation to donor type stimulator cells (Tomasoni et al., 2005). Although these studies support the strategy of targeting NF-κB in donor-type APCs, the efficacy of NF-κB inhibition in APCs to promote donor specific tolerance is yet to be established.

BAY11-7082 (BAY) has been described as an NF-κB inhibitor which binds to IκB proteins and prevents their phosphorylation thereby inhibiting NF-κB signal transduction (Pierce et al., 1997). To assess the role of NF-κB signaling in APC function, we used BAY to block the NF-κB pathway in APCs. The aim of this chapter was to examine the effects of BAY-mediated NF-κB inhibition in the capacity of APCs to stimulate allogeneic T cells. Pentoxifylline (Ptx), a cRel inhibitor, was also used to examine whether cRel plays a role allowing APCs to stimulate allogeneic T cells. Additionally, the current study also investigated whether priming of T cells by APCs whose NF-κB activity is inhibited in this manner could promote the development of alloantigen-specific tolerogenic T cells.

5.2 Confirmation of NF-κB signaling inhibition by BAY11-7082 and pentoxifylline treatment

Previous studies have demonstrated that BAY prevents NF-κB signaling by arresting IκB phosphorylation (Pierce et al., 1997). Pentoxifylline (Ptx) is a cRel inhibitor possibly acting through the inhibition of phosphodiesterase activity (Rao et al., 2003; Wang et al., 1997). To determine the effects of these inhibitors, cRel protein levels in cytoplasmic extracts from BAY-treated C57BL/6 splenocytes and Ptx-treated EL-4 T cell line were analyzed by western blot. EL-4 T cells were used for confirming Ptx absorption and cRel inhibition as we did not

observe obvious alteration in cRel cytoplasmic expression in Ptx-treated splenocytes.

Both untreated splenocytes and EL-4 cells expressed cytoplasmic cRel [Figure 5-1 (a) and (c)]. When stimulated with LPS/cytokines (IL-4, TNF-α and IFN-γ), both splenocytes and EL-4 cells exhibited increased cytoplasmic expression of cRel at 4 hours and 48 hours post-activation [Figure 5-1 (a)-(d)]. Culture with BAY did not affect basal expression of cytoplasmic cRel in splenocytes whereas Ptx treatment decreased cytoplasmic cRel expression in EL-4 Cells [Figure 5-1 (a) and (c)]. This confirmed Ptx-mediated inhibition of cRel. Inhibition of the NF-κB pathway by BAY was also confirmed following splenocyte activation. After the addition of LPS/cytokines to BAY splenocytes, no induction of cRel was observed in the cytoplasmic extract at 4 hours post-activation while control splenocytes treated with LPS/cytokines exhibited enhanced expression of cRel [Figure 5-1 (a) and (b)]. Interestingly, upregulation of cytoplasmic cRel expression was observed at 48 hours post-activation in BAY splenocytes [Figure 5-1 (a) and (b)]. Ptx treatment of EL-4 cells also inhibited the induction of cRel in the cytoplasm at 4 hours post-activation, but like BAY splenocytes, cRel cytoplasmic expression was strongly enhanced at 48 hour post-activation in Ptx-treated EL-4 cells [Figure 5-1 (c) and (d)]. Collectively, the data demonstrated that BAY treatment and Ptx treatment both reduces cytoplasmic cRel expression early stages after treatment, however, the inhibitory effects diminish by 48 hours post-treatment.

5.3 Inhibition of immunostimulatory property of splenocytes with BAY treatment

NF-κB signaling has been shown to play an important role in APC maturation and in the ability of APCs to prime alloreactive T cells (Mintern et al., 2002; Morelli et al., 2000). Lack of NF-κB signaling in APCs is known to render these cells incapable of stimulating T cells effectively (Mintern et al., 2002; Morelli et al., 2000). Based on these reports, the capacity of

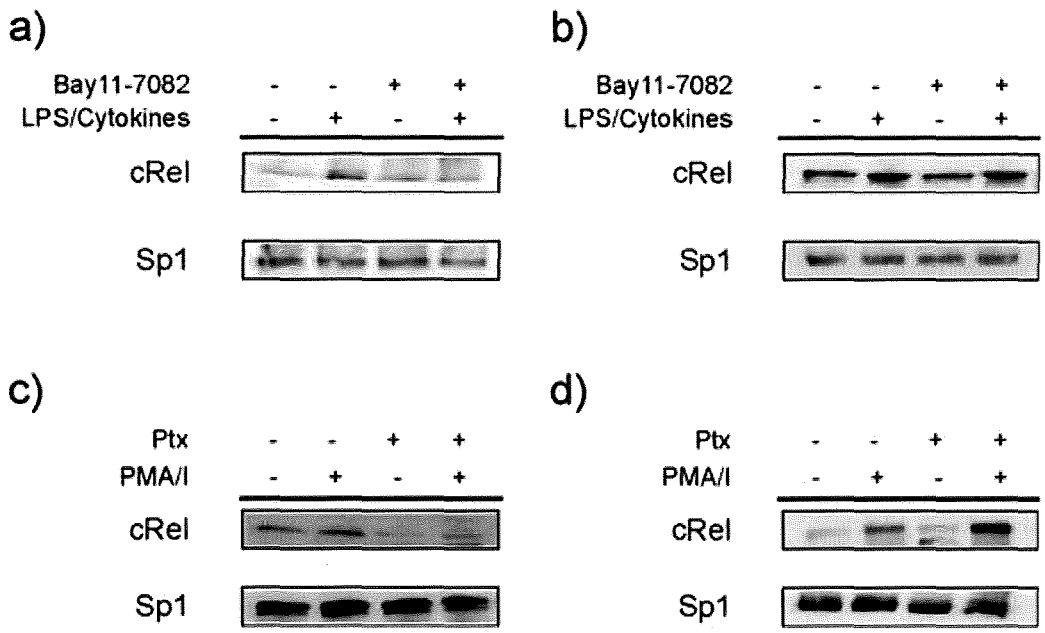


Figure 5-1: Western blot analyses of cRel cytoplasmic expression following Bay11-7082 and pentoxifylline treatment.

cRel cytoplasmic expression was examined in cytoplasmic extracts prepared from 10μM Bay splenocytes with or without LPS/cytokine stimulation [LPS (100ng/ml), IFN-γ (10ng/ml), TNF-α (10ng/ml), and L-4 (10ng/ml)] by Western blot analysis. (a) shows cRel cytoplasmic expression of Bay splenocytes at 4 hours post-LPS/cytokine activation and (b) at 48 hours post-LPS/cytokine activation. cRel levels were also analyzed in 6mg/ml Ptx treated EL-4 T cells with or without PMA/I activation. (c) shows cRel cytoplasmic expression of Ptx EL-4 cells at 4 hours post-PMA/I stimulation and (d) 48 hours PMA/I stimulation [PMA (20ng/ml) /calcium ionophore (1μM)].

BAY splenocytes (C57BL/6) to prime allogeneic T cells (CBA/H) was examined by MLRs.

Firstly, the optimal dosage of the inhibitor was investigated by titrating the dose of BAY on C57BL/6 splenocytes and coculturing the treated cells with CBA/H T cells in MLRs. Untreated C57BL/6 splenocytes were able to prime CBA/H T cells effectively (3[H] incorporation of CBA/H T cells in response to C57BL/6 splenocytes was 26111±771cpm at 1:1 stimulator/responder ratio) [Figure 2 (a)]. In contrast, treatment of splenocytes with 2.5μM BAY significantly reduced alloreactive T cell proliferation compared to that with untreated splenocytes (3[H] incorporation in response to 2.5μM BAY splenocytes was 5558±1286cpm; $p<0.01$) [Figure 2 (a)]. Suppression of responder T cell proliferation by BAY was dose-dependent and 10μM BAY completely abrogated the capacity of splenocytes to prime alloreactive T cells (3[H] incorporation in response to 5μM BAY splenocytes was 2508±86cpm; the response to 10μM BAY –treated splenocytes was 1557±28cpm) [Figure 2 (a) and (b)].

It could be speculated the lack of T cell priming was due to the inhibition of costimulatory molecule expression on BAY splenocytes as costimulatory molecules including CD40, CD80, CD86 and MHC molecules have been reported to be under NF-κB transcriptional control (Kopp and Medzhitov, 1999; Mintern et al., 2002; Speirs et al., 2004). Flow cytometry with a gate on H-2^b-positive cells was used to analyze costimulatory and MHC molecule expression on splenocytes treated with 10μM BAY harvested on day 3 of MLR. Untreated splenocytes and BAY splenocytes expressed comparable levels of CD40, CD80 and CD69 expression [Figure 5-3]. BAY splenocytes, however, showed approximately 2- and 6-fold reductions in CD86 and MHC Class II expression respectively in comparison to untreated splenocytes [Figure 5-3]. These results indicated that the capacity of BAY to inhibit priming by splenocytes correlated with impaired upregulation in the expression of CD86 and MHC

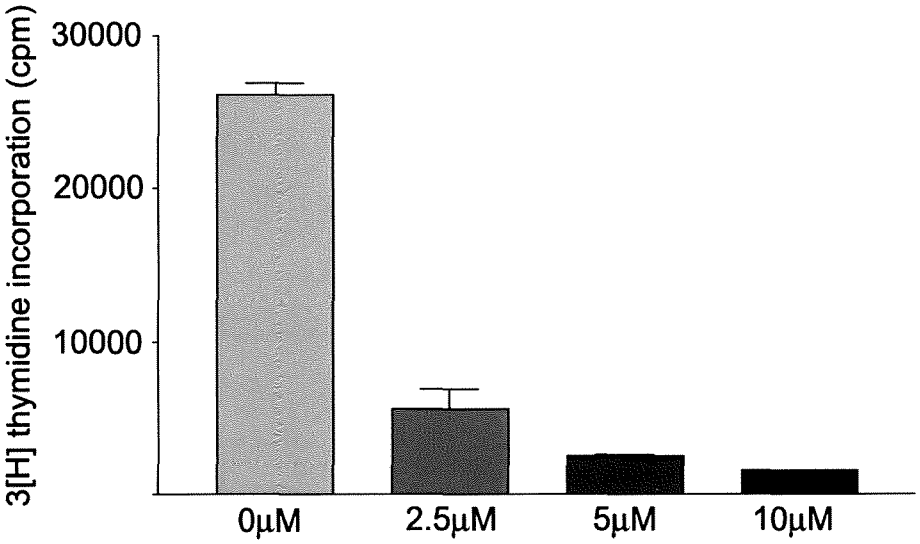


Figure 5-2: Titration of Bay11-7082 on splenocytes used as stimulators in MLR

C57BL/6 splenocytes were treated with 0-10μM concentrations of Bay11-7082 and their immunostimulatory capacity was examined. Bay splenocytes were mixed with CBA/H T cells and 3[H] incorporation was measured (cpm). Representative 3[H] thymidine incorporation (at 1:1 stimulator/responder ratio) on Day 4 is shown.

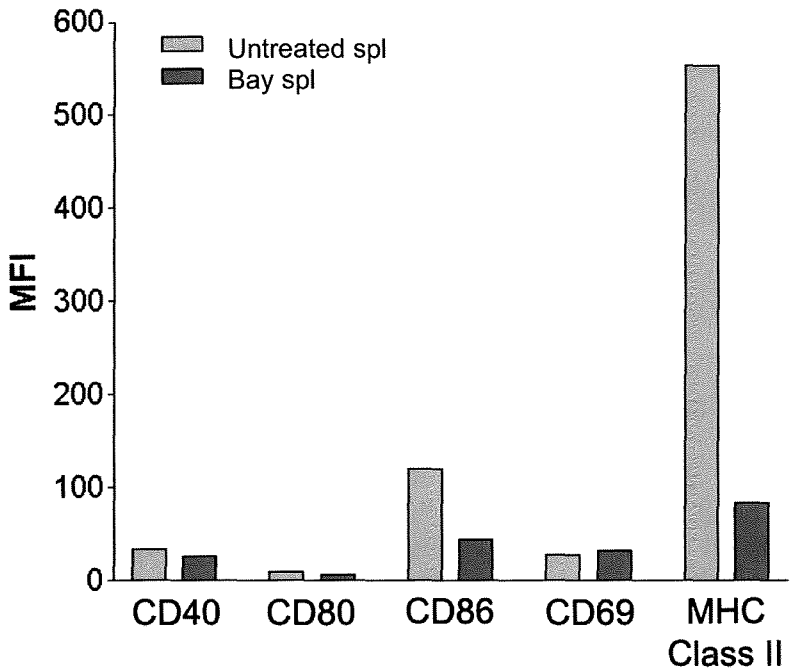


Figure 5-3: Phenotypic analysis of splenocytes treated with Bay11-7082

Control splenocytes and Bay splenocytes were recovered from Day 3 MLR culture. Expression of activation markers (CD40, CD80, CD86, CD69 and MHC Class II) on these populations was analyzed by 4 color flow cytometry. Experiments were repeated 3 times and representative phenotypes are shown.

Class II molecules.

5.4 Effects of BAY treatment in cytokine production

NF-κB signaling is known to be involved in the regulation of cytokine production such as IL-3, IFN-γ, TNF-α and IL-4 (Finn et al., 2001; Gerondakis et al., 1996; Ghosh and Karin, 2002; Mason et al., 2002) and has been suggested to regulate polarization of Th1/Th2 immune responses (Hilliard et al., 2002; Lederer et al., 1996). It was speculated that the observed inhibition of T cell priming by BAY splenocytes could also be due to an altered cytokine microenvironment in culture. We therefore examined the levels of Th1-type cytokines (TNF-α, IFN-γ and IL-2) and Th2-type cytokines (IL-4 and IL-5) present in the MLR culture supernatant on day 4.

Culture supernatants obtained from T cells alone, untreated splenocytes alone and BAY splenocytes alone contained TNF-α (culture with T cells alone contained 12.7pg/ml; untreated splenocytes alone contained 7.9pg/ml; BAY splenocytes alone contained 0.00pg/ml), IFN-γ (culture with T cells alone contained 1.4pg/ml; untreated splenocytes alone contained 0.0pg/ml; BAY splenocytes alone contained 0.00pg/ml), IL-2 (culture with T cells alone contained 8.9pg/ml; untreated splenocytes alone contained 3.4pg/ml; BAY splenocytes alone contained 0.00pg/ml) or IL-4 (culture with T cells alone contained 0.00pg/ml; untreated splenocytes alone contained 0.00pg/ml; BAY splenocytes alone contained 0.00pg/ml) [Figure 5-4]. Basal level of IL-5 was detected in culture supernatant obtained from T cells alone (7.2pg/ml IL-5) and BAY splenocytes alone (7.1pg/ml IL-5) [Figure 5-4]. No IL-5 was detected in the medium containing untreated splenocytes alone [Figure 5-4]. When untreated splenocytes were co-cultured with allogeneic T cells, between 17- and 2500-fold increase in cytokine levels was observed in TNF-α (86.0-fold increase), IFN-γ (2463.1-fold increase), IL-2 (173.0-fold increase) and IL-4 (16.9-fold increase)

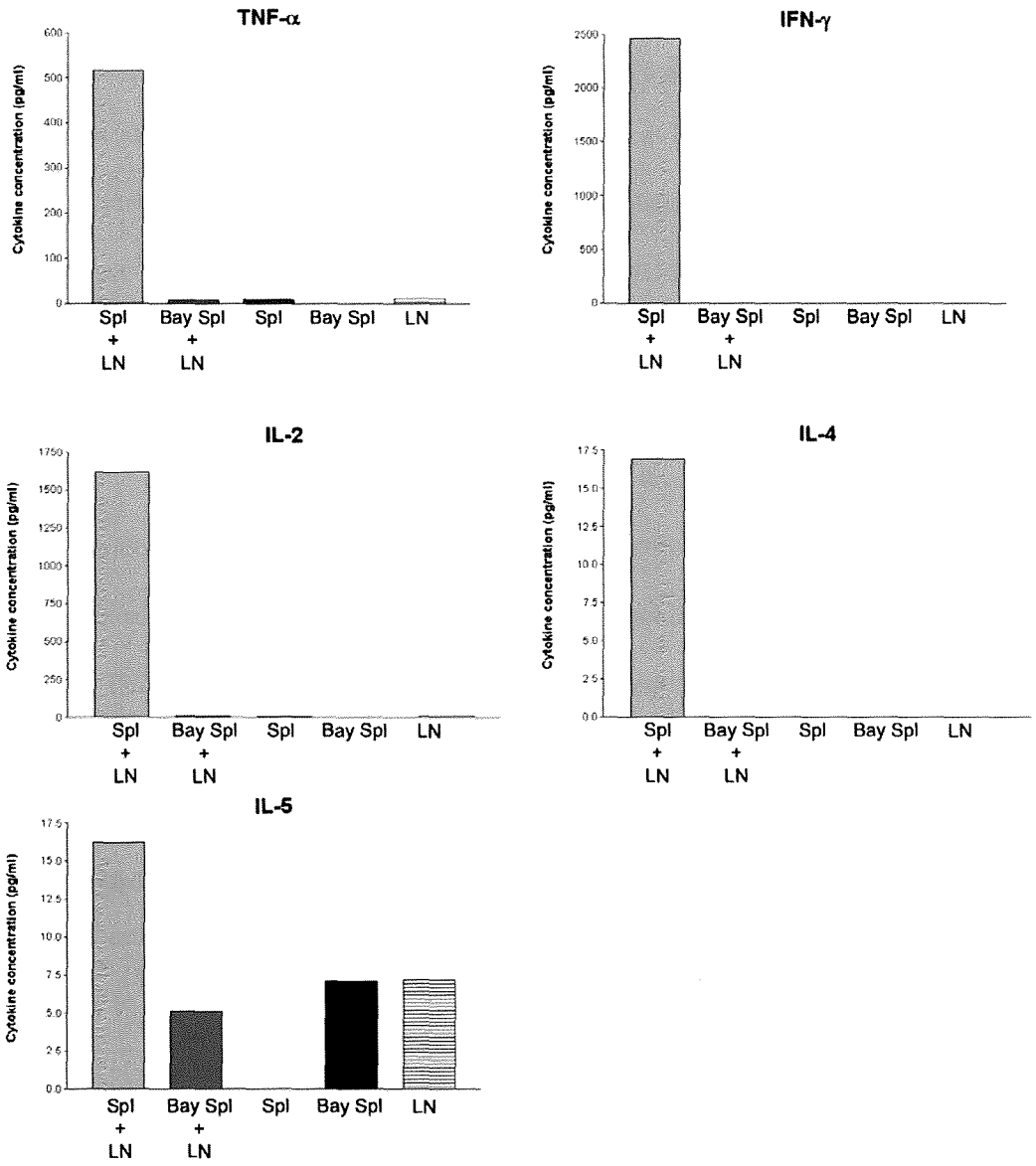


Figure 5-4: Levels of cytokines present in the culture supernatant in MLR using untreated splenocytes and Bay-splenocytes

IFN-γ, TNF-α, IL-2, IL-4 and IL-5 were measured in supernatants from untreated 10μM Bay treated C57BL/6 splenocytes cultured with or without CBA/H LN T cells or in supernatant from CBA/H LN T cell culture without splenocytes. Cytokine levels were measured using Mouse Th1/Th2 Cytokine Cytometric Bead Array Kit (BD Biosciences Pharmingen) (data is from single experiment).

(untreated splenocytes and T cells coculture contained 515.5pg/ml TNF-α; 2463.1pg/ml IFN-γ; 1618.4pg/ml IL-2; 16.9pg/ml IL-4) in comparison with cytokine concentration in supernatants of culture without T cells. Approximately a 2-fold increase in the quantity of IL-5 was also obtained in the untreated splenocyte and T cell co-culture compared with controls (untreated splenocytes and T cell coculture contained 16.2pg/ml IL-5) [Figure 5-4]. In contrast, no IFN-γ and IL-4 and little TNF-α and IL-2 were found in the supernatant obtained from BAY splenocyte and T cell co-culture (BAY splenocyte and T cell coculture contained 6pg/ml TNF-α; 0.00pg/ml IFN-γ, 9.3pg/ml IL-2; 0.00pg/ml IL-4); the quantity of IL-5 in the culture supernatant remained at the basal level (5.1pg/ml IL-5) [Figure 5.4]. These results indicated that the lack of immunostimulatory capacity of splenocytes treated with BAY correlated with inhibition of inflammatory cytokine production. Inhibition of both Th1 and Th2 cytokines also suggested that BAY treatment does not result in polarization to Th1 or Th2 cytokine production.

5.5 Effects of BAY treatment on splenocyte proliferation

It is well established that an inhibition of NF-κB in T cells leads to T cell proliferation arrest (Boothby et al., 1997; Ferreira et al., 1999; Mora et al., 2001; Ruland et al., 2001), however, it is not clear whether lack of NF-κB signaling affects APC proliferation. We therefore examined the effect of BAY treatment on splenocyte proliferation when co-cultured with allogeneic T cells by performing MLRs with or without splenocyte irradiation and comparing the overall 3[H] thymidine incorporation.

As expected, T cells proliferated strongly when mixed with irradiated untreated splenocytes, and T cell proliferation was not observed when T cells were co-cultured with irradiated BAY splenocytes (3[H] thymidine incorporation in response to irradiated untreated splenocytes was 12960±615cpm; in response to irradiated BAY splenocytes was 2390±50cpm; $p<0.01$)

[Figure 5-5 (a)]. T cell proliferation was also inhibited when T cells were treated with 10μM BAY, and cultured with irradiated splenocytes without BAY treatment (3[H] thymidine incorporation in response to irradiated untreated splenocytes was 386±44cpm)[Figure 5-5 (a)]. This observation was consistent with previous reports showing the lack of T cell proliferation in NF-κB deficient mice (Boothby et al., 1997; Ferreira et al., 1999; Mora et al., 2001; Ruland et al., 2001). When both splenocytes and T cells were treated with BAY, no 3[H] thymidine incorporation was observed (3[H] thymidine incorporation was 79±4cpm) [Figure 5-5 (a)]. In a parallel experiment, MLRs were performed without irradiating splenocytes. Unstimulated splenocytes without irradiation were able to stimulate T cells and there was up to 1.5-fold increase in the 3[H] thymidine incorporation compared with MLR using irradiated counterpart (3[H] thymidine incorporation in response to non-irradiated untreated splenocytes was 22748±367cpm; $p<0.01$)[Figure 5-5 (a) and (b)]. The increase in 3[H] thymidine incorporation was considered to be due to both splenocyte proliferation and T cell proliferation. In contrast, 3[H] thymidine incorporation in non-irradiated BAY splenocytes and T cell culture was low (3[H] thymidine incorporation in response to non-irradiated BAY splenocytes was 3170±208cpm) [Figure 5-5 (b)]. These results indicated that treatment with BAY leads to arrested splenocyte proliferation and a loss in T cell priming capability. MLRs using BAY treated T cells, and MLRs using splenocytes and T cells both treated with BAY showed no signs of proliferation (3[H] thymidine incorporation of BAY T cells in response to non-irradiated splenocytes was 468±150cpm; BAY T cell proliferation in response to non-irradiated BAY splenocytes was 179±13cpm)[Figure 5-5 (b)]. Collectively, these data showed that BAY treatment not only render splenocytes incapable of priming T cells but also the inhibited splenocytes proliferation. In addition, the lack of 3[H] thymidine incorporation in the culture with non-irradiated splenocytes and BAY treated T cells may indicate that splenocytes require positive feedback from responding T cells in order to proliferate. It is possible that BAY treatment disrupts splenocyte-T cell interactions.

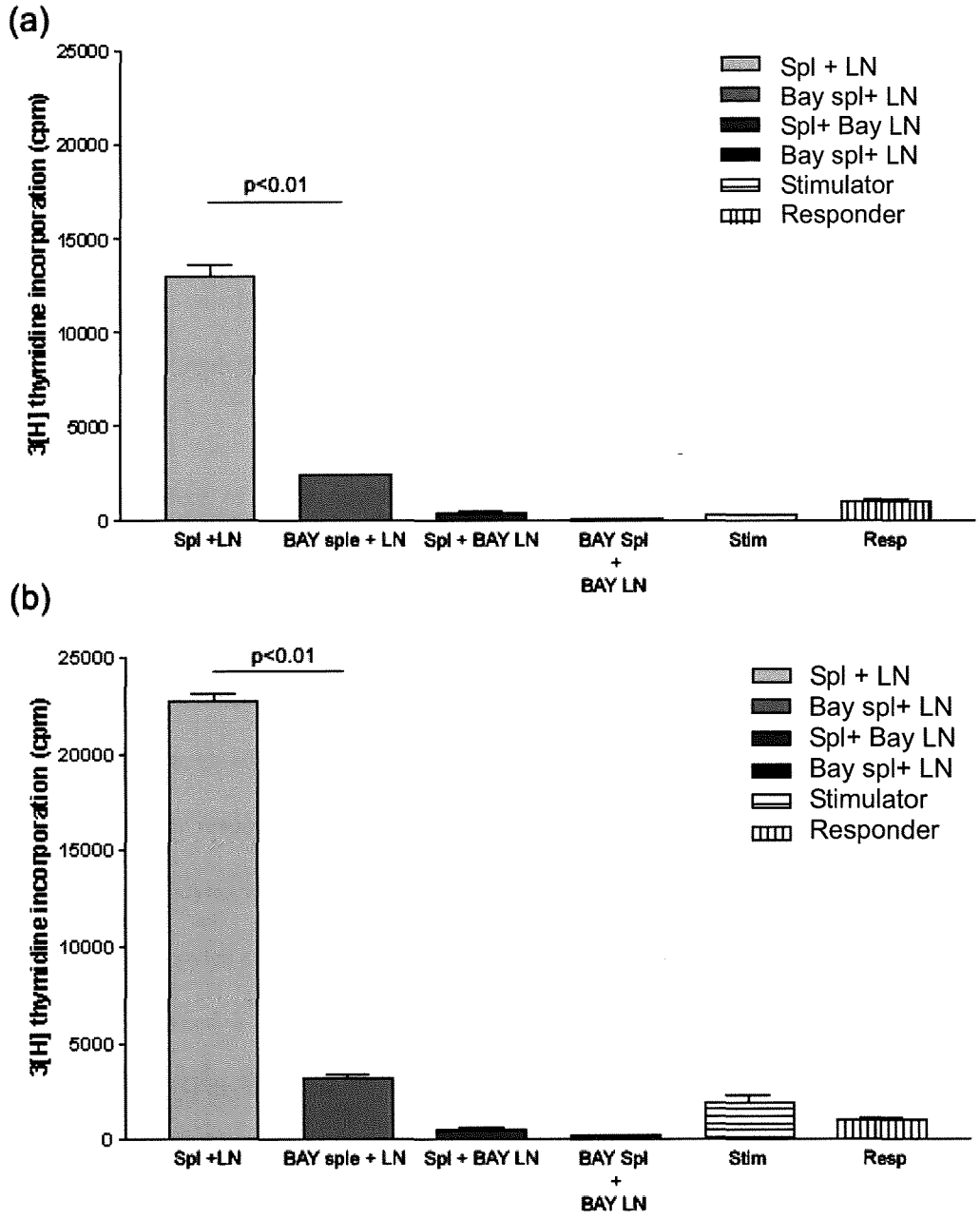


Figure 5-5: Effects of Bay treatment on splenocyte proliferation

Proliferation of splenocytes treated with 10μM Bay and untreated C57BL/6 splenocytes in response to allogeneic CBA/H LN T cells were measured. MLRs using combinations of splenocytes and LN cells with/without Bay treatment were carried out (a) with splenocyte irradiation or (b) without splenocyte irradiation. Representative 3[H] thymidine incorporation (at 1:4 stimulator/responder ratio) on Day 4 is shown.

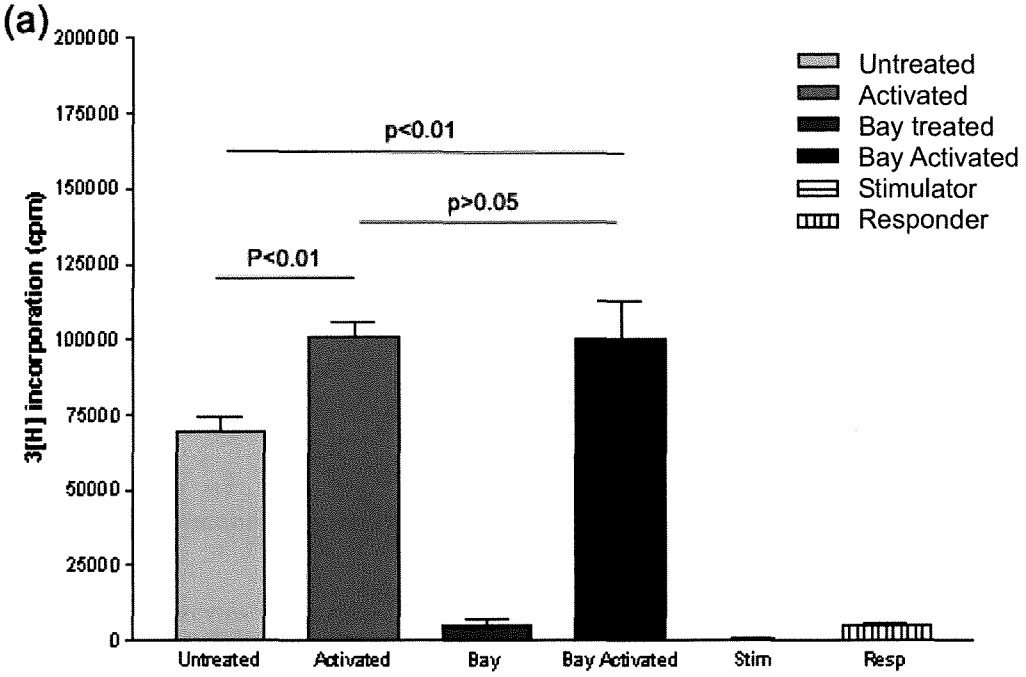
5.6 Effects of BAY in splenocytes at different stages of activation

Previous sections demonstrated that BAY treatment can inhibit naïve splenocytes from priming allogeneic T cells. To further dissect the effects of BAY treatment, we examined whether pretreatment with BAY can inhibit priming by previously LPS/cytokine activated splenocytes or whether BAY treatment can prevent splenocytes from activation upon LPS/cytokine stimulation.

Firstly, the effect of BAY treatment on already activated splenocytes was examined. Splenocytes which were activated with LPS/cytokines then treated with BAY (BAY Act splenocytes) were used in MLRs and their immunostimulatory capacity was investigated. Naïve splenocytes were able to prime allogeneic T cells and stimulation with LPS/cytokines significantly increased the capacity of splenocytes to prime T cells (3[H] thymidine incorporation in response to untreated splenocytes was 69286±5152cpm; in response to activated splenocytes was 100703±4842.000; $p<0.01$) [Figure 5-6 (a)]. While BAY-treatment on naïve splenocytes completely inhibited the proliferation of responding T cells, BAY Act splenocytes primed T cells significantly more effectively than both untreated and BAY splenocytes (3[H] thymidine incorporation in response to BAY splenocytes was 4920±2092; in response to BAY Act splenocytes was 99824±12910cpm) ($p<0.01$)[Figure 5-6 (a)]. The magnitude of T cell proliferation in response to BAY Act splenocytes was comparable to that observed in response to activated splenocytes without BAY treatment ($p>0.05$) [Figure 5-6 (a)]. This study showed that BAY treatment did not inhibit the immunostimulatory property of already-activated splenocytes.

Whether BAY-treatment can prevent activation of naïve splenocytes was examined by using

(a)


200000
175000
150000
125000
100000
75000
50000
25000
0
3[H] incorporation (cpm)
p<0.01
p>0.05
P<0.01
Untreated
Activated
Bay treated
Bay Activated
Stimulator
Responder
Untreated
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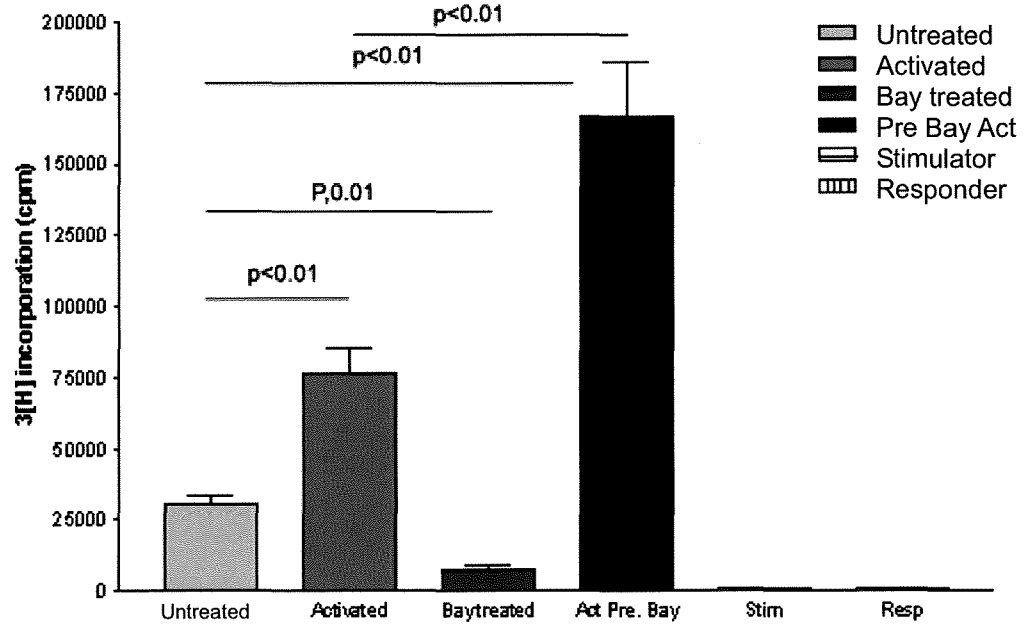
(b)


200000
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0
3[H] incorporation (cpm)
p<0.01
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Untreated
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Bay treated
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Stimulator
Responder
Untreated
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Figure 5-6: Immunostimulatory capacity of splenocytes treated with BAY pre- or post-activation.

C57BL/6 Splenocytes were treated with 10μM Bay pre- or post-LPS/cytokine activation and their immunostimulatory capacities were examined. (a) shows CBA/H LN T cell proliferation in response to untreated splenocytes, Activated splenocytes, Bay splenocytes, and Bay Act splenocytes. (b) shows CBA/H LN T cell proliferation in response to splenocytes, Activated splenocytes, Bay splenocytes and Pre-Bay Act splenocytes. Representative 3[H] thymidine incorporation on Day 4 is shown (1:32 stimulator/responder ratio).

splenocytes which were first pretreated with BAY then stimulated with LPS/cytokines (Pre-BAY Act splenocytes). It was found that BAY treatment did not prevent the activation of naïve splenocytes. Pre-BAY Act splenocytes induced allogeneic T cell proliferation approximately 5-fold more potently as compared with naïve unstimulated splenocytes (3[H] incorporation in response to naïve splenocytes was 30679±2652cpm; in response to Pre-BAY Act splenocytes was 167096±18813cpm; $p<0.01$) [Figure 5-6 (b)]. Interestingly, we also observed a 2.2-fold increase in T cell proliferation in response to Pre-BAY Act splenocytes compared to activated splenocytes without BAY treatment (3[H] incorporation in response to activated splenocytes was 76416±8929cpm; $p<0.01$) [Figure 5-6 (b)]. These data indicate that pretreatment with BAY may condition splenocytes to mount more potent T cell proliferation when a subsequent activation stimulus is received.

5.7 Effects of Pentoxifylline treatment on splenocytes

Section 5.3 described that general inhibition of NF-κB signaling by BAY disables splenocytes from priming alloreactive T cells. It is known that cRel is important for the production of proinflammatory cytokines such as IFN-γ, TNF-α, IL-2 and IL-12 (Boffa et al., 2003; Rao et al., 2003). These studies suggested that cRel could play an integral role in the immunostimulatory function. Ptx is a phosphodiesterase inhibitor that inhibits c-Rel dependent NF-κB signaling (Brettingham-Moore et al., 2005). To investigate whether a lack of cRel activity in splenocytes compromised their capacity to stimulate T cells, splenocytes were pretreated with Ptx and their immunostimulatory capacity was examined.

C57BL/6 splenocytes were treated with 0 - 9mg/ml concentrations of Ptx. Untreated splenocytes induced strong proliferation of allogeneic T cells (3[H] thymidine incorporation in response to untreated splenocytes was 66239±7059cpm) [Figure 5-7]. Treatment of splenocytes with Ptx resulted in no significant alterations in their capacity to prime

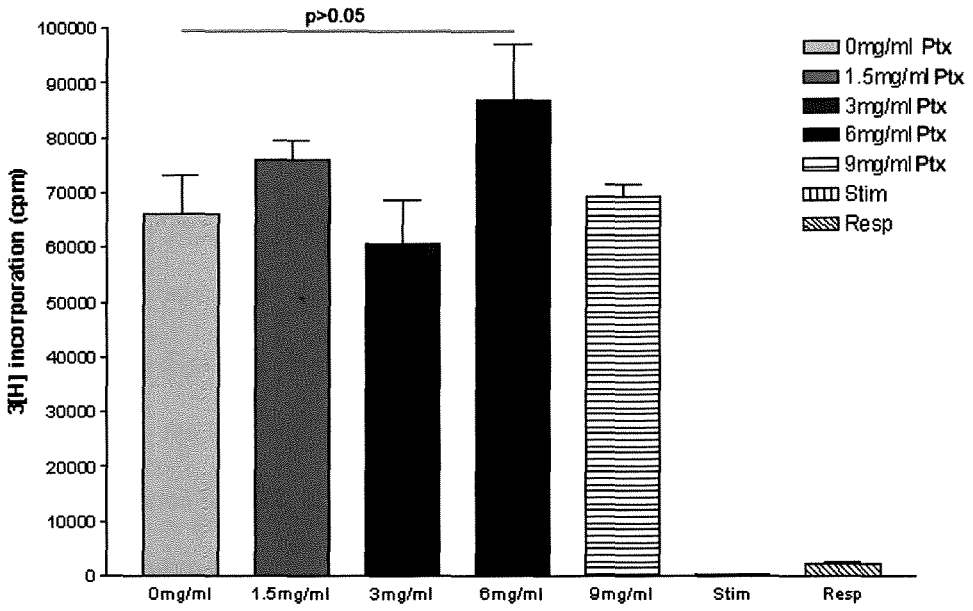


Figure 5-7: Allogeneic T cell response induced by Pentoxifylline (Ptx) treated splenocytes

C57BL/6 Splenocytes were treated with 0-9mg/ml concentrations of Ptx and their immunostimulatory capacity was examined by MLR. C57BL/6 splenocytes treated with Ptx were mixed with CBA/H LN T cells and 3[H] incorporation was measured (cpm). Representative 3[H] thymidine incorporation (at 1:1 stimulator/responder ratio) on Day 4 is shown.

alloreactive T cells, at all concentrations of Ptx tested (3[H] thymidine incorporation in response to splenocytes treated with 1.5mg/ml Ptx was 76210±3483cpm; in response to splenocytes treated with 3mg/ml Ptx was 60608±8127cpm; in response to splenocytes treated with 6mg/ml Ptx was 86922±10360cpm; in response to splenocytes treated with 9mg/ml Ptx was 69472±2197cpm; $p>0.05$) [Figure 5-7]. These results indicated that cRel inhibition was not the mechanism responsible for the inability of BAY splenocytes to stimulate T cell proliferation.

5.8 Effects of BAY on BMDCs

Among APC populations, DCs possess the most potent immunostimulatory capacity and they are crucial in T cell-mediated immunity (reviewed in (Banchereau et al., 2000)). To investigate whether the lack of T cell stimulation by BAY splenocytes was due to inhibition of antigen presentation by DCs, C57BL/6 bone marrow-derived DCs (BMDCs) were treated with 10μM BAY and co-cultured with allogeneic T cells in MLRs.

BAY-mediated inhibition of NF-κB signaling was able to reduce the capacity of BMDCs to prime T cells [Figure 5-8]. Relatively strong T cell proliferation was observed when allogeneic T cells were mixed with unstimulated BMDCs (3[H] thymidine incorporation in response to unstimulated BMDCs was 116016±1516cpm) [Figure 5-8]. The capacity of BMDCs to stimulate alloreactive T cells was significantly enhanced when BMDCs were activated with LPS/cytokine (3[H] thymidine incorporation in response to activated BMDC was 155118.±1994cpm; $p<0.01$) [Figure 5-8]. The magnitude of T cell proliferation in response to 10μM BAY BMDCs was significantly reduced in comparison with unstimulated BMDCs without BAY treatment (3[H] thymidine incorporation in response to BAY BMDCs was 85659±3337.518; $p<0.01$) [Figure 5-8]. BAY treatment of previously LPS/cytokine stimulated BMDCs (BAY Act BMDCs) did not affect the level of proliferation of responding

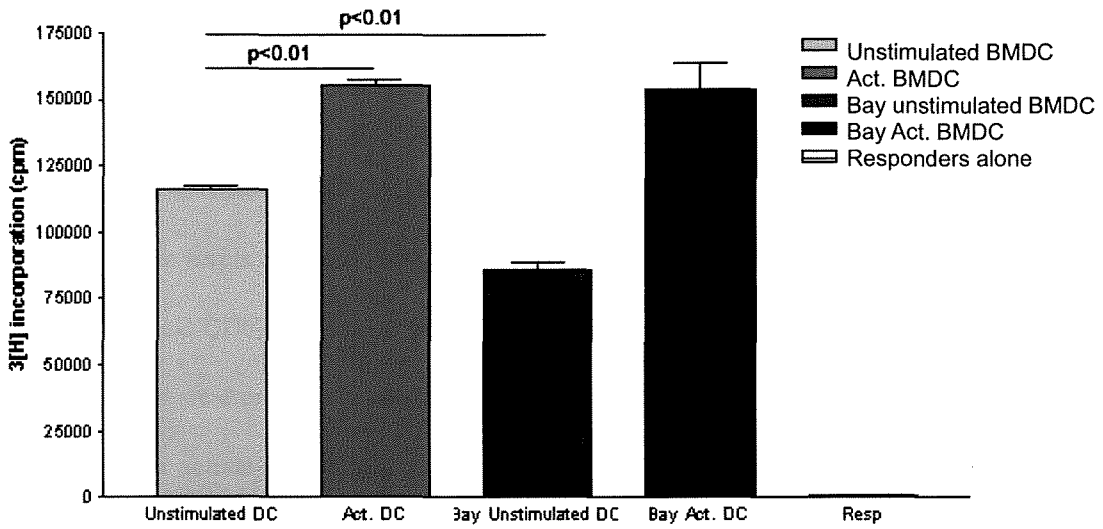


Figure 5-8: Immunostimulatory capacity of Bay11-7082 treated BMDCs.

C57BL/6 BMDCs were treated with 10μM Bay before and after LPS/cytokine stimulation and their immunostimulatory capacity was examined by MLR. C57BL/6 BMDCs, activated BMDCs, Bay BMDCs or Bay Act BMDCs were mixed with CBA/H T cells and 3[H] thymidine incorporation was measured (cpm). Representative 3[H] thymidine incorporation on Day 4 is shown (1:32 stimulator/responder ratio).

T cells and induced a similar level of T cell proliferation as activated BMDCs (3[H] thymidine incorporation in response to BAY Act BMDCs was 153731±10050cpm; $p>0.05$) [Figure 5-8]. Consistent with data obtained from splenocytes, this experiment indicated that NF-κB signaling is required for effective T cell stimulation by DCs. Similarly, it was also shown that BAY treatment did not prevent the ability of already activated DCs to stimulate the proliferation of allogeneic T cells.

To investigate whether there were any changes in costimulatory molecule expression following BAY treatment, FACS analysis was performed on BMDCs harvested from MLR culture on Day 4. Unstimulated BMDCs expressed low levels of CD40, CD69, MHC Class II, and moderate levels of CD80 and CD86 [Figure 5-9]. While there was little difference in CD40, CD80, and CD69 expression between unstimulated and activated BMDCs, CD86 and MHC Class II were dramatically upregulated showing approximately 6- and 14-fold increase respectively on activated BMDCs compared to unstimulated BMDCs [Figure 5-9]. BAY treatment did not alter the surface expression of CD40, and CD69 but increased the expression of CD80 and CD86 on BMDCs in comparison with unstimulated BMDCs without BAY treatment [Figure 5-9]. BAY Act BMDCs resulted in approximately a 2-fold reduction in the expression of CD86 and a 1.6-fold reduction in MHC Class II molecules in comparison with activated BMDCs without BAY treatment. However, BAY Act BMDCs still expressed 2.5- and 7-fold higher levels of CD86 and MHC Class II molecules compared to unstimulated BMDCs [Figure 5-9]. These results indicated that BAY treatment can dampen the expression of costimulatory molecules on both activated BMDCs. Although reduction of costimulatory molecule expression following BAY treatment correlated with a decrease in the T cell priming capacity of BAY BMDCs, relatively high costimulatory molecule expression on BAY Act BMDCs was sufficient to promote strong T cell proliferation as observed in Figure 5-8.

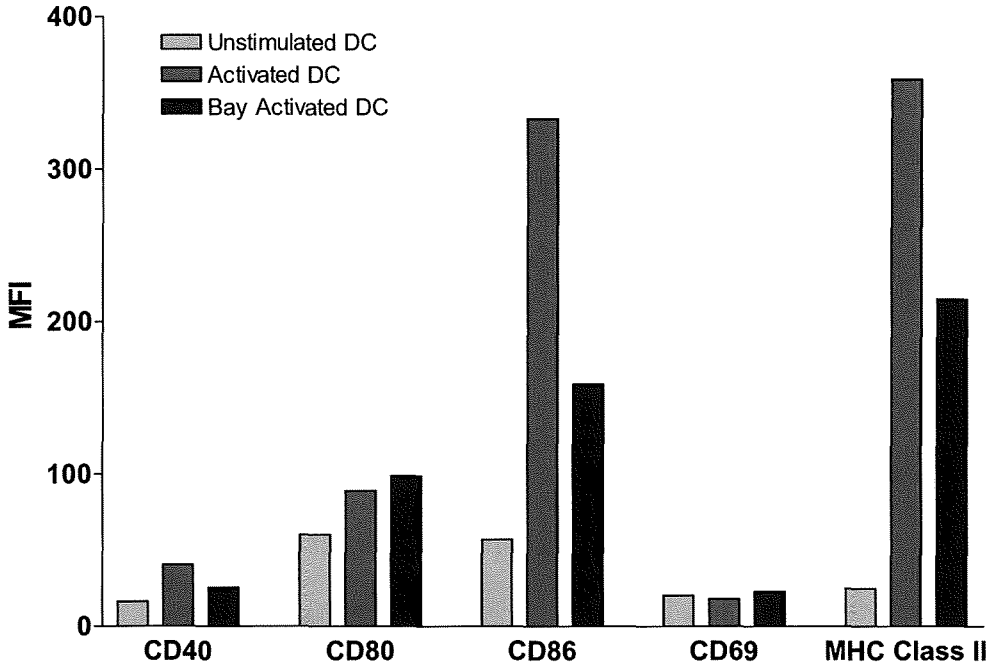


Figure 5-9: Phenotypic analysis of BMDCs harvested on Day 4 culture of MLRs.
Control BMDCs, Activated BMDCs, and Bay Act BMDCs were recovered from Day 3 MLR culture by gating on H-2K^b. Expression of activation markers (CD40, CD80, CD86, CD69 and MHC Class II) on these populations was analyzed by 4 color flow cytometry. Experiments were repeated 3 times and representative phenotypes are shown. Experiments were repeated 3 times and representative data is shown.

5.9 Characterization of T cells exposed to BAY splenocytes

Previous studies have shown that DCs deficient in NF-κB signaling can induce T cell anergy (Martin et al., 2003; Nouri-Shirazi and Guinet, 2002; Tan et al., 2005). It was also shown that DCs lacking costimulatory molecules, including CD80 and CD86, can induce T cell anergy and, in some cases, regulatory T cell differentiation (Martin et al., 2003; Nouri-Shirazi and Guinet, 2002; Tan et al., 2005). Studies in Section 5.3 have shown that BAY treatment of splenocytes inhibits alloreactive T cell stimulation and leads to a notable reduction in CD86 surface expression. Secondary MLRs using freshly isolated splenocytes as the secondary stimulus were performed to examine the nature of allogeneic T cells cocultured with BAY splenocytes in primary cultures.

In secondary MLR, T cells derived from primary MLR conducted with nonactivated splenocytes proliferate less compared to T cells derived from primary MLR conducted with activated splenocytes (3[H] thymidine incorporation of T cells previously stimulated with nonactivated splenocytes was 9853±249cpm; T cells previously stimulated with activated splenocytes was 30769±2057cpm; $p<0.01$) [Figure 5-10 (a)]. T cells which had been stimulated with Pre-BAY Act splenocytes in the primary culture also proliferated very strongly at a comparable level with T cells previously exposed to activated splenocytes in the primary culture (3[H] thymidine incorporation of T cells co-cultured with Pre-BAY Act splenocytes in the primary MLR was 29825±950cpm; $p>0.05$). These T cells were still viable 4 days after the primary MLR culture and were still able to respond to the secondary stimulus.

In the primary MLR culture, T cells did not proliferate in response to BAY splenocytes (See section 5.3) but the T cells recovered from this primary MLR were able to proliferate in response to the secondary stimulus at a similar intensity as naive T cells (3[H] thymidine

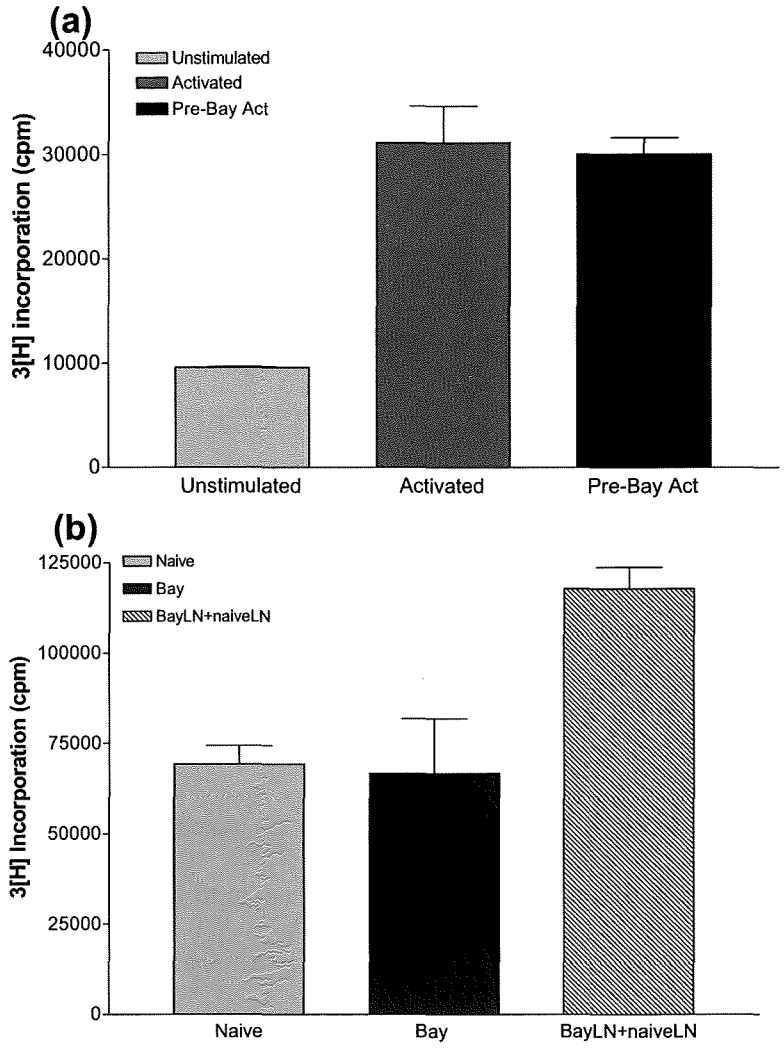


Figure 5-10: Secondary MLRs of T cells co-cultured with Bay splenocytes in the primary MLRs.

Proliferation of CBA/H LN T cells recovered from the primary MLRs was examined by a secondary challenge with freshly isolated C57BL/6 splenocytes. (a) CBA/H LN T cells previously exposed to C57BL/6 control unstimulated splenocytes, activated splenocytes, and Pre-Bay Act splenocytes were stimulated with freshly isolated C57BL/6 splenocytes and 3[H] incorporation was measured (cpm). (b) naïve CBA/H T cells, CBA/H T cells previously cultured with Bay splenocytes, and 1:1 mixture of naïve T cells and T cells previously exposed to Bay splenocytes, were challenged with freshly isolated C57BL/6 splenocytes and 3[H] thymidine incorporation was measured. Representative 3[H] thymidine incorporation on Day 4 is shown (1:1 stimulator/responder ratio).

incorporation by naïve T cells was 68928±2996cpm; T cells co-cultured with BAY splenocytes in the primary MLR was 66709±15148; $p>0.05$) [Figure 5-10(b)]. These results indicated that T cells previously exposed to BAY splenocytes were not anergic. To determine whether these T cells had acquired suppressor function following exposure to BAY splenocytes, a 1:1 mixture of naïve T cells and T cells, cultured with BAY splenocytes in the primary MLR, was challenged with a secondary stimulus. Approximately a 1.7-fold increase in the 3[H] thymidine incorporation was obtained with the mixed T cell population in comparison to the proliferation of naïve T cells, and that of T cells cultured with BAY splenocytes in the primary culture (3[H] thymidine incorporation was 117867±5938cpm; $p<0.01$) [Figure 5-11]. This 1.7-fold increase in 3[H] thymidine incorporation indicated that T cells co-cultured with BAY splenocytes in the primary MLR did not suppress the proliferation of naïve T cells.

The secondary MLRs showed that T cells cultured with BAY splenocytes in the primary MLR were not anergic nor able to suppress naïve T cell proliferation. To confirm this finding, T cells harvested from the primary MLR were restimulated with ConA and the proliferation of T cells was examined by 3[H] thymidine incorporation. Naïve T cells proliferated strongly in response to ConA stimulation (3[H] thymidine incorporation of naïve T cells was 80615±5002cpm) [Figure 5-12]. T cells, which were co-cultured with BAY splenocytes in the primary MLR, proliferated at a comparable level to naïve T cells (3[H] thymidine incorporation of T cells co-cultured with BAY splenocytes in the primary culture was 63169±7322cpm; $p>0.05$). In contrast, only a weak proliferation of T cells, which were stimulated with untreated splenocytes in the primary culture, was observed in response to ConA stimulated (3[H] thymidine incorporation was 10734±1621cpm) [Figure 5-12]. It is likely that this finding was due to the over-growth of T cells because these T cells would proliferate more readily than the naïve population as they had already been primed in the

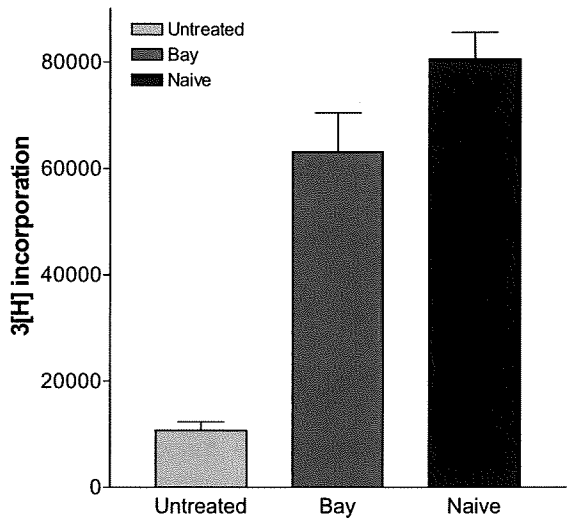


Figure 5-11: ConA stimulation of T cells previously co-cultured with Bay splenocytes in primary MLR.

Naïve T cells and T cells previously exposed to control splenocytes or Bay splenocytes in a primary MLR, were stimulated with ConA and 3[H] thymidine incorporation was measured (cpm) on Day 4. The data represent the mean of a triplicate experiment

primary culture. Due to the very rapid proliferation of T cells, depletion of nutrients in the culture medium might have occurred resulting in little proliferation on day 4. The lack of difference between the secondary responses of naïve T cells and T cells co-cultured with BAY splenocytes in the primary MLR further confirmed that there were no obvious alterations in the proliferative capacity of T cells previously exposed to BAY splenocytes

5.10 Proportion of $CD4^+$ and $CD8^+$ T cells in primary and secondary MLR

Previous gene knockout studies have associated cRel and p50 with the polarization of Th1 and Th2 type immune response respectively (Das et al., 2001; Hilliard et al., 2002). The current study has shown that BAY treatment of splenocytes abrogated their capacity to prime T cells. Whether the inhibition of NF-κB signaling in APCs selectively affects the subsequent proliferation of $CD4^+$ or $CD8^+$ T cells remains unresolved. To investigate the possibility of a biased T cell response, the proportion of $CD4^+$ and $CD8^+$ T cells amongst the T cells harvested at the end of primary and secondary MLRs were analyzed by flow cytometry.

Amongst the T cells responding to unstimulated allogeneic splenocytes in primary MLRs, 33.7% of allogeneic T cells were $CD4^+$ and 66.3% were $CD8^+$ [Figure 5-12 (a)]. Similar ratios of allogeneic $CD4^+$ and $CD8^+$ T cells were obtained in MLRs with BAY splenocytes, activated splenocytes, BAY Act splenocytes and Pre-BAY Act splenocytes (BAY splenocytes induced 30.1% $CD4^+$ and 69.9% $CD8^+$ T cells; activated splenocytes induced 30.0% $CD4^+$ and 70.0% $CD8^+$ T cells; BAY Act splenocytes induced 32.8% $CD4^+$ and 67.2% $CD8^+$ T cells; Pre-BAY Act splenocytes induced 35.5% $CD4^+$ and 64.5% $CD8^+$ T cells) [Figure 5-12 (a)].

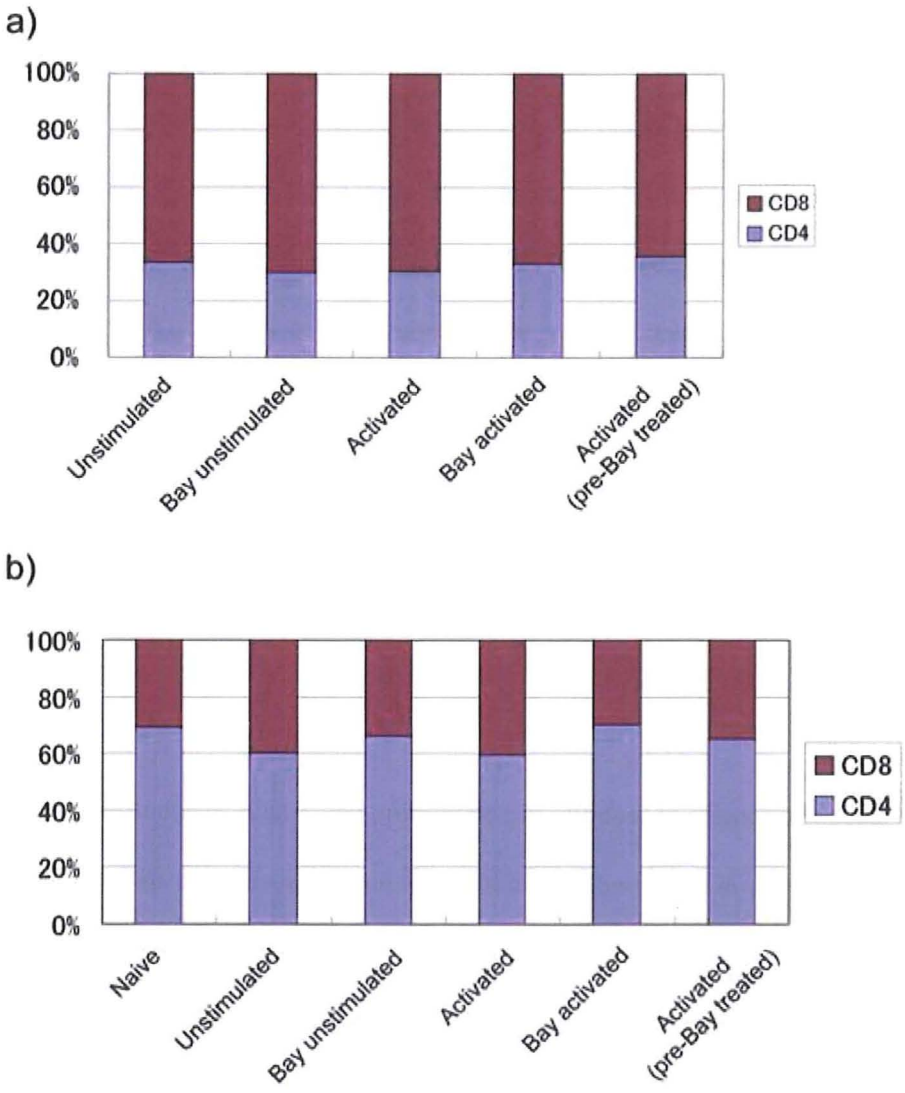


Figure 5-12: Proportion of $CD4^+$ and $CD8^+$ T cells in primary and secondary MLRs

T cells were recovered from primary and secondary MLR cultures at Day 4. Proportions of $CD4^+$ and $CD8^+$ T cells among H-2^k positive cells harvested from (a) primary and (b) secondary MLRs were analyzed by 4 color flow cytometry. Experiments were repeated 3 times and representative data is shown.

In the secondary MLRs, no notable differences in the proportion of $CD4^+$ and $CD8^+$ T cells were observed between T cell populations harvested from different primary culturing conditions. In response to the secondary stimulus, T cells co-cultured with naive splenocytes in the primary MLR, showed 59.9% $CD4^+$ and 39.1% $CD8^+$ [Figure 5-12 (b)]. T cells previously co-cultured with BAY splenocytes in the primary MLR exhibited similar proportion of $CD4^+$ and $CD8^+$ T cells in the secondary MLR (T cells co-cultured with BAY splenocytes in the primary culture were 66.2% $CD4^+$ and 35.8% $CD8^+$ in the secondary MLR) [Figure 5-12 (b)]. T cells co-cultured with activated splenocytes, BAY Act splenocytes, or Pre-BAY Act splenocytes, in the primary culture all showed similar proportions of $CD4^+$ and $CD8^+$ populations (T cells co-cultured with activated splenocytes in the primary MLR were 59.7% $CD4^+$ 40.3% and $CD8^+$; T cells co-cultured with BAY Act splenocytes in the primary MLR were 70.4% $CD4^+$ and 29.6% $CD8^+$; and T cells co-cultured with Pre-BAY Act splenocytes were 65.4% $CD4^+$ and 34.6% $CD8^+$ in the secondary MLR).

These results indicated that T cell proliferation in response to splenocytes treated with BAY pre- or post-LPS/cytokine activation were not biased towards $CD4^+$ or $CD8^+$ T cells in both primary and secondary MLRs. This result suggested that NF-κB inhibition in splenocytes does not influence the manner in which $CD4^+$ or $CD8^+$ T cell proliferate in response to a subsequent allogeneic stimulus.

5.11 Discussion

This chapter describes the immunomodulatory effects of BAY11-7082 inhibition of NF-κB signaling on the capacity of naive splenocytes to stimulate allogeneic T cells. This was achieved through downregulation of CD86 and MHC II expression and inhibition of IL-2, IL-4, IFN-γ and TNF-α production. Inhibition of cRel by Ptx revealed that cRel inhibition alone is not responsible for such inhibition of T cell priming by splenocytes. It was also

shown that BAY splenocytes do not induce T cell anergy or the differentiation of regulatory T cells.

Previous studies have shown the requirement of the NF-κB pathway for effector T cell priming by APCs (Baltathakis et al., 2001; Clark et al., 1999; Hofer et al., 2001; Neumann et al., 2000). Consistent with these reports, the present study shows that an inhibition of NF-κB activity by BAY treatment significantly reduces the immunostimulatory capacity of splenocytes [Figure 5-2]. BMDCs were then used to examine whether the inhibition of T cell priming by BAY splenocytes was through the inhibition of T cell priming specifically by DCs present in the splenocyte population. However, this issue was not clearly resolved. Failure to achieve complete inhibition of T cell priming by BAY BMDCs (Figure 5-8) suggested that the BMDCs might have been partially activated during the 7 day propagation from progenitors. It could be argued that since BAY treatment was only effective on naïve populations [Section 5.3], BAY treatment of partially activated BMDCs would not display optimal inhibition of T cell activation. It is possible that experiments using splenic DCs, instead of BMDCs, would have provided more appropriate approach for testing whether the inability of BAY splenocytes to prime T cells was due to the inhibition of NF-κB signaling in the DC sub-population of splenocytes.

FACS analyses and cytokine measurements in the MLR cultures indicated that the lack of T cell proliferation was associated with costimulatory molecule donwregulation , reduction in MHC Class II expression on APCs and suppression of inflammatory cytokine production [Figure 5-3 and Figure 5-4]. BAY treatment inhibited CD86 and MHC Class II induction on splenocytes. This finding was consistent with previous studies showing NF-κB to a critical regulator of costimulatory molecule expression on APCs (Ardeshna et al., 2000; O'Sullivan et al., 2000; Saemann et al., 2004; Yoshimura et al., 2001). Such reports have also described

that CD40 and CD80 expression is under NF-κB transcriptional control, however, reduction in CD40 and CD80 expression on BAY splenocytes was not observed [Figure 5-3]. BAY BMDCs, on the other hand, showed a reduction in CD80 expression as well [Figure 5-9] but no clear decrease in CD40 expression. Low CD40 expression on both splenocytes and BMDCs even after LPS/cytokine stimulation hindered our assessment of the effect of BAY treatment on CD40 expression. Production of inflammatory cytokines, including IL-2, IL-4, TNF-α and IFN-γ, were completely absent and IL-5 production was substantially reduced in the MLR culture supernatant of co-cultured BAY splenocytes and T cells [Figure 5-4]. This observation supports the involvement of NF-κB signaling in the production of these cytokines as reported previously (Finn et al., 2001; Grigoriadis et al., 1996; Mason et al., 2002; Mora et al., 2001; Yoshimura et al., 2001). This finding is also consistent with the previous notion that NF-κB signaling transduction in allogeneic DCs is critical in inducing T cell proliferation and associated IFN-γ production (Contreras et al., 1998). Since T cells derived from primary MLR conducted with BAY splenocytes can proliferate in the secondary MLR [Figure 5-10], it is unlikely that low cytokine concentration in co-culture with BAY splenocytes was not due to T cell death. The present study showed that BAY treatment inhibited the production of both Th1 and Th2 cytokines and this was consistent with previous reports showing the involvement of NF-κB signaling in the control of both Th1 and Th2 cytokines (Boffa et al., 2003; Calder et al., 2003). Control of cytokine production by the NF-κB pathway therefore is not Th1- or Th2-specific. The present study also showed that NF-κB signaling in APCs does not have a role in determining CD4- or CD8-biased T cell proliferation [Figure 5-12].

The failure of Ptx-treatment of allogeneic splenocytes to inhibit T cell activation indicated that cRel was not the central component in the observed inhibition of T cell priming by BAY splenocytes [Figure 5-2 and Figure 5-5]. Our observation that cRel inhibition alone did not

affect the capacity of splenocytes to induce T cell proliferation, contradicted a previous report by Boffa *et al.* which showed the selective loss of cRel in unstimulated DCs resulted in a significant reduction in subsequent T cell priming (Boffa et al., 2003). The study reported that a decrease in CD86 expression and inflammatory cytokine production correlated with the phenomenon (Boffa et al., 2003). However, it was also shown that cRel deficiency did not prevent DCs from undergoing maturation upon LPS stimulation and once activated, the cRel deficient DCs became capable of stimulating allogeneic T cells at a similar level to control DCs (Boffa et al., 2003). This report and the current study therefore support the notion that cRel is not essential for APCs to prime allogeneic T cells.

The current study shows that the BAY-treated-APC-mediated inhibitory effect in T cell priming by APCs is restricted to unstimulated splenocytes/BMDCs. It was shown that BAY treatment of already activated splenocytes/BMDCs or on splenocytes prior to LPS/cytokine activation was not able to prevent T cell priming [Figure 5-6 and Figure 5-8]. This finding was in contrast to a previous report demonstrating the inhibition of T cell priming by DCs which were treated with BAY and then received activation stimulus (Thompson et al., 2004). The discrepancy between this report and the current study could be due to the difference in the methodologies used for DC maturation. In the study by Thompson *et al.*, DCs received activation stimuli in the presence of BAY inhibitor in the culture (Thompson et al., 2004) while in the current study, splenocytes were stimulated after the removal of BAY from the culture medium. The binding of BAY to IκB proteins is irreversible, however, IκB proteins synthesized after the withdrawal of BAY treatment would not be subjected to inhibition (personal communication, Dr. Sudah Rao, JCSMR). It could therefore be speculated that IκB protein turnover in BAY splenocytes allowed the newly synthesized IκB to be phosphorylated by LPS/cytokine stimulus during 48 hours of LPS/cytokine stimulation. This hypothesis is supported by western blot analyses showing that cytoplasmic expression of

cRel in LPS/cytokine stimulated splenocytes pretreated with BAY was at a comparable level to that of LPS/cytokine stimulated splenocytes without a pretreatment of BAY (at 48 hours post-activation). A time course study of cRel induction in BAY splenocytes may be required to verify this hypothesis. In addition, BAY treatment of previously activated BMDCs showed strong T cell priming, yet, it resulted in a reduction of CD86 and MHC Class II expression and this may suggest that NF-κB signaling is also involved in maintaining a mature DC phenotype [Figure 5-9].

Previous studies of a number of cell types have suggested the importance of the NF-κB pathway in cell cycling (Bladh et al., 2005; Chen et al., 2001; Feng et al., 2004). For example, it was shown that cRel is directly involved in the transcriptional regulation of cyclin E expression, which is important in B cell cycling (Cheng et al., 2003; Feng et al., 2004). The present study demonstrated that BAY treatment prevented the proliferation of splenocytes and circumvented the without a need for their irradiation for MLRs [Figure 5-5] and thus further supports the participation of NF-κB in cell proliferation.

Several studies have shown that APCs lacking their NF-κB signaling could induce alloantigen-specific T cell anergy or regulatory T cell development (Martin et al., 2003; Tomasoni et al., 2005; Xu et al., 2004). These studies showed unresponsiveness of T cells to secondary stimuli and also dampening of the immune response. It was also suggested that reduction in the expression of CD86 and other costimulatory molecules on DCs was associated with the induction in T cell anergy and in the generation of regulatory T cells (Lutz et al., 2000; Martin et al., 2003; Yoshimura et al., 2001). The results presented in this chapter were, however, in contrast to these reports. Although BAY splenocytes did not elicit T cell proliferation in the primary MLRs and these splenocytes expressed a lower level of CD86, T cells previously exposed to BAY splenocytes were still able to proliferate in a

similar manner to naïve T cells in the secondary MLRs [Figure 5-10 (a) and (b)]. This clearly indicated that the T cells which encountered BAY splenocytes in the primary culture were not anergic. Proliferation of this T cell population in response to secondary stimuli also indicated that the lack of T cell proliferation in the primary MLR was not due to T cell apoptosis or T cell deletion. Furthermore, the proliferation of naive T cells in the presence of T cells previously co-cultured with BAY splenocytes in the secondary MLRs suggested that T cells previously exposed to BAY splenocytes did not gain suppressor function [Figure 5-10(b)]. It is possible that there was an insufficient number of DCs contained in the BAY splenocytes in the primary MLR to induce T cell anergy as DCs constitute only 0.2-0.5% of the total splenocyte preparation (Steinman et al., 1979). BAY treatment of BMDCs resulted in some reduction in T cell priming capacity, however, further studies, including secondary MLRs, are needed to understand the basis for this intermediate effect.

Whether there was any physical interaction between BAY splenocytes and T cells in the primary culture remains unclear. It appeared that there was no cell-to-cell engagement in the primary culture as T cells previously exposed to BAY splenocytes could interact normally with fresh, untreated allogeneic splenocytes in secondary MLRs [Figure 5-11 (b)]. This study supports the findings previously reported that NF-κB is required for DC-T cell interaction (Andreakos et al., 2003). The hypothesis that BAY splenocytes were invisible to allogeneic T cells in the primary culture was supported by the lack of detection of cytokines in the culture supernatant from primary MLRs [see Figure 5-4]. However, Thompson *et al.* showed that TCR signaling still occurs between BAY treated DCs and allogeneic T cells despite lack of T cell proliferation (Thompson et al., 2004). In that study, sensitization of T cells was achieved by DCs deficient in NF-κB, and these T cells produced higher levels of IFN-γ in response to a secondary stimulus (Thompson et al., 2004). Whether the exposure of BAY splenocytes or BAY BMDCs affected the levels of inflammatory cytokines produced by these T cells in

response to secondary stimulus is unknown. To examine whether the protocol used in this study resulted in BAY splenocytes to sensitize T cells as described by Thompson *et al.*, the levels of inflammatory cytokine production may need to be examined in the secondary MLR culture.

Studies in this Chapter using the inhibitors of the NF-κB pathway demonstrated that APCs stimulate allogeneic T cells in a NF-κB-dependent but cRel independent manner. Bay-treatment of splenocytes reduced costimulatory molecule and MHC Class II expression and inhibited inflammatory cytokine production. However, this inhibitory effect was restricted to unstimulated APC populations; BAY-mediated inhibition of NF-κB signaling could not prevent already activated APC populations from priming allogeneic T cells. Furthermore, pretreatment of splenocytes with BAY inhibitor could not prevent the splenocytes themselves from being activated by LPS/cytokines. It could be speculated that this was due to IκB protein cycling which allowed newly synthesized IκB proteins to be phosphorylated, thereby over-riding the inhibition. Secondary MLRs indicated that the lack of T cell proliferation in response to BAY splenocytes was not due to T cell anergy nor due to the acquisition of a regulatory phenotype in the primary culture. Instead, the absence of T cell proliferation may have been due to lack of effective interactions between BAY splenocytes and T cells. In addition, BAY treatment did not selectively alter proportion of $CD4^+$ or $CD8^+$ T cells, , indicating that NF-κB signaling in APCs does not bias $CD4^+/CD8^+$ T cell differentiation. Collectively, these results suggest that the inhibition of NF-κB signaling alone does not promote alloantigen tolerance via generation of regulatory T cells *in vitro*.

Essential signals for the induction of tolerance still remain to be elucidated. Secondary lymphoid organs other than DCs and T cell *in situ* may provide additional signals required for the generation of regulatory T cells *in vivo* and it is possible that this interaction was not

reproduced *in vitro*. In the following chapter, the capacity for BAY splenocytes or BAY BMDCs to alter alloresponses *in vivo* will be investigated. We will also investigate whether inhibition of NF-κB signaling in donor passenger leukocytes can promote allograft protection by preventing the activation of alloreactive T cells.

Chapter 6

Role of NF-κB in Allograft Rejection

6.1 Introduction

During acute allograft rejection, T cells primed by the direct pathway dominate the rejection process (Hornick et al., 1998). It has been demonstrated that APCs carried within the graft, so called passenger leukocytes, are the primary mediators of the rejection of cellular allografts (Benichou et al., 1999; Gould and Auchincloss, 1999). These donor-type APCs express MHC Class I and Class II alloantigens and costimulatory molecules and they are capable of activating recipient T cells. Lafferty *et al.* showed that removal of passenger leukocytes from the graft by culturing in a high oxygen gas phase resulted in indefinite survival of thyroid allografts (Lafferty et al., 1983). This study clearly indicated a need for controlling passenger leukocytes to achieve successful allotransplantation. The importance of passenger leukocytes in graft rejection was further highlighted by studies demonstrating rejection of passenger leukocyte-depleted allografts following the adoptive transfer of donor-type APCs (Lafferty et al., 1975; Lechler and Batchelor, 1982). Subsequent studies have shown that migration to the draining LNs and the antigen presentation by these APCs are crucial for initiation of the acute rejection process via the direct pathway (Lakkis et al., 2000). Despite a limited time-frame in which passenger leukocytes (donor APCs) can operate in the recipients, their impact on the outcome of graft survival is critical. However, current therapeutic strategies tend to focus more on facilitating the induction of donor antigen-specific tolerance by manipulating the recipients' immune system rather than by controlling the passenger leukocyte activity in the transplant tissue.

It was demonstrated in Chapter 5 that inhibition of NF-κB signaling in splenocytes abrogates their capacity to prime allogeneic T cells. This finding suggests that targeting NF-κB signaling in passenger leukocytes may protect tissue allografts from rejection. While Lafferty *et al.* succeeded in preventing allograft rejection by culturing mouse thyroid from cyclophospho-amide pretreated donors in a high oxygen environment for 14 days (Lafferty et

al., 1983), the poor survival of human islets under such condition limited the use of this *in vitro* method of immunomodulation (Benhamou et al., 1998). We hypothesized that treatment of allograft with BAY11-7082 may inhibit direct priming of recipient T cells, requiring a much shorter culturing time and may provide an alternative approach to controlling the immunostimulatory activity of passenger leukocytes. To assess this hypothesis, the aim of this chapter was to examine whether pretreatment of thyroid or pancreatic islets with BAY before transplantation can prolong allograft survival by inhibiting the T cell priming capacity of passenger leukocytes within grafts.

In addition, it was also shown in Chapter 5 that BAY splenocytes and BAY BMDCs did not induce the differentiation of regulatory T cells *in vitro*. These findings were in contrast to a previous study demonstrating regulatory T cell differentiation in response to BAY-treated BMDC administration (Martin et al., 2003). These regulatory T cells were found in *ex vivo* T cell population after treating mice with BAY BMDCs *in vivo* (Martin et al., 2003). Since the *in vitro* system employed in Chapter 5 does not fully replicate physiological conditions, it was possible that additional signals provided *in vivo* are required for regulatory T cell differentiation. To explore this possibility, this chapter investigates whether BAY-treated donor splenocytes or BAY BMDCs can mediate allograft protection *in vivo* via the induction of alloantigen unresponsiveness in recipient mice.

6.2 Identification of the optimal dose of BAY for thyroid tissue

Firstly, the optimal concentration of BAY to be used in thyroid cultures was determined. C57BL/6 thyroid tissue was cultured in Minimal Essential Medium supplemented with 10% heat-inactivated fetal calf serum and 0-1mM BAY for 12 hours in 5%CO_2, 95% air. Cultured thyroids were then examined histological to ascertain the maximum concentration of BAY which maintained tissue viability.

Control thyroid tissue, cultured in 5%CO_2, 95% air, showed loss of thyroid follicles and apparent necrosis [Figure 6-1]. 10μM BAY-cultured thyroids also exhibited similar morphology with few intact thyroid follicles remaining around the periphery of the tissue [Figure 6-1]. Thyroids cultured in 100μM and 1mM BAY also failed to show viable thyroid tissue [Figure 6-1]. The extensive damage to thyroid glands in culture without BAY indicated that the culture conditions employed here were not suitable for maintaining viability of thyroid tissue.

In the study conducted by Lafferty *et al.*, a long-term thyroid culture was achieved by employing at atmosphere of 95%O_2 5%CO_2 (Lafferty et al., 1983). We therefore cultured thyroid tissue in 95%O_2 5%CO_2 for 12 hours, and found preservation of intact follicles without signs of necrosis [Figure 6-2(a)]. Using these culturing conditions, 5μM BAY-cultured thyroid tissue exhibited intact thyroid architecture lacking any signs of thyroid damage after 12 hours of incubation [Figure 6-2(b)]. These results confirmed that the thyroid damage observed in the earlier experiments was likely caused by an unsuitable gas phase and that a high oxygen environment was appropriate for further experiments. These experiments also demonstrated that BAY at a 5μM concentration was not toxic to thyroid glands.

To allow exposure of the passenger leukocytes to maximum levels of BAY without toxic effects to the thyroid tissue, mouse thyroid was cultured with different concentration of BAY in a high oxygen gas phase. Following culture, thyroid viability was assessed by histology. Consistent with data obtained from the 12 hour culture, thyroid glands cultured without BAY or with 5μM BAY showed normal thyroid morphology after 24 hours of incubation [Figure 6-3 (a) and (b)]. Thyroids cultured in 10μM, 15μM or 20μM also remained intact and showed no signs of necrosis [Figure 6-3 (c)-(e)]. This observation was consistent with a

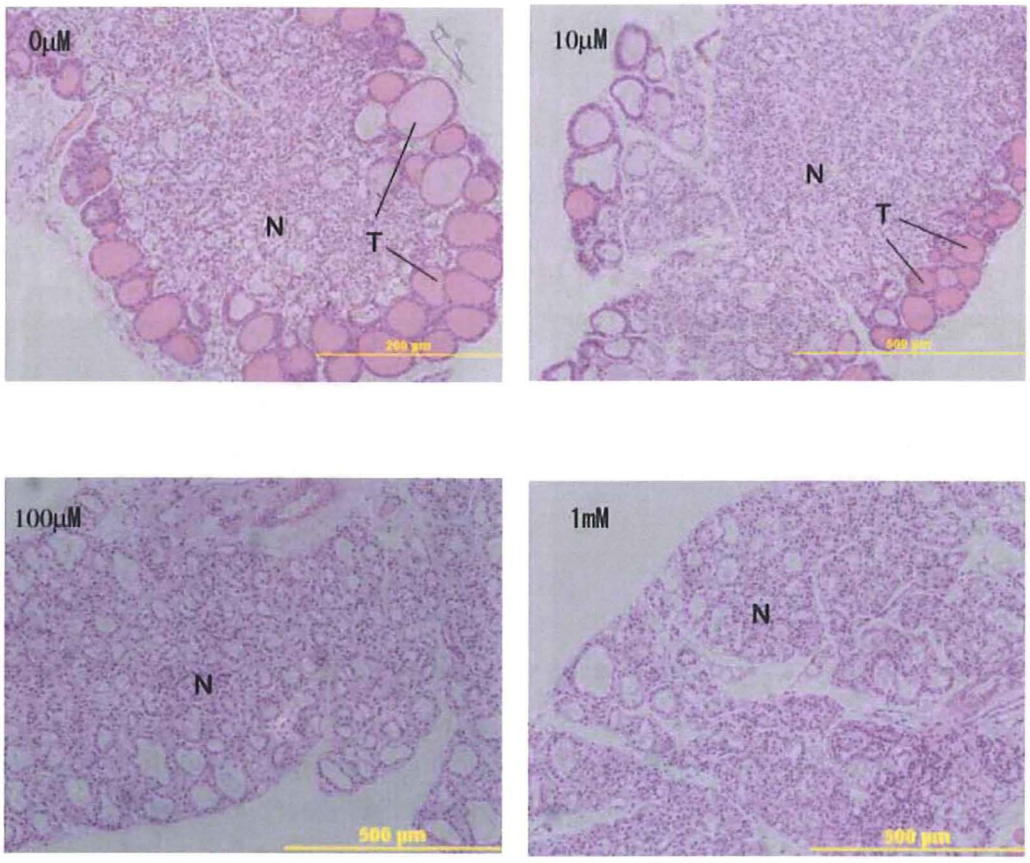


Figure 6-1: Histologic appearance of mouse thyroid tissue cultured with 0μM-1mM BAY11-7082 in 5%CO_2 95% air for 12 hours.

Thyroid glands were isolated from C57BL/6 mice and cultured in a medium containing (a) 0μM, (b) 10μM, (c) 100μM, and (d) 1mM BAY in 5%CO_2 95% air for 12 hours. Representative histology is shown for each culture condition. (T) shows thyroid follicles and (N) identifies necrotic tissue. Hematoxylin and eosin, × 20.

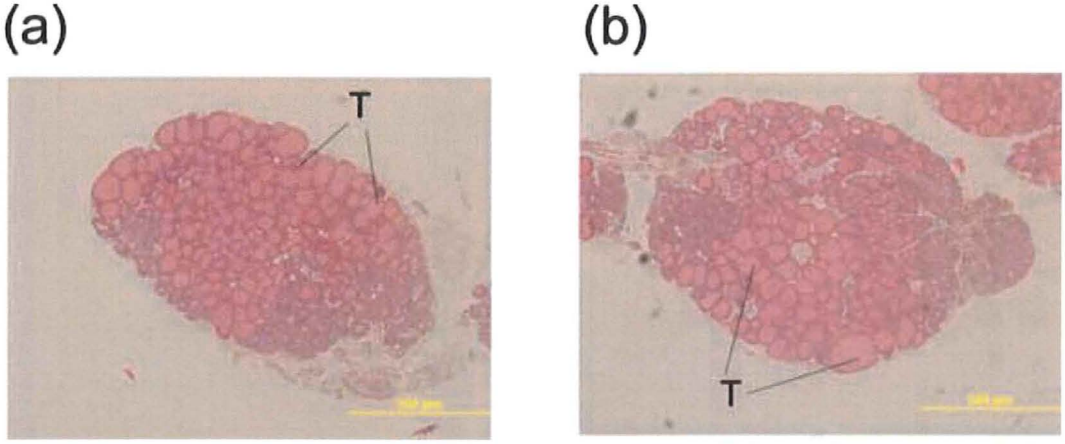


Figure 6-2: Histologic appearance of mouse thyroid tissue cultured with 0μM-5μM BAY11-7082 in 95%O_2 5% air for 12 hours

Thyroids were isolated from C57BL/6 mice and cultured in a medium containing (a) 0μM and (b) 5μM BAY in 95%O_2 5% CO_2 for 12 hours. Representative histology is shown for each culture condition. (T) identifies thyroid follicles. Hematoxylin and eosin, ×20.

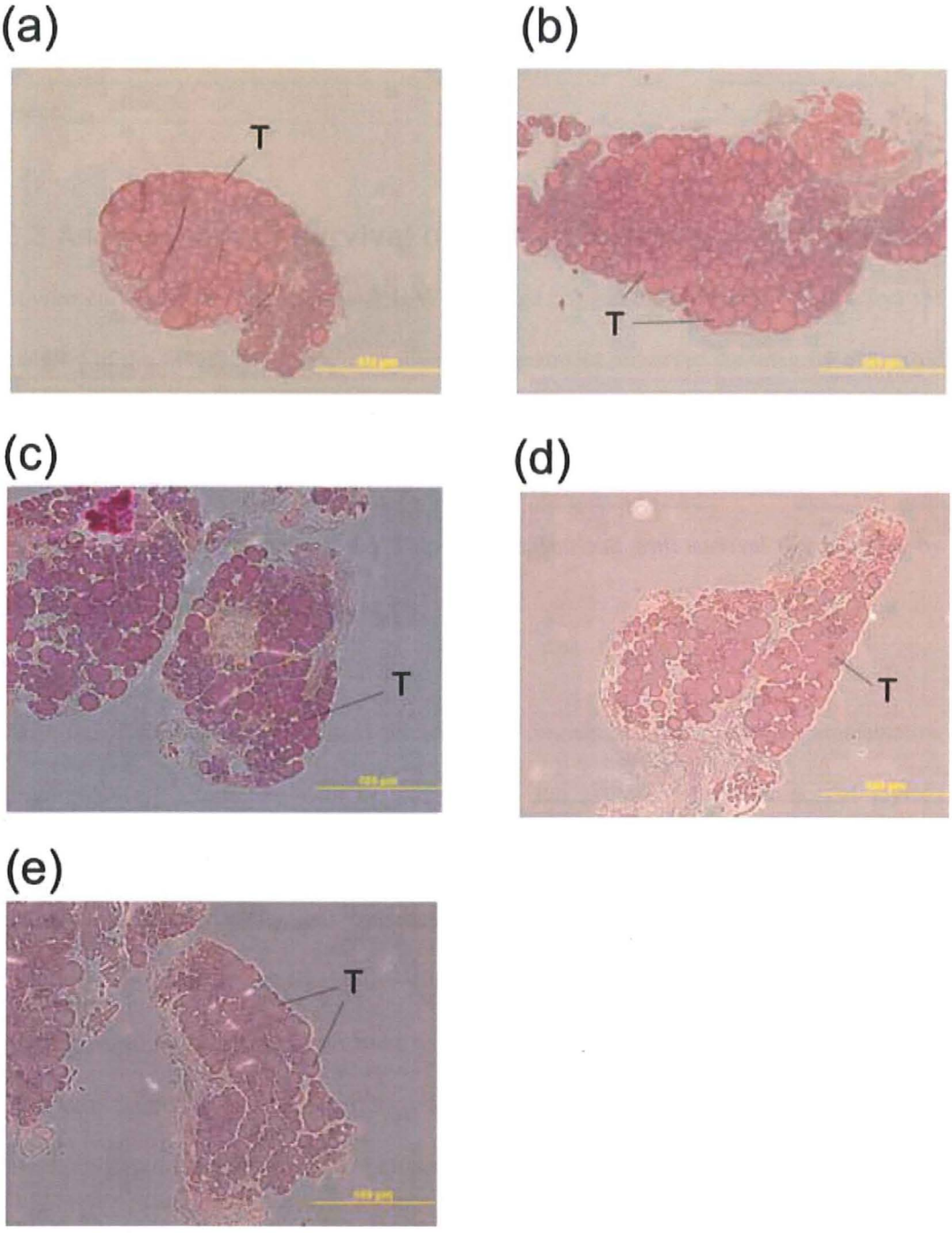


Figure 6-3: Histologic appearance of mouse thyroid tissue cultured with 0μM-20μM BAY11-7082 in 95%O_2 5%CO_2 for 24 hours.

Thyroid glands were isolated from C57BL/6 mice and cultured in medium containing (a) 0μM, (b) 5μM, (c) 10μM, (d) 15μM and (e) 20μM BAY in 95%O_2 5% air for 24 hours. Representative histology is shown for each culture condition. (T) identifies thyroid follicles. Hematoxylin and eosin, × 10.

previous report showing that up to 20μM BAY was not toxic for DCs (Lafferty et al., 1983); the current study suggests that treatment within ≤20μM BAY is not toxic for mouse thyroid tissue.

6.3 Assessment of survival of BAY-cultured thyroid allografts

In vitro culture of thyroid tissue with BAY in a high oxygen environment did not affect the viability of the tissue. To ascertain whether BAY treatment preserves the integrity of thyroid tissue *in vivo*, and determine the optimal concentration of BAY, C57BL/6 thyroid glands were precultured with 0-80μM BAY and transplanted into isogeneic recipients. Grafts were harvested from recipient mice at day 14 post-transplant and graft survival was assessed by histology and morphometric analysis.

Isografts of thyroid tissue cultured without BAY remained intact following implantation [Figure 6-4(a)]. Consistent with *in vitro* results, the histological appearance of thyroid isografts cultured with 5μM, 10μM, 20μM and 40μM BAY prior to transplantation exhibited no loss of tissue viability and consisted of intact thyroid follicles [Figure 6-4(b)-(e)]. Similarly, morphometric analyses of 0-40μM BAY-cultured isografts indicated no apparent graft damage, which was in accordance with histological appearance (percent intact follicles of control cultured thyroid isografts was 43.1±0.56; 5μM BAY-cultured isografts was 34.0±2.09; 10μM BAY-cultured isografts was 39.4±3.22; 20μM BAY-cultured isografts was 38.3±3.72; 40μM BAY-cultured isografts was 38.3±3.72) [Figure 6-5]. Thus, no significant differences were found between the percentages ($p>0.05$) of intact follicles obtained from grafts precultured without BAY and grafts precultured with up to 40μM BAY, indicating that thyroid viability was preserved. Although the difference in graft survival between 0μM and 5μM precultured thyroids was significant ($P=0.01$), the survival and histological appearance of thyroid grafts precultured in 10-40μM BAY indicated that 5-40μM concentration was not

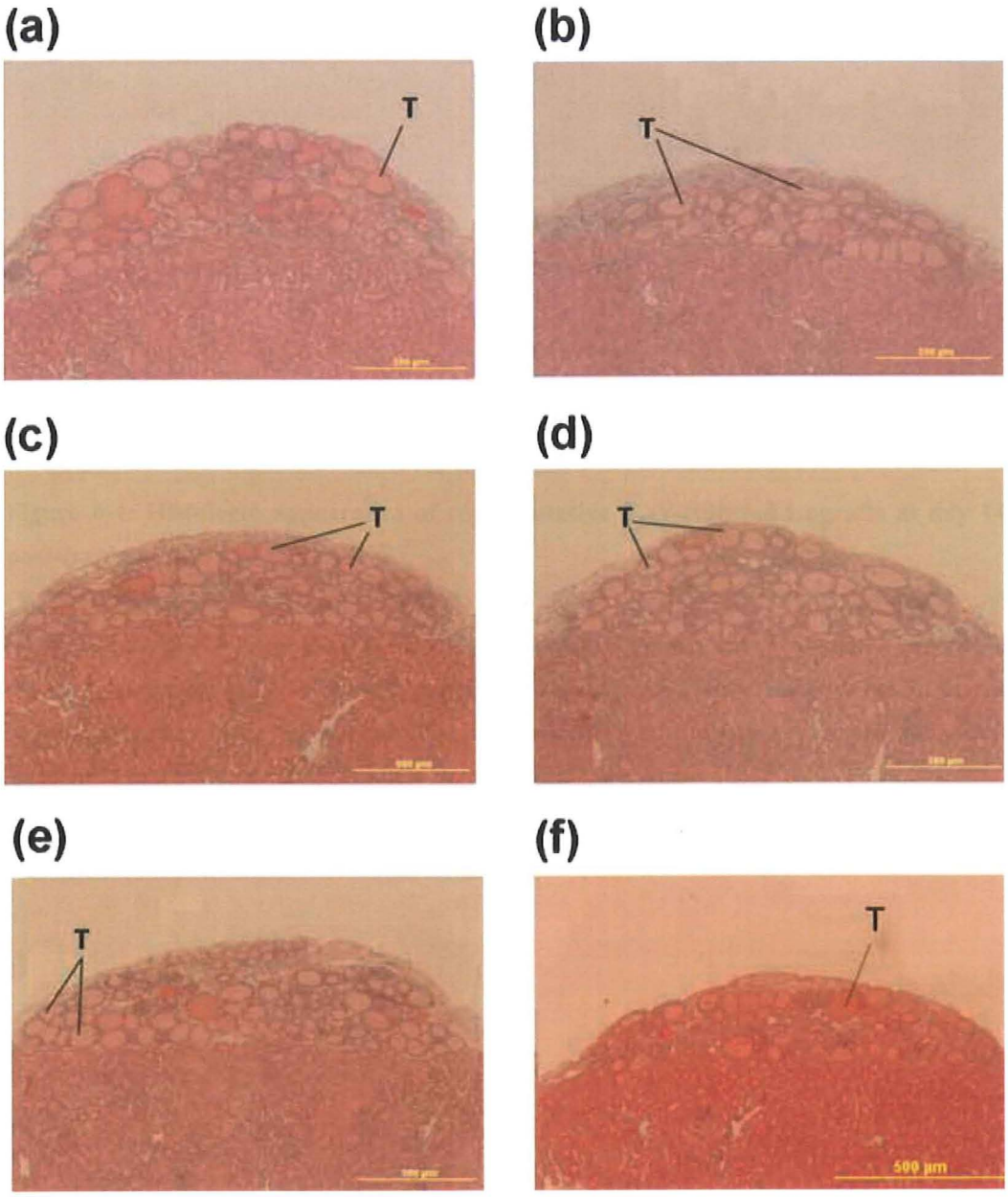

(a)
T
(b)
T
(c)
T
(d)
T
(e)
T
(f)
T
500 µm

Figure 6-4: Histologic appearance of representative BAY-cultured isografts at day 14 post-transplant to C57BL/6 mice.

C57BL/6 thyroid were cultured with (a) 0μM, (b) 5μM, (c) 10μM, (d) 20μM, (e) 40μM, and (f), 80μM BAY11-7082 in 95%O_2 5% CO_2 for 24 hours before transplantation. The thyroid tissue was then transplanted beneath the kidney capsule of C57BL/6 mice on day 0. Grafts were harvested at 14 days post-transplant. A representative histologic section of each group is shown. (T) identify thyroid follicles. Hematoxylin and eosin, × 10.

toxic to thyroid tissue. In contrast, histological appearance of thyroid isografts precultured in 80μM BAY showed some minor loss of intact follicles [Figure 6-4(f)], probably induced a low level of BAY toxicity. In support, morphometric analysis revealed a 29.5% reduction in percent intact follicles in 80μM BAY cultured thyroid isografts compared to grafts precultured without BAY (percent intact follicles of 80μM BAY thyroids was 30.4±0.39) [Figure 6-5]. In comparison to untreated grafts, grafts precultured with 40μM BAY displayed 11.1% reduction in percent intact follicles [Figure 6-5]. The significant difference ($p<0.05$) between the percentage of intact follicles in 80μM BAY precultured thyroids with all other treatments tested indicates that the optimal dose of BAY is <80μM. Collectively, these results demonstrate that BAY treatment (up to 40μM) of mouse thyroids does not affect the tissue survival *in vitro* and *in vivo*.

It was hypothesized that culture of thyroid tissue with BAY would allow the NF-κB inhibitor to act on passenger leukocytes carried within donor tissue. Since BAY treatment inhibits the T cell priming capacity of splenocytes *in vitro*, it was anticipated that culturing thyroid with BAY would also inhibit the ability of passenger leukocytes to stimulate recipient T cells, thus promoting allograft survival. Based on this hypothesis, C57BL/6 thyroid glands were cultured with 0-80μM BAY for 24 hours in a high oxygen gas phase then transplanted into CBA/H mice. Grafts were harvested on day 14 post-transplant and histological and morphometric analyses were performed.

Thyroid allografts cultured without BAY were acutely rejected exhibiting extensive cellular infiltration, hemorrhage, and with few intact follicles remaining in the graft [Figure 6-6(a)]. Thyroid allografts precultured with 5μM, 10μM, 20μM, 40μM, and 80μM BAY were also rejected in a similar manner to control allografts [Figure6-6 (b)-(f)]. Histological examination of these allografts showed no signs of improved graft survival. The features of

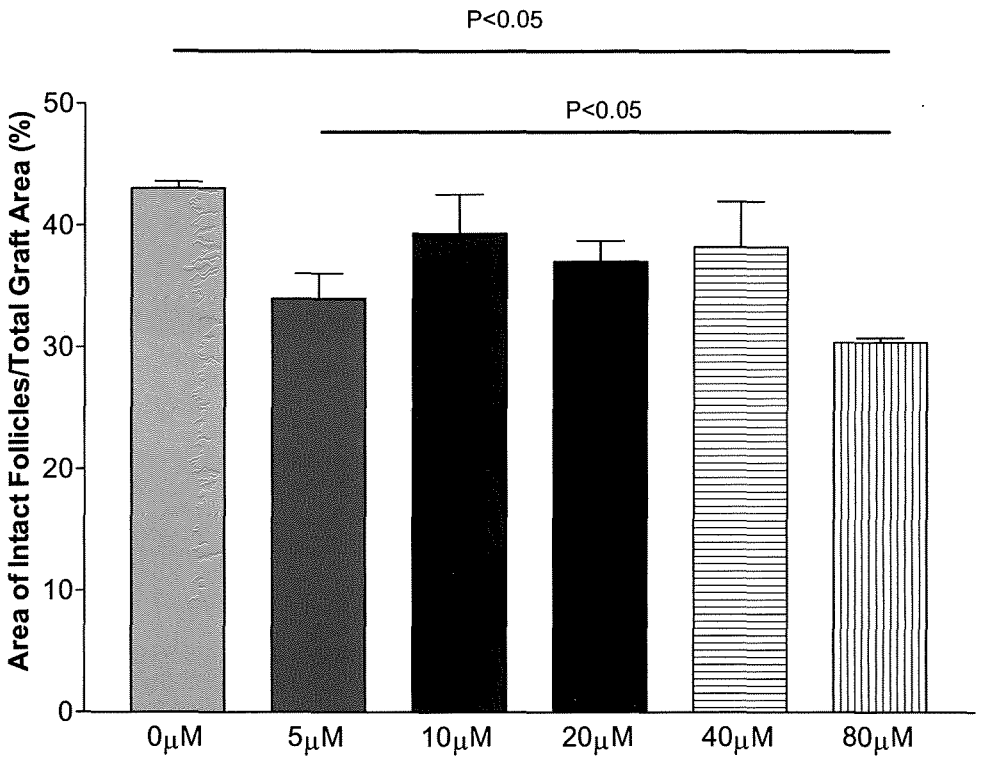
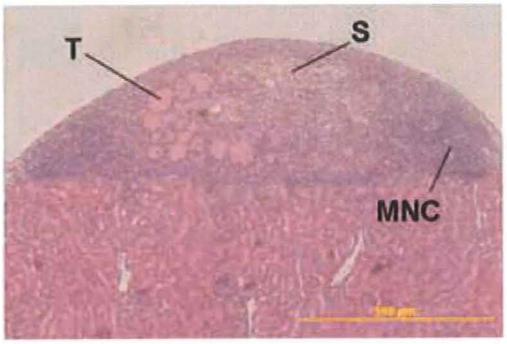


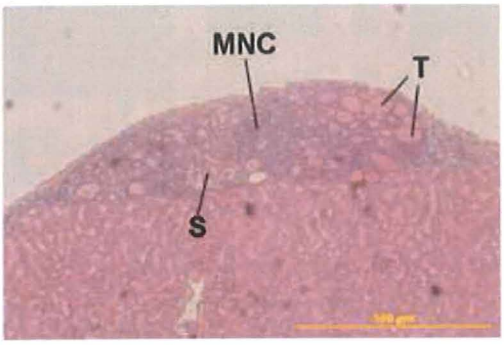
Figure 6-5: Morphometric analysis of C57BL/6 thyroid isografts cultured with 0μM-80μM BAY11-7082 at 14 days post-transplant.

C57BL/6 thyroid tissue was cultured with 0μM-80μM BAY11-7082 in 95%O_2 5%CO_2 for 24 hours prior to transplantation. The thyroid tissue was transplanted beneath the kidney capsule of C57BL/6 mice on day 0. The grafts were harvested at 14 days post-transplant. Percent intact follicles of the total graft area was calculated as described in Section 2.4.5. Data represent mean±SEM.

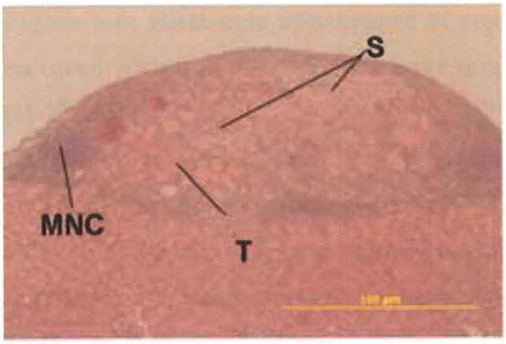
(a)



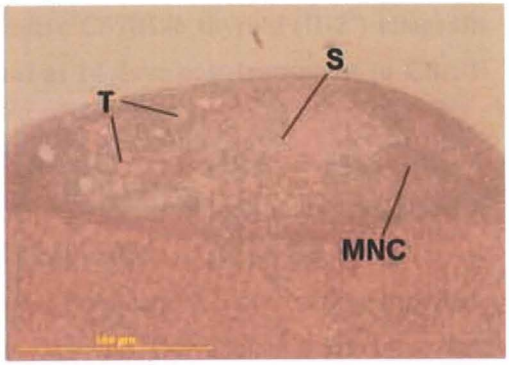
(b)



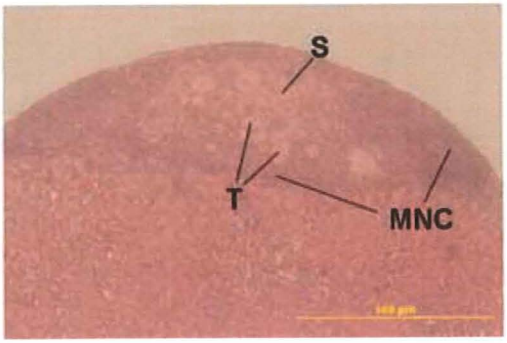
(c)



(d)



(e)



(f)

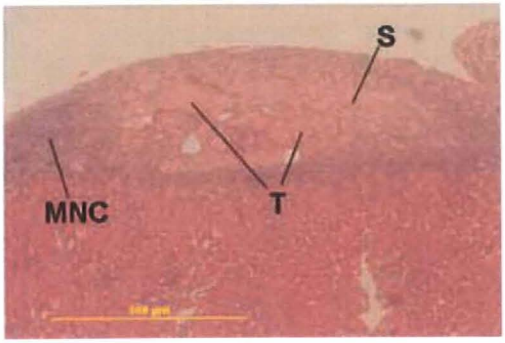


Figure 6-6: Histologic appearance of representative C57BL/6 thyroid (H-2^b) allografts cultured with 0-80μM BAY (24 hour incubation) at 14 days post-transplant to CBA/H (H-2^k) mice.

C57BL/6 thyroid tissue was cultured with (a) 0μM, (b) 5μM, (c) 10μM, (d) 20μM, (e) 40μM, and (f), 80μM BAY11-7082 in 95% O_2 5% CO_2 for 24 hours before transplantation. The cultured tissue was then transplanted beneath the kidney capsule of allogeneic (H-2^k) CBA/H mice on day 0. Grafts were harvested at 14 days post-transplant. A representative histologic section of each group is shown. (T) indicates thyroid follicles, (MNC) indicates mononuclear cell infiltrate and (S) identifies scarring. Hematoxylin and eosin, × 10

rejected BAY pretreated allografts included heavy infiltration, hemorrhage, scarring and extensive loss of intact follicles [Figure 6-6(b)-(f)]. Morphometric analyses confirmed that BAY treatment of thyroid tissue prior to transplantation was not effective in protecting allografts [Figure 6-7]. The percentage of intact follicles in the control allograft display normal kinetics of allograft rejection (percent intact follicles in control cultured allografts was 9.263±4.77; isografts was 43.1±0.56; $p<0.01$)[Figure 6-7]. Likewise, the percentages of intact follicles obtained from BAY-precultured allografts showed no significant difference in graft survival in comparison with the control (percent intact follicles in 5μM BAY cultured allografts was 3.90±2.78; 10μM BAY cultured allografts was 2.70±1.07; 15μM BAY cultured allografts was 8.49±2.69; 20μM BAY cultured allografts was 5.82±1.63; 40μM BAY cultured allografts was 4.99±1.84; 80μM BAY cultured allografts was 9.00±3.42; $p>0.05$) [Figure 6-7]. These results suggest that preculture of mouse thyroid tissue with BAY did not prolong allograft survival.

6.4 cRel$^{-/-}$ thyroid allotransplantation

Despite a report suggesting the absence of cRel in APCs weakens their T cell priming capacity (Boffa et al., 2003), our study showed that cRel deficiency does not affect the capacity of APCs to stimulate allogeneic T cells (Section 5.7). Allotransplantation of cRel$^{-/-}$ thyroid tissue (H-2^{b}) into CBA/H mice (H-2^{k}) was therefore performed to check that lack of cRel in donor passenger leukocytes does not facilitate allograft survival. Allografts were harvested on day 14 post-transplant and graft survival was assessed by histology and morphometric analysis.

cRel$^{-/-}$ thyroid allografts were rejected in a similar manner to WT allografts [Figure 6-8]. cRel$^{-/-}$ thyroid rejection was accompanied by heavy infiltration of leukocytes into the graft site, hemorrhage and loss of thyroid follicles [Figure 6-8]. No notable differences between

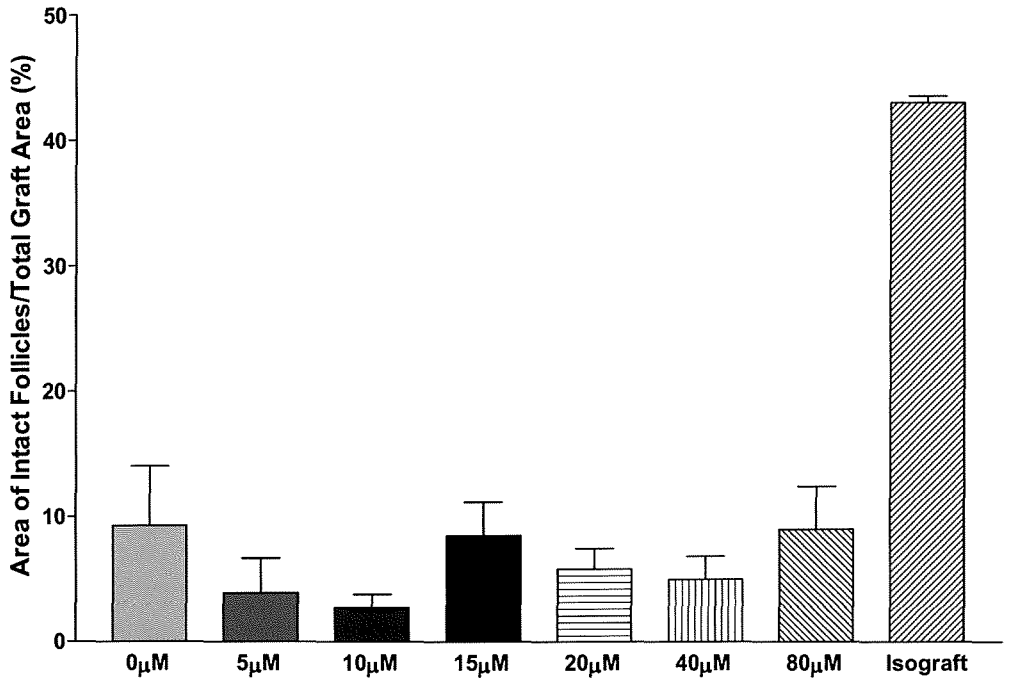


Figure 6-7: Morphometric analysis of C57BL/6 (H-2^b) thyroid allografts cultured with 0μM-80μM BAY11-7082 at Day 14 post-transplant to CBA/H (H-2^k) mice.

C57BL/6 thyroids were cultured with 0μM-80μM BAY11-7082 in 95%O_2 5% CO_2 for 24 hours prior to transplantation. The thyroid tissue was transplanted under the kidney capsule of CBA/H (H-2^k) mice on day 0. The grafts were harvested at 14 days post-transplant. Percent intact follicles of total graft area was calculated as described in Section 2.4.5. Data represent mean±SEM.

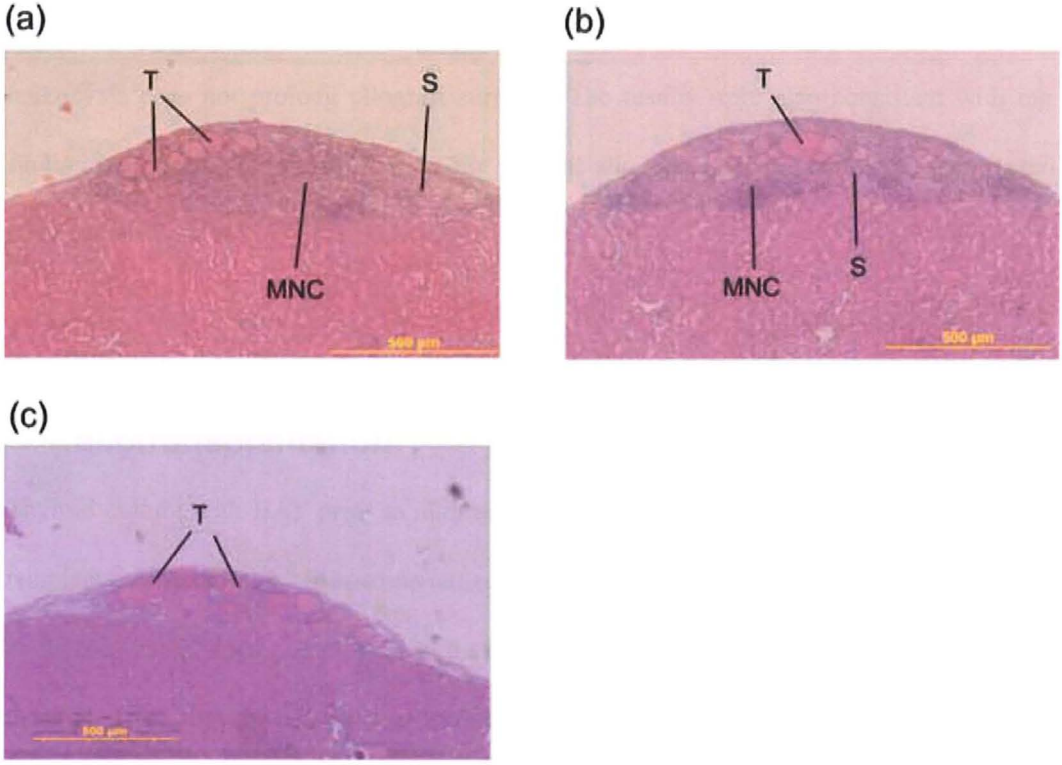


Figure 6-8: Histologic appearance of WT and cRel$^{-/-}$ (H-2^{b}) thyroid allografts at 14 days post-transplant to CBA/H (H-2^{k}) mice.

Thyroids isolated from (a) C57BL/6, (b) cRel$^{-/-}$ (C57BL/6 back ground) and (c) CBA/H mice were transplanted beneath the kidney capsule of CBA/H mice. Grafts were harvested at 14 days post-transplant. A representative histologic section of each group is shown. (T) shows thyroid follicles, (MNC) indicates mononuclear cell infiltrate and (S) shows scarring. Hematoxylin and eosin, × 10.

WT and cRel$^{-/-}$ thyroid allografts were observed histologically on Day 14 post-transplant [Figure 6-8]. Quantitative analysis by morphometry also revealed no significant difference in the presence of intact follicles in the grafted WT and cRel$^{-/-}$ thyroids (percentage intact follicles in WT thyroid allograft was 5.60±3.28; cRel$^{-/-}$ thyroid allografts was 10.86±3.82; $p>0.05$) [Figure 6-9]. These results indicate that the lack of cRel in donor passenger leukocytes does not prolong allograft survival. The results were also consistent with our finding that the inhibition of cRel did not prevent allogeneic T cell priming by Ptx-treated splenocytes (Section 5.7).

6.5 Effect of culturing adult islets with BAY11-7082 prior to allotransplantation

Thyroid culture with BAY prior to allotransplantation did not improve allograft survival in recipients. Although this observation suggests that NF-κB inhibition in passenger leukocytes in thyroid tissue does not prevent allo-stimulation, a possible explanation was that BAY penetrated the thyroid tissue mass inefficiently thereby limiting the capacity of BAY to inhibit immunostimulation by donor passenger leukocytes. Since adult islets represent much smaller units of tissue, adult islet allotransplantation with BAY pretreatment was investigated. Adult islets were isolated from C57BL/6 (H-2^b) mice and cultured in 5μM BAY for 24 hours before transplantation into CBA/H (H-2^k) recipients. Grafts were harvested on Day 14 post-transplant and the survival of islet allografts was assessed by histology and morphometric analysis.

Adult islet isografts cultured without BAY engrafted successfully and by Day 14 intact islets were present at the graft site without cellular infiltration [Figure 6-10(a)]. Isografts precultured with 5μM BAY prior to transplantation also showed intact islet tissue, indicating BAY treatment did not affect islet viability [Figure 6-10(b)]. Morphometric analysis

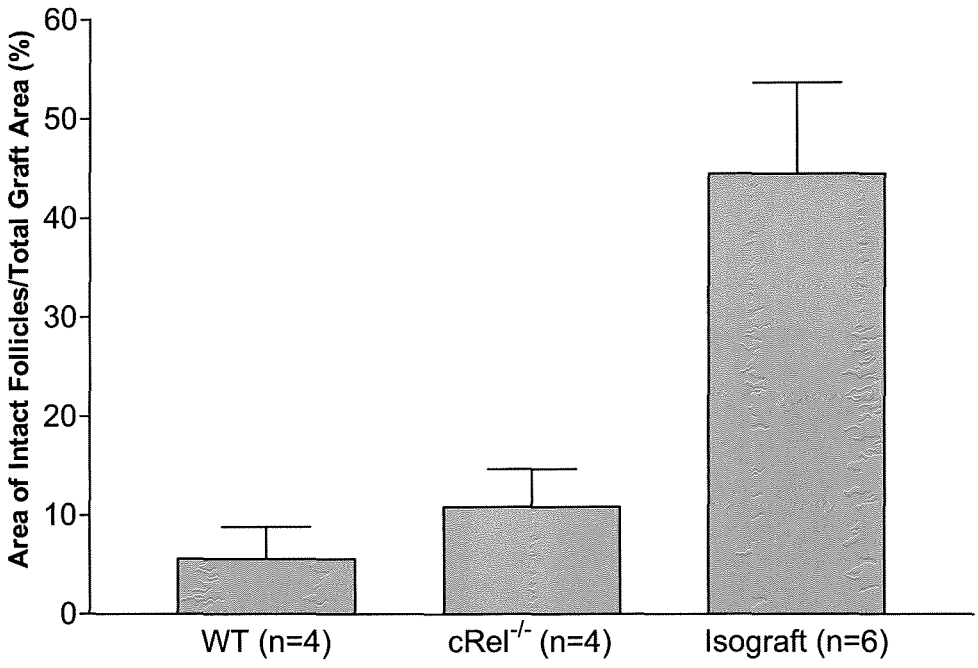


Figure 6-9: Morphometric analysis of WT and cRel$^{-/-}$ thyroid allografts at 14 days post-transplant to CBA/H mice

Thyroids isolated from C57BL/6 WT, cRel$^{-/-}$ and CBA/H mice were transplanted beneath the kidney capsule of recipient CBA/H mice on day 0. The grafts were harvested at 14 days post-transplant. Percent intact follicles of total graft area was calculated as described in Section 2.4.5. Data represent mean±SEM.

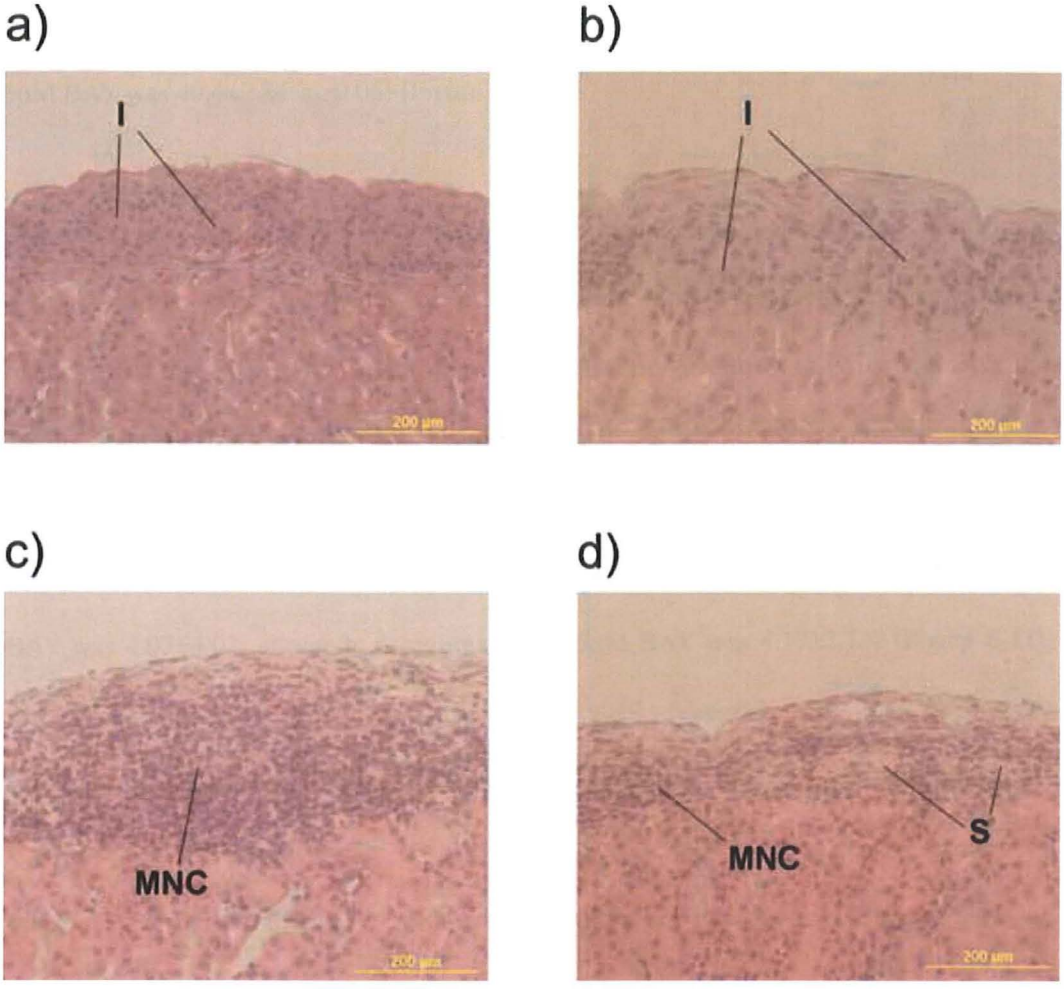


Figure 6-10: Histologic appearance of representative BAY-treated C57BL/6 (H-2^b) islet isografts and allografts at day 14 post-transplant to C57BL/6 or CBA/H (H-2^k) mice.
Adult islets were isolated from C57BL/6 mice. (a) uncultured adult islet isografts were transplanted underneath the kidney capsule of C57BL/6 mice. (b) Adults islets were cultured with 5μM BAY11-7082 in 10%CO_2 90% air for 24 hours prior to transplantation and then transplanted underneath the kidney capsule of C57BL/6 mice. C57BL/6 islets were also transplanted to allogeneic CBA/H (H-2^k) recipients with prior culture alone (c), or (d) with 5μM BAY11-7082 in 10%$CO_2$90% air for 24 hours prior to transplantation. Grafts were harvested at 14 days post-transplant. A representative histologic section of each group is shown. (I) indicates intact islets, (MNC) mononuclear cell infiltrate and (S) scarring. Hematoxylin and eosin, × 20.

confirmed preculture with BAY did not damage islets as there was no significant difference in percentage graft survival between isografts cultured with BAY and without BAY (percent intact islets in isografts precultured without BAY was 43.6±7.31; isografts precultured with 5μM BAY was 40.4±2.84; $p>0.05$) [Figure 6-11].

Allogeneic recipient mice rejected control 24 hr-cultured islets in an acute manner [Figure 6-10(c)]. Grafts contained heavy cellular infiltration and there were few intact islets remaining by day 14 post-transplant [Figure6-10(c)]. Prior treatment of islets with BAY did not improve allograft survival and histologically, the grafts resembled control allografts [Figure 6-10(d)]. Morphometric analysis showed that there was no difference between control and BAY-treated allografts (percentage islet tissue in allograft precultured without BAY was 4.07±4.07; allografts precultured with 5μM BAY was 4.17±2.14) [Figure 6-11]. There was a significant difference between the survival of isografts and allografts ($p<0.01$) but no significant difference between control and BAY-treated allografts at day 14 ($p>0.05$).

These results indicate that BAY treatment of islets did not improve islet allograft survival. The above data, together with thyroid allotransplantation with BAY pretreatment suggests either that the inhibition of NF-κB signaling in passenger leukocytes does not protect allografts from acute rejection or that BAY did not diffuse into isolated islets sufficiently to be taken up by intra-islet leukocytes.

6.6 Thyroid allotransplantation to recipient mice preconditioned with BAY-treated donor splenocyte or BMDC

In Chapter 5, we demonstrated that BAY-mediated inhibition of NF-κB signaling can prevent splenocytes and, to a lesser extent, BMDCs from priming allogeneic T cells (Section 5.3 and

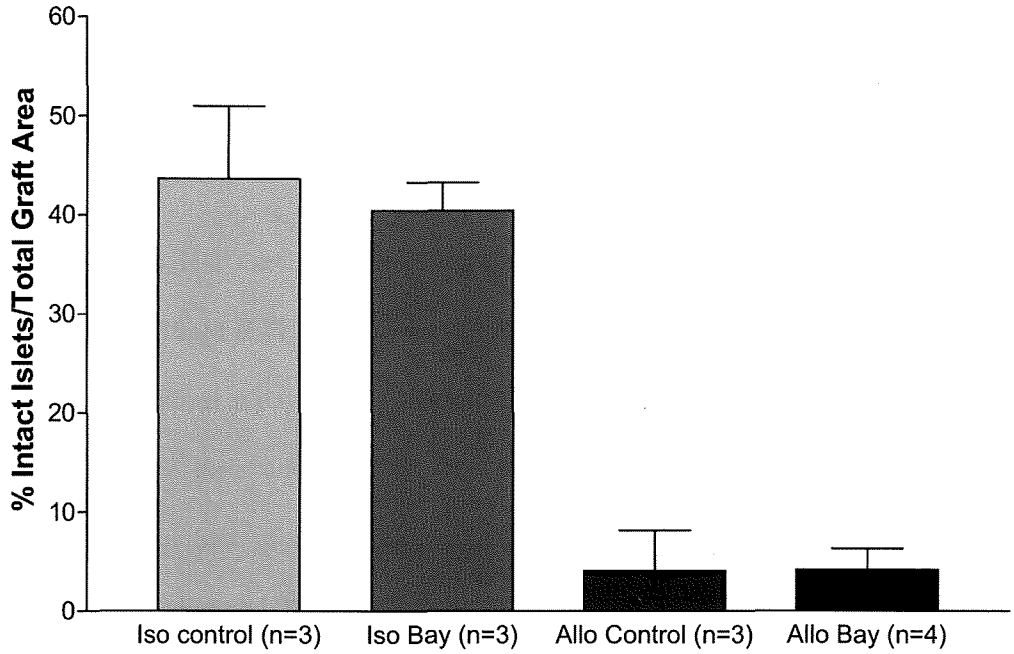


Figure 6-11: Morphometric analysis of C57BL/6 islet isografts and allografts cultured with or without BAY11-7082 prior to transplantation to C57BL/6 (H-2^b) or CBA/H (H-2^k) mice respectively.

Adult islets isolated from C57BL/6 mice were cultured for 24 hours with or without 5μM BAY11-7082 and transplanted underneath the kidney capsule of C57BL/6 (H-2^b) or CBA/H (H-2^k) mice. The grafts were harvested at 14 days post-transplant. Percent intact follicles of total graft area was calculated as described in Section 2.4.5. Data represent mean±SEM.

5.8). It was also demonstrated that BAY splenocytes were unable to render T cells capable of suppressing naïve T cell proliferation (Section 5.9). These observations were in contrast to a previous report demonstrating induction of regulatory T cells in response to BAY BMDC *ex vivo* (Martin et al., 2003). We hypothesized that this discrepancy might have been due to the lack of additional signals required by BMDCs and to their absence in the *in vitro* system. In order to examine this hypothesis, thyroid allotransplantation was performed in recipients pretreated with 3×10^6 donor-type BAY –treated splenocytes (BAY splenocytes) or BAY-treated BMDCs (BAY BMDCs).

Firstly, CBA/H (H-2^k) recipients were injected intravenously with control C57BL/6 (H-2^b) splenocytes or 10μM BAY splenocytes at 7 days prior to transplantation and the survival of grafts was examined at day 14 post-transplant. Mice injected with control donor splenocytes acutely rejected their allografts by day 14; the grafts contained cellular infiltration, hemorrhage and loss of intact follicles [Figure 6-12(a)]. In contrast, delayed allograft rejection was observed when recipients were treated with BAY splenocytes prior to transplantation [Figure 6-12(b)]. Despite cellular infiltration into the graft site, more residual intact follicles were seen in grafts harvested from mice injected with BAY splenocytes compared to the mice injected with control splenocytes [Figure 6-12(b)]. Morphometric analysis also showed a significant increase in the percentage of intact follicles in allografts harvested from BAY splenocyte treated recipients compared with control mice (percent intact follicles in allografts harvested from mice treated with control splenocytes treated mice was 4.54±3.25; 10μM BAY splenocytes was 18.40±1.425829; $p<0.01$) [Figure 6-13].

To elucidate whether the observed improvement in graft survival could also be mediated by immature DCs, 3×10^6 control donor-type BMDCs or 10μM BAY-treated donor–type BMDCs were administered to recipient mice 7 days before transplantation and the allograft

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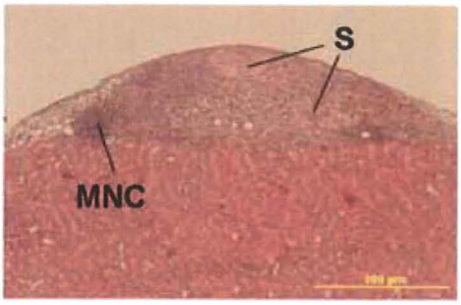

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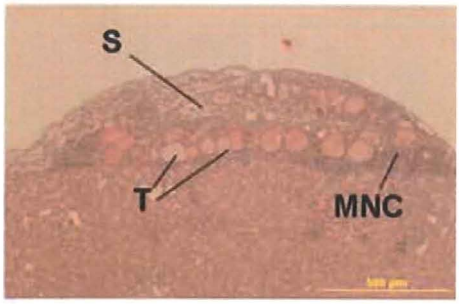

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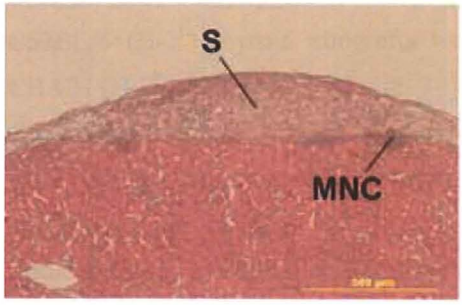
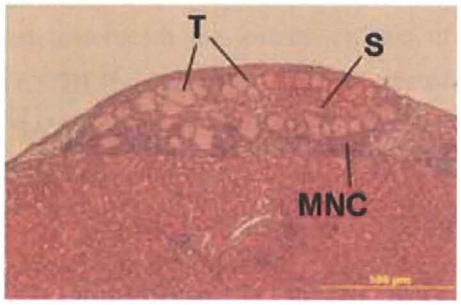


(d)



(e)

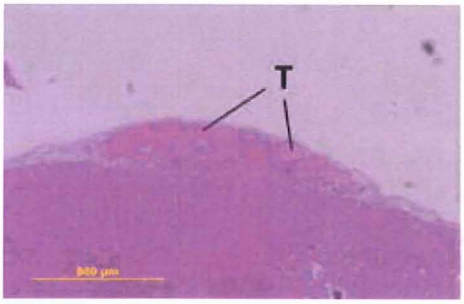


Figure 6-12: Histologic appearance of representative C57BL/6 (H-2^b) thyroid allografts at 14 days-post transplant to CBA/H (H-2^k) mice which received BAY splenocytes or BAY BMDCs 7 days prior to transplantation.

C57BL/6 (H-2^b) thyroid allografts were transplanted underneath the kidney capsule of CBA/H (H-2^k) mice pretreated with 3×10^6 (a) control C57BL/6 splenocytes, (b) BAY-treated C57BL/6 splenocytes, (c) C57BL/6 BMDCs, or (d) BAY-treated C57BL/6 BMDCs 7 days prior to transplantation. CBA/H thyroid isografts were also transplanted underneath the kidney capsule of (e) CBA/H mice as controls. Grafts were harvested at 14 days post-transplant. A representative histologic section of each group is shown. (T) indicates thyroid follicles (MNC) mononuclear cell infiltrate and (S) scarring. Hematoxylin and eosin, × 10.

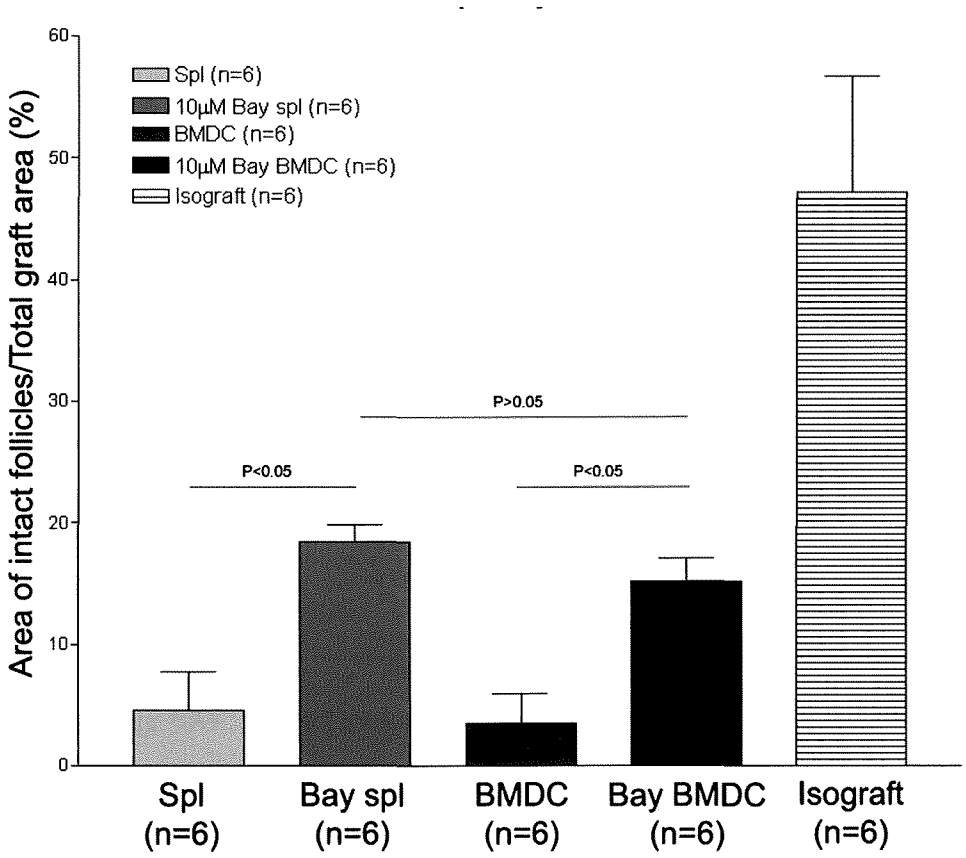


Figure 6-13: Morphometric analysis of C57BL/6 (H-2^b) thyroid allograft at 14 days post-transplant to CBA/H (H-2^k) mice which received BAY splenocytes or BAY BMDCs 7 days prior to transplantation.

CBA/H recipient mice were pretreated with 3×10^6 control C57BL/6 splenocytes, 10μM BAY splenocytes. Untreated BMDCs, or 10μM BAY BMDCs i.v. 7 days prior to thyroid transplantation. C57BL/6 thyroid was transplanted underneath the kidney capsule of CBA/H mice. The grafts were harvested at 14 days post-transplant. Percent intact follicles of total graft area was calculated as described in Section 2.4.5. Data represent mean±SEM.

survival was examined at day 14 post-transplant. The administration of control BMDCs did not prolong the survival of thyroid allografts [Figure 6-12 (c)]. However, pretreatment of host mice with donor BAY BMDCs improved allograft survival and the appearance of allografts was similar to that obtained with BAY splenocyte treated mice [Figure 6-12 (b) and (d)]. Morphometric analysis showed that there was a significant improvement in the percentage of intact follicles in grafts from BAY BMDC treated mice compared to control BMDC-treated hosts (percent intact follicles in allograft harvested from mice treated with control BMDCs was 3.51±2.39; BAY BMDCs was 15.12±1.93; $p<0.01$) [Figure 6-13]. Although complete protection of allografts was not achieved (percent intact follicle in isografts was 47.15±9.55; $p<0.01$), these results demonstrate that the administration of BAY splenocytes or BAY BMDCs prolonged allograft survival.

6.7 Discussion

This chapter describes that the inhibition of direct alloantigen presentation by passenger leukocytes using BAY does not prolong allograft survival. Allografts of both thyroid and islet tissue which were precultured with BAY were rejected, suggesting that direct contact between BAY and the donor leukocytes was required. The current study also demonstrate that pretreatment of allograft recipients with donor-type APCs pretreated with BAY, prolonged allograft survival..

It was anticipated that preculturing allografts with BAY prior to transplantation would have a similar immunomodulatory effect to culture in a high oxygen gas phase, as described by a previous report (Lafferty et al., 1983). However, treatment of allografts with BAY did not prolong graft survival. Results obtained in this chapter indicate that the inhibition of NF-κB signaling in passenger leukocytes either was not achieved or had no effect on the outcome of allografts *in vivo*. The NF-κB dependent anti-apoptotic pathway (Bonizzi and Karin, 2004;

Ferreira et al., 1999) in grafted tissues was unlikely to be affected by BAY treatment as BAY treated isografts survived without any signs of graft damage at day 14 post-transplant. Since BAY-treated APCs failed to prime allogeneic T cells in Section 5.3 and 5.7, the transplantation data suggests that BAY may not penetrate tissues efficiently. It was previously shown that thyroid allograft tolerance induced by long-term high oxygen organ culture can be reversed by administration of donor-derived splenocytes (Simeonovic et al., 1984). It is therefore informative to investigate whether long-term high oxygen organ culture of thyroid is rejected when recipient mice were reconstituted with donor splenocytes treated with or without BAY. Alternative explanation could be that the effect of BAY treatment in passenger leukocytes was limited by IκB protein turnover. Newly synthesized IκB protein in passenger leukocytes would not be subjected to inhibition (as discussed in Section 5.11) thus passenger leukocytes could be activated post-transplant and stimulated recipient T cells inducing allograft rejection.

This chapter demonstrated pretreatment of recipients with BAY-treated donor splenocytes prolonged allograft survival despite the inability of BAY splenocytes to induce T cell anergy or regulatory T cell differentiation *in vitro* (Section 5.9). The cell types responsible for delaying the allograft rejection process and the mechanism involved remain to be elucidated. The delay in allograft rejection in recipients pretreated with BAY-treated donor BMDCs suggested that the DC population contained in BAY splenocytes might be responsible for mediating the graft protective effect of the latter. Experiments using splenic DCs treated with BAY or removal of DCs from Bay-treated splenocytes however would more accurately test this notion. It is possible that BAY splenocytes or BAY BMDCs induced regulatory T cell differentiation *in vivo*. The capacity of BAY BMDCs to promote regulatory T cell differentiation has been previously reported (Martin et al., 2003). The study by Martin *et al.* demonstrated antigen-specific regulatory T cell proliferation *ex vivo* after pretreating mice *in*

vivo with BAY BMDCs (Martin et al., 2003). To assess regulatory T cell differentiation in the current model, phenotypic analysis of T cell recovered from BAY splenocyte- or BAY BMDC-treated allograft recipients would be required to determine whether $CD4^+CD25^+$ T cells were induced. Studies on expression of the regulatory T cell-specific transcription factor, Foxp3, may also address regulatory T cell development in response to BAY splenocytes/BMDCs. *Ex vivo* proliferation assays of transplant recipient T cells with naïve T cell populations may also be useful in assessing immunogenic or tolerogenic properties of T cells exposed to BAY splenocytes/BMDC treated mice *in vivo*. IL-10 and TGF-β are often associated with tolerance induction by regulatory T cells (Hara et al., 2001; Josien et al., 1998). If T cells were to obtain immunomodulatory properties by interacting with BAY splenocyte/BMDC *in vivo*, their capacity to secrete immunosuppressive cytokines including IL-10 and TGF-β could also need to be investigated. Alternatively, cell contact-dependent suppression of alloreactive T cells by surface bound TGF-β or CTLA-4 on BAY treated-APCs or T cell harvested from BAY-APC-treated mice, respectively may also require examination. In addition, if BAY-treated APCs have the capacity to promote allograft protection, possibly by inducing regulatory T cells, then passenger leukocytes within BAY-precultured thyroid tissue or isolated islets would similarly be rendered tolerogenic. Lack of prolonged allograft survival with BAY precultured thyroid and islets may indicate this protective effect mediated by BAY-treated APCs was dose-dependent and the absolute number of APCs carried within the graft might be insufficient to induce such a regulatory or protective effect.

The observed prolongation of allografts by pretreatment of host mice with BAY splenocytes/BAY BMDCs *in vivo* also indicates that these APCs are able to reduce T cell response towards a secondary donor-type stimulus. The discrepancy between *in vitro* and *in vivo* results suggests that signals in addition to those provided by BAY splenocytes or BAY

BMDCs might have contributed to the reduction of the anti-allograft T cell response. The source of these additional signals remains unclear. Previous studies suggested the secondary lymphoid tissues are important for regulatory T cell development (Garrod et al., 2006; Ochando et al., 2005). Saiki *et al.* reported that donor APCs migrated to draining LNs and interacted with recipient T cells at these sites *in vivo* (Saiki et al., 2001). Based on these studies, it can be hypothesized that BAY splenocytes and BAY BMDCs modulate recipient T cell at secondary lymphoid organs with additional signals provided at these sites. Regulatory T cell development following administration of BAY treated DCs *in vivo* (Martin et al., 2003), but not *in vitro* (Thompson et al., 2004), further supports the requirement of secondary lymphoid tissues in facilitating tolerance induction.

The current study did not provide sufficient evidence to determine whether prolonged graft survival was a consequence of reduced T cell function in a general or antigen-specific manner. In order to assess this question, simultaneous third party allograft transplantation may need to be performed. If the effect of BAY splenocytes or BAY BMDCs were antigen-specific, the current donor-recipient combinations would allow prolonged allograft survival while the kinetics of the third party allograft rejection would not be affected. MLRs of T cells, harvested from the recipients, with stimulators of different donor H-2 haplotypes would also clarify the antigen specificity of immune modulation mediated by BAY splenocytes or BAY BMDCs.

In summary, the current chapter demonstrates that preculturing thyroid tissue and adult islets with BAY before transplantation does not promote allograft survival. This finding suggests that either the NF-κB pathway was not inhibited in passenger leukocytes or has little effect on preventing the direct pathway of allograft rejection. Pretreatment of recipients with BAY splenocytes or BAY BMDCs, on the other hand, significantly delayed allograft rejection,

indicating APCs lacking NF-κB may be useful in inducing transplantation tolerance. However, the mechanism employed by these treatments in promoting prolonged allograft survival needs to be examined in future studies.

Chapter 7

General discussion

7.1 General discussion

This thesis investigated the potential of two molecular pathways, the Notch and NF-κB pathways, to generate tolerogenic DCs capable of protecting allografts. Notch signaling is well known to play critical roles during the development of the immune system (Amsen et al., 2004; Izon et al., 2001; Kim and Siu, 1998; Ohishi et al., 2001; Osborne and Miele, 1999; Pear and Radtke, 2003; Robey, 1999; Wong et al., 2003). Recent studies have revealed that Notch signaling may be important in tolerance induction (Anastasi et al., 2003; Hoyne et al., 2000; Hoyne et al., 2000; Wong et al., 2003). In an attempt to induce allograft-specific tolerance, Chapter 3 and 4 examined whether over-expression of the Notch ligands Jagged-1 and Delta-l1, or Notch signaling modifiers Mfng, and Lfng, promote the development of tolerogenic DCs. We used the pKMV-retroviral system of gene transfer to induce over-expression of Notch-related molecules on the immature DC line, JAWS II, and examined whether *in vivo* these transduced DCs modulated anti-allograft responses. The NF-κB pathway is another immunoregulatory pathway and it is well established that NF-κB signaling is crucial in T cells and DCs to mount effector responses (Andreakos et al., 2003; Caamano and Hunter, 2002; Ferreira et al., 1999; Finn et al., 2001; Ouaaz et al., 2002; Sha et al., 1995; Yoshimura et al., 2001). Recent developments have indicated that blockade of the NF-κB pathway in APCs may facilitate alloantigen-specific tolerance (Saemann et al., 2004; Tomasoni et al., 2005; Xu et al., 2004). The NF-κB inhibitors, BAY11-7082 and pentoxifylline (Ptx), were therefore used to examine whether inhibition of the NF-κB pathway in APCs can be utilized to prevent alloreactive T cell responses (Chapter 5) and to inhibit the rejection of thyroid and islet allografts (Chapter 6).

Notch signaling has been shown to contribute to the induction of antigen-specific tolerance. It was reported that over-expression of Jagged-1 on APCs promotes the differentiation of antigen-specific $CD4^+$ regulatory T cells in allergy and EBV models (Hoyne et al., 1999;

Hoyne et al., 2000; Vigouroux et al., 2003). Such tolerance was shown to be attributed to enhanced production of IL-10 by regulatory T cells (Vigouroux et al., 2003). In the context of transplantation, Wong *et al.* demonstrated that pretreatment of recipient mice with L-cell over-expressing Delta-l1 and alloantigen prolonged the survival of cardiac allografts by promoting the expansion of $CD8^+$ regulatory T cells *in vivo*. These studies led us to examine the capacity of immature DCs over-expressing Notch related molecules to induce allograft tolerance.

Retroviral-mediated gene transduction with centrifugal enhancement resulted in the enhanced expression of Jagged-1, Delta-l1, Mfng and Lfng on JAWS II cells and stable incorporation of the genes of interest without affecting their maturation status. MLRs with allogeneic T cells revealed that over-expression of these Notch-related molecules did not alter the capacity of JAWS II cells to prime alloreactive T cells *in vitro*. Pretreatment of thyroid allograft recipients with JAWS II cells over-expressing Notch-related molecules also failed to induce prolonged allograft survival. When cellular transplantation was performed to examine the immunogenicity and survival of transduced JAWS II cells in allogeneic recipients, JAWS cell allografts were acutely rejected. These findings confirmed that over-expression of Notch-related molecules on these immature DCs failed to modulate the recipient immune response against alloantigens, in contrast to previous reports that APCs over-expressing Jagged-1 or Delta-l1 are tolerogenic (Hoyne et al., 1999; Hoyne et al., 2000; Wong et al., 2003). It is proposed that 5(and6)-carboxy fluorescein diacetate succinimidyl ester (CFSE) labeling of JAWS II cells with or without Notch-related molecules, could be used to track JAWS II cells following systemic administration to allogeneic mice. Furthermore, since secondary lymphoid organs are important for mounting anti-graft response (Larsen et al., 1990; Saiki et al., 2001), tracking the migration of CSFE-labeled JAWS II cells in allograft recipients may identify the location of direct presentation of

allopeptide by donor APCs and hence alloactivation.

Although primary immature DCs instead of the JAWS II cell line would have been a more relevant method for assessing the capacity of Notch signaling to induce tolerance to alloantigen, poor retroviral transduction efficiency in primary BMDCs prevented this approach in the current project. Nevertheless, repeated transduction of BM cells could be used in future to enhace the efficiency of gene transduction. Recent studies indicate that another DC subset, plasmacytoid DCs, may also be important for inducing allograft tolerance. Transfusion of BM-derived plasmacytoid DC precursors before cardiac allograft transplantation induced prolonged survival of allografts by significantly reducing donor alloantigen specific T cell proliferation (Abe et al., 2005). Similarly, administration of plasmacytoid DC precursors to skin allograft recipient significantly prolonged allograft survival (Fugier-Vivier et al., 2005). Ochando *et al.* also demonstrated that allograft tolerance can be transferred to naïve syngeneic mice by plasmacytoid DCs isolated from LNs of cardiac allograft tolerant mice (Ochando et al., 2006). The mechanism was attributed to the expansion of $CD4^+CD25^+Foxp3^+$ T cells in the secondary hosts (Ochando et al., 2006). The study showed that migration of plasmacytoid DCs presenting donor-derived MHC peptide to to T cells in recipients' LNs, but not spleen, is critical for plasmacytoid DC-mediated induction of allograft tolerance (Ochando et al., 2006). Based on these reports, it would be worthwhile investigating whether over-expression of Notch-related molecules on plasmacytoid DCs enhances their tolerogenic properties in allotransplantation settings. Alternative approaches to examining the role of Notch signaling in induction of allograft tolerance might be analysis of Notch-related molecule expression on $CD4^+CD25^+$ regulatory T cells or plasmacytoid DCs isolated from tolerant mice.

Studies employing knockout mice established that NF-κB signaling is an essential

component for promoting DC maturation (Baltathakis et al., 2001; Boffa et al., 2003; Clark et al., 1999; Hofer et al., 2001; Neumann et al., 2000). In addition, other studies revealed that blockade of NF-κB signaling in DCs by NF-κB inhibitors, NF-κB decoy oligodeoxynucleotides (ODN) (Grigoriadis et al., 1996; Xu et al., 2004; Xu et al., 2004), pyrrolidine dithiocarbamate (PDTC) (Saemann et al., 2004), or gene targeting, or insertion of a dominant negative form of IKK2 (Tomasoni et al., 2005), generated tolerogenic DCs capable of inducing alloantigen-specific tolerance. These NF-κB signaling-deficient DCs were prevented from upregulating the expression of costimulatory molecules and from producing inflammatory cytokines such as IFN-γ, TNF-α and IL-10 (Tomasoni et al., 2005; Xu et al., 2004; Xu et al., 2004). Prolongation of allograft survival following administration of these DCs to allograft recipients in the present study indicated that inhibition of NF-κB in donor-derived APCs (DCs) may be beneficial in inducing tolerance to cellular allografts.

In agreement with previous reports, treatment of naïve APCs with BAY completely inhibited their capacity to prime T cells. This was found to correlate with downregulation of CD86 and MHC Class II expression and inhibition of the production of IL-2, IFN-γ, TNF-α, IL-4 and IL-5. This was consistent with previous findings that NF-κB is involved in the regulation of costimulatory molecules (Ardeshna et al., 2000; O'Sullivan et al., 2000; Saemann et al., 2004; Yoshimura et al., 2001) and cytokine production (Finn et al., 2001; Grigoriadis et al., 1996; Mason et al., 2002; Mora et al., 2001; Yoshimura et al., 2001). The inability of Ptx to inhibit the capacity of splenocytes to prime alloreactive T cells indicated that inhibitory effects of BAY on splenocytes were cRel-independent. This finding was supported by Boffa *et al.* who demonstrated the LPS-induced maturation of cRel deficient DCs and allogeneic T cell proliferation in response to DCs lacking cRel (Boffa et al., 2003). Interestingly, T cell proliferation in response to splenocytes which were first pretreated with BAY then stimulated with LPS/cytokines (Pre-BAY Act splenocytes) suggested that BAY did not prevent

activation induced by LPS/cytokine. Since splenocytes were stimulated for 48 hours after the withdrawal of BAY, it can be speculated that activation occurred as a consequence of IκB protein turnover. This may have allowed the newly synthesized IκB (which is not subject to irreversible BAY-mediated inhibition) to activate the NF-κB pathway, thereby activating APCs.

Although BAY treatment prevented splenocytes from inducing the proliferation of alloreactive T cells, these T cells were able to subsequently proliferate in response to naïve splenocytes (secondary stimulus), indicating that T cells exposed to BAY splenocytes did not become anergic. Inability of these T cells to prevent naïve T cell proliferation *in vitro* also revealed that there was no expansion of regulatory T cells in response to BAY treated splenocytes, however, pretreatment of thyroid allograft recipients with BAY splenocytes or BAY BMDCs induced prolonged survival of allografts *in vivo*. This observation was consistent with previous studies demonstrating that BAY-treated BMDCs were able to promote regulatory T cell differentiation *in vivo* (Martin et al., 2003) but not *in vitro* (Thompson et al., 2004). These studies collectively indicate additional signal(s) present *in vivo* that were absent *in vitro* are required for BAY BMDCs to induce allograft tolerance. Furthermore, previous reports have indicated that draining LNs are a critical requirement for facilitating the interaction of DCs with $CD4^+$ T cells and subsequent regulatory T cell differentiation (Bai et al., 2002; Ochando et al., 2006; Ochando et al., 2005). Whether allograft protection following administration of BAY-treated splenocytes was mediated by one or more DC subsets contained in BAY splenocytes is unresolved. Prolonged allograft survival achieved by pretreating recipients with BAY BMDCs suggests that BAY-treated splenic DCs should be tested for their capacity to induce allograft protection. Further study should also investigate how these APCs which are deficient in NF-κB signaling, act to reduce the capacity of allograft recipients to mount anti-graft responses. Phenotypic analyses

of BAY splenocytes and BMDCs indicated that they express reduced levels of CD86 and MHC Class II molecules. BAY splenocytes were unable to produce IL-2, IL-4, IFN-γ and TNF-α and produce less IL-5. Whether these BAY-treated APCs induce expansion of regulatory T cells still remains to be elucidated. The ability of BAY BMDCs to promote $CD4^+$ regulatory T cell differentiation *in vivo* (Martin et al., 2003) suggests that prolonged allograft survival in the current study may be mediated by the expansion of $CD4^+$ regulatory T cells. In addition, third party allografts may also be useful to further examine whether the observed allograft protection was donor alloantigen-specific.

It has been established that direct alloantigen presentation by donor passenger leukocytes together with their capacity to provide costimulatory signals play an important role in initiating the acute rejection of cellular allografts (Gould and Auchincloss, 1999; Lafferty et al., 1975; Lafferty et al., 1983; Simeonovic et al., 1984). Removal of passenger leukocytes by culturing thyroid or pancreatic islets in a high oxygen environment results in indefinite survival of allografts (Lafferty et al., 1975; Lafferty et al., 1983; Simeonovic et al., 1984). Since BAY splenocytes were not able to induce alloreactive T cell proliferation, thyroid and islet tissues were precultured with BAY to inhibit donor passenger leukocyte-mediated immunostimulation of host alloreactive T cells. Acute rejection of BAY cultured allografts indicated that BAY treatment for 24 hours is much less efficient than high oxygen culture for modulating tissue immunogenicity. It is also possible that BAY was not able to efficiently penetrate the cultured tissue thus direct contact between BAY and intragraft passenger leukocytes might not have been achieved. Extending the length of tissue culture with BAY may enhance the chance of direct contact between BAY and passenger leukocytes. An alternative approach would be to transplant high-oxygen cultured allografts and then administer donor splenocytes with or without BAY-treatment early post-transplant. This is based on a previous finding that indefinite survival of allografts precultured in a high oxygen

gas phase can be prevented by the early administration of donor splenocytes (Simeonovic et al., 1984), which thereby reconstitute recipient animals with donor passenger leukocytes. If the allografts are rejected following administration of BAY splenocytes, it may indicate BAY-pretreatment of thyroid or islet tissue prior to transplantation may not be an effective approach to promote allograft survival. Alternatively, if BAY-treated splenocytes prevented the induced allograft rejection response, it could be argued that acute treatment donor tissue with BAY *in vitro* is not effective in modulating the immunogenicity of passenger leukocytes e.g. due to inadequate penetration into the tissue mass.

Interaction between Notch and NF-κB pathways have been previously described (Bash et al., 1999; Nickoloff et al., 2002; Oswald et al., 1998; Wang et al., 2006). However, the nature of these signaling interactions remain to be further investigated. Recent studies reported that Notch-1 induced NF-κB expression by direct interaction with NF-κB1 in the nucleus (Shin et al., 2006). Moreover, siRNA-mediated silencing of Notch-1 was shown to inhibit NF-κB DNA binding activity (Wang et al., 2006). The synergistic relationship between Jagged-1-induced-Notch activation and NF-κB activation has also been reported (Bash et al., 1999; Nickoloff et al., 2002). Bash *et al* demonstrated a correlation between Jagged-1 and cRel expression in B cells (where cRel induces B cell activation via Jagged-1 expression) (Bash et al., 1999). In contrast, Oswald *et al*. reported that Notch-1 activation results in suppression of NF-κB2 via RBP-Jκ (one of the genes targeted by Notch signaling) activation in the Jerkat T cell line (Oswald et al., 1998). Whether interactions between Notch and NF-κB signaling occur to induce differentiation of regulatory T cells is not understood. If Notch and NF-κB signaling act in synergy, this would contradict previous findings that tolerogenic APCs/DCs can be generated by either over-expressing Notch ligands (Hoyne et al., 1999; Wong et al., 2003) or inhibiting the NF-κB pathway (Saemann et al., 2004; Tomasoni et al., 2005; Xu et al., 2004). The mechanism underlying the communication

between these two pathways requires further investigation. Since both pathways have been reported to be involved in regulatory T cell differentiation, it is possible that interaction between the Notch and NF-κB pathways occurs during induction of antigen-specific regulatory T cell development. It would therefore be worthwhile to investigate whether NF-κB-deficient DCs capable of promoting prolonged survival of allografts exhibit different levels of Notch ligand/modulator expression compared to non-tolerogenic DCs, suggestion a modified Notch signaling pathway.

In conclusion, this thesis investigated the induction of allograft survival by over-expressing Notch related molecules on DCs or by inhibiting NF-κB signaling in DCs. Analysis revealed that over-expression of Notch related molecules did not alter T cell responses to alloantigens and pretreating allograft recipients with JAWS II cells over-expressing Notch related molecules failed to promote allograft survival. Inhibition of the NF-κB pathway in APCs prevented their capacity to stimulate alloreactive T cells in a cRel-independent manner *in vitro*, however, this was not due to anergy or regulatory T cell differentiation. While inactivation of donor passenger leukocytes by preculturing thyroid or islets with BAY did not protect allografts from rejection, pre-transplant administration of BAY-treated splenocytes or BAY BMDCs which lack NF-κB signaling resulted in prolonged allograft survival. The graft-protective effects of BAY splenocytes and BAY BMDCs *in vivo* also indicate that additional signals present *in vivo* may be required for these DCs to facilitate such beneficial effects. Although the mechanism(s) by which the blockade of NF-κB in DCs facilitated allograft protection still requires further investigation, these findings present the NF-κB pathway as an attractive target for the induction of allograft tolerance.

Chapter 8

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8.1 Bibliography

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Appendix

Appendix 1 Media and Buffers

Medium	Contents
JAWS II medium	Dulbecco's Modified Eagle's Medium (DMEM) alpha medium (GibcoBRL Life Technologies, Gaitherberg, MD) with 20% low endotoxin level heat inactivated fetal calf serum (HIFCS) (Sigma, St Louis, MO), 60ng/ml penicillin G (Media and Washup Facility, JCSMR) ,100ng/ml streptomycin (Media and Washup Facility, JCSMR), 100ng/ml neomycin (Media and Washup Facility, JCSMR), and 5μg/ml GM-CSF (PeproTech Inc., Rocky Hill, NJ)
Phoenix Eco Medium	DMEM with 10% HIFCS (GibcoBRL), and 60ng/ml penicillin G (Media Unit, JCSMR), 100ng/ml streptomycin (Media and Washup Facility, JCSMR), 100ng/ml neomycin (Media and Washup Facility, JCSMR)
MLC medium	RPMI medium (GibcoBRL), 0.2% 2ME, 1% N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) Buffer (ARH Bioscience, Lenexa, KS), 1% 1mM L-Glutamine, 1% sodium pyruvate (GibcoBRL), 1% Non-essential amino acid (GibcoBRL), 10% HIFCS(GibcoBRL), 60g/ngml penicillin G (Media Unit, JCSMR), 100ng/ml streptomycin (Media and Washup Facility, JCSMR), 100ng/ml neomycin (Media and Washup Facility, JCSMR)
EL-4 Culture Medium	F15 medium, 5% HIFCS(GibcoBRL), 60g/ngml penicillin G (Media Unit, JCSMR), 100ng/ml streptomycin (Media and

		Washup Facility, JCSMR), 100ng/ml neomycin (Media and Washup Facility, JCSMR)
BMDC flush medium		RPMI1640 medium (GibcoBRL) with 5% HIFCS(GibcoBRL), 60g/ngml penicillin G (Media Unit, JCSMR), 100ng/ml streptomycin (Media and Washup Facility, JCSMR), 100ng/ml neomycin (Media and Washup Facility, JCSMR)
BMDC culture medium		RPMI1640 medium (GibcoBRL) with 10% HIFCS(GibcoBRL), 5μg/ml GMCSF, 60g/ngml penicillin G (Media Unit, JCSMR), 100ng/ml streptomycin (Media and Washup Facility, JCSMR), 100ng/ml neomycin (Media and Washup Facility, JCSMR)
Red cell lysis buffer (pH 7.3)		ddH_2O with 0.94% (w/v) ammonium chloride, 0.0037% (w/v) EDTA, 0.1% (w/v) sodium hydrogen carbonate (AJAX chemicals, NSW, Australia).
FACS Buffer		PBS (Media and Washup Facility, JCSMR) supplemented with 2%HIFCS(GibcoBRL) and 0.2% sodium azide (BDH Laboratory Supplies, UK, Cat:30111)
For adult islet preparation	Buffer A	HBSS (Media and Washup Facility, JCSMR)supplemented with 2% 1M HEPES(ARH), 3% BSA (Sigma), 0.1% 30μg/ml penicillin G (Media and Washup Facility, JCSMR), 0.1% 50μg/ml streptomycin (Media and Washup Facility, JCSMR), 50μg/ml neomycin (Media and Washup Facility, JCSMR), 10μg/ml DNase (Sigma, Cat: D5025-150KU)

	Buffer B	HBSS (Media and Washup Facility, JCSMR)supplemented with 2% 1M HEPES(ARH), 10% HIFCS(GibcoBRL), 60g/ngml penicillin G (Media Unit, JCSMR), 100ng/ml streptomycin (Media and Washup Facility, JCSMR), 100ng/ml neomycin (Media and Washup Facility, JCSMR)
Nuclear extraction	Buffer A	ddH_2O supplemented with 10mM Tris(pH7.5) (Sigma, Cat; T3253), 10mM $NaCl_2$ (AJAX), 3mM $MgCl_2$ (Sigma), 0.1mM EDTA (Sigma), and 0.5% NP-40 (IGEPAL CA-630; Sigma, Cat: 1886)
	Buffer A-NP40	ddH_2O supplemented with 10mM Tris(pH7.5) (Sigma,), 10mM NaCl, 3mM $MgCl_2$, and 0.1mM EDTA (Sigma)
	Buffer C	MilliQ H_2O supplemented with 400mM $NaCl_2$, 7.5 mM $MgCl_2$ (Sigma), 0.2 mM EDTA (Sigma), 0.1 mM EGTA (Sigma), and 1mM DTT (Sigma)
Western Blot	SDS Running Buffer	196mM glycine (Merck, VIC, Australia) , 0.1% SDS (Sigma) , and 50mM Tris-HCl pH 8.3 (Sigma)
	Transfer buffer	20% ethanol, with 25mM Tris (Sigma), and 192mM glycine (Merck)

Appendix 2 Primers used for PCR reactions and their conditions

Primer	Sequence (5'-3')	Conditions and Cycles
GFP forward	GCTCGAGATGGTGAGCAAGGGCGAG	94°C for 3 mins followed by 25, 30, and 35 cycles of 94 °C 30s; 64°C 1 min; 72°C 1min
GFP reverse	GCTTTACTTGTACAGCTCGTC	

mJag-1 forward	CAGAATGACGCCTCCTGTCG	95°C for 5 mins followed by 25, 30, and 35 cycles of 95°C 10s; 58°C 10s; 72°C 30s
mJag-1 reverse	TGCAGCTGTCAATCACTTCG	
mLfng forward	AAGACCACCAGAAAGTTTCACC	95°C for 5 mins followed by 25, 30, and 35 cycles of 95°C 10s; 58°C 10s; 72°C 30s
mLfng reverse	AAACCAAAAGTGGACAGGTCTC	
mMfng forward	TCACTGACAGCCCAGATGAAC	94°C for 5 mins followed by 25, 30, and 35 cycles of 94 °C 30s; 53°C 30s; 72°C 45s
mMfng reverse	TGCACTCGATGATGTAGCCC	
mDll-1 forward	ACCTCGGGATGACGCCTTTG	95°C for 2 mins followed by 25, 30, and 35 cycles of 95°C 20s; 60°C 30s; 72°C 1 min
mDll-1 reverse	AGACCACCACAGCAGCACAG	
GAPDH forward	ACCACAGTCCATGCCATCAC	94°C for 3 mins followed by 25, 30, and 35 cycles of 94 °C 30s; 60°C 30s; 72°C 45s
GAPDH reverse	TCCACCACCCTGTTGCTGTA	

Table 1: Primers used for RT-PCR analyses and their reaction conditions

Appendix 3 Primers used for PCR amplification of Dll-1 with attB sites on 5' and 3' ends and their reaction conditons

Primer	Sequence (5'-3')	Condition and Cycle
Dll-1 attB forward	GGGGACAAGTTTGTACAAAAAAGCAGGC TGGTACCATGGGCCGTCGG	94°C 2 mins followed by 35 cycles of 94°C 15 secs, 68°C 3 mins
Dll-1 attB reverse	GGGGACCACTTTGTACAAGAAAGCTGGG TTCTTACACCTCAGTCGCTATAACACACT CATC	

Table 3: Primers used for PCR amplification of Dll-1 with attB sites on 5' and 3' ends and their reaction conditions.

Appendix 4 Primers used for Sequencing

Primer	Sequence (5'-3')
mJag-1 F1	GTAACACCTTCAATCTCAAG
mLfng F1	CGTTCATCTTCACTGATG
mMfng F1	TCGTGGTCACCAACTGT
mDelta-l1 F1	GAACTCCTGCAGCTTCAGC

Appendix 5 Sequencing data

pKMV-GFP-mJag-1 Sequence

Sequence obtained from Jagged F1

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GCATGGGTCTTGTAAACTTCCAGGTGACTGCAGGTGCCAGTACGGTTGGCAGGGCCT
GTACTGCGACAAGTGCATCCCGCACCCAGGATGTGTCCACGGCACCTGCAATGAACC
CTGGCAGTGCCTCTGTGAGACCAACTGGGGTGGACAGCTCTGTGACAAAGATCTGAA
TTACTGTGGGACTCATCAGCCCTGTCTCAACCGGGGAACATGTAGCAACACTGGGCCT
GACAAATACCAGTGCTCCTGCCCAGAGGGCTACTCGGGCCCCAACTGTGAAATTGCT
GAGCATGCTTGTCT

Matching Sequence

Mus musculus jagged 1 (Jag1), mRNA

Length=5377

Score = 1283 bits (647), Expect = 0.0

Identities = 647/647 (100%), Gaps = 0/647 (0%)

Strand=Plus/Plus

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pKMV-GFP-mMfng Sequence

Sequence obtained from Manic F1

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TGCATCAACCGCCAACTGGCTTTGAAGATGGTGCCATGGGCCAGCGGCTCCCACTTTG
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CCCCTCCAGATTCCGCTCCCTCCATTGTCTCCTCTACCCAGACACACCCTGGTGTCCG
CTGCTGGCAGCGCCCTGAGCACCCAGCTTTCTTGTACAAAGTGGTGATGATCTCTCGA

Matching Sequence

Mus musculus manic fringe homolog (Drosophila) (Mfng), mRNA
Length=1846

Score = 1063 bits (536), Expect = 0.0
Identities = 536/536 (100%), Gaps = 0/536 (0%)
Strand=Plus/Plus

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pKMV-GFP-mLfng Sequence

Sequence obtained from Lunatic F1

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Matching Sequence

Mus musculus lunatic fringe gene homolog (Drosophila) (Lfng),
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pKMV-GFP-Dll-1 Sequence

Sequence obtained from Delta-1SeqF1

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CAAACCGAAAGCAAAAATTCAGACGGAGGCGGGAACTGTTTTAGGTTCTCGTCTCCT
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GTTCAGAACTCGTCAGTTCCACCACGGGTCCGCCAGATACAGAGCTAGTTAGCTAACT
AGTACAGACGCAGGCGCAAACATTAGATGCCGGCACAAACACGCACAAAGACAGAG
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AGTCATCAATCTGAGGAGACCCTCCCAAGGATCAGCGAGTCCACGATTCGGATGCCA
ACAGCAAGGAGGCTTTATGGGAATACGGTACCGGGCCGACGCAGTCTATCCGGAGGA
CTGCCGCCGAGTGAGGGTTGTGGCTCTTTTTTGAGCTCGGGGAGCGAGCGCGCAAAG
AAGCGGAGAGGAAACTGAATGGTTAATTTCCACTAG

Matching Sequence

Mus musculus delta-like 1 (Drosophila) (Dll1), mRNA

Length = 3209

Score = 634 bits (320), Expect = e-179

Identities = 320/320 (100%)

Strand = Plus / Plus

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Query: 1    ctcttacctgtgccggtgccaggctggcttctccgggaggtactgcgaggacaatgtgga 60
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Sbjct: 1619 ctcttacctgtgccggtgccaggctggcttctccgggaggtactgcgaggacaatgtgga 1678


Query: 61   tgactgtgcctcctccccgtgtgcaaatgggggcacctgccgggacagtgtgaacgactt 120
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Sbjct: 1679 tgactgtgcctcctccccgtgtgcaaatgggggcacctgccgggacagtgtgaacgactt 1738


Query: 121  ctcctgtacctgcccacctggctacacgggcaagaactgcagcgcccctgtcagcaggtg 180
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Sbjct: 1739 ctcctgtacctgcccacctggctacacgggcaagaactgcagcgcccctgtcagcaggtg 1798


Query: 181  tgagcatgcaccctgccataatggggccacctgccaccagaggggccagcgctacatgtg 240
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Sbjct: 1859 tgagtgcgcccagggctatggcggccccaactgccagtttctgctccctgagccaccacc 1918


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Sbjct: 1919 agggcccatggtggtggacc 1938
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