MANIPULATION OF DENDRITIC CELL MIGRATION AND FUNCTION IN RELATION TO ALLOIMMUNE REACTIVITY AND TRANSPLANT OUTCOME

by

Yuk Yuen Lan

Bachelor of Science, The University of Hong Kong, 2001

Master of Arts, The Hong Kong Polytechnic University, 1997

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This dissertation was presented

by

Yuk Yuen Lan

It was defended on

April 7, 2006

and approved by

Simon M. Barratt-Boyes, PhD, Associate Professor, Departments of Infectious Diseases and Microbiology and Immunology

Pawel Kalinski, MD, PhD, Assistant Professor, Departments of Surgery and Immunology

Charles R. Rinaldo Jr., PhD, Chairman and Professor, Department of Infectious Diseases and Microbiology

Russell D. Salter, PhD, Associate Professor, Department of Immunology

Dissertation Advisor: Angus W. Thomson, PhD, DSc, Professor, Departments of Surgery and Immunology

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Yuk Yuen Lan, M.A.

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The immediate challenge of transplant immunology is to induce donor-specific immune tolerance to improve graft outcome and to eliminate the need for dependency on immunosuppressive therapy. Cell-based therapy employing dendritic cells (DC) for induction of transplant tolerance has been researched intensely due to their critical roles in controlling and regulating immunity. As the most potent antigen (Ag)-presenting cells (APC), DC are well-equipped to capture, process and present Ags, and their unique migratory behavior *in vivo* is central to the direct and indirect allorecognition pathways of transplantation. This dissertation examined two approaches to exploiting the inherent plasticity of DC with respect to their migration and function to regulate immune responses and enhance allograft survival. First, FTY720 is a novel immunomodulator pro-drug known to cause blood lymphopenia. Its active metabolite, FTY720-phosphate (FTY720-P) is a receptor agonist of sphingosine 1-phosphate (S1P) that regulates cell growth, differentiation, and migration. Our hypothesis was that FTY720 would modulate DC trafficking and function, an effect that could contribute to FTY720-induced immunosuppression. Herein, we show that FTY720 retained DC in the circulation, and concomitantly reduced their number in lymph nodes and spleen, by down-regulating surface intercellular adhesion molecule and homing receptor expression on DC, likely via the $S1P_1$ signaling pathway. Second, we investigated the immunoregulatory capacity of alternatively-activated (AA) DC and their therapeutic efficacy in a fully MHC-mismatched cardiac allograft model. We hypothesized that AADC, generated in an immunosuppressive milieu and then 'activated' via Toll-like receptor (TLR) ligation, could exhibit tolerogenic properties and offer potential as therapeutic agents to promote long-term vascularized organ allograft survival. We demonstrated that the anti-inflammatory cytokines, IL-10 and TGF-β, impacted significantly on DC maturation in response to the TLR4 ligand LPS and impaired their ability to induce T cell proliferation. AADC prolonged allograft survival by induction of a tolerogenic cytokine environment and expansion/proliferation of $CD4^+$ regulatory T (Treg) cells, an effect that was

markedly potentiated by blockade of the B7-CD28 costimulation pathway. These novel data provide new insights into manipulation of DC migration and function for regulation of alloimmune reactivity and promotion of transplant tolerance by control of S1PR, cytokine, and TLR signaling.

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PREFACE

I came to Pittsburgh in the lovely summer of 2001. Sweet aroma filled the air, and my heart was full of hope and eagerness to learn. That was the time when my exciting PhD training was about to begin. Five years have gone by, and I am proud to say, "I've made it!" Who would have guessed that doctoral training was so much about struggles and frustration! Though, I have to admit that those rewarding moments of getting new data and having my first paper published have made it all worthwhile. The training was a marathon to prove my persistence and endurance; and yet it was not a race in which only personal achievement was recognized. As Angus has always emphasized, "Research is teamwork." Through the years of twists and turns, I have had great people around me. Whether it was a helping hand in experiments, a friendly critique, a smile or a pat on the shoulder, I am thankful.

I was fortunate to be Angus's student. He is a great scientist and leader, and he never fails us. Fixing experimental designs and revising manuscripts is certainly his expertise, but what I truly admire is his perceptive understanding of individual talents. He always knew when to give me a push to do things that would benefit my development—writing grant proposals, giving presentations, attending conferences. Perhaps it was demanding at the time, but in the end, I realized how much he had helped me to achieve. Sincerely, all the glory of my awards and accomplishments should belong to Angus, my most respected mentor.

My Dissertation Committee was the best a student could dream of. My Committee members are knowledgeable, fair, and committed. When meeting with them, I never felt pressure because I knew they only wanted to help me—they did—tremendously. To Simon, Pawel, Chuck, and Russ: many heartfelt thanks.

My days in the lab were made extra special by these great members of the Thomson Lab: Zhiliang Wang shared the same birthday with me (I am much younger at heart!) and took care of me like a brother; Miriam Meade was the most charming and determined administrative assistant; the 'Chemokine Queen' Bridget Colvin was my best help in migration experiments; Audrey Lau and I supported each other through our years of PhD training; Giorgio Raimondi dealt with my troubles with regulatory T cells; Alan Zahorchak taught me English by applying GRE words in our daily conversations; and Daisuke Tokita, whom I could not beat for being the last one to leave the lab at night. There are also the ex-Thomson Lab members whom I miss a lot. Toby Coates started the FTY720 project and led me through the difficult initial steps of my training. An de Creus, with whom I had the great pleasure of working, inspired me to take a big step forward with my research. Timucin Taner, a role-model PhD student of my senior, has been a great friend. Masanori Abe and Antonino Castellanata were, respectively, my Japanese and Italian tutors. My thanks also go to 'Master Adrian (Morelli)', who knew all the answers to my questions by heart.

The Thomas E. Starzl Transplantation Institute is prestigious and distinguished, and yet I had the honor to enjoy its personal touch. Beyond its excellent facilities and support, people at the Institute offered special care to their most junior colleagues, the students. I especially thank, Jennifer Woodward, Janet Coulter, and Elaine Sico, for their continuous assistance. I am also much indebted to Dr. Timothy Billiar (Chairman) and Dr. Adriana Zeevi of the Department of Surgery.

I started as a student in the Interdisciplinary Biomedical Graduate Program in the School of Medicine. Thank you, Dr. Steven Phillips, Cindy Duffy, Sandra Honick, and the staff of the Graduate Office, for your dedication to students. As a student in the Department of Immunology, I benefited from the great classes and seminars provided by the devoted faculty and staff under the leadership of our reputable and energetic Chair, Dr. Olivera Finn.

The research on FTY720 would not have been possible without the generous support of Dr Volker Brinkmann of the Novartis Institutes for Biomedical Research, Basel, Switzerland, leading expert in FTY720 research and co-author of my first publication. I would also like to thank American Heart Association for the award of a pre-doctoral fellowship.

Thanks to my good friends and classmates, Jian Huang (the second person I met here with whom I celebrated the same birthday), Qing Zhang, Louisa Ho, and Weixin Zhou, for their

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Dearly, to my husband, David (Man Kai Yeung), for his unwavering support in every step of my wild endeavors to excel. We jumped hurdles and climbed mountains. From him, I learned to stand tall against my fears. He often says, “The sky is the limit!” Here I am, another new height reached and many more dreams to come. He is my mentor for life!

This dissertation is dedicated to my family and David’s family in Hong Kong and Australia, especially to my mom and brother in heaven.

1.0 INTRODUCTION

1.1 DC IMMUNOBIOLOGY

DC are rare, ubiquitously distributed, migratory leukocytes derived from bone marrow progenitors (1). They reside virtually in all tissues including the blood, skin, interstitial spaces of many organs, primary and secondary lymphoid tissues, and afferent lymphatics (2). As sentinels of the immune system, DCs specialize in antigen uptake, processing, transport, and presentation to T cells (1, 3). They are regarded as the most potent antigen-presenting cells (APC) capable of activating naïve T cells to induce (allo)immunity. Conversely, they play critical roles in central and peripheral tolerance in the normal steady state, and promote tolerogenicity.

DC are commonly generated *in vitro* using granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 from bone marrow hematopoietic stems cells consisting of common myeloid progenitors (CMP) and common lymphoid progenitors (CLP). *In vivo* administration of GM-CSF or fms-like tyrosine kinase 3 ligand (Flt3L) expands DC in various tissues, including the blood, spleen, lymph nodes, liver and kidney (4).

Recognized for their heterogeneity in phenotype and function, several DC subtypes have been identified by cell surface marker expression. In mouse, 'lymphoid' and 'myeloid' DC are still commonly used to denote respectively $CD8\alpha^+$ (located in T cell areas) and $CD8\alpha^-$ DC (marginal zones) found in the lymphoid tissues (5). This characterization however, is not indicative of their lineage origin, as both $CD8\alpha^+$ and $CD8\alpha^-$ DC can arise from CMP (6), and they represent different maturation stages of the same DC population (7). The ontogeny of DC subtypes is still debated upon the ideas of separate lineage development and different activation states of a single lineage (Figure 1) (5). Langerhans cells act as an epidermal immunologic 'barrier' to the external environment. They renew in the skin under steady-state conditions (8) and their mature form is only found in the skin-draining lymph nodes (5). The interferon (IFN)-

α-producing, plasmacytoid DC (pDC) precursors ($B220^+$) differentiate from both lymphoid and myeloid lineages, but are mainly of myeloid origin (9).

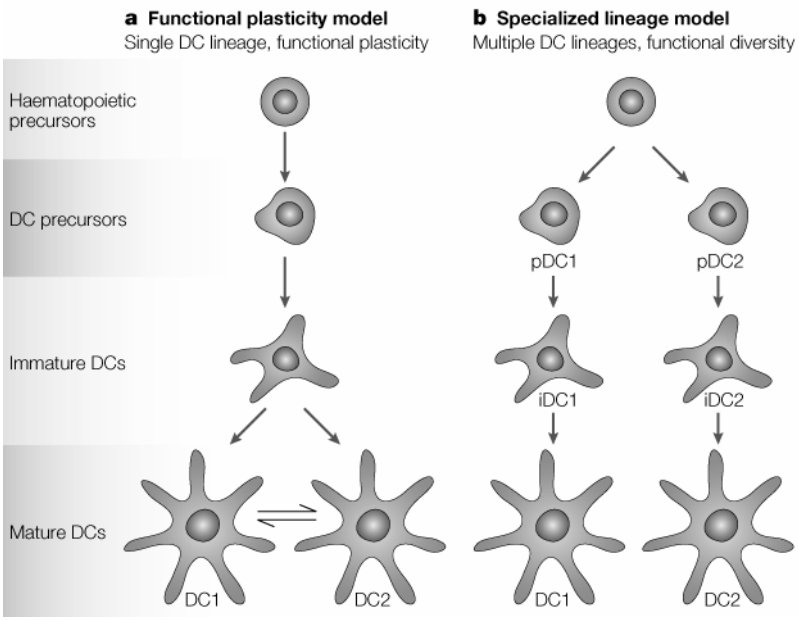

Figure 1. Alternative models for generation of distinct DC subtypes.

The functional plasticity model proposes that all DC belong to a single haemotopoietic lineage. The specialized lineage model proposes that different DC subtypes derive from early divergences in the developmental pathway. Adapted from Shortman, K. and Liu, Y.J. 2002. Mouse and human DC subtypes. *Nat. Rev. Immunol. 2, 151-160.*

DCs exhibit unique functions at the 'immature' and 'mature' stages of development[1]. Freshly-isolated tissue DC (from lymph node, spleen or liver) are immature, expressing low surface MHC class II and costimulatory molecules (CD40, CD80, and CD86) and exhibit poor naïve T cell stimulatory ability (1, 10). They are equipped to internalize exogenous antigens (Ags) efficiently by micropinocytosis, receptor-mediated endocytosis and phagocytosis. DCs mature under inflammatory influences of endogenous or exogenous mediators, including proinflammatory cytokines [i.e. GM-CSF, interleukin (IL)-1β, tumor necrosis factor (TNF)-α,

[1] The terms immature and immature DC are generally used with some imprecision, because they encompass different DC subtypes, DC found in different organs and DCs generated under different conditions (2). 'Semi-mature' DC have also been coined (10) to describe steady-state migrating DC that express high levels of MHC class II (signal 1) and costimulatory molecules (signal 2), but do not produce inflammatory cytokine IL-12p70 (signal 3). They are believed to be major components in immune homeostasis for active tolerance against self-Ags through T cell anergy or Treg cell induction (10).

interferon (IFN)-α], bacterial or viral components [e.g. lipopolysaccharide (LPS), viral double-stranded RNA] and CD40 ligand (L) (CD154), that act via Toll-like receptors (TLRs) or TNF receptor family members. Fully mature DC typically display extended dendrites and become professional APC characterized by dramatically up-regulated levels of MHC class II for presentation of processed Ags (Ag-dependent T cell receptor-mediated; signal 1), and costimulatory molecules (cognate interaction; signal 2) to stimulate clonal expansion of naïve and memory T cells. Their chemokine receptor (CR) expression is also remodeled with a loss of CCR5 due to receptor down-regulation or desensitization (11). By contrast, up-regulation of CCR7 expression promotes migration of mature DC via lymph or blood to T cell areas of secondary lymphoid tissues in response to "homing" or "constitutive" chemokines (12), i.e. CCL21 (secondary lymphoid tissue chemokine/SLC) and CCL19/MIP-3β secreted by stromal cells (13, 14).

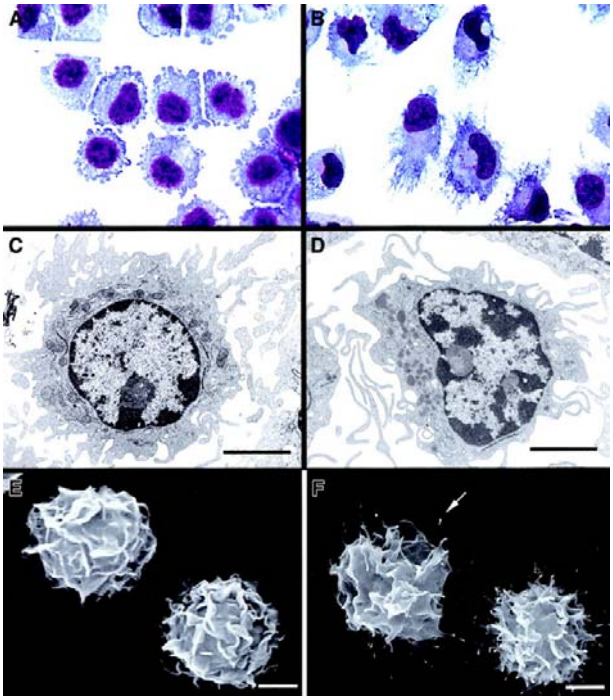

Figure 2. Morphology of immature and mature DC.

Immature DC display short, blunt prolongations (A), a round nucleus, multiple cytolplasmic vesicles (C), and typical 'veils' (E). After maturation, DC show a typical dendritic morphology, with an eccentric, indented nucleus (B, D), and a veiled surface with delicate filamentous projections (F). (A-B) May-Grunwald-Giemsa, (C-D) TEM ×6000, (E-F) SEM ×3500. Bar=5 μm. Adapted from Morelli, A.E. *et al.* 2001. Cytokine production by mouse

myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation. *Blood 98:1512-1523.*

Janeway's proposal (15) of incorporating the concept of costimulation in immunity has positioned DC as an essential link between innate and adaptive responses. DC discriminate 'infectious non-self' and 'non-infectious self' and perceive evolutionary pathogens via germline-encoded pattern recognition receptors (PRRs) that recognize conserved pathogen-associated molecular patterns (PAMPs) on micro-organisms (16). The major PRRs expressed on DC are TLRs for microbial antigens. Other receptor families include FcR for Ag-Ab complexes, and C-type lectin receptors (CLRs) for glycoproteins. Both TLRs and CLRs are able to distinguish self- and non-self-Ags (17). The immunogenic or modulating stimuli that DC encounter in the microenvironment critically determine the cytokine milieu produced by DC upon activation (soluble mediators for polarization signal 3) (18) and thereby the nature of the T cell response generated. TLR signaling on DC by microbial pathogen components such as LPS, CpG motifs and double-stranded RNA, mainly induces DC production of IL-12 and IL-18 that drives naïve T cells to differentiate into IFN-γ-producing Th1 cells (19, 20)[2]. Conversely, fungi, schistosomes and cholera toxin promote IL-4 production of DC and helps Th2 differentiation (18, 21)**.**

1.2 DC IN TRANSPLANTATION

1.2.1 DC trafficking, Ag presentation and allorecognition pathways

The long-standing self-non-self model of immunity predicts transplant rejection due to non-self Ags present on the graft (exogenous signals); whereas the danger model considers tissue damage and ischemic cell death involved in transplant surgical procedures (endogenous signals) sources of alarm signals that activate APC (22). On the contrary, the infectious non-self model proposed by Janeway (23) predicts that transplants should not be rejected because of the absence of

[2] DC are heterogeneous in terms of TLR expression. Differential expression of TLR on DC subtypes can induce dissimilar cytokine production profiles. Further, different TLRs may activate similar but distinct signaling pathways (20).

conserved ligands for the PRRs to recognize, and identification of requisite receptors for DC maturation under this sterile circumstance is awaiting (2). Either way, DC are well established as instigators of allograft rejection. Batchelor *et al.* (24) found that kidneys could be successfully grafted if depleted of donor APC. Elicitation of acute allograft rejection in recipients of Flt3L-treated liver is associated with enhanced trafficking of stimulatory donor DC (25).

The crucial roles of donor and recipient DC in allograft rejection have been well demonstrated with increased understanding of the molecular basis of allorecognition. DC migration is closely related to their maturation, cell surface CR expression, and chemokines present in the microenvironment. In the early post-transplant phase, passenger leukocytes migrate out of allografts into the recipient's spleen and/or lymph nodes (lymphoid tissues) to sensitize $CD4^+$ T cells (26-28). The vigorous alloresponse that follows is caused by direct priming of high frequency donor-reactive T cells (5-10% in the general repertoire in mice) (29) by donor DC due to the cross-reactivity of self MHC-restricted T cells with allo-MHC-peptide complexes in the direct allorecognition pathway. At the same time, "early" or "inducible" inflammatory chemokines in the graft tissue recruit immature migratory recipient DC that respond to these chemokines [e.g. CCL3/macrophage-inflammatory protein (MIP)-1α, CCL4/MIP-1β, CCL5/regulated on activation, normal T expressed and secreted (RANTES)] via their cognate chemokine receptors (CCR1-CCR5) (30). They traffick through the transplanted tissue capturing Ags from the graft (soluble MHC molecules or dying or apoptotic cells) and transporting these Ags to the secondary lymphoid tissues via coordinated up-regulation of CCR7 (11, 30), to present these donor-derived peptides on self-MHC molecules, and stimulate the relatively low frequency T cells via the indirect allorecognition pathway (31). In addition, the Lechler group (27) has proposed the 'semi-direct' pathway in which recipient DC can aquire and present intact donor MHC I to direct pathway $CD8^+$ T cells.

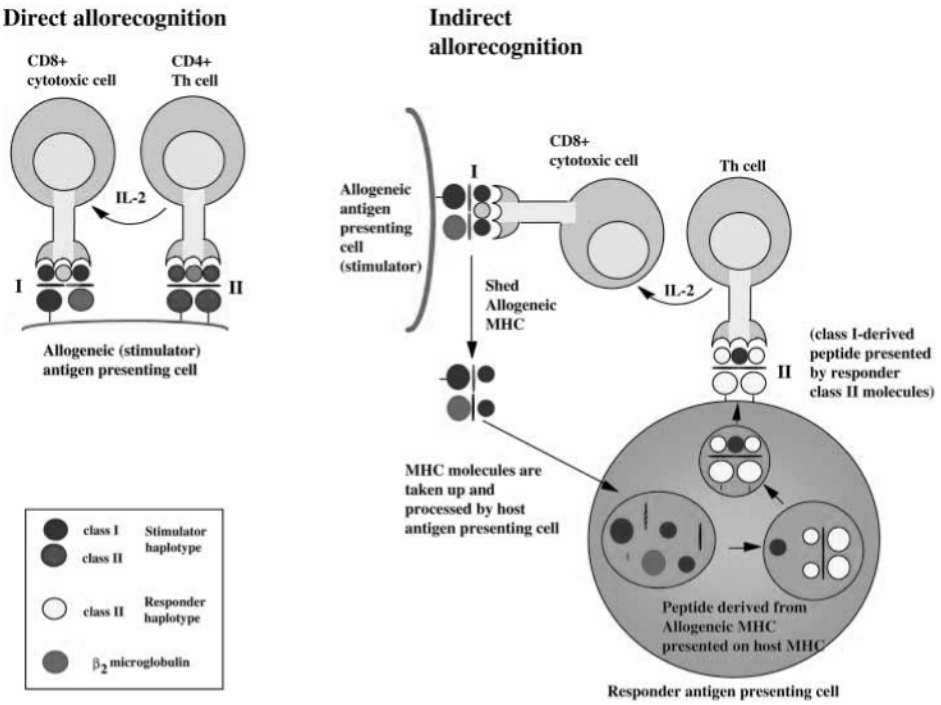

Figure 3. Direct and indirect allorecognition pathways.
Adapted from Rogers, N.J. and Lechler, R.I. Allorecognition. 2001. *Am. J. Transplant. 1(2), 97-102*.

In vivo trafficking of donor and recipient DC leads to initiation of alloimmune responses in the lymphoid tissue (32), and subsequent T cell infiltration results in allograft rejection. It is believed that the direct anti-donor responses dominating acute rejection are short-lived and decrease after transplantation as donor DC are eliminated with time (27). The indirect alloAg-presentation pathway as the important driver of chronic rejection, that contributes to continual graft damage and graft vessel disease is well supported by the literature (31, 33, 34).

1.2.2 DC as regulators of transplant rejection

The role of DC in mounting T cell immunity and initiating graft rejection is well established. Equally important are observations that reveal the tolerogenic potential of DC in prolonging allograft survival. Immature donor MHC class II^+ DC, deficient in costimulatory molecules can induce alloAg-specific T cell hyporesponsiveness and prolong donor-specific graft survival in the absence of immunosuppressive therapy (35-37). Certain DC subtypes are known to

downregulate immune response. Immature myeloid DC of donor origin prolong organ or pancreatic islet allograft survival (38). Splenic $CD8\alpha^+$ 'lymphoid-related' DC regulate primary $CD4^+$ T cell response via Fas (CD95)/Fas ligand (FasL)-induced apoptosis (39). Liver-derived DEC-205^+ DC induce low T cell responses in MLR, express IL-10 and IFN-γ transcripts, and prolong the survival of cardiac allografts (40). De Creus *et al.* (41) have also shown that low TLR4 expression on liver DC correlates with their reduced capacity to activate allogeneic T cells in the presence of LPS. Pre-operative infusion of pDC can enhance heart allograft survival significantly but non-specifically (42).

1.3 ROLE OF DC IN TOLERANCE INDUCTION

DC play major roles in defining immunologic self during the steady state to avoid horror autotoxicus (2). In the thymus, central tolerance is imposed when DC present self Ags to developing T cells and cause deletion of auto-reactive lymphocytes (43, 44). This Ag-driven intrathymic T cell clonal negative selection is systemic and is the most robust form of T cell tolerance in which most donor-reactive clones are deleted (45). In transplantation, intrathymic alloresistance can be overcome by thymic irradiation, repetitive T cell depletion or costimulation blockade (45). The limitations of central tolerance[3] necessitate control of self-reactive cells in the peripheral tissues through T cell deletion and unresponsiveness (46), and induction of Treg by immature (2) (or semi-mature) (10) DC. This reinstates the accepted idea that DC maturation is a control point for regulating tolerance and immunity (2). The immunological mechanisms underlying acquired tolerance towards an allograft are basically the same as those that maintain tolerance to self-antigens (45). Various mechanisms have been acknowledged: T cell anergy, deletion, induction of Treg, immune deviation (all involving DC-T cell interaction), or ignorance (47-49).

[3] Central tolerance is mainly developed during fetal life. Many self Ags may not access the thymus or they may not be expressed at the time when the T cell repertoire is formed (2).

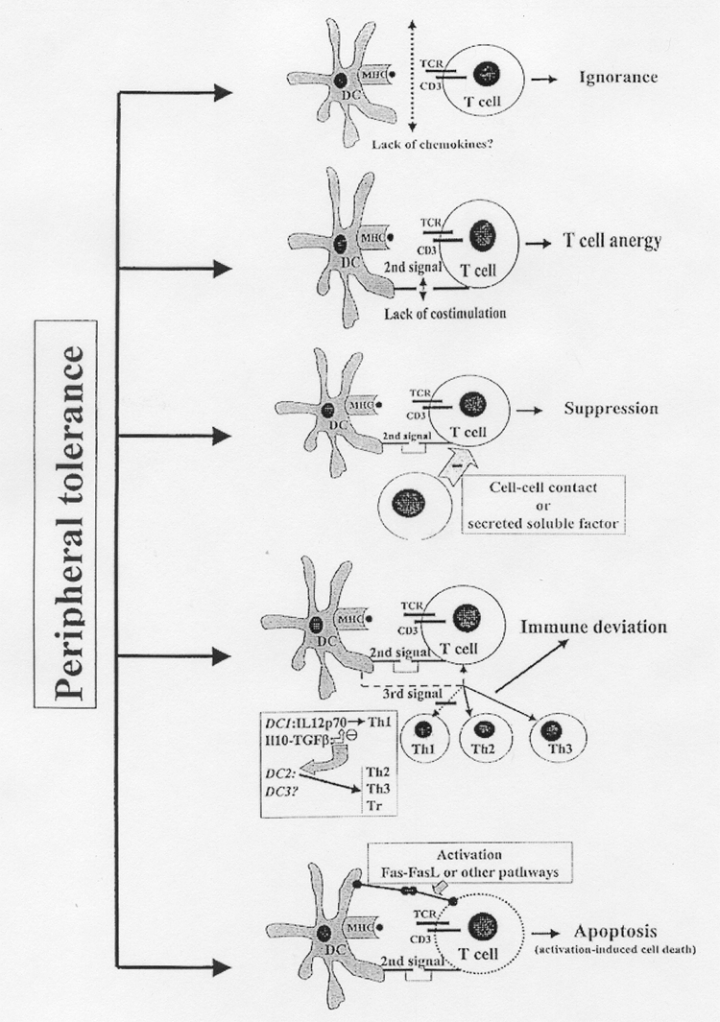

Figure 4. Mechanisms of peripheral tolerance.

Adapted from Rifle, G. and Mousson, C. 2002. Dendritic cells and second signal blockade: a step toward allograft tolerance? *Transplant. 73(1), S1-S2*.

1.3.1 Induction of T cell anergy

T cell anergy is a state of long-lasting, partial or total unresponsiveness induced by partial activation (50). It commonly occurs when TCR are triggered without costimulation or by partial agonists (50, 51). There are varying levels of anergy and anergic cells are unable to produce or respond to proliferative signals (50). Clonal anergy of previously activated T cells can be reversed by exogenous IL-2, whereas in vivo anergy of naïve T cells often involves inhibition of effector functions (52). Costimulatory blockade using anti-CD154 Ab (anti-CD40L) or CTLA4-

Ig to block respectively the CD40-CD154 and B7-CD28 interactions may therefore induce anergy (45). Long-term survival achieved in fully allogeneic skin grafts using both agents suggests independent regulation of the CD28 and CD40 pathways in T cell responses (53). Anergic T cells display impaired trafficking ability and remain localized in the lymph nodes at sites colonized by tolerogenic DC (54). They also inhibit the Ag-presenting function of DC via a cell contact-dependent mechanism (55).

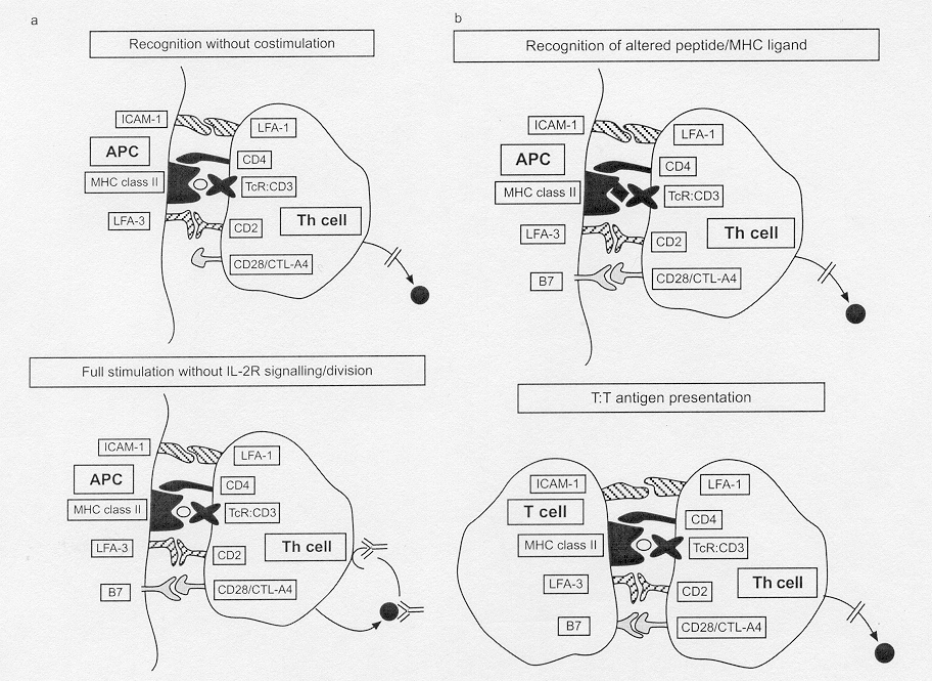

Figure 5. The different circumstances that induce T cell anery *in vitro.*

Adapted from Lechler, R. *et al* 2001. The contributions of T-cell anergy to peripheral T-cell tolerance. *Immunol. 103, 262-269*.

1.3.2 T cell deletion

T cell deletion has been implicated in the induction of central tolerance and maintenance of peripheral tolerance in the steady state. Using combined treatment of rapamycin (blocks IL-2 signaling, but does not inhibit priming for activation-induced cell death or apoptosis) and costimulation blockade, stable skin allograft tolerance is achieved (56). It is further suggested that deletion of activated T cells through activation-induced cell death requires both signal 1 and 2 of T cell activation (57) and T cell apoptosis is necessary to achieve peripheral transplantation

tolerance (58). DEC205$^+$ (CD205$^+$) DC generated in vitro and splenic CD8α^+ DC (59) uniformly express Fas ligand (FasL/CD95L) and are associated with low T cell proliferative responses (39). FasL-transfected DC induce apoptosis-mediated donor-specific hyporesponsisvenss and prolong cardiac allograft survival (60).

1.3.3 Induction of Treg

Recent studies have implicated the undisputed roles of the naturally anergic Treg (CD4$^+$CD25$^+$) in active suppression of self-reactive lymphocytes and immune response to alloAgs. On identifying the transcription factor Forkhead helix protein 3 (Foxp3) as a key regulator for their development, Treg are more specifically characterized as CD4$^+$CD25$^+$Foxp3$^+$ cells (61, 62). They are known to mediate transferable tolerance that prevents rejection in a donor-specific manner (63). Treg are persistently present beyond secondary lymphoid tissues in tolerated skin allografts (64) and are found as early as 5 days post-transplant in spleen and lymph nodes of tolerant recipient rats receiving donor-specific blood transfusion (65). They affect functions of other T cells, including inhibition of cytokine production and proliferation, either directly or indirectly through effects on DC (66). In the process of infectious tolerance (67), they convert CD4$^+$ T cells to a regulatory phenotype through contact-dependent interactions (68). Both steady state and mature DC induce proliferation of adoptively transferred CD4$^+$CD25$^+$ Treg and DC-expanded CD4$^+$CD25$^+$Foxp3$^+$ Treg cells are more potent suppressors of responses to major MHC Ags (69). A rather unique CR profile expressing CCR4, CCR5 and CCR8 guides the trafficking and tissue localization of these suppressive cells (70, 71).

1.3.4 Immune deviation

The accepted paradigm of Th1 (IL-2, IFN-γ, TNF-α) and Th2 (IL-4, IL-5, IL-10) cytokines in transplant models suggests that Th1 cytokine profile often associates with allograft rejection, whereas Th2 profile promotes tolerance (72). Strom *et al.* (73) propose that T cell growth factors cannot be rigidly classified as supporting either rejection or tolerance due to their redundancy in functions. In their opinion, there is a hierarchy of cytokine expression according to their ability

to block tolerance. Therefore, IL-2 (Th1 cytokine) is a strong barrier to tolerance, while IL-4 (Th2 cytokine) provides a weak barrier. Different pathogen products or stimuli have been shown to render DC more flexible to direct Th1 or Th2 development (18). IL-12-deficient DC generated in the presence of an inflammatory mediator PGE_2 promote Th2 cytokine (IL-4, IL-5) production (74). Further, mouse myeloid DC and pDC induce the development of effector cells depending on the dose of Ag (75). The Th1/Th2 cytokine profile of renal transplant recipients has been examined to evaluate the feasibility of cytokine analysis as a prognostic index of kidney transplant outcome (76).

1.3.5 Ignorance

Ignorance can occur when donor Ags from the graft are unable to reach the recipient lymphoid system (no priming), or when recipient lymphocytes fail to invade the graft (no effector function) (45). Lafferty *et al.* (77) attempted a reduction of tissue immunogenicity by depleting passenger leukocytes from the graft prior to transplantation. Indefinite cardiac allograft survival in splenectomized *aly/aly* mice demonstrates the principle of immunologic ignorance in the absence of secondary lymphoid tissues in the recipient (32). However, this mechanism does not often apply to vascularized organ transplants, as recipient cells can always reach the graft and donor cells migrate to recipient lymphoid tissues (45).

1.4 MANIPULATING DC FOR THERAPY

Due to their inherent plasticity and eminent role in immune control, much effort has been targeted on utilizing DC to meet therapeutic ends. Splenic DC isolated from tolerant graft recipients possess an immature phenotype of low levels of costimulatory molecules CD40 and CD86, and produce little IL-12 (78). Indeed, our group has employed costimulatory molecule-deficient DC to prolong allograft survival (35). However, the use of immature DC *in vivo* is limited by their instability to differentiate into mature immunostimulatory DC in an inflammatory environment. There have been numerous attempts to generate ‘designer’ DC *in*

vitro by irreversible modulation of terminally differentiated DC to improve transplant outcome. These approaches have focused on the critical parameters governing the tolerogenic properties of DC, most importantly their maturation and function. In addition, modulating the migratory behavior of DC has emerged as a focus of investigation.

1.4.1 Targeting DC maturation and function

DC can be manipulated pharmacologically, genetically (both described in this section), or biologically (section 1.61) to enhance their tolerogenicity by targeting their differentiation, allostimulatory and Ag-presenting functions, and cytokine production profiles. Diverse immunosuppressive drugs control allograft rejection by inhibiting lymphocyte activation, receptor signaling, and proliferation. They also act earlier in the immune response by affecting DC differentiation and maturation, *in vitro* and *in vivo*. Anti-proliferative drugs like mycophenolate mofetil (MMF) causes a dose-dependent reduction of costimulatory molecule expression on DC with concurrent decreased IL-12 production (79). Calcineurin inhibitors cyclosporine A (CsA) and tacrolimus (FK506) block the phosphatase activity of calcineurin, their common molecular target, and inhibit IL-2 transcription for signal transduction in T cells (79). CsA reduces cell surface expression of CD40, CD80, and CD86 without affecting MHC Ag expression (80), while tacrolimus shows no effect on DC phenotype (81). Both drugs decrease the allostimulatory capacity and inflammatory cytokine production of DC and block bidirectional DC-T cell interaction during Ag presentation (79). Rapamycin (RAPA; sirolimus) structurally resembles tacrolimus and inhibits T cell proliferation through the mammalian target of RAPA (mTOR) (79). Hackstein *et al.* (82) have demonstrated that RAPA inhibits IL-4-dependent DC maturation *in vitro* and impairs DC function and mobilization *in vivo*. The impairment of DC functions by other potent immunosuppressants (glucocorticosteroids, deoxyspergualin), anti-inflammatory agents (aspirin), and vitamin D_3 analogs have also been demonstrated (79). DC maturation critically involves activation of the nuclear transcription factor nuclear factor (NF)-κB that controls expression of MHC class II and costimulatory molecules on DC and their Ag presentation (83, 84). Several immunosuppressants such as CsA, tacrolimus and aspirin, block DC maturation through the NF-κB pathway (78, 80), and NF-κB decoy

oligodeoxyribonucleotides that blocks the action of NF-κB can prolong cardiac allograft survival (85).

DC can also be genetically engineered to express 'immunosuppressive' molecules through which desired gene products may be delivered locally to avoid systemic side effects (48). DC genetically modified to express CTLAg-4 immunoglobulin fusion protein (CTLA4-Ig), which inhibits the B7-CD28 costimulation, induce alloAg-specific T cell hyporesponsiveness and polarize Th2 cytokine production (86). IL-12p35 gene silencing in DC using small interfering RNA blocks IL-12 production and impairs DC allostimulatory functions (87). Indoleamine 2,3-dioxygenase (IDO)-expressing DC decrease the concentration of tryptophan, an essential amino acid required for cell proliferation, and suppress allogeneic T cell proliferation (88). DC that express FasL mediate apoptosis of Ag-specific T cells (89, 90).

1.4.2 Modulating DC migration

Chemokines have emerged as important regulators of DC migration and chemokine-targeted therapeutic strategies in transplantation have been tested with some success. Several approaches to block CCR5 (KO mice, anti-CCR5 mAb, or synthetic analogs), that is highly expressed on immature DC, have achieved indefinite graft survival (49, 91, 92). We have shown that IL-10-transduced DC exhibit lower levels of CCR7 and increased CCR5 expression, which appears to explain their impaired *in vivo* homing from peripheral tissue to secondary lymphoid organs (93). Considering migration of mature donor DC from the graft, blockade of CCR7-CCL19/21 interaction to interfere with the direct pathway may be more feasible for transplant therapy (49). Paucity of lymph node T cells (*plt*) mice lack secondary lymphoid expression of the CCR7 ligands, CCL19 and CCL21. Colvin *et al.* (94) and Wang *et al.* (95) found deficient migration of donor DC to draining lymph nodes of *plt* heart and islet allograft recipient mice, respectively. Studies of the immunomodulator FTY720 in this dissertation (Chapter 2) delineate a novel signaling pathway through which DC migration can be modulated *in vivo*.

1.5 THE NOVEL IMMUNOMODULATOR FTY720

FTY720 (2-amino-2[2-(4-octylphenyl)-ethyl]-1, 3-propane-diol, C19 H33 NO2: HCl) is a modified structural analogue of ISP-1 (myriocin), a fermentation product obtained from the culture of the ascomycete *Isaria sinclairii* (96, 97). It represents the first of a new class of immunomodulators. Unlike conventional immunosuppressive agents, it does not inhibit T or B cell activation or proliferation, or their effector functions (98, 99), including T cell cytokine secretion profiles (100); but alters their migration and homing (101).

1.5.1 FTY720 prolongs allograft survival

FTY720 has proved effective in prolonging graft survival in a dose-dependent manner in preclinical models of transplantation: rat (102) and mouse skin (103, 104), rat (105) and mouse heart (106), cynomolgus monkey (107) and canine kidney (108), rat (109) and canine liver (110), rat (111) and mouse small bowel (112), and rat cornea (113). It has achieved promising results in Phase I (114, 115) and II studies (116) conducted in stable renal transplant patients and is currently in phase III clinical trials. The drug has a high oral bioavailability (98), though intra-peritoneal administration has shown to be effective in experimental models (117). The pharmacokinetics of FTY720 reveals a long elimination half-life ($t_{1/2}$ = 89-157 h), moderate inter-patient variability, and good tolerance/safety following single or multiple dosing (118). There is no evidence of additional toxicities (mutagenicity, fertility, renal, hepatic, pancreatic or bone marrow) (98) except asymptomatic bradycardia (114). The drug also shows a synergistic effect with CsA, tacrolimus, RAD (everolimus) and RAPA (107, 108, 118, 119). Pre-operative administration of FTY720 is recommended although delayed administration also attenuates the progression of vasculopathy and tissue fibrosis in chronic heart graft rejection (100).

1.5.2 FTY720 as sphingosine 1-phosphate (S1P) receptor agonist

S1P is a platelet-derived lipid mediator synthesized *de novo* from sphingosine, a breakdown product of the membrane lipid sphingomyelin, upon sphingosine kinase (SK) activation (120,

121). It is found in high nanomolar concentrations in the blood (122) and induces important biological responses, including cell proliferation (123), differentiation (124), cytoskeletal reorganization (125), migration (126, 127), and protection from apoptosis (128). S1P plays dual messenger functions as a second messenger in intracellular signaling and as a natural ligand of extracellular G protein-coupled (GPC) S1P receptors (R) (129) of which 5 have been identified: $S1P_{1\text{-}5}$ (130). FTY720 is a sphingosine analogue that is readily phosphorylated by sphingosine kinases to its active metabolite FTY720-phosphate (FTY720P) (131). After oral administration, the level of FTY720P in blood rose to four times of that of parent compound (132). FTY720P shares structural homology with S1P (131), and binds to four of the five S1PR ($S1P_{1,\ 3,\ 4,\ 5}$) *in vitro* non-selectively with high affinity (96, 131).

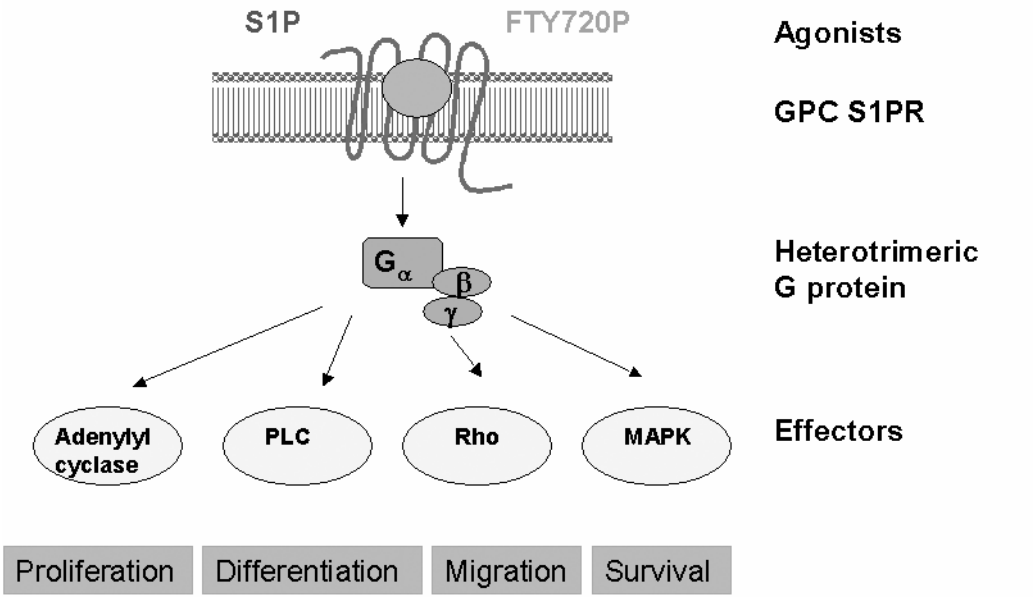

Figure 6. S1P signaling pathway.

S1P and its receptor agonist FTY720 activates surface GPCRs that signal downstream molecules effecting different biological processes. PLC=phospholipase C, MAPK=MAP kinase.

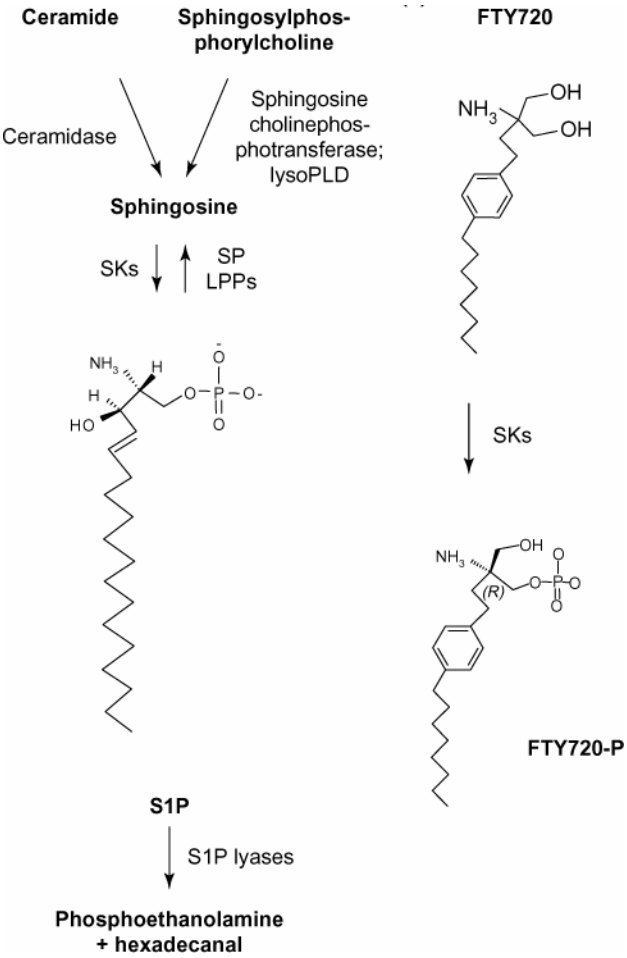

Figure 7. S1P and FTY720 structure and metabolism.

Adapted from Gardell, S.E. *et al.* 2006. Emerging medicinal roles for lysophospholipid signaling. *Trends Mol. Med. 12(2), 65-75*.

1.5.3 FTY720 modulates lymphocyte trafficking and function

FTY720 causes rapid and dramatic depletion of circulating lymphocytes by up to 85%, within 2-6 h of intake (114, 118), but does not affect monocytes or granulocytes (133). Mature lymphocytes are sequestered in lymph nodes and intestinal Peyer's patches, leading to dramatic decreases in lymphocytes in the thoracic duct lymph and spleen (101, 134), and thereby decreasing T cell infiltration within the allograft (100, 134) and inhibitng graft rejection. The depletion is selective and completely reversible (99, 131, 134). Trafficking of both naïve and effector-memory T cells ($CD44^{high}CD62L^{low}$) is altered (100, 135). Surprisingly, FTY720 also

causes peripheral lymphopenia in *aly/aly* mice (without lymph nodes or Peyer's patches) and splenectomized *aly/aly* mice (136).

Initially, it was hypothesized that FTY720 produced lymphopenia by inducing T cell apoptosis based on *in vitro* studies using relatively high dosage (micro-molar compared to nano-molar blood levels realized at therapeutic dosing (132)) of the drug (137, 138). Later studies showed that transferred CFSE-labeled T cells reappeared in the blood within 7-21 days after drug withdrawal (99), and the Sayegh group (100) recently demonstrated that FTY720 administration *in vivo* did not affect T cell apoptosis in a TCR transgenic mouse model. This renders apoptosis an unlikely mechanism of the action of FTY720.

The more popular model focuses on lymphocyte sequestration due to agonism or antagonism of $S1P_1$. Sanna *et al.* (139) show that $S1P_1$ regulates lymphocyte recirculation and $S1P_3$, dominantly expressed in heart tissue, regulates heart rate. Indeed, FTY720-mediated activation down-regulates $S1P_{1, 2, 5}$ on lymphocytes (140) and causes internalization of $S1P_1$ to inhibit lymphocyte egress from thymus and peripheral lymphoid organs (141). Mature T cells in $S1P_1$-transgenic mice preferentially distribute to the blood rather than secondary lymphoid organs (142). FTY720 also differentially affects the accumulation of $CD4^+CD25^+$ Treg in the blood and spleens of mice and increases their suppressive activity (143). Other mechanism-related studies indicate that the altered lymphocyte trafficking is independent of homing receptors, including CD62L (144), CCR7 (145) and CXCR5 (146); and yet splenic and peripheral lymph node T cells have distinct chemokine (CCL19, CXCL12) and S1PR stimulation requirements for transendothelial migration (147). Both the multidrug sphingosine transporter Abcb1 (Mdr1) and the cysteinyl leukotriene C_4 transporter multidrug resistance-associated protein 1 (Mrp1) must be active for FTY720-mediated T cell migration and LN homing (148). In addition, S1P enhances adherens junction assembly (124) and FTY720 inhibits vascular permeability (149), essential factors affecting lymphocyte trafficking.

The potential effects of FTY720 on DC migration are largely undefined. Recent data indicate that S1P induces chemotaxis of immature, monocyte-derived human DC and promotes Th2-type immunity (150). Since work for this dissertation commenced, Czeloth *et al.* (151), however, have demonstrated that S1P-mediated migration of mature mouse DC can be severely hampered by FTY720. The leukotriene C_4 transporter Mrp1, which is involved in FTY720-mediated T cell trafficking, regulates CCL19-dependent mobilization of skin DC to LN (152).

1.6 'ALTERNATIVELY-ACTIVATED' DC (AADC)

As their roles in central and peripheral tolerance are confirmed and better understood, the tolerogenic potential of DC is being explored in T cell-mediated disorders including autoimmune diseases and allograft rejection. DC differentiation is highly dependent on the inducing stimulus and signaling pathway. Many immunosuppressive agents block DC maturation and activation (Section 1.4.1). DC may also undergo 'alternative activation'[4] under specific conditions, and acquire unique features enabling them to carry out 'suppressive' functions (153). CD40-CD40L interactions in the presence of the glucocorticoid hormone dexamethasone (DEX) redirect the DC activation process, resulting in IL-10-producing DC that down-modulate Th1-type responses (154). With their suppressive activity, AADC present themselves as effective therapeutic tools. DEX-treated DC activated by LPS delay skin graft rejection in a fully-mismatched mouse strain combination (155). Sato *et al.* (156) used combined treatment with IL-10 and TGF-β to generate 'regulatory' (r) DC activated by LPS, that display low levels of costimulatory molecules. A single injection of recipient-matched myeloid rDC protects mice from acute graft-versus-host disease and leukemia relapse (156). Their tolerogenic effects are mediated via induction of T cell anergy and $CD4^+CD25^+CD152^+$ T cells *in vitro* and *in vivo* (157).

1.6.1 The immunosuppressive cytokines IL-10 and TGF-β

IL-10 is a multi-functional cytokine recognized for its ability to inhibit activation and effector function of T cells, monocytes, and macrophages (158). Its crucial role is to limit inflammatory responses (158). The best characterized IL-10 signaling pathway is the Jak/stat system, but IL-10 also inhibits NF-κB signaling (158). Administration of IL-10 to recipient mice pre-transplant

[4] The concept of 'alternative activation' was first introduced by the Gordon group to describe IL-4-transformed macrophages that displayed a phenotype and reduced proinflammatory cytokine secretion, distinct from IFN-γ-induced activation (154).

prolongs graft survival, whereas post-transplant treatment accelerates rejection (159). Groux *et al.* (160) demonstrated that IL-10 induced Ag-specific T cell anergy, while recent evidence showed that Treg cells requires IL-10 to mediate tolerance to alloAgs *in vivo* (161). IL-10 also imposes its effects on DC. Steinbrink *et al.* (162) found that IL-10 inhibited up-regulation of costimulatory molecules on DC and IL-10-treated DC induced alloAg-specific anergic $CD4^+$ T cells that secreted less IL-2 and IFN-γ. IL-10-treated DC produce less IL-12 and thereby skew the Th1/Th2 balance to Th2 *in vivo* (163). Notably, fully mature DC are completely resistant to the effects of IL-10 (162). IL-10 can inhibit DC differentiation in an autocrine/paracrine fashion (164, 165).

TGF-β is a well-described key regulator of inflammation. TGF-β-deficient mice suffer immune dysregulation, develop a rapid wasting syndrome with intense inflammation and die by 3-4 weeks of age (166). This may be related to uncontrolled innate immune mechanisms as TGF-β null mice show increased TLR4 mRNA expression in tissues (liver, lung, heart), associated with endotoxin hypersensitivity (167). The primary effect of TGF-β signaling in T cells is to block IL-2 production (168). It also imposes an additive effect with IL-10 to induce $CD4^+$ T cell alloAg-specific hyporesponsiveness (169). Recent evidence has pointed to its intriguing role in Treg suppression (168). $CD4^+CD25^+$ Treg express high and persistent levels of TGF-β on the cell surface that may allow their delivery of a regulatory signal to target responder cells via a cell contact-dependent process (170) (Figure 8). In addition, TGF-β converts $CD4^+CD25^-$ naïve T cells to $CD4^+CD25^+$ Treg by inducing their Foxp3 expression (171). TGF-β promotes DC generation *in vitro* by protecting progenitor cells from apoptosis (172), but inhibits DC maturation (173). However, certain DC subtypes are positively regulated by TGF-β (174). TGF-β is necessary for LC development *in vivo* (175). TGF-β-transduced myeloid DC markedly impair allo-reactive T cell proliferation *in vitro* and significantly extend cardiac allograft survival (176).

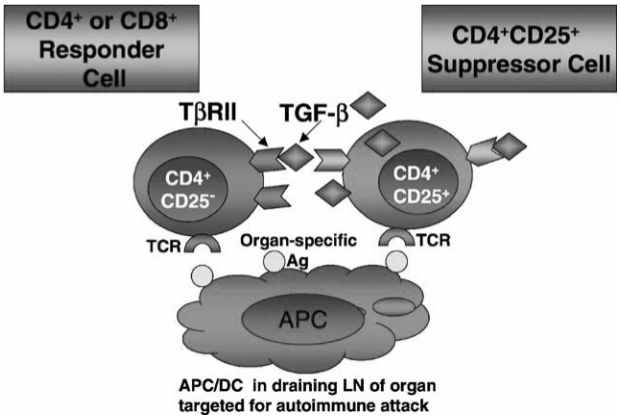

Figure 8. The role of TGF-β in $CD4^+CD25^+$-mediated immunosuppression.

Freshly isolated $CD4^+CD25^+$ T cells express cell surface TGF-β and TGF-β receptor II (TβRII), which are upregulated by TCR stimulation by APC. Naïve $CD4^+CD25^-$ responder T cells express neither TGF-β nor TβRII, but become positive for TβRII after TCR ligation. The interaction between surface TGF-β on $CD4^+CD25^+$ suppressors and TβRII on $CD4^+CD25^-$ responders may explain how the suppressors function. Adapted from Chen, W. and Wahl, S.M. 2003. TGF-β: the missing link in $CD4^+CD25^+$ regulatory T cell-mediated immunosuppression. *Cytokine & Growth Factor Rev. 14, 85-89*.

1.7 AIMS OF THE STUDY

Intense research is ongoing to unfold the multifaceted aspects of DC biology, ontogeny and therapy. Exciting potential of DC manipulation to improve clinical outcomes in cancer, transplantation and autoimmune disease reside in their inherent plasticity and unique capacity as APC to control and regulate immunity. Ample evidence has revealed multiple mechanisitic pathways to modify DC properties in experimental and therapeutic approaches. The studies in this dissertation focused on modifying DC migration and function. Two approaches were employed to modulate the immunoregulatory capacity of DC to favor inhibition of immune responses and to improve transplant outcome: (1) use of the novel immunodulator FTY720, and (2) conditioning of DC by the anti-inflammatory cytokines IL-10 and TGF-β.

1.7.1 Modulation of DC trafficking by FTY720

Numerous studies have confirmed the immunosuppressive effects of FTY720 due to altered lymphocyte trafficking and suggested different hypotheses to explain the underlying mechanisms. Few reports on the influence of S1P on DC have emerged, but the effects of its receptor agonist FTY720 on DC migration and function have not been fully elucidated. The *hypothesis* addressed is that FTY720 can modulate DC trafficking and function and thus contribute to the FTY720-induced immunosuppressive effects in prolonging allograft survival. Experiments performed in Chapter 2 utilized the pro-drug FTY720 to examine S1P signaling as a novel molecular pathway in DC migration and immunoregulation. The aim was to ascertain the influence of FTY720 on *in vivo* DC trafficking and alloimmune reactivity and to dissect the underlying mechanisms involved.

1.7.2 Influence of AADC on alloimmune responses and transplant outcome

Various inhibitory effects of immunosuppressive agents on DC maturation and function have been reported. The *hypothesis* addressed is that AADC, generated in an immunosuppressive milieu, can exhibit potent immunoregulatory properties and offer potential as therapeutic agents for modification of T cell-mediated immunity, as well as promotion of vascularized organ allograft tolerance. Chapter 3 investigated the alloantigenic immunoregulatory capacity of AADC and the mechanisms underlying the observed effects. Further, the therapeutic efficacy of AADC was studied in a fully MHC-mismatched, cardiac allograft model to optimize the necessary conditions using AADC for promotion of long-term allograft survival and donor-specific tolerance induction.

2.0 MODULATION OF DC TRAFFICKING BY FTY720[5]

The pro-drug FTY720 is undergoing phase III clinical trials for prevention of allograft rejection. After phosphorylation, FTY720 targets the G protein-coupled sphingosine 1-phosphate receptor-1 ($S1P_1$) on lymphocytes, thereby inhibiting their egress from lymphoid organs and their recirculation to inflammatory sites. Potential effects on dendritic cell (DC) trafficking have not been evaluated. Here we demonstrate the expression of all 5 S1P receptor subtypes ($S1P_{1-5}$) by murine DC. Administration of FTY720 to C57BL/10 mice markedly reduced circulating T and B lymphocytes within 24 h, but not blood-borne DC, that were enhanced significantly for up to 96 h, while DC in lymph nodes and spleen were reduced. Numbers of adoptively-transferred, fluorochrome-labeled syngeneic or allogeneic DC in blood were increased significantly in FTY720-treated animals, while donor-derived DC and allostimulatory activity for host naïve T cells within the spleen were reduced. Administration of the selective $S1P_1$ agonist SEW2871 significantly enhanced circulating DC numbers. Flow analysis revealed that CD11b, CD31/PECAM-1, CD54/ICAM-1 and CCR7 expression on blood-borne DC was downregulated following FTY720 administration. Transendothelial migration of FTY720-P-treated immature DC to the CCR7 ligand CCL19 was reduced. These novel data suggest that modulation of DC trafficking by FTY720 may contribute to its immunosuppressive effects.

[5] Data included in this chapter have been published: Lan, Y.Y., De Creus, A., Colvin, B.L., Abe, M., Brinkmann, P.T.H., Thomson, A.W. The sphingosine-1-phosphate receptor agonist FTY720 modulates dendritic cell trafficking in vivo. American Journal of Transplantation 2005; 5: 2649-2659. Dr Colvin performed the transendothelial migration assay (Fig. 17B), and Dr Abe assisted in the T cell allostimulation experiment (Fig. 14C). All co-authors contributed to revising the manuscript.

2.1 INTRODUCTION

The novel immunomodulatory pro-drug FTY720 is a structural analogue of ISP-1 (myriocin), a fermentation product of the ascomycete *Isaria sinclairii* (97, 132). FTY720=2-amino-2-[2-(4-octylphenyl-ethyl)]-1,3-propane-diol hydrochloride (C_{19} H_{33} NO_2: HCl) represents the first member of a new class of immunosuppressants. Unlike conventional immunosuppressive agents, it does not inhibit T or B cell activation or proliferation, or their effector function (98, 99). FTY720 causes rapid and dramatic depletion of circulating lymphocytes, but not granulocytes or monocytes, within 2-6 h of intake (114). Mature lymphocytes are sequestered in peripheral and mesenteric lymph nodes and intestinal Peyer's patches, leading to decreases also in lymphocytes in the thoracic duct lymph and spleen (101, 134). The sequestration is completely reversible and infused labeled lymphocytes reappear in the blood after drug withdrawal (99, 131, 134). FTY720 prolongs rodent heart and skin allograft survival, and acts synergistically with cyclosporine to suppress canine and non-human primate kidney graft rejection (102, 105, 107, 108). It has proved a promising anti-rejection agent in clinical renal transplantation (115), and is currently undergoing phase III clinical trials.

The exact mechanism(s) underlying the influence of FTY720 on leukocyte trafficking and its immunosuppressive action are still poorly understood. A recent theory suggests that FTY720 activates G-protein-coupled sphingosine-1-phosphate (S1P) receptors (R), known previously as endothelial differentiation gene (Edg) receptors, stimulation of which promotes leukocyte migration (96, 131, 177). Upon binding S1P, these receptors act downstream on the small GTPases Cdc42, Rac and Rho, and evoke a S1PR-dependent activation of Cdc42, Rac and Rho, that initiates cytoskeletal rearrangements that determine leukocyte morphology and motility (178, 179).

FTY720 is phosphorylated *in vivo* to its active metabolite FTY720-phosphate (FTY720-P), a structural homolog of S1P, that binds to four of the five S1PR ($S1P_{1,3,4,5}$) in vitro (131, 132). In vivo, FTY720-P acts as a high-affinity agonist at $S1P_1$ on lymphocytes, thereby inducing aberrant internalization of the receptor, rendering the cells unresponsive to S1P, and depriving them of an obligatory signal to egress from lymphoid organs (119). Consequently, FTY720 inhibits egress of lymphocytes from secondary lymphoid organs (SLO) into efferent

lymph and blood, thereby preventing recirculation of auto- and allo-aggressive effector T cells to peripheral inflammatory sites.

To date, there have been few reports concerning potential effects of FTY720 on dendritic cells (DC). DC are uniquely well-equipped bone marrow (BM)-derived migratory antigen-presenting cells, that induce and regulate immune responses (3, 48, 180-182). There is recent evidence that human DC express S1PR (150, 183), and that S1P and FTY720 or FTY720P induce changes in human DC migration, cytokine production profile and T cell stimulatory function in vitro (183). In the present study, we have investigated the influence of FTY720 and the selective $S1P_1R$ agonist SEW2871 on blood-borne and secondary lymphoid tissue DC in mice. The data suggest that FTY720 administration may impair DC migration from blood and downregulate the expression of key intercellular adhesion molecules and the CC chemokine receptor CCR7 required for DC transendothelial migration and homing to SLO. These effects on DC may contribute to the immunosuppressive action of FTY720.

2.2 MATERIALS AND METHODS

2.2.1 Mice

Male C57BL/10J (B10; $H2^b$) and C3H HeJ (C3H;$H2^k$) mice, 8-12 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the specific pathogen-free Central Animal Facility of the University of Pittsburgh. They received Purina rodent chow (Ralstan Purina, St. Louis, MO) and tap water *ad libitum*. Experiments were conducted in accordance with the National Institutes of Health Guide for use and care of laboratory animals and under an Institutional Animal Care and Use Committee-approved protocol.

2.2.2 FTY720 or SEW 2871 administration

Mice were injected with a single i.p dose of 3.5 mg/kg FTY720 (Novartis, Basel, Switzerland) or with 20 mg/kg SEW2871 (Calbiochem, La Jolla, CA). Administration of FTY720 by the i.p.

route has been shown previously to be associated with immunosuppression, reduction in circulating lymphocytes and suppression of graft rejection (184, 185). Normal controls were untreated.

2.2.3 Reagents and Abs

FTY720 was dissolved in distilled water and FTY720-P (Novartis) in DMSO/50 mM HCl, then diluted to the appropriate concentrations. The selective $S1P_1R$ agonist SEW2871 was dissolved in DMSO before dilution to the appropriate concentration. The DC poietin recombinant (r) human fms-like tyrosine kinase 3 ligand (Flt3L) (chinese hamster ovary cell-derived) used to mobilize DC, was a gift from Amgen (Seattle, WA). Recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 were gifts from Schering-Plough, Kenilworth, NJ. Complete medium (CM) comprised RPMI-1640 (BioWhittacker, Walkersville, MD) supplemented with 10% v/v fetal calf serum (Nalgene, Miami, FL), non-essential amino acids, L-glutamine, sodium pyruvate, penicillin-streptomycin and 2-mercaptoethanol (all Life Technologies, Gaithersburg, MD). BD PharM Lyse® NH_4Cl lysing reagent and all anti-mouse monoclonal antibodies (mAbs) except PE-anti-CCR7 (BioLegend, San Diego, CA) were obtained from BD PharMingen (San Diego, CA). Vybrant™ CFDA SE cell tracer kit was purchased from Molecular Probes (Eugene, OR). Lympholyte-M® was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). Collagenase IV was obtained from Sigma (St. Louis, MO). Magnetic microbeads and separation columns were from Miltenyi Biotech (Auburn, CA). Trizol reagent was purchased from R&D Systems (Minneapolis, MN) and Advantage™ RT-for-PCR kit from Clontech (Palo Alto, CA). Mouse r CCL19 was purchased from R&D Systems.

2.2.4 Leukocyte analysis

Heparinized blood collected from pairs of mice was pooled. Samples (200 μl) were then immunostained for T cells (FITC-anti-CD3), B cells (FITC-anti-CD19), NK cells (PE-anti-NK1.1) or DC (FITC-anti-CD11c) simultaneously with red blood cell (RBC) lysis using BD

PharM Lyse®, according to the manufacturer's instructions. Lymph node (submandibular, axillary and inguinal) and splenic mononuclear cells were isolated as described (186), then immunostained with PE-anti-CD11c, and FITC-anti-CD8α or FITC-anti-CD3 or FITC-anti-CD19 mAbs to determine DC subsets, T cell and B cell populations, respectively. Following flow analysis, absolute cell number was calculated by multiplying the total leukocyte number by the percentage (%) of each population of interest. Results were expressed as % normal control value ±1 standard deviation (SD).

2.2.5 DC propagation

DC were propagated from normal B10 mouse BM in GM-CSF+ IL-4 (187) then positively selected using anti-CD11c magnetic microbeads, as described in detail (82). Their purity was consistently >95%. The purified CDllc^{+} cells were characterized by 2-color flow cytometric analysis and were CD40^{lo}, CD80^{lo}, CD86^{lo} and MHC class II (IA^{b})^{lo}, as shown previously (188).

2.2.6 Detection of S1PR expression

BMDC and blood-borne DC were positively selected using anti-CD11c bead separation, as described (189). Spleen DC were first enriched using a 16% v/v nycodenz gradient (500g; 4°C; 20 min) before anti-CD11c bead separation. DC purity was >95%. Total RNA was extracted from the purified DC using the Trizol method (187). cDNA was synthesized from the RNA samples with Advantage™ RT-for-PCR kit. PCR primers were: $S1P_1$ (681 bp): $S1P_1$a (5'-GGG ACA CAA TTA GCA GCT AT-3', sense) and $S1P_1$b (5'-GTA GAG GAT GGC GAT GGA AAG-3', antisense); S1P2 (461 bp): $S1P_2$a (5'-TTA ACT CCC GTG CAG TGG TTT GC-3', sense) and S1P2b (5'-ACG ATC GTC ACC GTC TTG AGC A-3', antisense); S1P3 (681 bp): S1P3a (5'-CGC ATG TAC TTT TTC ATT GGC AA-3', sense) and S1P3c (5'-GGG TTC ATC GCG GAC TTC AG-3', antisense); S1P4 (445 bp): S1P4a (5'-GAG TCA TAC CCA CAG TTG C-3', sense) and S1P4b (5'-CAG TGT GAT GTT CAG CAG G-3', antisense); S1P5 (571 bp): S1P5e (5'-CTA CTG CTA CAG ACT GAC G`-3', sense) and S1P5f (5'-GCT CTG TTT CCT CTG TAG C-3', antisense). The PCR mix for $S1P_{1-3}$ was run 35 cycles (94ºC, 30 s; 54ºC, 30 s;

72ºC, 2 min), with a final extension step of 10 min at 72ºC. The PCR mix for $S1P_{4,5}$ was run 35 cycles (94ºC, 30 s; 58ºC, 30 s; 72ºC, 30 s), with a final extension step of 7 min at 72ºC. PCR samples were then analyzed on 1% w/v agarose gel stained with ethidium bromide.

2.2.7 Exposure of DC to FTY720-P in vitro

The active metabolite of FTY720, FTY720-P, used in *in vitro* experiments was diluted in CM from a stock solution of 10^{-3} M. BMDC were cultured in the presence of a clinically-relevant concentration of 10^{-6}M FTY720-P for 24 h.

2.2.8 Analysis of DC apoptosis

DC apoptosis was monitored by staining of phosphatidylserine translocation with FITC-Annexin V in combination with propidium iodide (PI) according to the manufacturer's instructions. Cells were co-stained with CD11c to allow specific analysis of DC by flow cytometry.

2.2.9 Analysis of CCR expression

CCR mRNA expression by highly-purified DC was determined by RNase protection assay (RPA) as described (186). Staining of DC for cell surface CCR7 and subsequent flow cytometric analysis was performed using anti-CCR7 mAb.

2.2.10 Analysis of DC migration in vitro

Migratory responses of DC to S1P were quantified in Transwell® chemotaxis assays, as described (186). For analyzing DC transendothelial migration in response to CCL19, resting murine endothelial cells (EC; MS1, a gift from Dr T. M. Carlos, Department of Medicine, University of Pittsburgh) were layered ($7.5\text{-}8 \times 10^4$) on each Transwell® filter (5 μm), 24-48 h before the addition of DC (10^5), and then the chambers incubated for 4 h Migrated DC were enumerated using a Coulter counter.

2.2.11 Quantitation of DC-endothelial cell interactions

Control or FTY720-P-treated (10^{-6}M; 24 h), bead-purified BMDC were labeled with CMFDA (15 μM). Five × 10^5 were then added to an EC monolayer (2.10^5) in 1.5 ml complete medium for 1.5 h at 37°C. The wells were washed gently with warm PBS, and the number of labeled DC adherent to EC in 10 randomly-chosen high-powered (×400) fields counted using a fluorescence microscope.

2.2.12 Analysis of adhesion molecule expression

Blood DC from control or FTY720-treated mice, or cultured BMDC were double-stained with PE-anti-CD11c and either FITC-anti-CD11b, -anti-CD31/PECAM-1, -anti-CD54/ICAM-1 or anti-CD62L and analyzed by flow cytometry.

2.2.13 CFSE labeling and in vivo monitoring of DC

Freshly-isolated BM cells were incubated in pre-warmed PBS containing CFSE (5 μM) for 15 min at 37°C, and washed extensively before use. Fifty million labeled cells were injected i.v. into syngeneic recipients via the lateral tail vein, 2 h after FTY720 administration. Mice were killed 18 h later. Blood samples were processed as described above, then stained with PE-anti-CD11c mAb. Flow cytometry was used to analyze the double-positive, CFSE-labeled $CD11c^+$ population.

2.2.14 Allogeneic BM transplantation

BM cells from Flt3L-mobilized B10 mice (10 μg i.p. for 10 days) comprising 15-30% $CD11c^+$ DC (81) were labeled with CFSE, as described above. Similar to the syngeneic DC tracking experiment, 50 × 10^6 labeled BM cells were injected i.v. into either untreated or FTY720-treated

allogeneic (C3H) mice. Blood and spleen samples were collected and processed as in the syngeneic cell transfer experiment.

2.2.15 Analysis of T cell allostimulatory activity

The capacity of C3H host spleen cells, harvested 24 h after i.v. infusion of $50x10^6$ Flt3L-mobilized allogeneic (B10) BM cells, to stimulate proliferative responses in freshly-isolated, naïve C3H splenic T cells was determined in 72 h MLR, as described (188).

2.2.16 Statistical analysis

Significances of differences between means were calculated using the Wilcoxon-Mann-Whitney test for small samples or Student's t-test as appropriate. Differences between groups were considered significant at $p < 0.05$.

2.3 RESULTS

2.3.1 DC from blood and secondary lymphoid tissue and BMDC express S1PR

In the first set of experiments, we employed RT-PCR to analyze the expression of $S1P_{1-5}$ (formerly Edg-1,-5,-3,-6 and -8, respectively) (120) by freshly-isolated (immature) B10 mouse blood and spleen DC and by in vitro-propagated, immature BM-derived myeloid DC. As shown in Fig. 9A, each DC population expressed all 5 S1PR. Expression of each S1PR was also evident following DC maturation induced by overnight culture in the presence of the Toll-like receptor (TLR) 4 ligand, LPS, with evidence of higher expression of $S1P_{1-3}$ on mature DC (Fig. 9B**,** C; data shown only for BMDC). We also examined the in vitro migratory responses of immature versus LPS-matured BMDC to S1P using Transwell® chemotactic assays, as described (186). As shown in Fig. 9D, mature BMDC exhibited modest but significant migratory responses to S1P, corresponding to the higher expression of $S1PR_{1-3}$ on these DC.

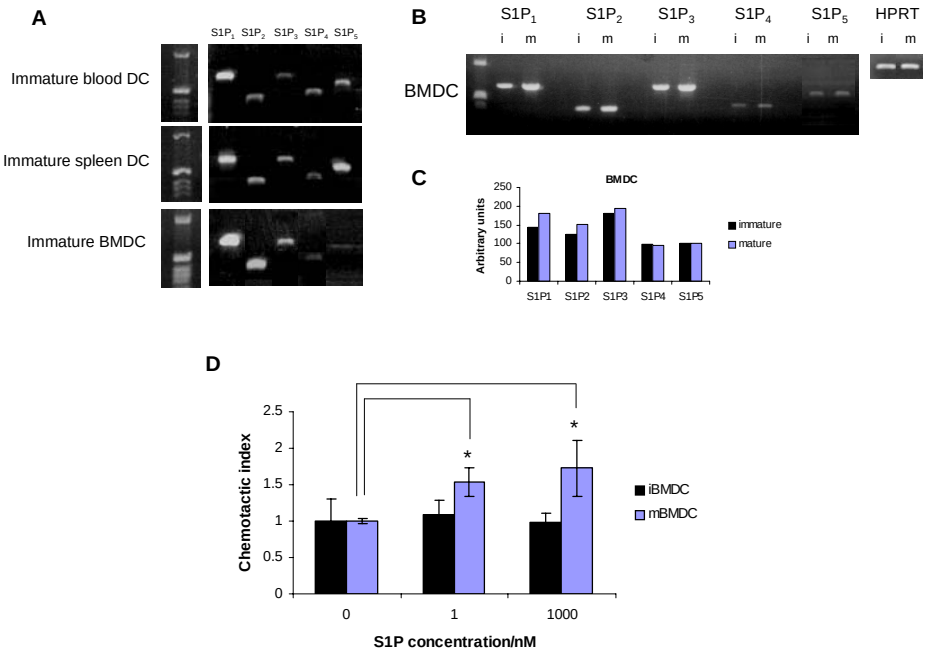

Figure 9. C57BL10 (B10) mouse DC express S1P receptors.

(A), RT-PCR analysis of S1P receptor ($S1P_{1\text{-}5}$) expression was performed on highly-purified, freshly-isolated C57BL/10 (B10) blood and spleen DC, and on immature, bone marrow-derived DC (BMDC), as described in the Materials and Methods. Data are from one experiment representative of two performed. 1Kb markers are displayed on the left. (B), both immature (i) and LPS-matured (m) BMDC express S1PR, with evidence (C), of higher expression of $S1P_{1\text{-}3}$ on mature DC. (D), although iBMDC did not migrate in vitro to the S1PR ligand S1P, mBMDC displayed a modest chemotactic response, corresponding to their higher expression of $S1P_{1\text{-}3}$. Values are means ±1SD. *, $p<0.05$. Data are from one experiment representative of three (B, C) or two performed (D).

2.3.2 FTY720-P does not interfere with DC differentiation or affect the apoptotic death of DC

To examine its influence on DC differentiation, FTY720-P (10^{-6}M) was added at the beginning of BMDC cultures and replenished every 2 days. No significant influence on the numbers of DC recovered from the cultures at day 7 (control: 4.8 ± 0.8 x 10^6/well; FTY720-P:4.7 ± 0.7 x 10^6/well) or on the expression of cell surface MHC class II (IA^b) or costimulatory molecules (CD40, CD80 and CD86) was observed (Fig. 10A). To ascertain whether FTY720-P might affect the apoptotic death of DC, BMDC were exposed to 10^{-6} M FTY720-P in CM (10% FCS-supplemented) for 24 h. As shown in Fig. 10B**,** no significant influence on the incidence of

Annexin V^+/PI^- cells, determined by flow analysis was found. When serum was removed from the medium in similar experiments, to enhance apoptotic cell death over the ensuing 72 h period, and the influence of FTY720-P reassessed, no significant effect was again observed (Fig 10B). Thus, under either steady-state or apoptosis-promoting conditions, FTY720-P did not affect the death of DC.

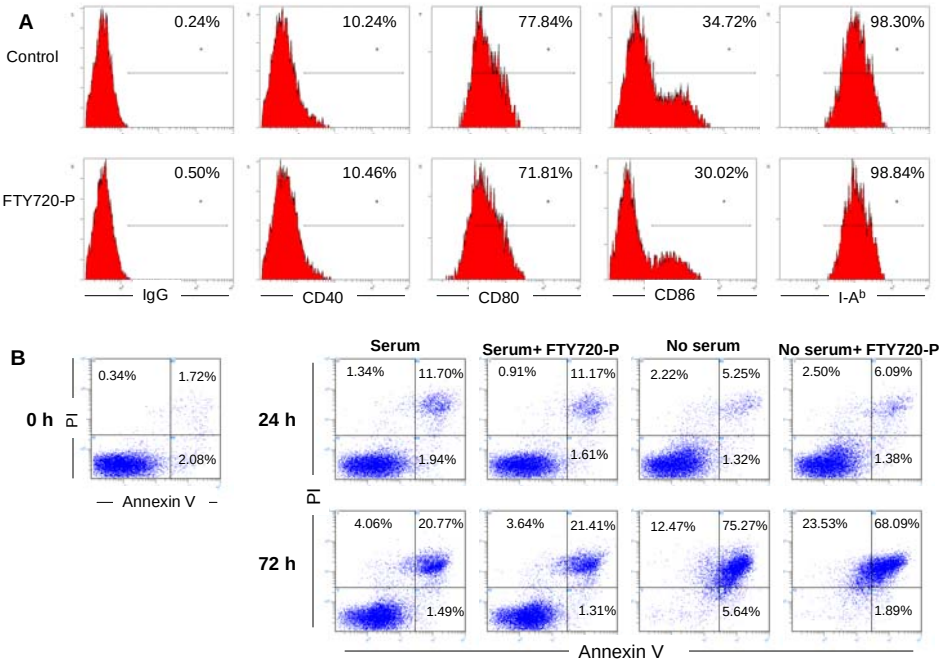

Figure 10. Exposure to FTY720-P does not affect DC differentiation or apoptosis.

(A), FTY720-P (10^{-6} M) was added at the start of 7-day B10 BMDC cultures and replenished every 2 days. Cell surface expression of MHC class II (IA^b) or costimulatory molecules, detected by flow cytometry at day 7, were unaffected. Percentages of positive cells are shown. All events were gated on $CD11c^+$ cells. (B), BMDC exposed to FTY720-P (10^{-6}M) in the presence or absence of serum for the indicated time periods, show no difference in the incidence of apoptotic cells compared with untreated controls. Apoptosis of $CD11c^+$ cells was detected by staining with FITC-Annexin V in combination with PI, as described in the Materials and Methods. Data shown are from one experiment and are representative of two performed.

2.3.3 FTY720 administration markedly depletes circulating T and B lymphocytes, but enhances blood DC

We next examined whether systemic administration of FTY720 might affect DC trafficking *in vivo*. Circulating lymphocytes (T, B and NK cells) and DC ($CD11c^+$ $NK1.1^-$) were analyzed, 24 h after drug administration to B10 mice, by single or two-color flow cytometry, as described in the Materials and Methods. As shown in Fig. 11, and as anticipated, FTY720 administration caused substantial reductions in the incidence of circulating $CD3^+$ T cells and $CD19^+$ B cells in blood. By contrast, an increased incidence of blood-borne $CD11c^+$ ($NK1.1^-$) DC was observed. The incidence of NK cells that did not co-express the DC-associated marker CD11c ($NK1.1^+$ $CD11c^-$) was unaffected.

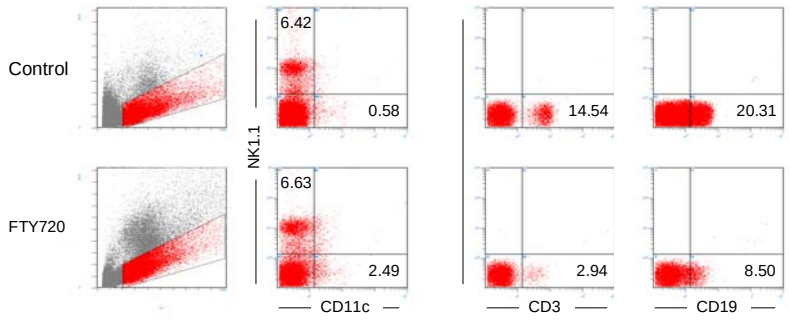

Figure 11. FTY720 administration depletes blood T ($CD3^+$) and B ($CD19^+$) lymphocytes, but not circulating DC ($CD11c^+$ $NK1.1^-$) or NK cells ($NK\ 1.1^+$).

Blood samples were obtained from groups of untreated control B10 mice, or from animals 24 h after FTY720 administration (3.5 mg/kg). Isolated leukocytes from pooled blood were immunostained with FITC-anti-CD3, FITC-anti-CD19, or PE-anti-NK1.1 and PE-anti-CD11c mAbs, then analyzed by flow cytometry, as described in the Materials and Methods. Data shown are from one experiment, and are representative of three performed.

2.3.4 FTY720 administration depletes DC in spleen and lymph node

To ascertain its influence on DC and lymphocyte populations in SLO, lymph node and spleen mononuclear cells were obtained 24 h-7 d after systemic FTY720 administration. Concomitant with their approximate, 2.5-fold elevation in the circulation at 24 h, that was sustained at 48 and 96 h, but no longer evident at 7 d, DC numbers were reduced modestly but significantly in the spleen (Fig. 12B). Similar reductions were evident in both classic myeloid ($CD11c^+$ $CD8\alpha^-$) and 'lymphoid-related' ($CD11c^+$ $CD8\alpha^+$) DC subsets (Fig. 12A). FTY720 also decreased the numbers of DC in lymph nodes at 48 and 96 h (Fig. 12B). There were concomitant reductions in the incidences of T and B lymphocytes in the spleen, whereas some increase in B cells in lymph nodes was observed (Fig. 12A).

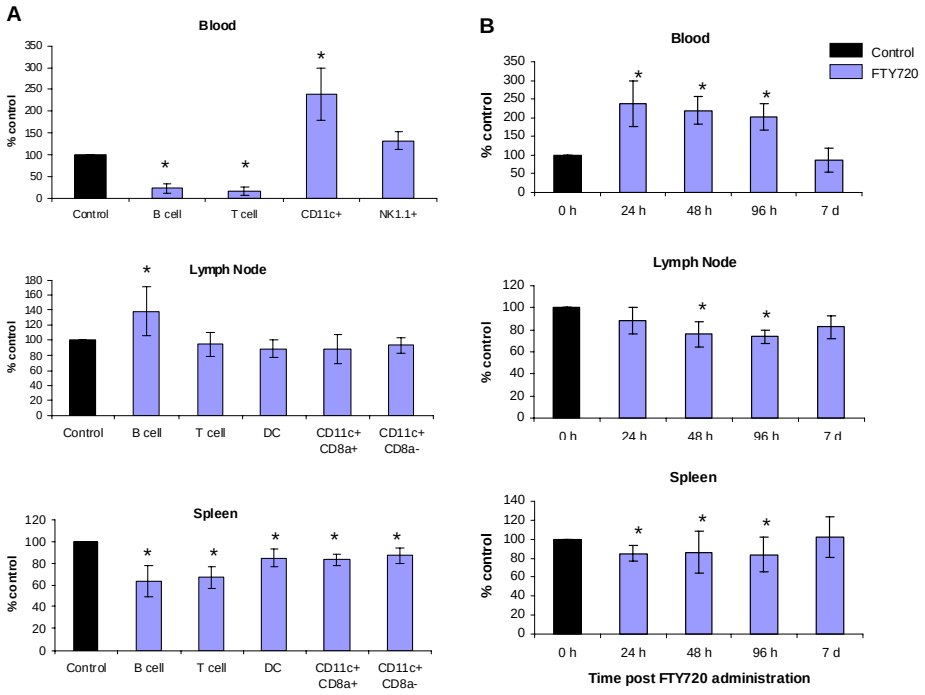

Figure 12. FTY720 adminstration depletes DC in the spleen and lymph nodes.

(A), Single cell suspensions from blood, lymph nodes or spleens of groups of control B10 mice or animals 24 h after FTY720 administration (3.5 mg/kg) were prepared as described in the Materials and Methods. The cells were then immunostained with FITC-anti-CD3, FITC-anti-CD19 or PE-anti-CD11c mAbs to assay T cell, B cell and DC populations respectively, by flow cytometry. Data were calculated as absolute cell number and shown as percent control values (absolute number x 100 ÷ absolute cell number in controls). DC in spleens decreased consistently, together with T and B cells, whereas DC numbers in lymph nodes appeared unaffected at 24 h. Both $CD8\alpha^+$

('lymphoid-related') and classic CD8α$^-$ (myeloid) DC subsets were modestly reduced. Data are mean values ±1SD obtained from three experiments performed. (B), Reduced numbers of DC were observed in both spleen and lymph nodes, 48 and 96 h after FTY720 administration, concomitant with the increased numbers of DC in blood. Cell numbers were restored to normal by day 7. Data are mean values ±1SD obtained from three experiments performed. *, $p<0.01$.

2.3.5 Greater numbers of infused syngeneic DC are retained in the circulation of FTY720-treated mice

To evaluate further the influence of FTY720 administration on circulating DC, we infused CFSE-labeled syngeneic B10 BM cells (50×10^6) i.v. into either untreated controls, or into mice 2 h after FTY720 administration, then monitored the fate of the labeled cells. As shown in Fig. 13, a higher proportion (approx. 7-fold) and absolute number (50% increase) of CFSE-labeled DC were retained in the circulation of FTY720-treated mice compared with normal controls when examined 18 h later. At the same time, splenic CFSE-labeled DC that trafficked to the spleen were reduced modestly, but significantly.

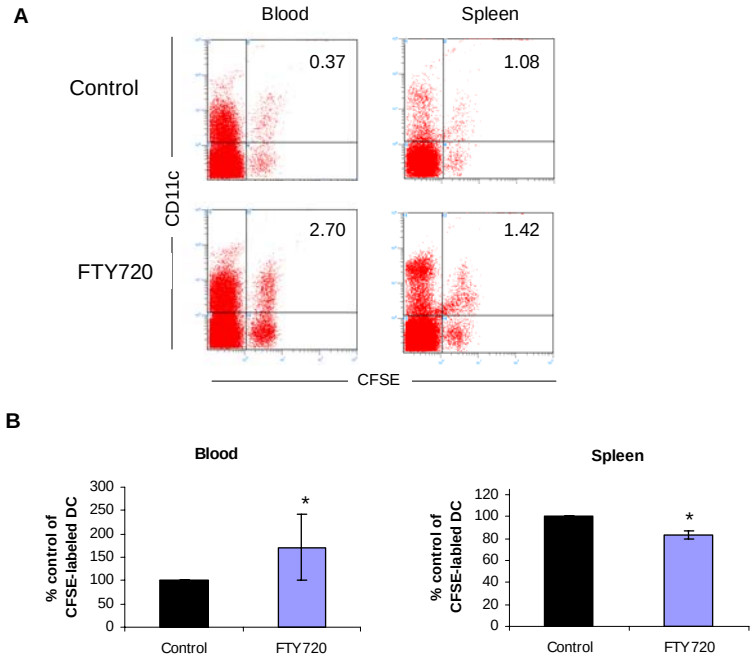

Figure 13. Enhanced numbers of donor DC are retained in the circulation of FTY720-treated mice following syngeneic BM cell infusion.

Fifty million CFSE-labeled B10 BM cells were injected i.v. into each normal syngeneic recipient via the lateral tail vein, 2 h after systemic FTY720 administration (3.5 mg/kg). Pooled blood samples from control or FTY720-treated mice were collected 18 h later. Cells were stained with PE-anti-CD11c mAb for subsequent flow cytometric analysis. Results were expressed as % control (absolute cell number x 100 ÷ absolute cell number in controls). (A), A higher incidence and (B), an approximate, 2-fold higher absolute number of double-positive, CFSE-labeled DC were observed in blood of animals treated with FTY720. A small but significant reduction in CFSE-labeled DC was observed concomitantly in the spleen. Data are from one experiment representative of 3 performed (A) or means ±1SD obtained from 3 separate experiments (B). *, $p<0.05$.

2.3.6 Greater numbers of donor DC are retained in blood of FTY720-treated animals following allogeneic BM transplantation: association with reduced naive T cell allostimulatory activity in host spleens

Following allogeneic BM or organ transplantation, donor-derived DC migrate via the blood to SLO and therein instigate anti-donor T cell responses (28, 81). To determine its influence on the migration of fully MHC-mismatched DC from the blood, 50×10^6 CFSE-labeled, Flt3L-mobilized B10 BM cells were injected i.v. into normal control or FTY720-treated C3H recipients. As shown in Fig. 14A, B, FTY720 administration enhanced retention of allogeneic DC ($CD11c^+$) in the blood 18 h later by approx. 2-3-fold, whereas CFSE-labeled $CD11c^+$ cells in the spleen were reduced concomitantly. The reduction in donor-derived DC within host spleens was associated with reduced allostimulatory activity of splenocytes for naïve host T cells (Fig. 14C), indicating that reduced trafficking of donor-derived DC to the spleens of FTY720-treated mice was associated with a reduced level of T cell priming activity.

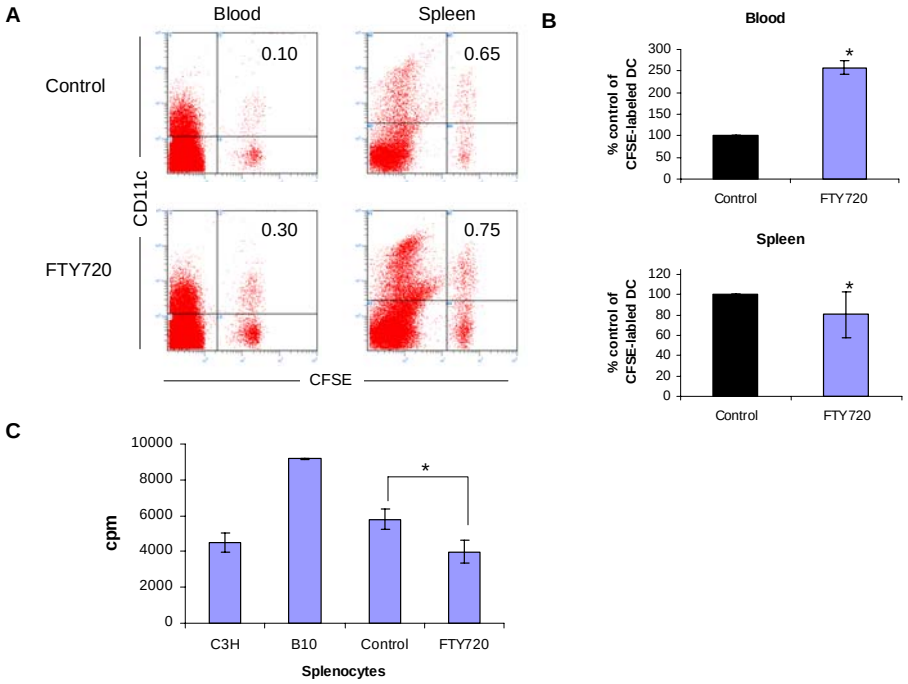

Figure 14. Enhanced numbers of donor DC are retained in the blood of FTY720-treated mice after allogeneic BM transplantation: association with reduced donor DC and naïve T cell allostimulatory activity within host spleens.

Fifty million allogeneic (B10; $H2^b$), Flt3L-mobilized CFSE-labeled BM cells were infused i.v. via the lateral tail vein of untreated control C3H ($H2^k$) mice or mice given FTY720 (3.5 mg/kg) 2 h earlier. Blood and spleen were collected 18 h later, and mononuclear cells isolated and stained with anti-CD11c mAb. (A), Dot plots show an increased incidence of labeled DC in the blood, but not in the spleen of treated mice. Percentages of CFSE-labeled $CD11c^+$ cells are shown. Data are from one experiment representative of three performed. (B), The absolute number of labeled DC increased 2-3 fold in the blood, but decreased concomitantly in the spleens of FTY720-treated mice compared with controls. % control = absolute cell number x 100 ÷ absolute cell number in controls. Data are means ±1SD obtained from three independent experiments. *, $p<0.01$. (C), the decrease in number of allogeneic DC reaching the spleen of FTY720-treated recipients was associated with reduced in vitro allostimulatory activity of host splenocytes for naïve C3H T cells, as determined by MLR. Data (means ±1SD) are from one experiment representative of two performed. *$p<0.05$.

2.3.7 The selective $S1P_1$ agonist SEW 2871 rapidly enhances the number of blood DC

To ascertain whether the influence of FTY720 on blood DC could be reproduced by a selective $S1P_1$ agonist, we administered SEW 2871 i.p. and determined blood and SLO DC levels 6 and

24 h later. As shown in Fig. 15, there were significantly increased levels of DC in the blood of SEW 2871-treated animals compared with controls at 6 h. This increase was no longer apparent at 24 h. No significant changes in splenic or lymph node DC numbers were observed following SEW 2871 administration.

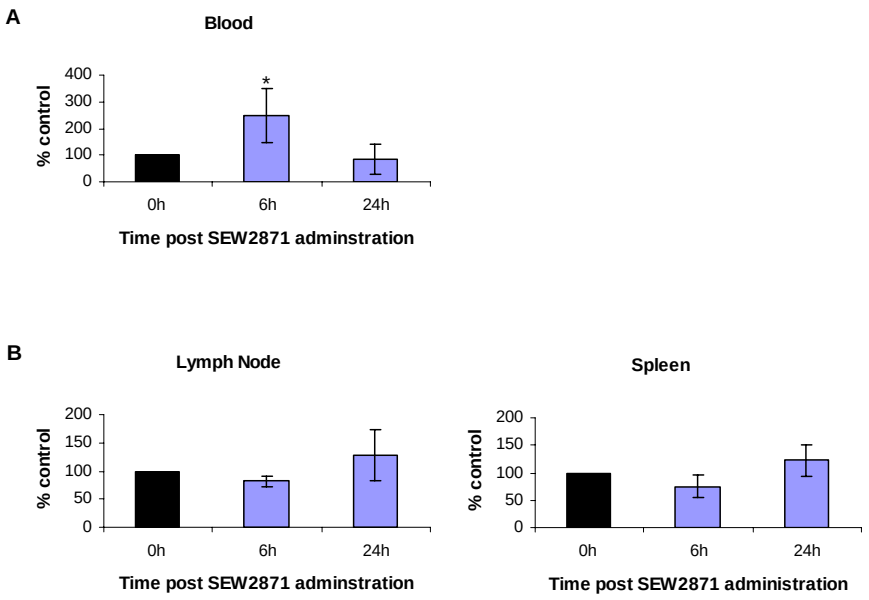

Figure 15. The selective $S1P_1$ agonist SEW2871 increases DC numbers in the blood.

B10 mice were injected i.p. with SEW2871 (20 mg/kg), then 6 h and 24 h later, $CD11c^+$ DC were quantified in (A) blood and (B), secondary lymphoid organs. SEW2871 significantly increased DC in blood at 6 h, but the effect was no longer apparent at 24 h. % control = absolute cell number x 100 ÷ absolute cell number in controls. Data are means ±1SD obtained from three experiments. *, $p<0.05$.

2.3.8 FTY720 administration reduces adhesion molecule expression on blood DC

To ascertain whether FTY720 might affect DC adhesion molecule expression *in vivo,* we treated mice with FTY720, then 24 h later, examined the expression of various cell surface adhesion molecules on blood DC. Immunostaining with PE-anti-CD11c and either FITC-anti-CD11b, -anti-CD31/PECAM-1, -anti-CD54/ICAM-1 or anti-CD62L was performed simultaneously. As shown in Fig. 16, FTY720 administration downmodulated expression of CD11b, CD31 and

CD54, but no consistent effect on surface CD62L expression was observed. When we examined directly the influence of 24 h FTY720-P pretreatment of immature BMDC on their subsequent adhesion to resting mouse endothelium, no significant effect was found (data not shown).

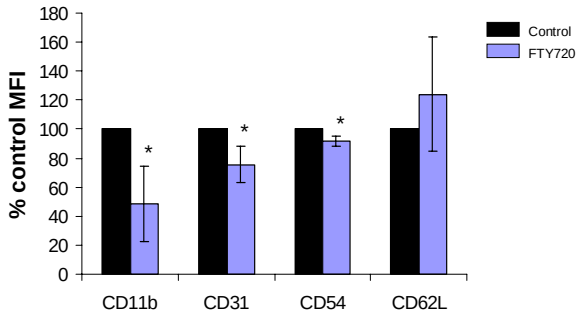

Figure 16. FTY720 administration downregulates adhesion molecule expression on circulating DC.

Pooled blood samples from normal control or FTY720-treated B10 mice were collected 24 h after FTY720 administration (3.5 mg/kg). Immunostaining of blood leukocytes with PE-anti-CD11c and either FITC-anti-CD11b, -anti-CD31/PECAM-1, -anti-CD54/ICAM-1 or –anti-CD62L was performed simultaneously with RBC lysis, as described in the Materials and Methods. The level of expression (mean fluorescence intensity; MFI) of CD11b, CD31 and CD54 was reduced significantly on DC from FTY720-treated mice compared with those from control animals. Results are means ±1SD. The experiment was repeated three times, with similar results. *, $p<0.05$.

2.3.9 FTY720-P or FTY720 administration down-modulates CCR7 expression on DC: association with reduced transendothelial migration to CCL19

Modulation of chemokine receptor expression on DC is important in regulating migratory responses of these cells to chemokines, expressed either constitutively or in sites of inflammation (11, 14). We considered that FTY720 might affect the expression of CC chemokine receptors that affect transendothelial migration by DC and their homing to SLO. RPA of highly-purified, unstimulated BMDC following their in vitro exposure to FTY720-P or S1P for 8 or 24 h was

performed as described (186). No significant influence of FTY720-P or S1P on CCR1, 5, 6, or 7 mRNA expression was detected (data not shown). We also examined cell surface expression of CCR7 on the FTY720-P-treated BMDC and on blood DC, 24 h after FTY720 injection. As shown in Fig. 17A, FTY720-P reduced the incidence of $CCR7^+$ immature BMDC (by approx. 30%) and to a lesser extent (approx. 10-15%) the incidence of $CCR7^+$ mature BMDC. Moreover, the percentage of blood DC expressing CCR7 was reduced (approx. 40%) in FTY720-treated animals. The decreased expression of CCR7 on BMDC was accompanied by a reduction in *in vitro* migratory responses to the CCR7 ligand CCL19 (Fig. 17B).

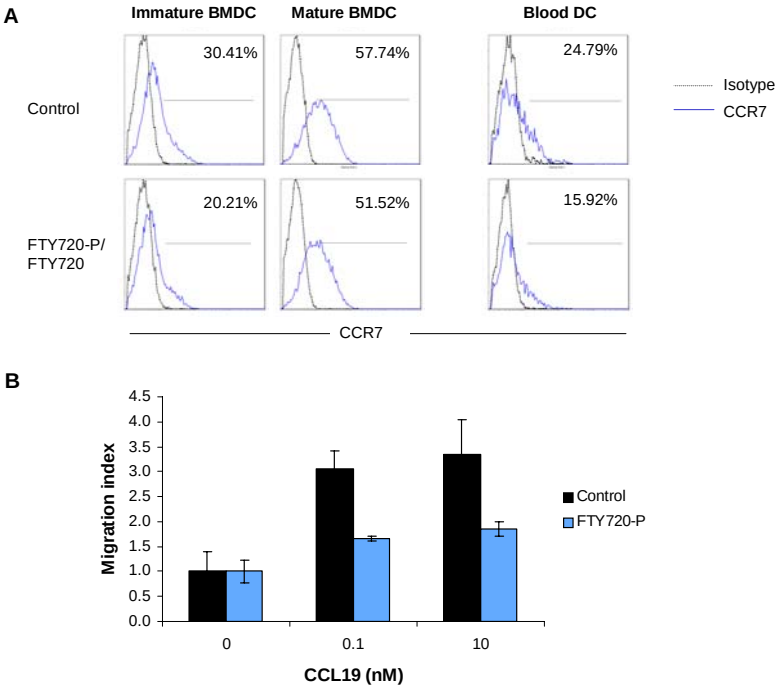

Figure 17. FTY720/FTY720-P down-modulates CCR7 expression on DC: association with reduced transendothelial migration in response to CCL19.

(A), Immature or LPS-activated mature BMDC were treated with FTY720-P (10^{-6}M) for 24 h, then stained with FITC-anti-CD11c and PE-anti-CCR7 mAbs. Blood samples from FTY720-treated B10 mice were lysed with PharM Lyse® NH_4Cl lysing reagent and stained in the same way. Controls were untreated. Flow cytometric analysis reveals reduced levels of CCR7 expression on both immature and mature BMDC and on blood DC, following FTY720-P or FTY720 treatment, respectively. Data are representative of three separate experiments; gated on $CD11c^+$ cells. (B), Transendothelial migration of untreated or FTY720-P (10^{-6}M)-treated immature BMDC in Transwell® assays. Migrated DC were enumerated 4 h after start of incubation using a Coulter counter. FTY720-P-

treated BMDC showed reduced migration to the CCR7 ligand CCL19 at 0.1 and 10 nM (B). The experiment was repeated three times, each with similar results.

2.4 DISCUSSION

Here we show for the first-time, that in normal animals, not only circulating T and B lymphocytes, but also numbers of blood DC are affected by systemic FTY720 administration. Freshly-isolated blood or spleen DC, or in vitro-propagated murine myeloid DC subsets, expressed all 5 S1PR subtypes. This finding is consistent with the recent demonstration of $S1P_{1-4}$ expression by human monocyte-derived DC (150, 183). Treatment of mice with a single dose of either FTY720, or the selective $S1P_1$ agonist SEW2871 (139), increased the number of DC in blood. By contrast, DC subsets were reduced significantly in the spleens and lymph nodes of FTY720-treated animals. Accordingly, systemic FTY720 administration resulted in enhanced retention of adoptively-transferred, unstimulated syngeneic or allogeneic DC in the blood. Increases in blood-borne DC were associated with down-modulation of specific cell surface adhesion molecules (CD11b, CD31 and CD54/ICAM-1) and the CC chemokine receptor CCR7. Reduction in CCR7 expression may contribute to the altered homing of DC to SLO. Moreover, lower numbers of donor-derived DC within the spleens of FTY720-treated mice were associated with reduced stimulatory (priming) activity for host naïve T cells in MLR, verifying the impact of altered DC migration on induction of alloimmune reactivity.

DC trafficking is determined by various factors, in particular cell maturation status, cognate G-protein-coupled receptor expression, chemokine signaling and expression of cell surface intercellular adhesion molecules (190-192). In the present study, exposure of immature BMDC (equivalent to those that circulate in the blood) to physiologic concentrations of FTY720-P did not affect their chemokine receptor (CCR1, 5, 6, and 7) mRNA expression significantly. However, cell surface expression of CCR7 on FTY720-P-treated immature BMDC and their migratory responses to the CCR7 ligand CCL19 were reduced compared with control cells. These latter observations correlate with the increased numbers of blood-borne DC and with the significant reductions in DC trafficking from blood to SLO in FTY720-treated mice. Thus we consider it unlikely that DC precursors/immature DC in the BM undergo enhanced

motility/migration into the blood in response to FTY720 administration. Indeed, FTY720-P-treated immature human DC show decreased chemotactic responses to RANTES and stromal-derived factor-1α (183), associated with a reduction in actin polymerization, a prerequisite for cell migration.

DC migration from the blood is regulated by cell surface adhesion molecules on the DC and their ligands on EC. CD11b/CR3 is important for DC adhesion to endothelium (193). In addition, blocking mAb against CD31/PECAM-1 inhibits DC migration across activated endothelium (194), whereas CD54/ICAM-1 mediates LFA-1-dependent monocyte migration through human umbilical vein EC (195). In the present study, blood-borne DC from FTY720-treated animals showed reduced surface expression of CD11b, CD31 and CD54, suggesting that modulation of intercellular adhesion molecule expression may underlie/contribute to the influence of FTY720 on the blood-tissue distribution of DC. Although we found no significant effect of FTY720 administration on CD62L expression by blood DC, the implications of this finding are uncertain. CD62L expression is usually associated with the migratory behavior of T cells, although CD62L has been implicated in regulation of human plasmacytoid DC migration from blood to SLO (196). Our failure to demonstrate a consistent effect of FTY720-P on adhesion molecule expression by, or adherence of BMDC to vascular endothelial cells in vitro does not exclude the possibility that, *in vivo*, intercellular adhesion and transendothelial migration by DC may be affected by FTY720 administration, particularly as *in vivo*, both DC and EC are exposed to the drug. Interestingly, while expression of these intercellular adhesion molecules on FTY720- or FTY720-P-treated human DC was not examined in the recent study of Muller et al (183), the authors did observe reduced expression of CD18, that forms heterodimers with CD11a, b and c, which could also interfere with the migratory activity of DC.

DC homing from blood to SLO clearly involves trafficking across multiple barriers. When circulating DC precursors fail to transmigrate, the number of DC reaching the lymph nodes and spleen decreases. Spleen DC have a rapid turnover [half-life, 1.5-2.9 days (197)], do not recirculate, and remain in the lymphoid tissue until they die. Thus, reduction in migration of immature DC from blood would quickly result in DC depletion in the spleen, while recovery (following drug withdrawal) would be expected to take several days, as observed in this study. Our *in vitro* data also reveal that FTY720-P does not induce DC apoptosis, either under steady-state conditions, or when cell death is enhanced *in vitro* by serum deprivation. This makes it

unlikely that (as with lymphocytes) (99), this mechanism accounts for the observed DC depletion in the spleens of FTY720-treated mice.

It is tempting to speculate that FTY720-P, the principal in vivo metabolite of FTY720, may down-modulate S1PR, particularly $S1P_1$, on DC, thereby depriving them of signals provided by serum S1P, that might be critical for the regulation of adhesion molecule expression. Agonistic interaction of FTY720-P with S1PR on DC could also lead to receptor internalization, particularly of $S1P_1$. This in turn, could deprive DC of an S1P signal required for migration from blood into SLO. In line with this view, it has been suggested that DC may upregulate $S1P_1$ upon maturation and Ag uptake, prior to migration into SLO, and that DC down-modulate $S1P_1$ once in lymphoid tissue (141). Our data indeed suggest that maturation of DC is associated with modest upregulation of $S1P_1$ expression at the mRNA level. While this finding contrasts with a recent report of similar amounts of mRNA for S1PR on immature and mature human DC (150), the apparent inconsistency may relate to the different experimental conditions employed.

The need to understand the contributions of specific S1PR to the mode of action of the relatively non-specific S1P receptor agonist FTY720 has become more urgent as, in clinical trials, its immunosuppressive action is accompanied by transient bradycardia (96, 116, 198). The discovery of selective S1P agonists, as exemplified by SEW 2871 (139), has led to the observation that agonism of $S1P_1$ alone is sufficient to control lymphocyte egress/recirculation. Use of selective agonism, together with $S1P_3^{-/-}$ mice, has revealed that $S1P_1$ and $S1P_3$ respectively, regulate lymphocyte circulation and heart rate (139). Our data obtaining using SEW2871 suggest that agonism of $S1P_1$ alone is also sufficient to affect DC numbers in blood and SLO, and rapidly enhances circulating numbers of these important APC.

We conclude that FTY720 promotes the retention of DC in the circulation, associated with downregulation of several cell surface intercellular adhesion molecules believed to be important for endothelial adhesion and transmigration. In addition, cell surface expression of the SLO homing receptor CCR7 was also downregulated. We propose that FTY720 induces these effects as the result of FTY720-P binding to S1PR, in particular $S1P_1$, that is expressed by murine DC. As with T-cells, FTY720 may functionally inactivate/internalize $S1P_1$ (141), thereby affecting the migration of DC to SLO. Understanding of how DC-endothelium interaction and the migratory ability of DC is affected by FTY720 may lead to improved means of regulating DC function and the role of these cells in immune reactivity and tolerance

3.0 INFLUENCE OF AADC ON ALLOIMMUNE RESPONSES AND TRANSPLANT OUTCOME[6]

Anti-inflammatory cytokines significantly impact the maturation of dendritic cells (DC) in response to TLR ligands and affect their ability to induce T cell activation and proliferation. In this study, we propagated myeloid DC from BALB/c ($H2^d$) mouse bone marrow progenitors in IL-10 and TGF-β, then stimulated the cells with LPS. These 'alternatively-activated' (AA) DC expressed lower TLR4 transcripts than LPS-stimulated control DC and were resistant to maturation, expressing comparatively low levels of MHC class II, CD40, CD80, CD86 and programmed death (PD)-ligand (L) 2 (B7-DC; CD273), whereas PD-L1 (B7-H1; CD274) and inducible costimulatory ligand expression was unaffected. AADC secreted much higher levels of IL-10, but lower levels of IL-12p70 compared with activated control DC. Their poor allogeneic (C57BL/10) T cell stimulatory activity and ability to induce alloAg-specific T cell hyporesponsiveness, was not associated with enhanced T cell apoptosis. Increased IL-10 production was variably induced in the alloreactive T cell population, wherein $CD4^+$ $Foxp3^+$ cells were expanded. In vivo migration of AADC to secondary lymphoid tissue was not impaired. A single infusion of AADC to quiescent recipients induced alloAg-specific T cell hyporesponsiveness and prolonged subsequent heart graft survival. This effect was potentiated markedly by CTLA4Ig, administered one day after the AADC. Transfer of $CD4^+$ T cells from recipients of long-surviving grafts (>100 days) prolonged the survival of donor-strain hearts in naïve recipients. These data enhance insight into the regulatory properties of AADC and demonstrate their therapeutic potential in vascularized organ transplantation.

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[6] Data presented in this chapter have been submitted for publication: Lan, Y.Y., Wang, Z., Raimondi, G., Wu, W., Colvin, B.L., De Creus, A., Thomson, A.W. 'Alternatively-activated' dendritic cells preferentially secrete IL-10, expand $Foxp3^+CD4^+$ T cells and induce long-term organ allograft survival in combination with CTLA4-Ig. Dr Wang and Dr Wu performed all the heart transplantations; Dr Raimondi assisted with Treg expansion experiments (Fig. 22); and Dr Colvin provided support for immunohistochemistry (Fig. 25D).

3.1 INTRODUCTION

Dendritic cells (DC) constitute a heterogeneous population of rare, uniquely well-equipped, bone marrow-derived antigen-presenting cells (APC), that play critical roles in central and peripheral tolerance in the normal steady state(1, 181). Due to their inherent plasticity, DC can instigate or regulate immune reactivity. In an effort to improve transplant outcomes and minimize dependency on anti-rejection drugs, various approaches have been adopted to generate tolerogenic DC with ability to regulate alloreactive T cell responses (48). Thus, immature myeloid DC, propagated from bone marrow precursors in low concentrations of GM-CSF and expressing low levels of MHC and costimulatory molecules, induce alloantigen (Ag)-specific T cell hyporesponsiveness (36) and prolong organ graft survival (35, 37). Pharmacologic agents, including aspirin (199), vitamin D_3 (200, 201), and various immunosuppressive drugs, e.g. corticosteroids (202), cyclosporine A (80), rapamycin (82) and mycophenolate mofetil (203), inhibit DC maturation, promote their tolerogenicity (204) and enhance their ability to induce long-term allograft survival (205, 206).

DC exposed to immunosuppressive cytokines exhibit potential for tolerance induction. Thus, IL-10, a multifunctional cytokine, with diverse effects on many cell types (158, 163), acts as an autocrine/paracrine inhibitor of DC differentiation (164, 165). Moreover, DC exposed to IL-10 or genetically modified to over express IL-10 (207) can induce Ag-specific T cell anergy (162, 208), while the differentiation of regulatory T cells (Treg) by immature DC may require IL-10 (209). TGF-β1 is essential for the maintenance of immune homeostasis (210). It blocks DC development from bone marrow progenitors (173), while protecting these progenitors from apoptosis (172). Like IL-10, TGF-β has also been implicated in the generation and expansion of Treg (171, 211). In addition to their influence on DC, IL-10 and TGF-β1 exert an additive, suppressive effect on suppressing Ag-specific T cell responses. Thus, IL-10 and TGF-β1-treated $CD4^+$ T cells, but not T cells treated with either cytokine alone, exhibit alloAg-specific hyporesponsiveness and have markedly impaired ability to induce graft-versus-host disease (GVHD) (169). Significantly, however, neither IL-10 nor TGF-β-transduced DC exert a strong inhibitory effect on allograft survival (176, 212).

Recently, evidence has emerged that regulatory or ‘alternatively-activated’ (AA) DC generated in the presence of both IL-10 and TGF-β1, then treated with LPS, protect mice from

lethal GVHD (156, 157) or experimental endotoxemia (213). These recipient-matched DC display low levels of costimulatory molecules and impair allogeneic effector T cell functions. Importantly, their ability to regulate T cell function is retained *in vitro* and *in vivo*, even under inflammatory conditions. Here, our goal was to extend insight into the functional biology of these AADC, and their capacity to modulate alloreactive T cell responses. In addition, we assessed, for the first time, their influence on organ allograft survival. Our data indicate that AADC not only induce alloAg-specific T cell hyporesponsiveness, but also expand $CD4^+CD25^+$ forkhead winged helix protein-3^+ ($Foxp3^+$) Treg. Moreover, one infusion of AADC into prospective graft recipients could prolong MHC-mismatched heart transplant survival significantly, and, together with B7-CD28 pathway blockade, could induce graft survival indefinitely. This regulatory effect could be transferred by $CD4^+$ T cells.

3.2 MATERIALS AND METHODS

3.2.1 Mice

Male BALB/c ($H2^d$) C57BL/10J (B10; $H2^b$) and C3H ($H2^k$) 8-12-wk old mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the specific pathogen-free Central Animal Facility of the University of Pittsburgh. They received Purina rodent chow (Ralstan Purina, St. Louis, MO) and tap water *ad libitum*. Experiments were conducted in accordance with the National Institutes of Health Guide for use and care of laboratory animals and under an Institutional Animal Care and Use Committee-approved protocol.

3.2.2 Reagents and mAbs

Recombinant (r) mouse GM-CSF was a gift from Schering-Plough, Kenilworth, NJ, whereas r murine IL-10 and r human TGF-β were obtained from PeproTech Inc. (Rocky Hill, NJ). Complete medium (CM) comprised RPMI-1640 (BioWhittacker, Walkersville, MD) supplemented with 10% v/v FCS (Nalgene, Miami, FL), non-essential amino acids, L-glutamine,

sodium pyruvate, penicillin-streptomycin and 2-ME (all Life Technologies, Gaithersburg, MD). Anti-mouse mAbs were from BD PharMingen (San Diego, CA), unless specified. Vybrant™ CFDA SE cell tracer kit was purchased from Molecular Probes (Eugene, OR). Magnetic microbeads and separation columns were from Miltenyi Biotech (Auburn, CA). Trizol reagent was purchased from R&D Systems (Minneapolis, MN) and Advantage™ RT-for-PCR kit from Clontech (Palo Alto, CA). Quantikine®M IL-10, IL-12p70, and IFN-γ ELISA kits were from R&D Systems. Human CTLA4-Ig was a gift from Bristol-Myers Squibb Pharmaceutical Research Institute (Candiac, Quebec, Canada).

3.2.3 Generation of AADC

BM cells were flushed from femurs and tibias of normal BALB/c mice and subjected to red cell lysis. The cells were then plated at a density of 0.2×10^6/ml in petri dishes. As described by Sato *et al.* (156, 157), DC were generated in 10 ml of CM, supplemented with GM-CSF (1000 U/ml), IL-10 (20 ng/ml) and TGF-β (20 ng/ml). Fresh medium and cytokines were added on day 4. On day 7, the non-adherent cells were collected, washed with PBS, and DC positively selected with anti-CD11c magnetic microbeads (purity >90%). The purified DC were re-plated at the same density in CM with *E. coli* LPS (1 μg/ml; serotype 026:B6; Sigma, St. Louis, MO) for 24 h. On day 8, the LPS-stimulated DC (AADC) were collected and washed extensively in PBS before use. Control DC (referred to hereafter as GMDC) were propagated under the same conditions in GM-CSF alone, before exposure to LPS for the final 24 h of culture.

3.2.4 Cytospin preparations

Immunobead-purified, $CD11c^+$ GM- or AADC (50,000 cells) were spun onto glass slides using a Shandon cytocentrifuge (Shandon, Cheshire, U.K.) at 230 × g for 5 min, air dried, and stained with Accustain Geimsa (Sigma) according to the manufacturer's protocol.

3.2.5 Detection of TLR4 expression

Total RNA was extracted from purified GM- or AADC using the Trizol method, as described (187). cDNA was synthesized from the RNA samples with Advantage™ RT-for-PCR kit. PCR primers were: 5'-GCA TGG CTT ACA CCA CCT CT-3', sense, and 5'-GTG CTG AAA ATC CAG GTG CT-3', antisense. The PCR mix was run for 35 cycles (94°C, 30 s; 61°C, 30 s; 72°C, 30 s), with a final extension step of 7 min at 72°C. PCR samples were then analyzed on 1% w/v agarose gel stained with ethidium bromide.

3.2.6 Flow cytometric analysis

DC were double-stained with PE-anti-CD11c and either FITC-anti-CD40, -CD80, -CD86, -IA^d, -programmed death (PD)-1 ligand PD-L1 (B7-H1; CD274), -PD-L2 (B7-H2;CD273) or –ICOSL for phenotypic analysis. For surface adhesion molecule or chemokine receptor expression, the DC were stained with PE-anti-CD11c and either FITC-anti-CD11b, -CD31, -CD44, -CD54, or -CCR7. The incidence of positive cells and mean fluorescence intensity (MFI) were determined by flow cytometry using an EPICS Elite flow cytometer (Beckman Coulter, Hialeah, FL).

3.2.7 Cytokine quantitation

Culture supernatants were stored at -80°C and levels of IL-10 and IL-12p70 measured using ELISA kits, according to manufacturer's instructions. The sensitivity limits for IL-10 and IL-12p70 were 30 pg/ml and 7.8 pg/ml, respectively.

3.2.8 MLR

Nylon wool-purified, allogeneic T cells (B10; 2×10^5) were co-cultured with γ-irradiated (20 Gy), BALB/c GMDC or AADC at various T cell: DC ratios for 72 h in 96-well, round-bottom plates (Corning, Corning, NY). [^{3}H]-thymidine (Amersham Pharmacia Biotech, Uppsala, Sweden) was added to each well 18 h prior to cell harvesting and determination of T cell

proliferation using a liquid scintillation counter. Data are expressed as mean counts per minute (cpm) ±1SD.

3.2.9 Analysis of T cell apoptosis

GM- or AADC were co-cultured with allogeneic (B10) nylon wool-purified T cells at a ratio of 1:10 for 1-5 days. T cell apoptosis was monitored by staining of phosphatidylserine translocation with FITC-Annexin V, in combination with propidium iodide (PI), as described (214) and according to the manufacturer's instructions (BD PharMingen, San Diego, CA). Cells were co-stained with PE-anti-CD4/CD8 mAb to allow analysis of T cell subsets by flow cytometry.

3.2.10 In vitro T cell restimulation

Three-day co-cultures of nylon wool-purified splenic T cells (B10; 2×10^5) and irradiated GMDC or AADC (BALB/c) were set up in 96-well, round-bottom plates at a DC: T cell ratio of 1:10. DC were then depleted by negative CD11c immunobead separation and the T cells rested for 3 days in CM supplemented with low concentration IL-2 (5 U/ml; Genetics Institute, Cambridge, MA). Primed T cells (2×10^5) were restimulated with irradiated donor (BALB/c) or third party (C3H) bulk splenocytes, with or without exogenous of IL-2 (100 U/ml) for 3 days. T cell proliferation was measured by [^{3}H]-thymidine incorporation, as with primary MLR. To quantify cytokine production by primed T cells upon restimulation in the absence of exogenous IL-2, supernatants were collected at 72 h and levels of IL-10 and IFN-γ determined by ELISA.

3.2.11 In vitro expansion of $CD4^+$ Treg

Immunobead-purified, allogeneic $CD4^+$ T cells (B10; 2×10^5) were co-cultured with non-irradiated, GMDC or AADC (BALB/c; 2×10^4) for 5 days. The T cells were then surface-stained with Cyc-anti-CD4 and FITC-anti-CD25 mAbs and resuspended in Fix/Perm Buffer (eBioscience, San Diego, CA). Intracellular staining with PE-anti-Foxp3 mAb (eBioscience) and estimation of the incidence of $CD4^+CD25^+FoxP3^+$ Treg was then determined by flow cytometry,

as described (215). To quantify Treg-induced proliferation, purified allogeneic $CD4^+$ T cells were labeled with CFSE as described (215) prior to 5-d co-culture with GM- or AADC. T cells collected were surface-stained with Cyc-anti-CD4 mAb, then intracellularly with PE-anti-Foxp3 mAb. Proliferation of $CD4^+FoxP3^+$/$FoxP3^-$ cells was evaluated from the CFSE dilution profile.

3.2.12 DC trafficking

Three million CFSE-GM- or AADC, labeled as described (186), were injected subcutaneously into the rear footpads of normal allogeneic (B10) recipients. $CFSE^+$ cells in the draining (popliteal) lymph nodes were determined 18 h later, by rare-event, flow cytometric analysis. Non-draining (inguinal) lymph node cells served as negative controls.

3.2.13 Ex vivo T cell restimulation

Two million GMDC or AADC were injected i.v. into normal allogeneic (B10) recipients. Seven days later, primed T cells (2×10^5) from recipient spleens were purified using nylon wool columns, then restimulated with irradiated donor (BALB/c) or third party (C3H) bulk splenocytes, at various ratios. T cell proliferation was determined by [^{3}H]-thymidine incorporation during the final 18 h of culture.

3.2.14 Heart transplantation

Heterotopic (intra-abdominal) vascularized heart transplantation was performed from BALB/c to B10 mice as described (188), under methoxyflurane inhalation anesthesia (Medical Development, Springvale, Australia). The heart was transplanted into the abdomen with end-to-side anastomosis of aorta to aorta and pulmonary artery to vena cava. Two million GM- or AADC were injected i.v. via the lateral tail vein, 7 days before transplant (d-7). Human CTLA4-Ig (250 μg/mouse) was administered i.p. on d-6. Graft survival was assessed by daily transabdominal palpation. Rejection was defined as total cessation of graft contraction and confirmed by histological examination.

3.2.15 Immunohistochemistry

Cryostat sections were fixed in ethanol, treated with goat serum and the avidin/biotin blocking kit (Vector, Burlingame, CA) and incubated with CD4 mAb (BD PharMingen). Slides were then incubated with Cy^{TM} 2F $(ab')_2$ anti-rat IgG (Jackson, West Grove, PA) and anti-Foxp3 mAb (Alexis, San Diego, CA), followed by $Cy^{TM}3F(ab')_2$ anti-rat IgG. Nuclei were stained with DAPI (Molecular Probes, Eugene, OR).

3.2.16 Adoptive cell transfer

$CD4^+$ T cells were isolated from lymph nodes and spleens of long-surviving (>100 d) graft recipients or control B10 mice using immunomagnetic beads (Miltenyi). Ten million cells were adoptively transferred by i.v. injection (lateral tail vein) to naïve, syngeneic recipient mice (B10), that received BALB/c heart allografts 24 h later. Graft survival was assessed as described above.

3.2.17 Statistical analyses

Significance of differences between means was calculated using the paired Student's t-test. Differences between groups were considered significant at $p<0.05$. Graft survival data were compiled using Kaplan-Meier analysis, and compared by log-rank test.compiled using Kaplan-Meier analysis, and compared by log-rank test.

3.3 RESULTS

3.3.1 Exposure to IL-10 and TGF-β alters the surface phenotype, TLR4 expression and cytokine secretion pattern of LPS-stimulated, BM-derived myeloid DC

We first examined the influence of IL-10 and TGF-β on DC development from replicating BALB/c BM progenitors. IL-10 and TGF-β did not affect DC viability, that ranged from 90-96% over the culture period. Prior to LPS activation, differences in surface phenotype were observed between the control and cytokine-treated populations, with lower expression of MHC II (IA^d), CD80, PD-L2 and ICOSL on the cytokine-treated DC (Fig. 18). Stimulation with LPS for 24 h increased levels of MHC II and classic costimulatory molecules (CD40 and CD80) on both populations (referred to hereafter as control GMDC and AADC), while the incidence of cells expressing MHC II, CD40, CD80 and CD86 and the MFI for each of these molecules was consistently greater on GMDC (Fig. 18). This indicated that AADC were comparatively resistant to maturation in response to TLR4 ligation. Levels of the B7 family cosignaling molecules PD-L1 and ICOSL were similar on LPS-stimulated GMDC and AADC, but AADC expressed less PD-L2 than GMDC. Both $CD11c^+$ GMDC and AADC exhibited characteristic DC morphology, with prominent, eccentric, reniform or multi-lobulated nuclei, though the latter DC appeared more rounded, with shorter dendrites (Fig. 19A). As it has been reported that TLR4 mRNA expression is increased in TGF-β null mice (167) and down-regulated in TGF-β-treated murine DC (216), we considered that exposure to both IL-10 and TGF-β might also inhibit TLR4 expression. Indeed, when compared with GMDC, AADC showed significantly lower expression of TLR4 mRNA, as determined by RT-PCR (Fig. 19B).

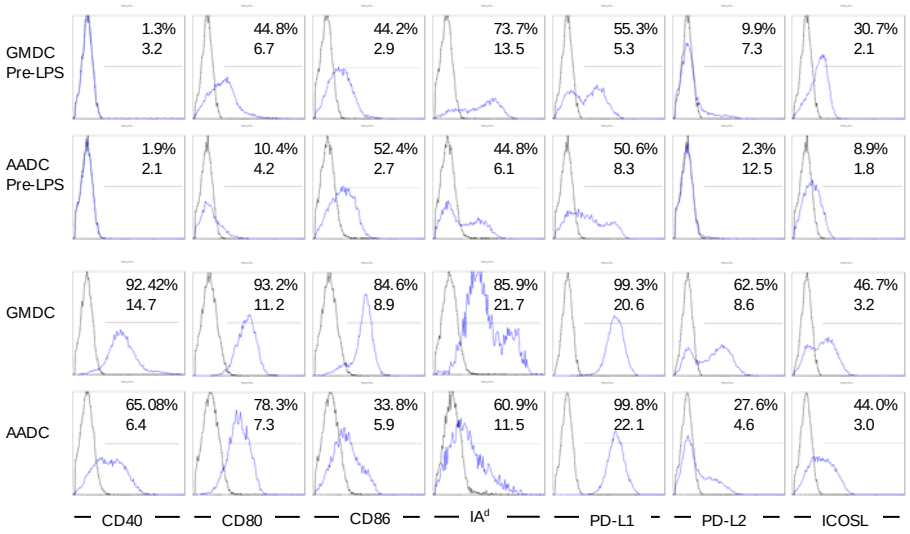


Figure 18. DC propagated in IL-10 and TGF-β are less mature than control DC and more resistant to LPS-induced maturation.

BALB/c DC propagated from BM in IL-10 and TGF-β were co-stained with mAbs against CD11c and either MHC II (IA^d) or TNFR (CD40) or B7 family members (CD80, CD86, PD-L1 [CD274], PD-L2 [CD273] and ICOSL) before and after LPS stimulation and analyzed by flow cytometry. Histograms shown were gated on the $CD11c^+$ population and both % positive cells and MFI are indicated. The results are from one representative experiment of three performed.

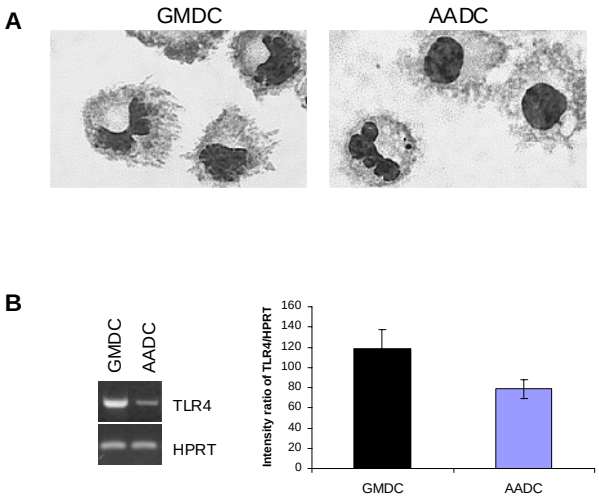


Figure 19. Morphology and TLR4 expression by AADC and control DC (GMDC).

(A), cytospin preparations of both types of DC showed a high nucleus-cytoplasm ratio and reniform or multi-lobulated nuclei. Dendrites were abundant and well-developed on GMDC. AADC were more rounded, with less prominent dendrites. Giemsa, ×200. (B), TLR4 expression by GMDC and AADC was examined by RT-PCR. AADC expressed a lower level of TLR4 mRNA. Densitometric values for TLR4-specific bands are normalized to HPRT. The experiment was performed three times and the data shown are means ±1SD.

3.3.2 AADC produce more IL-10 and less IL-12p70 than GMDC and exhibit markedly impaired T cell allostimulatory ability

When we examined the cytokine production pattern of the LPS-stimulated DC over 24 h, AADC produced less IL-12p70 but substantially more IL-10 compared with GMDC (Fig. 20A). Based on their comparatively low surface expression of classic co-stimulatory molecules, high expression of PD-L1 [implicated in suppression of alloreactive T cell responses by DC (42)] and enhanced secretion of IL-10 compared with GMDC, we postulated that AADC would be less capable of naive allogeneic T cell stimulation. When tested in primary MLR, BALB/c AADC induced much weaker proliferative responses in B10 T cells compared with GMDC, at all DC:T cell ratios examined (Fig. 20B).

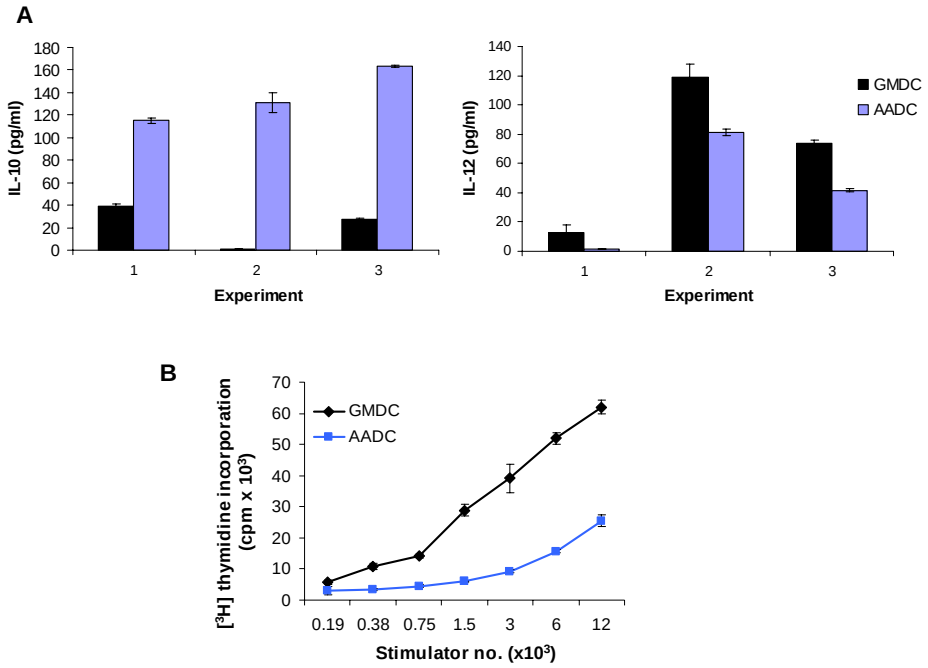


Figure 20. AADC produce substantially more IL-10 and less IL-12p70 than GMDC and are comparatively poor stimulators of allogeneic T cell proliferation.

(A), supernatants of LPS-stimulated GMDC or AADC cultures were harvested at 24 h, and levels of IL-10 and IL-12p70 determined by ELISA. Data from 3 separate experiments are shown. (B), γ-irradiated GMDC or AADC were co-cultured with nylon wool-purified allogeneic B10 T cells (2×10^5) at different ratios in 72 h MLR. T cell proliferation was measured by [^{3}H] thymidine uptake during the last 18 h and expressed as counts per minute (cpm ± 1SD). The results are from one experiment representative of four performed.

3.3.3 AADC do not enhance allogeneic T cell apoptosis, but promote Ag-specific T cell hyporesponsiveness

To further address possible mechanisms underlying the much inferior capacity of AADC to induce T cell proliferation, T cell apoptosis was examined in 1-5-day co-cultures of GM- or AADC with allogeneic (B10) T cells. The incidence of Annexin V^+/PI^- (apoptotic) $CD4^+$ or $CD8^+$ T cells at day 2 (Fig. 21A) and at other time points (data not shown) was similar in both GMDC- and AADC-stimulated cultures. Thus, pre-exposure to IL-10 and TGF-β did not affect the influence of LPS-activated DC (AADC) on the apoptotic death of allogeneic T cells. We also examined whether T cell stimulation by AADC could lead to alloAg-specific T cell

hyporesponsiveness. As shown in Fig. 21B, AADC-primed (p) B10 T cells (pT) were hyporesponsive to restimulation by donor (BALB/c) Ag. This hyporesponsiveness was donor-specific, as proliferative responses to third party (C3H) stimulators were unimpaired. Hyporesponsiveness to donor was not reversed by exogenous IL-2 (Fig. 21B). When specific cytokine levels were assayed, increased levels of IL-10 were variably found in those cultures in which AADC-stimulated (compared with GMDC-stimulated) B10 pT were re-stimulated with donor alloAg. (Fig. 21C). Levels of IFN-γ production in cultures of AADC-stimulated pT were unaffected compared with GMDC-pT cultures.

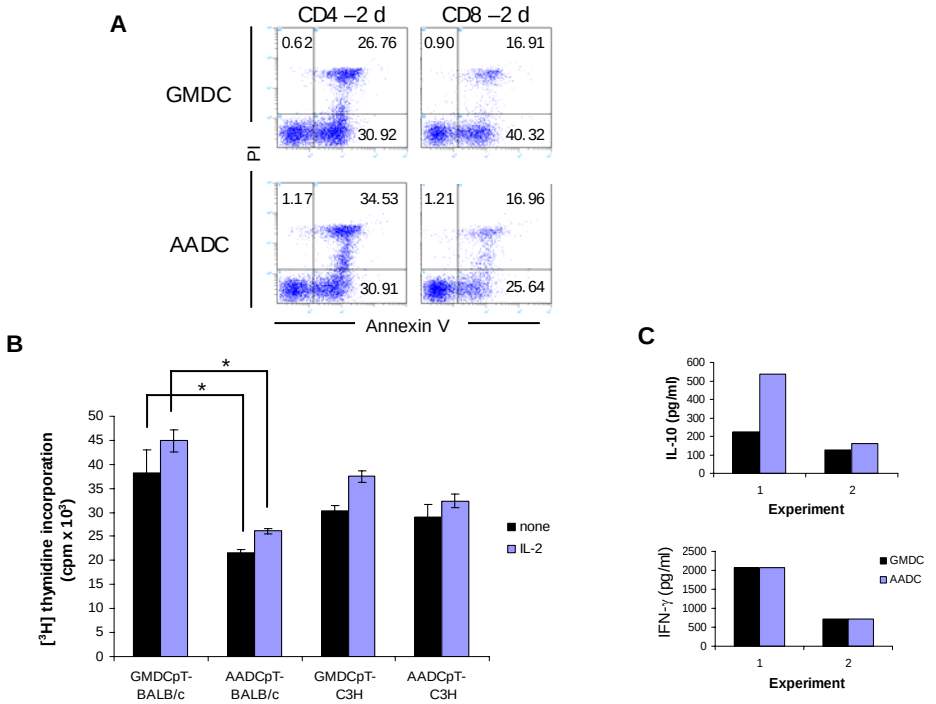


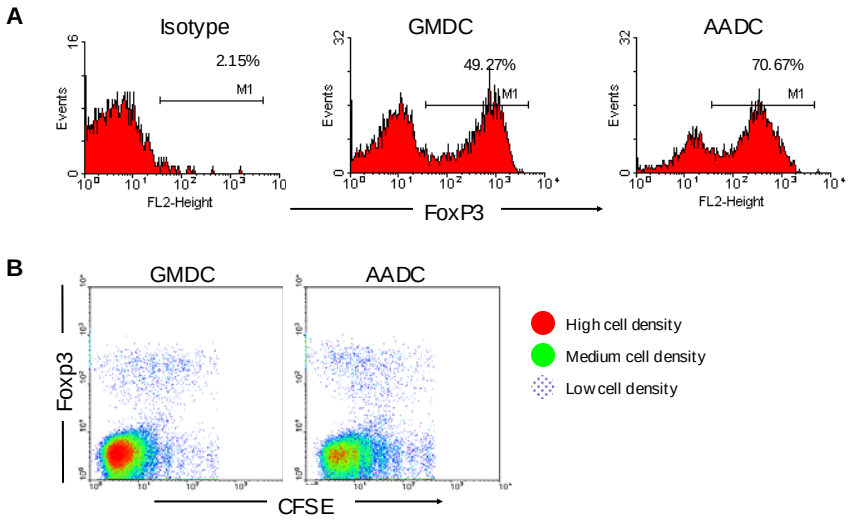
Figure 21. AADC do not enhance allogeneic T cell apoptosis but induce alloAg-specific T cell hyporesponsiveness.

(A), γ-irradiated GMDC or AADC (2×10^4) were co-cultured with nylon wool-purified, allogeneic B10 T cells (2×10^5) for 2 days. Apoptosis of $CD4^+$ or $CD8^+$ T cells was analyzed by staining with FITC-Annexin V in combination with PI, as described in the Materials and Methods. Percentages of apoptotic T cells (Annexin V^+/PI^-) in GMDC, or AADC-stimulated cultures are shown. Data are from one experiment and are representative of two performed. (B), nylon wool-purified, allogeneic T cells (B10; 2×10^5) were co-cultured with γ-irradiated GMDC or AADC (BALB/c; 2×10^4) for 72 h. DC were then depleted by negative immunobead selection and T cells rested for 72 h in CM supplemented with low concentration IL-2 (5 U/ml). Primed T cells (pT) were then restimulated with irradiated, allogeneic (BALB/c) or third party (C3H) bulk splenocytes, at a ratio of 1:1, with or without exogenous

IL-2 (100 U/ml) in secondary 72 h co-culture. T cell proliferation was measured by [^{3}H]-thymidine incorporation, as in primary MLR. AADC-stimulated pT demonstrated alloAg-specific hyporesponsiveness. This hyporesponsiveness was not reversed by addition of IL-2. Data shown are from one representative experiment of two performed, *$p<0.01$. (C), supernatants were harvested from the 72 h T cell restimulation cultures (no added IL-2) and levels of IL-10 and IFN-γ determined by ELISA. The results of two separate experiments are shown.

3.3.4 AADC expand $CD4^+CD25^+Foxp3^+$ Treg in vitro

We next investigated whether the tolerogenic properties of AADC might be correlated with specific interactions with $CD4^+CD25^+$ Treg. GMDC or AADC were cultured with normal, allogeneic (B10) $CD4^+$ T cells for 3 days, then the frequency of $CD4^+CD25^+Foxp3^+$ Treg analyzed by flow cytometry. A higher incidence of $Foxp3^+$ cells was detected consistently in AADC-stimulated cultures (Fig. 22A). This could indicate an ability of AADC to promote the survival and/or expansion of $CD4^+$ Treg, relative to effector $CD4^+$ T cells. To distinguish between these possibilities, we analyzed the level of proliferation of $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cells in MLR using CFSE-labeled allogeneic $CD4^+$ T cells as responders. As indicated in the density plots (Fig. 22B), AADC retained the ability to induce proliferation of $Foxp3^+$ Treg (comparable to GMDC), while $Foxp3^-$ T cell proliferation was markedly impaired. Consequently, stimulation of allogeneic T cells with AADC resulted in an increased ratio of Treg over effectors (Fig. 22C) that could contribute to their control of alloreactivity.



C

	GMDC				AADC			
	Expt. 1	Expt. 2	**Average**	SD	Expt. 1	Expt. 2	**Average**	SD
% Foxp3+ gated on proliferating CD4+	6.6	7.5	7.05	0.64	8.3	9.1	8.7	0.57
% Foxp3- gated on proliferating CD4+	74.2	70.3	72.25	2.76	55.2	50.5	52.85	3.32
Ratio of Foxp3+/Foxp3-	0.0889	0.107	**0.098**	0.01	0.15	0.18	**0.165**	0.02

Figure 22. AADC preferentially expand Foxp3$^+$ Treg in proliferating CD4$^+$ T cell cultures.

(A), Non-irradiated GM/AADC (2 x 10^4) were co-cultured with bead-purified allogeneic B10 CD4$^+$ T cells (2 x 10^5) for 5 days. Cells were surface-stained with Cyc anti-CD4 mAb and then stained intracellularly with PE-anti-Foxp3 mAb to determine the frequency of CD4$^+$CD25$^+$Foxp3$^+$ Treg. A higher incidence of Foxp3$^+$ cells was detected consistently in AADC-stimulated cultures. Histograms gated on CD4$^+$CD25$^+$ cells are from one representative experiment of two performed. To examine the level of proliferation of Foxp3$^+$/Foxp3$^-$ CD4$^+$ T cells, CFSE-labeled allogeneic CD4$^+$ T cells were used as responders. AADC retained the ability to induce proliferation of Foxp3$^+$ Treg (comparable to GMDC), but markedly impaired Foxp3$^-$ T cell proliferation (B). Proliferating CFSE-labeled cells are shown in the density plots, representative of two experiments performed. This resulted in an increased ratio of Treg over effectors in AADC-stimulated cultures compared to GMDC (C). The results are from two separate experiments performed.

3.3.5 AADC express similar levels of surface adhesion molecules but lower CCR7 than GMDC and migrate to secondary lymphoid tissue of allogeneic hosts

AADC and GMDC uniformly expressed similar surface levels of the intercellular adhesion molecules CD11b, CD44 and CD54 that are important for DC transendothelial migration whereas only minimal expression of CD31 was detected (Fig. 23A). Surface levels of CCR7, that promotes homing to secondary lymphoid tissue in response to cognate chemokines (CCL19 and

CCL21) were lower on AADC (Fig. 23A). To evaluate the migratory properties of the DC in vivo, we injected CFSE-labeled GMDC or AADC s.c. into the hind footpad of normal allogeneic (B10) recipients and compared the numbers of labeled DC that reached the draining (popliteal) lymph node within 18 h. Similar absolute numbers of labeled GMDC and AADC were found (Fig. 23B), which indicated that despite lower surface CCR7, AADC could migrate as well as GMDC to draining secondary lymphoid tissue.

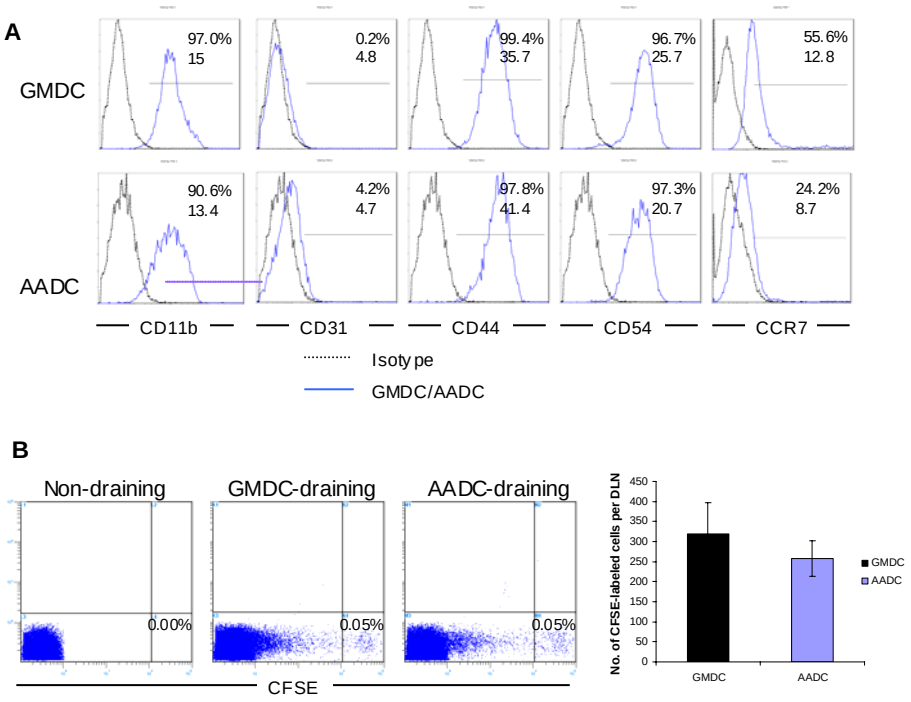


Figure 23. AADC express similar levels of surface adhesion molecules but lower CCR7 than GMDC and migrate to secondary lymphoid tissue of allogeneic hosts.

(A), surface adhesion molecule and CCR7 expression on GMDC and AADC were examined by two-color flow cytometry. Histograms shown are gated on $CD11c^+$ populations, with the incidence of positive cells and MFI indicated. Results shown are from one experiment representative of two performed. (B), three million CFSE-labeled BALB/c GMDC or AADC were injected s.c. into the left and right footpad respectively, of the same allogeneic B10 recipient. Labeled cells in the draining (popliteal) lymph nodes were determined 18 h later, by rare-event analysis. Percentages of labeled GMDC/AADC were similar, as shown in the dot plots. The mean number of CFSE-labeled cells per popliteal lymph node in GMDC- compared with AADC-injected mice (n=3) did not differ significantly: GMDC: 319±78; AADC: 258±45. Labeled cells did not reach the non-draining (inguinal) lymph nodes that were analyzed for comparison. Results shown are from one experiment representative of two performed.

3.3.6 AADC induce alloAg-specific T cell hyporesponsiveness in vivo

To better understand the influence of AADC on T cell responses in mice receiving BALB/c GMDC or AADC infusions prior to heart transplant, we isolated primed T cells (pT) from the spleens of (B10) mice, 7 d after DC injection and quantified the ex vivo T cell proliferative response upon alloAg restimulation. Primed T cells from AADC-treated mice were less responsive to restimulation by donor splenocytes than those from GMDC-treated animals (Fig. 24). By contrast, stimulation by third party (C3H) alloAg evoked a similar, much lower response by T cells from GMDC- and AADC-treated mice.

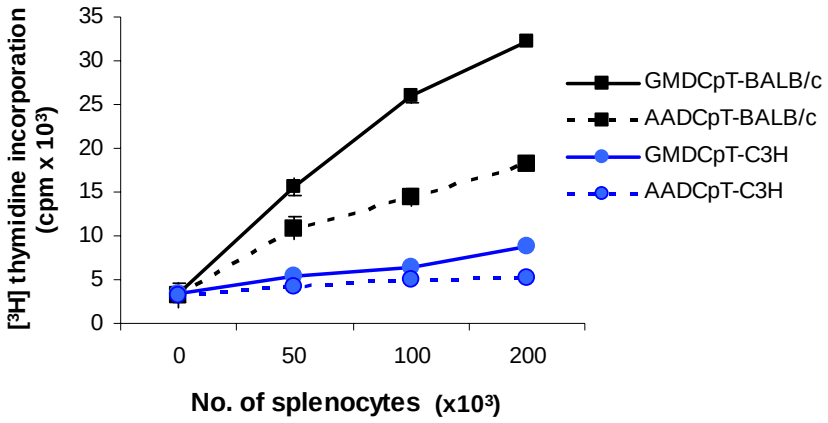
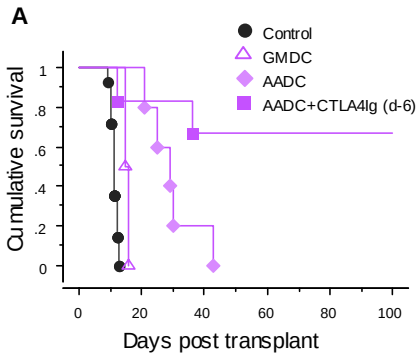


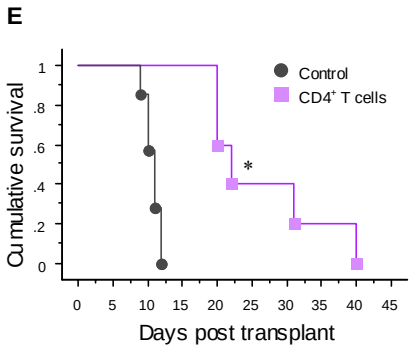
Figure 24. AADC induce alloAg-specific T cell hyporesponsiveness in vivo.

Two million BALB/c GM/AADC were infused i.v. into groups of 2 normal allogeneic (B10) recipients. Primed T cells (pT) were then isolated from recipient spleens, 7 d later and restimulated ex vivo with γ-irradiated donor (BALB/c) or 3rd party (C3H) bulk splenocytes (2×10^5) in 72 h MLR. T cell proliferation was determined by [^{3}H]-thymidine incorporation for the final 18 h. Primed T cells from AADC-treated recipients were less reactive to donor Ags. Results (means ± 1SD) are from one representative experiment of two performed.

3.3.7 Infusion of AADC in combination with CTLA4-Ig can induce long-term (>100 d) heart graft survival; transfer of $CD4^+$ T cells to naïve recipients confers resistance to rejection

We then studied the therapeutic efficacy of AADC in the fully MHC-mismatched (BALB/c→B10), vascularized heart allograft model. Two million BALB/c GMDC or AADC were infused i.v. into quiescent B10 recipients, 7 days before transplantation (on day 0). Graft survival in AADC-treated recipients, but not GMDC-treated recipients, was prolonged significantly (Fig. 25A, B). This effect was enhanced by a single injection of the B7-CD28 pathway blocking molecule CTLA4-Ig, 24 h after the AADC, resulting in a median graft survival time of >100 d. Histological examination of these long-surviving grafts revealed minimal parenchymal injury, with absence of significant vasculopathy (Fig. 25C). Immunohistochemical staining for CD4 and Foxp3 revealed $CD4^+$ $Foxp3^+$ cells within the tolerated grafts, but not within normal or rejecting hearts (Fig. 25D). Moreover, adoptive transfer of $CD4^+$ T cells (10×10^6 i.v.) from lymphoid tissues of long-term (>100 d) graft survivors, but not from normal B10 mice, to naïve B10 recipients, one day before transplant, significantly prolonged donor strain (BALB/c) but not third party heart graft survival (Fig. 25E).





B

Group	Treatment	n	Graft survival time (d)	Mean	SD	MST	p value to control	p value to Gp 3
1	Control	14	9, 10, 10, 10, 11, 11, 11, 11, 11, 12, 12, 12, 13, 13	11.1	1.2	11	N/A	N/A
2	GMDC (d-7)	4	15, 15, 16, 16	15.5	0.6	16	N/S	N/A
3	AADC (d-7)	5	21, 25, 29, 30, 43	29	8.3	30	<0.01	N/A
4	AADC+CTLA4Ig (d-6)	6	12, 36, >100, >100, >100, >100	>74.7	40	>100	<0.01	<0.05

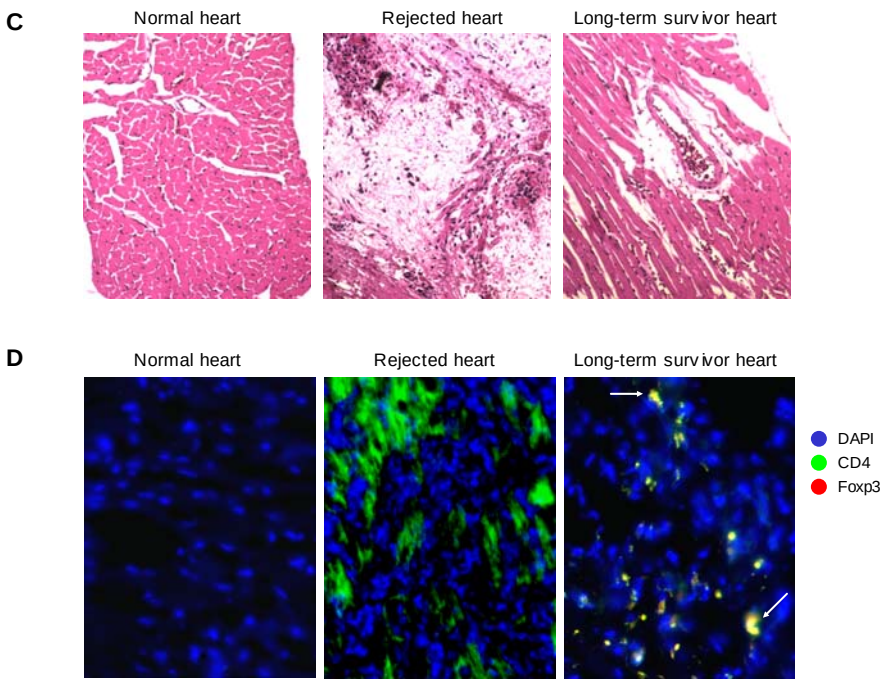


Figure 25. Infusion of AADC in combination with CTLA4-Ig can induce long-term (>100 d) organ allograft survival; $CD4^+$ T cells transfer resistance to rejection.

(A, B), BALB/c GMDC or AADC (2×10^6) were infused i.v. into normal B10 recipients, 7 d before vascularized heart transplant. Graft survival was prolonged significantly in AADC-treated recipients. Additional treatment of recipients with CTLA4-Ig one day later resulted in long-term graft survival (>100 d) in a high proportion of recipients. (C), Histological examination of long-surviving heart grafts (>100 d) in mice treated with AADC and CTLA4Ig revealed a low level of mononuclear cell infiltration, minimal parenchymal damage and the absence of vasculopathy. H & E, ×200. (D), Immunohistochemical staining for CD4 and Foxp3 revealed $CD4^+$ $Foxp3^+$ cells in the tolerated heart allografts, but not in normal or rejecting hearts. ×200. (E), Adoptive transfer of $CD4^+$ T cells (10×10^6, i.v., d-1) from lymphoid tissues of long-surviving graft (>100 d) recipients significantly prolonged donor-strain heart graft survival in naïve B10 recipients. $p<0.005$. MST=median graft survival time. The p value is based on the Log-rank test compared to control.

3.4 DISCUSSION

Exposure of replicating myeloid DC progenitors (156) or DC line cells (155) to anti-inflammatory agents, followed by their 'activation' in response to LPS, is a comparatively novel approach to the generation of tolerogenic (or 'regulatory') DC for cell-based therapy of allograft

rejection or GVHD. The present study was prompted by recent observations that such 'alternatively-activated' DC (AADC), propagated from mouse BM in IL-10 and TGF-β, then stimulated with LPS, exhibit potent immunoregulatory properties. Thus, it has been reported that a single systemic injection of AADC can control the ability of transplanted T cells to induce lethal GVHD (157) or protect mice against lethal endotoxemia and peritonitis (213). Such potent regulatory DC appear to be more effective in controlling adverse inflammatory/immune responses in vivo than DC 'conditioned' in IL-10 or TGF-β alone, or modified to express transgenes encoding these cytokines and that exert a comparatively modest effect on allograft rejection without immunosuppression therapy (176, 212, 217). The influence of these reputedly potent AADC on organ allograft survival has not previously been examined. Our goal was to further assess the functional biology of these cells, particularly in relation to alloreactive T cell responses and to assess their impact on graft outcome in a vascularized heart transplant model.

In this study, we found that identical conditioning of murine myeloid DC propagated from the same strain (BALB/c) as reported previously (157) with IL-10 and TGF-β, followed by LPS activation, resulted in comparatively low levels of TLR4 and cell surface MHC class II and classic costimulatory molecule (CD40, CD80 and CD86) expression. By contrast, no significant effects on surface expression of the newer B7 family cosignaling molecules, PD-L1 and ICOSL-implicated in immune regulation (218), were detected compared with LPS-stimulated control GMDC. In addition, we found that AADC secreted much higher levels of IL-10, but lower levels of IL-12p70 than GMDC.

Decreased expression of TLR4, the receptor that interacts with bacterial cell wall LPS to initiate an NFκB-dependent signaling pathway, was associated, in AADC, with LPS hyporesponsiveness. This resulted in reduced secretion of the Th1-inducing cytokine IL-12p70 and increased production of IL-10. Conversely, it has been shown that increased TLR4 mRNA in TGF-β1 null mice is associated with LPS hyperresponsiveness (167) TGF-β blocks NFκB activation and interferes with TLR ligand-induced responses (219). Moreover, Fujita et al (213) have observed that in AADC, activation of LPS-induced signaling events involving MAPK and NFκB are impaired. They have suggested that potent expression of IκB proteins in AADC may suppress NFκB-mediated production of pro-inflammatory cytokines, such as IL-12 (as shown in the present study), IL-1β, TNF-α and IL-6. The preferential autocrine production of IL-10 by AADC that we observed, and that appears to involve cAMP-mediated activation of PKA (213),

may be involved in the defective production of these pro-inflammatory cytokines. This view is strengthened by the observation that AADC propagated from IL-10 knockout mice show reduced impairment of proinflammatory cytokine production (213).

Compared with control GMDC, the ability of AADC to induce normal allogeneic T cell activation/proliferation was markedly impaired, reflecting both comparatively low levels of classic TNFR (CD40) and B7 family (CD80, CD86) costimulatory molecules and the preferential secretion of IL-10/reduced IL-12p70 production by these cells. However, like control GMDC, AADC exhibited uniformly high levels of surface PD-L1, a novel B7 family molecule (220) that has been implicated in downregulation of T cell responses. Indeed, our recent studies (42) show that comparatively high levels of PD-L1 relative to CD80/CD86 expression on murine (plasmacytoid) DC inhibits their ability to drive allogeneic T cell proliferation. Furthermore, there is evidence that PD-L1:PD-1 interactions may influence alloreactive T cell responses by inducing regulatory cells, and that blocking of PD-L1, but not PD-L2 signaling, prevents their induction (221). Interestingly, ICOSL expression, that was observed at comparatively low levels on both AADC and control GMDC in the present study, is not (unlike CD80 and CD86) dependent on NFκB activation (222).

Our data indicate that AADC do not enhance apoptotic death of alloactived T cells in vitro. Interestingly, it has been reported (213) that AADC treatment of mice with systemic inflammation reduces thymocyte apoptosis. Thus, modulation of T cell apoptosis does not appear to account for the poor allostimulatory activity of AADC. In our study, AADC induced alloAg-specific T cell hyporesponsiveness that was not reversed by IL-2, variably enhanced IL-10 production by alloactivated T cells and expanded $CD4^+CD25^+Foxp3^+$ (Treg) cells in short-term culture. Such rapid expansion of Treg, within days of allostimulation, has recently been reported *in vivo* (65). These observations extend the functional characterization of AADC generated in IL-10 and TGF-β and strongly suggest their potential to regulate alloimmune reactivity *in vivo*. IL-10 can directly affect DC to enhance their tolerogenic functions (208) and also promote the differentiation of Treg (209). We observed a higher proportion of $CD4^+CD25^+Foxp3^+$ Treg in AADC-stimulated allogeneic T cell cultures, consistent with poor effector ($Foxp3^-$) T cell proliferation, compared with that observed in response to GMDC. The mechanism responsible for expansion of $CD4^+CD25^+$ Treg from $CD4^+$ T cells primed with allogeneic AADC is unclear,

but may involve conversion of naïve $CD4^+$ T cells to $CD4^+CD25^+$ Treg. Alternatively, allogeneic AADC may preferentially expand naturally-existing $CD4^+CD25^+$ Treg.

Our data show that AADC expressed similar levels of surface intercellular adhesion molecules to GMDC, but reduced CCR7 expression,- the latter consistent with their more immature phenotype and reduced responsiveness to CCL19 (213). However, AADC appeared to traffic normally in vivo to draining lymph nodes of quiescent, prospective organ allograft recipients. This is consistent with the observations of Sato et al (156), who reported no change in the secondary lymphoid tissue homing ability of AADC following their infusion into allogeneic recipients. Homing of potentially tolerogenic DC to recipient lymph nodes, as observed in these studies, has been underscored as important for maximization of their therapeutic potential (223).

We found that a single infusion of allogeneic AADC to quiescent animals induced alloAg-specific T cell hyporesponsiveness and prolonged subsequent organ allograft survival significantly in the absence of immunosuppressive therapy, but no grafts survived >43 days. This contrasts with the ability of similar numbers of recipient AADC, administered once only, 2 days after allogeneic BM transplantation, to prevent lethal GVHD and permit host survival for >60 days (156). However, the therapeutic effect of AADC in our model was markedly potentiated by a single injection of CTLA4-Ig, one day after the AADC. Thus, blockade of the B7-CD28 pathway led to >100 day graft survival in the majority of AADC-treated heart graft recipients. Costimulation blockade with CTLA4-Ig or anti-CD154 mAb has been shown previously to potentiate the tolerogenicity of donor DC (206, 217) and this strategy has been linked to induction of Treg (206). The present data are the first to show that long-term organ allograft survival can be achieved by host conditioning with AADC plus costimulation blockade. Sato et al (156) found that IL-10-producing $CD4^+$ T cells and $CD4^+CD25^+CD152^+$ Treg cells were increased in AADC-treated recipients. Consistent with this finding, we showed that AADC could expand $CD4^+CD25^+$ $Foxp3^+$ T cells. We further detected $Foxp3^+$ cells in the heart allografts of long-term survivors and demonstrated that $CD4^+$ T cells could transfer regulation of alloimmune reactivity in naive recipients, thus implicates $CD4^+$ Treg in the long-term maintenance of allograft survival.

In conclusion, the present study extends insight into the functional biology of AADC and their interactions with allogeneic T cells. It also extends potential therapeutic applications of AADC to organ transplantation, where a single pre-transplant infusion of AADC, plus one

injection of CTLA4-Ig, can lead to long-term allograft survival, an effect that appears to be mediated at least in part, by $CD4^+$ Treg cells.

4.0 CONCLUSIONS

Over the past several decades, there have been remarkable advances in transplantation medicine, with improved mechanistic understanding of transplant rejection and development of more effective and safer chemical immunosuppression (79). Introduction of the calcineurin inhibitors CsA (1979) and tracrolimus (FK506; 1989)—the most efficacious immunosuppressive agents (224), into clinical use greatly enhanced patient survival after organ transplantation (225). Currently used immunosuppressive regimens have supported outstanding short-term, 1-year graft survival (80-95%) (224). However, the major challenge remains chronic allograft dysfunction that can be caused by both immunological ('true' chronic rejection) and non-immunological mechanisms (including immunosuppressive drug toxicity and associated side-effects of renal dysfunction, diabetogenicity, hypertension, hyperlipemia, infection, cancer and atherosclerosis) (45). Needless to say, the continual problem facing transplantation is the severe organ shortage (224), which is specifically addressed by steadfast endeavors in xenotransplantation research (226).

The immediate challenge of transplantation therefore, is to induce donor-specific (to leave the rest of the immune response intact) immune tolerance to improve graft outcome and eliminate the need for continued (life-long) immunosuppression. True tolerance equates acceptance of donor tissue without graft pathology in the absence of chronic immunosuppressive therapy, and has been more precisely defined to include the absence of donor-specific alloantibodies, absence of destructive lymphocyte infiltration, and proof of donor-specific unresponsiveness with retention of third party responses in functional assays *in vitro* (45).

Clarification of the mechanisms underlying the actions of new immunosuppressive drugs is crucial in defining their potential use and safety in prevention and therapy of allograft rejection. These agents act through different targets or pathways (Figure 26) and are being investigated as a part of true tolerance-inducing protocols (224). For cell-based experimental

therapy of tolerance induction, manipulation of DC for tolerogenicity remains a highly attractive strategy due to their unique potential for Ag-specific monitoring and migratory capacity *in vivo*.

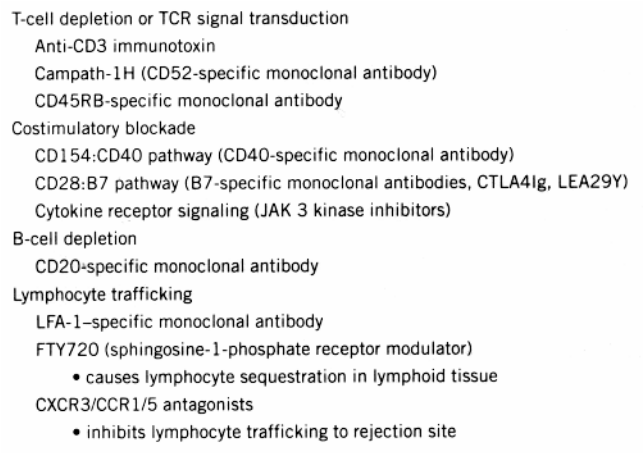


Figure 26. Promising agents for clinical development.

Adapted from Lechler, R.I. *et al.* 2005. Organ transplantation—how much of the promise has been realized? *Nature Med. 11(6):605-613.*

Understanding of DC immunology has advanced swiftly to facilitate their potential therapeutic applications. In a recent discovery, DC were found to communicate via a physically connected network of tunneling nanotubules (227). Morelli *et al.* (228) have developed a 'second-generation' DC-based tolerance induction strategy to deliver donor alloAgs to quiescent recipient DC *in situ* by donor APC-derived exosomes—nanovesicles rich in MHC molecules. Alternative molecules/pathways that may regulate DC tolerogenicity by distinct mechanisms have also been described.

The new members of the B7 gene family PD-L1 (B7-H1/CD274) and PD-L2 (B7-DC/CD273) are expressed on DC, whereas the expression of their cognate receptor PD-1 on T cells is inducible (218). PD-L1/2 ligation leads to inhibition of T cell proliferation and cytokine secretion (229). The relative levels of inhibitory PD-L1 and costimulatory B7-1/B7-2 signals on DC may determine the threshold between tolerance and immunity (230). PD-1 ligation by PD-L1.Ig under submaximal TCR or costimulatory signals can prevent allograft rejection (231). Encoded by a tightly-regulated gene responsive to inflammatory mediators, indoleamine 2,3-dioxygenase (IDO) is a tryptophan-degrading enzyme, and its activity correlates with reduced T cell-mediated responses in tissue transplant rejection (232). IDO^+ DC that may represent a

regulatory subset of APC in humans can be detected *in vivo* (233). IDO expression in DC, which can be triggered by B7-CTLA4 ligation (234), suppresses T cell clonal expansion (235).

Studies on the S1PR agonist FTY720 are directed by two important facts: the effects of immunosuppressive agents on DC mobilization and migration have not been fully investigated; and it has been argued that the key event in graft acceptance is cell migration and relocation (236). Regarding the immunosuppressive effects of FTY720, we have revealed lysophospholipid S1P signaling as a novel pathway that regulates DC migration and function as reflected in the concurrent effects of DC redistribution and lymphocyte sequestration in FTY720-inhibited allograft rejection. To summarize, we have demonstrated that FTY720 administration *in vivo* retains blood-borne DC, while dramatically depleting T and B cells in the circulation. DC were reduced concomitantly in the secondary lymphoid tissues (LNs and spleen), associated with reduced T cell priming in host spleen following allogeneic bone marrow cell infusion. The inhibitory effect on DC migration was due to down-regulation of surface intercellular adhesion molecule and homing receptor expression, likely via the $S1P_1$ signaling pathway. We believe that FTY720 may inhibit blood-borne DC transmigration across the endothelial barrier to reach secondary lymphoid tissues, where T cells are sequestered (except in the spleen). The relatively high T cell and low DC numbers results in a sub-optimal level of DC-T cell interaction. In addition, FTY720 may directly hinder DC allostimulatory capacity, leading to its immunosuppressive action in transplantation. Our data provide a more comprehensive understanding of the action of FTY720 on DC and complement onging lymphocyte research.

The lysophospholipids (LPs; lysophosphatidic acid and S1P) are simple phospholipids long recognized as components in the biosynthesis of cell membranes (237). Their cognate GPCRs have been identified as molecular targets with therapeutic potential as altered LP signaling has been implicated in the etiology of various diseases. These include cardiovascular and respiratory disorders, cancer, autoimmune diseases, neuropathic pain, psychiatric disorders, reproductive pathology, and obesity (121). Entry of FTY720 into clinical trials as an efficacious immunosuppressant with novel mechanisms of action for kidney transplantation and multiple sclerosis has shed light on treatment options aimed at S1P signaling.

Being a non-selective S1PR agonist, the major drawback of FTY720 is its negative chronotropic effect predominantly on atrial myocytes (98) via $S1P_3$ (139). New synthetic immunosuppressants are being designed to restrict specificity to $S1P_1$, that mediates lymphocyte

recirculation (139). Thus, KRP-203, of similar molecular structure to FTY720, prolongs rat skin and heart allograft survival and significantly attenuates bradycardia as an adverse effect (238).

As the prototype of a novel class of immunosuppressant, FTY720 presents clear benefits. It is not burdened with many of the side-effects of existing immunosuppressants (as described earlier) (98). FTY720 acts synergistically with calcineurin antagonists (CsA, tacrolimus/FK506) and T cell proliferation signal inhibitors (sirolimus/RAPA, everolimus) to favor sub-therapeutic drug doses (98). Using median effect analysis, the combination indices for FTY720/CsA (0.1/1.0 mg/kg/d), FTY720/RAPA (0.5/0.08 mg/kg/d), and FTY720/CsA/RAPA (0.1/0.5/0.08 mg/kg/d) were 0.15, 0.28, and 0.18 respectively (values <1.0 means synergistic interactions) in rats (98). Synergistic effects have been demonstrated in small animal and non-human primate transplant models (98), indicating the use of FTY720 in combined regimens to reduce therapeutic dosing. FTY720 may mitigate ischemia-reperfusion injury and graft vascular disease, two major transplantation obstacles that impair long-term graft survival (98). It does not prevent T cell activation and effector-memory generation that can preserve more intact immunity (induction and expression of immune responses) against infections. Its use in sensitized transplant patients with donor-specific memory cells seems feasible because trafficking of pre-existing, activated effector-memory T cells is also affected by FTY720 (100).

An important question is whether FTY720 can promote tolerance. Low, multiple doses of FTY720 induce permanent acceptance of heart grafts with allochimeric class I MHC Ag treatment (239). FTY720 failed to enhance marrow engraftment (or donor chimerism) in canine non-myeloablative stem cell transplantation, in which stable donor/recipient chimerism was uniformly achieved (240). In another bone marrow transplant tolerance model of allogeneic mixed chimerism using costimulation blockade, FTY720 had no negative effect on chimerism or tolerance development (241). The major influence of FTY720 on cell trafficking seems to link closely with the key event in allograft acceptance, as stated earlier. The key concept of chimerism in transplant tolerance describes alloengraftment as a product of a double immune reaction of co-existing donor and recipient immune systems, in which the mechanisms of non-responsiveness are governed by the migration and localization of the respective Ags (242). Our findings clearly indicate that FTY720 primarily affects DC migration *in vivo*. Future studies of how FTY720 may affect donor leukocyte chimerism and recipient DC relocation in a

vascularized allograft transplant model may provide valuable insights into its potential role in tolerance induction.

Generation of AADC with the anti-inflammatory cytokines IL-10 and TGF-β and their subsequent stimulation by the TLR4 ligand LPS has demonstrated the plasticity of DC to become tolerogenic via an alternative activation pathway. Characterized by an 'immature' phenotype with low levels of costimulatory molecules, AADC also expressed less TLR4 transcripts than GMDC, which suggested their regulation through activation of LPS-induced signaling events involved in the TLR pathway. They preferentially produced the Th2 cytokine IL-10 in culture and induced IL-10 production in allogeneic responder T cells, a tolerogenic cytokine profile. Their poor T cell allostimulatory activity and ability to induce alloAg-specific T cell hyporesponsiveness was not associated with enhanced T cell apoptosis, but with expansion of $CD4^+Foxp3^+$ Treg. The therapeutic efficacy of AADC was confirmed when pre-transplant AADC infusion prolonged subsequent cardiac allograft survival, an effect that could be markedly potentiated by CTLA4-Ig injection.

Alternative activation of DC has been achieved by exposure of the cells to pharmacologic agents. Monocyte-derived DC generated in vitamin D_3 and activated by LPS show inhibited IL-12 and IL-10 secretion and allostimulatory ability (243). Rea *et al.* (154) investigated the tolerogenic properties and functions of CD40-triggered, monocyte-derived DC pretreated with the glucocorticoid DEX (24 h), and found that DEX profoundly inhibited the CD40-dependent maturation of these AADC and impaired their ability to stimulate Th1-type responses *in vitro*—similar to our observations with cytokine-modified AADC. Roelen *et al*. (155) used the same method to generate DEX-exposed, LPS-activated AADC from a murine immature splenic DC line and demonstrated their efficacy in prolonging skin allograft survival following a single infusion of 1×10^6 cells. In comparison, in our AADC transplant experiments, we adoptively transferred 2×10^6 cells together with B7-CD28 blockade to achieve long-term graft survival. The effect of cell number (1×10^6 cells), timing (3 or 1 d pre- or post-transplant), and of multiple infusions of AADC alone or in combination with immunosuppressive agents (tacrolimus, RAPA) should be studied in future experiments to optimize the conditions for designing therapeutic tolerance protocols.

Both human and murine AADC have been generated by cytokine modification and the potent therapeutic ability of murine AADC demonstrated in allogeneic BM transplantation (156)

and xenogeneic graft-versus-host disease models (157). The therapeutic potential of AADC in promoting transplant tolerance is enormous in view of their potent tolerogenic properties exhibited *in vitro* and efficacy in animal models; the possibility of exploiting other immunosuppressive agents (RAPA, everolimus) and activation pathways (CD40-CD40L, B7-CD28, TLR4-LPS, TLR9-CpG) combinations; and their extended application to other DC subtypes. In a recent report, Sato *et al.* (213) further reported their potential value in protecting mice against inflammatory responses associated with endotoxemia and peritonitis, in addition to applications in BM transplantation.

Strategies to utilize DC for therapy have been concerned mainly with donor DC. Similarly, we have centered our AADC investigation on employing donor-derived DC for the purpose of promoting long-term graft survival via the direct pathway. Nevertheless, indirect allorecognition by recipient DC becomes dominant in chronic rejection once the role of donor DC subsides. Use of recipient DC is also clinically relevant considering the time restraints in obtaining deceased donor tissues for culture. Studies exploiting recipient DC in transplant experimentation are important. Permanent acceptance of rat cardiac allografts was achieved by intra-thymic injection of allopeptide-pulsed host DC, in combination with transient anti-lymphocyte serum immunosuppressive therapy (244). Injection of recipient immature DC (NF-κB oligodeoxyribonucleotide decoy-treated) pulsed with donor-derived apoptotic cells significantly prolonged vascularized allograft survival and skewed intragraft cytokine expression toward Th2-type (245). Recipient-derived tolerogenic (TGF-β-treated) DC, deficient in surface costimulatory molecules, prevented acute graft-versus-host disease by inhibiting Th1 responses (246). Our group has utilized RAPA-treated, host-derived DC pulsed with whole donor cell lysates to produce long-term surviving allografts, with a short post-transplant course of tacrolimus to suppress early T cell activation via the direct pathway (205). Continued efforts to examine the efficacy of recipient-derived AADC in vascularized organ transplantation will shed light on mechanisms involved in tolerance induction specifically via the indirect pathway and facilitate design of clinically applicable cell-based therapy.

This dissertation has explored two exciting ways to manipulate DC migration and function to improve allograft survival and ultimately promote donor-specific tolerance. Novel pathways to regulate DC migratory behavior and to enhance their tolerogenic properties are revealed. The S1PR pathway is currently being investigated in other disease models due to the

pleiotropic functions of its natural ligand S1P. Research on TLR signaling has expanded rapidly as the linkage between innate and adaptive immunity by germline-encoded PRRs has become widely recognized. FTY720-mediated T cell chemotaxis requires the leukotriene C_4 transporter (148), that regulates CCL19-dependent mobilization of DC to lymph nodes (152). Our data on AADC also suggest cytokine modification of TLR expression by IL-10 and TGF-β. How cytokine activation may connect to S1PR and TLR signaling is still unknown. Cross-talk between these pathways is highly probable, and its elucidation will enhance mechanistic understanding of DC manipulation to deliver cell-based tolerance-enhancing protocols for transplantation therapy.

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