DENDRITIC CELLS, RAPAMYCIN and TRANSPLANT TOLERANCE

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Dendritic cells (DC) are uniquely well-equipped, professional antigen-presenting cells (APC), with the ability to initiate and regulate immune responses. In transplantation, DC of both donor and host origin contribute to graft rejection by inducing T cell activation and proliferation, via the direct and indirect pathways of allorecognition, respectively. Evidence has also accumulated, however, that DC, particularly in an immature state, can promote tolerance induction and prolong organ allograft survival. Rapamycin is a potent immunosuppressant pro-drug that is well-recognized for its inhibitory effects on T cell proliferation. Despite extensive research on rapamycin's impact on lymphocytes, little is known to date regarding its effects on DC. The central hypothesis in these studies was that**, rapamycin interferes with the DC maturation and enhances their tolerogenic potential.** We first analyzed the influence of rapamycin, in pharmacologically-relevant concentrations, on the maturation, functional activation and T cell stimulatory potential of murine myeloid DC. Herein we show that rapamycin targets DC antigen (Ag)-uptake and IL-4-mediated maturation both *in vitro* (in bone marrow-derived DC), and *in vivo* (in freshly-isolated DC, following *in vivo* administration of rapamycin). Exposure to rapamycin impairs inflammatory cytokine production and effective T cell stimulation by DC. Furthermore, rapamycin-treated DC induce Ag-specific T cell anergy. Next, we determined that presentation of alloAgs to T cells by rapamycin-pretreated DC of host origin, under *in vivo* (pre-transplant) steady-state conditions, could induce hyporesponsiveness to subsequent challenge and prolong organ (heart) graft survival. A single infusion of these cells, seven days prior to

transplant, led to a significant improvement in transplant outcome in an Ag-specific manner. Furthermore, repeated infusion resulted in marked prolongation of graft survival. These studies demonstrate, for the first time, that the immunosuppressive action of rapamycin can be ascribed, in part, to its inhibitory effects on DC and that rapamycin can potentiate the tolerogenic properties of DC. They also reveal the potential of rapamycin-treated DC as therapeutic vectors of transplant tolerance.

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1. CHAPTER ONE[1]

INTRODUCTION

1.1. Dendritic cells

1.1.1. Origins, Subsets and Functions

Dendritic cells (DC) are a heterogeneous group of professional antigen-presenting cells (APC) that are essential for the immune system to recognize and react to antigens (Ag). These latest members of the immune system to be identified[2] are recognized for their diversity and remarkable developmental and functional dynamism at a single cell level. It is only fitting then, that this dynamism is also reflected on the -ever-evolving- information acquired about their origins, functions and different subsets. Currently, DC are known to induce immune responses to i) a variety of infectious agents, ii) tumors, iii) self Ags, prompting autoimmune reactions, and iv) foreign tissue Ags, instigating transplant rejection. In addition to this now well-recognized immunostimulatory role, DC are increasingly more acknowledged for their bridging role between the innate and adaptive immune responses. DC also take part in induction of central (thymic) and peripheral tolerance. Hence, DC appear to be critically positioned in the immune system where the decision of 'if' and 'how' to respond to an Ag is made.

DC are derived from $CD34^+$ bone marrow (BM) hematopoietic stem cells. Both common myeloid progenitors (CMP) and common lymphoid progenitors (CLP) can give rise to DC (2). *In vitro*, either of these progenitor populations can be induced to differentiate into DC, using granulocyte/macrophage colony-stimulating factor (GM-CSF) and/or fms-like tyrosine kinase 3

[1] This chapter contains modified excerpts from reviews co-written by the author in the journals *Transplantation* and *Current Opinion in Molecular Therapeutics*.
[2] With the exception of epidermal Langerhans cells that were first identified in 1868 (1).

ligand (Flt3L). Blood monocytes also give rise to DC when cultured with GM-CSF and interleukin (IL)-4, presenting a possible intermediary cell in DC development (3). *In vivo*, GM-CSF or FLt3L administration results in expansion of DC. Step-by-step developmental pathways of different subsets of DC (discussed below), however, remains to be determined *in vivo*.

In the mouse, DC in the secondary lymphoid organs are comprised of at least three different subsets: $CD8\alpha^-$ classic myeloid (M)DC, $CD8\alpha^+$ 'lymphoid-related' (L)DC and $CD45/B220^+$ plasmacytoid (p)DC. Whereas human counterparts of MDC and pDC exist, $CD8\alpha^+$ DC have not been demonstrated in humans. MDC are found primarily in the marginal zones of the spleen and the outer edges of the paracortex in the lymph nodes (4). In contrast $CD8\alpha^+$ DC are embedded deep in the T cell areas. Beside the differences in surface markers, these subsets appear to have different functions. These differences seem to be associated with how different antigenic stimuli are handled by the immune system. Plasmacytoid DC are known to produce large amounts of type I interferons (IFN) (e.g. IFN-α) in response to viral infection (5), whereas bacterial cell wall components stimulate MDC and LDC to secrete IL-12 (6). $CD8\alpha^+$ cells are superior to other subsets in taking up dying cells, contributing to tolerance induction to self Ags (7-10). In addition to these well-defined lymphoid tissue populations, DC are found in virtually every organ, usually embedded in the interstitium, including the skin (epidermal Langerhans cells and dermal DC), mucosa of alimentary and respiratory tracts, reproductive system, liver, kidney and heart. It should be noted that, regardless of embryologic origin of the tissue they are found in, all DC (dendritic leukocytes) are BM-derived.

The nature of the response initiated by DC is determined mainly by their differentiation stage, termed as maturation. Maturation is a complex process of end-stage DC differentiation, entailing several important changes in their phenotype and function (Figs. 1 & 2). When freshly-isolated

from *in vivo* sources, or in the early stages of *in vitro* differentiation, DC are immature. In the immature state, DC have the ability to capture Ags through a variety of mechanisms. They take up soluble Ags by means of micropinocytosis and receptor-mediated endocytosis, and particulate Ags by phagocytosis. Despite the similarities with macrophages in the Ag uptake mechanisms, immature DC differ from the former in several ways: DC are up to four times more efficient than macrophages in micropinocytosis (11), presenting a quantitative advantage; and Ags taken up by receptor-mediated endocytosis by DC are directed to the major histocompatibility complex (MHC) class II processing pathway, as opposed to lysosomes for destruction in the macrophages, representing a qualitative advantage (12). Ag uptake is almost always followed by initiation of DC maturation that increases the efficiency of DC to present the Ag. During maturation, DC upregulate surface expression of MHC and T cell costimulatory molecules, and increase production of inflammatory cytokines such as IL-12, all leading to an enhanced immunostimulatory capacity. Maturation also leads to a major shift in chemokine receptor expression by DC, such that the Ag-bearing DC traffic to the T cell areas of the lymph nodes where T cell priming occurs. This localization is associated with upregulation of CCR7 (13) and downregulation of CCR5 that mediates chemoattraction of the immature/semi-mature DC to the inflammatory sites, where Ag encounter takes place (14). Therefore DC are uniquely well-equipped to recognize, take up and convey Ags -especially those that are pathogen-associated, to T cells to induce an immune response. With these properties, DC are now regarded as the sentinels of the immune system. Their role, however, is not limited to inflammatory situations. In the steady state, DC continuously scavenge for self and non-self Ags. Uptake of apoptotic cells in the quiescent state by DC triggers CCR7 upregulation without stimulating maturation (15). Presentation of Ags by the immature DC leads to induction of unresponsiveness of the

corresponding T cells to subsequent antigenic challenge (16), preventing autoimmune reactions that would occur otherwise. Therefore DC maturation is a key control mechanism whereby the appropriate immune response to an Ag is initiated.

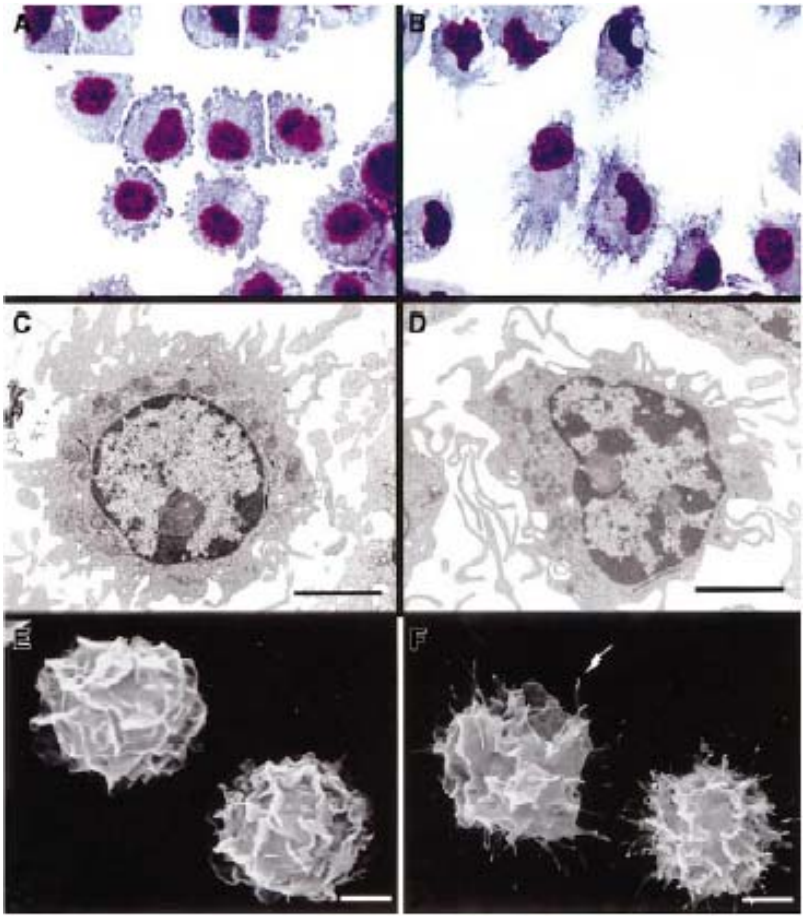


Figure 1. Morphology and ultrastructure of immature and mature murine BM-derived DC

Immature DC possess short, blunt prolongations (A), a round nucleus with prominent nucleoli, multiple cytoplasmic vesicles, mitochondria, few lysosomes (C), and typical 'veils' (E). After maturation, $CD86^{+}$ DC show typical dendritic morphology, with eccentric, indented nuclei (B, D) and a veiled surface with delicate filamentous projections with knob-like tips (F, arrow). (A-B) May-Grunwald-Giemsa. (C-D) TEM x6000 (E-F) SEM x3500. Bar, 5µm. *Copyright American Society of Hematology, used with permission.*

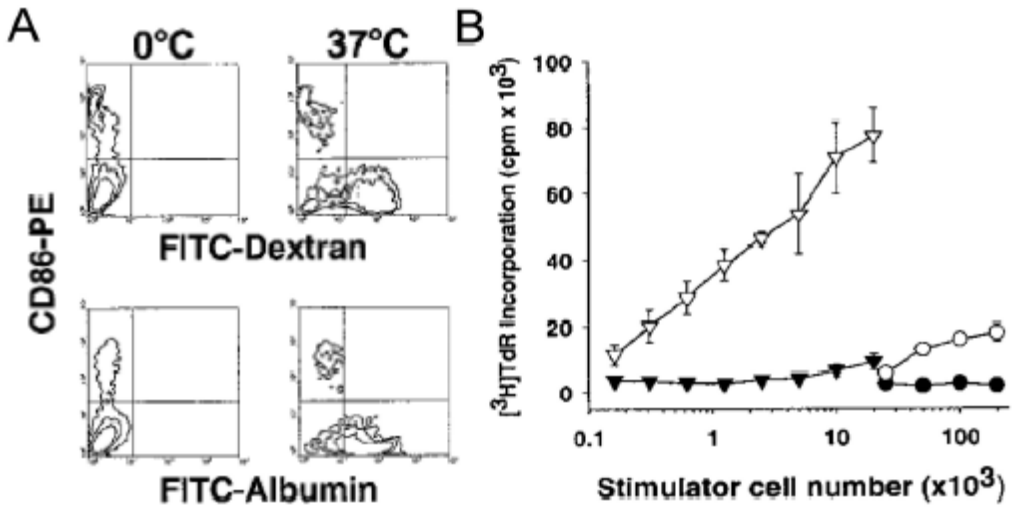


Figure 2. Immature and mature BM-derived DC function.

(A) FITC-Dextran and FITC-Albumin uptake by murine $CD11c^+$ bead-sorted $CD86^-$ (immature) and $CD86^+$ (mature) C57BL/10 mouse BM-derived DC. Only immature DC internalized FITC-Dextran and FITC-Albumin at 37ºC, a phenomenon downregulated at 0 ºC. Results are representative of three independent experiments. (B) Allostimulatory activity of γ-irradiated, FACS-sorted immature (closed triangles) or mature DC (open triangles), assessed using C3H splenic T cells as responders. The MLR stimulatory activity of freshly isolated allogeneic (B10; open circles) or syngeneic (C3H; closed circles) bulk spleen cells is also shown. *Copyright American Society of Hematology, used with permission.*

Several distinct types of stimuli lead to DC maturation. Thus, maturation can be induced by inflammatory cytokines (IL-1, IL-4, tumor necrosis factor [TNF]-α and IFNγ) or microbial constituents (lipopolysaccharide [LPS], unmethylated CpG oligonucleotides). The latter are recognized by DC through pattern recognition receptors (PRR). PRRs are evolutionarily-conserved receptors of the innate immune system, that detect microbial pathogen-associated molecular patterns. Both human and murine DC express numerous PRRs, including Toll-like receptors (TLR), C-type lectins and mannose receptors (4). Among these, TLR are the best characterized, and at least 10 different TLR have been demonstrated in DC. DC can recognize

Gram(+) bacterial wall peptidoglycans by TLR2, Gram(-) bacterial LPS by TLR4, flagellins by TLR5 and unmethylated CpG motifs of bacterial DNA by TLR9. Viruses are detected by TLR3 (double-stranded RNA), TLR7 (single-stranded viral DNA) and TLR9. Expression of these receptors varies with DC subset and maturation status. Human monocyte-derived immature DC express TLR1, 2, 4 and 5, all of which are downregulated upon maturation (17). In the mouse, all splenic DC subsets express TLR1, 2, 4, 6, 8, 9. Interestingly, both human and murine pDC lack TLR3 expression, but can detect viruses effectively through TLR7 and 9, and secrete high concentrations of type I IFNs during viral infection. Taken together, DC, by virtue of maturation through TLR, appear to be a major link between innate and adaptive immune responses. Apart from the cytokine and microbial ligands, several other stimuli in the inflammatory microenvironment can drive DC to maturation. Necrotic cells -possible by release of heat shock proteins (18) or high mobility group box protein 1 (HMGB1) (19), and CD40 ligand (CD154) expressed on activated T cells, induce DC maturation. The latter accounts for an important feedback mechanism, as ligation of CD40 on T cells stimulates their proliferation (20).

1.1.2. DC in Transplantation

1.1.2.1. DC as instigators of transplant rejection

To understand the critical role of DC in transplant rejection (or survival), it is important to point out the mechanisms by which host T cells recognize the graft as 'foreign'. Allorecognition occurs through two distinct pathways; direct and indirect. Although programmed in the thymus to recognize only self MHC molecules, 1-10% of mature T cells in the periphery can respond to intact allogenic MHC molecules directly (21), presumably due to homology in either the MHC molecule or the peptide presented in the peptide-binding groove. This direct recognition leads to activation of multiple T cell clones, resulting in a high frequency T cell

response against the graft (22, 23). By contrast, a much smaller fraction of T cells recognize foreign MHC Ags when these are internalized, processed and presented on the recipient APC though self MHC. Hence, this 'indirect' pathway of allorecognition resembles the recognition of any given Ag in the periphery. Indeed, only <0.01% of all T cells react to a given autoAg (24) and <1% of reactive T cells recognize alloAgs presented indirectly (25). Although other cell types may contribute to activation of T cells through either pathway, donor-derived (direct) and host DC (indirect) are the main instigators of alloreactive T cell activation, because i) they possess ample amounts of MHC for recognition, ii) are equipped for efficient Ag-presentation, and iii) unlike other cells, are capable of providing necessary costimulation (signal 2). Accordingly, in a rat renal transplant model, restoration of donor DC causes rejection of otherwise permanently accepted donor passenger leukocyte-depleted grafts (26). Recent evidence suggests that these two pathways are not mutually exclusive, and interactions occur between them: first, in a mouse cardiac transplant model where donor DC can only provide MHC but not costimulation, 'in trans' costimulation by the host DC was shown to mediate acute rejection (27). Second, DC can acquire intact allogeneic MHC on their cell surface and present these to T cells *in vitro* or *in vivo*, -this has been termed the 'semidirect pathway' by Lechler's group (28). Strikingly, DC are the main players in either case. It is also known that indirect alloreactivity regulates, negatively or positively, direct activation of T cells (29-31).

The direct pathway was long thought to be the central mechanism instigating allograft rejection. However, it is now widely accepted that the direct pathway mediates mainly early (within the first several months post-transplant) alloimmune responses, whereas the indirect pathway contributes to chronic rejection (32). This concept is based on several studies that have demonstrated that direct alloreactivity diminishes with time from transplant, as donor DC are

depleted. These observations, performed on human organ recipients, have been made for a variety of organ grafts (renal, cardiac and lung transplants) and show that, the indirect pathway response increases over time with increased frequencies of T cells with indirect specificities (33-37). In the mouse model, T cells reactive to single self-restricted allopeptides (i.e. the indirect pathway) mediate rejection of skin grafts with characteristic chronic, fibrotic changes (38). In human lung transplant recipients, there is a strong correlation with chronic allograft rejection and development of indirect alloreactivity against donor MHC class I or class II peptides (33, 34). In addition, there is increasing evidence that development of humoral immunity (de novo donor-specific anti-HLA antibodies) is strongly associated with chronic rejection (39). It should be noted, however, that the indirect pathway is not merely a 'late' response, as indirect recognition by itself can induce acute rejection of skin grafts in mice (40-42), depending presumably, on the extent of donor/recipient MHC mismatch.

1.1.2.2. DC as a therapy for transplant rejection

The concept of cell therapy in human organ transplantation dates back to the original studies demonstrating the beneficial effect of blood transfusion before clinical renal transplantation (43). With the advancement of knowledge in DC biology and their role in the transplant rejection, it is now thought that the tolerogenic effect of blood transfusion may be due mainly to DC (44). In parallel with the initial characterization of rejection as a result of predominantly the direct pathway, the tolerogenic potential of donor (as opposed to recipient) DC in transplantation was examined first. Our group first demonstrated that intravenous (i.v.) administration of *in vitro*-propagated, donor-derived, immature MDC, seven days prior to transplantation, resulted in significant prolongation of murine pancreatic islet or cardiac graft survival in the absence of immunosuppression (45, 46). Since these original observations,

immature donor DC infusion has been shown to prolong solid organ transplantation survival, in some cases indefinitely, in different rodent models (47-49). By contrast, studies using recipient DC as part of a therapeutic regimen to prevent transplant rejection have been limited, until recently. Prolongation of cardiac or pancreatic islet graft survival in anti-lymphocyte serum (ALS)-pretreated rats after i.v. infusion of immunodominant donor class I MHC peptide-pulsed recipient DC (50, 51) remain the only such reports in the literature.

1.1.3. DC and tolerance induction

Although recognized initially solely for their potential in instigating immune responses, the role of DC in tolerance is now well-documented. Over the past two decades, DC have been demonstrated to induce both central and peripheral tolerance in animal models, as well as in humans. The first evidence regarding DC tolerogenicity was the establishment of their roles in generation of intrathymic self-tolerance (52, 53). The $CD8\alpha^+$ thymic DC mediate negative selection of developing T cells in the thymic medulla (54). This process constitutes an important central checkpoint against development of self-reactive T cells. Neonatal intrathymic injection of Mls (minor lymphocyte-stimulating locus)-incompatible spleen or thymic DC can induce tolerance via T cell clonal anergy (55), -defined conventionally as T cell receptor (TCR)-mediated inactivation of certain potential responses, in the absence of suppressor cells or inhibitory cytokines (56). Similar results have been reported in BM chimeric and transgenic (tg) mice (57, 58). Intrathymic inoculation of alloAg results in tolerance and this appears to be dependent on thymic DC (59). Furthermore, BM-derived host MDC, pulsed with allopeptide, and injected intrathymically, can induce organ or pancreatic islet transplant tolerance in ALS-conditioned hosts (60, 61).

DC are also involved in the maintenance of peripheral tolerance (62-64). There is ample evidence that the presentation of peripherally-derived Ag by DC within secondary lymphoid tissue is not only effective for T cell priming, but also for the induction of T cell tolerance to self-Ags expressed exclusively by peripheral (extralymphoid) tissues (65, 66). Initially assigned to the $CD8\alpha^+$ LDC, this immunoregulatory function is now believed to be possessed also by MDC (66). Splenic $CD8\alpha^+$ LDC are found in T cell areas of murine lymph nodes and they express high levels of MHC class II/self peptide (62, 64). They kill $CD4^+$ T cells via Fas (CD95)-mediated apoptosis (67) and exhibit tolerogenic activity *in vivo* (68). In the eye, an immune privileged organ, apoptotic cells enter the circulation and are captured by splenic $CD8\alpha^+$ $CD11c^+$ LDC, which are responsible for tolerance induction when these apoptotic cells are coupled to a model Ag (trinitrophenyl) (69, 70). In contrast to MDC, mouse $CD8\alpha^+$ LDC lack the myeloid marker CD11b, and express high levels of the multilectin receptor CD205. In mice, $CD8\alpha^-$ MDC induce T cell proliferation without concomitant cytokine (IL-2, IL-3, IFN-γ, GM-CSF) production (63, 71). Based on these properties of $CD8\alpha^+$ LDC, speculation arose that $CD8\alpha^+$ LDC and MDC were specialized for induction of tolerance and immunity, respectively (72). MDC, however, have also been shown to induce tolerance, in several animal models. For instance, i.v. inoculation of immunodominant peptide-pulsed MDC of host origin induces long term, Ag-specific survival of pancreatic islet transplants in a rat model (51). Furthermore, influenza matrix peptide- or keyhole limpet hemocyanin-pulsed immature autologous MDC induce tolerance to these Ags in healthy human volunteers (73). In mice, adoptive transfer of Ag-pulsed $CD8\alpha^+$ LDC or MDC appears to induce predominant Th1 or Th2 responses, respectively (74, 75). The converse has been reported for human (plasmacytoid) DC2 and

(monocytoid) DC1 *in vitro* (76). Thus, it appears that either subset can induce tolerance, depending on the experimental conditions, animal models, species and the Ag.

Apart from the above-mentioned differences in DC subsets, the capacity of DC to stimulate or suppress immune reactivity may depend on their level of maturity, the route of their *in vivo* administration, the temporal relationship between their administration and that of Ag, the number of cells injected, the amount and physical condition (soluble or particulate) of Ag presented by DC. Thus, DC loaded with low doses of tumor Ag enhance tumor rejection, while those loaded with high doses, or injection of large numbers of tumor Ag-pulsed DC, inhibit their anti-tumor effect (77). BM-derived MDC can prolong skin allograft survival when administrated via the portal vein, but not after i.v. immunization (78). By contrast, subcutaneously (s.c.)-injected, trinitrobenzenesulphate (TNBS)-pulsed DC sensitize for contact hypersensitivity in syngeneic recipients, whereas i.v. injected DC do not (79). Murine immature MDC, infused systemically, can prolong cardiac or islet allograft survival, whereas mature MDC from the same donor strain, accelerate rejection (45, 46). Similarly, in the mouse model where LDC are responsible for cross-tolerization to apoptotic cell-bound Ag, immunity rather than tolerance develops if agonistic CD40 mAb is administered concomitantly, indicating that the fate of the immune response depends on the level of maturity of DC. In the same system, tolerance induction ensues when CD40/CD40L interaction is blocked with anti-CD40 mAb, keeping DC at an immature stage (70).

Several mechanisms by which DC regulate immune reactivity have been proposed over the last few years: induction of T cell anergy and/or apoptosis, Th1/Th2 immune deviation and induction of regulatory T cell (Treg) subsets (80-85). These mechanisms, at large, concur with those that are widely accepted for the induction of tolerance (86). Evidence for each mechanism

is summarized below. It should be noted that the mechanisms postulated may not be mutually exclusive, and may collectively contribute to tolerance induction.

1.1.3.1. Induction of T cell anergy or apoptosis

DC maturity, as discussed above, is an important determinant of the immune response generated against the Ags presented by DC. Several groups have demonstrated that when maturation of DC is suppressed by blockade of costimulatory molecule expression (87) or by exposure to anti-inflammatory cytokines (IL-10 and transforming growth factor [TGF]-β) (88-91), they induce Ag-specific T cell hyporesponsiveness. This is in accordance with the current model of tolerance induction that postulates that T cells, when activated in the absence of Signal 2, become anergic and therefore unresponsive to further stimuli. In the quiescent state, immature DC convey self Ag and induce T cell anergy/deletion in the periphery, resulting in tolerance to the particular self-Ag (92). In a similar fashion, presentation of tumor-associated and self-Ag peptide by DC can induce T cell anergy *in vivo* (93). Interestingly, in mice, intrathymic injection of soluble Ag (ovalbumin; OVA), leading to interaction with thymic DC (presumed LDC), results in peripheral T cell anergy (94), possibly mediated by DC migrating from the thymus (95).

DC have also been shown to promote T cell apoptosis, through various death-associated molecules. Thus, Fas ligand (FasL; CD95L) (67, 96) or nitric oxide (NO) (97) expressing DC can promote activation-induced T cell death. FasL-Fas pathway appears to be important in the DC-induced apoptosis of alloreactive T cells, following blockade of the B7/CD28 pathway by CTLA4Ig (96). DC expressing influenza virus hemagglutinin (HA) can induce tolerance in the periphery by inducing anergy and deletion of naïve mature T cells (98).

1.1.3.2. Selective activation of Th2 cells (immune deviation)

Immune deviation (skewing of T cells toward the Th2 type) is another mechanism that has been shown to underlie tolerance induction. Several groups have shown that DC can induce immune deviation in autoimmune disease and transplant models. DC that are rendered incapable of IL-12p70 synthesis have been shown to activate Th2 cells selectively (99). CTLA4Ig-pretreated splenic DC, when pulsed with myelin basic protein (MBP) and infused systemically, block development of experimental allergic encephalitis (EAE) in rats. (100). The protective mechanism in this model appears to be immune deviation, as immunohistology of the central nervous system demonstrates almost complete inhibition of IL-2 and IFNγ, with upregulation of IL-4 and IL-13 production (100). Thus, tolerogenic DC, obtained by blocking costimulatory molecule expression with soluble protein, appear to lose their capacity to induce Th1 responses/immunity, and can selectively activate Th2 cells. IL-10, a signature cytokine for Th2 response, appears to provide positive feedback to DC, as it skews the Th1/Th2 balance to Th2 cells by blocking IL-12p70 synthesis by DC (88). Accordingly, DC of any origin/lineage can favor stimulation of either Th1 or Th2, mostly depending on the nature of the stimulus, experimental and local environmental conditions (101). For instance, in the presence of high dose Ag, both MDC and pDC induce Th1 development, whereas low Ag dose favors Th2 development regardless of the DC subset (102). In contrast to several reports demonstrating the beneficial effect of immune deviation in different autoimmune disease models, the role of this mechanism in transplant tolerance remains controversial (103, 104). The graft prolongation observed in some models is presumably due mainly to the dampened Th1 response, since Th2 cells are equally effective in the allograft rejection process (105, 106).

1.1.3.3. Induction of Treg Cells

Since the recent repopularization of the concept of 'extrinsic' T cell suppression by Treg cells, the role of DC in the induction of these regulatory cells is increasingly being recognized. Strikingly, this role entails promotion of not only a single type, but a variety of Treg cells in different models (107, 108). *In vitro*, repeated stimulation of human naïve T cells with allogeneic immature DC results in IL-10 producing $CD4^+$ T cell population with regulatory capacity (109). Moreover, in humans an Ag-specific, IL-10 secreting $CD8^+$ T cell subset can be induced by immature, Ag-pulsed DC (73). In the mouse, a murine liver-derived B cell-like DC ($CD205^+B220^+CD19^-$) can induce allogeneic T cells with a cytokine profile resembling Tr1 cells (110). Based on these and similar studies, a paradigm has evolved over the last several years whereby DC maturation is a control point for the generation of effector (by mature DC) vs. regulatory T cells (by immature DC) (111). This paradigm, however, has been challenged recently by reports that demonstrate induction of Treg cells by mature myeloid (112) and plasmacytoid-like ($CD11c^{low}CD45RB^{high}$) DC (113) in mouse. Taken together, these observations provide evidence that DC can promote development of Treg cells and potentially be used for therapy in transplantation, since Treg cells have been repeatedly isolated from tolerated allografts and draining lymphoid tissues in animal models (114).

1.1.4. DC plasticity

With the development of culture methods that allowed researchers to generate large numbers of DC *in vitro*, much information has accumulated in recent years about their functions under different environmental conditions. These observations have also revealed the remarkable plasticity of DC. This plasticity allows DC generated from a single source to be modified to either boost immunity -as in cancer and infectious diseases, or induce tolerance -in autoimmunity

and transplantation. Different approaches to 'program' DC for tolerance induction include genetic (transgene insertion), biologic (differential culture conditions, anti-inflammatory cytokine exposure) and pharmacologic manipulation (23, 115) (Fig. 3).

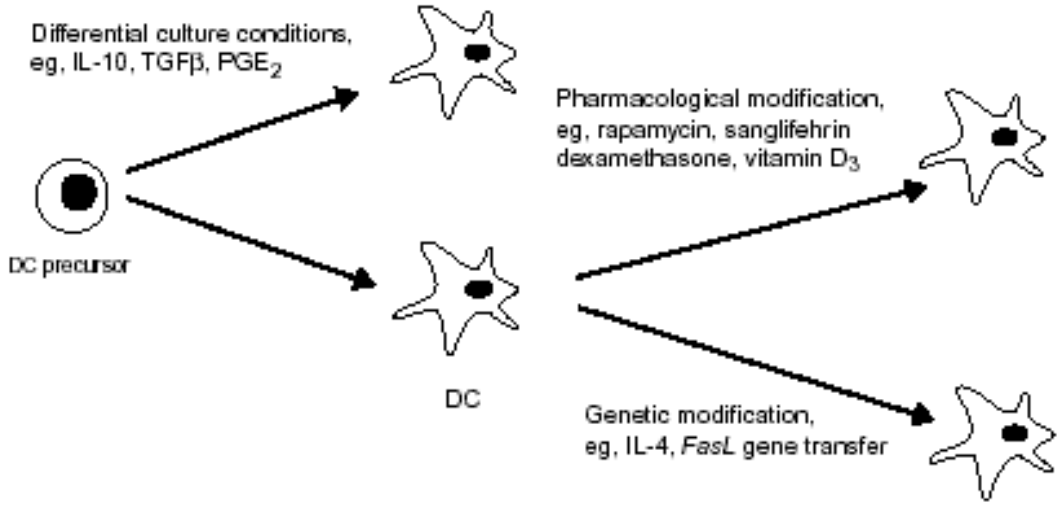


Figure 3. Strategies for generating regulatory/tolerogenic DC.

Regulatory DC can be designed by biological (differential culture conditions), pharmacological or genetic modification of *in vitro* generated DC. These approaches allow researchers to tailor the tolerogenic properties of DC, exploring their use for immunotherapy in immune-mediated conditions. *Modified from (115).*

1.1.4.1. Biologic modification of DC

Presently, DC, particularly of the myeloid subset, can be generated from either BM precursors (BMDC) or human peripheral blood monocytes (MoDC). Both approaches use combinations of GM-CSF and IL-4 as the baseline DC growth factors. During the past decade, several different culture methods have been introduced to produce DC capable of regulating T cell responses. When cultured in low-dose GM-CSF, in the absence of IL-4, BM precursors give rise to immature DC, which can prolong allograft survival, when infused into recipients prior to

transplant (46, 48). Both human MoDC and BMDC acquire regulatory functions when cultured with IL-10 (88-90) and/or TGF-β1 in addition to GM-CSF and IL-4 (116, 117). These DC induce T cell anergy through either the direct or the indirect pathway (118), and show protective effects when infused 2 days after BM transplantation in mice. Interestingly, the regulatory action of these DC is twofold: they inhibit mature DC-mediated T cell stimulation and induce generation of $CD4^+CD25^+$ Treg cells constitutively expressing CD152 (CTLA-4). Even if these two mechanisms may not be mutually exclusive, it is important to note that Treg cells can be generated by IL-10-conditioned regulatory DC in the presence of mature DC. Murine BMDC matured with short-term (4 h) TNF-α treatment become poor producers of the inflammatory cytokine IL-12, despite their mature phenotype. These DC induce IL-10-producing $CD4^+$ T cells and prevent EAE in the mouse model (119). Together, these studies underscore the importance of stably immature DC that fail to produce Signal 2 (i.e. costimulation) or Signal 3 (i.e. inflammatory cytokine secretion), in promoting T cell tolerance.

When prostaglandin E_2 (PGE_2) is used (as a part of maturation stimuli) in DC cultures, the cells become severely impaired in their ability to produce IL-12p70 (99, 120). These DC are refractory to further stimuli, and promote selective Th2 activation. Like PGE_2, PGD_2 inhibits IL-12 secretion by both BMDC and MoDC, and promotes a type 2 response. (121, 122). Similarly, addition of adenosine (123) and Atrial Natriuretic Peptide (ANP) (124) into DC cultures promotes generation of DC with ability to selectively induce Th2 activation. MoDC stimulated with adenosine upregulate expression of MHC and costimulatory molecules, but become poor producers of IL-12p70, TNF-α and CXCL10 (ligand for CXCR3, which is preferentially expressed by Th1 cells) in response to LPS. Adenosine also enhances LPS-induced IL-10 and CCL17 (ligand for CCR4, which is expressed preferentially by Th2 cells) secretion by DC.

1.1.4.2. Genetically engineered DC

Their unique role in either generating or regulating the immune responses, coupled with the potential to deliver the desired gene products locally makes DC highly attractive vectors for all immunotherapeutic approaches. Significant findings have been reported recently regarding genetically modification of DC to enhance their tolerogenic potential. One of these immunoregulatory transgenes, IL-4, has been introduced successfully into DC. IL-4 is a Th2-driving cytokine that inhibits IL-2 and IFNγ production by Th1 cells and inflammatory cytokine production by macrophages. Hence, DC producing IL-4 provide potential in situations where selective Th1 silencing is required. DC transfected adenovirally to express IL-4 prevent the onset of diabetes in non-obese diabetic (NOD) mice and alter the Th1:Th2 cytokine ratio in favor of the latter in the pancreas (125). These findings support earlier reports showing inhibition of murine collagen-induced arthritis (CIA) by IL-4-transduced DC (126, 127). In contrast, research from our group has revealed that IL-4-transduced DC produce increased amounts of IL-12, become better stimulators of T cells and accelerate graft rejection when infused 7 days prior to transplantation. Combined, these reports illustrate the distinct responses to the same approach to DC manipulation in different animal models and underline the differences between syngeneic (i.e. autoimmunity) vs. allogeneic (i.e. transplantation) systems. In a similar manner, DC transduced to express FasL have been reported to improve pre-established CIA after a single i.v. infusion (128), whereas they may induce rejection of skin grafts after s.c. injection (129), demonstrating the importance of their route of delivery. Indolamine 2,3-dioxygenase (IDO), an enzyme involved in catabolism of tryptophan, has lately become the focus of attention due to its role in controlling autoimmunity. When MoDC are made to overexpress IDO, they lose their T cell allostimulatory capacity, presumably due to increased T cell death exerted by tryptophan catabolites (130). At present, it is not clear if this is the main mechanism of immunoregulation by

IDO-expressing DC. Nevertheless, these *in vitro* effects merit exploration in transplant and autoimmunity models. In a different approach, attempting to interfere with their maturation by inhibiting NF-κB, MoDC transduced to overexpress IκBα were found to lose their stimulatory activity for Ag-specific T cells *in vitro* (131). Interestingly, the mechanism underlying this effect was not anergy, but increased T cell apoptosis. When IL-12 production by DC is targeted by small interfering (si)RNA technology, IL-10 production is significantly upregulated and these DC promote Th2 polarization, both *in vitro* and *in vivo* (132). In the rhesus monkey, adenoviral transduction of TGF-β1 in MoDC inhibits their T cell allostimulatory capacity and makes them capable of abrogating Ag-specific immune responses induced by mature DC (133).

1.1.4.3. Pharmacologic modification of DC

The onset of increased popularity in altering DC function by means of pharmacologic intervention coincides with interest in influence of immunosuppressive drugs on DC. Although most of the immunosuppressive agents used in clinics owe their approval to earlier studies performed predominantly on lymphocytes, their potent effects on DC have now been well-established. Once studied extensively, this approach has the potential of using the immunosuppressive agents in a more selective, specifically targeted way, by means of manipulated DC. In addition to the immunosuppressives, a wide variety of pharmacologic agents (e.g. Vitamin D, Aspirin, N-acetyl cysteine) are being investigated for their impact on DC. In these studies, typically DC are either generated *in vitro* and exposed to the agent of interest for different durations, much as the differential culture methods discussed above, or they are isolated freshly from animals after *in vivo* administration of the agent.

Cyclosporine A (CsA) has been shown to inhibit the maturation and allostimulatory capacity of mouse MDC, by inhibiting NF-κB translocation (134). CsA also impairs IL-6 and IL-

12 production by DC, and DC-triggered production of IFNγ, IL-2 and IL-4 by T cells in the bi-directional DC-T cell system (135). By contrast, human MoDC appear resistant to the inhibitory effects of CsA on their maturation and allostimulatory capacity (136). Similarly, another calcineurin inhibitor, FK506, has been reported to have heterogenous effects on DC maturation, depending on the stimuli used to trigger DC maturation in each experimental system (137). Glucocorticoids inhibit LPS- or CD40L-induced DC maturation, and DC production of IL-12 and TNF-α. DC exposed to dexamethasone fail to prime Th1 cells efficiently, and repeated stimulation of T cells with these DC generates IL-10-producing Treg cells (138). Sanglifehrin, a novel immunophilin-binding immunosuppressive agent, targets DC more selectively by suppressing IL-12 and TNF-α production without affecting differentiation, maturation and cell viability (139). In addition to these immunosuppressant agents, several other pharmacologic agents with anti-inflammatory properties have been shown to target DC function (137). Hence, Vitamin D (140), aspirin (141), N-acetyl cysteine (142), glatiramer acetate (143) and BAY (an NF-κB translocation blocker) (144) have all been shown to inhibit DC maturation, stimulatory function and/or their ability to produce IL-12. However, the advantage of using the above-mentioned classic immunosuppressives to 'program' tolerogenic DC lies in the fact that this approach provides a relatively safe passage into pre-clinical large animal (non-human primate) and –potentially- clinical trials, as these agents currently constitute the mainstream therapy for graft rejection.

1.2. Rapamycin

1.2.1. Clinical development

Rapamycin, a macrolide antibiotic pro-drug derived from *Streptomyces hygroscopicus*, was first isolated from a soil sample taken from Easter Island (Rapa Nui) in 1973, during a discovery program for new antimicrobial agents (145). It was first recognized for its potent antifungal actions. Shortly after, however, its immunosuppressive properties were documented by Dr. Suren Sehgal and his collegues at the Ayerst Laboratories, where the drug was first characterized. In the first animal experiments, rapamycin was demonstrated to inhibit the development of adjuvant arthritis and EAE in rodent models (146). It was later shown to prevent onset of type-1 diabetes in NOD mice, prolong survival in MRL/l mice (model of systemic lupus erythematosus [SLE]) and inhibit incidence and severity of polyarthirits in CIA mouse (147). Rapamycin's antirejection activity in organ transplantation was first described by Sir Roy Calne in rats receiving heart and in pigs receiving kidney grafts (148). After numerous single- and multi-center clinical trials, rapamycin was approved by the FDA in 1999 to be used for kidney transplant rejection, as part of a combination therapy.

1.2.2. Mechanism of Action

Similar to the earlier discovered immunosuppressant FK506, rapamycin forms a complex with the intracellular immunophilin FK506 binding protein (FKBP12). Unlike FK506, however, rapamycin does not inhibit calcineurin phosphatase activity. Instead, the rapamycin-FKBP12 gain-in-function complex inhibits the function of the serine/threonine kinase mammalian target of rapamycin (mTOR), a central effector protein that is shared by several signal transduction pathways (Figure 4) (145). The TOR protein is highly conserved from yeast to mammals. It controls cap-dependent translation initiation by phosphorylating eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1). In the unphosphorylated form, 4E-BP1 binds and

inhibits activation of eIF4E. Phosphorylation by TOR promotes dissociation of 4E-BP1 and eIF4E (149, 150). When released, eIF4E, along with other initiation factors, forms the eIF4F complex. This complex is required for translation initiation of 5'-capped mRNAs in eukaryotes. TOR also phosphorylates -either directly or indirectly- and activates the ribosomal p70 S6 kinase (S6K). S6K activation is associated with translation of 5' terminal oligopyrimidine tract (TOP) mRNAs, which exclusively encode for proteins involved in translational machinery (all ribosomal proteins, elongation factors, and the poly-A binding protein). Activation of eIF4e and S6K is induced by serum, growth factors and insulin, and blocked by rapamycin. A recently-identified adaptor protein named regulatory associated protein of TOR (raptor) binds to TOR and its two downstream effectors S6K and 4E-BP1. It appears that, at least *in vitro*, raptor is required for efficient phosphorylation of 4E-BP1 and S6K (150). Rapamycin disrupts the mTOR-raptor complex, thereby preventing downstream pathways (151). Complete inhibition of TOR by rapamycin causes only partial inhibition of TOP mRNA translation (152), but blocks eIF4F-mediated cap-dependent translation initiation (153). Despite comparatively limited suppression of protein translation, due to their selective properties, inhibition of TOR leads to an arrest in cell growth, both in size (by mimicking nutrient deprivation) and in number (by inhibition of cell cycle). Thus, activated eIF4E and S6K cooperate to control cell size (154) and to promote cell cycle progression (155). Selective translation of mRNAs encoding growth factors such as the fibroblast growth factor (FGF) and the vascular endothelial growth factor (VEGF) may also be prompted by eIF4E, accounting for another mechanism whereby TOR inhibition leads to the arrest of cell growth (156).

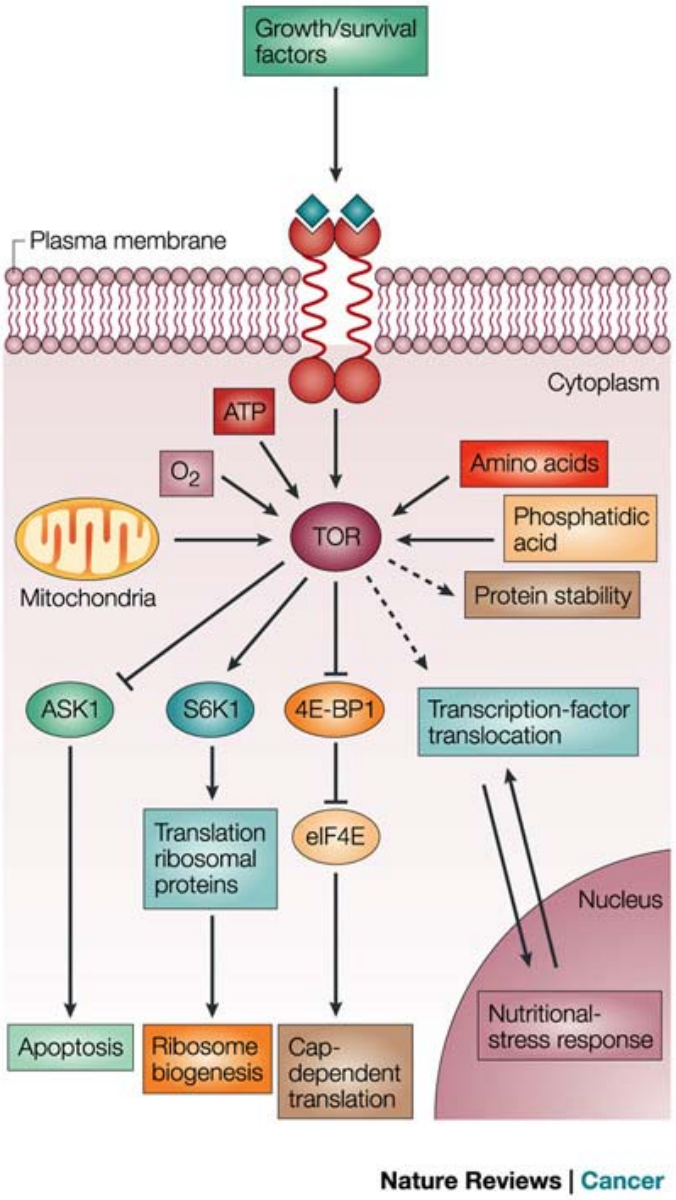


Figure 4. TOR is a central regulator of cell growth and proliferation in response to environmental and nutritional conditions.

TOR signaling is regulated by growth factors, amino acids, ATP and O_2 levels; second messengers (for example, phosphatidic acid); and, possibly, mitochondrial stress. Signaling through TOR seems to regulate several downstream pathways that impinge on cell-cycle progression, translation initiation, transcriptional stress responses, protein stability and survival. Dashed lines indicate pathways that are best described in yeast. ATP, adenosine triphosphate; ASK1, apoptosis-signal-regulating kinase 1; S6K1, ribosomal p70 S6 kinase 1; eIF4E, eukaryotic translation initiation factor 4E; 4E-BP1, eIF4E-binding protein 1. *Figure taken from (149).*

1.2.3. Immunosuppressive and tolerogenic effects

The immunosuppressive effects of rapamycin are associated mainly with inhibition of T cell activity (145). This inhibition occurs through several distinct mechanisms. During T cell activation, ligation of CD28, the best described costimulatory receptor on T cells, results in activation of the transcription factor Rel-1, a CD28 response element-binding factor. In the absence of CD28-mediated costimulation, Rel-1 is sequestered in the cytoplasm by the regulatory protein IκBα. Ligation of CD28 leads to phosphorylation and subsequent degradation of IκBα, allowing Rel-1 translocation to the nucleus. Downregulation of IκBα is blocked by rapamycin, therefore inhibiting CD28-mediated transcription in T cells (157). In addition, rapamycin inhibits IL-2-mediated T cell mitogenesis by inhibiting activation of S6K and cyclin-dependent kinase (cdk) through elimination of the cdk inhibitor protein p27kip1 (158), hence blocking their entry into S phase (159). By and large, rapamycin prevents T cell proliferation in response to a variety of stimuli, including mitogens, cytokines and CD3-CD28 crosslinkage, in both humans and rodents (160-163). Stimulation of T cell clones in the presence of rapamycin leads to an anergic state (164). Furthermore, in the anergic T cell clones, reversal of anergy by IL-2 is blocked by rapamycin (165). Apart from these well-defined potent suppressive activities on T cells, rapamycin also inhibits B cell proliferation (166) and differentiation into immunoglobulin (Ig)-producing plasma cells (167); spontaneous and FGF-mediated proliferation of endothelial cells and fibroblasts (168), and basic (b)FGF and platelet-derived growth factor (PDGF)-driven proliferation of vascular smooth muscle cells (169, 170) (Table 1). The latter effects provide rapamycin an advantage over other commonly-used anti-rejection agents in controlling chronic allograft rejection. In fact, rapamycin stops the progression of graft vascular disease (GVD) in a non-human primate model (171) and slows cardiac transplant vasculopathy progression in humans (172).

Cell Type	Stimulus	Function	Response to Sirolimus
T lymphocytes	Mitogen, antigen	Proliferation	Inhibition
		Production of IL-2, IL-3, IL-4, IL-5, GM-CSF, and IFN-γ	No effect
	Antibodies to cell-surface molecules (αCD28, αCD2)	Proliferation	Inhibition
	IL-2, IL-4, IL-7, IL-12, IL-15	Proliferation	Inhibition
	Activation-induced apoptosis	Apoptosis	No effect
B lymphocytes	Anti-immunoglobulin, LPS	Proliferation	Partial inhibition
PBMC	Pokeweed mitogen	Proliferation	Inhibition
		Production of IgG, IgM, and IgA	Inhibition
		Production of IgE	Inhibition
Mast cells	IL-4, IL-3 ± IL-4	Proliferation	Inhibition
Smooth muscle cells	Growth factors	Proliferation	Inhibition
Fibroblasts	Growth factors	Proliferation	Inhibition

Table 1. *In vitro* cellular effects of rapamycin (sirolimus).

Table taken from (145), with permission from Elsevier.

Strikingly, rapamycin has emerged as a "tolerance-sparing" immunosuppressant in recent years. Currently, two lines of evidence support this paradigm. First; unlike other commonly-used immunosuppressive agents, rapamycin does not interfere with tolerance induction in different animal models (173, 174). This unique ability is attributed, at least partly, to rapamycin's capacity to allow activation-induced cell death (AICD) in T cells. In mice, AICD is a crucial component of tolerance induction (175). Therefore, administration of rapamycin facilitates peripheral tolerance in mice (174). Rapamycin blocks the proliferative effects of IL-2 on T cells, without interfering with its pro-apoptotic actions (176). Second, rapamycin acts synergistically with costimulation blockade in animal models, a unique action that is not shared by the conventional immunosuppressive agents like corticosteroids and FK506, which have a negative impact on the efficacy of costimulation blockade (177).

SCOPE OF THIS THESIS

As organ transplantation has become a common practice around the world, transplant recipients on life-long immunosuppression, with compromised defenses against life-threatening infections and cancers, have increased in number. There is a clear and urgent need to better understand the mechanisms underlying allograft rejection, and to develop strategies that promote the induction of Ag-specific transplant tolerance. Until recently, the influence of commonly-used immunosuppressive agents on DC have been ignored, due mainly to their well-known, potent anti-lymphocyte activities. In this thesis, we have investigated systematically the inhibitory activities of rapamycin on events leading to T cell activation, focusing on the functional immunobiology of DC. **In Chapter Two**, we present our findings regarding the impact of rapamycin on mouse BM-derived DC (BMDC), using mainly *in vitro* approaches. **In Chapter Three**, we expand our observations to DC freshly-isolated from (both steady-state and growth factor-mobilized) rapamycin-treated mice. Here, along with the *in vivo*/*ex vivo* analyses, we employ adoptive transfer of DC to naïve animals in order to evaluate their tolerogenic function. Based on our findings in these two chapters, **in Chapter Four**, we examine the impact of exposure of BMDC to rapamycin on their subsequent capacity for alloAg presentation and tolerance induction via the indirect pathway. We have also tested the efficacy of pre-transplant infusion of these cells in a clinically-relevant transplant model in order to evaluate their potential as therapeutic vectors.

2. CHAPTER TWO[3]

IMPACT OF RAPAMYCIN ON *IN VITRO*-GENERATED DC

2.1. ABSTRACT

MDC with potent allostimulatory capacity can be generated in large numbers from BM precursors with GM-CSF ± IL-4 (BMDC). Given the recently-reported 'tolerance-sparing' impact of the immunosuppressant rapamycin, and the paucity of information on its impact on DC, here, we have investigated the effects of rapamycin on the phenotype and functions of BMDC. Rapamycin, in clinically-relevant doses (1-10 ng/ml), inhibited maturation and allostimulatory capacity of GM-CSF + IL-4 expanded DC, without interfering with their differentiation *in vitro*. These effects were strictly IL-4 dependent, and not observed when DC were expanded with GM-CSF alone. Studies to explore the underlying mechanism revealed that rapamycin suppressed surface expression of both subunits of the IL-4 receptor complex on DC. In addition, rapamycin inhibited macropinocytosis and receptor-mediated endocytosis by DC, nonetheless, in an IL-4-independent manner. These effects were not due to increased apoptotic cell death and by contrast, rapamycin had a protective effect against LPS-induced apoptotic DC death. Strikingly all these effects were mediated through FKBP12 binding and mTOR inhibition, as molar excess of FK506 antagonized the suppressive effects of rapamycin on DC.

[3]Data presented in this chapter is excerpted from (178) and (179).

2.2. INTRODUCTION

Rapamycin is now a commonly used immunosuppressive agent for prevention and treatment of transplant rejection. Owing to its broad inhibitory action on the cell cycle and protein synthesis, rapamycin or its derivatives are also in clinical trials for treatment of several tumors and atherosclerotic coronary artery disease (149). During the clinical development of rapamycin, much like other immunosuppressants, T cells had been considered the principal therapeutic targets (180). A much less studied aspect is the possible impact of rapamycin on DC, and their ability to present Ag to T cells prior to Ag-specific lymphocyte activation and proliferation. As discussed above, DC are ubiquitously distributed APC that play critical roles as initiators and modulators of immune responses (181). Among the most striking features underlying the efficiency of DC as APC is their unsurpassed capacity to take up Ags via constitutive macropinocytosis and mannose receptor-mediated endocytosis (11) and to subsequently process and present MHC-Ag complexes on their surface (181). The capacity of DC to endocytose and to present Ag is under tight developmental control: immature DC are excellent at internalizing Ag, but express low surface MHC class II, whereas mature DC downregulate endocytotic activity and upregulate MHC class II and costimulatory molecules (CD40, CD80, CD86) that promote T-cell activation. The ability of DC to initiate an immune response depends on their transition from Ag-processing to Ag-presenting cells. This transition constitutes an important checkpoint in mounting an immune response, as immature DC not only fail to prime T cells effectively (87, 182), but also serve to promote tolerance induction (46, 73, 109, 115).

In this chapter, we have systematically investigated the influence of rapamycin on DC functions *in vitro*. For this, we used mouse BMDC generated in GM-CSF ± IL-4, that are known for their remarkable allostimulatory capacity (183). Our results reveal that rapamycin, while

allowing DC generation from BM precursors, interferes with DC function at various levels, impairing immune reactivity at the earliest stages. We show that DC, when exposed to rapamycin *in vitro*, exhibit an immature phenotype and fail to stimulate allogeneic T cells. Strikingly, rapamycin treatment inhibits Ag uptake by DC, dissociating this important function from DC maturation. The inhibitory effect of rapamycin on functional DC maturation appears due, at least partly, to interference with IL-4 signaling. These novel insights into the action of rapamycin are likely to contribute to the understanding of the immunosuppressive action of this agent in transplantation, and aid the development of this and other agents that impact on DC function for the treatment of autoimmune diseases. In addition, rapamycin appears to be a good candidate to enhance the tolerogenic potential of DC, that could potentially be used as therapeutic vectors to induce tolerance.

2.3. MATERIALS and METHODS

2.3.1. Animals

Eight-to 12-week-old C57BL/10 (B10; $H2K^b$) and C3H/HeJ (C3H; $H2K^k$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the specific pathogen-free Central Animal Facility of the University of Pittsburgh Medical Center. IL-4 receptor (R) α-deficient mice (BALB/c background) (184) were obtained from the Institute for Clinical Microbiology and Immunology, University of Erlangen, Germany.

2.3.2. Generation of BMDC

BMDC were generated as described previously (141). Briefly, B10 BM cells were flushed from femurs and tibias using PBS. These cells then were cultured for 7 days in RPMI-1640 with 10% heat-inactivated fetal calf serum (FCS), L-glutamine, non-essential amino-acids, sodium pyruvate, penicillin-streptomycin, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid), 2- mercaptoethanol (all from Life Technologies, Gaithersburg, MD), 1000 U/ml recombinant (r) murine GM-CSF (Schering-Plough, Kenilworth, NJ) ± 1000 U/ml r murine IL-4 (R&D Systems, Minneapolis, MN). 1-100 ng/ml rapamycin (Sigma, St. Louis, MO) ± 10-100 ng/ml FK506 (tacrolimus; Prograf® for i.v. use, Fujisawa Healthcare, Deerfield, IL) was added at day 2. Every 2 days, 75% supernatant was replaced with fresh, cytokine-containing medium (± rapamycin or FK506). On day 4, non-adherent cells were removed; on day 7, ≥50% of the non-adherent cells expressed CD11c.

2.3.3. Phenotypic analysis of DC

DC surface Ag expression was analyzed by flow cytometry on day 7 of BM culture. Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, CyChrome-conjugated or biotinylated monoclonal

antibodies (mAbs) used to detect expression of CD11c (HL3), CD40 (HM40-3), CD54 (ICAM-1; 3E2), CD80 (16-10A1), CD86 (GL1), IA^b β chain (25-9-17), $H2K^b$ (AF6-88.5), IA^d (AMS-32.1), CD124 (Institute for Microbiology and Immunology, University of Erlangen, Germany) or CD132 (TUGm2), as well as isotype-matched control mAbs and Streptavidin-CyChrome, were purchased from BD PharMingen (San Diego, CA), unless otherwise noted. Five × 10^5 cells were blocked with 10% v/v normal goat serum (10 min; 4 °C) then stained with mAb (30 min; 4 °C). Appropriate isotype-matched Igs were used as negative controls. The cells were analyzed using an EPICS Elite flow cytometer (Beckman Coulter, Hialeah, FL).

2.3.4. Mixed Leukocyte Reaction (MLR)

For evaluation of their T cell allostimulatory activity, graded numbers of γ-irradiated (20Gy), immunomagnetic bead-purified B10 BMDC were cocultured with nylon-wool column-enriched allogeneic (C3H) splenic T cells for 72h, in a volume of 200 μl in round-bottom plates. For the final 18h, cells were pulsed with 1 μCi [^{3}H]/well. The amount of radioisotope incorporated was determined using a β-scintillation counter.

2.3.5. RNase protection assay

The procedure adopted for RNase protection assay was performed as described in detail (185). Briefly, RNA was isolated from 5 × 10^6 snap-frozen, immunomagnetic-bead sorted DC using a total RNA Isolation Kit (BD PharMingen). The RNase protection assay was performed using the RiboQuant Multi-Probe RPA System (BD PharMingen) with ^{32}P-UTP-labeled antisense RNA probes specific for CD124, CD132 and the housekeeping genes L32 and glyceraldehyde-3-phosphatedehydrogenase (GAPDH) according to the manufacturer's instructions. Mouse RNA and RNA degradation controls were included. Yeast tRNA served as negative control.

2.3.6. Analysis of apoptosis

DC were stimulated with LPS (1 μg/ml RPMI culture medium) or left without any stimuli and apoptosis was analyzed over time by staining of phosphatidylserine translocation with FITC-Annexin-V in combination with the vital dye 7-AAD (BD PharMingen) according to the manufacturer's instructions. Cells were co-stained for CD11c to allow specific analysis of DC by flow-cytometry.

2.3.7. Endocytosis

Quantitative analysis of endocytosis was performed as described (141) with minor modifications. Five × 10^5 cells were incubated with 5 μg/ml FITC-Albumin (MW 66,000, Sigma) or 0.1 mg/ml FITC-Dextran (MW 42,000, Sigma) at either 37 °C or 4 °C for 60 min. Endocytosis was stopped by two washes in ice-cold 0.1% sodium azide/1% FCS/PBS. Cells were stained for CD11c (HL3), and in some experiments, for MHC class II expression (IA^b β-chain, 25-9-17) as described (mAbs from BD PharMingen).

2.3.8. Statistical analyses

Statistical analyses were performed using Student's '*t*' test or the Wilcoxon rank sum test. All tests were performed two-tailed; $P<0.05$ was considered significant. Normal distribution of values, a prerequisite for using Student's '*t*' test, was established by employing the Kolmogorov-Smirnov test.

2.4. RESULTS

2.4.1. Rapamycin does not inhibit DC differentiation *in vitro*

To investigate the impact of rapamycin on *in vitro* DC generation, we first compared the cell yield in cultures grown in the presence or absence of rapamycin. When added on day 2 of the 7-day DC cultures, rapamycin concentrations within the human whole blood trough therapeutic range (186) inhibited *total cell expansion* in a dose-dependent manner, but did not significantly block the *differentiation* of precursor cells into $CD11c^+$ DC as evidenced by the similar yield of $CD11c^+$ DC. CD11c is a very reliable marker for murine DC (187) and is not expressed in significant amounts by murine macrophages (188). The decrease in total cell number was antagonizable by a 20-fold molar excess of FK506. Achievement of similar DC yields was due to consistently increased percentages of $CD11c^+$ DC with characteristic DC morphology in the rapamycin-treated cultures, as observed by flow-cytometry and under the microscope, respectively.

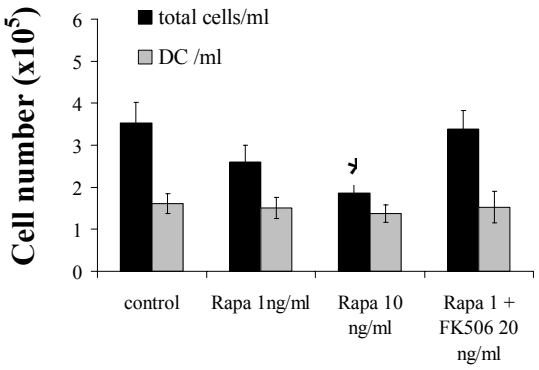


Figure 5. Numbers of DC are comparable in normal vs. rapamycin cultures

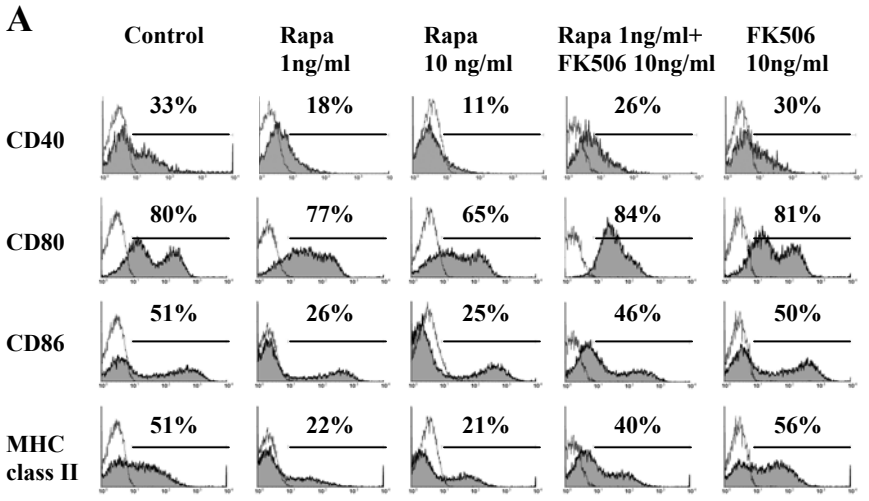
BMDC were propagated for 7 days, as described in Materials and Methods; rapamycin (Rapa) was added on day 2 (± FK506) at the concentrations indicated. Cultures were harvested on day 7, and both total cell and DC ($CD11c^+$) numbers were compared. Differences between paired cultures were assessed by two-tailed Student's '*t*' test for paired samples (**P*<0.05 vs. control). Results are representative of 6 separate experiments.

2.4.2. Rapamycin inhibits maturation of BMDC in an IL-4 dependent manner

As outlined in the introduction, DC maturation is an important check point by which the outcome of the immune reaction is determined. Thus, next we examined the impact of rapamycin on DC maturation. Rapamycin (1-10 ng/ml) decreased surface expression of CD40, CD80, CD86 and MHC class II molecules on GM-CSF+IL-4 expanded DC harvested on day 7 of culture (Fig. 6A). Accordingly, the T cell allostimulatory activity of CD11c immunobead-purified DC was also markedly impaired by rapamycin in a dose-related manner, whereas the structurally-related macrolide immunosuppressant FK506 had no effect on DC phenotype or function (Fig. 6A, B). Rapamycin, when added late, at day 5 of culture, inhibited DC maturation similarly as compared to day 2, though to a slightly lesser extent (e.g. 57% of control DC vs. 30% of rapamycin-treated cells expressed MHC class II, 50% vs. 32% expressed CD86, and 47% vs. 16% expressed CD40); ruling out the possibilities that, i) rapamycin affects the generation of a certain DC subtype in culture, and ii) its inhibitory effect on DC maturation is limited to earlier stages of differentiation.

Next we analyzed whether the inhibition of DC maturation by rapamycin was IL-4-dependent, since IL-4 promotes DC maturation (87, 189) and performed similar experiments in the absence of IL-4. For this, DC were generated from BM precursors with GM-CSF alone, in the presence or absence of rapamycin. Generation of murine DC in GM-CSF is a well-established culture method (183). The results (Fig. 6C) demonstrated that rapamycin's suppressive effect on DC maturation was IL-4 dependent as no significant inhibition of upregulation of MHC class II or costimulatory molecules was observed in the absence of the cytokine. Additionally, the T cell allostimulatory activity was unaffected (Fig. 6D). DC maturation was also not impaired when BMDC from IL-4R α-deficient animals were expanded with GM-CSF + IL-4 in the presence of rapamycin (Fig. 6E). These data indicate that rapamycin

inhibited IL-4 mediated DC maturation. Importantly, in all experiments, cells were gated on the DC-specific marker CD11c in order to analyze costimulatory molecule expression specifically on DC and to rule out confounding effects due to different total numbers of DC.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Control
Rapa 1ng/ml
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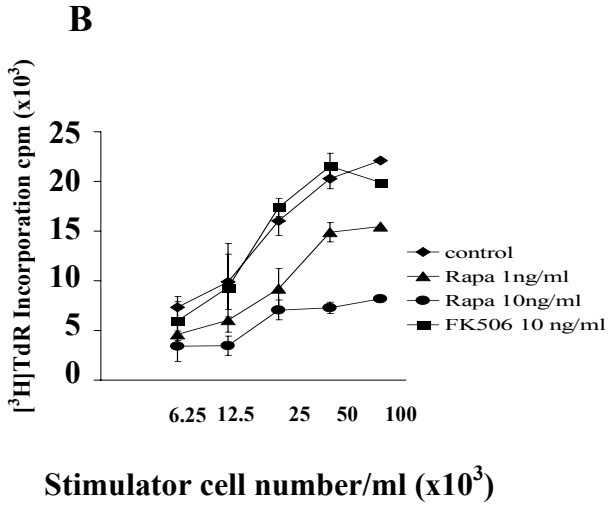
Rapa 1ng/ml+ FK506 10ng/ml
FK506 10ng/ml
CD40
33%
18%
11%
26%
30%
CD80
80%
77%
65%
84%
81%
CD86
51%
26%
25%
46%
50%
MHC class II
51%
22%
21%
40%
56%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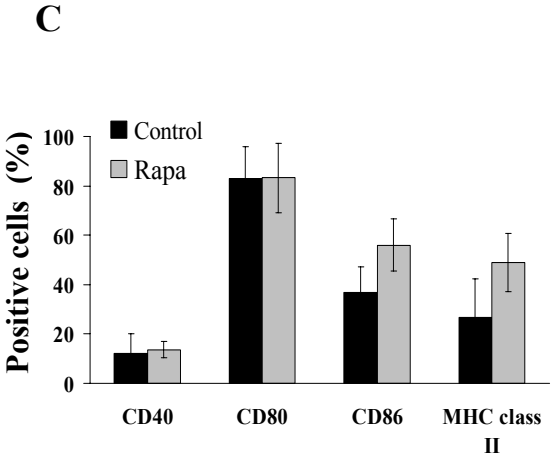
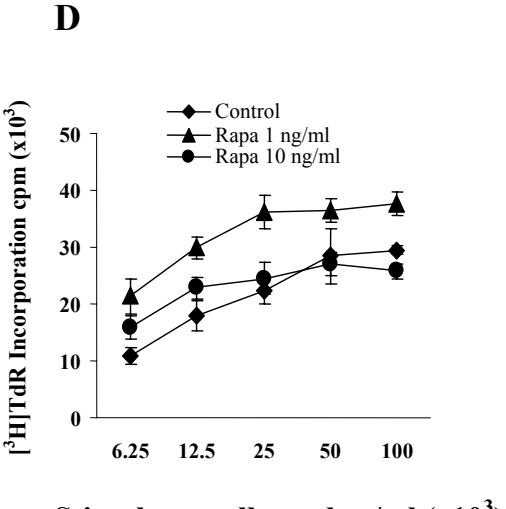
6.25 12.5 25 50 100
control
Rapa 1ng/ml
Rapa 10ng/ml
FK506 10 ng/ml
Stimulator cell number/ml (x10^3)


C
Positive cells (%)
100
80
60
40
20
0
Control
Rapa
CD40
CD80
CD86
MHC class II


D
[3H]TdR Incorporation cpm (x10^3)
50
40
30
20
10
0
6.25 12.5 25 50 100
Control
Rapa 1 ng/ml
Rapa 10 ng/ml
Stimulator cell number/ml (x10^3)

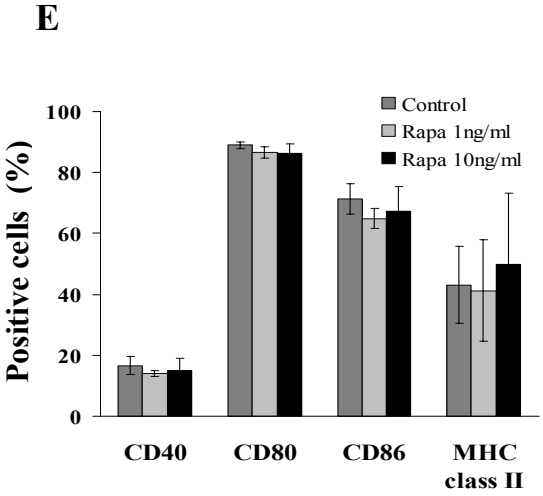

E
Positive cells (%)
100
80
60
40
20
0
Control
Rapa 1ng/ml
Rapa 10ng/ml
CD40
CD80
CD86
MHC class II

Figure 6. The inhibitory effect of rapamycin on DC maturation is IL-4-dependent and mediated via FKBP-12 binding.

BMDC were generated with GM-CSF ± IL-4 and analyzed on day 7. **(A-B)** In the presence of IL-4, rapamycin (Rapa) inhibited cell surface expression of CD40, CD80, CD86 and MHC class II molecules and the allostimulatory activity of purified $CD11c^+$ DC, whereas FK506 exhibited no effect. Competition for rapamycin's intracellular receptor FKBP12 by a molar excess of FK506 (A; second column from right) antagonized the inhibitory effects of rapamycin on DC maturation. **(C-E)** In the absence of IL-4 **(C-D)**, or in IL-4 receptor-α deficient mice **(E)**, rapamycin exerted no inhibitory effect on DC surface expression of CD40, CD80, CD86, or MHC class II molecules, or T cell allostimulatory activity. **(A, C, E)** Cells were gated on CD11c. The incidence of $CD11c^+$ cells expressing the Ag of interest is indicated. Results show representative data from ten (A), three (B, E), five (C), and two (D) similar experiments.

2.4.3. Rapamycin downregulates post-transcriptional expression of the functional IL-4 receptor complex on DC

To elucidate the underlying mechanism of IL-4 mediated DC maturation inhibition in the presence of rapamycin, we investigated expression of both chains of the functional IL-4 receptor (IL-4R) complex on purified DC at both the transcriptional and posttranscriptional levels by RNase protection assay and flow cytometry, respectively. The high-affinity functional IL-4R is a heterodimer composed of the IL-4R α chain (IL-4Rα; CD124) and the common cytokine receptor γ chain (CD132). The results revealed that while rapamycin suppressed cell surface expression of both CD124 and CD132 subunits, their mRNA expression was not altered (Fig. 7). These data suggest that rapamycin targets IL-4-mediated myeloid DC maturation via post-transcriptional inhibition of both chains of the functional IL-4R complex and is in agreement with rapamycin's potent inhibitory effects on protein translation (145, 190) .

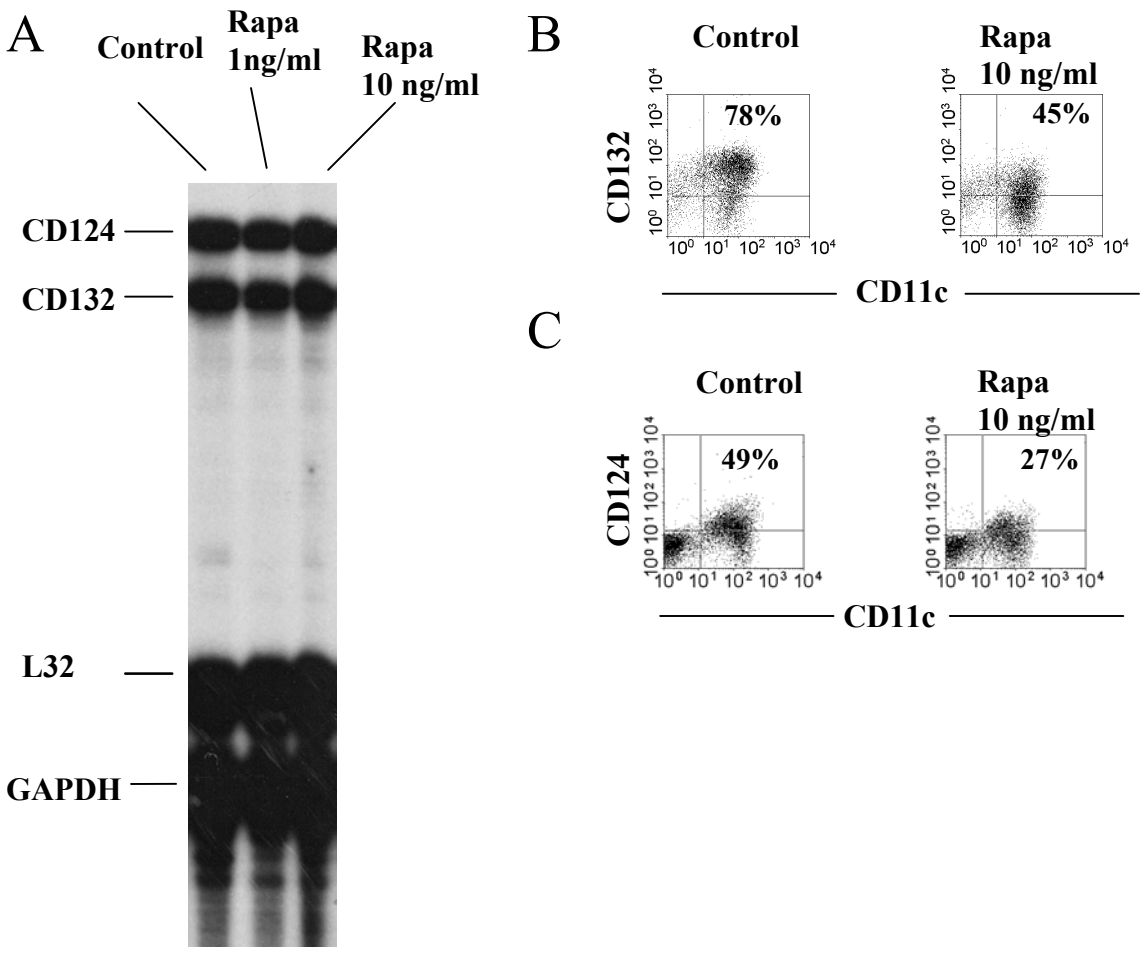


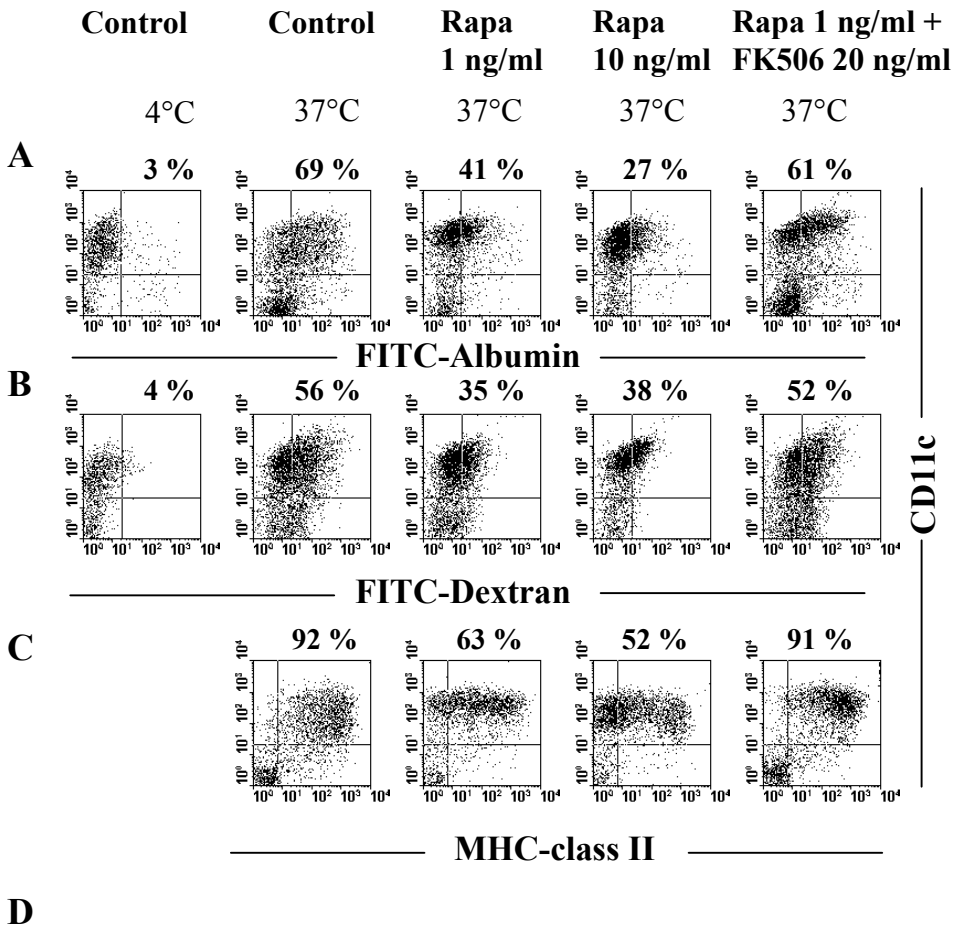
Figure 7. Rapamycin suppresses DC high-affinity IL-4 receptor complex expression at the posttranscriptional level.

BMDC were generated with GM-CSF + IL-4, purified by immunomagnetic-bead sorting and subjected to RNase protection assay or directly analyzed by flow cytometry. **(A)** Comparative RNase protection assay analysis indicates no effect of rapamycin (Rapa) on CD124 or CD132 mRNA expression. L32 and GAPDH represent internal controls. **(B,C)** Downregulation of CD124 and CD132 cell surface expression by rapamycin. Cells were gated on CD11c. The incidence of $CD11c^+$ cells expressing the Ag of interest is indicated. Results show representative data of three (A, B) and five (C) experiments.

2.4.4. Rapamycin inhibits endocytosis by DC in an IL-4 independent manner

After observing the inhibitory activity of rapamycin on DC maturation, we analyzed the Ag uptake ability of rapamycin-treated DC. As discussed in Section 1.1.1., DC maturation entails a sequence of events, including marked downregulation of Ag uptake (11). Immature DC, in turn, are known for their remarkable endocytotic capacity. Analysis of GM-CSF+IL-4 expanded, BM-derived DC harvested at day 7 of culture revealed a reduced capacity of cells exposed to rapamycin to exhibit macropinocytosis of FITC-Albumin and mannose-receptor mediated endocytosis of FITC-Dextran. This was evident both with respect to the incidence (Fig. 8A, B) and the mean fluorescence intensity (MFI) of $CD11c^+$ cells (Fig. 8D).

Since previous experiments indicated that the inhibitory effect of rapamycin on DC maturation was IL-4 dependent (Section 2.4.2.), we expanded DC with GM-CSF only. Additionally, in order to determine more precisely the endocytotic activity of homogenous DC at the same stage of maturation, we specifically analyzed immature MHC II^{low} DC. These experiments confirmed the inhibitory effects of rapamycin on DC endocytosis and indicated that they were not IL-4 related (Fig. 9A-C). Again, low concentrations of 1 ng/ml rapamycin (1.1 nmol) were sufficient to significantly and markedly suppress endocytotic activity. Using 1 ng/ml rapamycin, the relative MFI of immature MHC class II^{lo} $CD11c^+$ DC compared to controls was <42% and <32% with respect to FITC-Albumin and FITC-Dextran respectively (Fig. 9C). Given that the mean *trough* whole blood level of rapamycin in renal transplant patients is 17.3 ng/ml (5 mg rapamycin/day) (186) and that the free plasma fraction is 8%, these concentrations are clinically relevant. When rapamycin was added at day 6 of culture, it still inhibited DC endocytosis, but the overall effect was weaker (relative MFI 69% and 53% for FITC-Albumin and FITC-Dextran respectively at 5 ng/ml rapamycin).



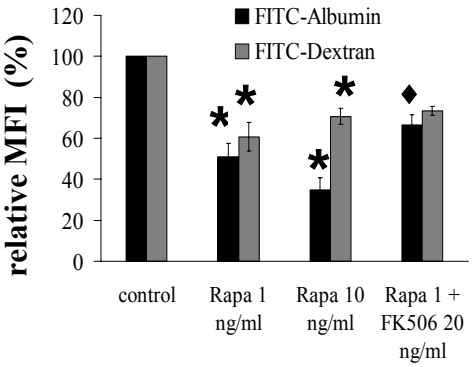


Figure 8. Rapamycin inhibits endocytosis by GM-CSF+IL-4 expanded DC.
(A-D) BMDC were expanded for 7 days as described in the Materials and Methods; rapamycin (Rapa) was added at day 2 (± FK506) at the concentrations indicated. FITC-Albumin and FITC-Dextran internalization at 37° C and 4°C (negative control), MHC class II (IA^b β-chain) and CD11c expression were analyzed by flow cytometry. In all experiments, $CD11c^+$ cells were analyzed to determine endocytosis and MHC class II expression specifically in DC. (A-C) Numbers indicate the percentage of $CD11c^+$ cells that were positive for the marker indicated. (D) Data represent mean values (± SE) of $CD11c^+$ cells after subtraction of background fluorescence (4°C). (D) Differences between paired cultures were compared using two-tailed, Student's '*t*' test for paired samples (*P<0.05 vs. control; ♦P<0.05 vs. Rapa 1 ng/ml). (A-D) Results are representative of four (FITC-Dextran) and six (FITC-Albumin) separate experiments.

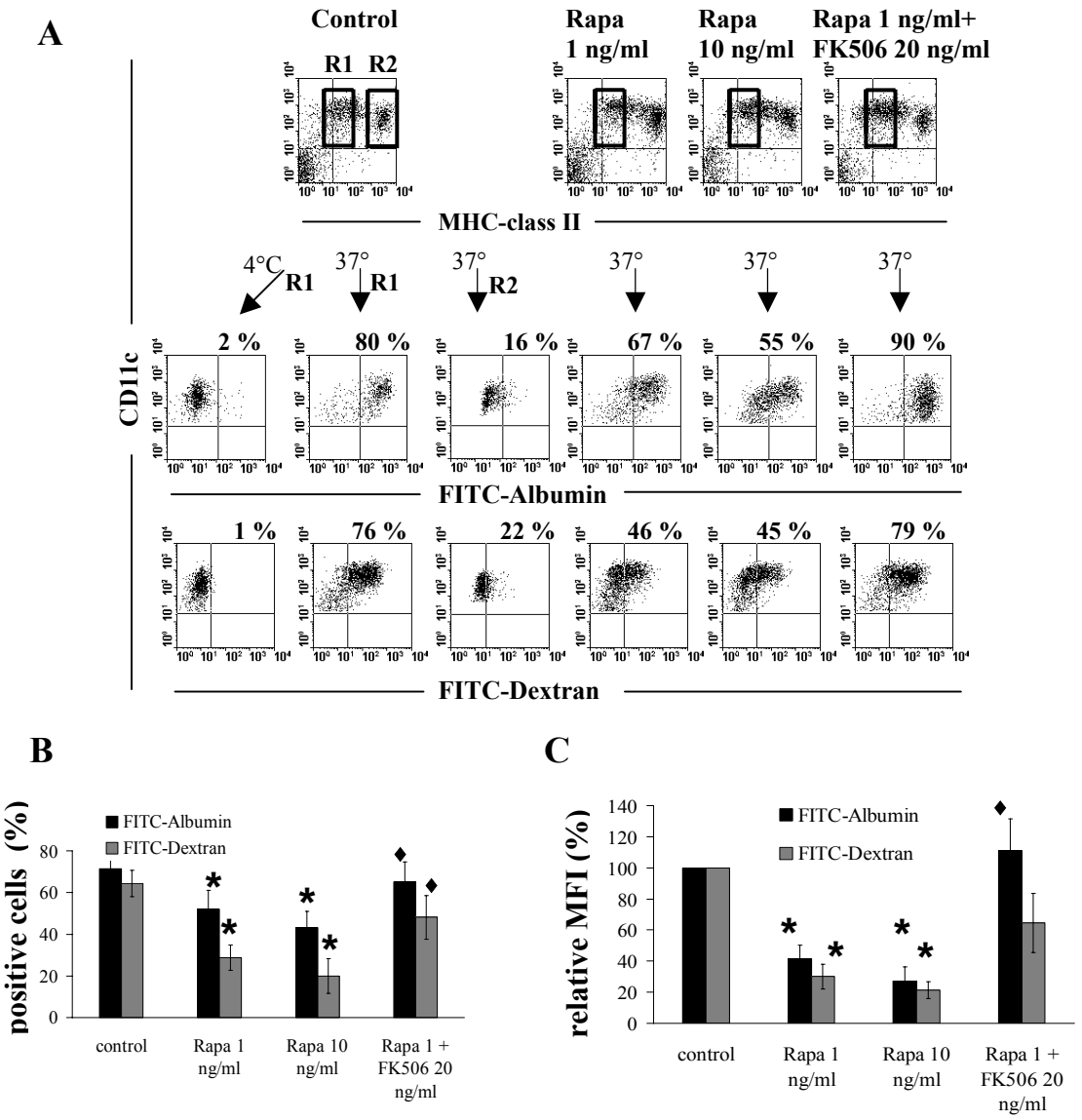


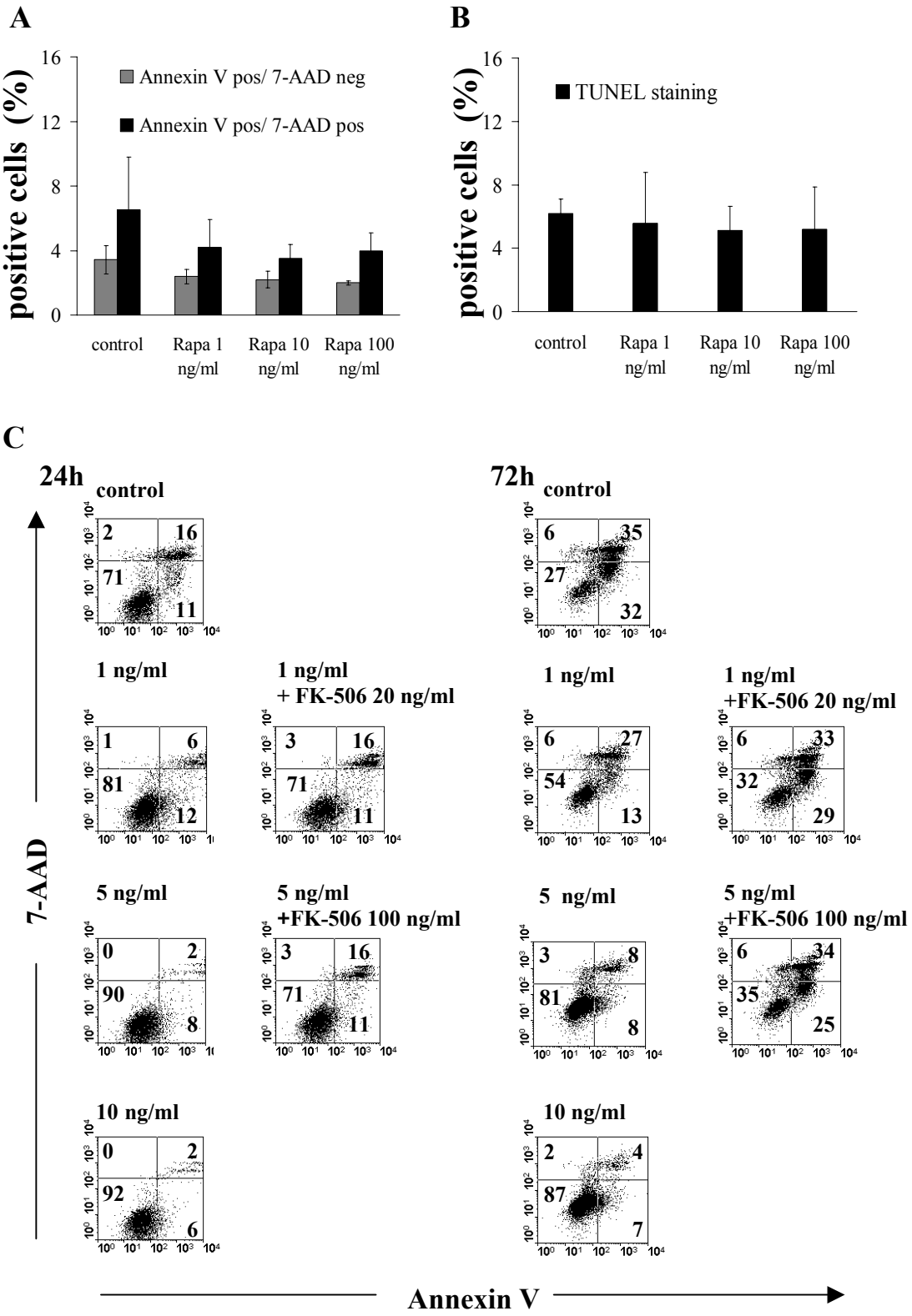
Figure 9. Rapamycin inhibits endocytosis by GM-CSF expanded immature BMDC.

(A-C) BMDC were expanded for 7 days with GM-CSF only as described in the Materials and Methods; rapamycin (Rapa) was added at day 2 (± FK506) at the concentrations indicated. (A-C) FITC-Albumin and FITC-Dextran internalization at 37° C and 4°C (negative control), MHC class II (IA^b β-chain) and CD11c expression were analyzed by 3 color flow cytometry. $CD11c^+$, MHC class II^{lo} cells were gated to determine endocytosis specifically in immature DC. Regions R1 and R2 show endocytotic activity of immature MHC class II^{low} and mature MHC class II^{high} DC, respectively. (A, B) Numbers indicate the percentage of $CD11c^+$ MHC class II^{low} cells positive for the marker indicated. (B, C) Data represent mean values (± SE) of $CD11c^+$ MHC class II^{lo} cells after subtraction of background fluorescence (4°C). Differences between paired cultures were compared using the two-tailed, Student's '*t*' test for paired samples (**P*≤0.01 vs. control; ♦*P*<0.05 vs. Rapa 1 ng/ml).

2.4.5. Rapamycin's inhibitory effects on DC maturation and endocytosis are not due to increased apoptosis

Having established that rapamycin inhibited DC maturation and endocytosis, we analyzed whether these effects were due to increased apoptotic cell death. In contrast to a previous report regarding rapamycin-treated human monocyte- and CD34-derived DC (191), the incidence of apoptosis at day 7 of culture was consistently low (<10%), and was not affected significantly by rapamycin, even at a 'supra'-pharmacological dose of 100 ng/ml, as determined independently by Annexin-V/7-AAD and TUNEL staining (Fig. 10A, B). Similar results were obtained with GM-CSF+IL-4 expanded DC and at day 4 of culture (data not shown).

To ascertain whether rapamycin increased the susceptibility of DC to apoptosis induction, we either stimulated DC propagated in GM-CSF+IL-4 with LPS only (in the absence of cytokines) or cultured DC with medium alone in the absence of any stimuli (LPS, cytokines), then analyzed apoptosis in $CD11c^+$ DC, 24 and 72 hours later by Annexin-V/7-AAD staining. The results show that rapamycin inhibited DC death when cells were stimulated for an extended period with LPS only (Fig. 10C) but did not significantly affect DC death when cells were cultured for an extended period in the absence of LPS (Fig. 10D). Thus, it is unlikely that in these experiments rapamycin acts primarily on DC via apoptosis induction.


A
positive cells (%)
16
12
8
4
0
Annexin V pos/ 7-AAD neg
Annexin V pos/ 7-AAD pos
control
Rapa 1 ng/ml
Rapa 10 ng/ml
Rapa 100 ng/ml
B
positive cells (%)
TUNEL staining
C
24h
72h
control
2
16
71
11
6
35
27
32
1 ng/ml
1 ng/ml + FK-506 20 ng/ml
1 ng/ml +FK-506 20 ng/ml
1
6
81
12
3
16
71
11
6
27
54
13
6
33
32
29
5 ng/ml
5 ng/ml +FK-506 100 ng/ml
0
2
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3
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35
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2
92
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4
87
7
7-AAD
Annexin V

D

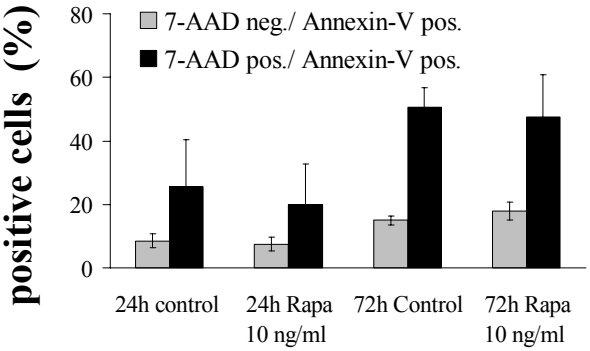


Figure 10. Rapamycin does not induce DC apoptosis under steady state conditions, but protects DC from LPS-induced apoptosis

BMDC were expanded for 7 days with GM-CSF + IL-4. **(A, B)** Apoptosis of $CD11c^+$ DC on day 7 was determined by annexin-V/7-AAD staining (A) and the TUNEL assay (B). Numbers represent mean values (± SE). Incidences of apoptotic (annexin-V^+/7-AAD^- or TUNEL positive) or late apoptotic/necrotic (annexin-V^+/7-AAD^+) cells were consistently lower than 10%. **(C)** Cell death is induced when DC are stimulated on day 7 with LPS (1μg/ml). Rapamycin (Rapa) inhibits LPS-induced DC apoptosis and death in a dose- and time-dependent manner, **(D)** but does not affect the incidence of cell death when DC are cultured for an extended time in the absence of any stimuli. (C) Incidence of cells in each quadrant is indicated. Results show representative data from two (A), three (B), four (C) and three (D) experiments. Similar results were obtained with DC generated in GM-CSF only.

2.4.6. Suppressive effects of rapamycin on DC maturation, endocytosis and apoptosis are antagonized by competition for FKBP12 binding

To reveal whether the suppression of DC maturation and apoptosis were specific rapamycin related effects, we performed additional control experiments in the presence of a molar excess of the immunophilin ligand FK506. This structurally similar macrolide competes for rapamycin's intracellular receptor FKBP12 and thus prevents specific interaction of the rapamycin-FKBP12 complex with mTOR and subsequent inhibition of TOR signaling (145). In fact, binding of rapamycin to FKBP12 and TOR inhibition can be antagonized *in vitro* by FK506 (192). Addition of a ≥ 10-fold molar excess of FK506 antagonized the inhibitory action of rapamycin on DC maturation (Fig. 6A), apoptosis (Fig. 10C) and endocytosis (Fig. 8A, B, D; 9A-C), indicating that these were related to FKBP12-mediated TOR inhibition. Although a near-complete antagonism could be achieved for most of rapamycin's effects on DC, in some cases the antagonistic effect of FK506 was incomplete, especially with respect to mannose receptor-mediated endocytosis. In this particular case, similar results were obtained over a wide range of drug concentrations (1-20 ng/ml rapamycin; 10-250 ng/ml FK506) and at different FK506/rapamycin ratios (10-55 molar). This suggested that other FKBP might also be involved in endocytosis inhibition. One candidate is FKBP25, that has >100 times greater binding affinity for rapamycin than FK506 (193).

2.5. DISCUSSION

Numerous studies have demonstrated rapamycin's potent suppression of the effectors of immune responses, -T and B lymphocytes (145, 194), but its influence on DC, the most specialized inducers of immune responses, are not well understood. Here we provide comprehensive *in vitro* evidence that rapamycin potently targets functional DC activation. The principal findings of this study provide novel insight into the immunopharmacology of this agent and have implications with respect to the therapeutic application of rapamycin.

First, rapamycin targets responsiveness to the key DC regulatory cytokine IL-4. This effect is associated with downregulation of the high-affinity IL-4R complex, which consists of the IL-4R α-chain and the common cytokine receptor γ-chain. The inhibitory effects on DC maturation are mediated through rapamycin's intracellular receptor FKBP12. However, inhibition of IL-4-mediated DC activation is only one of several aspects by which rapamycin can interfere with DC function. Downregulation of the common cytokine receptor γ-chain, which is an indispensable component not only of the IL-4R, but also the functional IL-2R, IL-7R, IL-9R, IL-15R, and IL-21R complexes (195-198), may have important additional implications with respect to the immunosuppressive effects of rapamycin on DC and other cells. This finding matches with a recent series of reports regarding rapamycin's inhibitory impact on various cytokine signaling pathways in DC. Thus, rapamycin has recently been reported to inhibit GM-CSF-induced human DC survival by disrupting this cytokine's signaling pathways inside DC (199), whereas in murine DC, rapamycin suppresses autocrine IL-12 signaling through inhibition of Jak2/Stat4 pathway (200).

Second, rapamycin inhibits DC endocytosis, providing evidence that it may interfere with immune responses at a very early stage. Strikingly, rapamycin disrupts the sequential pairing of

DC Ag uptake and immaturity, thereby rendering them unable to both acquire and present Ag. This is in contrast to other anti-inflammatory drugs, like corticosteroids (201) or salicylates (141), that have recently been shown to suppress DC maturation and as a consequence, enhance their endocytotic activity. Our findings regarding inhibition of Ag-uptake were confirmed recently in rapamycin-treated human monocyte-derived DC (202). The precise mechanism(s) by which rapamycin inhibits DC endocytosis remains to be determined. In this context, it is important to note that the Rho GTPases CDC42 and Rac that interfere with the endocytotic activity of DC (203, 204) complex with and activate the p70 S6 kinase (205) that belongs to the central signaling pathway disrupted by rapamycin (145). In addition, rapamycin's inhibition of TOR signaling downregulates protein translation and has been demonstrated to suppress actin synthesis (206). These novel findings, coupled with parallel results in the human system, may provide further incentive for the use of rapamycin in clinical settings other than transplantation, e.g. in autoimmune disease.

Third, rapamycin does not affect DC differentiation and viability *in vitro*. In contrast to a recent report indicating that rapamycin induced apoptosis specifically in DC in the human system (191), we found the frequency of apoptotic or dead cells to be consistently less than 10% in BM-derived, *in vitro*-generated, rapamycin-treated DC. Moreover, when DC were stimulated with LPS, rapamycin inhibited cell death in a dose-dependent, FKBP12-mediated manner. Thus, it is unlikely that rapamycin acts primarily on DC via apoptosis induction. It should be noted that there have been several reports that rapamycin can exert anti-apoptotic effects on different cell populations (207-210). It is of particular importance that comparatively large numbers (comparable to normal DC cultures) of rapamycin-treated, immature DC can be generated *in*

vitro, since these cells could potentially provide a source as therapeutic vectors to be used in transplant tolerance induction regimens (see Chapter Four).

In conclusion, we show in this chapter, for the first time, that rapamycin can enhance the tolerogenic potential of murine BMDC *in vitro* by interfering with their functional activation at different levels. These observations, while they need to be confirmed *in vivo* to further analyze the mechanistic actions of rapamycin as an immunosuppressant, provide insight into better and perhaps broader use of this agent in situations where immune dysregulation occurs.

3. CHAPTER THREE[4]

IMPACT OF RAPAMYCIN ON DC FOLLOWING *IN VIVO* ADMINISTRATION

3.1. ABSTRACT

Immunosuppressive agents are commonly and increasingly used in clinical medicine, not least because of the growing numbers of organ transplant recipients. Due to the complex nature of the immune system, studies that dissect the impact of immunosuppressants on each component of the system may potentially aid in better use of these agents. Here, building on our findings presented in Chapter Two, we have analyzed the influence of rapamycin on DC development and function *in vivo*. For these studies, normal or Flt3L-treated mice were injected with rapamycin (0.5 mg/kg/day) or the drug vehicle for 7-10 days. The data show that rapamycin significantly decreased (40-50% reduction; $P<0.002$) DC numbers in BM and spleen, both under steady-state conditions and Flt3L-mediated DC expansion. Freshly-isolated DC from rapamycin-treated mice were impaired in their ability to upregulate surface costimulatory molecules (CD80, CD86) and secrete TNF-α and IL-12p70. *In vivo* exposure to rapamycin also inhibited DC Ag uptake. Furthermore, T cells of mice that had been injected with freshly-isolated, purified DC from rapamycin-treated donors, showed significantly reduced proliferation, and IL-2 and IFNγ production upon restimulation with donor alloAgs.

[4] Data presented in this chapter is excerpted from (178) and (179).

3.2. INTRODUCTION

Organ transplantation remains the only definitive treatment for various end-stage organ failures. Despite significant advances in this field, -especially in short-term graft outcomes-, long-term outcome continues to be limited due to several reasons. Transplant recipients almost invariably rely on life-long heavy immunosuppression and its devastating side effects. On the other side of the spectrum, lowering the level of immunosuppression almost always brings the risk of rejection. In addition, current immunosuppressive regimens have limited protective impact on generation of chronic rejection. In theory, it would be ideal to program the recipient's immune system in advance to tolerate the new organ. Ag-specific tolerance can be achieved through several approaches in experimental animal models, however this 'holy grail' of transplant immunology remains far from reach in clinical transplantation at the moment, except for a minority of cases -particularly liver recipients-, for reasons and mechanisms still to be determined. Unmistakably, any attempt to induce donor Ag-specific tolerance needs to be evaluated in animal models under cover of clinically available immunosuppression, as this would be the strict condition when these approaches are translated into clinical trials. It is therefore critical to determine the optimum conditions of immunosuppression, under which tolerance could be achieved.

Current immunosuppressive regimens rely on inhibition of lymphocyte activation and proliferation, and not on DC:lymphocyte interaction where the crucial fate of the immune response appears to be determined. Since most of the mechanisms of tolerance induction require initial activation of lymphocytes, immunosuppressive drugs that disrupt pathways associated with T cell activation can interfere with the development of tolerance (175, 211-213). One exception is rapamycin, which allows AICD in alloreactive T cells, an effect that promotes tolerance induction (174, 175). This tolerance-sparing effect of rapamycin may partially be due

to its impact on DC functions. In the previous chapter, we have shown, for the first time, that exposure to clinically relevant doses of rapamycin inhibits maturation and allostimulatory potential of BMDC. In this chapter, we investigated the impact of rapamycin on DC *in vivo*. Our aims were three-fold: 1) to test if the observed impact of rapamycin on DC *in vitro* can be translated *in vivo*; 2) to examine if *in vivo* administration of rapamycin has any effect on DC differentiation; and 3) to assess the immunomodulatory effect of i.v.-infused rapamycin-treated DC on alloimmune responses. The latter investigation bridges this chapter with the next, where rapamycin-treated DC will be used as therapeutic vectors for transplant tolerance induction.

Our results show that *in vivo* administration of rapamycin interferes with DC antigen uptake, maturation, allostimulatory function and bioactive IL-12p70 production. Furthermore, these DC, when adoptively transferred, induce T cell hyporesponsiveness. We also show that rapamycin blocks the *in vivo* effects of Flt3L, a potent endogenous DC growth factor that also regulates the proliferation of hematopoeitic precursor/stem cells and monocytic precursors (214, 215). These novel observations provide new insights into actions of rapamycin and have significant implications for the development of new therapeutic strategies in disease processes in which DC may play a crucial immunopathologic role.

3.3. MATERIALS AND METHODS

3.3.1. Animals

Eight-to 12-week-old C57BL/10 (B10; H2K^b) and C3H/HeJ (C3H; H2K^k) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the specific pathogen-free Central Animal Facility of the University of Pittsburgh Medical Center.

3.3.2. *In vivo* DC expansion and rapamycin administration

The *in vivo* effects of rapamycin were investigated in normal animals and in mice in which DC were expanded by administration of r human Flt3L (Chinese hamster ovary [CHO] cell-derived; 10μg/day, intraperitoneally (i.p.); d1-10; Immunex, now Amgen, Seattle, WA). Rapamycin (Wyeth-Ayerst, Princeton, NJ) was dissolved in 51% PEG300, 5% polysorbate 80, 5% ethanol (vehicle, all reagents from Sigma). Mice were injected with rapamycin (0.5 mg/kg/d; i.p.) or vehicle for 7 or 10 days (d3-d10; d1-10). Due to the long elimination half-life, mice received a loading dose on day 1 (1.5 mg/kg), according to the recommendation of Mahalati and Kahan (216).

3.3.3. DC isolation and purification

Spleens were injected with 100 U/ml type IV collagenase (Sigma) in RPMI-1640, disrupted and chopped with fine scissors and the resulting cell suspension kept at 4°C. The remaining tissue fragments were digested in 400 U/ml collagenase/RPMI-1640 solution for 45 min at 37°C. Finally, the cells were pooled, passed through a strainer and washed in sterile, ice-cold, Ca^{2+}-free PBS. DC were further enriched by density gradient centrifugation using 16% w/v metrizamide (Sigma) in PBS at 1200 x g for 20 min at 4°C. BM cells were isolated from femurs and tibias and subjected to density gradient centrifugation. To obtain highly-purified DC

populations for analysis of allostimulatory activity, cytokine production or their adoptive transfer, the cells were labeled with magnetic bead-conjugated anti-CD11c mAb (Miltenyi Biotec, Auburn, CA) followed by positive selection through paramagnetic columns (LS columns, Miltenyi Biotec) according to the manufacturer's instructions. DC purity of 90-95% was consistently achieved.

3.3.4. Phenotypic analysis of DC

DC surface antigen expression was analyzed by flow cytometry as described in the previous chapter. FITC-, PE, CyChrome-conjugated or biotinylated mAbs used to detect expression of CD11c (HL3), CD40 (HM40-3), CD54 (ICAM-1; 3E2), CD80 (16-10A1), CD86 (GL1), IA^b β chain (25-9-17), $H2K^b$ (AF6-88.5), as well as isotype-matched control Igs and Streptavidin-CyChrome, were purchased from BD PharMingen (San Diego, CA). Five × 10^5 cells were blocked with 10% v/v normal goat serum (10 min; 4 °C) then stained with mAb (30 min; 4 °C). Appropriate isotype-matched Igs were used as negative controls. The cells were analyzed using an EPICS Elite flow cytometer (Beckman Coulter, Hialeah, FL).

3.3.5. Cytokine quantitation and allostimulatory activity

IL-12p70 and TNF-α production were measured in 24h supernatants of LPS-stimulated (2 μg/ml), immunomagnetic bead-purified DC (10^6/ml) using ELISA kits (Quantikine, BD PharMingen). LPS stimulation was performed in the presence of low dose GM-CSF (Schering-Plough, Kenilworth, NJ; 50 U/ml RPMI-1640 culture medium) and graded concentrations (0-1000 U/ml) of IL-4 (R&D Systems, Minneapolis, MN), where indicated. IL-2, IL-4, IL-10, and IFNγ were quantified in 72h supernatants of MLR cultures using reagents and procedures

recommended by the manufacturer (BD PharMingen). Graded numbers of γ-irradiated (20 Gy), magnetic bead-sorted B10 DC were used as stimulators in 72h MLR with nylon-wool column purified allogeneic (C3H) splenic T cells as responders (2×10^5/ml) as described.

3.3.6. Endocytosis

Quantitative analysis of endocytosis was performed as described in Chapter Two. Five × 10^5 cells were incubated with 500 μg/ml FITC-Albumin (Sigma) or 1 mg/ml FITC-Dextran (MW 42,000, Sigma) at either 37 °C or 4 °C for 40 min. Endocytosis was stopped by two washes in ice-cold 0.1% sodium azide/1% FCS/PBS. Cells were stained for CD11c (HL3), and in some experiments, for MHC class II expression (IA^b β-chain, 25-9-17) as described (mAbs from BD PharMingen).

3.3.7. Statistical analyses

Statistical analysis was performed using Student's '*t*' test or the Wilcoxon rank sum test. All tests were performed two-tailed; $P<0.05$ was considered significant.

3.4. RESULTS

3.4.1. Rapamycin suppresses DC generation *in vivo*

To address its *in vivo* effects, we first analyzed the impact of rapamycin on DC generation in normal mice. Mice were injected with 0.5 mg/kg/day rapamycin for 10 days, then DC in spleen were purified and quantitated. Rapamycin administration significantly reduced (>50%) the number of DC in comparison to animals injected with the drug vehicle (Fig. 11A). To explore the effects of rapamycin on DC expansion *in vivo* under dynamic conditions, we expanded DC injecting the endogenous DC growth factor Flt3L for 10 days in combination with either rapamycin or vehicle. Using this model, we confirmed that rapamycin impaired DC expansion, as evidenced by 40-50% reduction in BM and spleen DC numbers (Fig. 11B, C). In Flt3L-treated animals, the inhibitory effect of rapamycin on cell expansion was apparent from the appearance and significant reduction in weights of the spleens (Fig. 11D, E). To ascertain whether this difference was due to an ongoing induction of DC death in response to rapamycin, we analyzed the incidence of cell death ex vivo. The rate of apoptosis in DC freshly-isolated from rapamycin-treated animals was consistently low (≤ 8%, n=3/group), similar to data we obtained previously from *in vitro* BMDC cultures (Chapter Two).

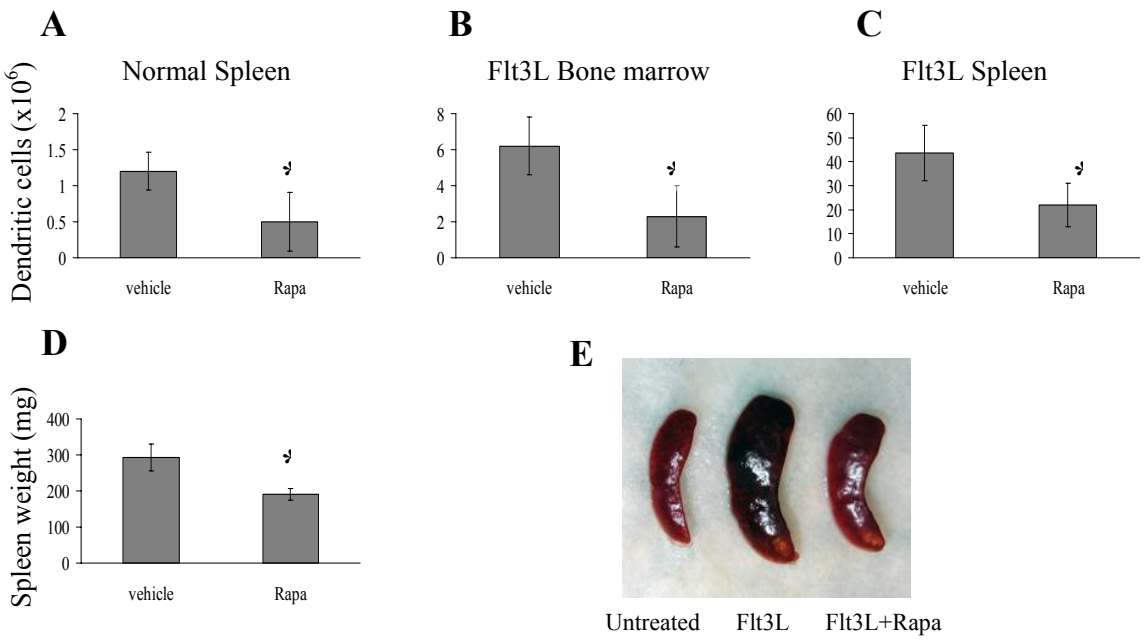


Figure 11. DC generation in the steady-state and under dynamic conditions.

In vivo administration of rapamycin (Rapa) suppresses DC generation under **(A)** steady-state and **(B, C)** dynamic conditions. (A-C) Effect of rapamycin or drug vehicle on the number of $CD11c^+$ DC/tissue on day 10 (with or without Flt3L). Results are representative of 8-10 animals/treatment group. **P*=0.005 vs. vehicle (A, normal spleen), *P*=0.003 vs. vehicle (B, Flt3L BM), *P*=0.002 vs. vehicle (C, Flt3L spleen); two-tailed Student's '*t*' test. **(D, E)** Effect of rapamycin or drug vehicle on spleen weight and appearance (8 animals/treatment group) in Flt3L-treated animals on day 10. **P*=0.004 vs. vehicle; two-tailed Student's '*t*' test.

3.4.2. *In vivo* administration of rapamycin impairs upregulation of costimulatory, but not MHC class II molecules on DC, and inhibits their T cell stimulation capacity

Freshly-isolated DC from rapamycin and vehicle-injected animals displayed an immature phenotype. To test whether *in vivo* administration of rapamycin affected the upregulation of costimulatory molecules, we stimulated DC from rapamycin- or vehicle-treated animals with LPS (50 ng/ml). DC from rapamycin-injected animals showed significantly impaired ($P<0.01$) upregulation of the costimulatory molecules CD80, CD86, and the adhesion molecule CD54,

whereas the expression of MHC class II was unaffected (Fig. 12A, B). This pattern of reduced costimulatory molecule expression but unaffected MHC class II expression in response to LPS was detected reproducibly in spleen and BM DC (freshly-isolated) from both normal and Flt3L-treated animals. To assess the *in vivo* effects of rapamycin on the capacity of DC to stimulate T cells on a per cell basis, animals were treated with 0.5 mg/kg/d rapamycin or vehicle (7-10 days) and DC purified by density gradient centrifugation and immunomagnetic-bead sorting to >90% purity, as determined by $CD11c^+$ staining. DC generated *in vivo* in the presence of rapamycin were found to be less efficient stimulators of fully allogeneic naïve C3H T cells in 72h MLR (Fig. 12C).

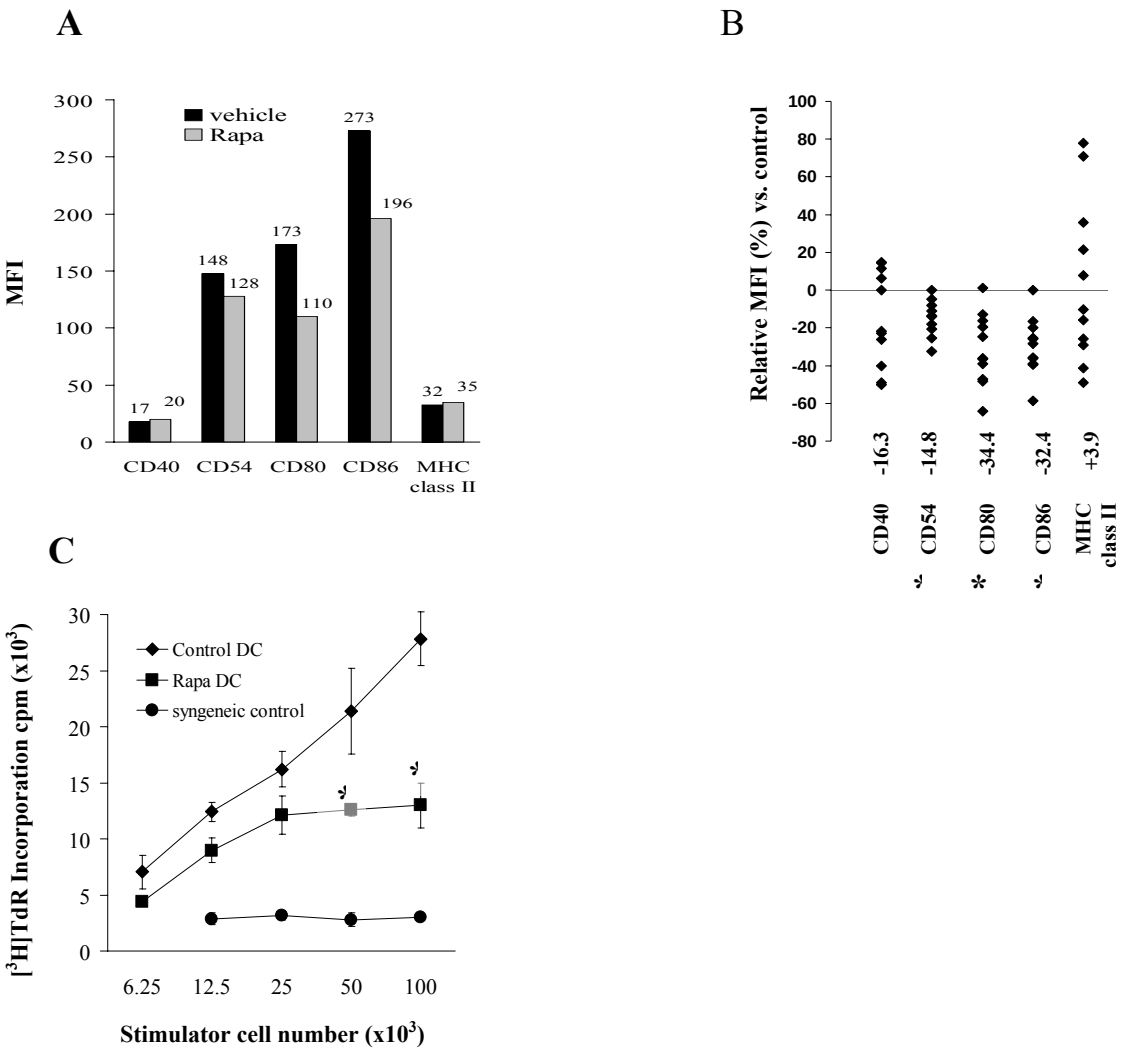


Figure 12. Effect of *in vivo* rapamycin administration on the surface phenotype and allostimulatory capacity of DC.

Mice were injected with either rapamycin (Rapa) or the drug vehicle for 10 days (with or without Flt3L). Splenic DC were purified and stimulated ex vivo with LPS. The MFI **(A)** and relative MFI **(B)** of $CD11c^+$ cells expressing the antigen of interest in comparison with cells from drug vehicle-treated control animals is indicated. (A) Typical data from one representative experiment on day 10 of treatment. (B) Each point represents a single experiment with 3-6 animals (with or without Flt3L per treatment group after *in vivo* administration of rapamycin. **P*<0.01 vs. vehicle (Wilcoxon test). **(C)** Allostimulatory activity of freshly-isolated, immunogenic bead-purified B10 DC ($H2K^b$, IA^b) from rapamycin- or drug vehicle-treated (10 days) animals. Mean proliferative activity of fully allogeneic C3H responder T cells ($H2K^k$, IA^k) in 72h MLR (± SD) is shown. **P*<0.05 vs. control DC.

3.4.3. *In vivo* administration of rapamycin inhibits DC endocytosis

To investigate the *in vivo* relevance of DC endocytosis inhibition observed during our studies presented in Chapter Two, we analyzed endocytotic activity in splenic DC of animals that were injected with rapamycin (0.5 mg/kg/day, 10 days i.p.) or vehicle, and in which DC were expanded with Flt3 ligand as described earlier. After 10 days, the animals were killed and FITC-Albumin and FITC-Dextran uptake by freshly-isolated splenic $CD11c^+$ DC was analyzed. The phenotype of these DC was immature in both treatment groups. As shown in Fig. 13, $CD11c^+$ DC of rapamycin-treated animals displayed a significantly reduced macropinocytotic activity, both with respect to the number of positive cells and the relative MFI (*P*=0.001 and *P*=0.002, respectively). Similar findings were obtained with respect to FITC-Dextran uptake (41.5% positive cells vs. 24.8% in rapamycin injected animals, *P*<0.05; relative MFI 74.7%, *P*<0.05).

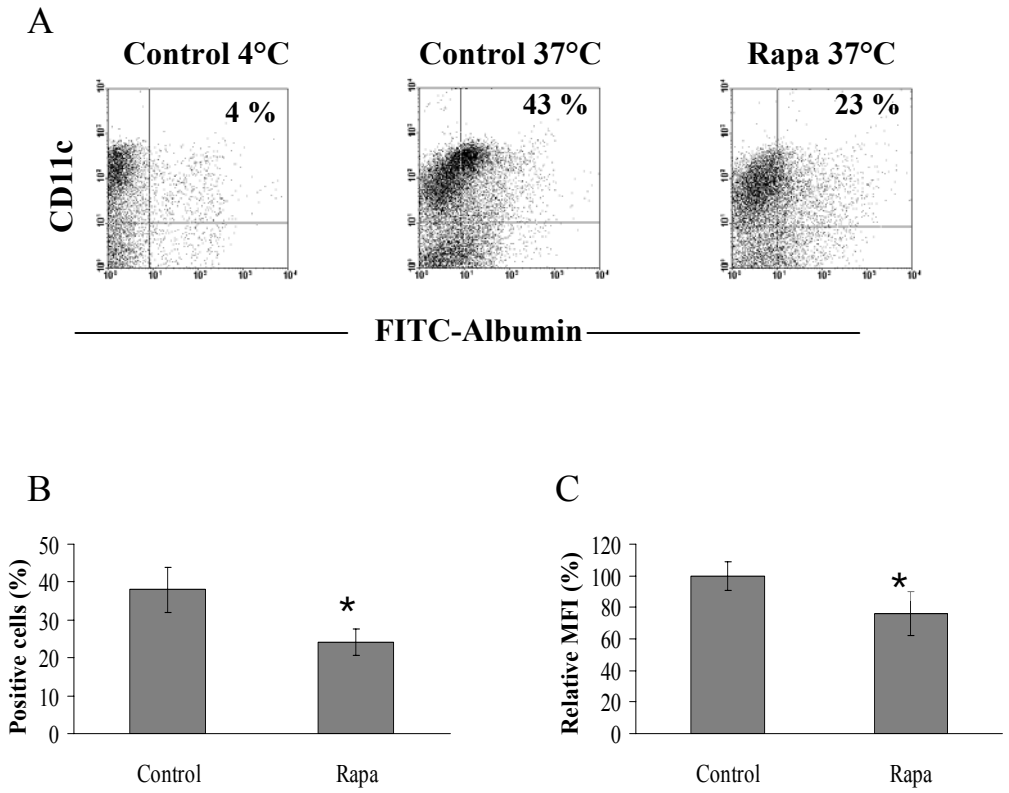


Figure 13. *In vivo* administration of rapamycin inhibits endocytosis by Flt3L-expanded splenic DC.

(A-C) DC were expanded *in vivo* with Flt3L and animals were injected with either rapamycin (Rapa) or the drug vehicle. Endocytotic activity was measured by analyzing FITC-Albumin uptake at 37ºC and 4ºC (negative control) and relative MFI in comparison with animals injected with vehicle. Results are representative of 8 animals/treatment group, and were obtained in five independent experiments. (B) **P*=0.001 and (C) **P*=0.002, Student's '*t*' test. Similar findings were obtained with respect to FITC-Dextran uptake.

3.4.4. DC exposed to rapamycin *in vivo* induces allogeneic T cell hyporesponsiveness after adoptive transfer

Next, we investigated the *in vivo* priming ability of DC isolated from rapamycin-injected animals by performing adoptive i.v. transfer of $5x10^5$ purified DC into naïve, allogeneic C3H recipients (Fig. 14A). Control recipients were injected with DC purified from age- and sex-matched control mice that had been injected with drug vehicle. Two weeks later, the animals were killed, and recipient T cells were restimulated with donor splenocytes. T cells of mice that had been injected with DC purified from rapamycin-treated donors showed markedly reduced T cell proliferative responses compared to controls indicating that rapamycin impaired the *in vivo* priming efficacy of the DC (Fig. 14B, C), regardless to whether they are from normal or Flt3L-treated animals. We also examined the cytokine profile of challenged T cells primed by rapamycin-exposed DC. These T cells displayed significantly reduced IL-2 and IFNγ production (Fig. 15A, B), whereas IL-4 and IL-10 (Fig. 15C, D) secretion was not significantly affected in response to restimulation with donor alloAgs.

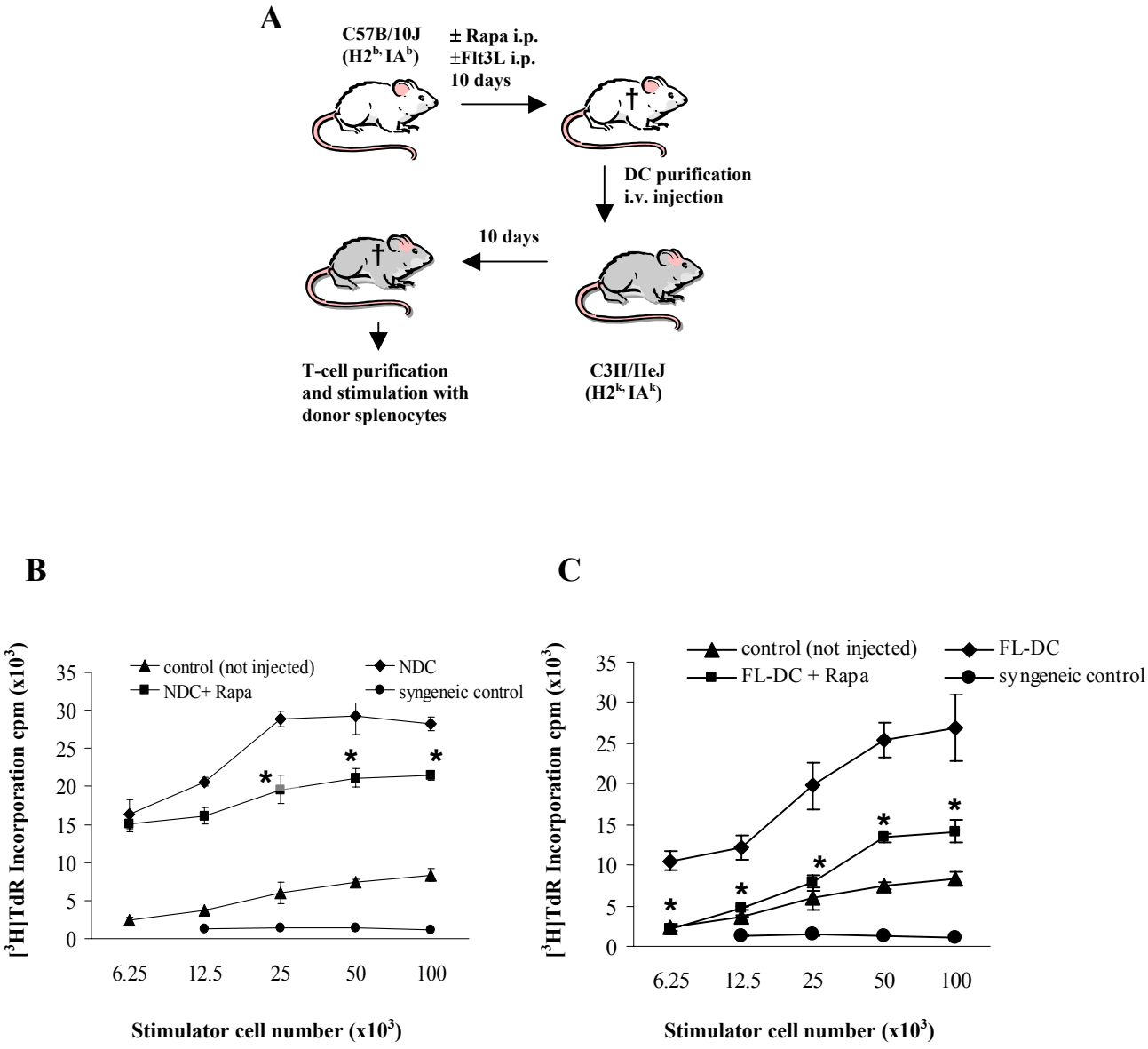


Figure 14. DC exposed to rapamycin *in vivo* induce allogeneic T cell hyporesponsiveness.

(A) Adoptive transfer of freshly-isolated splenic B10 DC from animals (**B**, normal; **C**, Flt3L (FL)-treated) that were injected with rapamycin (Rapa; 7 days) or the drug vehicle into fully allogeneic C3H recipients. C3H mice received $5x10^5$ immunomagnetic bead-purified B10 DC (i.v.). Ten days later, the mice were killed and splenic T cells restimulated with graded numbers of γ-irradiated donor splenocytes. (B, C) Mean proliferation of C3H responder T cells (3 animals/group) in 72h MLR is shown (± SD). T cells from non-immunized animals ("naive"), from animals given DC from drug vehicle-injected controls, as well as syngeneic splenocytes ("syngeneic control") were used as controls. **P*<0.05 vs. vehicle, two-tailed Student's '*t*' test.

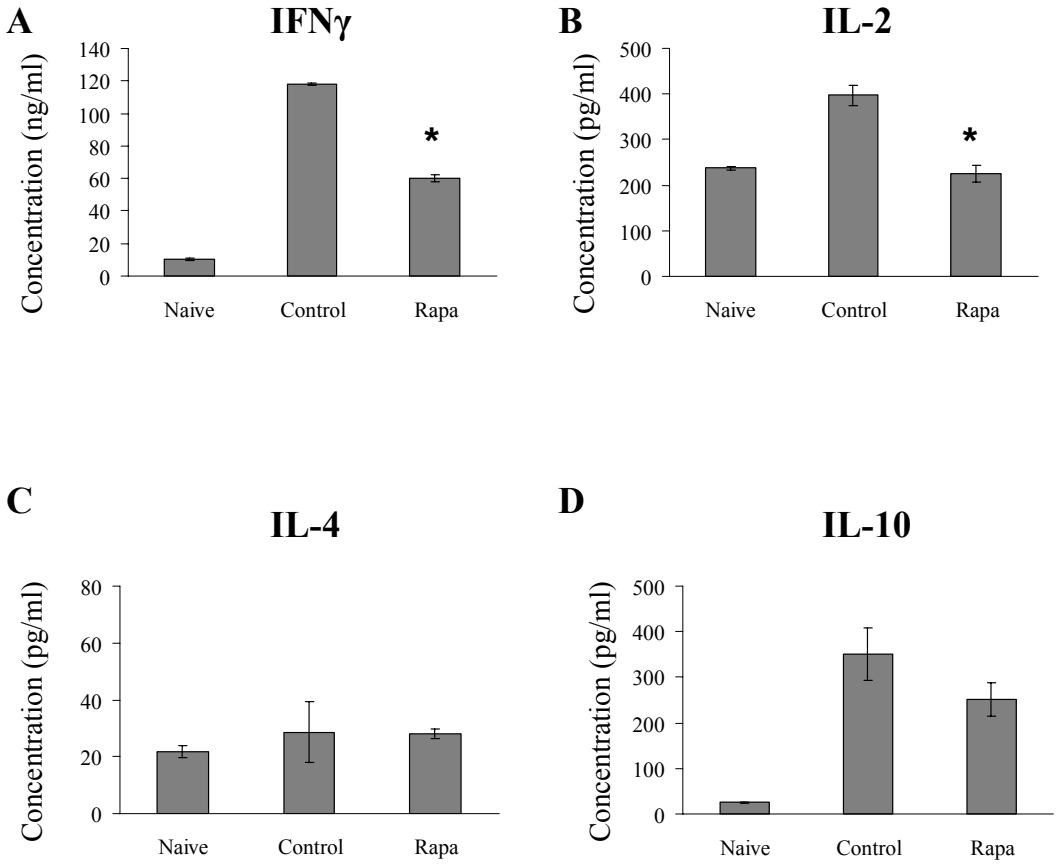


Figure 15. Effect of adoptive transfer of DC on IFNγ, IL-2, IL-4 and IL-10 production by recipient T cells after restimulation with donor alloantigen (splenocytes).

Freshly-isolated splenic B10 DC ($5x10^5$, i.v.) from animals that were injected with rapamycin (Rapa; 7 days) or the drug vehicle, and Flt3L were adoptively transferred into fully allogeneic C3H recipients. Ten days later, the mice were killed and splenic T cells restimulated with graded numbers of γ-irradiated donor splenocytes. Mean levels of cytokine production by C3H responder T cells (n=3 animals/group) in a 72h MLR are shown (± SD). **P*<0.05 vs. vehicle, two-tailed Student's '*t*' test.

3.4.5. *In vivo* administration of rapamycin promotes IL-4 hyporesponsiveness of DC and dramatically impairs TNF-α secretion

Based on our *in vitro* finding indicating that rapamycin only affected IL-4-dependent DC maturation, we hypothesized that *in vivo*-generated DC might be hyporesponsive to IL-4. To address this question, we took account of the finding that IL-4 is a major inducer of bioactive IL-12p70 production in DC. Splenic DC harvested from animals injected with drug vehicle were then stimulated with increasing IL-4 concentrations in the presence of LPS, exhibited a striking increase in IL-12p70 production, in agreement with Hochrein et al. (217). By contrast, when DC from rapamycin-injected animals were stimulated in the same manner, IL-4-induced production of IL-12p70 was abrogated, in an IL-4 dependent manner (Fig. 16A, B). We then analyzed production of TNF-α, a second major pro-inflammatory cytokine induced in DC by LPS stimulation. In contrast to the IL-12p70 data, TNF-α production by purified DC was dramatically impaired at all IL-4 concentrations. This finding (Fig. 16C) suggested IL-4-independent suppression of TNF-α production by rapamycin.

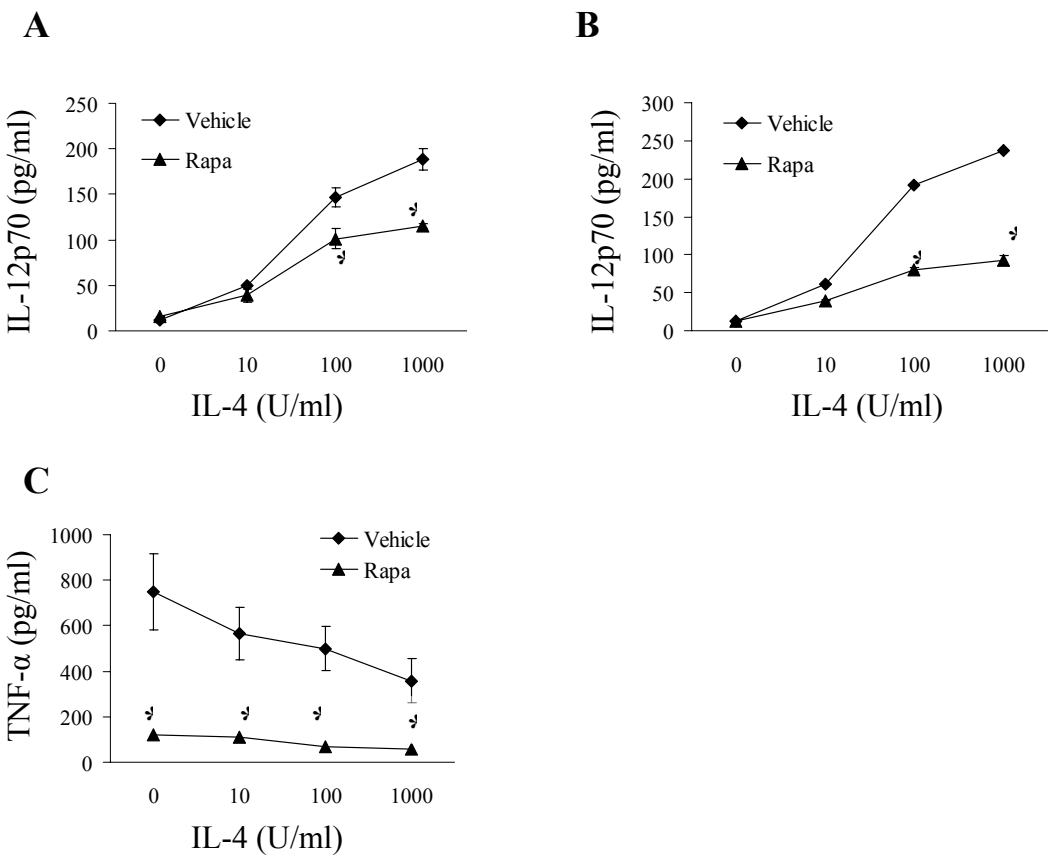


Figure 16. *In vivo* administration of rapamycin promotes IL-4 hyporesponsiveness of DC and suppresses TNF-α production.

Animals were treated with rapamycin or the drug vehicle (+Flt3L, 10 days). Splenic DC were purified by density gradient centrifugation, immunomagnetic-bead sorting and stimulated with LPS for 24h. **(A, B)** Titration of the effect of IL-4 (in the presence of LPS) on the production of bioactive IL-12p70. Mean IL-12p70 production (± 1 SD) by DC from mice given rapamycin for 7 (A) and 10 days (B) vs. drug vehicle-injected controls (3 animals/group). **(C)** Effect of rapamycin on TNF-α production. Mean TNF-α production (± SD) by DC from animals that received rapamycin for 10 days versus drug vehicle-injected controls (3 animals/group). **P*<0.05 vs. vehicle, two-tailed Student's '*t*' test.

3.5. DISCUSSION

Evidence accumulated in the past two decades has provided insight into how masterfully DC orchestrate the immune system, with their unique abilities to sense and react to various 'danger' signals. Albeit mostly beneficial -e.g. recognizing microorganisms-, this master role can, in some instances, lead also to immune dysregulation causing autoimmunity or transplant rejection. Therefore there is a clear need for identifying agents that can modulate DC functions *in vivo*. In this chapter, we have studied the impact of rapamycin on DC generation and functions *in vivo*. Our results show that *in vivo* administration of rapamycin inhibits upregulation of costimulatory molecule expression, as assessed by flow cytometry, and the *in vivo* priming capacity of DC in naïve, fully allogeneic recipients, as tested by adoptive transfer experiments. These findings were obtained in two different *in vivo* models, and suggest that rapamycin is effective at impairing DC activation. In addition, we found markedly reduced production of the pro-inflammatory cytokine IL-12p70 and TNF-α by DC isolated from animals injected with rapamycin. Our results also suggest that rapamycin can interfere with immune responses at a very early stage by inhibiting DC endocytosis, confirming the observations made *in vitro*. Thus, rapamycin targets a unique function of DC that influences the induction of immunity against microbial pathogens (e.g. Salmonella (218)) and allergens (219). This effect may also suppress indirect alloantigen processing following transplantation.

Pharmacological suppression of DC proliferation and activation by rapamycin has important implications for impairment of immune responses at the level of the APC. These cells are of pivotal importance in pathological conditions where DC perpetuate chronic inflammatory immune responses, e.g. chronic graft vasculopathy (220), atherosclerosis (221) and autoimmune diseases (222). One disease that may be especially amenable to rapamycin's inhibitory action on

DC is SLE. Intriguingly, Blanco et *al*. demonstrated that serum from SLE patients contained elevated levels of IFN-α that induced normal monocytes to differentiate into DC (223). They were able to correlate disease activity with the capacity of patients' serum to induce DC differentiation, and concluded that unabated induction of DC by IFN-α may drive the immunopathologic process in SLE (223). Farkas et al (224) reported that pDC, which represent the natural IFN-α producing cells, accumulate in cutaneous SLE lesions. In this context it is of interest that rapamycin has been reported to arrest pathophysiological changes in murine SLE (225, 226). Thus, based on the pathogenetic importance of altered DC function in SLE, results from animal models, and our present findings, we contend that rapamycin merits evaluation for the treatment of SLE.

Interestingly, rapamycin inhibits not only the *in vivo* generation of DC under steady-state conditions in normal animals, but also Flt3L-induced *in vivo* expansion of DC. Through reduction of the total DC pool, this action compounds the inhibitory effect of rapamycin independent of its effect at the single cell level. As discussed in Chapter Two, we carefully tested the hypothesis that the observed effects might be related to apoptosis. Similar to results obtained *in vitro*, the frequency of apoptotic or dead cells was consistently < 10% in *in vivo*-generated DC obtained from animals injected with rapamycin. These findings indicate unexpectedly the potential of rapamycin for the treatment of hematological malignancies where increased Flt3 signaling is involved in disease pathogenesis. Our data demonstrate that rapamycin effectively inhibits Flt3L-induced DC expansion *in vivo*, as well as accompanying splenomegaly. With respect to this finding, it is of interest that activating Flt3 mutations are present in >25% of patients with acute myelogenous leukemia and the most common form, internal tandem duplications, confers a poor prognosis (227-231). Therefore, in addition to the recently-published

therapeutic effects of rapamycin on solid tumor progression and metastasis (232), this finding provides an incentive to examine the therapeutic effects of rapamycin in models of acute myelogenous leukemia.

In summary, by using two different *in vivo* models, we have provided evidence that rapamycin potently targets functional DC activation and expansion *in vivo*, mirroring its effects *in vitro*. These findings provide new insight into the immunopharmacology of rapamycin, and also have significant implications for the development of new therapeutic strategies in disease processes in which DC may play a crucial role.

4. CHAPTER FOUR[5]

RAPAMYCIN-TREATED DC AS THERAPEUTIC VECTORS TO INDUCE TRANSPLANT TOLERANCE

4.1. ABSTRACT

Tolerogenic properties of DC, particularly those in the immature state, and their therapeutic potential are increasingly being recognized. Among several distinct approaches to generate stably immature DC, pharmacologic manipulation stands out as a promising and clinically applicable option. In the previous chapters we have demonstrated that rapamycin can inhibit DC maturation and their effector functions. Here, we examined the impact of rapamycin exposure on subsequent alloAg presentation by MDC via the indirect pathway. Rapamycin-treated, allogeneic donor cell lysate-pulsed host DC (Rapa-DC) were inferior stimulators of syngeneic T cells, compared to donor cell lysate-pulsed, otherwise untreated DC. Rapamycin exposure did not block alloAg uptake by DC in *extended cultures* nor impair their *in vivo* homing to splenic T cell areas after adoptive transfer. T cells primed by rapamycin-treated, alloAg-pulsed DC showed decreased capacity to produce IL-2 and IFNγ, and were hyporesponsive to subsequent challenge via both the direct and indirect pathways, in an Ag-specific manner. When infused one week before transplantation, Rapa-DC significantly prolonged alloAg-specific heart graft survival. This effect was reversed by systemic IL-2 administration in the early post-operative period, but enhanced by either repeated infusion of the cells, or a short post-transplant course of FK506. These therapeutic effects, achieved by targeting both major pathways of allorecognition, provide the basis for a clinically-applicable strategy to suppress graft rejection.

[5] Parts of the data presented in this chapter is excerpted from (233).

4.2. INTRODUCTION

As discussed elsewhere in this thesis, DC are BM-derived professional APC with the unique ability to both initiate and regulate immune responses (16, 80). The nature of the immune response elicited by DC depends on their state of maturation and functional differentiation, that is influenced by microenvironmental factors (microbial products, cytokines and cyclooxygenase metabolites) (181). In the immature state, DC are inherently tolerogenic (66) and can suppress T cell responses to self or foreign Ag (16). By contrast, their maturation in response to inflammatory stimuli is associated with the acquisition of potent immunostimulatory function, linked to upregulated expression of cell surface MHC and costimulatory molecules. This dichotomous function of DC, combined with their remarkable plasticity, provides a basis for the design of DC-based therapeutic applications to either boost or regulate the immune reaction (23, 137, 234). Owing mainly to the initial immunostimulatory role assigned DC, there has been considerable advance in the use of DC-based vaccines for cancer immunotherapy. Thus far, nearly 100 clinical trials using this approach proved the potential of DC-based, tailored cancer therapy, although optimization of this approach still remains to be achieved (234). In these studies, typically tumor Ag-pulsed, mature DC are used to induce Ag-specific antitumor T cell responses.

By contrast, there has been little clinical testing of DC-based tolerogenic strategies. Immature DC can mature once administered *in vivo*, limiting their tolerogenic potential. It is conceivable that if immature, tolerogenic DC can be stabilized at that stage, then by mimicking cancer studies, Ags to which tolerance is desired can be derived through DC-based 'negative vaccination' strategies. We have shown in previous chapters that *in vitro* or *in vivo* exposure of DC to rapamycin inhibits their maturation, inflammatory cytokine (bioactive IL-12p70 and TNF-α) secretion and T cell allostimulatory capacities. Thus, rapamycin appears to be a good

candidate for pharmacological suppression of DC functions and the generation of DC with tolerogenic/immunoregulatory activity. Indeed, as demonstrated in Chapter Three, rapamycin-treated DC induce T cell hyporesponsiveness to donor Ag following their injection into allogeneic recipients.

In this chapter, we have further explored the immunoregulatory capacity of rapamycin-treated DC in the context of alloimmune reactivity. More specifically, we have examined the function of rapamycin-treated DC in indirect alloAg presentation and their role in modulation of organ transplant rejection. Our results show that these cells can be loaded effectively with Ag derived from donor cell lysates to induce alloAg-specific T cell hyporesponsiveness *in vivo*. Furthermore, infusion of these rapamycin-treated, alloAg-pulsed DC (Rapa-DC) prior to transplantation prolongs fully MHC-mismatched heart allograft survival, in some cases indefinitely, in otherwise untreated mice. The regulatory effect of these DC is more marked in animals given a short, postoperative course of subtherapeutic FK506. These novel findings provide insight into clinically applicable strategies for immunomodulation using pharmacologically-modified DC of host origin for therapy of graft rejection, with implications for use of regulatory DC in other immune-mediated disorders.

4.3. MATERIALS and METHODS

4.3.1. Animals

Eight- to 12-week-old C57BL/10 (B10; $H2K^b$), C3H/HeJ (C3H; $H2K^k$) and BALB/c ($H2K^d$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the specific pathogen-free Central Animal Facility of the University of Pittsburgh Medical Center. Experiments were conducted under an institutional animal care and use committee-approved protocol and in accordance with National Institutes of Health-approved guidelines.

4.3.2. Generation of BM-derived DC

BMDC were propagated as described in Chapter Two. Briefly, BM cells were removed from femurs and tibias of C3H mice and depleted of erythrocytes by hypotonic lysis. Erythroid precursors, B lymphocytes and granulocytes were removed by complement depletion using a cocktail of mAbs (anti-TER-119 [TER-119], anti-B220 [RA3-6B2] and anti-Gr1 [RB6-8C5]; BD PharMingen, San Diego, CA) followed by incubation (45 min; 37°C) with low-toxicity rabbit complement (Cedarlane, Hornby, ON, Canada). The cells were cultured for 7 days in RPMI-1640 with 10% v/v heat-inactivated FCS, L-glutamine, non-essential amino acids, sodium pyruvate, penicillin-streptomycin, HEPES, 2-mercaptoethanol (all from Life Technologies, Gaithersburg, MD), 1000 U/ml r murine GM-CSF (Schering-Plough, Kenilworth, NJ) and 1000 U/ml r murine IL-4 (R&D Systems, Minneapolis, MN). On day 2, 10 ng/ml rapamycin (Sigma, St Louis, MO) was added. Every 2 days, 75% of the culture supernatant was replaced with fresh cytokine-containing medium (with or without rapamycin). On day 4, non-adherent cells were removed; on day 7, 50% or more of the non-adherent cells expressed CD11c.

4.3.3. Phenotypic analysis of DC

DC surface Ag expression was analyzed by flow cytometry on day 8 of BM culture, 24h after alloAg pulsing. Stimulation was performed with LPS (0.1 to 1 μg/ml, *Escherichia coli* serotype 026:B6; Sigma) in the presence of low-dose GM-CSF (50 U/ml) in RPMI-1640 for 16h at 37°C. FITC-, PE- or CyChrome-conjugated, or biotinylated mAbs used to detect expression of CD11c (HL3), CD80 (16-10A1), CD86 (GL1), IA^k α chain (11-5.2), $H2K^b$ (AF6-88.5) as well as isotype-matched control Igs and streptavidin-CyChrome, were purchased from BD PharMingen, unless otherwise noted. Cells ($5x10^5$) were blocked with 10% v/v normal goat serum (Vector, Burlingame, CA) (10 min; 4°C) then stained with mAb (30 min; 4°C). Appropriate isotype-matched IgGs were used as negative controls. The cells were analyzed using an EPICS Elite flow cytometer (Beckman Coulter, Hialeah, FL).

4.3.4. Pulsing of DC and autologous MLR

CD11c immunomagnetic bead (Miltenyi Biotec, Auburn, CA)—purified DC were incubated with allogeneic splenocyte lysates at a DC: splenocyte equivalent ratio of 1:10 for 24 h at 37°C. Normal B10 splenocyte lysates were obtained by three cycles of rapid freeze/thaw exposure in PBS. To disrupt DC:lysate clumps, the cells were washed extensively (3x, 700x g, 5 min) with PBS containing 5 mM ethylenediamine tetra-acetic acid (EDTA) following pulsing. Graded numbers of γ-irradiated (20 Gy) DC were then used as stimulators in 72 h MLRs with nylon-wool column-enriched syngeneic (C3H) splenic T cells as responders ($2x10^5$/ml) in 96-well, round-bottom plates. For the final 18 h, individual wells were pulse-labeled with 1 μCi [^{3}H] thymidine. The amount of radioisotope incorporated was determined using a β scintillation counter. Recombinant murine IL-2 (R&D Systems; 100 U/ml) was added at the beginning of cocultures, where indicated. Results are expressed as mean c.p.m. ± 1 SD of triplicates.

4.3.5. Analysis of Ag uptake

Uptake of cell lysates by C3H DC was analyzed by flow cytometry. Thus, B10 splenocytes ($H2K^{b+}$) were labeled with carboxyfluoroscein succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) according to the manufacturer's protocol, prior to lysis. CFSE enters the cells freely and is cleaved by non specific esterases once inside. Cleaved CFSE binds to cellular proteins irreversibly for days to months. Following pulsing, the percentage of $CD11c^{+}CFSE^{+}H2K^{b-}$ cells was quantified as Ag-loaded DC.

4.3.6. Analysis of T cell apoptosis and intracellular cytokine expression

T cells cocultured with DC at a 10:1 ratio were harvested on day 3 of MLR. Apoptosis was analyzed over time by staining externalized phosphatidylserine with FITC-annexin-V, in combination with the vital dye 7-amino-actinomycin D (7-AAD; BD PharMingen) according to the manufacturer's instructions. Cells were costained for CD3 (anti-CD3, 17A2; BD PharMingen) to allow specific analysis of DC by flow cytometry. For intracellular cytokine analysis, harvested T cells were restimulated with plate-bound anti-CD3 and soluble anti-CD28 (37.51; BD PharMingen) in the presence of Brefeldin A (Sigma), -the latter to block cytokine secretion, for 5 h. After extracellular staining with fluorochrome-conjugated anti-CD3, CD4 or CD8 mAbs, cells were permeabilized with 1% saponin and stained for IL-2, IL-4, IL-5, IL-10 or IFNγ (BD PharMingen). Appropriate isotype-matched IgGs were used as negative controls.

4.3.7. *In vivo* imaging of labeled DC and immunohistochemical staining of tissue sections

DC were labeled green with PKH-67 (Sigma), according to the manufacturer's protocol and infused i.v. ($1.5x10^6$ in 0.1 ml PBS) via the lateral tail vein. Spleen blocks were embedded in Tissue-Tek OCT (Miles Laboratories, Elkhart, IN), snap frozen in isopentane/liquid nitrogen, and stored at -80°C. Cryostat sections (8 μm) were fixed in 96% ethanol (10 min), blocked with 10%

v/v normal goat serum, and incubated overnight (4°C) with biotinylated-anti-CD3 mAb. As a second step, slides were incubated with 1:3000 Cy3-streptavidin (Jackson Immunoresearch Lab, West Grove, PA), for 30 min at room temperature. Cell nuclei were stained with DAPI (4,6 diamidino-2-phenylindole; Molecular Probes, Eugene, OR). Slides were fixed in 2% paraformaldehyde, mounted in glycerol/PBS, and examined with a Zeiss Axiovert 135 microscope equipped with appropriate filters and a cooled CCD camera (Photometrics CH250, Tucson, AZ). Signals from different fluorochromes were acquired independently, and montages edited using the Adobe Photoshop software program (Adobe Systems, Mountain View, CA).

4.3.8. Vascularized heart transplantation

Heterotopic (intra-abdominal) heart transplantation was performed from B10 to C3H mice, as described (46). Briefly, the heart was transplanted into the abdomen with end-to-side anastomosis of aorta to aorta, and pulmonary artery to vena cava, under methoxyflurane inhalation anesthesia (Medical Development, Springvale, Australia). Graft survival was assessed by daily transabdominal palpation. Rejection was defined as total cessation of cardiac contraction and was confirmed by histological examination. Animals received either no treatment or were injected i.v. with $1.5x10^6$ immunobead-sorted alloAg-pulsed DC (day –7, or days –10, -3 and 0). A subtherapeutic dose of 1 mg/kg/day FK506 (Prograf® for i.v. use; Fujisawa Healthcare, Deerfield, IL) was administered i.m. for 10 consecutive days (days 0 to 9) in two groups. For the IL-2 treatment protocol, recipient mice were treated with 60,000 U rhIL-2 (Proleukin®, Chiron Therapeutics, Emeryville, CA) i.p every 8 h for 3 days, starting 8 h posttransplant (235). Graft survival was assessed by daily transabdominal palpation. Rejection was defined by the complete cessation of cardiac contraction, and was confirmed histologically.

4.3.9. Immunofluorescence staining of tissue sections

Blocks from heart grafts were embedded in Tissue-Tek OCT (Miles Laboratories, Elkhart, IN), snap frozen in isopentane/liquid nitrogen, and stored at -80°C. Cryostat sections (8 μm) were fixed in 96% ethanol (10 min), blocked with 10% normal goat serum, and incubated overnight (4°C) with each of the following biotin-mAbs: anti-CD4, anti-CD8α, anti-Gr1 (all from BD PharMingen) or anti-F4/80 (Bachem). As a second step, slides were incubated with 1:3000 Cy3-streptavidin (Jackson Immunoresearch Lab, West Grove, PA), for 30 min at room temperature. Cell nuclei were stained with DAPI (4,6 diamidino-2-phenylindole; Molecular Probes, Eugene, OR). Slides were fixed in 2% paraformaldehyde, mounted in glycerol/PBS, and examined with a Zeiss Axiovert 135 microscope equipped with appropriate filters and a cooled CCD camera (Photometrics CH250, Tucson, AZ). Signals from different fluorochromes were acquired independently, and montages edited using the Adobe Photoshop software program (Adobe Systems, Mountain View, CA).

4.3.10. RNase protection assay

The procedure was performed as described previously. Briefly, RNA was isolated from $5x10^6$ snap-frozen, magnetic-bead sorted DC using a total RNA Isolation Kit (BD PharMingen). RNase protection assay was performed using the RiboQuant Multi-Probe RPA System (BD PharMingen) with ^{32}P-UTP-labeled antisense RNA probes specific for IL-2, IL-4, IL-10, IL-15, IFNγ and the housekeeping genes L32 and GAPDH according to the manufacturer's instructions. Mouse RNA and RNA degradation controls were included. Yeast tRNA served as negative control. Quantification of bands was performed by densitometry (Personal densitometers 1; Molecular Dynamics, Sunnyvale, CA). The signals from specific mRNAs were normalized to

signals from housekeeping genes (L32 and GADPH) run on each lane to adjust for loading differences.

4.3.11. Statistical analyses

Statistical analysis was performed using the two-tailed Student's '*t*' and Mann-Whitney tests, and a *P* value of <0.05 was considered significant. Graft survival data were compared by Kaplan-Meier analysis and the log-rank test. Results are expressed as means ± 1SD.

4.4. RESULTS

4.4.1. Rapamycin inhibits DC maturation and their subsequent capacity to stimulate T cells through the indirect pathway

DC generated from C3H BM in the presence or absence of a clinically relevant concentration of rapamycin (10 ng/ml) were incubated overnight on day 7 with freeze-thaw lysates of B10 splenocytes. Preliminary experiments revealed that a DC:splenocyte equivalent ratio of 1:10 was optimal for pulsing DC, and gave the most consistent results (data not shown). After pulsing, DC were analyzed for both spontaneous and LPS-induced maturation by flow cytometry. AlloAg-pulsed, gated $CD11c^+$ Rapa-DC showed decreased surface expression of CD80, CD86 and MHC class II (IA^k) molecules compared with alloAg-pulsed untreated control DC (Fig. 1A). These DC were then washed extensively, purified by CD11c magnetic beading, and then used as stimulators of naïve C3H splenic T cells. Rapamycin-treated, alloAg-pulsed DC were poorer stimulators of T cells compared to alloAg-pulsed control DC (Fig.1B). Lysates alone did not exhibit a significant stimulatory capacity for allogeneic naïve T cells, demonstrating that the direct pathway, a possible contributing factor that may arise from contamination of lysates with intact splenocytes or membrane fragments, was not a significant contributor to the induction of T cell proliferative responses.

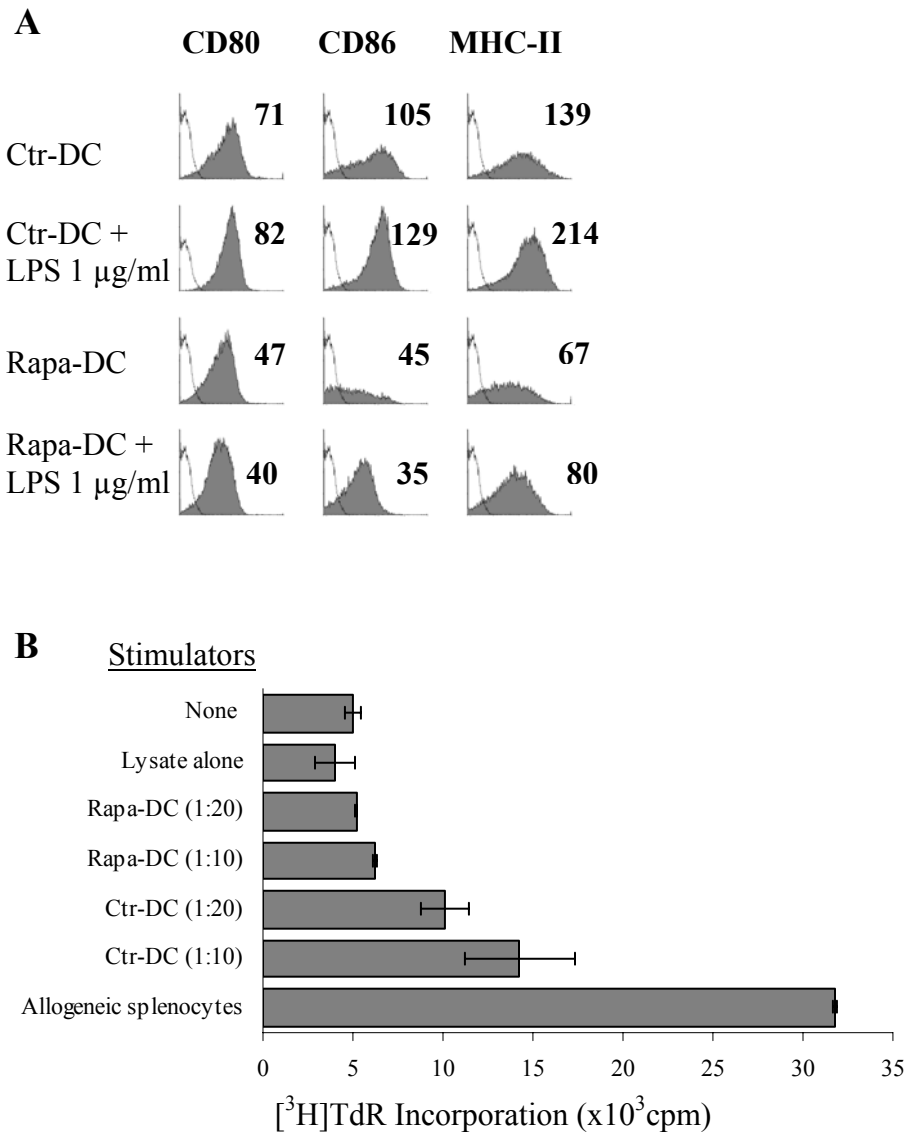


Figure 17. Inhibition of DC maturation and T cell allostimulatory function via the indirect pathway.

(A) C3H BM-derived DC were generated as described in the 'Materials and Methods', in the presence or absence of 10 ng/ml rapamycin. Rapamycin inhibited cell surface expression of CD80, CD86 and MHC-II on gated $CD11c^+$ cells on day 8, following alloAg pulsing. Upregulation of these molecules in response to LPS (1µg/ml) was also suppressed by exposure of DC to rapamycin. The mean fluorescence intensity of $CD11c^+$ cells expressing the marker of interest is indicated. **(B)** B10 alloAg-pulsed, Rapa-DC were inferior stimulators of naïve syngeneic C3H T cells, compared to alloAg-pulsed control (Ctr) DC. The ratio of DC:T cells is indicated in parentheses. Data are representative of results obtained from three (A) and four (B) similar experiments.

4.4.2. Rapamycin treatment does not interfere with lysate uptake by DC

To ensure that the suppressed T cell stimulatory activity observed was due to active regulation and not simply to decreased uptake of lysates by Rapa-DC, we pre-labeled B10 splenocytes with CFSE, a fluorescent reagent that binds stably to intracellular proteins, prior to cell lysis. Using the standard overnight pulsing protocol, we examined the uptake of lysates by $CD11c^+$ cells using flow cytometry. To eliminate false-positive co-staining due to DC-lysate cell surface adherence, we used a fluorochrome-conjugated mAb against MHC class I expressed by the allogeneic lysates ($H2K^b$) and compared $CD11c^+CFSE^+H2K^{b-}$ populations of control and Rapa-DC ($H2K^b$ is expressed by the B10-derived lysates, but is absent on the surface of C3H DC). As seen in Fig. 18, Rapamycin treatment did not significantly block lysate uptake by DC when the DC were incubated with allogeneic cell lysates for an extended time period.

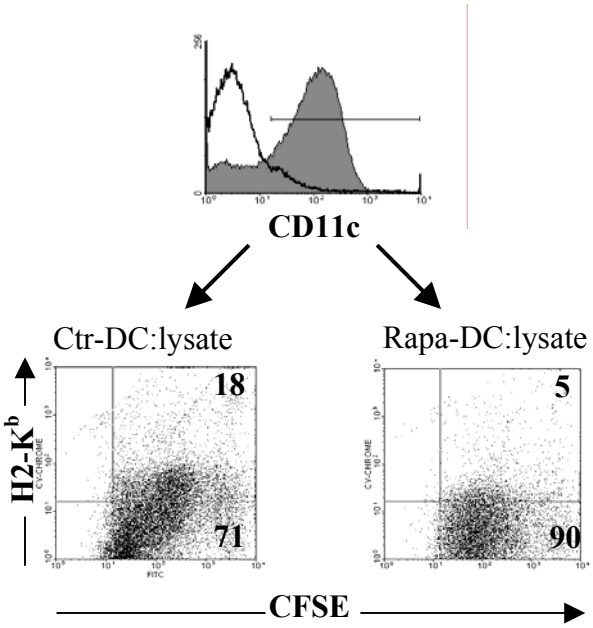


Figure 18. Exposure to rapamycin does not block alloAg uptake by DC.

C3H BM-derived DC were cultured with or without rapamycin. On day 7, they were harvested and incubated overnight with CFSE pre-labeled B10 splenocyte lysates. At the end of pulsing period, the DC were stained for CD11c and colabeling with CFSE was analyzed to determine alloAg uptake. Additional $H2K^b$ staining was performed to quantify DC-lysate clusters. The incidence of positive cells is indicated in each quadrant. Results are representative of three similar experiments.

4.4.3. Rapamycin treatment does not affect homing of DC to the spleen after their adoptive transfer, but confers capacity to suppress alloAg-specific responses

Next, to explore their potential for delivery of tolerogenic signals *in vivo,* we examined the *in vivo* migratory capacity of Rapa-DC. Following i.v. infusion ($1.5\text{-}2x10^6$) into naïve C3H recipients, C3H Rapa-DC labeled (green) with the lipophilic marker PKH-67 localized to T cell areas in spleen (Fig. 19B) as efficiently as control DC (Fig. 19A), as our laboratory has shown previously for immature DC (236). To investigate the *in vivo* T cell priming ability of Rapa-DC, we isolated T cells from the spleens of recipient mice, 7 days after the adoptive transfer of these cells. The T cells were challenged *ex vivo* by either DC of B10 origin (Fig. 20A) or B10 lysate-pulsed, C3H-derived DC (Fig. 20B). T cells that had been primed *in vivo* by alloAg-pulsed Rapa-DC showed markedly decreased proliferative responses to secondary stimulation, via either the direct- or indirect pathways of allorecognition. This hyporesponsiveness was alloAg-specific, as T cells responded strongly to third party (BALB/c; $H2K^d$) DC (Fig. 20A). Addition of 100 U/ml of exogenous rIL-2 at the start of cultures abrogated this hyporesponsiveness and restored the T cell proliferative responses to levels similar to those of T cells primed by alloAg-pulsed, control DC.

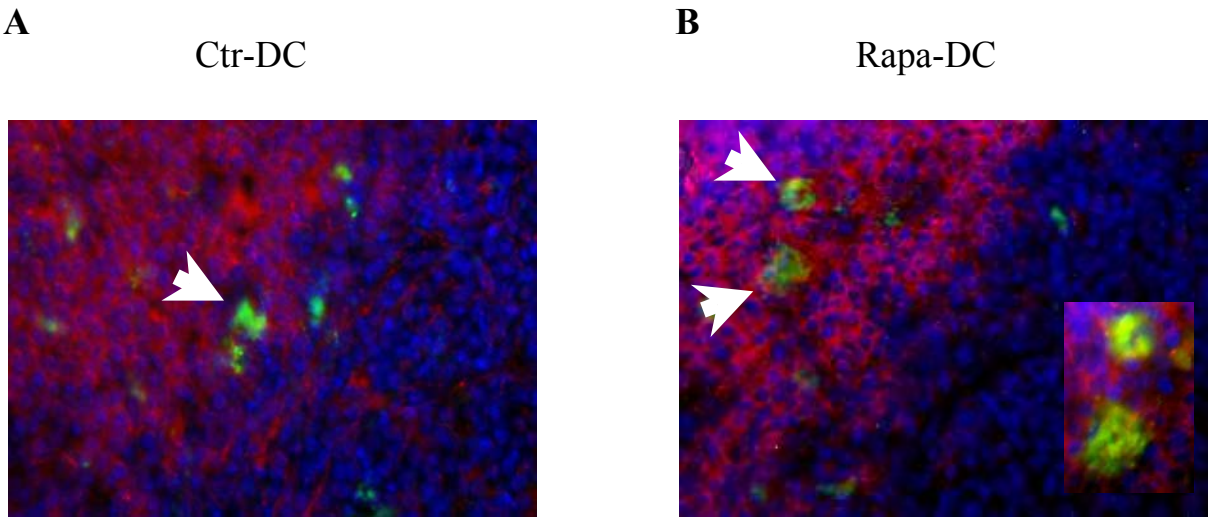


Figure 19. Rapamycin treatment does not affect the homing of DC to splenic T cell areas after adoptive transfer.

Immunobead-purified, C3H BM-derived DC were labeled with PKH-67 (green) and injected i.v. into naïve syngeneic animals (1.5 x 10^6/animal). Twenty four hours later, recipient animals were killed, and the spleens harvested. Frozen sections were stained with anti-CD3 mAb (red). Control DC (**A**; arrowhead) and Rapa-DC (**B**; arrowheads and inset) both localized to $CD3^+$ T cell areas of the spleen. Nuclei were counterstained with DAPI (blue). Original magnification for both panels, 400x; inset B, 1000x. Images are representative of multiple sections at different levels for two animals/group analyzed.

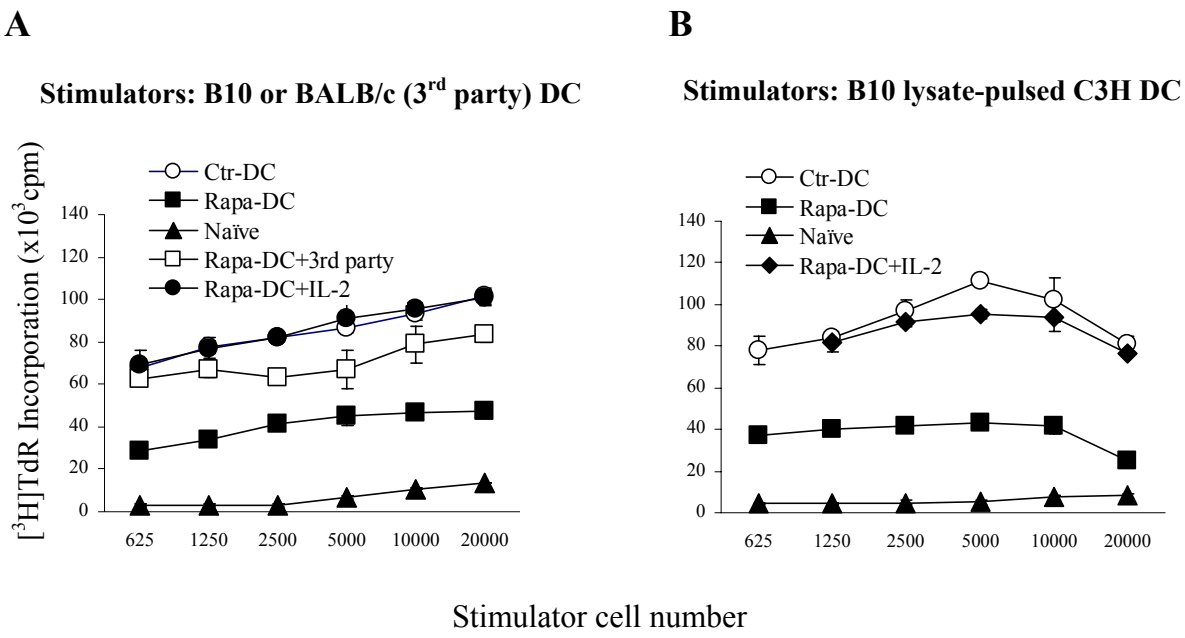


Figure 20. AlloAg-pulsed, rapamycin-treated DC induce Ag-specific T cell hyporesponsiveness *in vivo*.

AlloAg-pulsed (B10), immunobead-purified control (Ctr)- or Rapa-DC were adoptively transferred (i.v) to syngeneic C3H mice (1.5 x 10^6/animal). Seven days later, recipient animals were killed and splenic T cells challenged ex vivo with graded numbers of γ-irradiated allogeneic (B10) DC or third party (BALB/c) DC **(A)**, or alloAg-pulsed syngeneic (C3H) DC **(B)**. Mean proliferation of responder T cells in 72 hour MLRs is shown (± 1 SD). At the beginning of cocultures, 100 U/ml rmIL-2 was added to the groups indicated. T cells from non-immunized animals (naïve) were used as controls. Results are from a single experiment representative of four (each) experiments performed.

4.4.4. Rapamycin-treated, alloAgs-pulsed DC do not increase the incidence of T cell death but markedly inhibit T cell IL-2 and IFNγ production

To investigate other possible mechanisms underlying the regulatory influence of Rapa-DC on alloreactive T cell responses, we tested their potential to promote T cell apoptosis *in vitro*. We harvested T cells from DC:T cell cultures on day 3 and assessed the percentage of early apoptotic (Annexin-V^+/7-AAD^-) and late apoptotic/necrotic (Annexin-V^+/7-AAD^+) T cells. As seen in Fig. 21A, there was no difference in the incidence of T cell death between cultures in which control or rapamycin-treated, alloAg-pulsed DC were used as stimulators. We also performed intracellular cytokine staining of T cells from these MLR cultures. Whereas a significant proportion of T cells primed indirectly by alloAg-pulsed control DC expressed IL-2 and IFNγ, production of these cytokines was markedly reduced in T cells stimulated by alloAg-pulsed Rapa-DC (Fig. 21B, C). Expression of the Th2 signature cytokines IL-4, IL-5 and IL-10 was also decreased, but to a lesser extent, in these allostimulated T cells (Fig. 21D).

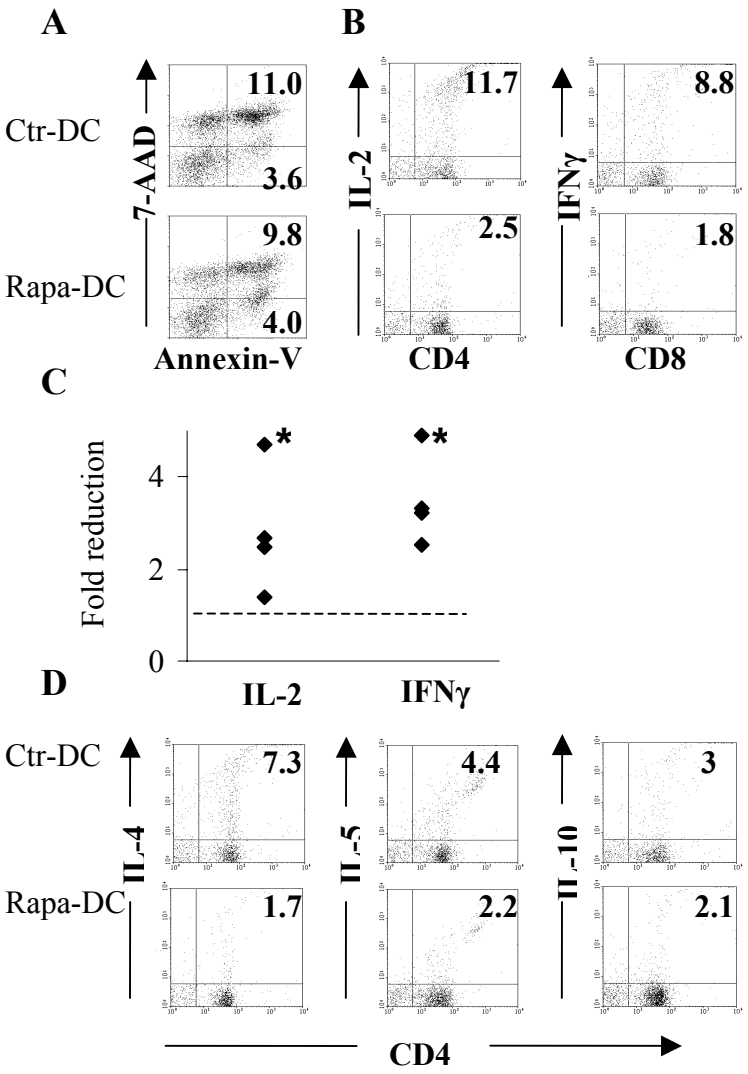


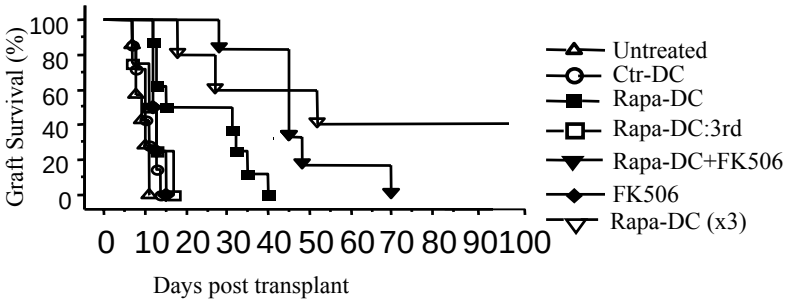
Figure 21. Rapa-DC suppress IL-2 and IFNγ production by T cells, without affecting the incidence of T cell death.

Naïve T cells were cultured with alloAg-pulsed control (Ctr)- or Rapa-DC at 10:1 ratio for 3 days. **(A)** Cells were then analyzed after staining of phosphatidylyserine externalization with FITC-annexin-V in combination with the vital dye 7-AAD. The incidence of $CD3^+$ cells expressing the marker of interest is indicated. **(B-D)** T cells were also permeabilized and analyzed for intracellular cytokine production. T cells primed *in vitro* by alloAg-pulsed Rapa-DC produced lower amounts of IL-2 and IFNγ (B, C); nevertheless this was not selective for these Th1 cytokines as IL-4, IL-5 and IL-10 production (D) were also decreased compared to T cells primed with Ctr-DC. Numbers in upper right quadrants indicate percent positive $CD3^+$ cells for the cytokines indicated. (C) Results of four different experiments are shown. Results are representative of three (A), four (B, C) and three (D) experiments. **P*<0.05 vs. Ctr-DC (Mann-Whitney test).

4.4.5. A single infusion of alloAg-pulsed, rapamycin-treated DC prolongs alloAg-specific heart graft survival; multiple infusion leads to long-term survival

Next, to explore the therapeutic potential of alloAg-pulsed, Rapa-DC *in vivo*, we employed a fully MHC-mismatched (B10 to C3H) heterotopic vascularized heart transplant model. In this model, B10 heart grafts are rejected by C3H recipients within 7-11 days (MST=9.1 days) without immunosuppressive treatment. Graft survival was improved minimally, although not significantly, when alloAg-pulsed, control syngeneic DC were infused i.v. on day –7 (Fig. 22). By contrast, a single infusion of donor alloAg-pulsed, rapamycin-treated DC prolonged graft survival significantly (MST=23.8 days; $P<0.005$). This effect was Ag-specific, as no graft prolongation was observed in the group that received Rapa-DC pulsed with third party (BALB/c) alloAg, and was reversed by systemic administration of 8-hourly rIL-2 for 3 days, commencing 8h post transplant. In addition, the beneficial effect of donor alloAg-pulsed Rapa-DC was improved to a MST of 46.8 days ($P=0.0005$ vs. untreated; and $P=0.0029$ vs. Rapa-DC alone) by short-term administration of a subtherapeutic dose of FK506 (1 mg/kg/day, i.m., days 0-9), which alone did not prolong graft survival. Strikingly, repeated infusion of donor alloAg-pulsed Rapa-DC, but not Ctr-DC (x3; days -10, -3 and 0) led to indefinite graft survival (>100 days) in 40% of otherwise unmodified graft recipients (Fig. 22).

A



B

Group	n	Graft Survival (days)	MST (days)
1 Untreated	7	7, 8(x2), 9, 10, 11(x2)	9.1
2 Ctr-DC	7	7, 8, 10(x2), 11, 13, 14	10.4
3 Rapa-DC	8	12, 13(x2), 15, 31, 32, 35, 40	23.8[a]
4 Rapa-DC+IL-2	4	6, 6, 6, 8	6.5
5 Rapa-DC:3rd party	4	7, 11, 13, 17	12
6 Rapa-DC+FK506	6	28, 45(x3), 48, 70	46.8[b,c]
7 FK506	4	12(x2), 13, 15	13
8 Ctr-DC (x3)	4	9, 9, 11, 15	11
9 Rapa-DC (x3)	5	18, 27, 52, >100 (x2)	>59[d,e]

[a]$P<0.0001$ compared with group 1; [b]$P=0.0005$ compared with group 1 and [c]$P=0.0029$ compared with group 3; [d]$P=0.0014$ compared with group 1 and [e]$P=0.0464$ compared with group 3
MST: mean survival time

Figure 22. AlloAg-pulsed Rapa-DC prolong heart graft survival.

AlloAg-pulsed, control (Ctr)- or Rapa-DC of host origin were injected i.v. into syngeneic C3H mice, 7 days before the transplantation (day 0) of B10 heat grafts. FK506 (1 mg/kg/day) was administered i.m. for 10 days (days 0-9) and IL-2 (60000 U/animal/8h) was administered i.p. for 3 days from the time of transplant, where indicated. Untreated, third party (BALB/c) lysate-pulsed Rapa-DC-treated mice were used as control recipients. Alternatively, the alloAg-pulsed Rapa-DC or alloAg-pulsed Ctr-DC were infused i.v. x3 (days -10, -3 and 0) into otherwise unmodified graft recipients. Statistical differences are analyzed by the log-rank test.

4.4.6. Analysis of mechanisms involved in prolonged graft survival in response to alloAg-pulsed Rapa-DC

In order to elucidate the mechanisms underlying prolonged graft survival, we repeated transplant experiments. Recipient mice that were pre-infused once (on day -7) with alloAg-pulsed control- or rapamycin-treated DC, or those that were otherwise untreated, were killed on day 7, and grafts as well as spleens were harvested. Splenic T cells of Rapa-DC-treated recipients were hyporesponsive to challenge with donor alloAg (Fig. 23A), confirming that this effect observed previously in adoptive transfer experiments was contributing to graft prolongation. Graft histology of the same group revealed minimal tissue disruption (Fig. 23B), whereas grafts from the otherwise untreated controls were severely damaged, with heavy cellular infiltration. Further analysis of the grafts confirmed that on day 7, grafts were infiltrated by granulocytes, macrophages, $CD4^+$ and $CD8^+$ cells (Fig. 24). Strikingly, grafts taken from Rapa-DC-preteated recipients had markedly reduced levels of infiltration. Furthermore, the latter showed significantly reduced levels of IFNγ gene expression, compared to control grafts (Fig. 25 A, B). In all cases, pretreatment with alloAg-pulsed control DC appeared to have an immunomodulatory effect, albeit less than that of Rapa-DC, that can account for the insignificant prolongation of graft survival following their administration (MST of 10.4 days vs 9.1 days in the untreated group; Fig. 22).

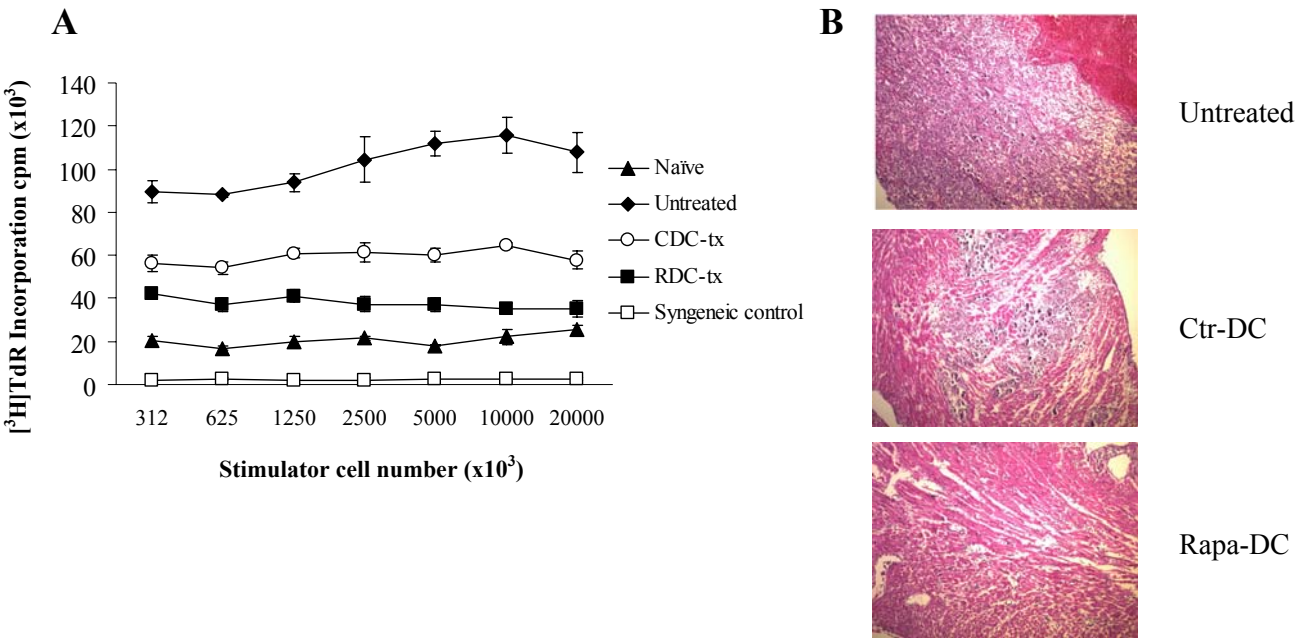


Figure 23. Rapa-DC-pretreated graft recipients show T cell hyporesponsiveness to donor alloAg and near-normal graft histology.

AlloAg-pulsed, control (Ctr)- or Rapa-DC of host origin were injected i.v. into syngeneic C3H mice, 7 days before the transplantation. On day 7, animals were killed and tissues harvested. **(A)** Splenic T cells were challenged with graded numbers of γ-irradiated allogeneic (B10) DC. Mean proliferative responses (± SD) of T cells in 72h MLRs are shown. **(B)** Sections obtained from the grafts were stained with hematoxylin-eosin (H&E).The observations are representative of multiple sections at various levels from two animals in each group.

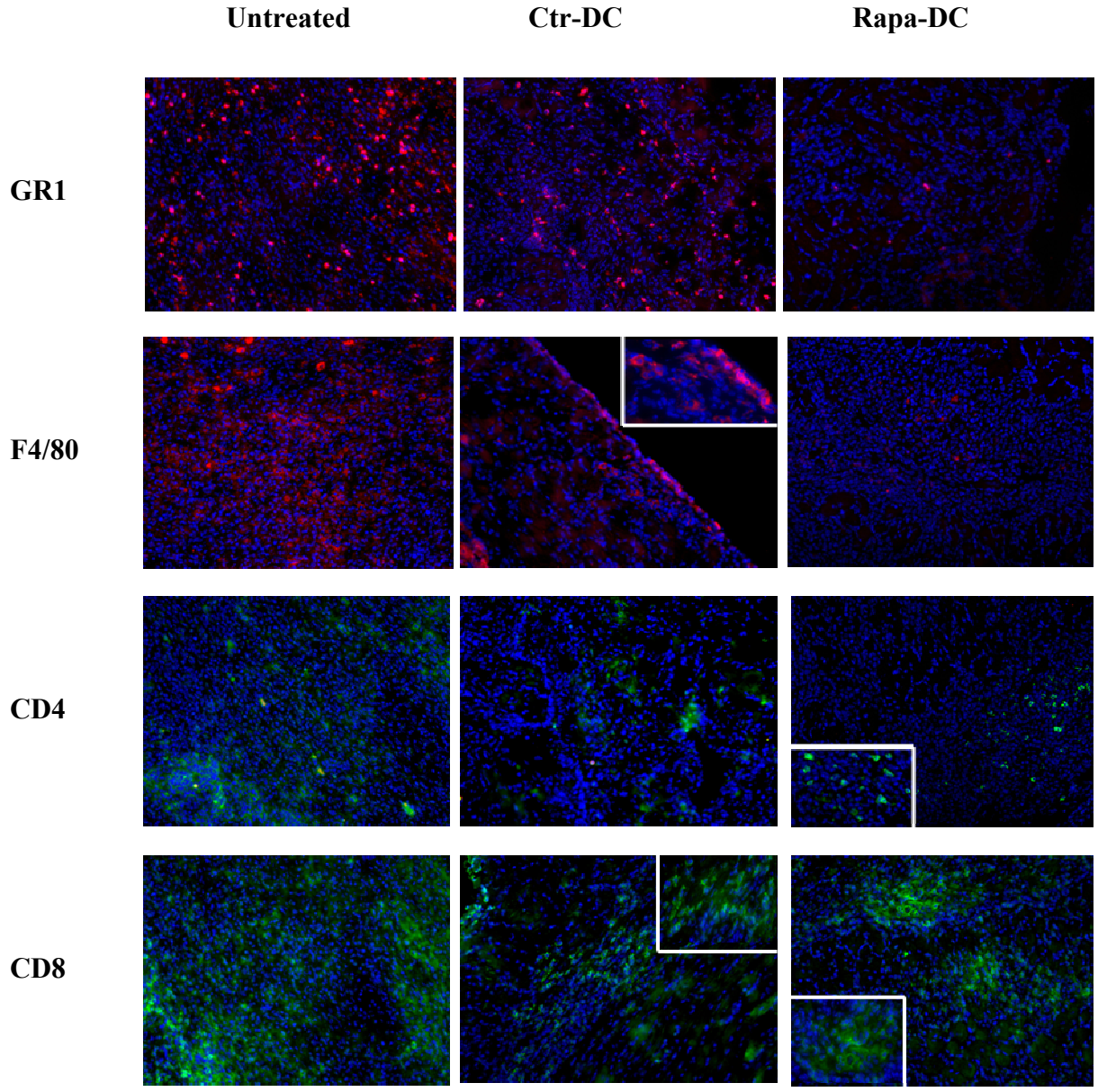


Figure 24. Inflammatory cell infiltration into grafts on day 7 is reduced in Rapa-DC-pretreated heart graft recipients.

Immunofluorescence staining of heart grafts harvested from otherwise untreated (LH column) or control DC- (middle column) or Rapa-DC- (RH column) pretreated recipients. Decreased infiltration of $GR1^+$ (red, top row), $F4/80^+$ (red, second row), $CD4^+$ (green, third row) and $CD8^+$ (green, fourth row) cells into graft is evident following treatment with Rapa-DC. Results are representative of multiple sections at various levels from two animals in each group.

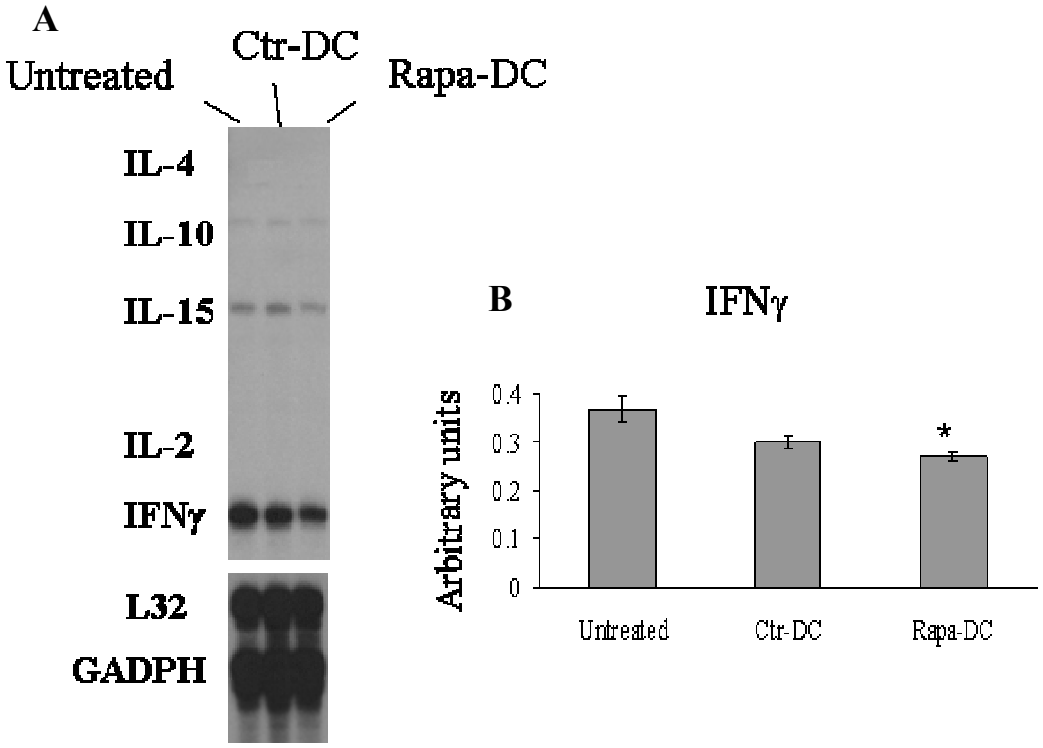


Figure 25. Intragraft IFNγ gene expression is decreased in Rapa-DC pretreated recipients.

Seven days post transplant, recipients were killed and heart grafts harvested. Total RNA was isolated and analyzed for common inflammatory cytokine signals by RNase protection assay. There was a significant reduction in IFNγ gene expression in the Rapa-DC group vs. the untreated group (**P*<0.05, Student's '*t*' test).

4.5. DISCUSSION

DC, once known almost exclusively for their unrivalled capacity to stimulate immune responses, are now recognized increasingly for their roles in immune regulation and the induction/maintenance of tolerance (16, 23, 66). In the immature state, DC exhibit inherent tolerogenic properties, as they fail to provide adequate costimulation for T cell activation. They can increase pancreatic islet (45) and vascularized organ transplant survival (46, 48). DC can also delay the onset or regulate the severity of autoimmune diseases in animal models (91, 119, 237, 238). In the transplantation setting, both donor and recipient DC contribute to immune responses that lead to allograft rejection (25, 26, 239-241). Direct alloAg presentation, mediated by donor APC, results in the activation of host T cells by allogeneic MHC and costimulatory molecules expressed on these donor APC. This in turn, leads to vigorous T cell proliferation and anti-graft effector immune responses. As donor DC undergo attrition, their role as presenters of alloAg subsides, and recipient DC, that can traffic to the graft, become the predominant APC (242). Emerging evidence suggests that, once recipient DC populate the graft, they convey and present alloAg indirectly to T cells, in much the same way as other Ags in the periphery are presented in the normal steady state (243).

Reports of the induction by DC of peripheral tolerance to self or MHC class II Ags encountered in the absence of inflammatory stimuli (62, 98), coupled to evidence that direct alloAg presentation diminishes with time after transplantation, provide a strong impetus for targeting indirect allorecognition via DC-induced cross-tolerance. In healthy human volunteers, subcutaneous injection of Ag (influenza matrix peptide or keyhole limpet hemocyanin)-pulsed autologous, immature, monocyte-derived DC led to specific inhibition of Ag-specific effector T cell functions (73). Moreover, IL-10-treated human monocyte-derived DC induce T cell anergy via cross-presentation of phagocytosed necrotic fragments (118). Similarly, immature DC

pretreated with N-acetyl-L-cysteine induce alloAg-specific T cell hyporesponsiveness after loading with apoptotic cells (244). Further, while mice infused i.v. with alloAg-pulsed peritoneal exudate cells (PEC) develop a delayed-type hypersensitivity (DTH) response similar to that of allograft recipients after challenge with the alloAg, i.v. infusion of PEC pulsed with alloAg in the presence of IL-10/transforming growth factor (TGF)-β results in an anti-inflammatory, "allograft acceptor-like" DTH (245). Herein we show for the first time, that DC exposed to rapamycin become resistant to maturation and induce Ag-specific T cell hyporesponsiveness via indirect Ag presentation. In this model, T cells cross-primed by alloAg-pulsed, Rapa-DC become hyporesponsive to subsequent challenge with the same Ag, not only through the indirect pathway, but also the direct pathway, providing evidence for regulation of both these major pathways of allorecognition by pharmacologically-modified DC.

The Ag-specific T cell hyporesponsiveness induced by our approach appeared to be the result of Rapa-DC trafficking to secondary lymphoid tissue and active regulation of T cell function that was reversible by exogenous IL-2. Freshly-isolated immature DC differ from mature DC in that they cannot provide adequate costimulation (signal 2) and inflammatory cytokine (e.g. IL-12p70) support (signal 3) in conjunction with their ability to ligate TCR through Ag presentation (signal 1). Whereas stimulation of T cells by mature DC results in their activation, priming by immature DC causes T cell anergy (246). Different approaches to generation of either stably immature or "alternatively-activated" DC, such as those conditioned with IL-10 (247, 248), have been reported over the past few years. IL-10-conditioned DC (90) and Vitamin D_3-treated DC (140) both induce allogeneic T cell anergy. Repetitive stimulation of human T cells by allogeneic immature DC generates anergic T cells with limited resistance to IL-2 stimulation (109). In a similar manner, rapamycin-treated DC become stably immature, and

as demonstrated in previous chapters, lose their capacity to produce IL-12p70 and TNF-α upon LPS stimulation. Importantly, after exposure to rapamycin, DC retain their capacity to take up cell lysate Ag *in extended overnight culture* (16-24 h), in contrast to previous reports by us (Chapters Two and Three) and others (202) that concern inhibition of Ag uptake *over much shorter periods* (≤ 2h). Moreover, it should be noted that the overnight pulsing with cell lysates was performed in the absence of rapamycin.

The T cell hyporesponsiveness induced by Rapa-DC was not the sole result of the cells' inability to prime T cells effectively. It was rather due to immunomodulatory activity of these cells since T cells primed by Rapa-DC later responded strongly to 3rd party DC challenge, but responded to DC from the same donor comparatively poorly. These *in vitro* and *ex vivo* observations were confirmed by Ag-specific prolongation of organ graft survival following systemic infusion of alloAg-pulsed Rapa-DC. We also observed a striking inhibition of the two main mediators of the type 1 T cell response, i.e. IL-2 and IFNγ production by T cells stimulated with Rapa-DC. Although there was a clear balance shift in type 1 vs. type 2 T cell biosignatures in the MLR cultures, there was no increase *per se* in the expression of the latter (IL-4, IL-5 and IL-10). This universal T cell cytokine downregulation is likely a contributing mechanism to the effects of Rapa-DC in transplant models, as Th2 cells play a role in alloimmune responses (25) and can be sufficient for graft rejection (105, 106, 249). Furthermore, IL-4 is recognized for its stimulatory effect on IL-12p70 production by DC (217). Thus it is possible that by reducing IL-4 production, activation of "bystander" APC in the recipient is also inhibited by Rapa-DC. In our studies, we were not able to demonstrate an increase in incidence or activity of T cells with previously defined Treg cell phenotype such as $CD4^+CD25^+$, in response to either *in vitro* or *in vivo* stimulation by Rapa-DC. It should be emphasized that the Treg cell analyses were done 7

days after priming of T cells by Rapa-DC, and in light of current evidence showing requirement for repeated stimulation for generation of Treg cells, we cannot rule out their induction by Rapa-DC as a possible mechanism in long-term graft survivors.

Strategies to utilize DC for the induction of donor-specific tolerance and the prolongation of organ allograft survival have until now been concerned mainly with donor DC (46, 48, 87, 250, 251), whereas studies exploiting recipient DC in therapeutic approaches to graft rejection have been limited. One exception is the use of DC-DC hybrids, that express MHC of both parental APC, to delay the onset of alloAg-specific graft-versus-host disease (GVHD) when engineered to overexpress CD95L (252). Recently, in a rat kidney transplant model, preoperative infusion of dexamethasone-treated immature F1 DC, followed by CTLA4Ig and a short postoperative course of calcineurin inhibition, was found to promote indefinite graft survival and immune regulation via the indirect pathway (253).

It has also been shown that intrathymic or i.v. injection of recipient BM-derived or thymic DC, pulsed with immunodominant alloMHC-I-derived peptide, prior to transplantation, prolongs cardiac or pancreatic islet allograft survival in ALS-treated rats (50, 60, 61, 254). These latter effects have been attributed, in part, to the induction of central tolerance by the allopeptide-pulsed DC, as they were abrogated when the recipient rats were thymectomized prior to cell infusion (51). Clinical application of this strategy, however, is limited by the necessity to identify unrelated donor MHC peptides. The advantage and comparative simplicity of the present model lies in the use of whole donor cell lysates, which constitute the complete library of donor alloAgs to be presented indirectly by Rapa-DC. This approach also permits Ag-specific immunomodulation, as opposed to alternative approaches that target recipient DC via pharmacologic treatment of the host, -a method shown to induce tolerogenic DC in graft

recipients (255). The incomplete prolongation of graft survival we observed uniformly following a single infusion of the DC was presumably due to activation of T cells via the direct pathway, since once this early response was suppressed, albeit minimally, by a transient subtherapeutic dose of FK506 (which alone did not prolong allograft survival), rejection was more markedly and significantly delayed. Calcineurin inhibition might also work in other ways to augment the beneficial effect of pretreatment with alloAg-pulsed Rapa-DC, e.g. by inhibiting donor DC migration (256). Notably, we were able to enhance the tolerogenic potential of Rapa-DC by repeated infusion of the alloAg-pulsed cells, -40% of recipients in this protocol exhibited long-term graft survival, confirming the hyporesponsiveness achieved to both direct and indirect challenge *in vitro.*

In summary, rapamycin-treated, alloAg-pulsed host DC can induce Ag-specific T cell hyporesponsiveness *in vivo* and prolong the survival of MHC-mismatched vascularized heart allografts. This approach, that obviates systemic delivery of the DC-modifying pharmacologic agent, constitutes a clinically applicable, cell-based 'negative vaccination' strategy for suppression of allograft rejection.

5. SUMMARY

With the advancement in surgical techniques and modern pharmacological immunosuppression, human organ transplantation, -merely an experimental procedure 50 years ago, has become the therapy of choice for end-stage vital organ failure. This relatively novel 'therapy', however, is far from being the ideal 'treatment' for the majority of allogeneic organ graft recipients, as they rely on life-long immunosuppression and become chronically immunosuppressed. Thus, the next frontier in transplant immunology is to achieve therapeutic, drug-free, Ag-specific tolerance. Activation of T cell clones reactive to self Ags (or to 'newly-self' Ags in the case of transplantation) is believed to be the main mechanism underlying initiation of autoimmunity and transplant rejection. Although most known autoimmune diseases have a genetic component, due to involvement of complex environmental factors, it is hard to predict whether an individual will develop autoimmune reactivity before the disease is manifest. This, in turn, limits Ag-specific therapeutic approaches aimed at autoimmunity. By contrast, in transplantation, Ags to which the host will react can be predicted, even before they are introduced to the host's immune system. Indeed in the U.S., live kidney donation (usually from a relative or spouse) exceeds cadaveric kidney donation. This 'predictability' in transplantation presents an advantage in this field for trials of tolerance induction. It is now well-documented that Ags, when introduced to the immune system under steady-state conditions, can lead to tolerance. Studies by others and us have consistently shown that immature DC can induce tolerance to Ags that they express *in vivo*. By virtue of their remarkable plasticity however, immature DC-based therapeutic tolerance approaches face the risk of instigating immunogenic responses should the DC 'mature' in vivo. In transplantation, for instance, both the inflammatory responses due to surgical trauma and ischemia-reperfusion injury can promote DC maturation and generation of subsequent

alloimmune responses. It is therefore important to devise approaches to predictably prevent DC maturation *in vivo*.

We have demonstrated herein that the commonly used immunosuppressant rapamycin inhibits spontaneous and TLR4-mediated maturation of MDC, without interfering with their ability to present Ag to T cells. Rapamycin-treated DC, either *in vitro* or *in vivo*, lose their capacity to produce inflammatory cytokines (IL-12p70 and TNF-α), and induce Ag-specific T cell anergy. This study, for the first time, demonstrates that stably-immature DC can be pulsed with complex antigenic mixtures (257), and used as therapeutic vectors to induce allograft tolerance. Under steady-state conditions before organ transplant, administration of rapamycin-conditioned, donor Ag-loaded DC prolongs heart graft survival significantly. This effect is enhanced when the initial acute inflammation, presumably due to surgery, is controlled by a short, post-operative course of FK506, and abrogated by administration of exogenous IL-2.

Rapamycin was found to inhibit DC maturation and inflammatory cytokine production in two different strains (B10 and C3H) and under two different experimental conditions (*in vitro* and *in*/*ex vivo*) in the mouse. DC pretreated with rapamycin remain immature after exposure to both LPS and allogeneic cell lysates. Rapamycin treatment also inhibits the DC response to IL-4 (*in vitro*) and Flt3L (*in vivo*). In Chapter Two, we showed that the suppression of the IL-4-mediated in vitro maturation of murine BMDC is due to downregulation of the IL-4R complex on these cells upon exposure to rapamycin. Due to the broad mechanism of action of rapamycin, however, it is likely that this effect is only partially responsible for the IL-4 hypo-/unresponsiveness and intracellular IL-4 signaling pathways may also be affected. Indeed, rapamycin may be targeting a common signaling pathway for IL-4R and Flt3, hence blocking the DC differentiation and maturation associated with ligation of these receptors, as discussed in

Chapters Two and Three. One possible common target is the JAK/Stat pathway that is active in signal transduction pathways of both receptors.

We believe that data presented in this thesis provide a framework on which novel strategies to use tolerogenic DC as therapeutic vectors may be based. In the light of these findings in mice, we propose to use rapamycin-conditioned DC-based regimens to promote transplant tolerance in a clinically-relevant large animal model. Based on this work, and to achieve a reliable outcome, several important parameters still need to be evaluated. Although rapamycin-treated DC induce Ag-specific T cell hyporesponsiveness *in vivo*, suggesting they retain their immature phenotype after i.v. administration, we have not formally evaluated their response to maturation stimuli, other than those mentioned above. Therefore, it will be of interest to analyze the expression of TLR4 and other TLRs on the DC surface following their exposure to rapamycin, and their response to TLR ligands and CD40 agonism (CD40L). In order to achieve predictable, long-term graft survival, the dose, frequency and timing of rapamycin-conditioned DC administration will need to be optimized. Data presented in Chapter Three provide evidence for use of rapamycin *in vivo* to control activation of 'intrinsic' DC (of host and donor origin) post-transplant, that may synergize with the tolerogenic potential of pre-administered Rapa-DC. At present, the main predictable hurdle with translation of this approach to the clinic is the induction of apoptosis in human DC by rapamycin. Studies by two different groups (191, 202) have demonstrated that rapamycin, while not affecting differentiation of either monocyte-derived or $CD34^+$ hematopoietic progenitor-derived human DC, reduces cell recovery up to 50%, under concentrations comparable to those used in this thesis (10 ng/ml). This difference could be attributed to the fundamental differences between mouse and human systems, and the distinct starting population in DC cultures. However, it should also be noted that timing and duration of

rapamycin exposure are different in those and the current study, such that rapamycin is added at the beginning of human DC cultures, whereas we have opted to begin exposing murine cells to rapamycin on day 2.

Studies presented in this thesis investigating mechanisms underlying prolonged graft survival will also need to be repeated in long-term graft (tolerant) survivors. As discussed in Chapters Two and Three, DC exposed to rapamycin either *in vitro* or *in vivo* have a potent, yet incomplete inhibitory impact on alloreactive T cell proliferation and inflammatory cytokine production. This is in parallel to the limited, albeit statistically significant reduction of intragraft IFNγ expression post-transplant, following a single injection of Rapa-DC. Since multiple pre-transplant infusions of these cells lead to long-term graft survival in a fraction of the recipients -in a strain combination where spontaneous graft acceptance never occurs-, it is reasonable to presume that mechanisms other than induction of T cell anergy by rapamycin-pretreated DC are in effect, e.g. generation/expansion of T cells with regulatory capacity. Pharmacologically-modified (with dexamethasone) DC expressing both donor and host alloAgs (F1) have recently been shown to induce indefinite survival of rat renal allografts, through induction of indirect pathway Treg cells (253). It would be interesting, then, to look for Treg cells in bulk T cell populations stimulated repeatedly with Rapa-DC. Additionaly, Rapa-DC need to be investigated for their capacity to expand naturally occurring (e.g. $CD4^{+}CD25^{high}Foxp3^{+}$) Treg cells.

Although we have used exclusively donor alloAg to pulse DC in our work, other Ags can potentially be introduced to the immune system through 'tolerogenic' DC. Recent evidence has suggested that indirect pathway stimulation with autoAg in the context of transplantation may be an important mechanism leading to immune-mediated allograft injury. Development of autoimmunity to the autoAg cardiac myosin (CM) (in human cardiac transplant recipients) (258)

and type V collagen (in human lung transplant recipients) (259) has been correlated with destructive immunity against the graft. It has been suggested that these autoAgs, which are usually 'hidden' from immunologic surveillance, may be released during ischemia-reperfusion injury or during alloimmune-mediated injury to the graft. $CD4^+$ T cells reactive against CM have been isolated in a mouse model of heart transplantation (258). Furthermore, anti-CM autoimmune responses appear to be associated with the development of chronic heart graft rejection. Given the potential pathologic implications of autoimmunity to CM in heart transplant recipients, the use of rapamycin-conditioned DC to tolerize recipients to CM may be advantageous.

In addition to demonstrating its role in potentiating DC tolerogenicity, our findings also expand knowledge of rapamycin's mechanism of action as an immunosuppressant. As outlined in Chapter One, rapamycin has been noted traditionally for inhibiting signaling pathways associated with activating/inflammatory cytokines in T cells. In light of our data, we hereby argue that rapamycin may also suppress production of these cytokines by T cells upon stimulation by rapamycin-pretreated DC. Since, *in vivo*, all immune cells are exposed to the agent, this effect is likely a component of overall immusuppression, demonstrating the importance of designing studies dissecting the impact of immunosuppressants on different cell types, rather than concentrating solely on effector T cell populations.

In summary, the studies presented herein have provided insight into the fields of pharmacologic immunosuppression and DC manipulation. Our discovery that rapamycin enhances the tolerogenic potential of DC, and that these cells can be loaded with complex Ag mixtures to promote tolerance, will likely provide impetus to further investigate this and similar approaches to develop DC-based therapeutic vectors for the treatment of allograft rejection.

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