REGULATION OF IMMUNE RESPONSES BY GENETICALLY-ENGINEERED DENDRITIC CELLS AND EXOSOMES

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Approximately 5-8% of Americans are affected by some type of autoimmune disease. The current standard of care for these patients involves pharmacologic therapy to induce systemic immunosuppression, which carries significant side effects. There is a need for therapies that effectively restore immune-mediated tolerance towards the body, but do not cause inappropriate immunosupression.

The following dissertation details the synthesis, characterization, and performance of tolerogenic dendritic cells (DC), as well as the exosomes that they secrete. Exosomes are small (40-100 nm) membrane-bound microvesicles produced by reverse budding of the membrane of the multivesicular endosome in DC, as well as many other cell types. DC were transduced with adenoviral vectors expressing target genes in order to manipulate their function. After transduction, DC and their exosomes were examined for the ability to modulate disease-induced inflammation in the NOD mouse model of type 1 diabetes and the delayed-type hypersensitivity (DTH) model.

DC transduced with an IL-4 expressing vector are capable or preventing the onset of hyperglycemia when administered to 12-weeks-old and 12-16 week-old prediabetic mice. Treated mice demonstrate modulation of T-cell mediated β-cell autoimmunity, including reduced insulitis, increased FoxP3 expression in the islet-draining lymph nodes, and Th2 skewing of islet-antigen induced cytokine secretion profile.

We further demonstrate that transduction of DC with adenoviral vectors expressing indoleamine 2,3-diooxygenase (IDO) and CTLA-4-Ig are efficient means to generate IDO+ DC *in vitro,* and that these DC effectively reduce paw swelling in the DTH model of antigen-specific inflammation. The exosomes secreted by these IDO+ DC contain IDO protein, and suppress DTH responses at a level comparable to their parental DC in a manner dependent on B7-1 and B7-2. To confirm the role of B7 costimulatory molecules on tolerogenic DC and exosomes, we further demonstrate that B7-1 and B7-2, but not PD-L1 or PD-L2, are required for *in vivo* suppressive activity of rIL-10 treated DC and their secreted exosomes.

This endeavor has increased the knowledge regarding therapeutic DC and exosomes. We have demonstrated strategies to generate tolerogenic DC and exosomes, as well as factors that are required for their activity *in vivo.*

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NOMENCLATURE

ANOVA: analysis of variance

APC: antigen presenting cell

cDNA: complementary deoxyribonucleic acid

CFA: complete Freund's adjuvant

CpG: cytosine guanine dinucleotide

CTL: cytotoxic lymphocyte

CTLA: cytolytic T lymphocyte-associate Antigen

DAMP: damage associated molecular pattern

DC: dendritic cell(s)

DMEM: Dulbecco's modified Eagle's medium

DNA: deoxyribonucleic acid

DTH: delayed-type hypersensitivity

EAE: experimental autoimmune encephalomyelitis

ELISA: enzyme-linked immunosorbent assay

ELISPOT: enzyme-linked immunospot

FACS: fluorescence-activated cell sorter

FoxP3: forkhead box p3

GAD: glutamic acid decarboxylase

GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor

Hsp: heat shock protein

i.p.: intraperitoneal(y)

i.v.: intravenousl(y)

IDO: indoleamine 2,3-dioxygenase

ILN: inguinal lymph node

IFN: interferon

KLH: keyhole limpet hemocyanin

KO: knockout

LPS: lipopolysaccharide

MHC: major histocompatibility complex

MFI: mean fluorescence intensity

mRNA: messenger RNA

mAb: monoclonal antibody

PLN: pancreatic lymph node

NF-κB: nuclear factor κB

NOD: nonobese diabetic

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PAMP: pathogen associated molecular pattern

PBS: phosphate buffered saline

PFU: plaque-forming unit

qRT-PCR: quantitative Reverse Transcriptase Polymerase Chain Reaction

rpm: revolutions per minute

SD: standard deviation

SEM: standard error of the mean

SFC: spot forming cells

siRNA: small interfering RNA

TLR: toll-like receptor

TNF: tumor necrosis factor

Tr1: T regulatory-type 1 cells

PREFACE

I would like to express my gratitude to my advisor, Dr. Paul Robbins, for his guidance and for helping me to mature as a scientist. I would also like to thank the members of my committee: Drs. Mohammad Ataai, Michael Lotze, Adrian Morelli, and Johnny Huard for their advice and support throughout this project.

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Much of scientific work is accomplished in collaboration, and this work is no exception to that. I would like to recognize that portions of this manuscript have been published in part or whole or are currently under review. Previously published data can be found in:

1.) Bianco NR, Kim SH, Ruffner MA, Robbins PD. "Therapeutic effect of exosomes from Indoleamine-2,3 Dioxegenase Positive Dendritic Cells in Collagen-Induced Arthritis and Delayed-Type Hypersensitivity Models." *Arthritis & Rheumatism* 2009. 60(2):380-9

2.) Ruffner MA, Kim SH, Bianco NR, Francisco LM, Sharpe AH, Robbins PD. "B7-1/2, but not PD-L1/2 molecules, are required on IL-10-treated tolerogenic DC and DC-derived exosomes for *in vivo* function." *Accepted, European Journal of Immunology*. DOI 10.1002.

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1.0 INTRODUCTION

The immune system is responsible for defense of the body against harmful pathogens by mounting appropriate responses to eliminate the problematic organism. However, another important task of the immune system is differentiating harmless from injurious stimuli in order to prevent unnecessary and potentially dangerous reactions to innocuous organisms or parts of the host organism. Indeed, a wide variety of research has elucidated mechanisms whereby the immune system senses exogenous and endogenous signals and differentiates whether there is potential for harm [1].

As professional antigen presenting cells (APC), dendritic cells (DC) play a central role in determining whether the response of the immune system will be directed towards immunity or tolerance. DC are derived from CD34+ hematopoeitic stem cells, and are distributed throughout all tissues where they convey antigen from peripheral sites into the secondary lymphoid tissues. They are equipped with numerous pattern recognition receptors, such as TLR4 (recognizes the bacterial product lipopolycaccharide (LPS), extracellular breakdown products of hyaluron, and endogenous Hsp70), TLR2 (recognizes DNA CpG sequences), which enable them to detect danger signals released by injured cells as well as pathogens [2]. The cumulative effect of the signals received by APC results in alterations in their final maturation state when they present antigen to T cells within the secondary lymphoid tissues.

APC encountering antigen in the presence of danger signals will stimulate immunity. It is important to consider that these danger signals are not exclusively delivered by infections, but can occur in the setting of cell injury, setting up a vicious cycle in pathological conditions such as autoimmune diseases whereby inflammation-induced cell damage contributes to the ongoing inflammation. DC maturation occurs following binding of damage-associated molecular patterns (DAMPs) or pattern-associated molecular patterns (PAMPs) bind their pattern-recognition receptor on the DC, which results in intracellular signaling changes within the DC. This results in the release of inflammatory cytokines from DC, such as IL-12 and interferon-α (IFN-α) [3, 4]. Mature DC are characterized by an increase in major histocompatibility complex (MHC) presentation of antigen, as well as upregulation of costimulatory molecules such as B7-1 (also known as CD80) and B7-2 (also known as CD86), as well as an increase in the production of inflammatory cytokines and chemokines [5, 6]. Mature DC are capable of priming T cell responses towards infections and tumors [7, 8], and polarizing T cell immunity towards the Th1, Th-17, or Th2 pathways [9-11].

In the absence of pathogenic threats, DC continue to sample antigen from the body. In these conditions, however, the antigen will be derived from the repertoire of host-expressed proteins, and therefore in these conditions it is critical that DC prevent unnecessary autoimmune activation that may lead to self-destructive autoimmunity. DC play critical roles in both thymically-developed central tolerance, as well as peripheral tolerance. In the thymus, thymic and peripheral DC present self-derived antigen to thymocytes during clonal deletion, a process whereby autoreactive T cells are eliminated [12, 13]. However, there is a growing body of evidence that demonstrates that in most individuals some autoreactive T cells persist despite the process of central deletion. Therefore, the mechanisms of peripheral tolerance are necessary to

prevent the development of autoimmune disease. These mechanisms are varied and include: immunological ignorance of the antigen, apoptosis (or peripheral deletion) of the self-reactive clone following exposure to excessive levels of antigen, induction of anergy, or induction of regulatory T cells [14]. A subset of peripheral DC termed 'tolerogenic' DC play a significant role in the final two mechanisms, and a growing body of evidence suggests tolerogenic DC play important roles in autoimmune disease, allergy, and transplantation, among others and will be discussed in further detail below.

The goal of this research is to investigate the therapeutic potential of DC which have been engineered to be tolerogenic using adenoviral vectors, as well as the exosomes secreted by these DC, for the treatment of type 1 diabetes. The methods used to generate tolerogenic DC and evaluated in this proposal have the potential to be therapeutic in numerous different autoimmune diseases. However, the proposed work will be carried out focusing on two model systems. The NOD mouse, which spontaneously develops type 1 diabetes will be employed as it is the most widely accepted model of type 1 diabetes. The delayed-type hypersensitivity model, which is a model of acute, antigen-specific inflammation, will be used in order to allow us to conduct some mechanistic studies that would not currently be possible in the NOD model. The specific aims of the studies detailed in this dissertation are summarized below.

1.1 SPECIFIC AIMS

1.1.1 Specific Aim 1: Investigate the therapeutic benefit of DC genetically-engineered to overexpress IL-4 (DC/IL-4) and exosomes secreted by these DC (exo/IL-4) for type I diabetes using a late-stage NOD mouse model, and elucidate the cellular mechanisms by which they modulate inflammation.

We have previously demonstrated that DC transduced with an adenoviral vector expressing Interleukin-4 (IL-4) reduce disease severity in murine models of arthritis and hypersensitivity, and are able to prevent diabetes onset when administered at early ages to NOD mice [15, 16]. Further, it is known that the β cell destruction in type 1 diabetes is mediated by a Th1-type T cell response, with CD8+ T-cells being responsible for the majority of beta cell damage. Successful therapies alter this process significantly, therefore we will investigate the effect of DC and exosome therapy on T cell function and phenotype.

Hypothesis 1.1: DC/IL-4 and exo/IL-4 have the ability to reduce pancreatic insulitis and prevent the onset of hyperglycemia in NOD mice following adoptive transfer at 12 weeks of age.

Hypothesis 1.2: Increased levels of IL-4 in secondary lymphoid organs following adoptive transfer of DC/IL-4 and exo/IL-4 will result in alteration of the pathogenic Th1/Th2 balance seen in the NOD mouse.

Hypothesis 1.3: Mice treated with DC/IL-4 as well as exo/IL-4 will have higher levels of regulatory T cells following treatment when compared to untreated mice.

1.1.2 Specific Aim 2: Examine the molecular mechanisms conferring the in vivo suppressive effects of DC and exosomes.

The experiments for Aim 2 will be conducted using a delayed-type hypersensitivity (DTH) model in C57Bl/6 mice instead of the NOD model because of the greater availability of knockout strains in a C57Bl/6 background. Our greater understanding of which modifications and doses of DC and exosomes are effective in this model make DTH a superior model compared to the NOD mouse to determine specific molecules that are necessary for the suppression of inflammation using our DC and DC-derived exosome treatments. There has been increasing evidence to support the importance of indoleamine-(2,3)-diooxygenase IDO expression in DC in establishing peripheral tolerance [17, 18]. However, efficient means to generate IDO+ DC *in vitro* have yet to be elucidated. Further studies demonstrating a requirement for B7-1 in the surface of exosomes for function *in vitro* drew our attention to the role of costimulatory molecules on exosomes, and our previous studies in the DTH model indicate that the *in vivo* suppressive activity of exosomes rely on the presence of MHC II and FasL on the exosome surface [19, 20]. These previous studies led us to suspect a role for direct interaction between exosomes and T cells *in vivo,* which may translate into a requirement for B7 family costimulatory molecules for *in vivo* suppressive activity. Therefore in the studies that follow we will examine:

Hypothesis 2.1: Adenoviral transfer of IDO results in a population of DC capable of suppressing DTH responses and secreting exosomes which also reduce DTH.

Hypothesis 2.2: Adenoviral transfer of CTLA-4-Ig to DC results in IDO+ DC and exosomes which are capable of suppressing DTH.

Hypothesis 2.3: Expression of B7-1 and B7-2 T cell costimulatory molecules are critical on the surface of DC and DC-derived exosomes for *in vivo* regulation of inflammation in the DTH model.

Hypothesis 2.4: Loss of PD-L1 and PD-L2 T cell costimulatory molecules, which tend to confer inhibitory signals to T cells, on DC and DC-derived exosomes will result in loss of ability to abrogate paw swelling in the DTH model.

1.2 TOLEROGENIC DENDRITIC CELLS

1.2.1 Mechanisms of DC-Mediated Tolerance

Dentritic cells (DC) are best known for their ability to induce strong innate and adaptive immune responses towards foreign antigen *in vivo.* However, a growing body of evidence illustrates that DC also play an important role in the maintenance of tolerance. This functional dichotomy is governed chiefly by the phenotypic state of the DC itself, as DC are equipped with multiple mechanisms by which they are able to sense the status of their surrponding microenvironment. These mechanisms include the toll-like receptors (TLR) 2, 3, 4, 7, and 9, which sense a wide variety of microbial and endogenous danger signals and sense DC maturation into a phenotypically mature and stimulatory state [21]. In the absence of danger signals, DC continue to sample self-antigen, but in this setting there are low costimulatory signals expressed on the DC, resulting in poor stimulation of naïve T cells [22, 23]. Studies have further elucidated the mechanisms by which tolerogenic DC function *in vivo,* and these will be discussed in further

detail below. Collectively, these data imply that tolerogenic DC possess a number of redundant mechanisms whereby they can regulate tolerance.

1.2.1.1 Induction of Regulatory T Cells

DC can promote the induction of regulatory T cells, including the thymically-differentiated CD4+CD25+ as well as the T regulatory-type 1 cells (Tr1 cells), which are CD4+ and express IL-10 and TGF-β. Repetitive *in vitro* stimulation with immature, allogeneic DC leads to the production of non-proliferating, IL-10-producing Treg cells [24]. Further, DC can expand antigen-specific CD4+CD25+ Treg populations by triggering their division [25]. DC cultured in the presence of IL-10, IL-4 or TGF-β assume a regulatory phenotype that is capable of generating CD4+ and CD8+ regulatory T cells [26]. Further pharmacologic treatment to induce tolerogenic DC have also been shown to produce DC capable of inducing T cell populations. When injected into mice post-transplant, rapamycing treated-DC promote the infiltation of allografts with regulatory T cells, which prevents vasculopathy and promotes graft tolerance [27].

1.2.1.2 Induction of T cell Apoptosis or Anergy

DC with reduced maturation characteristic have reduced ability to induce T cell anergy or apoptosis, or induce antigen-specific hyporesonsiveness. These DC are typically have reduced expression of the costimulatory molecules, which can be achieved by selective blockade, culture with cytokine cocktails, or treatments with pharmacological agents.

Evidence from use of CTLA-4-Ig further demonstrates the importance of B7 molecule expression on tolerogenic DC. CTLA-4-Ig can directly compete with CD28, which activates T cells, for binding of B7-1 and B7-2 on DC [28]. However, independently of this function it also

functions through reverse cell signaling into B7-1 and B7-2 containing cells to result in the upregulation of IDO [29]. IDO is a tryptophan metabolizing enzyme which indirectly inhibits T cell activation and induces T cell apoptosis [30]. Therefore, in addition to the costimulatory molecule profile of the DC, its expression of IDO is also a critical factor in mediating tolerance.

Another important feature of DC interactions with reactive T cells is the cytokine profile which they secrete. T cell polarization is driven by the cytokine profile present on initial antigen presentation, with high production of IL-12 being critical for the development of Th1-driven responses [31]. DC transduced to express IL-10, TGF-β, or CTLA-4-Ig induce Ag-specific T-cell hyporesponsiveness, inhibits the generation of cytotoxic T cells, and promotes Th2-skewing of donor responses [32].

1.2.1.3 Negative Selection Within the Thymus

Although central tolerance is less of a target for *ex vivo* modified DC, nonetheless there are a few studies supporting a role for manipulation of central tolerance via manipulation of DC. Thymic DC are located in the medulla, and both these DC and the medullary epithelium are capable of deleting autoreactive T cells [33]. This selection is dependent on the expression of antigen within MHC complexes [34]. Little work has been done to date to explore the ability of modified DC to manipulate central tolerance. A recent study as examined the ability to transduce thymic DC *in vivo* following thymic injection with recombinant adenoviral vectors, and find that this reduced disease severity in a model of bacterial peritonitis [35]. Further, intrathymic injection of alloantigen prevents allograft rejection in rate in a manner dependent on the presence of host APC within the thymus [36].

1.2.2 Phenotype of Tolerogenic DC

DC maturation is a critical component in determining the ability of DC to either confer tolerance or stimulate immunity. Maturation of DC occurs in response to extracellular stimuli, and allows DC to tailor their responses according to their microenvironment. Table 1 contrasts some important phenotypic differences between immature and mature DC [14, 21]. Mature DC are potent stimulators of T cell proliferation and polarization toward the Th1 or Th17 phenotype, whereas immature DC can differentiate both naturally-occurring and IL-10 secreting CD4+ Tr1-type regulatory T cells *in vivo* [37]. Diverse strategies have been examined as methods to generate tolerogenic DC *in vitro*, and these will be reviewed in detail in the following section. Variations in the phenotype of the resultant DC occur based on the type of treatment used in culture with the DC. In general, the aim of these "tolerizing" regimens is to produce DC which mimic the phenotype, and more importantly, the function, of immature DC closely. Specifically, the tolerogenic DC should not stimulate T cell responses but rather should promote tolerance [14, 21, 38]. This can be accomplished by controlling the culture conditions of DC with cytokines and pharmacologic agents in a manner which renders them maturation-resistant, or by providing DC with a single predominant feature, such as overexpression of IL-10, which results in a DC which induce tolerance when administered *in vivo.*

1.2.3 *In Vitro* Generation of Tolerogenic DC for Adoptive Transfer Therapy

Various methods to manipulate DC *in vitro* in order to generate tolerogenic DC have been tried. The goal of these *ex vivo* manipulations is to generate a maturation-resistant, immature DC phenotype that is capable of conferring tolerance when used in patients. Although the desired

Table 1: Phenotypic Characteristics of Immature and Mature DC

	Immature DC	Mature DC
MHC I	Low	High
MHC II	Low	High
B7 family		
B7-1	Low	High
B7-2	Low	High
PD-L1	Present/High	High
PD-L2	Present/High	High
ICOSL	Low/Present	High
CD40	Low	High
Adhesion molecules		
ICAM-1	High	Low
LFA-1	High	Low
NF-κB	Cytoplasmic	Activated/Nuclear
Polarizing cytokines		
IL-12 **(Th1)**	none/low	produced
IL-1β (**Th17 ad Th2)**	none	produced
IL-6 **(Th17)**	none	produced
TNF-α **(Th17)**	none	produced
Indoleamine 2,3-dioxygenase (IDO)	High	Low

phenotype of these DC is described in more detail above, they tend to express MHC, with a low ratio of costimulatory to inhibitory signals and low overall production of Th1-polarizing cytokines such as IL-12. Various methods have been used to induce the desired DC phenotype, and are detailed below.

1.2.3.1 Treatment with Biological Molecules

Following exposure to a number of cytokines, DC may assume a tolerogenic phenotype. Multiple studies have demonstrated a role for both IL-10 and TGF-β1 in the generation of a tolerogenic DC phenotype. These two cytokines have been of particular interest because they are both expressed by the Tr1-type regulatory T cell, and have been shown to mediate tolerance in multiple models, including autoimmunity and transplant [39]. Treatment of DC with IL-10 does not alter the ability of DC to take up soluble antigen, however, the antigen presentation properties of the DC are altered significantly as the levels of MHC II and costimulatory molecules are decreased on the surface of IL-10 treated DC [40, 41]. However, if IL-10 is applied to DC culture at early stages to monocyte cultures, it can inhibit DC maturation and promote the differentiation of macrophages [42, 43]. TGF-β treatment of DC resulted in induction of a CD8+ regulatory T cell population and amelioration of disease in mice with experimental autoimmune encephalomyelitis [44]. Recent studies have shown that autocrine TGF-β signaling in CD8+ DC results in sustained activity of IDO, whereas treatment of CD8- DC with TGF-β can result in IDO induction and induction of a regulatory DC phenotype [45].

In addition to IL-10 and TGF-β, other biological compounds have been used to generate tolerogenic DC. A large number of studies have looked at the ability of $1\alpha,25(OH)_2D_3$, the active form of vitamin D3, to generate tolerogenic DC *in vitro*. $1\alpha,25(OH)_2D_3$–treated DC were

LPS-induced maturation resistant, and alloreactive CD4+ T cells secreted lower levels of IFN-γ in mixed lymphocyte reactions with the $1\alpha,25(OH)_2D_3$-treated DC compared to control DC [46]. Vitamin D analog treatment of DC has further been shown to have aa significant impact on the gene expression within treated DC via binding to the Vitamin D receptor [47].

Recently, vasoactive intestinal peptide (VIP)-treated DC have been shown to express IL-10, maintain low levels of costimulatory molecules CD40, CD80, and CD86 upon stimulation with LPS, and induce Tr1 CD4+ and CD8+ CD28- CTLA-4+ CD8+ regulatory T cells when co-incubated with naïve cells [48, 49]. Other biological molecules that have been demonstrated to have an effect on DC phenotype include prostaglandin E2, histamine, β2 agonists, glucosamine, N-acetyl-ι-cysteine, and cobalt protoporphyrin, which induces expression of heme-oxygenase-1 [38].

1.2.3.2 Treatment with Pharmacologic Agents

Many immunomodulatory drugs have been investigated for their ability to modify DC function *in vitro* or *in vivo* [50]. Conventional immunosuppressive medications, such as cyclosporin A, FK506, rapamycin and corticosteroids have been tested. Cyclosporin A and tacrolimus (FK506) are both calcineurin inhibitors. Cyclosporin A inhibits NF-κB translocation, thereby inhibiting the production of IL-6 and IL-12, and maturation of the DC [51]. FK506 inhibits T-cell allostimulatory capacity of the DC, however, the phenotype of DC following treatment with FK506 seems to vary based on the culture conditions [52]. Glucocorticoid treatment inhibits the production of inflammatory cytokines such as IL-6, TNF-α, and IL-12p40 in response to ligation of CD40 or treatment with LPS, whereas the secretion of IL-10 remained unchanged [53]. Treatment of DC with LF15-0195, a deoxyspergualine analog prevents the TNFα/LPS

dependent translocation of NF-κB in DC, and these DC are promoted the differentiation of Th2 cell phenotype from naïve T cells *in vitro* [54]. Finally, rapamycin treatment of DC cultures reduces DC maturation and T cell stimulatory capacity both *in vitro* and *in vivo,* and the rapamycin treated DC have enhanced ability to stimulate the activity of CD4+ CD25+ FoxP3+ T regulatory cells [27, 55]. It is hoped that in the setting of transplantation, treatment of DC *ex vivo* with immunomodulatory pharmacotherapy could have the potential to spare patients form the severe systemic side effects associated with these agents, while administration of the tolerogenic DC generated could help to establish tolerance to the autograft.

1.2.3.3 Manipulation of Gene Expression

A number of viral and non-viral methods have been examined for the delivery of transgenes to DC, and are outlined in Table 2.

Different strategies have been employed to generate tolerogenic DC using gene therapy techniques. The majority of the studies focus on the delivery of a transgene with known biological properties, such as cytokines whose immunomodulatory properties are known to direct T cells responses away from the Th1 pathway. Some examples include IL-10, TGF-β, IL-4, or CTLA-4-Ig [32]. Other studies have focused on the inhibition of pro-inflammatory signals, such as siRNA targeting of IL-12, and the use of an NF-κB decoy to prevent DC maturation [56-58].

One potential pitfall to the use of viral vectors, however, is that the viruses themselves interact with the DC and are capable of modulating the DC characteristics . Each virus has its own characteristics of interaction with the host immune system which must be taken into account, in addition to the inherent advantages and disadvantages of the vector itself for gene

Table 2: Successful strategies for gene transfer to DC.

Viral Vectors:
Adenovirus [16, 62-68] Retrovirus [32, 69, 70] Lentivirus [71, 72] Vaccinia Virus [73] Herpes Simplex Virus [74, 75]
Non-Viral:
Plasmid DNA [76] Antisense oligonucleotides [77] NF-κB decoy oligodeoxyribonucleotides [58, 78] siRNA [56, 57]

transfer purposes. In the case of adenoviral vectors, invasion of the viral capsid in to the cell, as well as recognition of viral CpG motifs by pattern recognition receptors trigger a characteristic process of DC maturation [59, 60]. This results in the upregulation of costimulatory molecules, increased production of pro-inflammatory cytokines, and is dependent on NF-κB translocation [61]. Therefore, great care must be taken to assess the phenotype of DC following viral transduction.

1.3 EXOSOMES

Exosomes are small vesicles approximately 60-100 nm in diameter originating in the multivesicular endosome. These small vesicles were originally described as part of the process of reticulocyte maturation [79], as it was originally theorized that the exosomal pathway was an method to eliminate waste protein from the cell during the reticulocyte maturation process. However, it has since been demonstrated that antigen presenting cells such as dendritic cells (DC) secrete exosomes from the cell surface and further, these exosomes are functional and capable of modulating the immune responses [80]. In addition to DC, many other cell types including B cells [81], mast cells [82], macrophages, intestinal epithelial cells [83], and multiple malignant cell types including melanoma [84], lymphoma [85], and mesotheliomas produce exosomes [86]. Exosomes have been documented in human serum [87], urine [88], bronchoalveolar fluid [89], and malignant effusions [90, 91]. Following further investigation since their discovery in the 1980s, a growing body of literature demonstrates that exosomes are a mechanism for cell-cell communication.

Within the multivesicular endosome, exosomes are formed by reverse budding of the late endosomal membrane, resulting in membrane-bound vesicles that contain cytosolic proteins and expose the extracellular domain of membrane proteins. Thery et al. demonstrated using mass-spectroscopy that DC-derived exosomes carry MHC I and II, as well as the B7 costimulatory molecules B7-1 and B7-2 [92]. In addition, DC derived exosomes carry other molecules that modulate immune responses, such as FasL [93, 94], TNFα [95], and, PD-L1 [96]. Adhesion molecules, such as integrins, tetraspannins, and lactadherin (MFG-E8) are also present on the exosomal membrane, and heat shock proteins (hsc70, hsp82), enzymes, and cytoskeletal proteins have been demonstrated within the intraexosomal space [92]. To date, studies have demonstrated the presence of hundred of different proteins within exosomes, and the function of these are only beginning to be explored [80, 97]. Exosomes also carry biomarkers such as tumor antigens Mart-1 and MelCAM-1 [84, 98] and proteins associated with acute kidney failure [99], suggesting that exosomes are possible targets for diagnostic testing. Additionally, exosomes carry mRNA and microRNA, and studies have demonstrated that the mRNA carried in exosomes is functional and capable of inducing changes in gene expression in recipient cells [100].

1.3.1 Exosomes play a role in the regulation of immune responses.

Recently exosomes have been shown to be involved in regulating certain biological processes, although the mechanisms by which this immune regulation can occur remain unclear. Exosomes may transfer proteins to cells through membrane fusion. Mack et al. reported that microvesicles can transfer the co-receptor for HIV, CCR5, from CHO cells to CD4+ T cells that do not carry CCR5, rendering the cells susceptible to HIV infection [101]. Secretion of IL-1ß, a pro-inflammatory cytokine, can occur via microvesicles that appear to be similar or identical to

exosomes [102]. In addition, it has been suggested that exosomes are able to transfer MHC class II molecules through clustering and fusion to follicular DCs, cells that normally are devoid of MHC Class II [103].

There is also evidence that exosomes also are internalized and processed by certain types of DC, in a mechanism similar to the uptake of apoptotic cell debris. DC-derived exosomes are internalized via endocytosis by specific DC subsets both in cell culture as well as *in vivo* [104]. This internalization is dependent on LFA-1 on recipient CD8+ DC, which interacts with ICAM-1 on exosomes to mediate uptake and internalization of exosomes [105]. MFG-E8 (lactadherin) associates with the phosphatidyserine rich membrane of exosomes via its C1C2 domain, and can mediate exosomes phagocytosis following binding off its N-terminal domain to $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins on macrophages [106]. However, this interaction seems to be redundant as loss of lactadherin on the exosome surface does not inhibit the ability of cells to internalize exosomes. Furthermore, novel phosphatidylserine recptors, Tim1 and Tim4 (T cell immunoglobulin domain and mucin domain proteins 1 and 4) have been identified on activated phagocytes, which may play an additional role in the capture of exosomes [107]. How internalized exosomes regulate DC activity is unclear, but it is possible that the endocytosed exosomes are able to back fuse with the multivesicular endosome to mediate transfer of MHC class II and other proteins to the cell surface.

A number of studies have examined the ability of exosomes to interact directly with T cells. As, with DC, LFA-1 addresses DC-derived exosomes to T cells in a manner that is dependent on T cell activation and not T cell receptor specificity [108]. Furthermore, exosomes produced by DC pulsed with tumor antigen peptides were able to stimulate an anti-tumor response in mice as efficiently as the DC themselves [109]. There are conflicting reports

regarding the ability of DC derived exosomes to stimulate T cells directly in vitro. The ability of the exosomes carrying a specific MHC class I peptide to stimulate T cell proliferation in vitro required non-pulsed dendritic cells, suggesting that exosomes are able to transfer the MHC class I complex to the DC [110]. Others have demonstrated that pregnancy derived exosomes are capable of interacting directly with T cells and down regulating zeta-chain signaling [93]. Tumor-derived vesicles have been shown to inhibit T cell function in a manner dependent on FasL [94].

1.3.2 Exosomes Can Suppress Immune Responses.

There have been numerous reports in the literature that describe an ability of exosomes for different sources to suppress immune responses, although the mechanism of exosome-mediated suppression remains unclear in many cases. Small particles produced by rat intestinal epithelial cells cultured in the presence of INF-gamma and digested ovalbumins were able to induce a low level of antigen specific tolerance after injection. Similarly, small particles isolated from the serum of rats fed ovalbumin (OVA) were able to suppress an OVA-specific delayed type hypersensitivity (DTH) response through a MHC-dependent mechanism [111]. These small vesicles isolated from rat serum, termed tolerosomes, appear to be similar or identical to exosomes and are MHC Class II+. These results are similar to observations by our lab, which have shown that intra-dermal immunization with a specific antigen results in the generation of MHC Class II+ exosomes in the serum, able to suppress the DTH response following injection into the mouse footpad in an antigen-specific manner [19]. These results are consistent with the observation that placental-derived exosomes in the peripheral circulation of pregnant women induce apoptosis in T lymphocytes through a Fas/FasL, PD-L1/PD-1 and possibly IDO-

dependent mechanism [93]. Similarly, certain T cell subsets and tumor cells generate exosomes that contain FasL on their surface able to stimulate T cell apoptosis [112, 113]. Moreover, high levels of tumor-derived FasL positive exosomes can be found in the serum of cancer patients and tumor-derived vesicles can modulate the phenotype and function of monocytes. Thus exosomes derived from certain cell types, under certain conditions, are able to suppress the immune response.

Consistent with the observations that exosomes can suppress the immune response, we have shown in both murine DTH and collagen induced arthritis (CIA) models that DC-derived exosomes are able to suppress the immune response [114, 115]. Moreover, it appears as if the genetic modification of the DC to express IL-4, vIL-10, FasL or IDO results in exosomes that are able to suppress the DTH response more effectively. The suppression of the immune response, at least in a murine DTH model, appears to be MHC class I independent, but MHC Class II, and Fas/FasL-dependent, although it seems that FasL is able to regulate the immune response through a non-apoptotic mechanism. Taken together, the results examining immune regulation by exosomes suggests that exosomes derived from not only DC, but other cells types as well serve to regulate immune responses to foreign and possibly auto-antigens.

Despite the interesting observations regarding the ability of immunosuppressive exosomes to regulate immune responses, very little is known about the requirements for their activity *in vivo*, or their exact mechanism of action. Therefore, it seems that DC-derived exosomes are a poorly characterized, but potentially highly relevant and clinically useful agent for the treatment of not only autoimmune diseases, but potentially cancer, allergy, and other diseases. One possible advantage to the use of immunosuppressive exosomes for the treatment of autoimmune diseases, in contrast to immunosuppressive DC, is that exosomes are thought to

be unable to undergo phenotypic changes following administration. In theory, an exosome purified from a tolerogenic DC should retain its "tolerogenic" potential following injection, unlike immature DC that could undergo maturation if they receive stimulatory signals from the microenvironment *in vivo*. This may therefore make them more attractive as a therapeutic agent, if their efficacy and safety can be demonstrated in a convincing manner. However, there may be disadvantages to using exosomes as therapies, such as low product yield or product purity compared to the relatively high doses that may be required to achieve a therapeutic effect. Therefore, since both DCs and exosomes have advantages and disadvantages, we will investigate both as portential therapies in these studies.

1.4 TYPE I DIABETES

Type 1 diabetes (T1D), also known as insulin-dependent diabetes mellitus, is recognized as a rapidly growing health threat worldwide. The CDC estimates that 15,000 young people in the United States per year are diagnosed with T1D, with 19 new cases per 100,000 youth each year [116]. Worldwide, the incidence of diabetes varies greatly by geographical location, ranging from 0.1 per 100,000/year in China and Venezuela to 40.9 per 100,000/year in Finland, and has been increasing since the 1950's [117]. From 1990-1999, the incidence of T1D worldwide increased 2.8%, confirming the need for new methods to address this growing public health concern [117].

T1D is a polygenic autoimmune disease characterized by the T-cell-mediated destruction of insulin-producing β cells in the pancreatic islets of Langerhans, leading to insulin deficiency. T1D is a chronic disease that is known to exist for years in a preclinical phase before

the classic manifestations are observed. This preclinical phase, known as insulitis, is characterized by the infiltration of leukocytes into the islets. The actual onset of diabetes occurs after most β cells have been killed, resulting in insulin deficiency and secondary hyperglycemia [118].

Autoreactive T-cells that react specifically to β cell expressed proteins or auto-antigens such as glutamic acid decarboxylase (GAD) and insulin play a key role in the pathogenesis of T1D. β cell autoantigens are processed by antigen presenting cells (APC), including macrophages, dendritic cells, and B cells, in the pancreatic islets. APC then present processed peptides to autoreactive CD4+ T cells, cells which have survived negative selection in the thymus, in the pancreatic lymph node (PLN) and other peripheral draining lymphoid organs. Activated autoreactive CD4+ T cells secrete cytokines and activate β cell-specific cytotoxic CD8+ T cells (CTL). The activated CTL then migrate to the islets where the actively kill β-cells as well as produce inflammatory cytokines, which activate more CTL, as well as macrophages, further contributing to the destruction of β □cells[119, 120]. In general, T_H1 cytokines, such as IL-2, IFN-γ, and TNF-α, promote T1D development, while T_H2 or T_H3 cytokines, such as IL-4, IL-10, and TGF-β, prevent T1D onset. β cell death is the direct result of cytotoxic cytokines and other agents released from CTL and macrophages, including IL-1β, TNF-α, IFN-γ, oxygen free radicals, granzyme and perforin. Additionally, Fas and TNF receptors, known as death receptors, mediate apoptosis, thus playing a role in β cell death [119].

T1D patients are rarely diagnosed before onset of hyperglycemia and loss of the majority of pancreatic β cells, and typically present with symptoms associated with a combination of low insulin levels and high blood glucose, including: weight loss, polyuria, polydipsia, and polyphagia. This is a significant challenge to immunotherapy strategies, both because it is

difficult to predict which at-risk patients will ultimately develop diabetes, but also because very little β□cell mass remains at the time of diagnosis. Due to the current lack of therapies which halt the progression of β□cell destruction, T1D patients ultimately require lifelong insulin replacement therapy, and are at risk of developing significant complications associated with hyperglycemia, including: retinopathy, neuropathy, nephropathy, and accelerated peripheral vascular and coronary artery disease. A major focus of existing therapy is to improve the insulin therapy regimens of T1D patients in order to prevent the onset or progression of secondary complications.

1.4.1 Insulin Therapy for Type 1 Diabetes

The Diabetes Control and Complications Trial (DCCT) was the first large-scale trial to demonstrate the beneficial therapeutic value of intensive insulin control compared to conventional insulin therapy on the development and progression of complications in patients with T1D [121]. In the intensive insulin control group, patients monitored their blood glucose ≥4 times per day, and received insulin injections ≥3 times per day with the goal of achieving a target glycosylated hemoglobin (A1c) within the normal range (≤ 6.05%), whereas patients in the conventional therapy group had no specific goal except to prevent hyperglycemia or hypoglycemia, and received 1-2 daily self-injections of insulin. The baseline A1c for both groups was 9.1%, but at the conclusion of the study the intensive insulin therapy group's mean A1c had decreased to 7.4% compared to no change in the conventional insulin therapy group. In the primary prevention cohort the risk of developing retinopathy was reduced by 76% (95% CI: 62-85%) and in the secondary prevention cohort the progression of retinopathy was slowed by 54% (95% CI: 14-67%) in the intensive insulin therapy groups. Collectively, these studies laid

the groundwork for encouraging tight glycemic control in all diabetic patients in order to minimize the complications associated with hyperglycemia.

However, there are still shortcomings which remain to this approach. A major concern with the use of an intensive insulin regimen is the risk of severe hypoglycemic episodes, with accompanying risks of injury or neurological damage. In the DCCT, the risk of severe hypoglycemic episodes was approximately three times greater in the intensive insulin therapy group compared to the conventional therapy group. Additionally, the DCCT investigators observed a 33% increase in the mean adjusted risk of becoming overweight in the intensive therapy group compared to the conventional therapy group. Furthermore, practical application of intensive insulin regimens is often difficult in clinical settings due to the relatively large resources needed for patient education as well as their fundamental dependence on patient motivation and compliance. Intensive regimens also require more frequent monitoring and insulin administration, and although the development of insulin pumps have made insulin administration more simple there is still significant daily time and care involved. Finally, especially during adolescence some patients are not compliant with therapy, and during these times tight hyperglycemic control may be especially difficult [122].

Several general fields of research have become prominent in the search for alternatives to lifelong insulin therapy for type I diabetics: particularly islet transplantation to restore lost β cell mass and insulin production, and immunotherapy to establish tolerance and allow for endogenous β-cell regeneration. Although allogeneic islet transplantation has shown some evidence of success, the allo- and autoimmune response against the islets often leads to their destruction [123, 124]. Therefore patients receiving grafted islets require lifelong immunosuppressive therapy, which carries significant side effects. Due to the need for lifelong

immunosuppression, whole-pancreas transplant is rarely performed alone, and more frequently performed in T1D patients undergoing kidney transplant for diabetic nephropathy. The widespread applicability of both of these techniques is limited by the supply of islets from cadaveric donors, and in general both procedures have been offered only to T1D patients who have hypoglycemic unawareness, severe metabolic or secondary complications, or are unable to follow an insulin regimen [125]. Immunotherapy strategies, which promote tolerance toward the β cells, are currently an area of intense research because of their potential ability to save the patient's own remaining β cell mass, to possibly allow for β cell regeneration or pancreatic islet transplant.

1.4.2 Immunomodulatory Therapy for Type 1 Diabetes

Over the past 30 years, significant efforts have been made to test the efficacy of immunomodulating therapies in T1D. An ideal immunomodulating agent for diabetes would specifically halt β cell destruction without causing systemic immunosuppression or inhibiting the process of β cell regeneration. It has been demonstrated in both humans and NOD mice that at the onset of clinical diabetes there is still significant residual β-cell mass, and early intervention with an effective immunotherapy during this period has the potential to restore tolerance and allow endogenous cells to regenerate the islets [126]. However, some immunosuppressive drugs used in common islet transplant protocols have been shown to inhibit spontaneous regeneration of β cell mass *in vivo,* and therefore drugs intended for this application must be carefully tested for their effects on β cell growth *in vivo* [127]. Two general approaches to immunomodulation have been tried in T1D: non-specific agents as well as islet antigen-specific approaches to promote tolerance to β cells.

Non-specific agents such as cyclosporine, methotrexate, anti-thymocyte globulin, and BCG vaccination have been tested for the prevention of T1D onset, and have proven unsuccessful either due to poor side effect profiles or lack of efficacy [128]. Recent trials of the anti-CD3 immunoglobulin have been promising, as patients treated with a single dose of the anti-CD3 antibody had increased C-peptide responses to meals for 1 year or longer after treatment, reduced hemoglobin A1c levels, and reduced need for insulin supplementation [129]. However, there are also reports of side effects with anti-CD3, notably an influenza-like syndrome and Epstein-Barr viral mononucleosis [128]. Trials are currently underway investigating the use of rituximab, an anti-CD20 antibody that downregulates the B cell signaling of T cells. In preclinical trials using NOD mice, rituximab was shown to prevent and reverse diabetes onset by altering the levels of diabetogenic T cells [130, 131].

Preventative vaccination to self-antigens is an extremely attractive idea for the treatment of autoimmune diseases such as diabetes, but several hurdles remain before these therapies can be applied clinically. First, an antigen-specific vaccination product must have an excellent safety profile, including the ability to reliably tolerize and never stimulate the recipient's immune response towards autoimmunity. Second, despite extremely promising preclinical results in the NOD model of T1D, the therapeutic efficacy of these products for diabetes have yet to be shown in clinical trials with diabetes patients. For example, in the diabetes prevention trial (DPT-1), systemic or oral administration of insulin given to high-risk relatives of T1D patients had no efficacy in preventing diabetes onset when compared to matched control group [132]. Other trials examining the benefits of an intranasally administered insulin, as well as an intramuscular alum-Gad65 preparation, have shown some initial promise and work is ongoing to investigate the ultimate benefit of these methods [133, 134]. A recent clinical trial examining subcutaneous

injection of an Alum-GAD formulation showed a 15-month delay in the loss of secreted C-peptide levels, but ultimately did not impact the insulin requirement of patients receiving therapy [135]. Therefore, although tolerance induction to islet-specific antigen remains an intriguing possibility, there has not been a convincing demonstration of the efficacy of this approach.

Collectively, these studies illustrate the challenges facing scientists in finding appropriate immunotherapy for T1D. At this point, it remains unclear which of these agents are the most beneficial at halting the progression of autoimmunity as many underlying aspects of the pathogenesis of T1D have yet to be elucidated. An additional challenge in developing human therapies is the clinical heterogeneity of human disease compared to the homogeneity of inbred mouse models. Indeed, many interventions that have prevented diabetes in preclinical models such as the NOD mouse ultimately prove unsuccessful as clinical therapy [136]. As a result of this dismaying fact, Roep, Atkinson, and von Herrath have suggested that results from animal models such and the NOD mouse and knockout strains be considered similarly to clinical case reports, and that prospective therapies should be verified in multiple animal models prior to transitioning into clinical studies [137].

1.4.3 Role of DC in Type 1 Diabetes

Recent research is elucidating an important role for DC in the initiation and maintenance of autoimmunity in T1D and other autoimmune diseases [138]. This may in part be a consequence of defects in the DC themselves in autoimmune-prone individuals. An increasing number of studies have demonstrated that in both T1D patients and the NOD mouse model the DC have defects which make them more prone to stimulate autoimmunity than tolerance [139]. DC from NOD mice have been demonstrated to have defects in their maturation status, as well as

hyperactive NF-κB signaling due to increased activity of the I-κB kinase activity [140, 141]. Further, DC from NOD mice have a defect in tryptophan catabolism in response to IFN-γ, which renders them less able to confer tolerence [142]. A small study in which T1D patients failed to mount appropriate antibody responses following administration of DC pulsed with experimental antigen seems to confirm the results seen in NOD mice, and suggests that T1D patients as well also have some defects in antigen-presentation ability of DC [143].

Given the data to support the role of DC in mediating between immunity and tolerance, it is perhaps unsurprising to find that imbalanced DC function contributed to the pathogenesis of T1D. Given the role DC play in regulating autoimmunity in the periphery, tolerogenic DC therapy is especially promising in autoimmune diseases such as T1D because it has the potential to reestablish antigen-specific peripheral tolerance.

1.4.4 Tolerogenic DC Therapy for Type 1 Diabetes

Dendritic cells have been of particular interest for applications in T1D because of their unique role in regulating T cell responses. Both adenoviral and lentiviral gene transfer of IL-4 to DC have been shown to have protective effects in the NOD model of T1D [15, 72]. In collaboration, our lab has shown that DC transduced with an adenoviral vector expressing IL-4 (DC/IL-4) are able to prevent the onset of disease in 5 and 10 week old NOD mice, and this was accompanied by an increase in the IL-4:IFN-γ and IL-10:IFN-γ cytokine expression rations within the pancreata of mice treated with DC/IL-4 [15]. Cruesot et al have demonstrated similar results using DC transduced with a lentivirus expressing IL-4, and further demonstrate that the ability of DC/sIL-4 to prevent diabetes onset required MHC II expression on the DC [72].

Others have shown that modifying DC with an adenoviral vector encoding for galectin-1, a lectin with regulatory effects on T cells, could delete diabetogenic T cells *in vivo* [144]. Furthermore, non-viral approaches for transduction of DC have also been particularly successful. NF-κB decoy oligoneucleotide treatment of DC was shown to inhibit DC activation and maturation, inhibit the expression of CD40, CD80 (B7-1), and CD86 (B7-2) costimulatory molecules, and prevent the onset of diabetes in the NOD mouse model [145]. Further studies in NOD mice examining the ability of DC treated with antisense oligonucleotides directed at CD40, CD80 (B7-1) and CD86 (B7-2) costimulatory molecules have proven particularly successful, and these studies have progressed into clinical trials [77].

To date, few proposed gene therapy strategies for T1D have progressed to the point of clinical trials. However, a Phase 1 clinical trial to examine safety is currently ongoing using genetically modified DC in which antisense oligonucleotides (AS-ODN) targeting of mRNA for the costimulatory molecules CD40, CD80 (B7-1), and CD86 (B7-2) results in a tolerogenic DC phenotype with very low expression of the targeted costimulatory molecules. In preclinical studies using the NOD mouse model of T1D, investigators demonstrated that the AS-ODN treated bone-marrow derived DC were able to delay the incidence of diabetes after a single injection, and observed that the AS-ODN treated DC resulted in an expansion of a CD4+CD25+CD62L+ regulatory T cell population [77]. It is believed that the lack of costimulatory molecules on the DC result in an anergizing signal to diabetogenic T cells, as well as expansion of regulatory T cells. In the ongoing clinical study, autologous DC are generated from T1D patient's leukocytes *in vitro* after harvest by leukapheresis. The DC are cultured and engineered using the AS-ODN to CD40, CD80, and CD86 and then injected intradermally at a site distal to the pancreas to allow for DC migration into the lymph nodes. At the time of

writing, the Phase I trial is currently ongoing and is focused on the safety of this approach in a patient population that is over 18 and has had insulin-dependent T1D for greater than five years [146]. If successful, the next step will be a Phase II trial focused on efficacy of treatment in newly-diagnosed T1D patients, fitting with the paradigm that early intervention with immunotherapies is most effective at saving any residual β□cell mass.

1.5 DELAYED TYPE HYPERSENSITIVITY

There are four types of immune-mediated hypersensitivity reactions, which as a group cause significant disease burden. Types I, II, and III are mediated by antibodies and can be distinguished from each other based on the type of antigen involved as well as the antibody class effecting the hypersensitivity response. Type IV hypersensitivity reactions are T cell mediated and can be divided into three groups based on the T cell effector population. A summary of the different types of hypersensitivity reactions can be found in Table 3.

As shown in Table 3, DTH responses are mediated by CD8+ T cells and Th1 CD4+ T cells. There are two phases to DTH responses: sensitization and elicitation (summarized in [147, 148]). During sensitization, resident cutaneous DC, termed Langerhan's cells, take up antigen and migrate to the draining lymph node where they activate resident T cells, which generates a memory T cell response. Subsequent migration of these antigen-specific memory T cells results in their distribution throughout the dermis. During the elicitation phase of the DTH response, re-exposure to the sensitizing antigen results in the release of cytokines such as IFN-γ and IL-17 from memory T cells once recognized within the MHC complex. This stimulates the release of inflammatory mediators from nearby keratinocytes, such as IL-1, IL-6, TNF-α , GM-CSF, and

Table 3: Coombs and Gell classification system for hypersensitivity reactions.*

	Type I	Type II	Type III	Type IV	
Example of hypersensitivity reaction	Asthma, allergic rhinitis, systemic anaphylaxis	Some drug allergies (ie: hemolytic anemia), chronic urticaria (involving antibody formation to FCεR1α)	Serum sickness, Arthus reaction	"classic" DTH (ie: tuberculin skin reaction), contact dermititis	Contact dermitits
Immune Reactant	IgE	IgG directed at antigens on tissues	IgG directed at soluble antigens	Th1 cells	CTL
Antigen	Soluble	Cell- or matrix-associated	Soluble	Soluble	Cell-associated
Effector Mechanism	Mast-Cell Activation	Complement, FcR+ Cells (Phagocytes)	Complement, FcR+ Cells (Phagocytes)	Macrophage Activation	Cytotoxicity

***Modified from "Janeway's Immunobiology," 7th ed. Murphy K, Travers P, Walport M (eds.), p 556.**

chemokines, including CXCL8 (IL-8 in humans, MIP-2 in mice), CXCL11 (IP-9), CXCL10 (IP-10), and CXCL9 (Mig). These cytokines and chemokines induce the migration of additional monocytes and T cells into the lesion, amplifying the response.

Both the classical DTH response (that seen in the purified protein derivative (PPD), or tuberculin skin test) and contact hypersensitivity are examples of delayed-type hypersensitivity reactions. The classical DTH response is characterized by local erythema, induration and dermatitis. It is used clinically for diagnostic purposes to test the presence of memory T cells to antigen, such as tuberculosis-derived antigen in the case of the tuberculin skin test. Contact hypersensitivity is also characterized by erythema, but due to the more superficial distribution of antigen within the skin, indraepidermal abscesses and vesicle formation occurs. Contact hypersensitivity can occur in response to a wide variety of antigens, but a classical example is the reaction that develops in response to the chemical pentadecacatechol produced by the poison ivy plant. Finally, gluten-sensitive enteropathy, or celiac disease is also a form of delayed-type hypersensitivity which is directed at the gliadin antigen in wheat and other grains [149].

Given the multicellular and complex nature of the DTH response, it is perhaps not surprising that it can be disrupted at multiple points during both the sensitization and effector phases. Induction of DTH can be impaired by deficiencies in antigen presentation, for example young male SJL mice do not initiate DTH responses, however following adoptive transfer of a I-A+ Mac-1+ Mac-2- Mac-3+ cell population from female mice effective sensitization and normal DTH response occurs [150]. Splenic DC or Langerhan's cells pulsed with KLH antigen *ex vivo* effectively prime mice for the DTH response when injected subcutaneously, but elicit the production of IgG and do not prime for the DTH response if injected intravenously [151, 152].

These studies illustrate the importance of APC and presentation of antigen in the local lymph nodes in order for sensitization to antigen to occur.

Experiments comparing the ability of Th1 and Th2 cell subsets to participate in the effector phase of the DTH response have demonstrated that only Th1 cells elicit antigen-specific swelling [153], and that treatment with anti-IFN-γ blocking antibody is able to partially inhibit the DTH response [154, 155]. Due to its critical role in the production if IFN-γ, IL-12 is also necessary during the effector phase of the DTH response, and IL-12 knockout mice have significantly reduced DTH responses compared to wild-type controls [156]. Furthermore, exposure to UV radiation induces release of cytokines including IL-4 and IL-10, which can downregulate the levels of inflammation seen following challenge with sensitized antigen. Therefore, it is possible to limit either the sensitization and elicitation phases of the DTH response are effective methods to reduce the severity of disease.

2.0 DENDRITIC CELLS TRANSDUCED TO EXPRESS IL-4 PREVENT THE ONSET OF HYPERGLYCEMIA IN NOD MICE

2.1 ABSTRACT

Interleukin-4 (IL-4) has been demonstrated to be of significance in the pathogenesis and treatment of type 1 diabetes. We and others have demonstrated that transduction of DC with IL-4-expressing vectors can prevent the onset of diabetes in mice, however, there has yet to be a demonstration that DC transduced with IL-4 could reverse disease once defects in glucose homeostasis were demonstrated. DC were generated from the bone marrow of NOD mice and transduced with adenoviral vectors encoding soluble murine IL-4 (DC/sIL-4), a membrane-bound IL-4 construct, or empty vector control. Female NOD mice were segregated into normoglycemic (<150mg/dL) and prediabetic groups (between 150 and 250 mg/dL) on the basis of blood glucose measurements, and randomized for adoptive transfer of DC or exosome therapy via a single i.v. injection. We find a single injection of DC/sIL-4, when administered to normoglycemic 12-week old NOD mice, is capable of significantly preventing disease onset. Furthermore, DC/sIL-4 also prevent the onset of disease in prediabetic NOD mice between the ages of 12-16 weeks of age. This requires the production of soluble IL-4, as DC transduced with the membrane-bound IL-4 vector had no therapeutic effect. We further demonstrate that DC/sIL-4 significantly reduce islet mononuclear infiltration, and increase the expression of

FoxP3 in the pancreatic lymph nodes of a subset of animals. Further, DC/sIL-4 treatment alters the antigen-specific Th2:Th1 cytokine profiles as detected by ELISPOT of splenocytes in treated animals as compared to control animals. Exosomes derived from transduced DC were not capable of delaying diabetes onset when adoptively transferred to 12-week-old mice. We conclude that adoptive transfer of DC transduced to express IL-4 offers significant promise for the treatment of autoimmune diabetes, and should be further developed.

2.2 INTRODUCTION

Type I diabetes (T1D) is a polygenic autoimmune disease characterized by destruction of insulin-secreting β-cells in the islets of Langerhans by a chronic inflammatory infiltrate. In both humans and the nonobese diabetic (NOD) mouse, there is evidence that destructive insulitis is a T-cell mediated phenomenon, however, a growing body of evidence suggest that other cells types, such as NKT cells and antigen presenting cells (APC), play important roles in modulating disease onset and progression [138, 157, 158]. Although the exact pathogenesis remains unclear at this point, a combination of factors including a Th1-skewed CD4+ response as well as a deficiency of regulatory T cells are considered to be important hallmarks of the disease [157].

A shift in the Th1/Th2 balance of T cell responses towards characteristic Th1 properties such as the release of IFN-γ plays an important role in the initiation and perpetuation of the inflammatory response in diabetes. Furthermore, it has been demonstrated that shifting the balance of CD4+ responses towards a Th2-mediated response can have a therapeutic role. Numerous studies have demonstrated that delivery of exogenous IL-4, which polarizes T cell

responses towards a Th2 phenotype, has therapeutic potential for the treatment of type 1 diabetes. Systemic administration of recombinant IL-4 in NOD mice has been demonstrated to increase Th2-mediated responses in the spleen, thymus and pancreatic islets [159]. Transgenic NOD mice expressing IL-4 in the pancreatic islets are protected from the development of diabetes, and we have previously reduced onset of hyperglycemia in NOD mice after regulated delivery of IL-4 to pancreatic β cells following transduction with a double-stranded AAV vector expressing IL-4 under the control of the mouse insulin promoter [160]. Furthermore, we and others have shown that adoptive transfer of dendritic cell (DC) transduced *ex vivo* with IL-4-expressing viral vectors traffic to the spleen and pancreatic lymph node of NOD mice and are capable of preventing diabetes onset [15, 72].

DC are APC which play a critical role in the development of central tolerance and the maintenance of peripheral tolerance by presenting foreign- or self-derived antigens in the context of instructive secondary signals to T cells. Results from both NOD mice and patients with type 1 diabetes document abnormalities in DC function such as increased NF-κB activity, decreased expression of indoleamine-2,3-dioxygenase, and altered costimulatory and cytokine secretion profiles [141, 142, 161, 162]. Further, DC are responsible for the presentation of islet-cell derived antigens to diabetogenic T cells, as well as to regulatory T cell populations within the pancreas and pancreatic lymph node [163-165]. Thus, there is considerable interest in the development of DC-based therapy for type 1 diabetes. In addition to transduction with IL-4, others have demonstrated therapeutic benefit of other DC modifications, including: transduction with galectin-1, treatment with NF-κB-specific oligodeoxyribonucleotide, and antisense oligonucleotide blockade of costimulatory molecule expression [77, 78, 144]. At the time of writing, Phase I safety trials are currently underway testing costimulatory molecule-depleted DC

in type 1 diabetes patients, and trials of DC-based therapies in cancer patients have demonstrated the overall feasibility of *ex vivo* DC modification for immunotherapy [146, 166].

In the NOD model, insulitis typically begins around 3-4 weeks of age, and diabetes occurs in 60-80% of female mice between 12-18 weeks of age depending on the housing conditions. The NOD model has been used to test a large number of potential therapeutic interventions, many of which have ultimately had little to no clinical benefit [136]. One reason for this is that while many interventions are tested and successful in preventing diabetes onset in younger mice, they fail to prevent diabetes in older NOD mice with more advanced insulitis or are ineffective in clinical trials. We were therefore particularly interested in examining the therapeutic potential of DC in late stages of disease in the NOD mouse.

In this study we engineered syngeneic bone-marrow derived DC to overexpress soluble IL-4 (DC/sIL-4). In addition, exosomes were derived from DC/sIL4 and the abilities of these DC and DC-derived exosomes to prevent the onset of diabetes in 12-week old NOD mice were examined. We demonstrate that these DC/sIL-4 induce changes in the balance of Th2 vs Th1 effector profiles in splenocytes of treated mice, and induce upregulation of FoxP3 expression in the PLN of a subset of treated mice. We further demonstrate that DC/sIL-4 prevent the onset of hyperglycemia when administered to 12-16 week-old NOD mice that demonstrate impaired response to glucose challenge consistent with prediabetes. Lastly, we examined the exosomes secreted by the genetically engineered DC in order to determine if they could similarly regulate disease progression when systemically administered to 12-week old NOD mice, however our results indicate that DC-derived exosomes have little therapeutic effect in this model. Taken together, our results indicate that DC/sIL-4 prevents islet destruction in late stages of type 1 diabetes through modulation of T cell responses.

2.3 RESULTS AND DISCUSSION

2.3.1 Characterization of DC transduced with IL-4-expressing vectors.

We and others have previously shown that adenovirus transduction is an efficient means to transfer genes to DC, and that DC transduced to express IL-4 are therapeutic in preventing disease onset using mouse models of collagen-induced arthritis and delayed-type hypersensitivity [16, 167]. After transduction with an adenoviral vector expressing soluble murine IL-4 we observe significantly higher levels of IL-4 expression in the DC/sIL-4 than in the control or membrane IL-4 transduced DC culture conditions (Figure 1). This is consistent with efficient gene transfer to DC using the adenoviral vector.

We next examined the phenotype and maturation status of the DC by FACS in order to determine if infection with adenovirus resulted in significant differences in DC maturation. Our culturing method results in a population of DC that is highly CD11c (>70%) and CD11b (>50%) positive (data not shown). We examined CD40 expression in all four populations, and find that ~40% of DC were positive for CD40 regardless of whether they non-transduced or transduced with any one of the three vectors examined (data not shown). As shown in Figure 2, we further observe that transduction with a control adenoviral vector (DC/ψ5) increases surface expression of B7-1, B7-2, and PD-L2, and decreases the expression of PD-L1. DC/mbIL-4 demonstrated a similar pattern of changes in surface costimulatory molecule expression. However, DC/sIL-4 have decreased expression of B7-1 when compared to the DC/ψ5 or DC/mbIL-4, to levels more similar to those seen in the non-transduced DC. Although transduced DC upregulate costimulatory molecules due to adenoviral infection, the high levels of IL-4 secreted by the DC/sIL-4 may have an autocrine regulatory effect on the balance of B7 molecules expressed by

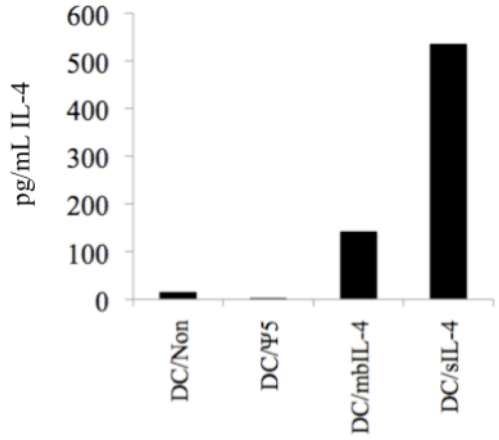

Figure 1: DC Transduced with Ad.sIL-4 Secrete High Levels of IL-4 in Culture.

After harvest on day 8, $1x10^6$ DC were plated per well in OPTI-MEM media in a 96-well plate. Following 24 hours of culture, supernatants were analyzed by ELISA for levels of IL-4 secretion.

the DC. This is consistent with previous findings whereby IL-4 regulates cytotoxic CD8+ T cell responses is by modulating the levels of B7-1 and B7-2 on DC [168].

2.3.2 DC expressing soluble IL-4 delay the onset of hyperglycemia in 12-week-old NOD mice.

Female NOD/ShiLTJ mice were screened beginning at 11 weeks of age for the development of hyperglycemia. Most NOD mice will develop severe insulitis by 10 weeks of age, and in our experiments a small percentage (<10%) of mice will develop overt hyperglycemia by 12 weeks of age [157]. At 12 weeks, mice with blood glucose measurements <150 mg/dL were randomized to receive a single tail vein injection of 10^6 DC/sIL-4, 10^6 DC/mbIL-4, 10^6 DC/ψ5, or saline alone. Blood glucose levels of mice were followed weekly and the percentage of mice developing hyperglycemia is shown in Figure 3. Treatment with DC/sIL-4 conferred significant protection ($p=0.05$ using the log-rank test, $p=0.02$ using the Wilson rank test), as at 30 weeks of age only 50% of mice treated with DC/sIL-4 had developed diabetes as compared to >90% of saline-treated mice. There was no therapeutic benefit seen in mice treated with DC/mbIL-4 or DC/ψ5, which were not statistically significant from mice treated with saline alone ($p>0.05$ for both groups using log-rank and Wilson rank tests).

2.3.3 Evaluation of insulitis by histological analysis.

12-week old normoglycemic mice received a single tail vein injection of 10^6 DC/sIL-4, 10^6 DC/mbIL-4, 10^6 DC/ψ5, or saline alone and sacrificed at 15 weeks of age. Insulitis was evaluated by histology in order to determine the effect of treatment with DC/sIL-4 on

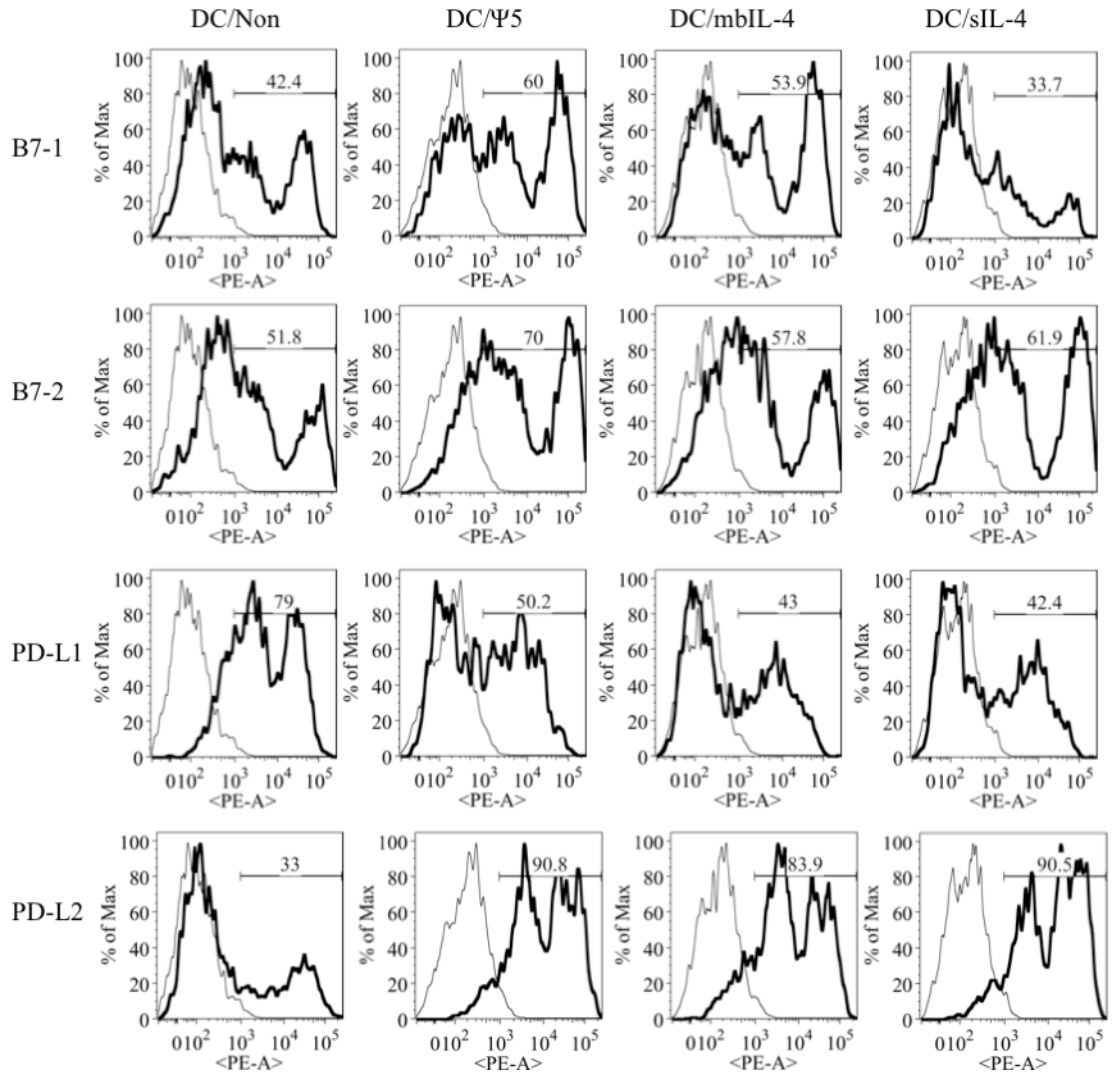

Figure 2: Characterization of DC Used for Adoptive Transfer.

DC transduced with adenoviral vectors expressing soluble IL-4 (DC/sIL-4) or membrane-bound IL-4 (DC/mbIL-4) or empty adenoviral vector (DC/ψ5) were compared to non-transduced DC (DC/Non). After harvest on day 8, $1x10^6$ DC were plated per well in OPTI-MEM media a 96-well plate. Following 24 hours of culture, supernatants were analyzed by by FACS. Plots based on live DC as determined by FSC vs. SSC profiles. Thin lines = isotype control stained samples, bold lines = anti-B7-1, -B7-2, -PD-L1, or -PD-L2 antibodies, as shown.

mononuclear cell infiltration into the islets. Three weeks following treatment, mice treated with DC/sIL-4 had sparsely infiltrated islets, with a majority of islets demonstrating no mononuclear cell infiltration or peri-insulitis (Figure 3A & B). In contrast, mice treated with DC/ψ5 had infiltrative insulitis in slightly greater than half of the islets (Figure 3C & D). Mice receiving saline alone had infiltrative insulitis in greater than 75% of all islets examined, with 50% of all islets completely infiltrated (Figure 3E &F). The insulitis score (Figure 3G) at 15 weeks of age in DC/sIL-4 recipient mice was lower (1.45 ± 0.93) than in mice receiving DC/ψ5 (2.51 ± 0.95) or saline (3.19 ± 0.86) and there was a statistically significant difference between the insulitis scores of the DC/sIL-4 treated and saline treated animals (p=0.024). The percentage of total intact islets and those with mild peri-insulitis in the DC/sIL-4 treatment group was 60%, which was twice the amount seen in the DC/ψ5 treated group (33.6%) and three times as many as that seen in the saline treated group (17.7%).

2.3.4 DC/sIL-4 treatment increases IL-4 secretion by islet-antigen specific T cells.

The balance of Th1 and Th2 cytokines plays an important role in the progression of autoimmune diseases, and in type 1 diabetes, a Th1-skewed balance of T cell responses contributed to the pathogenesis and progression of the disease [169-171]. Treatment with IL-4 has been shown to maintain Th2 CD4+ T cell responses that inhibit the progression of diabetes, and therefore we investigated whether DC/sIL-4 treatment had a beneficial effect on Th1/Th2 balance [159]. Normoglycemic 12-week-old NOD mice were treated with 10^6 DC/sIL-4, 10^6 DC/mbIL-4, 10^6 DC/ψ5, or saline alone, and at 15 weeks of age the frequency of splenic cells secreting IFN-γ or

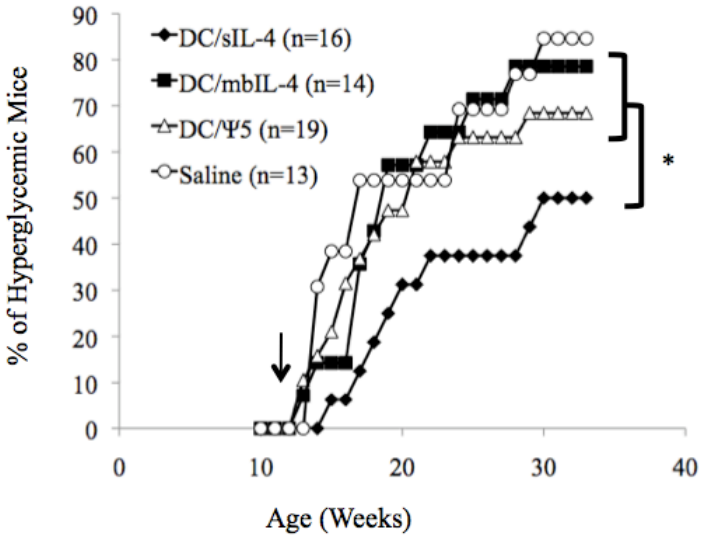

Figure 3: Incidence of Diabetes Following i.v. Administration of DC Therapy to 12-week-old Normoglycemic Female NOD Mice.

Mice were screened beginning at 11-weeks of age, and only mice with blood glucose measurements less than 150 mg/dL were randomized to receive a single tail vein injection of either $1x10^6$ DC/sIL-4, $1x10^6$ DC/mbIL-4, $1x10^6$ DC/ψ5, or saline. Combined results from 3 identical, independent experiments are shown. * denotes significance of p≤0.05 by Wilcoxon's test of DC/sIL-4 compared to DC/mbIl-4, DC/ψ5, and saline-treated groups. The p-value of comparisons between the DC/mbIl-4, DC/ψ5, and saline-treated groups is greater than 0.05.

IL-4 in response was assessed by ELISPOT. Insulin, $Gad65_{206-222}$, and NIT-1 cell lystate (a NOD-derived β-cell line) were used to stimulate islet-specific cytokine secretion in the splenocytes from treated mice. As shown in Figure 5A, we did not see significant changes in the numbers of splenocytes secreting the Th1-accociated cytokine IFN-γ between treatment groups. In contrast, splenocytes from DC/sIL-4 treated mice exhibited a significant increase in the secretion of the Th2-associated cytokine IL-4 in response to all three antigens tested (Figure 5B). Therefore, we observed significant increases in the ratio of Th2/Th1 specific T cells after treatment with DC/sIL-4 in all three stimulation conditions, indicating that administration of DC/sIL-4 is able to induce changes in Th1/Th2 balance within NOD mice.

2.3.5 DC/sIL-4 treatment upregulates FoxP3 expression in a subset of mice

Three and four weeks following treatment with DC/sIL-4, mice were sacrificed and splenocytes analyzed by FACS to determine the proportion of CD4+ CD25+ FoxP3+ regulatory T cells within the spleen. Cells were analyzed for the expression of FoxP3 and results are expressed as a ratio of CD4+ CD25+ FoxP3+ to the total number of CD4+ splenocytes. As shown in figure 6A, our results indicate that DC/sIL-4 treatment did not have an effect on the levels of FoxP3+ regulatory T cells within the spleen.

We next examined the relative changes in gene expression in the pancreatic lymph nodes of mice three weeks after treatment with DC/sIL-4 or controls. The pancreatic lymph nodes (PLN) play an important role in the priming of inflammation in type I diabetes, and it has been demonstrated in numerous studies that adoptively transferred DC are capable of homing to both the spleen and PLN [144, 172]. In a recent studies comparing the gene expression profiles of NOD and NOD.B10 mice, it has been demonstrated that NOD mice loose PLN expression of IL-

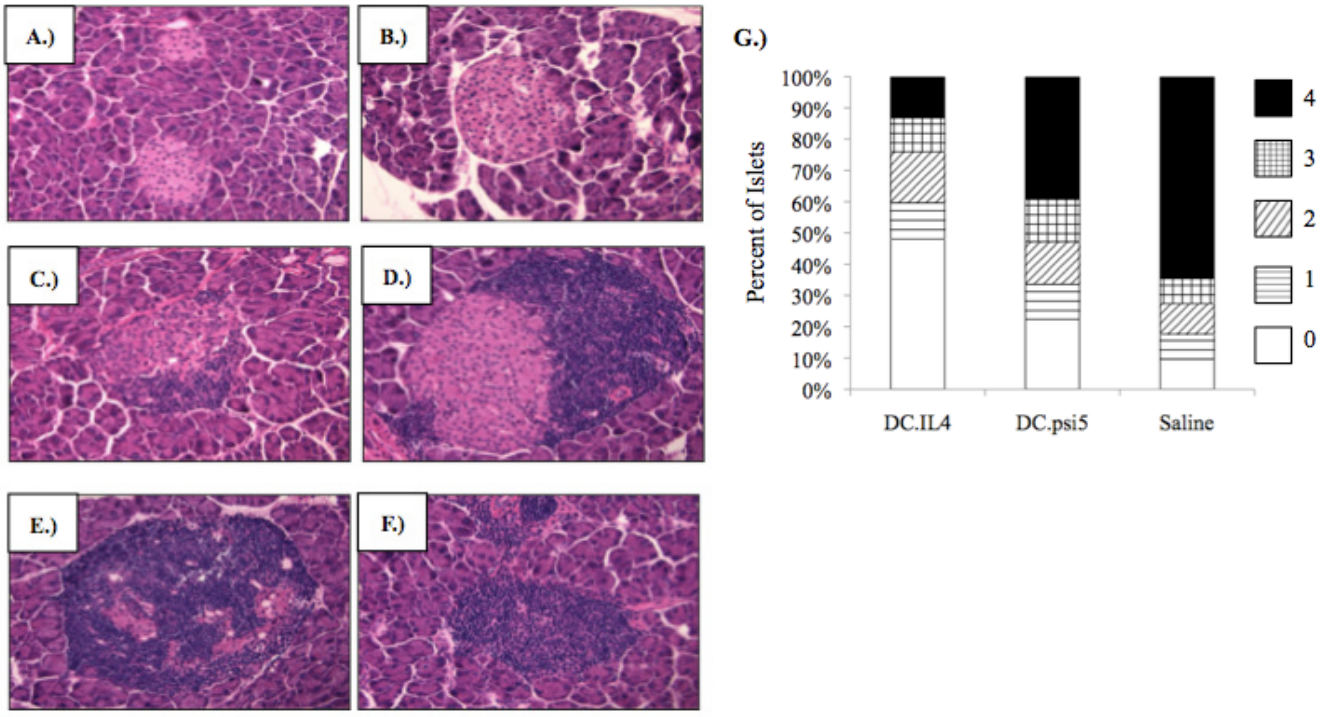

Figure 4: Histological analysis of insulitis in mice following adoptive DC gene therapy.

Normoglycemic mice received a single tail vein injection of either $1x10^6$ DC/sIL-4, $1x10^6$ DC/ψ5, or saline at 12 weeks of age and were monitored until 15 weeks, when they were sacrificed. Photomicrographs were taken of hematoxylin and eosin sections of pancreata collected from these mice. Mice treated with DC/sIL-4 show little or mild mononuclear infiltration surrounding the islets (A-B), whereas DC/ψ5 treated mice have moderate islet infiltration in the majority of islets (C-D), and the majority of mice treated with saline have severe insulitis (E-F). (G) The insulitis score was determined by a blinded investigator using the following scale: 0=no lymphocytic infiltration; 1=peri-insultits; 2=insulitis affecting less than 33% of the islet area; 3=insulitis affecting 33%-66% of the islet area; 4=insluitis affecting greater than 66% of the islet area. A minimum of 30 islets were evaluated per mouse on a minimum of 3 slides at least 100 mm apart. Statistical comparison between the groups using Student's t-test demonstrates: DC/sIL4 vs. Saline p=0.024, DC/sIL/4 vs. DC/ψ5 p=0.167, DC/ψ5 vs. Saline p=0.293.

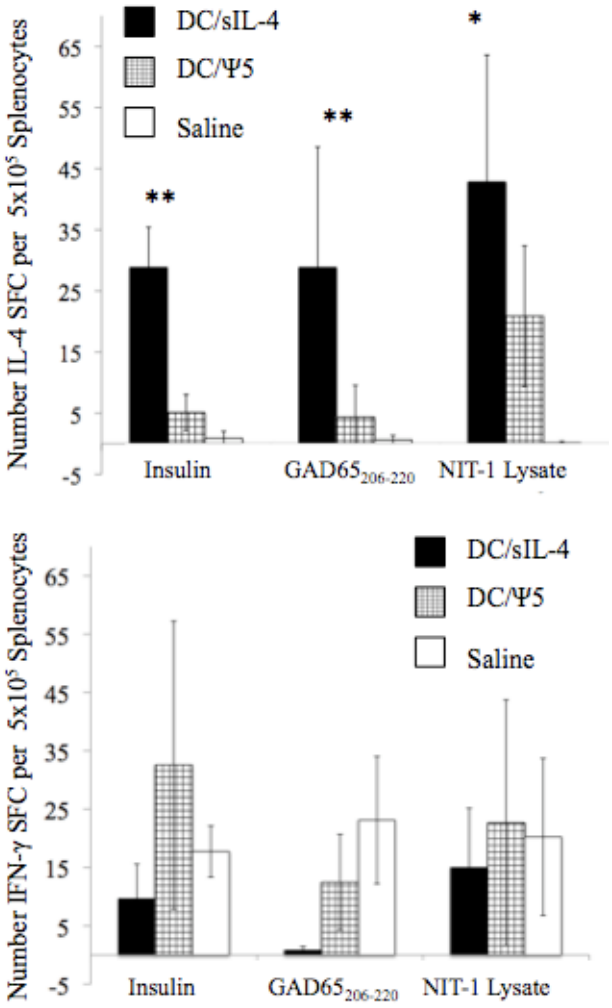

Figure 5: Antigen-specific IL-4 secretion by splenic T cells following treatment with DC overexpressing IL-4.

Normoglycemic mice received a single tail vein injection of either $1x10^6$ DC/sIL-4, $1x10^6$ DC/ψ5, or saline at 12 weeks of age and were monitored until 15 weeks of age, when they were sacrificed. Splenocytes were obtained from mice and assessed by ELISPOT for the frequency of splenic T cells secreting (A) IFN-γ, or (B) IL-4 in response to stimulation with $GAD65_{206-220}$, insulin, and NIT-1 cell lysate. Results shown are representative of three mice per group. Using student's t-test, ** denotes significance at $p<0.05$ of DC/sIL-4 treated group compared to both DC/ψ5 and saline treated groups, * denotes significance at $p<0.05$ of DC/sIL-4 group compared to saline alone.

4 over time compared to their NOD.B10 counterparts [72], therefore we were interested in determining if treatment with DC/sIL-4 would result in corresponding changes in the levels of gene expression when compared to DC/ψ5 or saline treated animals. We examined levels of IL-4, IFN-γ, and FoxP3 expression in the PLN and inguinal lymph nodes (ILN) of treated mice by quantitative real-time PCR (qRT-PCR). We examined the inguinal lymph nodes in order to ensure that baseline values of gene expression in the lymph nodes were not affected by DC-based gene therapy, and indeed in all of the experiments, the levels of gene expression in the inguinal lymph nodes of mice was equivalent between groups. Our results indicate that there are no changes in levels of IL-4 or IFN-γ expression within the PLN of treated animals, but we did detect robust upregulation of FoxP3 in the PLN of half of the DC/sIL-4 treated mice (Figure 6B). This could be consistent with induction of a regulatory T cell phenotype, or recruitment of regulatory T cells into the local environment.

2.3.6 A single administration of DC/sIL-4 prevents onset of hyperglycemia in prediabetic mice.

Although many interventions prevent the onset of type 1 diabetes in animal models, few are effective when administered late in the disease process [173]. This is problematic, as the clinical reality of diabetes treatment is that patients present once they are experiencing symptoms, and at that point they typically have advanced disease. Therefore, it is crucial to demonstrate that a therapy can be effective at preventing the onset of hyperglycemia when administered in the later stages of diabetes.

To this end, we monitored 12-16 week-old female NOD/ShiLTJ mice for the development of prediabetes, which we define as repeat random blood glucose measurement

between 150 mg/dL and 250 mg/dL. As shown in Figure 7A, intraperitoneal glucose tolerance testing of 14-week-old mice identified as prediabetic using this criteria have a reduced response to glucose during challenge, with higher levels of blood glucose at all time points compared to mice in the normoglycemic group. At 90 minutes, the blood glucose levels of the prediabetic group had still not returned to baseline, indicating an advanced state of insulitis and islet dysfunction.

While DC/sIL-4 was capable of preventing disease in mice with advanced insulitis, we were interested to see whether this therapy was efficacious in this prediabetic population of NOD mice. To this end, 12-16 week old mice prediabetic mice were randomized to receive a single tail vein injection of 10^6 DC/sIL-4, 10^6 DC/ψ5, or saline alone immediately after being classified as prediabetic. Mice were monitored weekly for the onset of hyperglycemia, and the results are shown in Figure 7B. DC/sIL-4 treatment significantly reduced the number of mice developing hyperglycemia compared to mice receiving DC/ψ5 or saline, indicating that even in more advanced stages of disease DC/sIL-4 can be an effective therapy to prevent the onset of type 1 diabetes.

2.3.7 Systemic administration of DC-derived exosomes does not delay the onset of hyperglycemia in 12-week-old NOD mice.

We have previously demonstrated that exosomes secreted by DC transduced with IL-4 are therapeutic in mouse models of delayed-type hypersensitivity and arthritis [16]. Despite a growing number of studies of exosome-based therapy for autoimmune disease, there have been no studies exploring the therapeutic potential of exosomes for type 1 diabetes to date. Therefore, we were interested in testing whether systemic administration of DC-derived exosomes would

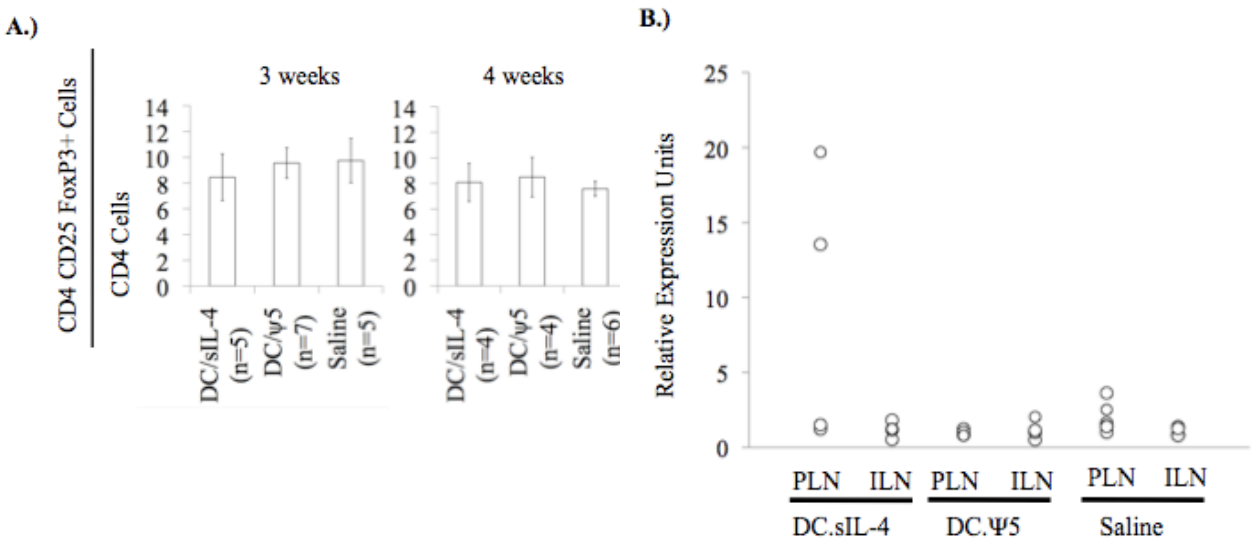

Figure 6: Analysis of regulatory T cell induction by DC/sIL-4 treatment.

Normoglycemic mice received a single tail vein injection of $1x10^6$ of either DC/sIL-4 or DC/ψ5, or saline at 12 weeks of age and were monitored until 15 or 16 weeks of age. (A) Splenocytes were obtained from mice 3 and 4 weeks post-treatment and analyzed by FACS for expansion of the CD4+CD25+FoxP3+ regulatory T cell compartment. Splenocytes were stained using isotype control and monoclonal antibodies to CD4, CD25 and FoxP3. Gates were set around live cells using FSC vs. SSC, then around CD4+ cells. Isotype control staining was used to set the FoxP3 gating, and the number of CD4+CD25+FoxP3+ cells per sample were collected and are represented as a ratio of total CD4+ cells counted in each sample. (B) FoxP3 gene expression from pancreatic (PLN) and inguinal lymph nodes (ILN) obtained from mice at 15 weeks of age was analyzed using real-time PCR. Expression of FoxP3 was normalized to the expression of beta-actin to give the relative expression, and samples from all mice were then normalized to the mean of the saline-treated ILN group.

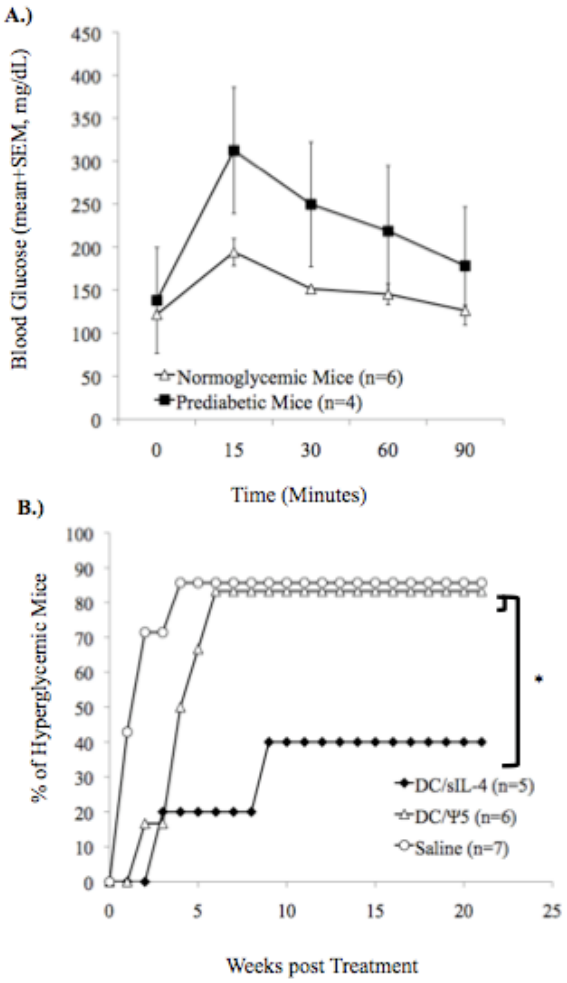

Figure 7: Incidence of diabetes following i.v. administration of DC therapy to 12-16 week-old prediabetic female NOD mice.

Mice were screened beginning at 11-weeks of age, and mice with blood glucose measurements between 150 and 250 mg/dL were considered prediabetic while mice with blood glucose measurements below 150 mg/dL were considered normoglycemic. (A) Normoglycemic and prediabetic mice were injected with 2mg of L-Dextrose per g of body weight, and their blood glucose measurements were measured at 15, 30, 60, and 90 minutes post injection. (B) Female prediabetic mice between 12 and 16 weeks of age were randomized to receive a single tail vein injection of either $1x10^6$ DC/sIL-4, $1x10^6$ DC/ψ5, or saline, and were then monitored for the development of hyperglycemia. * denotes significance of $p \leq 0.05$ by Wilcoxon's test of DC/sIL-4 compared to DC/ψ5, and saline-treated groups. The p-value of comparisons between the DC/ψ5 and saline-treated groups is greater than 0.05.

delay or prevent the onset of diabetes. Exosomes were isolated from the enriched culture media of DC using differential centrifugation. At 12 weeks, mice with blood glucose measurements less than 150 mg/dL were randomized to receive a single tail vein injection of 1 μg Exo/sIL-4, 1 μg Exo/mbIL-4, 1 Exo/ψ5, or saline alone. We have previously observed a dose of 1 μg to be effective at reducing disease when administered systemically by tail vein in collagen-induced arthritis and experimental autoimmune encephalomyelitis models. As shown in Figure 8, systemic administration of DC-derived exosomes had no long-term protective effects. Although there may be some marginal early benefit observed in the Exo/mbIL-4 groups, this effect is not robust and by 30 weeks of age there are no statistically significant differences between the groups.

2.4 CONCLUSIONS

A large number of interventions are effective at preventing the onset of diabetes in young NOD mice. However, very few are effective at preventing the onset of hyperglycemia when administered after 10 weeks of age or when administered once disease onset has occurred [136, 173]. Similar to previous results, we demonstrate the ability of IL-4 overexpressing DC to prevent diabetes onset in the NOD model [15, 72]. Our data indicate that DC/sIL-4 are capable of delaying disease onset (Figure 2) and reducing mononuclear infiltrate (Figure 3) in normoglycemic, but insulitic 12-week old mice. Our results are consistent with a recent report demonstrating that transduction of DC with a lentiviral vector expressing IL-4 prevents the onset of diabetes in 12-week old NOD mice [72]. However, we now have demonstrated that IL-4 transduced DC can prevent the onset of hyperglycemia in prediabetic mice (Figure 6B).

Screening NOD mice for elevated blood glucose allowed us to efficiently select mice with impaired response to glucose challenge, which is indicative of more advance disease (Figure 6A) [174]. Interestingly, our results indicate that DC/sIL-4 are as or more effective at reversing disease onset in the prediabetic NOD mice (60% diabetes free at 32 weeks of age compared to 14% of saline-treated mice) than 12-week-old normoglycemic mice (50% diabetes free at 32 weeks of age compared to 14% of saline-treated mice). Further, the therapeutic effect of the DC/sIL-4 is dependent upon their ability to secrete soluble IL-4, as we do not see any therapeutic benefit in mice receiving the DC/mbIL-4.

Use of replication-deficient adenoviral gene vectors to deliver IL-4 to DC resulted in increased IL-4 expression by DC as well as an altered DC costimulatory molecule phenotype (Figure 1). Our data indicate that adenoviral infection results in upregulation of the costimulatory molecules B7-1, B7-2, and PD-L2, consistent with other studies evaluating adenoviral gene transfer to DC [61, 175]. We further observed that the high levels of IL-4 secreted into the culture media result in nearly identical expression levels of B7-1 on the DC/sIL-4 and non-transduced DC. This is consistent with a previous report indicating that IL-4 treatment of DC leads to upregulation of B7-2 and downregulation of B7-1, and that DC treated with recombinant IL-4 decrease in the activity of diabetogenic CD8+ cells in a manner dependent on B7-2 on the DC [168]. Although viral transduction induces changes in expression of DC costimulatory molecules, we hypothesize that overexpression of IL-4 mitigates some of these effects, and the resulting increased B7-2:B7-1 ratio may contribute to the overall therapeutic effect of the DC/sIL-4 *in vivo*.

Our analysis of the mechanism of the therapeutic effect of DC/sIL-4 demonstrated that DC/sIL-4 administration to NOD mice results in increased islet-antigen specific secretion of the Th2-cytokine IL-4, whereas there were no changes in the levels of secreted Th1 cytokine IFN-γ

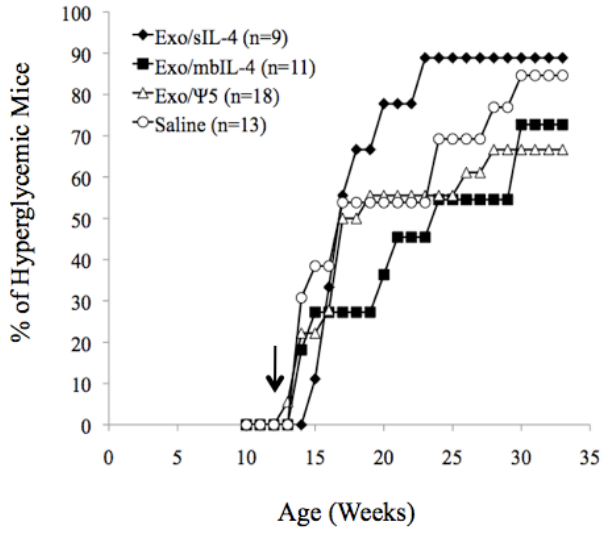

Figure 8: Incidence of diabetes following i.v. administration of DC-derived exosome therapy to 12-week-old normoglycemic female NOD mice.

Exosomes were isolated from the enriched media of DC cultures as described and resuspended in 500 μL of PBS at a dose of 1 μg. Mice were screened beginning at 11-weeks of age, and only mice with blood glucose measurements less than 150 mg/dL were randomized to receive a single tail vein injection of either 1 μg Exo/sIL-4, 1 μg Exo/mbIL-4, 1 μg Exo/ψ5, or saline. Combined results from 3 identical, independent experiments are shown. The p-value of comparisons between the Exo/sIL-4, Exo/mbIl-4, Exo/ψ5, and saline-treated groups is greater than 0.05.

(Figure 4). We hypothesize that this represents an alteration of the T cell responses within the treated animal, opposed to persistence of the DC/sIL-4 *in vivo* for several reasons. First, ELISPOT assays were conducted three weeks following the administration of the DC/sIL-4. Previous studies using DC expressing firefly luciferase as a reporter gene indicate that DC selectively traffic to the spleen and pancreatic lymph node of NOD mice and expression of luciferase in these tissues suggests the numbers of DC peak in these tissues between day 1 and day 3 and then wane significantly by day 8 post-injection [72]. Secondly, the frequency of the observed IL-4 spot forming units in our assays significantly exceeds the original dose of 10^6 DC/sIL-4 administered to the mice.

qRT-PCR analysis revealed that FoxP3 was significantly upregulated in the PLN of half of the mice receiving DC/sIL-4 (Figure 5B). IL-4 has been shown to play a role in the extrathymic induction of FoxP3+ regulatory T cells [176]. In addition, we have previously shown that transduction of the islets with an AAV vector expressing IL-4 under the control of the mouse insulin promoter results in expansion of the percentage of CD4+CD25+FoxP3+ regulatory T cells as opposed to saline-treated control animals [160]. However, splenocytes of DC/sIL-4 treated mice showed no significant changes in the percentages of CD4+CD25+FoxP3+ T-cells when compared to control treated animals either three or four weeks following treatment (Figure 5A). Thus the induction of regulatory T cells in the PLN may predict which animals respond positively to DC/sIL-4 therapy.

Lastly, we examined whether a single injection of 1 μg of exosomes derived from the supernatant of DC/sIL-4, DC/mbIL-4, or DC/ψ5 cultures would have any effect on the development of diabetes in normoglycemic 12-week-old NOD mice. However, in the NOD model, we did not see any remarkable therapeutic effects using a dose of 1 μg per mouse. There

are a number of reasons why this may be the case. While our previous results from the collagen-induced arthritis model indicate that systemic injection of DC-derived exosomes are capable of modulating inflammation and reducing the severity of disease in that model, a single dose of 1μg may be insufficient and larger quantity of exosomes or repeat dosing may be necessary [16, 115, 177, 178]. Furthermore, studies indicate that following i.v. injection, exosomes are internalized by splenic DC and hepatic kuppfer cells, which may be appropriate targets for some autoimmune diseases, such as arthritis, but not for others [104].

Collectively, our data demonstrate that adoptive transfer of DC/sIL-4 not only prevents the onset of diabetes in 12-week-old NOD, but can also maintain euglycemia in prediabetic mice ages 12-16 weeks of age that have demonstrated abnormalities in glucose homeostasis. This is the first demonstration that DC/sIL-4 are capable of preventing disease onset at such a late stage of diabetes, and provides further confirmation that this approach is clinically relevant. A patient's own DC could be harvested and transduced with adenovirus *ex vivo,* then administered via intravenous injection. As identification of patients at risk for type 1 diabetes improves, it may become increasingly possible to use a cell-based therapy such as DC/sIL-4 to prevent onset of disease in at risk individuals, or perhaps to intervene at the time of clinical diagnosis.

2.5 MATERIALS AND METHODS

2.5.1 Mice.

Female NOD/ShiLTJ mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free facility at the University of Pittsburgh according to US Department of Agriculture and National Institutes of Health guidelines. All experiments were conducted under protocols reviewed and approved by the Institutional Animal Care and Use Committee.

2.5.2 Vector construction and Adenovirus Generation.

Adenoviruses expressing soluble murine IL-4 (Ad.sIL-4) and membrane-bound (Ad.mbIL-4) and were constructed, propagated, and titered according to standard protocols as previously described [177]. The membrane-bound IL-4 construct is composed of IL-4 fused to the transmembrane domain of CD80. Recombinant adenoviruses were generated by homologous recombination in 293 cells expressing Cre recombinase (CRE8 cells), after co-transfection with a plasmid (ψ5) expressing the adenovirus 5-derived, E1- and E3-deleted adenoviral backbone and pAdlox, the adenoviral shuttle vector that expresses either sIL-4 or mbIL-4. Recombinant adenoviruses were purified by CsCl gradient ultracentrifugation, dialyzed in sterile virus storage buffer, aliquoted and stored at – 80^{o}C.

2.5.3 DC Generation.

BMDC were prepared following a bulk-culture protocol modified from Son, et al. as previously described [179, 180]. Briefly, bone marrow was collected from tibias and femurs of 6- to 7-week-old female NOD/ShiLTJ mice. Contaminating erythrocytes were lysed with ACK cell lysing buffer (Mediatech, Herdon, VA). Monocytes were collected from the interface after centrifuging on Nycoprep (NycoMed, Roskilde, Denmark) at 600 x g for 20 min at RT. Cells were cultured for 24 h in complete media (RPMI 1640 containing 10% FBS, 50 μM 2-β-Mercaptoethanol, 2 mM glutamine, 0.1 mM nonessential amino acids, 100 IU/mL penicillin/streptomycin) to remove adherent macrophages. Non-adherent cells were placed in fresh growth media (complete media containing 1000 U/mL of GM-CSF and IL-4) to generate DC. Cells were cultured for 4 days then harvested for adenoviral (Ad) transduction. For Ad infection, 1 X 10^6 DC were incubated with 5 X 10^7 PFU of the virus in 1mL of serum-free media for 2 hours, and then 10 mL complete media was added to the cells. After overnight incubation, DC were washed vigorously three times in PBS to remove excess virus and cultured for an additional 48 h in growth media. On day 8, culture supernatant was collected for exosome purification and DC were recovered. This infection method routinely gives us ~70-80% transfection efficiency using Ad.eGFP as a control [181].

2.5.4 FACS Analysis of DC.

Dendritic cells were preincubated with anti-CD16/32 (eBioscience, San Diego, CA), then stained with 1-2 μL of PE-labeled mAbs (B7.1 (16-10A1), B7.2 (PO3.1), PD-L1 (MIH5), PD-L2 (TY25), CD40 (1C10), CD11b (M1/70), and CD11c (N418), all from eBioscience, San Diego,

CA) in 100 µL total volume of ice-cold PBS containing goat serum. Following staining, cells were washed twice in FACS buffer, resuspended in 400 µL FACS buffer, and examined by FACS (FACScan, BD Biosciences, San Jose, CA). Results were analyzed using FlowJo (Treestar Inc. Ashland, OR).

2.5.5 ELISA Analysis of DC Supernatant.

Supernatants from DC were collected and analyzed using a sandwich ELISA to detect IL-4 (eBioscience San Diego, CA) according to the manufacturer's protocol. The limits of detection were 4 pg/mL.

2.5.6 Exosome Isolation.

Exosome isolation procedure has previously been described [115]. Briefly, DC culture supernatants were collected and centrifuged at 300 g for 10 min, 1200 g for 20 min, and 10,000 g for 30 min, and then ultra-centrifuged at 100,000 g for 1 h. Exosome pellet was washed in sterile PBS, centrifuged at 100,000 g for 1 h, and resuspended in 120 ml of PBS for further studies. Exosome batch protein content was quantified and standardized by a micro Bradford protein assay (Bio-Rad, Hercules, CA). 1 mg exosomes were suspended in 20 ml of PBS for *in vivo* mouse studies.

2.5.7 Adoptive Transfer of DC and Exosomes to NOD/ShiLTJ mice.

Blood glucose levels of NOD/ShiLTJ mice were monitored using blood drawn from the tail vein and analyzed via a glucometer (Ascencia BREEZE; Bayer). Mice with blood glucose readings below 150 mg/dL were considered normoglycemic. Mice with repeat measures between 150 and 250 mg/dL on consecutive days were considered prediabetic, and mice with repeat measures greater than 300 mg/dL on consecutive days were considered hyperglycemic.

To test the ability of treatment to delay diabetes onset, normoglycemic 12-week old female NOD/ShiLTJ mice were randomized to received a single tail vein injection containing one of the following: 10^6 DC therapy, 1 μg exosomes therapy, or PBS alone. For studies examining the ability of DC therapy to prevent diabetes in prediabetic mice, mice 12-16 weeks of age and with repeat blood glucose measurements between 150 and 250 mg/dL were randomized to received a single tail vein injection containing either 10^6 DC therapy, or PBS alone.

2.5.8 Intraperitoneal Glucose Tolerence Test.

Mice were fasted for 12 hours, and then weighed. Baseline blood glucose measurement was determined prior to i.p. injection with L-Dextrose (Sigma-Aldrich, St. Loius, MO) suspended in PBS at a dose of 2 mg per gram of body weight. Repeat blood glucose measurements were determined at 15, 30, 60, and 90 minutes following dextrose injection.

2.5.9 Histology and Islet Grading.

Pancreata were collected from 15-week old mice following DC therapy at 12 weeks of age, and were fixed, and embedded. 5 mm sections were deparaffinized, stained with hematoxylin and eosin, and evaluated by a blinded investigator for infiltrating mononuclear cells within the islets. Individual islets were assigned scores based on their appearance: 0=no lymphocytic infiltration; 1=peri-insultits; 2=insulitis affecting less than 33% of the islet area; 3=insulitis affecting 33%-66% of the islet area; 4=insluitis affecting greater than 66% of the islet area. The histology score index per mouse was calculated by dividing the sum of all individual islets scored by the total number of islets evaluated, and was used to represent each mouse in statistical testing. A minimum of 30 islets were evaluated per mouse on a minimum of 3 slides at least 100 mm apart.

2.5.10 Evaluation of splenic antigen-specific responses by ELISPOT and splenic regulatory T cell levels.

15-week old mice were sacrificed following DC therapy at 12 weeks of age. Spleens were collected and splenocytes immediately extracted. Contaminiating erythrocytes were lysed with ACK lysis buffer (Mediatech, Herdon, VA).

For ELISPOT assay, $5x10^5$ splenocytes per well were stimulated with $GAD65_{206-220}$ (50 ug/mL, Anaspec, Freemont, CA), insulin (50 ug/mL Sigma-Aldrich, St. Loius, MO), NIT-1 freeze-thaw cell lysate (20 ug/mL), or OVA protein (50 ug/mL Sigma-Aldrich, St. Loius, MO) in serum-free media in 96-well ELISPOT plates precoated with an anti-IL-4 or anti-IFN-γ mAbs. ELISPOT plates were cultured for 36 hours followed by incubation with biotin conjugated-anti-IL4 or anti-IFN-γ mAb, and development with streptavidin-peroxidase and 3-amino-9-

ethylcarbazole according to manufacturer's recommendations (BD Biosciences, San Jose, CA). Spots were counted with an ImmunoSpot counter (Cellular Technology, Shaker Heights, OH). Stimulations were conducted in triplicate per mouse per antigen.

To evaluate levels of splenic T regulatory cells, splenocytes were surface stained with antibodies for CD4 (clone RM4-5) and CD25 (clone PC61.5), for 30 minutes at 4°C according to the manufacturer's recommendations (eBioscience, San Diego, CA). Cells were washed then incubated in the dark at 4°C in fixation/permeabilization buffer overnight. Cells were again washed and resuspended in permeabilization buffer, and incubated in the dark at 4°C for 1 hr with an anti-FoxP3 monoclonal antibody (clone FJK-16s, eBioscience). Cells were washed twice with permeabilization buffer, then resuspended with FACS buffer and examined by FACS (FACScan, BD Biosciences).

2.5.11 Quantitative PCR.

15-week old mice were sacrificed following DC therapy at 12 weeks of age. Pancreatic lymph nodes (PLN) were harvested and RNA isolated according to manufacturer's recommendations using an RNAqueous-Micro Isolation Kit (Ambion, Austin TX). 375 ng RNA was used as a template to generate cDNA using SuperScript III First-Strand Synthesis Kit for qRT-PCR (Invitrogen, Carlsbad, CA). Real-time PCR was done using SYBR GreenER qPCR SuperMix (Invitrogen) kit on an iQ5 device (Biorad, Hercules, CA). The SuperMix kit uses Fluorescein to normalize the fluorescence fluctuations between reactions. Primers used for qRT-PCR were: GACGGCCAGGTCATCACTAT (ß-actin forward), AAGGAAGGCTGGAAAAGAGC (ß-actin reverse), TCTTGCCAAGCTGGAAGACT (FoxP3 forward), GGGGTTCAAGGAAGAAGAGG (FoxP3 reverse).

2.5.12 Statistics.

Survival analysis was conducted using Kaplan-Meier analysis and a Wilcoxon's test for significance between groups on Stata statistical software (College Station, TX). Other results were compared by Student's t test or ANOVA with Fisher's Least Significant Difference post-hoc test using SPSS statistical software (Chicago, IL). P values$\leq$ 0.05 were considered statistically significant.

3.0 THERAPEUTIC ROLES OF INDOLEAMINE-2,3-DIOXYGENASE AND B7 FAMILY MEMBERS IN TOLEROGENIC DC AND EXOSOMES

3.1 ABSTRACT

We have demonstrated previously that dendritic cells (DC), modified with immunosuppressive cytokines, as well as exosomes derived from these DC can reduce inflammation in murine collagen induced arthritis (CIA) and delayed-type hypersensitivity (DTH) models. Although we have previously shown that these effects are dependent on MHC II and FasL, much work remains to be done to understand the mechanism underlying the ability of tolerogenic DC and the exosomes they secrete to confer suppression *in vivo.* Herein we examine the roles of Indoleamine 2,3-dioxygenase (IDO) and the T cell costimulatory molecules B7-1, B7-2, PD-L1 and PD-L2 on the surface of tolerogenic DC and exosomes, as these have been demonstrated to be two key mechanisms involved in DC-mediated tolerance. In this study, we first examine the immunosuppressive effects of bone marrow derived DC and DC exosomes, genetically modified to express IDO. Bone marrow derived DC were adenovirally transduced with IDO or CTLA4-Ig (an inducer of IDO), and the resulting DC and exosomes were tested for their immunosuppressive ability in the DTH model. We demonstrate that both DC and exosomes derived from DC overexpressing IDO are anti-inflammatory the DTH model, and that the suppressive effects of exosomes from IDO-overexpressing DC were partially dependent on B7

costimulatory molecules. In order to determine the role of B7 family members in tolerogenic DC and exosomes in general, we examined the contributions of B7-1/2 and PD-L1/2 to the function of IL-10-treated, immunosuppressive DC as well as therapeutic exosomes derived from these DC. We demonstrate that absence of B7-1 or B7-2 on donor DC results in a loss of ability of IL-10 treated DC and their exosomes to suppress the delayed-type hypersensitivity (DTH) response, whereas IL-10 treated DC deficient in PD-L1/2 as well as their secreted exosomes retained the ability to suppress DTH responses. These results demonstrate that both IDO expressing DC and DC-derived exosomes are immunosuppressive and anti-inflammatory, and may represent a novel therapy for autoimmune disease. We further conclude that B7-1 and B7-2, but not PD-L1 and PD-L2, on DC and DC-derived exosomes play a critical role in immunosuppressive functions of both DC and exosomes.

3.2 INTRODUCTION

Dendritic cells (DC) are professional APC which play a crucial role in mediating the balance between immunity and tolerance *in vivo,* and therefore have been increasingly studied for therapeutic applications in allergy, infection, and autoimmunity. Significant efforts in the field of autoimmunity have been made to understand the genesis and function of DC that are immunosuppressive rather than stimulatory, and to understand their underlying phenotype [182]. Previously, we have demonstrated several effective modifications of dendritic cells (DC) which confer suppressive abilities to DC *in vivo* in delayed-type hypersensitivity (DTH) and collagen-induced arthritis (CIA) models. These include adenoviral gene transfer of IL-4 and FasL to DC, as well as rIL-10 treatment of DC [16, 67, 115, 177]. Furthermore, these studies demonstrated

that exosomes produced by modified DC could also be used to suppress inflammation *in vivo* in the DTH and CIA models. The exosomes secreted by the modified DC have been equally effective as the DC at decreasing disease. However, these exosomes are likely a better therapeutic avenue compared to DC, as exosomes are believed to be of a stable phenotype once secreted, while DC may undergo phenotype switching towards pro-inflammatory phenotype and thus worsen rather than mitigate disease.

Exosomes are small (40-100nm) membrane vesicles that originate in the multivesicular endosome of many cell types. Exosomes derived from APC, in particular DC, have the potential to promote immunity or tolerance in disease models of cancer, transplant or autoimmunity [183]. Exosomes carry molecules important for antigen presentation, also found on DC, such as major histocompatibility complex (MHC) class I and II, and B7 family costimulatory molecules such as B7-1, B7-2, and PD-L1, which allow them to regulate antigen specific immune responses [92, 96]. Exosomes are capable of interacting directly with T cells [184, 185], but also can act as a potential source of antigen or MHC complexes for APC presentation [186, 187]. Our previous studies demonstrated that *in vivo* mechanism of action of suppressive exosomes relies on MHC II and FasL, but not MHC I, while others have shown that B7-1 is important for exosome function *in vitro* [19, 20]. However, the mechanisms by which exosomes mediate their effects on host cells *in vivo* remains largely unclear at this time.

Indoleamine-2,3-Dioxygenase (IDO) is a tryptophan-catabolizing enzyme that catalyzes the rate-limiting first step in the degradation of tryptophan to a series of products, termed L-kynurenines. IDO plays a critical role in the maternal tolerance of the fetus [188], and has been demonstrated to be of importance in maintenance of tolerance in numerous settings including: cancer, rheumatoid arthritis, and transplantation [189-191]. IDO exists in two forms, IDO-1 and

IDO-2, and studies have shown that although both are expressed in DC, only IDO-1 mediates tryptophan conversion in DC [192]. IDO is upregulated in DC following several different stimuli, including IFN-γ, IFN-α, TNF-α, glucocorticoid-induced tumor-necrosis factor receptor (GITR), CD40L, and importantly, through reverse signaling following binding between B7 molecules on DC and CD28/CTLA-4 on CD4+ helper T cells [29, 193]. The exact mechanisms by which IDO confers tolerance are complex and as yet not fully elicited; however, two theories have been proposed. First, depletion of the essential amino acid tryptophan may reduce effector T cell proliferation by limiting the availability of this essential nutrient. It has been demonstrated that T cells can sense IDO-mediated depletion of tryptophan via GCN2 kinase, resulting in T cell anergy [194]. Other studies suggest that IDO does not function through depletion of tryptophan alone, and suggest that kynurines may be capable of conferring IDO-mediated signals from cell to cell [195]. However, both mechanisms may play important roles in conferring the tolerogenic effects of IDO.

As stated one of the mechanisms of IDO activation is via activation of costimulatory molecules. These signaling molecules play a critical role in determining immune system specificity and self-tolerance. The best characterized costimulatory molecules are B7-1 (CD80) and B7-2 (CD86), which are primarily expressed on APC and can either stimulate or antagonize T cell function via interaction with CD28 or CTLA-4 respectively [196]. B7-1 and B7-2 expression on APC is critical for homeostatic balance in not only effector T cell populations, but also in DC and regulatory T cell populations as well. CTLA-4 ligation of B7 signals bidirectionally, sending signals both into the T cell and inducing DC expression of IDO, which inhibits T cell function [197]. Novel members of the B7 family are also integral in controlling the DC and T-cell responses. Programmed Death-1 (PD-1) is expressed on activated T-cells

while its ligands PD-L1 and PD-L2 are expressed on numerous cell types including DC. The PD-1:PD-L1/2 signaling pathway plays an important negative regulatory role in T cell function and regulates peripheral T cell tolerance at multiple checkpoints [198]. Programmed Death-1 (PD-1) is upregulated on T cells upon activation, and its ligands, Programmed Death 1-Ligand 1 (PD-L1, also known as B7-H1) and Programmed Death 1-Ligand 2 (PD-L2, also known as B7-H2), have distinct expression patterns, with PD-L1 being expressed much more broadly than PD-L2. Both PD-L1 and PD-L2 are expressed on DC. Of significant interest, recent work indicates that PD-L1 and PD-L2 may not only regulate T cell responses by engaging PD-1 and modify TCR signaling, but also may act via reverse signaling and deliver signals into the PD-L1 and PD-L2-expressing cells. Treatment of DC with soluble PD-1Ig, inhibited their activation, resulted in increased IL-10 production, leading to a suppressive DC phenotype, which was independent of IDO upregulation. These effects could be prevented by neutralization of PD-1Ig with anti-PD-1, indicating that this phenotype is PD-1 specific and implicating PD-L1 or PD-L2 binding [199]. Because of their demonstrated abilities to confer suppressive properties to DC, we were particularly interested in examining the roles of B7-1/2 and PD-L1/2 costimulatory molecules in the context of immunosuppressive DC therapy.

Due to the immunosuppressive activity of IDO^+ DC, I have examined whether exosomes derived from IDO-expressing DC would be therapeutic in blocking inflammation in a footpad DTH model of antigen-specific inflammation. Herein, I document that exosomes derived from IDO+ DC are anti-inflammatory and therapeutic in the DTH model. In addition, I demonstrate that exosomes from CTLA4-Ig expressing DC reduced inflammation in an manner dependent on IDO in transduced DC, IDO dependent manner, suggesting that IDO either exogenously expressed or induced endogenously in recipient DC results in a suppressive phenotype.

Moreover, these results demonstrate that IDO expression in DC results in the generation of immunosuppressive exosomes.

In addition to DC derived exosomes altering the phenotype of host DC, there is data suggesting that DC derived exosome may interact and alter T-cell function within the host. Previous data by Kim et al. implicated MHC II and FasL, but not MHC I in the in vivo suppressive qualities of exosomes [19, 115]. Further data implicating a role for B7 signaling in the induction of IDO drew our interest to the role of B7 molecules and exosomes [19, 115]. Many of the factors important for exosome suppressive qualities including B7, MHC II and FasL are T-cell ligands [19, 20]. Thus, we hypothesized that direct interaction of exosomes with T-cells may play an important role in immune regulation of inflammation in the DTH model. In addition, the B7 family of costimulatory molecules may be required for these *in vivo* suppressive effects. Herein we demonstrate that the suppressive effects of exosomes derived from IDO+ DC in the DTH model were partially dependent on B7-1 and B7-2 costimulatory molecules, as exosomes from B7-1 and B7-2 knockout mice had reduced ability to confer the therapeutic effect. We further demonstrate that B7-1 and B7-2 are necessary for the *in vivo* suppressive function of both IL-10 treated DC and their secreted exosomes in the DTH model. This indicated that regardless of the method used to generate suppressive exosomes, B7-1 and B7-2 play a significant role in conferring their therapeutic effects. In contrast, we show that PD-L1 and PD-L2 on DC and DC-derived exosomes are not necessary for their immunosuppressive activity in the DTH model.

3.3 RESULTS AND DISCUSSION

3.3.1 Inhibition of inflammation in the DTH model by local administration of DC genetically modified to overexpress IDO and exosomes from IDO+ DC

It has been previously demonstrated that upregulated levels of IDO in DC *in vivo* are associated with tolerance in models of transplantation, cancer and autoimmunity [29]. Therefore, we were interested in methods to generate IDO+ tolerogenic DC *ex vivo*. In Bianco et al, we show that adenoviral transduction of DC with an IDO vector results in DC which are capable of ameliorating disease in the CIA model of rheumatoid arthritis [68]. CTLA-4-Ig is a synthetic fusion protein that binds with high affinity to B7-1 and B7-2, resulting in the upregulation of IDO [29]. To determine the effect of CTLA-4-Ig and IDO transduction on DC, DC were infected with IDO and CTLA-4-Ig expressing vectors and then cultured *in vitro* for an additional 3 days prior to the harvesting of DC or derivation of exosomes from the enriched supernatant. Our results indicate that transfection of DC with either IDO or CTLA-4-Ig expressing vector is an effective means to generate IDO+ DC *ex vivo,* as western blotting confirmed that CTLA-4-Ig transfected DC upregulated IDO compared to non-treated control DC (Figure 9A). We have previously demonstrated that the exosomes derived from the CTLA-4-Ig expressing DC are therapeutic in the CIA model [68]. We have further demonstrated that IDO overexpression in DC via transfection with IDO or CTLA-4-Ig adenoviral vectors results in slight, but detectable levels of IDO in the exosomes, whereas no IDO can be detected in control exosomes (Figure 9B). FACS analysis demonstrated there were no significant differences between the transduced and non-transdueced DC used in this study (Table 4).

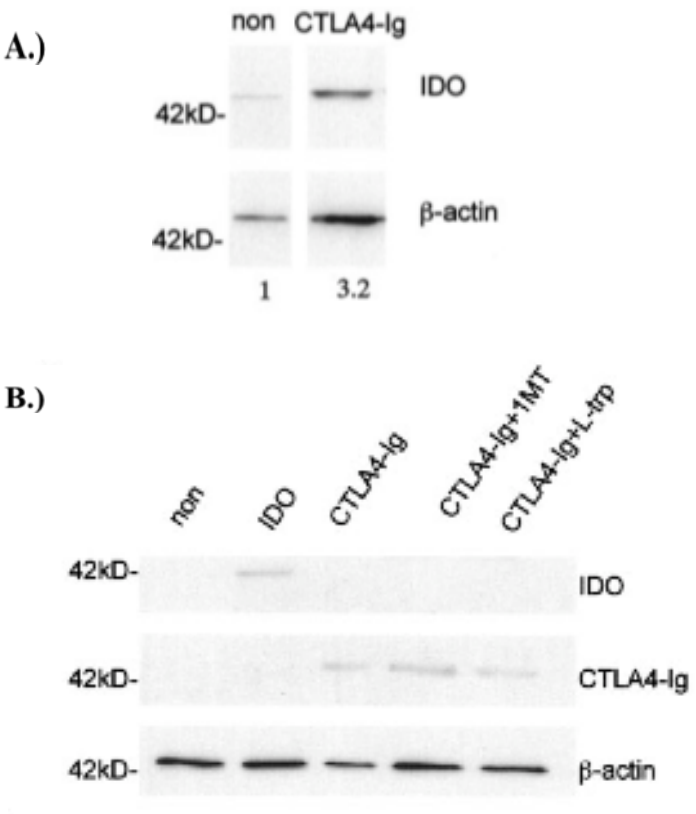

Figure 9: Transduction with IDO or CTLA-4-Ig vectors results in expression of IDO in DC and DC-derived exosomes.

(A) DC extracts (2 μg) from non-infected Ad.CTLA4-Ig cells were run on a 12% SDS-PAGE gel and immunoblotted for IDO or β-actin. Numbers indicate relative semi-quantitative expression of IDO after normalization to β-actin using the NIH software program Image J. (B) Exosomes were isolated from bone marrow derived DC that were previously infected with Ad.CTLA4-Ig and treated with ±1-MT or L-Trp, or non-infected. Five μg of total exosome protein was run on a 15% SDS-PAGE gel and immunoblotted for IDO, CTLA4-Ig, or β-actin.

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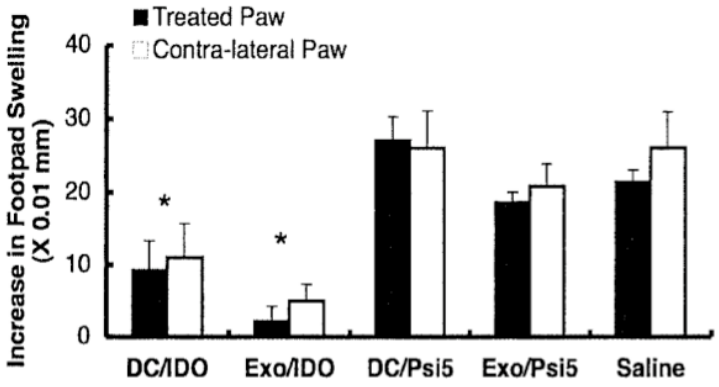

Figure 10: Suppression of DTH by IDO$^+$ DC and DC-derived exosomes.

Exosomes were isolated from BM-DC that were either infected with the Ad.Ψ5 (control) or Ad.IDO. The purified DC or exosomes were injected into the right footpad of KLH-immunized C57 BL/6 mice concurrently as KLH was injected into both hind footpads and the extent of swelling was measured at 48 h. Dark bars represent difference of footpad thickness in the treated paws and white bars in the non-treated contra-lateral paws (n=5). * denotes significance of ≤0.01 compared to DC/Psi5, Exo/Psi5, and Saline controls for both treated and contralateral paws.

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Table 4: FACS analysis of DC used in this study*

	CD80	CD86	Class I MHC	Class II MHC
Uninfected DCs	23.65	27.89	74.84	34.87
ψ5-positive DCs	29.86	45.28	54.66	45.38
IDO-positive DCs	24.26	36.60	61.18	35.10
CTLA-4Ig–positive DCs	31.55	49.39	53.61	48.90
CTLA-4Ig–positive DCs treated with 1-MT	30.85	48.62	52.56	48.31
CTLA-4Ig–positive DCs treated with L-tryptophan	27.81	42.15	51.81	42.10

*** DC were stained with PE-conjugated monoclonal antibodies against murine surface molecules (CD80, CD86, H-2Kb, I-Ab, and appropriate isotype controls). Isotype-matched irrelevant mAbs were used as negative controls (control=1.12%).**

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To determine if exosomes from DC genetically-modified to express IDO were therapeutic in a model of antigen-specific inflammation, a mouse model of DTH was used. To induce a Th1-driven DTH response, mouse were immunized to keyhole limpet hemocyanin (KLH) in complete freund's adjuvant at a single dorsal site two weeks prior to the introduction of antigen in the hind footpads. Either 10^6 or 1 μg of exosomes were concomitantly injected into the right hind footpad with the KLH antigen at the time of secondary antigen challenge in order to assess the ability of the DC or DC-derived exosomes to modulate the inflammatory response. Local injection of DC transfected with an IDO adenoviral vector (DC/IDO) significantly reduced paw swelling in both the treated and contralateral paw (Figure 10). Exosomes derived from the DC/IDO (Exo/IDO) were equally effective as the parental DC at reducing paw swelling in the treated as well as contralateral paws in the DTH model (Figure 10).

These results suggest that both DC/IDO and Exo/IDO are effective at suppressing inflammation at local as well as distant sites of inflammation. DC and DC-derived exosomes injected into the hind footpads of mice traffic to the ipsilateral draining popliteal lymph node, where they have been shown to interact with CD11c+ DC and F480+ macrophages [16, 67]. However, the mechanism whereby exosomes modulate inflammation *in vivo* both locally and at distant sites remains unclear.

We have further demonstrated that DC transfeted with CTLA-4-Ig (DC/CTLA-4-Ig) suppress paw swelling in the DTH model (Figure 11A). Exosomes derived from these DC (Exo/CTLA-4-Ig) are also capable of ameliorating inflammation in the treated and contralateral paws at levels comparable to the parental DC (Figure 11A). The addition of 1-MT, an inhibitor

of IDO, but not excess L-tryptophan, to the DC cultures resulted in inhibition of the anti-inflammatory effect of CTLA-4-Ig (Figure 11B). As seen in Figure 11B, exosomes derived from 1-MT treated DC/CTLA-4-Ig are not capable of suppressing paw swelling. This is consistent with the known ability of CTLA-4-Ig to generate a suppressive DC phenotype by upregulation of IDO, and our results indicating that upregulation of IDO does indeed occur in the DC/CTLA-4-Ig. Furthermore, this suggests that expression of IDO and not CTLA-4-Ig is critical for the in vivo suppressive effects seen in Exo/CTLA-4-Ig Altogether, these results support the conclusion that exogenous induction of IDO over-expression in DC results in a suppressive DC population, and that these DC as well as the exosomes they secrete can be used therapeutically in the DTH model.

3.3.2 Cells expressing IDO produce L-kynurenines, which can be detected in culture supernatants, but not in exosomes

IDO is the rate-limiting first enzymatic step in the catabolism of tryptophan to degradation products termed kynurenines. Belladonna et al. have demonstrated that in the presence of IFN-γ, kynurenines are capable of inducing tolerogenic properties in $CD8^-$ DC, demonstrating that the paracrine production of kynurenines is an important cell-cell signaling mechanism [195]. To determine whether it is plausible that exosomes from IDO-overexpressing DC could confer their suppressive effect by delivery of kynurenines to target cells *in vivo,* we performed an assay for the levels of L-kynurenine in purified exosomes and exosome-free supernatant. For this experiment, we used TA3 Hauschka cells (TA3 cells), a mouse mammary carcinoma line, due to their relative ease of adenoviral transduction and ability to produce large quantities of exosomes. The TA3 cells were infected with Ad.IDO, Ad.eGFP as a control, or left uninfected. As shown

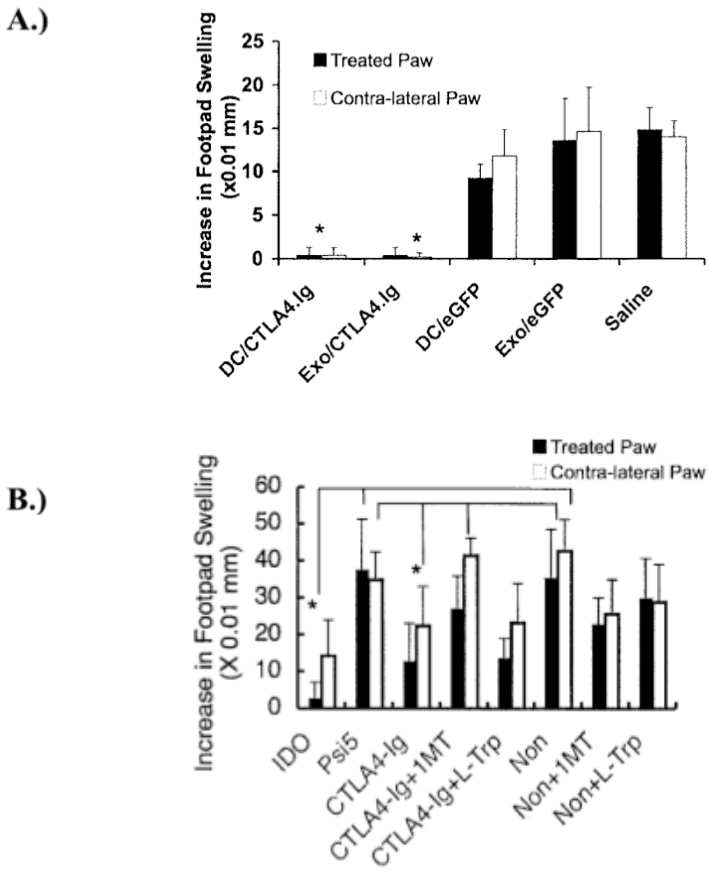

Figure 11: CTLA4-Ig^{+} DC and Exo suppress DTH in an IDO-dependent manner.

Exosomes were isolated from BM-DC that were infected with Ad.CTLA4-Ig and treated with ± 1-MT or L-trp. Purified DC or exosomes were injected into the right footpad of KLH-immunized C57 BL/6 mice concurrently as KLH was injected into both hind footpads and the extent of swelling was measured at 24 h (B) or 48 h (A). Note that (B) is exosomes only. Dark bars represent difference of footpad thickness in the treated paws and white bars in the non-treated contra-lateral paws (n=5). (A)* denotes significance of P≤0.001 compared to DC/eGFP, Exo/eGFP, and Saline Control Groups for both treated and contralateral paws (B) * denotes significance of P≤0.05 of CTLA4-Ig compared to controls Psi5, CTLA4-Ig+1MT and Noninfected controls for the treated paws, as well as significance of P≤0.05 of IDO group compared to all groups excepting the CTLA-4-Ig and CTLA-4Ig+1MT for both treated and contralateral paws.

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in Figure 12, we observe high levels of L-kynurenine production only in the exosome-free supernatant of the Ad.IDO infected cells as compared to the control groups. We do not detect L-kynurenine in any of the exosome groups. It therefore seems unlikely that delivery of kynurenines to target cells is a main mechanism by which exosomes form IDO-overexpressing cells confer their effects.

3.3.3 Anti-inflammatory effect of exosomes from DC/IDO are dependent on B7-1 and B7-2 for their mechanism of action

As shown in Figure 9, Exo/IDO also contain IDO, and delivery of IDO to target cells may be one mechanism by which they confer suppression *in vivo*. However, we were also interested in quality is not a consequence of differing levels of IDO between groups but that the immunosuppressive function of exosomes derived from IDO-overexpressing DC depend on B7-1 and B7-2 on the exosomes surface.

Given that B7-1 and B7-2 on the DC surface are known to have intracellular signaling capability which upregulate IDO [29], it is unclear whether or not the requirement for B7 molecules on the surface of tolerogenic exosomes is related in some way to the fact that they were generated form IDO-overexpressing DC. Therefore, in the following experiments, we set out to determine if the requirement for B7-1 and B7-2 for *in vivo* efficacy of BMDC and DC-derived exosomes was related to the specific adenoviral therapies used in these previous studies, or represented a more general phenomenon that could be generalized to other populations of suppressive DC and exosomes.

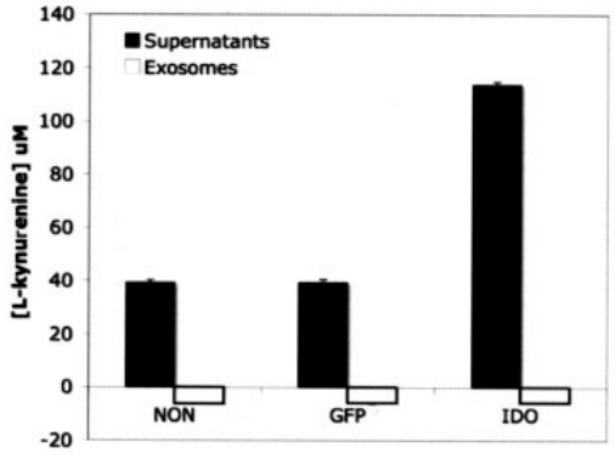

Figure 12: Exosomes from IDO$^+$ cells do not contain trp metabolites.

TA3 cells were either non infected (non) or infected with rAd containing expression cassettes for either eGFP or IDO. Exosomes were collected from the culture supernatant, and verification of IDO expression was conducted by western blot (data not shown. Exosomes (1 μg) or exosome-free supernatants (60μl) were assayed for levels of L-kynurenine.

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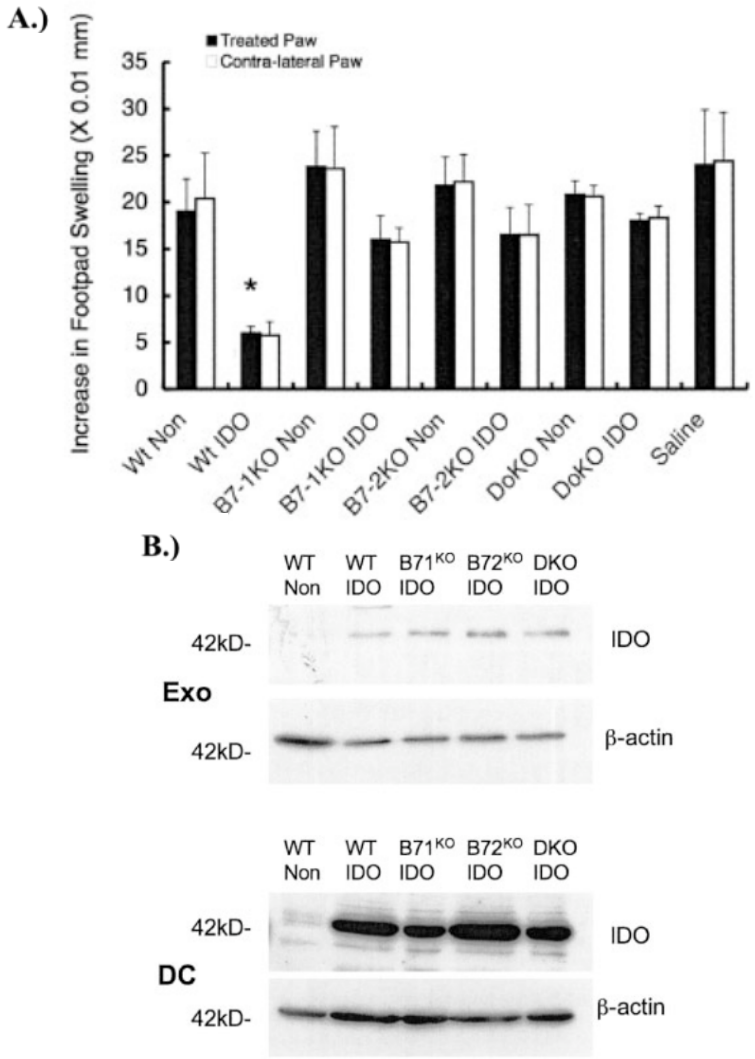

Figure 13: DTH suppression by exosomes is dependent on B7-1 and B7-2.

BM-DC from wild type C57BL/6 mice or B7-1^{KO}, B7-2^{KO}, or double knockout (DKO) were transduced with Ad.IDO or non-infected. (A) Exosomes were isolated from the DC media. Purified exosomes were injected into the right footpad of wild-type KLH-immunized mice concurrently with injection of KLH antigen into both hind footpads, and the extent of swelling was measured at 24 h. Dark bars represent difference of footpad thickness in the treated paws and white bars in the non-treated contra-lateral paws (n=5). * denotes significance of $P \leq 0.05$ compared to all other groups for both treated and contralateral paws. (B) Five μg of total DC or exosome protein extract was run on a 15% SDS-PAGE gel and immunoblotted for IDO or β-actin.

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3.3.4 IL-10 treatment of BMDC results in tolerogenic phenotype

The levels of the B7-1/2 and PD-L1/2 costimulatory molecules on DC before and after 24 hours of treatment with IL-10 were examined. As shown in figure 1A, IL-10 treatment of BMDC resulted in highly decreased levels of MHC I (H-2k^{b}), B7-1, B7-2, as well as lowered levels of MHC II (IA/IE) and PD-L2 after 48 hours. Similarly, Western blotting demonstrated that IL-10 treated DC have decreased overall expression levels of B7-1 and B7-2 (Figure 1B), in addition to decreased surface expression. These observations are consistent with published reports regarding the ability of IL-10 treatment to downregulate levels of costimulatory molecules on DC [15, 16], thereby generating a tolerogenic DC phenotype. We also examined the secreted levels of the proinflammatory cytokines IL-6, IL-23(p19/p40), IL-12p70, and IL-12/IL-23 total p40 by ELISA from supernatants of IL-10 and non-treated DC, plus/minus 24 hours of stimulation with 100ng/mL of LPS. We found that DC treated with IL-10 do not have increased IL-12 or IL-23 production when stimulated with LPS. In addition, although the LPS-stimulated DC.IL10 produce an increased amount of IL-6, it was significantly less than the amount produced by the non-treated control DC in response to LPS stimulation (Figure 1C). Collectively, these results confirm that an immunosuppressive phenotype of DC can be generated after 24 hours of treatment with IL-10.

3.3.5 DC-derived exosomes contain costimulatory molecules

Exosomes were purified from the supernatants of untreated and IL-10-treated DC cultures for analysis of costimulatory marker expression. To analyze by FACS, exosomes were first absorbed onto anti-CD81 coated latex beads, and then stained with labeled antibodies to

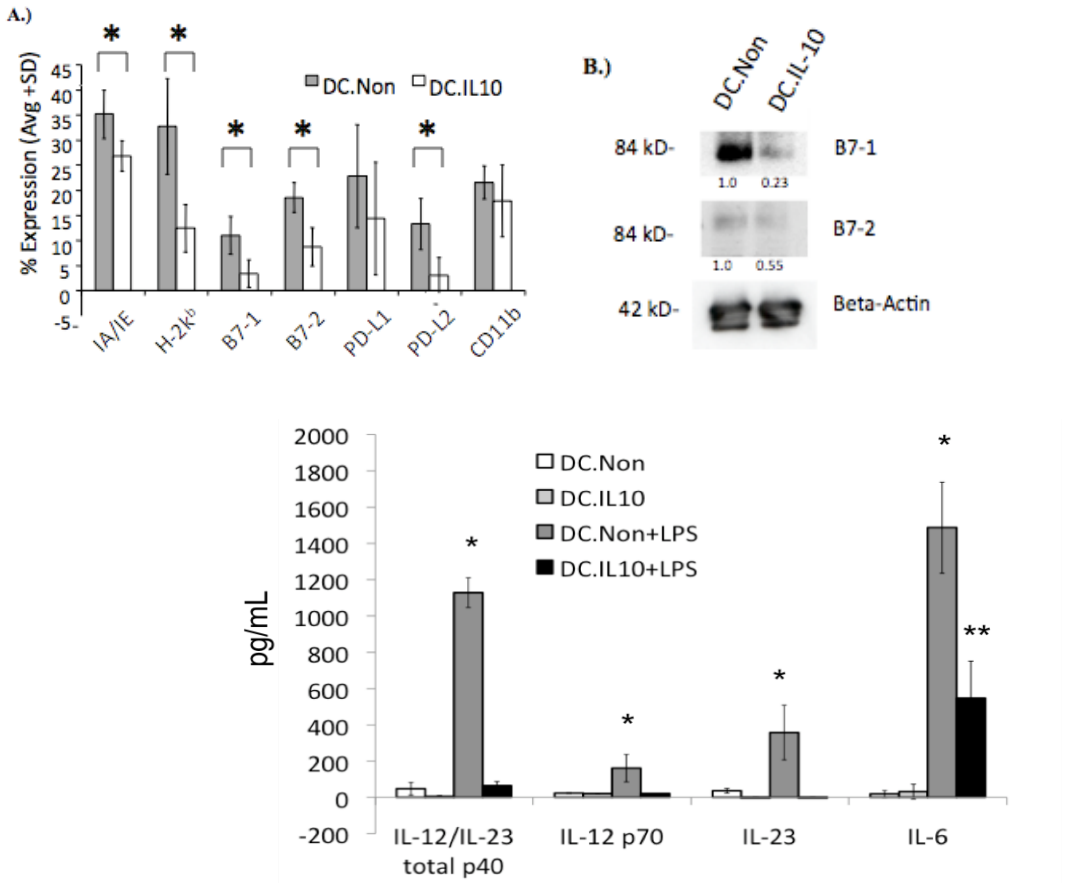

Figure 14: Characterization of rIL10-treated tolerogenic DC.

(A) DC were stained with PE-conjugated mAbs and analyzed by FACS. Percent of positively stained cells was compared to isotype controls. Results representative of four independent experiments. * denotes significance at $P \leq 0.05$ of expression levels of IL-10 treated DC compared to non-treated controls. (B) 10 µg of DC lysates were run on SDS-PAGE gels under non-reducing conditions and immunoblotted for B7-1, B7-2 or beta-actin as control. NIH Image J was used to analyze protein band density. (C) IL-10 treated and non-treated DC were stimulated with 100ng/mL LPS overnight on day 7 of culture. Cytokine levels in culture supernatants were determined on day 8 using ELISA. Significance at $p \leq 0.05$ is represented on the figure by a single asterisk for DC.Non + LPS group compared to the other three groups, and double asterisk for the DC.IL-10+LPS group compared to the other three groups.

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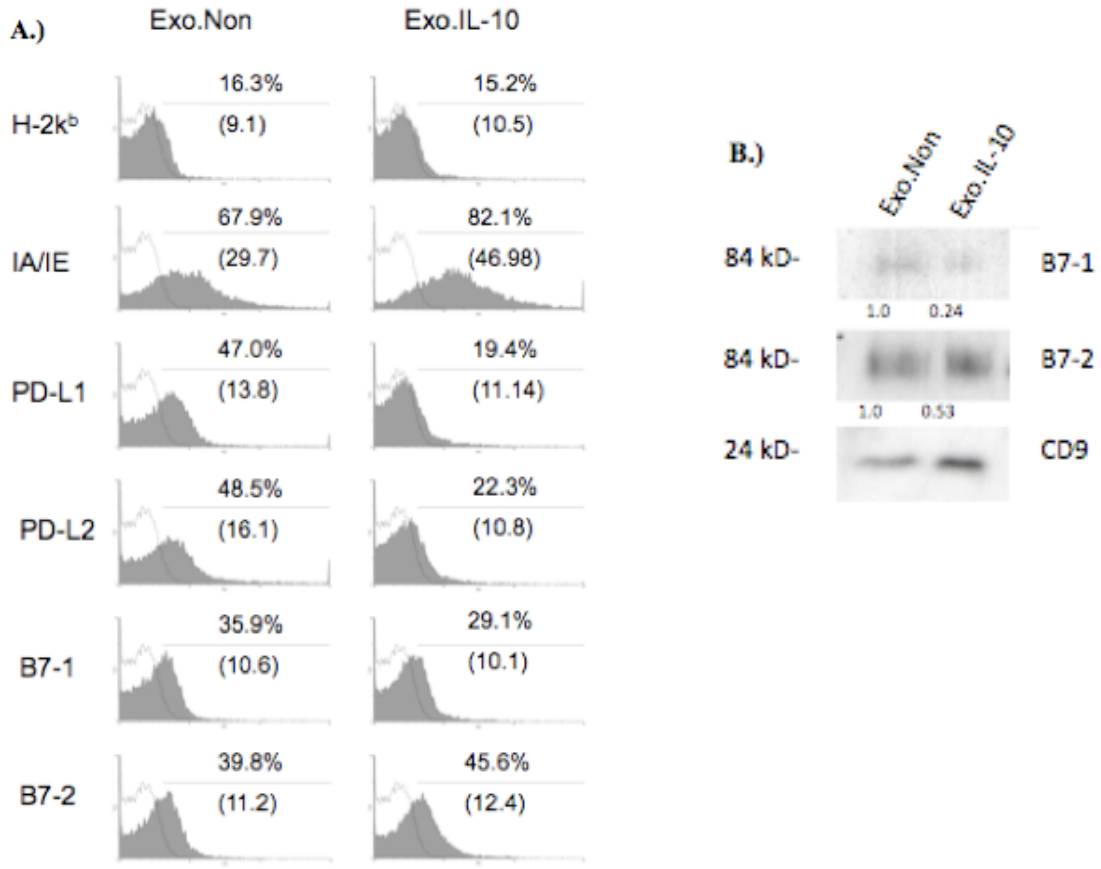

Figure 15: Characterization of exosomes from tolerogenic DC.

Exosomes were purified from supernatant of control or rIL-10 treated DC. (A) Anti-CD81 mAb was preabsorbed onto latex beads which were subsequently incubated with 10 µg of DC-derived exosomes. Samples were stained with PE-conjugated mAbs specific for IA/IE, H2k^b, PD-L1, PD-L2, B7-1, and B7-2, or isotype control (represented by the open black line). Results shown are representative of three independent experiments. Percentage of beads containing positive signal, as well as MFI (in parentheses) are displayed. (B) 5 µg of exosomes were run on SDS-PAGE gels under non-reducing conditions and immunoblotted for B7-1, B7-2 or CD9 as a loading control. NIH Image J was used to analyze protein band density.

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MHC I (H-2k^b), MHC II (IA/IE), B7-1, B7-2, PD-L1, and PD-L2 (Figure 2A). We observe a significant decrease in the levels of PD-L1, PD-L2, and B7-1 on exosomes secreted by IL-10 treated DC. The decrease in the levels of B7-1 was confirmed by Western blot along with a decrease in the levels of B7-2 when compared to the level of the CD9 loading control (Figure 2B). It has been demonstrated previously that exosomes secreted from mature DC are phenotypically and functionally different than those secreted by immature DC [17]. However, this is the first demonstration of phenotypic changes in the exosome population secreted by DC following treatment with cytokines to induce a tolerogenic phenotype. Our data indicate that qualitative changes in costimulatory molecules levels on exosomes secreted by DC occur following treatment with IL-10, suggesting that the phenotypic response of DC to IL-10 treatment is also seen in the exosomal compartment. IL-10 treatment decreased the overall levels of MHCI, MHC II, B7-1, B7-2, and PD-L2 on the surface of the DC whereas the level of MHC II appears to increase on exosomes in contrast to the decrease in the levels of B7-1, B7-2, PD-L1, and PD-L2.

3.3.6 *In vivo* therapeutic effects of IL-10 treated DC and exosomes require B7-1 and B7-2

In the preceeding sections we have demonstrated that gene transfer of IDO to BMDC results in DC and DC-derived exosomes that are therapeutic in DTH and CIA models [180]. We also demonstrate that exosomes from DC transduced with a CTLA-4-Ig expressing adenoviral vector were suppressive *in vivo* in an IDO-dependent manner. As shown in Figure 13, loss of B7-1 and B7-2 significantly decreased the ability of exosomes from IDO-transduced DC to suppress inflammation in the DTH model. However, it is unclear if the requirement for B7-1 and B7-2 for *in vivo* efficacy of BMDC and DC-derived exosomes is related to the specific method of IDO

overexpression used to generate the suppressive DC and exosomes, or represents a more general phenomenon that can be generalized to other populations of suppressive DC and exosomes. Therefore, in the following experiments, we set out to determine if exosomes derived from IL-10 treated DC also demonstrate a dependence on B7-1 and B7-2 for *in vivo* suppressive activity.

As shown in Figures 14 and 15, DC and DC-derived exosomes normally contain both B7-1 and B7-2. To determine whether these are essential components for tolerogenic DC function, DC were isolated from B7-1-/-, B7-2-/-, or B7-1/B7-2-/- mice and treated with rIL-10 or left untreated. Exosomes were isolated from the enriched media. IL-10 treated donor DC lacking B7-1 or B7-2, as well as the exosomes that they secrete, were significantly less effective at reducing paw swelling than the IL-10 treated wild-type controls (Figure 16A). Although the therapeutic ability of the knockout exosomes were greatly reduced, we observed some remaining ability of exosomes from IL-10 treated knockout DC to reduce paw swelling by approximately 20%. A 20% reduction in paw swelling was also observed in mice receiving the IL-10 treated B7-2 -/- DC.

These results demonstrate that both B7-1 and B7-2 are required on DC and DC-derived exosomes for reduction in inflammation in the DTH model. This is consistent with data demonstrating that B7-1 and B7-2 on DC play critical roles in the generation of tolerance by interacting with CD4+CD25+ cells [203], altering effector T cell cytokine profiles [204], and regulating DC function through CTLA-4 signaling. Furthermore, the ability of the B7-2-/- DC to suppress the DTH response 20% more efficiently than DC lacking B7-1 is of interest given that B7-1 and B7-2 are known to play different roles *in vivo* [205, 206].

Given the reported suppressive functions of PD-L molecules, and our observation that levels of PD-L1 and PD-L2 on DC and DC-derived exosomes are regulated by IL-10 treatment, we

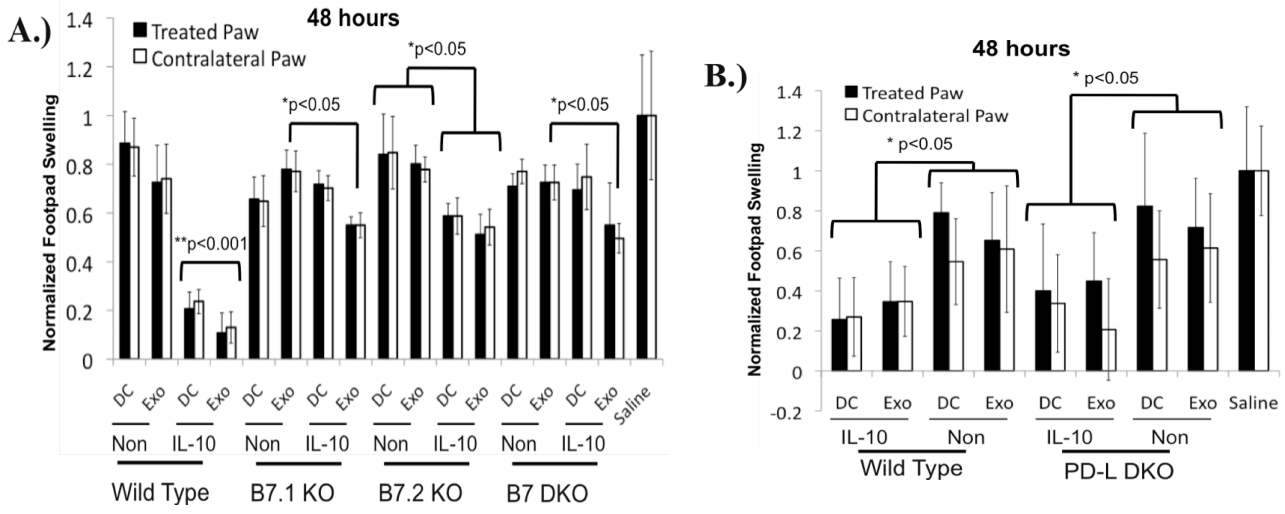

Figure 16: B7-1 and B7-2 are required on DC and DC-derived exosomes for therapeutic effect in the DTH model, whereas PD-L1 and PD-L2 are not.

Wild-type C57BL/6 or knockout strain-derived DC were treated with rIL-10 or left untreated, and exosomes were isolated from the enriched media. DC or exosomes were injected into the right hind footpad of KLH-immunized wild-type mice simultaneously with KLH challenge to both hind footpads. Dark bars = swelling post challenge in treated (right) paws, white bars = swelling in contralateral (left) untreated paws. (A) Loss of B7-1 or B7-2 on DC or DC-derived exosomes abrogates ability to suppress inflammation in the DTH model. **$p \leq 0.001$ for wild-type IL-10-treated DC and their secreted exosomes compared to all other groups. *$p \leq 0.05$, exosomes from IL-10-treated B7-1-/-, B7-2-/-, and double knockout DC conditions as compared to the wild type and their respective knockout untreated controls. B7-2-/- IL-10 treated DC are also significant at $p \leq 0.05$ compared to the B7-2-/- non-treated DC as well as the non-treated wild type DC. (B) PD-L1 and PD-L2 are not required on DC and DC-derived exosomes for therapeutic effect in the DTH model. * $p \leq 0.05$ for the treated paw of the IL-10-treated wild-type and PD-L DKO samples compared to controls. There are no statistical differences between the wild-type and PD-L DKO IL-10 treated DC and exosome groups.

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hypothesized that these molecules may also be required for DC and exosome function in the DTH model. BMDC were generated from PD-L1/PD-L2-/- mice, treated with IL-10 or left untreated, were evaluated in the DTH model. As shown in Figure 16B, IL-10 treated PD-L1/2-deficient DC and their exosomes were still able to suppress inflammation compared with wild-type DC and exosomes treated with IL-10. These results are consistent with data from bone marrow chimera studies that demonstrated expression of PD-L1 and PD-L2 on APC alone was not sufficient to ameliorate early-onset diabetes in PD-L1/PD-L2 -/- NOD mice [207].

3.4 CONCLUSIONS

Our results indicate that adenoviral transduction of DC with an IDO- or CTLA-4-Ig expressing vectors are both efficient means to exogenously generate IDO-overexpressing DC. These results with CTLA-4-Ig are consistent with those observed by others in the literature, as it has been previously reported that CTLA-4-Ig ligation of B7-1 or B7-2 on DC results in upregulation of IDO [208]. We further demonstrate that DC generated by either method produce exosomes that contain higher levels of IDO protein than untreated DC. As the mechanisms of exosomes-mediated signaling have not been fully elucidated, the functional significance of this finding is unclear. Exogenous IDO within exosomes may function by delivering IDO to IDO deficient antigen presenting cells, or IDO+ exosomes may interact directly with T cells in a manner that is capable of depleting them of essential tryptophan or producing kynureines in these cells. Studies have shown that IDO-mediated DC signaling to T cells results in suppression of effector CD8+ T cells and induction of a regulatory T cell population via GCN2-kinase dependent induction of FoxP3 in naïve CD4+CD25- cells [209, 210]. Although we detected production of L-kynurenine

in the exosomes-free supernatants, we did not detect L-kynurenine in the exosomes, suggesting that delivery of tryptophan metabolites is not a mechanism whereby exosomes from IDO-overexpresing cells confer suppression.

We next determined the therapeutic efficacy of IDO-overexpressing DC and the exosomes they secrete in a DTH model of antigen-specific inflammation. Our results indicate that both adenoviral transfer of IDO and CTLA-4 to DC result in a DC population capable of suppressing inflammation in the DTH model. We have further demonstrated that these genetically-modified DC secrete exosomes which are as effective as their parental DC at decreasing paw swelling in DTH mice. The IDO inhibitor 1-MT reduced the suppressive qualities of CTLA-4-Ig when added to DC culture, suggesting that the effects of CTLA-4-Ig transduction on the DC are dependent on IDO activity. However, in these experiments 1-MT was removed before injection of DC or exosomes into the mice, which implies that there is a critical role for IDO on the DC themselves which render them capable of regulating inflammation during the *ex vivo* culture period.

Therefore, we hypothesize that IDO-overexpression in the DC modifies them in some other way(s) to render them tolerogenic. Specifically, we demonstrate a critical role for B7-1 and B7-2 expression on the exosomes secreted by the IDO-overexpressing DC. This result suggests that exosomes may directly interact with T cells *in vivo,* and is consistent with previous findings that exosomes require MHC II and FasL for *in vivo* activity in the DTH model [19]. As B7 signaling is known to induce IDO activity within the DC, B7 may only have be integral in supporting IDO mediated suppression of DTH in exosomes, but not in mediating signaling in other suppressive DCs. Thus, we wanted to further explore the role of B7 using another type of suppressive exosome.

To do so, we first confirmed that IL-10 treatment generates a tolerogenic DC phenotype and demonstrated that IL-10 treated DC secrete a modified population of exosomes that contain decreased levels of B7-1, B7-2, PD-L1 and PD-L2 as compared to exosomes secreted by control DC. This data regarding phenotypic difference between tolerogenic and control exosomes is of particular significance given the growing interest in exosomes for therapeutic applications, as it is critical to the immunomodulatory mechanisms of exosomes. Our data also demonstrate that the phenotypic changes seen after treatment with IL-10 on the cell surface of DC and on the exosomes secreted by DC are different. This suggests that regulation of protein trafficking in these compartments differs, and may reflect an underlying difference in the physiologic role of DC and exosomes *in vivo.*

Using the DTH model, we further demonstrate a requirement for B7-1 and B7-2, but not PD-L1 or PD-L2 for *in vivo* function of IL-10 treated DC and the tolerogenic exosomes they secrete. This is consistent with our previous result demonstrating that B7-1 and B7-2 are partially required for *in vivo* function when suppressive DC and exosomes were generated using IDO gene transfer. Taken together, these results suggest a critical role for B7-1/2 on DC and DC-derived exosomes *in vivo,* regardless of the method by which the therapeutic DC or exosomes are generated. It is interesting to note, while B7 molecules are necessary to confer immunosuppressive qualities of exosomes, they are present in reduced levels on suppressive Exo/IL-10. Thus, this supports the hypothesis that these molecules allow for interaction with endogenous T-cell populations, and promote tolerance when expressed at low levels.

Our findings indicate that the majority of *in vivo* suppressive activity of exosomes is dependent on B7-1 and B7-2. We do not observe any differences in the requirement for B7 based on the method by which the suppressive DC are generated, as exosomes from IDO-

overexpressing DC and IL-10 treated DC demonstrate similar loss of function when B7 knockout cells are used. Our observations that exosomes require B7-1 and B7-2 for function *in vivo* are consistent with *in vitro* findings suggesting that both ICAM-1 and CD28/B7 molecules are critical for APC-derived membrane vesicles to directly activate T cells, but contradictory to findings that only ICAM-1, and not B7 molecules are required for exosomes to stimulate T-cell responses when mediated through DC uptake and representation [211, 212]. However, as yet it remains unclear whether the primary *in vivo* mechanism of exosomes is mediated through APC or involves direct interaction with T cells. Likely, there are multiple mechanisms of significance by which exosomes act depending on the particular conditions. While our results demonstrate the importance of both B7-1 and B7-2 molecules on exosomes for *in vivo* suppression of inflammation in the DTH model, in the B7 knockout conditions the exosomes from the IL-10 treated or IDO-overexpressing knockout DC retain approximately 20% of their suppressive ability. This suggests that while B7 signaling is highly important for the *in vivo* suppressive activity of exosomes, redundant mechanisms of exosome-host cell communication may be utilized to confer suppressive effects *in vivo*.

3.5 MATERIALS AND METHODS

3.5.1 Mice

Female C57BL/6 (H-2K^{b}) and B7 knockout mice (B7-1/B7-2 -/- = B6.129S4-Cd80^{tm1Shr} Cd86^{tm2Shr}/J, B7-1 -/- = B6.129S4-Cd80^{tm1Shr}/J, B7-2 -/- = B6.129S4-Cd86^{tm1Shr}/J) were purchased at 7-8 weeks of age (Jackson Laboratory, Bar Harbor, ME). PD-L1/PD-L2 -/- mice

have been previously described [207]. Mice were maintained in a pathogen-free facility according to institutional and National Institutes of Health (NIH) guidelines.

3.5.2 Vector Construction and Adenovirus Generation

Adenoviruses expressing murine IDO (Ad.IDO) and CTLA4-Ig (Ad.CTLA4-Ig) were constructed, propagated, and titered according to standard protocols as previously described [177]. Briefly, the recombinant adenoviruses were generated by homologous recombination in 293 cells expressing Cre recombinase (CRE8 cells), after co-transfection of DNA, an adenovirus 5-derived, E1- and E3-deleted adenoviral backbone (psi5) and pAdlox, the adenoviral shuttle vector. The inserted cDNA sequences are expressed under the human CMV promoter. The recombinant adenoviruses were purified by CsCl gradient ultracentrifugation, dialyzed in sterile virus storage buffer, aliquoted and stored at – 80^{o}C until use. The CRE8 cells were grown and maintained in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum.

3.5.3 DC Generation

Bone-marrow derived DC were prepared following a bulk-culture protocol modified from Son, et al. as previously described [179, 180]. Briefly, bone marrow was collected from tibias and femurs of 6- to 7-week-old mice. Contaminating erythrocytes were lysed with ACK cell lysing buffer (Mediatech, Herdon, VA). Monocytes were collected from the interface after centrifuging on Nycoprep (NycoMed, Roskilde, Denmark) at 600 x g for 20 min at RT. Cells were cultured for 24 h in complete media (RPMI 1640 containing 10% FBS, 50 μM 2-β-Mercaptoethanol, 2 mM

glutamine, 0.1 mM nonessential amino acids, 100 IU/mL penicillin/streptomycin) to remove adherent macrophages. Non-adherent cells were placed in fresh growth medial (complete media containing 1000 U/mls of GM-CSF and IL-4) to generate DC. Cells were cultured for 4 days and harvested.

3.5.4 Adenoviral infection of BMDC

On day 5 of BMDC culture, 1 X 10^6 DC were mixed with 5 X 10^7 PFU of the viruses in total volume of 1 ml serum-free media. After a 2 hr incubation with virus, 10 ml complete media was added to the cells. In certain experiments, the cells were also treated with 1-methyl-D-tryptophan (1-MT)(Sigma)(200μM) or L-trp (245 μM) at this time. After incubation for 24 hr, DC were washed intensively three times and cultured for a further 48 h. On day 8, culture supernatant was collected for exosome purification and recovery of the Ad-transduced DC. This infection method routinely gives us ~70-80% transfection efficiency using Ad.eGFP as a control [181]. There was no toxic effect of 1-MT or L-trp on the cells as observed by trypan blue exclusion and overall cell count.

3.5.5 IL-10 treatment of BMDC

On day 5 of BMDC culture, 1 X 10^7 DC were incubated for 2 hours with 1 μg/mL of the recombinant murine IL-10 (Cell Sciences, Canton, Massachusetts) in total volume of 1 mL serum-free media, then diluted 1:5 in CM for further incubation. After incubation for 12 hr, DC were washed three times in PBS and cultured for 48 h in growth media without rIL-10. On day 8, culture supernatant was collected for exosome purification and DC recovery.

3.5.6 Exosome Isolation

Exosome isolation procedure has previously been described [115]. Briefly, DC culture supernatants were collected and centrifuged at 300 g for 10 min, 1200 g for 20 min, and 10,000 g for 30 min, and then ultra-centrifuged at 100,000 g for 1 h. Exosome pellet was washed in sterile PBS, centrifuged at 100,000 g for 1 h, and resuspended in 120 μl of PBS for further studies. Exosome batch protein content was quantified and standardized by a micro Bradford protein assay (Bio-Rad, CA). 1 μg exosomes were suspended in 20 μl of PBS for *in vivo* mouse studies.

3.5.7 DTH Model

Female C57BL/6 of 7-8 weeks of age were sensitized by injecting 100 μg of Keyhole Limpet Hemocyanin (KLH) antigen emulsified 1:1 in CFA at a single dorsal site. 14 days later, the right hind footpad of immunized mice were injected with 10^6 DC or 1 μg of exosomes suspended in 50μL total volume PBS containing 20μg of KLH antigen. Contra-lateral footpad received 50μL total volume PBS containing 20μg of KLH antigen, without any DC or exosomes. Footpad swelling was measured bilaterally using a spring-loaded caliper at 24, 48 and 72 hours after injection. Results are expressed as the difference in swelling (X 0.01 mm), before and after Ag boost injection. Experiments were performed with 5 or 6 mice per group and repeated at least twice to ensure reproducibility.

3.5.8 Western Blot Analysis

For western blot, exosomal proteins (3-10μg) were separated by 12% or 15% SDS-PAGE, semi-dry transferred onto PVDF and detected by western blotting using an enhanced chemiluminescence detection kit. Primary antibodies used for western blot were: rabbit polyclonal anti-GFP (Santa Cruz), mouse monoclonal anti-hsc70 (Santa Cruz), rabbit polyclonal anti-beta actin (abcam), mouse monoclonal anti-IDO (Upstate), hamster monoclonal anti-B7-1 (clone 16-10A1), rat monoclonal anti-CD9 (clone KCM8), (both from BD Pharmigen, San Jose, California), and rat monoclonal anti-B7-2 (clone GL1, R&D systems, Minneapolis, Minnesota). Semi-quantitative analysis of the protein density bands was performed using the NIH Image J program.

3.5.9 Measurement of kynurenine concentrations

Kynurenine was measured in exosomes (1 μg) or exosome-free supernatants (60μl) using a spectrophotometric assay. Samples were mixed 2:1 with 30% trichloroacetic acid, vortexed, and centrifuged at 3716 x g for 10 minutes. 75μl of supernatant was then added to an equal volume of Ehrlich reagent (2% 4-(dimethylamino)benzaldehyde in glacial acetic acid) in a 96 well microtiter plate. Samples (in triplicate) were analyzed against a standard curve of L-kynurenine (Sigma)(0-5 mM). Absorbance was measured at 490nm using a microplate reader.

3.5.10 FACS Analysis

Anti-CD81 labeled-latex beads were generated by incubating 250 uL of 4 μm latex beads overnight at 4° C in a solution containing 60 ug anti-CD81 mAb (EBioscience, San Diego, CA) and 250 μL BSA in MES buffer. After washing beads three times in PBS, 10 μg of exosomes were incubated with 15 μL of the anti-CD81 labeled-latex beads in 500 uL of PBS. Samples were centrifuged at 700g for 5 min, resuspended in ice-cold PBS containing goat serum and stained with 1-2 μL of PE-labeled Ab in 100 μL total volume.

Dendritic cells were washed and stained with 1-2 μL of PE-labeled mAbs (B7.1 (16-10A1), B7.2 (PO3.1), PD-L1 (MIH5), PD-L2 (TY25), IA-IE (M5/114.15.2), H2-k^b (AF6-88.5), all from eBioscience, San Diego, CA) in 100 μL total volume of ice-cold PBS containing goat serum. Exosome-coated beads and DC were washed twice in FACS buffer, resuspended in 400 μL FACS buffer, and examined by FACS (FACScan, BD Biosciences, San Jose, CA). Results were analyzed using WinMDI 2.9.

3.5.11 ELISA

Supernatants from LPS- and mock-stimulated BMDC were collected and analyzed using a sandwich ELISA to detect IL-23 (R&D systems, Minneapolis, Minnesota), IL-6, IL-12/IL-23 total p40, and IL-12p70 (all from eBioscience, San Diego, CA) according to the manufacturer's protocol. The limits of detection were 2 pg/mL for IL-12/IL-23 total p40 , 4 pg/mL for both IL-6 and IL-12p70, and 1 pg/mL for IL-23.

3.5.12 Statistics

Results were compared by analysis of variance (ANOVA) with Fisher's Least Significant Difference post-hoc test. When appropriate, the Kruskal-Wallis non-parametric test was used to compare means between groups. P values less than 0.05 were considered statistically significant, and all tests were conducted using SPSS statistical software (SPSS, Chicago IL).

4.0 GENERAL DISCUSSION

4.1 SUMMARY

Molecular, cellular, and organ engineering has grown in significance to become an important part of the field of bioengineering. Although this is perhaps most obvious when one looks at the explosion of interest in the field of tissue engineering, where the goal is to regenerate an entire organ or tissue, its principles can nonetheless be employed to generate novel therapeutics at the single-cell, protein, or gene level. It is a multi-disciplinary field that utilizes knowledge from medicine, molecular biology, genetics, and engineering design principles in order to manipulate the structure-function relationships of the biological target. As our knowledge of cellular function has expanded, our ability to make purposeful modifications to cells has multiplied. For example, the discovery of DNA in 1953 led rapidly to an increase in the knowledge of basic cellular functions, and paved the pathway to clinical trials of gene therapy techniques by the 1990s [213]. The field is constantly incorporating new discoveries, as evidenced by the explosion of studies utilizing siRNA-targeted knockdown in the brief period of time following the description of the RNA-interference phenomena in 1998 [214].

This studies described herein fall within the field of cellular engineering, and are similarly multi-disciplinary. A main goal of this work is to genetically engineer DC so that they are reprogrammed to down-regulate inflammation. Adenoviral gene transfer techniques have

been used to manipulate bone-marrow derived DC, and increase the expression of anti-inflammatory genes, specifically IL-4, IDO, and CTLA-4-Ig, which confer tolerogenic properties.

The data presented in Chapter 2 demonstrate that DC overexpressing IL-4 are able to reduce insulitis and prevent the onset of diabetes in NOD mice. We observe an upregulation of FoxP3 in the pancreatic lymph node and an alteration in increase in the ratio of Th2 to Th1 cytokine secretion by splenocytes following stimulation with islet antigens. Taken together, these results imply that DC transduced to express IL-4 are capable of reestablishing peripheral tolerance in a proportion of the NOD mice. These results are consistent with previously published observations examining the ability of DC transduced with IL-4-expressing gene therapy vectors to prevent diabetes onset in 5- and 10-week old , as well as 12-week old NOD mice [15, 72]. When using the NOD model, the age at which a therapy is tested is very important as a larger number of immunomodulatory therapies are able to prevent autoimmune diabetes when administered at early ages, whereas comparably few prevent disease onset if administered after 12-weeks of age [137].

In Chapter 2, we report for the first time that DC transduced to express IL-4 are capable of preventing the onset of hyperglycemia when administered to NOD mice after they have developed prediabetes. In our studies, mice were screened by random blood glucose measurement for the development of prediabetes, defined here as a repeat random blood glucose measurements between 150 and 250 mg/dL. These criteria differentiate mice with elevated baseline blood glucose and increased fluctuations in blood glucose [174]. We confirm that these mice demonstrate an impaired response to glucose challenge, with elevated peak levels and prolonged return to baseline blood glucose following administration of L-dextrose. In the NOD

model this is indicative of islet dysfunction secondary to progressions in autoimmune diabetes. However, despite this advanced state of disease, a single i.v. administration of one million DC/sIL-4 resulted in significant protection from the disease progression and the onset of hyperglycemia.

In chapter 3 we demonstrate that exogenous gene transfer of IDO to DC can be used to efficiently generate tolerogenic DC *ex vivo.* Despite the known tolerogenic properties of IDO, few studies have examined exogenous gene transfer of IDO to DC. Previous studies have shown that genetically-engineered IDO+ DC inhibit T cell responses *in vitro* [215], and prolong cardiac allograft survival following adoptive transfer *in vivo* [63]. Our results indicate that transduction with both an IDO adenoviral vector, as well as a CTLA-4-Ig expressing vector results in the production of IDO+ DC. Following incubation with 1-MT, a specific IDO inhibitor, DC/CTLA-4-Ig were no longer suppressive *in vivo* (data not shown), and the exosomes they secreted were no longer able to reduce paw swelling in the DTH model.

However, there remains significant interest in the mechanisms whereby tolerogenic DC and exosomes regulate immune responses. In particular, little is known regarding the nature of exosome signaling *in vivo.* In Chapter 3, we address one aspect of these questions by examining the role of costimulatory molecules on our DC and exosomes. We demonstrate that B7-1 and B-72 are required on DC and exosomes for tolerogenic activity in the DTH model. By examining exosomes secreted from IDO-transduced and rIL-10 treated DC, we conclude that this requirement for B7 is not dependent on the method used to generate the tolerogenic DC. Further, we observe that exosomes lacking B7 molecules retain a reduced ability to decrease paw swelling in the DTH model, suggesting non-B7-dependent mechanism(s) of exosome-mediated

suppression *in vivo*. Surprisingly, loss of PD-L1 and PD-L2 from DC and exosomes had little effect on their function, which was contrary to our initial expectations.

4.2 CLOSING COMMENTS

The results presented in this thesis lay the groundwork for future endeavors in multiple areas. We demonstrate the efficacy of DC transduced to express in IL-4 in prediabetic NOD mice, however, many patients are fully diabetic at the time of diagnosis. Similarly, we demonstrate that generation of IDO+ DC using adenoviral vectors expressing IDO or CTLA-4-Ig is possible and can be used to reduce inflammation in DTH and collagen-induced arthritis [68]. Significant progress was made in examining gene transfer-based strategies for the development of tolerogenic DC and exosomes, however additional work will be required in preclinical models in order to determine their utility in treating human disease. A first step forward would be to examine the ability of DC/sIL-4 to treat NOD mice with recent-onset hyperglycemia, as many patients have are similarly hyperglycemic at the time of diagnosis.

Tolerance induction is a complex process, requiring the interaction of the proper cell types in the appropriate microenvironment and anatomical location. The balance between tolerance and immunity is very sensitive and maintained by the interplay of all of these factors. In this work, we demonstrate a critical role for B7-1 and B7-2 expression on tolerogenic DC and exosomes for the function *in vivo*. In itself, this is interesting as it runs contrary to the prevailing models of tolerogenic DC which postulate that tolerance induction is best achieved with DC with the lowest possible levels of B7 costimulatory molecules. However, it also raises a series of questions as well. Of particular interest is why exosomes have a B7 requirement, specifically,

which cells do they interact with and what are the effects of exosomal B7? However, the small scale of exosomes compared to the limits of currently available technology make these difficult issues to pursue. Nonetheless, this work suggests a role for both DC and exosomes in the induction of tolerance, and suggest that further investigation into their therapeutic applications for autoimmune disease such as type 1 diabetes are justified.

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