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Modulation of Dendritic Cells with the Interleukin-10 Gene on Polycation-Modified Polymeric Particles

Liang Jia

MODULATION OF DENDRITIC CELLS WITH THE INTERLEUKIN-10 GENE ON POLYCATION-MODIFIED POLYMERIC PARTICLES

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By

Liang Jia

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Approved October 30, 2009

Wilson S. Meng Associate Professor of Pharmaceutics (Committee Chair)	Christianah Moji Adeyeye Professor of Pharmaceutics (Committee Member)
James K. Drennen, III Associate Professor of Pharmaceutics (Committee Member)	Christopher K. Surratt Associate Professor of Pharmacology Division Head of Pharmaceutical Sciences (Committee Member)
Alan W. Seadler Associate Academic Vice President for Research (Committee Member)	David A. Johnson Director of Graduate Studies Associate Professor of Pharmacology
J. Douglas Bricker Dean of the Mylan School of Pharmacy Associate Professor of Pharmacology	

ABSTRACT

MODULATION OF DENDRITIC CELLS WITH THE INTERLEUKIN-10 GENE ON POLYCATION-MODIFIED POLYMERIC PARTICLES

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Gene therapy has emerged as a field to modulate cell functions by introducing genes of interest to target cells. An emerging focus in this field is to employ non-viral vectors to deliver immunosuppressive cytokines to dendritic cells (DCs) to attenuate damaging immune responses. DCs serve as potential targets for suppression of T cell responses. In this work, we investigated the ability of polycation-modified polymeric particles complexed with interleukin-10 (IL-10) gene to modulate DCs. The delivery systems (designated as PS_{O10H6} and $PLGA_{O10H6}$) were formed by coating cationic peptide O10H6 (O: ornithine; H: histidine) on the polystyrene (PS) and poly (lactic-co-glycolic acid) (PLGA) particulates. A mouse IL-10 encoding plasmid (pIL-10) was loaded on the surface of PS_{O10H6} and $PLGA_{O10H6}$ via ionic interactions. Physical characterization of these particles revealed stable colloidal dispersions (diameters: 297.2±14nm in

$PLGA_{O10H6}$-pIL-10 and 126.0±8nm in PS_{O10H6}-pIL-10). DNA molecules carried by PS_{O10H6} and $PLGA_{O10H6}$ were protected from serum digestion. Results from *in vitro* gene transfection studies showed two-fold enhancement of IL-10 expression in bone marrow-derived DCs transfected with PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 compared to untransfected DCs. Their suppressive functions were evaluated in an *in vitro* mixed lymphocyte model. Results indicated that PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 modified DCs elicited weakest proliferation of allogeneic bulk T cells as well as CD4 and CD8 T cells among all the delivery modes. Using cell-embedded Matrigel as a surrogate graft, we showed that IL-10 gene-modified DCs suppressed host cell infiltration *in vivo*. These data suggested PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 deliver an overriding suppressive signal to T cells. Further studies revealed T cells stimulated by the IL-10 gene-modified DCs exhibited characteristics of regulatory T (Treg) cells, as evident by up-regulation of a Treg cell marker forkhead-type transcription factor 3 (Foxp3). This result was concomitant with an increase in of transforming growth factor β (TGF-β) production.

Taken together, this work demonstrated that PS_{O10H6} and $PLGA_{O10H6}$ are effective in delivering pIL-10 to modulate DCs to suppress T cell responses. Collectively, the results raise the prospects of using PS_{O10H6} and $PLGA_{O10H6}$ as vectors to deliver immunosuppressive genes to modulate T cell responses *in vivo*.

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CHAPTER 1

INTRODUCTION

Statement of problem

The major function of mammalian immune system is to protect the host from a broad range of pathogenic microorganisms including viruses, bacteria, and other pathogens. However, excessive immune responses towards auto- and alloantigens can lead to host tissue damages, causing inflammation, autoimmune diseases, and transplant rejections. Anti-inflammatory and immunosuppressive agents (e.g. tacrolimus, cyclosporine, rapamycin, glucocorticoids) that can systematically suppress T cell responses have been used. But these drugs are associated with systemic suppression of normal immune functions, resulting in malignant and infectious diseases after years of administration. The toxicity and the life-threatening side effects associated with these agents demand the development of safe and effective therapeutic modalities that can attenuate damaging immune responses.

Dendritic cells are highly specialized antigen presenting cells that interact with T lymphocytes to trigger adaptive immune responses and suppression. DC's ability to induce immune suppression is currently being investigated. These DCs often constitute tolerogenic properties including lower level of co-stimulatory B7 molecules (e.g. CD80, CD86) and/or higher tendency to expand CD4+ Treg cells in animal models (Bellinghausen, Brand et al. 2001; Muller, Muller et al. 2002; Steinbrink, Graulich et al. 2002). Treg cells are identified by their capacity of suppressing proliferation and

cytotoxic activities of effector T cells. They are marked by their constitutive expression of Interleukin-2 (IL-2) receptor α chain (CD25) and Foxp3 (a member of the forkhead/winged-helix family of transcription regulators). Because Treg cells play an important role in damping excessive immune responses towards auto- and alloantigens, the capacity of DCs to induce or expand Treg cells becomes a key measurement of their tolerogenic properties.

A variety of studies suggest that immunosuppressive cytokines including IL-10 and TGF-β render DCs into tolerogenic phenotype. IL-10 is a pleiotropic, anti-inflammatory cytokine that acts on a variety of immune cells, including T cells, natural killer (NK) cells, and antigen presenting cells (APCs). Owning to its exclusive role in initiating adaptive immune responses and tolerance via the interaction with naïve T cells, DC is believed to be the primary mediator for IL-10 suppressive functions. Thus, IL-10 conditioned DCs have been extensively studied as therapeutic modalities to suppress harmful immune actions toward auto- and alloantigens.

IL-10 directly injected *in vivo* demonstrated disadvantages such as short half-life and instabilities. Therefore, efforts have been made to genetically engineer DCs to produce IL-10 in such suppressive signals that might dominate in locale where lymphocytes are programmed. A major approach in advancing this strategy is to develop effective vehicles to deliver IL-10 plasmid to DCs. Recombinant viral vectors have been used to achieve high gene transfection efficiency *in vivo*. However, the potential systemic toxicity of viral vectors and the viral-specific immune elimination by host cells hamper

their use in clinical applications.

Specific aims

Synthetic non-viral vectors fabricated from non-lipid polymers have gained substantial interests in recent years due to their noninflammatory nature and the relative ease in controlling surface functionalities. Among these vectors, submicron polymeric particulates are believed to be most favorable for delivery tasks targeted to DCs because of the fact that DCs constantly take up particulates in the surrounding tissue through phagocytosis and endocytosis due to their role as an immune sentinel.

Polycation-modified polymeric particles have been developed in our lab to deliver plasmid DNA molecules to DCs. O10H6, an ornithine-histidine peptide that binds and condenses plasmid DNA molecules through ionic interactions is adsorpted on the surface of polymeric particles. The **objective** of this work was to investigate the performance of the polycation-modified polymeric particles loaded with a murine IL-10 gene to modulate DC functions. In particular, submicron particles fabricated from two polymeric materials (a schematic diagram of the construct is shown in Figure 1.1), PLGA and polystyrene have been investigated. The former is biodegradable and thus suitable for *in vivo* or *ex vivo* gene transfer. The latter can be used for *ex vivo* transfection of graft DCs, which does not require repeat *in vivo* administrations. With respect to macromolecule delivery, the majority of studies within the literature employ PLGA and polystyrene in the context of vaccination and immune stimulation (O'Hagan, Singh et al. 2004). Whether these polymers are suitable delivery platforms for immunosuppressive cytokines has rarely

been investigated.

Hypothesis

The central **hypothesis** of this work is that the particulate systems (designated as $PLGA_{O10H6}$ and PS_{O10H6}) can effectively deliver pIL-10 to DCs to induce suppression of T cell responses via the expansion of Treg cells. The underling mechanisms are described in the following two aspects. First, the O10H6 modified polymeric particles can bind and stabilize DNA molecules on the surface of the delivery vectors, therefore facilitating their uptakes by DCs. Second, the forced expression of IL-10 in the transfected DCs can induce expansion of Treg cells, thus triggering the suppression of effector T cell responses. Ultimately, this work may raise the prospect of using $PLGA_{O10H6}$ and PS_{O10H6} to deliver suppressive genes to induce immune tolerance *in vivo*.

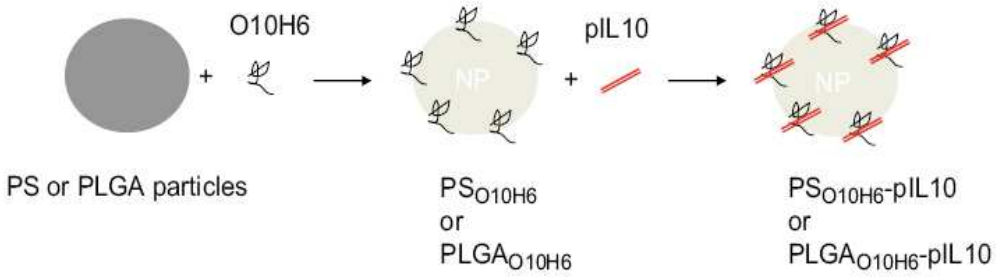


Figure 1.1: Schematic depiction of PLGA$_{O10H6}$ and PS$_{O10H6}$ particles loaded with DNA condensates on the surface.

CHAPTER 2

LITERATURE REVIEW

Dendritic cells

Origin and differentiation of dendritic cells

DCs were first discovered as Langerhans cells (LCs) in skin in 1868. Nevertheless, characterization of DCs began only 35 years ago when Steinman and Cohn identified a population of spleen cells having a distinct antigen (Ag) presenting function and morphological features with dendrites, and named these cells dendritic cells.

DCs differentiate from hematopoietic progenitors in the bone marrow and home to blood as well as peripheral non-lymphoid/lymphoid tissues and thymus via a broad pattern of chemokine receptors and adhesion molecules on their surfaces. Depending on their functionality and surface markers, DCs can be classified into mature and immature state.

Immature DCs are characterized by their highly endocytic and phagocytic activities permitting Ag capture, low secretion of pro-inflammatory cytokines, and poor antigen presentation capacities. These DCs lack major histocompatibility complex (MHC) molecules and the requisite accessory markers for antigen presentation and T-cell activation, such as CD40, CD54, CD80 and CD86, but are extremely well equipped with antigen-capturing receptors such as macrophage mannose receptor 7, DEC-205, and Fc receptors to capture antigens (Jiang, Swiggard et al. 1995). DCs in non-lymphoid

peripheral tissues such as skin (e.g. Langerhans cells and dermal DCs), lung, liver, kidney, heart, and mucosa of the digest and respiratory tract (mucosal surface-associated DCs) exist in immature state and provide a sentinel system at the front line of pathogen entry (Banchereau, Briere et al. 2000). Following a microbial infection and tissue damage, immature DCs migrate to infection or damage regions to capture antigens in response to the chemokines released from these tissues.

Mature DCs are characterized by several properties such as loss of endocytic and phagocytic capacities (Garrett, Chen et al. 2000), high expression level of MHC class II molecules on the cell surface and increased expression of co-stimulatory molecules such as CD80 and CD86, which correlate with the ability to induce the activation of the Ag-specific or allogeneic T cells. Additionally, mature DCs reprogram their responsiveness to chemokines by modifying expression of chemokine receptors, including down-regulation or desensitization of CCR1, CCR5, and CCR6 to decrease their responsiveness to inflammatory cytokines (e.g. CCL3, CCL5, and CCL20), and up-regulation of CCR7 to improve their responsiveness to homeostatic chemokines such as CCL19 and CCL21. These changes in DC's characteristics down-regulate their capacity to take up antigens but advance their antigen presenting ability and stimulatory activity towards T cells. Consequently, after antigen sampling, DCs will migrate from periphery to the draining lymph nodes, where they present antigens to specific T cells and induce their activation and differentiation into effector cells to initiate primary immune responses (Banchereau, Briere et al. 2000).

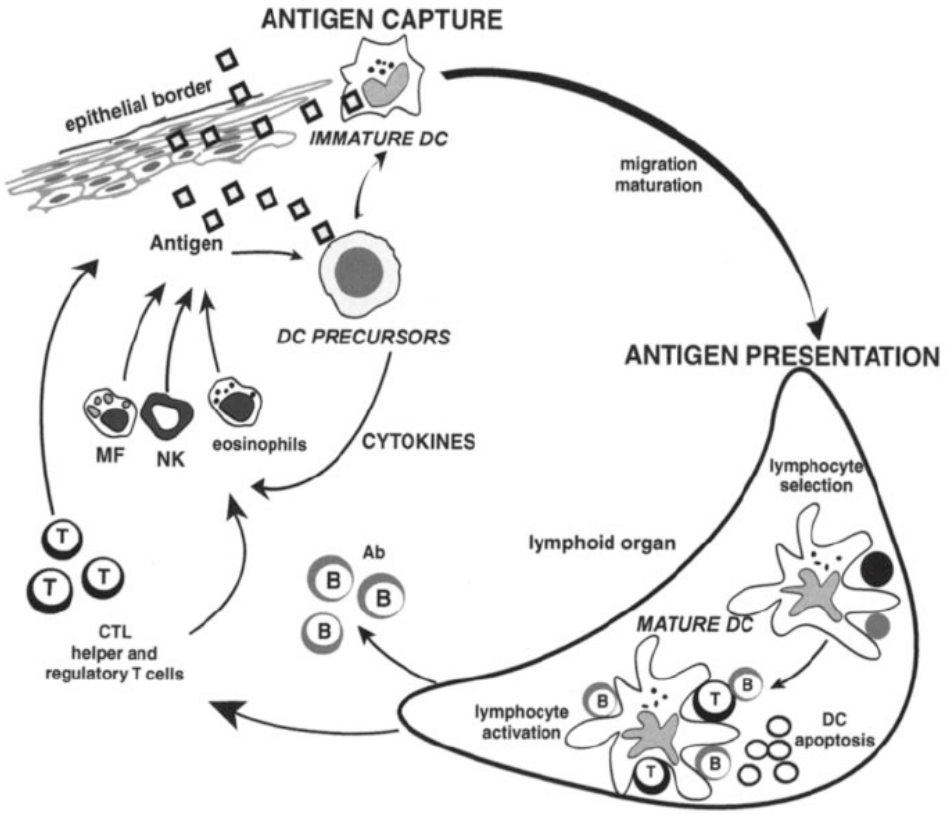


Figure 2.1: The life cycle of dendritic cells.

Dendritic cell subsets

Multiple DC subsets have been defined based on their phenotypes and functions. CD8, which is expressed in the form of an αα-homodimer on DCs, has been used to define DCs subsets, in which CD8α+ DCs have been referred to as "lymphoid DCs" and CD8α− DCs as "myeloid DCs". Combining CD8 with the other four markers, including CD4, CD11b (the integrin-α_M chain of macrophage receptor 1 [Mac-1]), CD11c (the integrin-αx chain of Mac-1), and CD205, five DC subsets are found in the lymphoid tissue of mice (Shortman and Liu 2002). Spleen contains three of these subsets, including CD4−CD8α+ (20% of spleen DCs), CD4+CD8α− (40% of spleen DCs), and CD4−CD8α- (15% of spleen DCs). Among thymic DCs, the CD4−CD8α^{high} CD11b−CD205+DC is the dominant subtype. Lymph nodes (LNs) contain two extra DC subtypes that are not normally found in spleen, which have apparently arrived in the LNs through the lymphatic system (Iwasaki and Kelsall 2000). Between them, CD4−CD8α−CD11b+CD205+ DC represents the mature form of interstitial tissue DCs. CD4−CD8α^{low}CD11b+CD205^{high} DC, found only in skin draining LNs, is believed to be the mature form of Langerhans cells (Iwasaki and Kelsall 2000).

The CD8α− DC subset is efficient stimulators of CD4+ and CD8+ T cells *in vitro*, and can direct immune response *in vivo* (Yasumi, Katamura et al. 2004). Both mature interstitial tissue DCs and mature LCs appear to be fully activated and efficient stimulators of naïve CD4+ T cells.

DCs subsets including CD4−CD8α^{high}CD11b−CD205+ lymphoid DCs and B220+

DCs can promote T-cell tolerance. It has been shown that CD4−CD8α^{high}CD11b−CD205+ DCs have a regulatory effect on T cells, in which they can induce apoptosis in CD4+ T cells and a limited CD8+ T cell response (Anjuere, Martin et al. 1999; Vremec, Pooley et al. 2000). DCs that express B220, a CD45 isoform in mice, can be found in all lymphoid organs of mouse. It has been proposed that B220+ DCs are involved in the maintenance of T-cell tolerance by promoting T regulatory cell differentiation (Martin, Del Hoyo et al. 2002).

Table 1 | **Lymphoid tissue distribution of mouse dendritic cell (DC) subtypes**

	'Lymphoid' DCs $CD4^-CD8^{hi}$ $CD205^{hi}CD11b^-$	'Myeloid' DCs $CD4^+CD8^-$ $CD205^-CD11b^+$	'Myeloid' DCs $CD4^-CD8^-$ $CD205^-CD11b^+$	'Myeloid' DCs $CD4^-CD8^-$ $CD205^+CD11b^+$	'Langerhans' DCs $CD4^-CD8^{lo}$ $CD205^{hi}CD11b^+$
Percentage total DCs in					
Spleen	23	56	19	< 4	< 1
Thymus	70				
Mesenteric lymph nodes	19	4	37	26	< 4
Skin-draining lymph nodes	17	4	17	20	33

Table 2.1. Lymphoid tissue distribution of mouse dendritic cell subtypes.

Dendritic cells in antigen presentation

Dendritic cells reside in most peripheral tissues, particularly at sites of interface with the environment (skin and mucosae) (Banchereau and Steinman 1998; Banchereau, Briere et al. 2000), where they take up self and non-self antigens from pathogens, infected cells, dead cells, or their derived products. Internalized antigens are then processed into proteolytic peptides, and these peptides are loaded onto MHC class I and II molecules. This process of antigen endocytosis, degradation, and loading is called antigen presentation.

There are at least four endocytic pathways exist distinguished based on the size of the cargo internalized and the mechanism of internalization: macropinocytosis, phagocytosis, and receptor mediated endocytosis (including clathrin-mediated endocytosis and caveolae-mediated endocytosis) (Trombetta and Mellman 2005).

Macropinocytosis is characterized by the bulk fluid uptake of soluble antigens through the actin skeleton by DCs and macrophages (Norbury 2006). This form of endocytosis is down-regulated in DCs upon maturation.

Phagocytosis, which is believed to be the first pathway associated with host defense, involves engulfment of large particulates (> 1 μm in diameter) derived from both pathogen and endogenous antigens. Immature DCs can phagocyte a broad range of particulates, including microbes, apoptotic cells, inert particles, and liposomes.

Langherans cells and bone marrow-derived dendritic cells at an early stage of their development are shown to internalize particulate antigens by phagocytosis (Steinman and Swanson 1995). DC's endocytic receptors including Fcγ receptors, Scavenger receptors (SRs), complement receptors, and a variety of lectins contribute to phagocytic process due to their ability to signal actin assembly and drives pseudopod extension and particle engulfment after binding to their ligands. Additionally, some microbes can promote their own entry into cells by shedding vesicles that may be internalized with the absence of endocytic receptors (Cossart and Sansonetti 2004).

Endocytosis is characterized by the uptakes of antigens, smaller vesicles or particulates (less than 1 μm) through clathrin-coated vesicles or by caveolin-containing invaginations. These antigens will be processed and presented in DCs through a sequence of events in clathrin-mediated or caveolae-mediated endocytic pathway, starting with transporting the antigen to an acidic endosomal/lysosomal compartments within the cell that allows antigen degradation into peptides fragments. Alternatively, a fraction of caveolae may fuse with the endoplasmic reticulum (ER) following internalization. From the ER, antigens can gain access to endosomal compartments by normal recycling or by fusion of ER with endosomal or phagosomal membrane (Tsai, Ye et al. 2002).

As antigens are partially degraded into peptides fragments, vesicles coated with nascent MHC class II molecules fuse with the lysosome. Peptides that have high affinity for the binding site located within the MHC class II heterodimer will displace the peptide on MHC class II molecules. The resulting MHC/Ag-derived peptide complexes are then

transported to the DC cell membrane, where they will be presented to Ag-specific CD4+ T cells (Figure 2.2; (Virella 2007).

One thing that needs to be pointed out is the maturation of DC down-regulates macropinocytosis and phagocytosis, but it seems to have little effect on clathrin-mediated endocytosis pathway (Garrett, Chen et al. 2000). Thus, antigen or particulates can still be internalized in mature DCs.

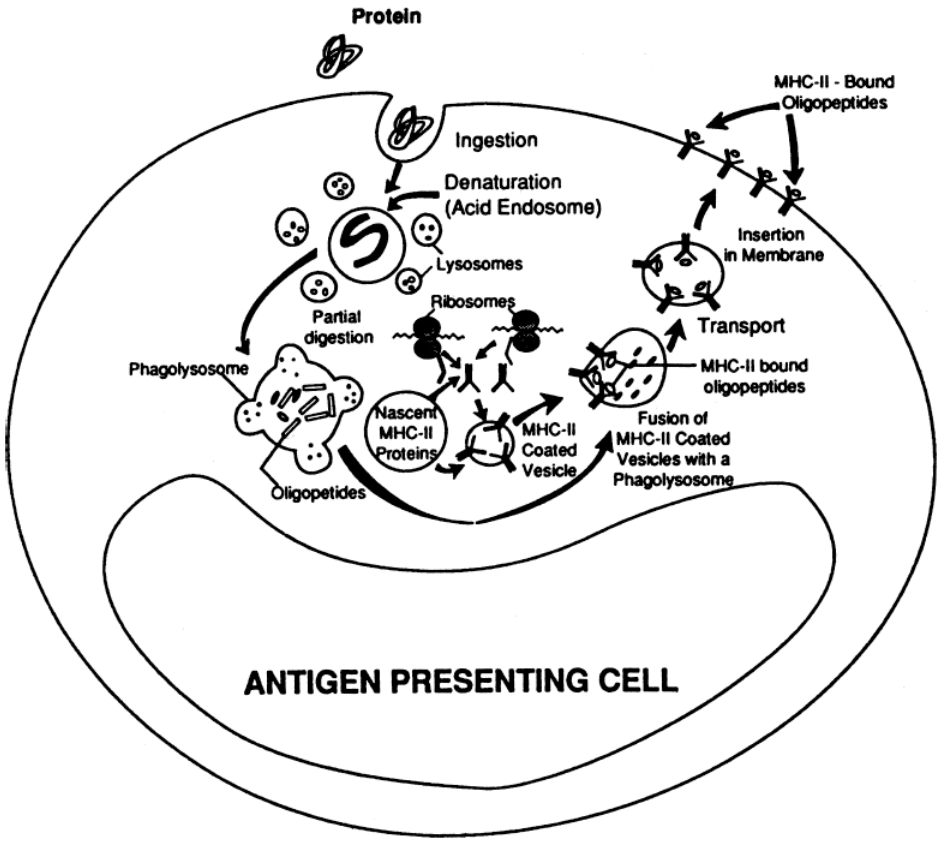


Figure 2.2: Schematic representation of the general steps in antigen processing.

Dendritic cells in T cell activation

The interaction between MHC class II/Ag-derived peptide complex on DCs and the complementary T cell receptor (TCR) on CD4+ T cells (signal 1, Figure 2.3) is the initial step to trigger a coordinated sequence of signals that induce T cell activation. In an acute transplant model, the miss match between MHC class I molecules on graft DCs and TCR on host CD8+ T cells will trigger T cell activation. Additionally, co-stimulatory pathways are required to deliver secondary signals to activate T cells. These pathways are mediated by interaction between CD28 on T cells and its correspondent B7 molecules (e.g. CD80, CD86) on DCs (signal 2, Figure 2.3). Mature DCs up-regulate co-stimulatory molecules, which will improve DC's T cell priming ability.

MHC class I and MHC class II molecules interact exclusively with CD8+ cytotoxic T cells (CTLs) and CD4+ helper T cells, respectively. The engagement between MHC class II complex and CD4+ helper T cells induces downstream signals to activate and translocate transcription factors, such as the nuclear factor-kappa B (NF–κB) and the nuclear factor of activated T cells (NFAT). Once entering the nucleus, these factors trigger expression of IL-2, IL-2 receptor, and the release of pro-inflammatory cytokines [e.g. interleukin-12 (IL-12), interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α)]. These changes will stimulate T cells proliferation and activation into effector T cells, which in turn orchestrate cell-mediated immunity and promote inflammation. CTLs will undergo clonal expansion with the presence of IL-2 (Bennett, Carbone et al. 1997). While exposed to antigen-specific somatic cells, CTLs will release perforin and granzyme, or up-regulate Fas ligand to kill target cells. Specifically, perforin forms pores

in the plasma membrane of target cells that allows granzyme, a serine protease, to enter the cytoplasm of these cells, which in turn leads to a caspase cascade that induces their apoptosis (program cell death). Fas ligand expressed on activated CTLs can bind to Fas receptor on target cells to induce apoptosis.

Figure 2.3. T-cell activation by antigen presenting cells. Reprinted by permission from Macmillan Publishers Ltd: Journal of Investigative Dermatology (2006) 126, 32–41, copyright 2006.

Dendritic cells in cell-mediated and humoral immunity

Antigens and lymphocytes had long been focused in the study of immunity in past decades. It was found that immune actions do not always been triggered by the simple presence of those two parties. APCs, especially DCs play a very significant role in initiating and modulating immune functions, including controlling T-cell and B cell immunities, and developing immune tolerance to self-antigen.

DCs are efficient in triggering cell-mediated immunity, in which target cells with specific antigens are recognized and killed by T cells that circulate in the blood stream. This type of T cell response is typically challenged by the small amount (typically one hundred or less per cell) of antigen presented on target cells (e.g. tumor, infection cells), and the rare Ag-specific T-cell clones (typically at a frequency of 1/100,000 or less) in the host. DCs provide a means of solving these challenges by capturing, internalizing and processing these antigens, and displaying them at their surface, along with up-regulation of co-stimulatory surface markers. DCs then migrate to lymphoid organs including spleen and drain lymph nodes, where they deliver activating signals to engage T cells in antigen recognition, leading to the clonal expansion and activation of Ag-specific T cells. More importantly, DCs are very effective in stimulating Ag-specific T cells: one DC is capable of turning 100 to 3,000 T cells into active T cells.

Besides their effects on cell-mediated immune response, DCs are also known to stimulate humoral immunity by presenting antigen peptides to specific T-helper cells and orchestrating them into Type 2 helper T cells (Th2). Th2 secrete cytokines such as

interleukin-4 (IL-4) and interleukin-5 (IL-5), and stimulate the proliferation and activation of specific B cells. Activated B cells subsequently produce antibodies that inhibit pathogens. Additionally, DCs in interstitial tissues can directly interact with B cells to stimulate the production of antibodies including immunoglobulin A2 (IgA2) (Banchereau, Briere et al. 2000).

Dendritic cells in immune tolerance

DCs play a key role in orchestrating cell-mediated and humoral immunity. Additionally, there is emerging evidence that DCs induce Ag-specific tolerance by taking up antigens in peripheral tissues and presenting antigens in the context of MHC molecules to T cell. (Morelli and Thomson 2003; Steinman, Hawiger et al. 2003). An addition study demonstrated that effective deletion of auto-reactive thymocytes was found in developing thymus injected with Ag-bearing DCs, but not with other APCs, including macrophages (Zal, Volkmann et al. 1994). In peripheral tissues, DCs can capture and present self-antigens that are exclusive to thymus to T cells in draining lymph nodes to induce tolerance as a result of T-cell anergy or deletion. There is also evidence that DCs contribute to the expansion and differentiation of regulatory T cells.

The control of immunity requires that DCs undergo differentiation or maturation in response to stimuli. In mature DCs, phenotypic changes such as increased expression of MHC-peptide complexes, co-stimulatory molecules and IL-2 contribute to their abilities to process antigens and activate T cells. Immature DCs, which express low level of MHC and co-stimulatory molecules, capture and present antigens to targeting naïve T

cells and induce their tolerance (Steinman, Turley et al. 2000; Roelen, Schuurhuis et al. 2003). One strategy that delivers defined antigens to specific populations of immature DCs to induce T cell tolerance *in vivo* has been explored (Jonuleit, Schmitt et al. 2000; Hawiger, Inaba et al. 2001; Barrat, Cua et al. 2002; Liu, Iyoda et al. 2002).

Interestingly, it has been demonstrated that some population of mature DCs can promote T cell tolerance both *in vitro* and *in vivo* (Albert, Jegathesan et al. 2001; Menges, Rossner et al. 2002; Verhasselt, Vosters et al. 2004). Therefore, DC maturation is no longer a distinguishing feature of immunogenic activity. Literature data suggested that both immature and mature DCs can expand Treg cells *in vitro* and *in vivo* under certain conditions, which is associated with the suppressive property of these DCs (Yamazaki, Iyoda et al. 2003; Tomasoni, Aiello et al. 2005). Specifically, DCs that express immunoglobulin like transcript 3 (ILT3) contribute to the induction of Treg cells. Studies also showed that IL-10 producing DCs isolated from lung or gut mucosal–associated lymphoid tissue suppress immune responses, as IL-10 enhances the formation of mouse (Barrat, Cua et al. 2002) and human (Levings, Sangregorio et al. 2002) Treg cells. Administration of IL-10 producing pulmonary DCs can suppress immunity following adoptive transfer of hyporesponsive antigen into mice (Akbari, DeKruyff et al. 2001).

It has been suggested that autoimmunity, certain inflammatory diseases, and transplant rejection can be influenced by the Ag-specific tolerogenic role of DCs. Two major approaches that use DCs to induce immune tolerance are under investigation: 1) use of immature DCs or control of DC maturation, and 2) engineering DCs using

immunosuppressive cytokines. In the first approach, clinical use of immature DCs may be limited by the graduate maturation of those cells under inflammatory conditions. In the second approach, immunosuppressive cytokines such as IL-10 can induce DCs into suppressive phenotype and expand the population of Treg cells. A strategy that utilizes this approach is to genetically modify therapeutic DCs *ex vivo* and re-infuse them to the host tissue or organ, where they can down-regulate immunity and expand regulatory T cells. Therefore, development of genetically modified DCs expressing IL-10 could be a suitable method for the induction of immune tolerance.

Interleukin-10

IL-10 was originally recognized as cytokine-synthesis-inhibiting factor (CSIF) when it was discovered in 1989 because of its ability to suppress the production of pro-inflammatory cytokines in macrophages and Type 1 helper T cells (Th1). Further studies revealed that IL-10 profoundly acts on a variety of immune cells to block the expression of pro-inflammatory cytokines and co-stimulatory molecules, leading to the suppression of T cell activation during an immune response.

Structure and biological activities of human and mouse Interleukin-10

The primary structures of human IL-10 (hIL-10) and mouse IL-10 (mIL-10) were determined by cloning cDNAs encoding the cytokines (Vieira, de Waal-Malefyt et al. 1991). Nucleotide sequence of mIL-10 has a significant degree (more than 80%) of homology with hIL-10 throughout their entire length (Howard and O'Garra 1992). The major difference between the two sequences is the insertion of a human *Alu* repetitive sequence element in the 3'-untranslated region of the hIL-10 cDNA clone. hIL-10, which contains 160 amino acid residues, has a primary structure comprising of an 18.5 kDa polypeptide that lacks detectable carbohydrate. mIL-10 is N-glycosylated at a site near its N-terminus that is missing from hIL-10. This glycosylation is heterogeneous, resulting in a mixture of 17, 19, and 21 kDa species (Moore, Vieira et al. 1990). However, the N-glycosylation of mIL-10 is not required for biological activity since mutants lacking the N-linked site are still active.

The secondary structure of hIL-10 and mIL-10 comprises of four helices in each subunit (Shanafelt, Miyajima et al. 1991). Disulfide bonds between cysteine (cys) residue 1, 3 and cys 2, 4 maintain helical structures of the subunit and subsequently the biological integrity of IL-10. The tertiary structure of hIL-10 is a V-shaped homodimer with six helices (four derived from one subunit and two from the other) in each half (Syto, Murgolo et al. 1998), while the structure of mIL-10 is a monomer with four helices.

It has been demonstrated that hIL-10 is active on mice. However, mIL-10 has not yet been found to cross-react significantly on human. Recombinant mlL-10 and hIL-10 have been expressed in cells from different species, including COS7 cells, mouse myeloma cells, Chinese hamster ovary (CHO) cells, baculovirus expression systems, and E. coli. The biological activities of these recombinant IL-10 proteins are so far indistinguishable.

Regulation of IL-10 by immune cells

IL-10 was originally described as a product of Th2 cells. It was soon discovered that many other T cell types (e.g. Th1 cells, Treg cells) and non–T cell hematopoietic cells (e.g. DCs, natural killer cells, monocytes, and macrophages) also produce IL-10. Currently, immunoregulators responsible for the generation of IL-10 have not been completely characterized and the mechanism by which these agents stimulate the production of IL-10 in a variety of cell types are under investigation. It is shown that different cells respond differently to IL-10 stimulus, and the response can depend on the

microenvironment of the cell. For example, IL-12 has been described to induce the generation of Th1 cell clones that produce large amounts of IFN-γ and IL-10. Lipopolysaccharide (LPS) has been reported to stimulate IL-10 production by human monocytes and alveolar macrophages concomitant with activation of p38 and Jun N-terminal (JNK) mitogen-activated protein (MAP) kinases, which involve in cell differentiation and apoptosis (Chanteux, Guisset et al. 2007). In addition, Interferon alpha (IFN-α) and interferon beta (IFN-β) have been demonstrated to increase IL-10 production by monocytes and CD4+ T cells (Porrini, Gambi et al. 1995); (Ho, Kaufman et al. 1996; Schandene, Del Prete et al. 1996; Platzer, Docke et al. 2000). Systemic release of TNF-α also induces IL-10 through a negative feedback using NF-κB dependent pathway (Barsig, Kusters et al. 1995). Literature data also suggested that TGF-β up-regulates IL-10 mRNA in Lewis rat with experimentally induced uveitis (Li, Sun et al. 1996). Interleukin-22 (IL-22), a cytokine produced by activated T cells, has been shown to increase IL-10 production by colon epithelial cells via the activation of signal transducer and activator of transcription 3 (STAT3) pathway (Nagalakshmi, Rascle et al. 2004). Studies also suggested that IL-2 enhances IL-10 production by CD4+CD25+ Treg cells or NK cells through STAT5 or STAT4 activation, respectively (Grant, Yao et al. 2008; Tsuji-Takayama, Suzuki et al. 2008).

Other cytokines including IL-4, interleukin-13 (IL-13) and interleukin-27 (IL-27) have been shown to down-regulate IL-10 production by monocytes via STAT1 pathway (Chomarat, Rissoan et al. 1993; de Waal Malefyt, Figdor et al. 1993). In addition, I kappa B (I-κB) family member Bcl-3, a regulatory protein for NF-κB pathway, has been

demonstrated to inhibit IL-10 expression in macrophages (Riemann, Endres et al. 2005). Also, IL-10 can down-regulate IL-10 mRNA production in a negative feedback manner (de Waal Malefyt, Abrams et al. 1991).

IL-10 signal transduction

It has been elucidated that IL-10 exerts its biological functions through the activation of IL-10 receptor complex on target cell surface. The IL-10 receptor complex comprises of a high affinity (dissociation constant [K_d]~35–200 pM) IL-10 receptor 1 (IL-10R1) that binds ligands and a low affinity IL-10 receptor 2 (IL-10R2) that initiates signal transduction (Liu, Wei et al. 1994). Consistent with IL-10's role as an immune inhibitor, down-regulation of IL-10R1 expression is associated with T cell activation (Liu, Wei et al. 1994). However, no evidence for regulation of IL-10R2 expression has been found in associated with immune cell activation (Gibbs and Pennica 1997).

As of now, limited knowledge is available regarding the IL-10 intracellular signaling transduction pathway. The best-characterized IL-10 signaling pathway is the Janus tyrosine kinase (JAK)/STAT system. In details, the difference of the affinity between IL-10R1 and IL-10R2 triggers a sequential binding mechanism, by which IL-10 first binds to IL-10R1 and subsequently engages IL-10R2 (Walter 2002). This interaction induces cross-phosphorylation of two tyrosine residues on the intracellular domain of IL-10R1 and subsequently engages the JAK family tyrosine kinases 2 (TYK2), which are constitutively associated with IL-10R1 and IL-10R2, respectively (Finbloom and Winestock 1995). The activated tyrosine kinase mediates the direct interaction between

STAT3 and IL-10 receptor complex, and subsequently phosphorylates latent transcription factors STAT1, STAT5 in cytoplasma with the formation of different DNA binding complexes (Weber-Nordt, Riley et al. 1996; Wehinger, Gouilleux et al. 1996). These complexes translocate into nucleus, bind to specific sites on DNA and trigger the activation/regulation of specific genes. Among genes activated by IL-10 are suppressors of cytokine synthesis (SOCS)-1 and SOCS-3, which play key roles in the inhibitory effects of IL-10 on immune cell activation (Cassatella, Gasperini et al. 1999; Berlato, Cassatella et al. 2002). It has been shown that SOCS-1 silences signaling by binding to the activation loop of JAK and physically occluding the kinase active site, preventing it from phosphorylating its substrates (Morse, Bidwell et al. 1999).

Both *in vitro* and *in vivo* studies in gene-deficient mice have linked the biological function of IL-10 to JAK/STAT signaling pathways. It was demonstrated that macrophages from JAK1 knockout mice do not respond to IL-10 (Rodig, Meraz et al. 1998), which indicates that JAK1 plays a key role in IL-10 response. In this pathway, STAT3 is served as an obligatory mediator of IL-10 signaling. STAT3 is recruited directly to the IL-10/IL-10R complex via tyrosine residues in the IL-10R1 cytoplasmic domain and phosphorylated in response to IL-10 (Weber-Nordt, Riley et al. 1996). Study showed that mice, in which STAT3 is abolished in macrophages and neutrophils, develop chronic enterocholitis, and their macrophages are completely resistant to the effects of IL-10 (Riley, Takeda et al. 1999).

In addition to JAK/STAT pathway, a number of studies demonstrated that IL-10

inhibits NF-κB pathway, which controls the synthesis of pro-inflammatory cytokines (Clarke, Hales et al. 1998; Ehrlich, Hu et al. 1998; Schottelius, Mayo et al. 1999). Further mechanistic studies revealed two different mechanisms: 1) IL-10 blocks NF-κB translocation in monocytes and macrophages by inhibiting IKK, a I-κB kinase, thus inhibiting degradation of I-κB, a NF-κB inhibitor; and 2) IL-10 inhibits DNA binding of NF-κB present in the nucleus. It was also shown that IL-10 inhibits the synthesis of interleukin-1 (IL-1), interleukin-6 (IL-6) and TNF-α by promoting the degradation of their mRNA (Wang, Wu et al. 1995). IL-10 can also stimulate phosphatidyl inositol-3 and p70 S6 kinases, which play important roles in promoting the expression of IL-10 (Crawley, Williams et al. 1996).

Immunosuppressive functions of IL-10

Literature data revealed that IL-10 suppresses immune action in damaging immune responses. It was demonstrated that both IL-10 monoclonal antibody (mAb) treated and IL-10 gene knockout mice developed exaggerated inflammatory responses to peritonitis or endotoxemea (van der Poll, Marchant et al. 1995; Lang, Rutschman et al. 2002).

The immunosuppressive action of IL-10 can be achieved both directly and indirectly on a number of immune cells. IL-10 acts on monocytes and macrophages to inhibit the synthesis of a number of cytokines that stimulate T cell responses, such as IL-1α, IL-1β, IL-6, IL-2, IL-12, IFN-γ, TNF-α GM-CSF, G-CSF, and M-CSF (Pestka,

Krause et al. 2004). Among them, IL-1 and IFN-γ are crucial for inflammatory activities due to their synergistic effect on inflammatory pathway. IL-10 also inhibits chemokines secreted by activated monocytes, including MCP-1, MIP-1, MIP-2, Rantes, MDC and IP-10 (Kopydlowski, Salkowski et al. 1999). These chemokines are implicated in the recruitment of monocytes, dendritic cells, neutrophils, and T cells in immune responses. Moreover, IL-10 enhances expression of natural antagonists of these cytokines. For instant, IL-10 enhanced production of IL-1 receptor antagonist (IL-1RA) and soluble p55 and p75 TNFR, which inhibit expression of IL-1RI and IL-1RII (Dickensheets and Donnelly 1997) of monocytes.

More importantly, IL-10 can modulate DC phenotypes and functions to suppress T cell activation and proliferation. Earlier studies showed that IL-10 down-regulates surface expression of MHC class II and co-stimulatory B7 molecules (e.g. CD80 and CD86) as well as IL-12 production by various types of DCs (Kubin, Kamoun et al. 1994; Willems, Marchant et al. 1994; Tong, Toshiaki et al. 2005). Addition of IL-10 during the culture of bone marrow derived DCs *in vitro* with GM-CSF and IL-4 inhibits co-stimulatory molecule and IL-12 elevation of these DCs by LPS and CD40 ligand (Fortsch, Rollinghoff et al. 2000). Moreover, observations in recent decades have shown that IL-10 treated DCs, including fully matured DCs ($B7^{high}$), can expand Treg cells or induce an anergy state in Ag-activated T cells (Takayama, Nishioka et al. 1998; Zeller, Panoskaltsis-Mortari et al. 1999; Mahnke and Enk 2005). These effects will lead to the suppression of T cell responses. Additional study demonstrated that treatment of DCs with glucocorticosteroids, vitamin D3, and prostaglandins in T cell co-cultures resulted in

IL-10-producing regulatory T cells (Penna and Adorini 2000). In general, the effects of IL-10 on DCs are consistent with inhibition of Th1 immune response and can be achieved by inhibitory effects on DCs, or by induction of T cell anergy or regulatory T cell expansion.

Besides its regulatory effects on DCs, IL-10 is capable of inhibiting cytokine production and proliferation of CD4+ T cells by directly affecting their functions. It has been shown that IL-10 suppressed anti-CD3 mAb stimulated T cell proliferation via inhibition of IL-2 production as well as IL-5 secretion by T cells (de Waal Malefyt, Yssel et al. 1993; Schandene, Alonso-Vega et al. 1994). Other studies showed that IL-10 inhibited CD28 tyrosine phosphorylation and abrogated CD28 downstream signaling to down-regulate co-stimulatory pathway and promote T cell anergy (Akdis, Joss et al. 2000; Joss, Akdis et al. 2000; Yang, Li et al. 2006). Additionally, study has demonstrated that naïve T cells that exposed to IL-10 *in vitro* developed into Treg cells (Levings, Sangregorio et al. 2001).

Owing to the suppressive function of IL-10, it has been used to hamper damaging immune responses that are prone to be found in autoimmunity, inflammation, as well as transplantations. Recent studies showed that administration of IL-10 to cardiac or cornea allograft models in rats or rabbits can prolong their survival after transplantation (Furukawa, Oshima et al. 2005; Yang, Li et al. 2006; Gong, Pleyer et al. 2007).

Regulatory T cells

T cells that can suppress immune response were first reported in the 1970s: when animals were injected with high doses of immunogen, a tolerant T cell population that is specific to that immunogen was developed (Chatenoud, Salomon et al. 2001). It was not until the 1990s that the concept of regulatory T cells was introduced. Treg cells are a subset of T lymphocytes involved in the maintenance of immune tolerance to auto- and alloantigens. Disordering in the development and function of Treg cells is related to the occurrence of autoimmune and inflammatory diseases in humans and animals. Emerging data suggests that adaptive immune response involves the recruitment of both effector cells and Treg cells. The balance between the two populations is critical for the establishment of tolerance to self- and non-self-antigens. Conversely, the literature has demonstrated that the immunosuppressive potential of Treg cells can be utilized therapeutically to treat autoimmune diseases and facilitate transplantation tolerance (Hawiger, Inaba et al. 2001). It was shown that Treg cells (CD25+CD4+) enriched from normal mice suppress allergy, establish tolerance to organ grafts, and prevent graft-versus-host diseases after bone marrow transplantation (Sakaguchi 2005).

Natural and adaptive regulatory T cells

Regulatory T cells are classified into naturally occurring and adaptive Treg cells. Naturally occurring Treg cells are generated within the thymus and exit to the peripheral tissue with fully functional suppressive phenotype. These Treg cells are CD4+ and express high affinity IL-2 receptor α chain (CD25) on the cell membrane surface. They

constitute approximately 5-10% of peripheral CD4+ T cell population. Naturally occurring Treg cell's function of maintaining self-tolerance has been demonstrated in many *in vitro* and *in vivo* experiments (Takahashi, Kuniyasu et al. 1998; Belkaid, Piccirillo et al. 2002; Nishimura, Sakihama et al. 2004). For instance, depletion of naturally occurring Treg cells by thymectomy in both neonatal and adult mice results in the appearance of tissue-specific auto antibodies in blood circulation and autoimmune damage in various organs (e.g. stomach, thyroid, ovaries, islets, or testes) (Sakaguchi 2000). Additionally, direct depletion of naturally occurring Treg cells results in excessive immune response to non-self-antigens, leading to inflammatory diseases such as bowel syndromes (Singh, Read et al. 2001).

Unlike naturally occurring Treg cells, adaptive Treg cells are developed in the periphery. They can be generated or expanded by CD4+CD25+ naturally Treg cells or by altering the activity of CD4+CD25- T cells in the presence of a number of suppressive cytokines, such as TGF-β, IL-10 and IL-35 (Groux, O'Garra et al. 1997; Chen, Jin et al. 2003; Kretschmer, Apostolou et al. 2005). These cytokines can transfer naïve T cells into different Treg cell subtypes including Foxp3+ Treg cells, IL-10 secreting Tr1 regulatory T cell (Tr1) (Chen, Kuchroo et al. 1994; Groux, O'Garra et al. 1997), and TGF-β secreting T cells (Th3) cells. Those adaptive Treg cells demonstrate the same immune suppressive function as naturally occurring Treg cells in many *in vitro* and *in vivo* studies (Groux, O'Garra et al. 1997; Zheng, Wang et al. 2004); (Khattri, Cox et al. 2003; Yamazaki, Iyoda et al. 2003; Fehervari and Sakaguchi 2004).

Regulatory T cell markers

An optimal approach of characterizing Treg cells is to identify a unique functional Treg cell marker. $CD5^{high}$ and $CD45RB^{low}$ were first identified in the 80's as surface markers of CD4+ T cell with suppressive function (Sakaguchi, Fukuma et al. 1985; Morrissey, Charrier et al. 1993). These findings prompted the search for a more specific marker in defining such immune suppressive CD4+ T cells.

It was not until 1995 that the discovery of CD25 expressed on a subset of T cells with regulatory properties was revealed (Sakaguchi, Sakaguchi et al. 1995). CD25 (the IL-2 receptor α-chain) positive T cells, which constitute 5%–10% of peripheral CD4+ T cells and less than 1% of peripheral CD8+ T cells in normal naïve mice and humans, are contained in the $CD5^{high}$ and $CD45RB^{low}$ fraction of CD4+ T cells. It was shown that when spleen cell suspensions from normal mice were transferred to T cell–deficient mice, animals that received suspensions depleted of CD25+ cells developed autoimmune diseases consisting of gastritis, oophoritis, thyroiditis, insulitis, and arthritis. Addition of purified CD4+CD25+ T cells prior to transfer prevented the autoimmunity. Further investigation demonstrated that CD4+CD25+ T cells had dominant suppressive effects on conventional CD4+ CD25− T cells *in vitro*, and their survival and growth relied on IL-2. Although these studies were performed in mice, but existence of a similar CD4+CD25+ T cell population was subsequently confirmed in humans (Ng, Duggan et al. 2001).

CD25 is a useful marker for obtaining a highly enriched population of Treg cells in normal naïve mice (Sakaguchi, Sakaguchi et al. 2001). However, CD25 is not unique

to Treg cells owing to the fact that conventional T cells also up-regulate CD25 marker when activated through their TCR. Nevertheless, within the CD4+CD25+ T cell population there exist cells with potent suppressive capabilities.

Recent discovery of the Treg-specific transcription factor Foxp3 supported the identification of Treg cells as a lineage potentially distinct from other T cells. Being a master control gene of Treg cells, Foxp3 transcription factor orchestrates a wide range of biological processes that are critical for the development, differentiation, and function of Treg cells (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003; Khattri, Cox et al. 2003). Therefore, Foxp3 is currently one of the most reliable Treg markers.

Constitutive expression of Foxp3 gene can be used to characterize Treg cells. Foxp3 gene was first identified as the defective gene in the mouse strain Scurfy, with an X-linked recessive mutant. The mutant male mouse exhibits hyperactivation and overproduction of CD4+ T cells, resulting in autoimmune disease and dysregulated T cell function that leads to its death within a month after birth (Brunkow, Jeffery et al. 2001). A human counter part of Scurfy, which has the mutation of the human FOXP3 gene, was also found to develop autoimmune disease (Kim, Rasmussen et al. 2007; Lahl, Loddenkemper et al. 2007). Foxp3 transfected T cells exert suppressive function *in vitro* and inhibit the development of autoimmune and inflammatory disease *in vivo* (Hori, Nomura et al. 2003; Khattri, Cox et al. 2003). Literature data indicated that Foxp3 is related to CD4+CD25+ Treg cell development, evidenced by studies that Foxp3-deficient mice harbor few CD4+CD25+ Treg cells, and transfection of Foxp3 gene in

CD4+CD25− T cells can up-regulate expression of CD25 (Fontenot, Gavin et al. 2003). Additionally, CD4+CD25+ T cells in both thymocyte and peripheral compartment of normal mice express high levels of Foxp3, whereas effector T cells do not (Hori, Nomura et al. 2003; Khattri, Cox et al. 2003).

The cellular and molecular mechanisms by which Foxp3 orchestrates Treg cell development and function have not been completly understood. It has been proposed that Foxp3 interacts with transcription factors that facilitate the activation and differentiation of T cells into effectors, overriding their transcription machinery, thus converting them to Treg cells. Such transcription factors include NFAT, NF-κB, acute myeloid leukemia-1 (AML1)/ runt-related transcription factor 1 (Runx1), and the histone acetyl transferase (HAT)/histone deacetyl transferase (HDAC) (Schubert, Jeffery et al. 2001; Bettelli, Dastrange et al. 2005). For example, NFAT and NF-kB promote the expression of IL-2 and IFN-γ, thus contribute to the activation of effector T cells (Rao, Luo et al. 1997). Interaction between Foxp3 and NFAT could restrain expression of IL-2, and promote expression of CD25. Recent studies demonstrate that Foxp3 directly or indirectly controls hundreds (~700) of genes (Marson, Kretschmer et al. 2007; Zheng, Josefowicz et al. 2007) that encode cytokines (e.g. IL-2) and cell-surface molecules (e.g. FasL).

Other Treg cells markers include cytotoxic T cell associated antigen-4 (CTLA-4) and glucocorticoid-induced TNF receptor family-related gene/protein (GITR). It was found that regulatory activity detectable in the CD25-CD4+ T cell population in normal naive mice was attributed to CTLA-4+ and GITR^{high} cells. Depletion of CTLA-4+ and

GITR^{high} T cells from normal spleen cells produced more severe forms of autoimmune diseases in a wider spectrum of organs (M. Ono and S. Sakaguchi, manuscript in preparation).

In general, the most specific marker for naturally occurring Treg cells is the Foxp3, which is expressed by the majority of CD4+CD25+ T cells and a fraction of CD4+CD25- T cells (Figure 2.4) (Sakaguchi 2004).

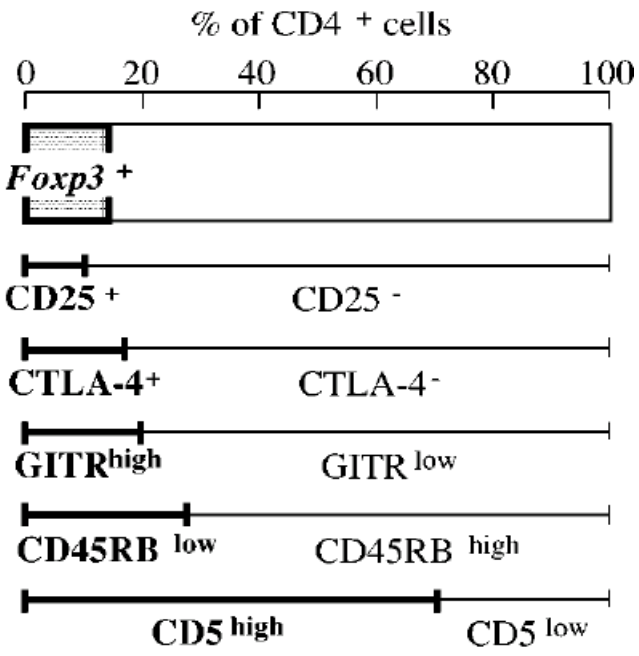


Figure 2.4: Cell surface markers for naturally occurring CD4+ Treg cells.

Regulatory T cell functions

Treg cells suppress proliferation and the cytotoxic activities of effector T cells (CD4+ and CD8+), natural killer cells, and DCs. The mechanisms by which Treg cells suppress the activity of these effectors remain unclear. Several of them have been proposed, including increase of immunosuppressive cytokines production, cell-contact-dependent suppression, and functional modification of APC by Treg cells (Asseman, Mauze et al. 1999; Seddon and Mason 1999).

Early studies reported that secretion of TGF-β or IL-10 by Treg cells could contribute to the suppression of effector T cell proliferation (Takahashi, Kuniyasu et al. 1998; Thornton and Shevach 1998). These cytokines may directly mediate suppression of responder T cells, or maintain Foxp3 expression and suppressive activity in Treg cells (von Boehmer 2005). IL-10 has a wide range of immunosuppressive effects such as decreasing T cell cytokine production, and decreasing antigen presentation and co-stimulatory molecule expression on DCs. TGF-β is a pleiotropic cytokine that acts on a variety of cells and regulates a wide range of biological activities, including wound healing, cell adhesion and migration, extracellular matrix formation, cell cycle and cell death (Nakamura, Kitani et al. 2001). More importantly, TGF-β plays a key role in regulating immune actions, evidenced by the report that TGF-β gene knock out mice developed aggressive autoimmune diseases in the first few weeks of life (Nakamura, Kitani et al. 2001). It was demonstrated that not only TGF-β is expressed by Treg cells, but also contributes to the expansion and differentiation of Treg cells. Local expression of TGF-β within the pancreas islets of non-obese diabetic (NOD) mice can inhibit the

autoimmune disease by promoting the expansion of the intra-islet CD4+CD25+ T cells (Javelaud and Mauviel 2004). Studies also showed that naïve human T cells could be differentiated into Foxp3+ regulatory cells when activated in the presence of TGF-β (Yamagiwa, Gray et al. 2001; Zheng, Wang et al. 2004). Finally, TGF-β may exert its effects indirectly by helping to maintain Foxp3 expression in Treg cells in peripheral tissues (Marie, Letterio et al. 2005).

Treg cells also hamper the activations between responder T cells and DCs in a cell-to-cell contact dependent manner (Thornton and Shevach 1998). This is supported by the finding that separation of CD4+CD25+ T cells and effector T cells with a semi-permeable membrane *in vitro* impeded the suppressive effect of Treg cells to effectors (Takahashi, Kuniyasu et al. 1998; Thornton and Shevach 1998). A recent study suggested that this *in vitro* suppressive cell contact was related to the delivery of a negative signal such as up-regulation of intracellular cyclic AMP to responder T cells (Tang and Bluestone 2008).

Treg cells may also hamper or down-modulate DC functions and thereby make DCs unable to activate effector T cells. For example, studies showed that Treg cells impeded stable contacts between responder T cells and DCs (Tadokoro, Shakhar et al. 2006; Tang, Adams et al. 2006), leading to anergy/inactivation of responder T cells, thereby suppressing immune response. Some studies suggested that CD4+CD25+ T cells down-regulated the expression of co-stimulatory molecules on DCs (Cederbom, Hall et al. 2000) .

Delivery of IL-10 to DCs

Introduction

Recombined IL-10 (rIL-10) has been tested in healthy volunteers, organ transplant patients, and rheumatoid arthritis patients for its inhibition of pro-inflammatory cytokine release (Wissing, Morelon et al. 1997; Keystone, Wherry et al. 1998; Boyer and Marcellin 2000; Colombel, Rutgeerts et al. 2001; Moore, de Waal Malefyt et al. 2001; Herfarth and Scholmerich 2002). The drug was well tolerated in healthy adults when administered intravenously at doses up to 25 µg/kg (Chernoff, Granowitz et al. 1995). Pharmacokinetic studies showed that rIL-10 was cleared through the kidney fairly rapidly following I.V. administration and yielded a terminal phase with a short half-life (~2 h) (Moore, de Waal Malefyt et al. 2001). Thus, *in vivo* administration of rIL-10 often resulted in low IL-10 concentrations in drug action sites, leading to little or no clinical improvement (Fedorak, Gangl et al. 2000; Narula 2000; Colombel, Rutgeerts et al. 2001). Increasing the dose of systemically administered IL-10 (>25 µg/kg) will cause side effects including anemia, headache, and flu-like symptoms (Moore, de Waal Malefyt et al. 2001; Herfarth and Scholmerich 2002).

Gene therapy provides a unique approach by introducing genes of interest to target tissue or cells. Genetically modifying DCs to express IL-10 becomes an attractive strategy to induce immune tolerance. A successful delivery system should protect DNA molecules from degradation, facilitate gene uptake and expression in target cells, and have minimal potential for inflammatory responses *in vivo*.

Gene delivery barriers

Delivery of therapeutic genes faces a variety of physical and biological obstacles that are categorized into extracellular and intracellular barriers.

Based on the target tissue, extracellular barriers can be extracellular glycocalyx barriers, mucus in apical membrane, glycoaminoglycan in tissue interstitum, and endothelial barriers as exemplified by the blood brain barrier. Studies also showed that DNA or oligonucleotide complexes are degraded by endonuclease in the serum and rapidly cleared from blood circulation after I.V. injection (Maurer, Mori et al. 1999). Others reported that several serum proteins such as bovine serum albumin (BSA), macroglobulin, high-density lipoproteins (HDL) and low-density lipoproteins (LDL) interact with cationic lipids in DNA delivery complexes, altering their physicochemical properties (Zelphati, Uyechi et al. 1998).

Intracellularly, three major processes, which are endocytosis, endosomal escape and nuclear entry, can affect gene delivery efficiency. DNA complexes can enter cells through a number of routes, including membrane fusion, phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and other endocytic pathways. The majority of DNA complexes enter target cells by clathrin-mediated endocytosis. During this process, DNA complexes bind to surface receptors and trigger the formation and accumulation of clathrin-coated vesicles that invaginate near the surface of the plasma membrane. The vesicles are delivered rapidly to early endosomes (pH 6.0), and then transferred to late endosomes (pH 5-5.5) that become more acidic as

ATP-dependent proton pumps transport hydrogen ions into the vesicle lumen. Eventually, late endosomes become lysosomes with even lower pH (~5.0), which accomplishes intracellular digestion using a variety of hydrolytic enzymes (Schmid, Fuchs et al. 1988). Thus, delivery vectors incorporating a mechanism of endosomal escape have been developed to avoid lysosomal degradation of carried DNA molecules in this cellular entry pathway. Lack of an endolytic feature in delivery vectors usually results in low transfection efficiency even when a high cellular uptake is achieved. Nuclear entry can be easily achieved in rapidly dividing cells and is often the rate-limiting step in slow or non-dividing cells. DNA fragments in the cytoplasm can accumulate in the nucleus by diffusion through the nuclear pore. The size of the nuclear pore complex is about 25 to 30 nm in diameter and allows the passage of molecules with a molecular weight lower than 40-60 kDa (Pante and Aebi 1996). Larger DNA molecules enter nucleus either during the process of cell division, or via a mechanism of nuclear pore targeting or active nuclear transport, such as nuclear localization sequence (NLS) peptides (Imamoto 2000).

Delivery of IL-10 gene by viral vectors

Currently available DNA delivery systems can be classified into two categories: viral and non-viral vectors. Viral vectors (e.g. retroviruses and adenoviruses), which utilize viral machinery to express carrying genes in the host cells, are capable of inducing highly efficient gene delivery. For instance, replication-deficient adenoviruses have been used to successfully deliver IL-10 gene in preclinical studies (Bromberg, Boros et al. 2002). However, viral vectors including adenoviruses also come with the increased risk

of triggering a viral specific immune response and subsequence immune-associated pathology in human. This may result in viral vector elimination from the host and a rapid immune response with potentially dangerous consequences under extreme circumstances (Sen, Hong et al. 2001; Buonocore, Van Meirvenne et al. 2002). Additionally, some viral vectors (e.g. retroviruses) are able to randomly integrate into the host cell genome and induce mutagenesis, which may be followed by development of cancer, especially upon chronic administration. Therefore, non-viral delivery systems become more attractive because they are less hazardous in terms of immunogenicity and mutagenesis.

Delivery of plasmid IL-10 gene by direct injection

A plasmid is a small circular piece of DNA molecule in bacteria that is capable of replicating independently of the chromosomal DNA. A DNA fragment that contains a gene of interest can be inserted into a plasmid and the resulting vector can be used to import DNA into a host cell. Since plasmid DNA is less immunogenic and is generally considered to be safer compared to viral vectors currently used, it has been widely used as a non-viral gene delivery vector. Direct administration of naked plasmid IL-10 to experimental animals was evaluated in the past. Nakagome et al. demonstrated the delivery of pIL-10 in mice challenged with ovalbumin (OVA) antigen through I.V. injection (Nakagome, Dohi et al. 2005). Experimental data indicated that expression of serum IL-10 was increased in mice after pIL-10 injection and antigen-specific immune response was suppressed subsequently. The authors further confirmed that suppressive effect of IL-10 is induced by attenuation of DC functions, including their antigen-presenting capacity and cytokine productions. However, naked plasmid DNA is

susceptible to endonuclease degradation and is more difficult to cross cell membranes, which reduce the quantity of DNA molecules taken up by target cells, thereby limiting their efficiency of gene expression. A very high dose of plasmid DNA (>100 mg per mouse) is usually required for direct injection to achieve a therapeutic response. However, such high dose is not suitable for DNA delivery into small organs that have lower capacity, such as mouse thyroids. Moreover, such large amounts may lead to Th1 cytokine IFN-γ synthesis in response to immunostimulatory sequences present in plasmid DNA (Roman, Martin-Orozco et al. 1997), which should be avoid especially when immunomodulation is expected.

Delivery of plasmid IL-10 by polymer-based non-viral vectors

Non-viral vectors have been developed to deliver DNA-based therapeutics. Based on the synthetic material used, they can be classified into two major categories: polymer-based and liposomal delivery systems.

In polymeric delivery systems, natural and synthetic cationic polymers or peptides such as chitosan, poly-L-lysine (PLL), polyethyleneimine (PEI) and polymethacrylates have been widely used (Nguyen, Green et al. 2008). These materials are usually biodegradable and/or biocompatible, and their physicochemical properties can be modified for different delivery applications. Cationic polymers can condense anionic DNA molecules into positively charged, nano-scaled complexes (polyplexes) via electrostatic interaction. The smaller size of cationic polyplexes and the electrostatic interaction between the polyplex and the cell membrane facilitate DNA intracellular

uptakes. Additionally, polyplexes can protect DNA molecules from endonucleases and improve their biological stability *in vivo*. These polymers can also be modified with functional groups that promote DNA release from endosomal compartment into cytoplasm.

PEI has been investigated as a prominent cationic polymer for gene delivery due to its ability to condense plasmid DNA molecules and protect them from nuclease degradation. The amine groups of PEI display proton buffer capacity at endosomal pH and act as a proton sponge that disrupt endosomal compartment to allow release of DNA into cytoplasm. Lee et al. (Lee, Park et al. 2006) used PEI as a carrier to intravenously deliver plasmid IL-10 into 5-week old nonobese diabetic (NOD) mice at a dose of 50 mg of DNA per mouse. The self-assembly polymer/DNA complexes were prepared by mixing PEI (25,000 Da) with pIL-10 in 5% glucose solution at a 5/1 N/P (nitrogen of PEI/phosphate of DNA) ratio. Experimental results showed that mice I.V. injected with PEI/pIL-10 complexes had persistent IL-10 gene expression greater than 5 weeks compared to PEI injected-only, and noninjected control mice. Assay of serum glucose levels showed that PEI/pIL-10 injection prevented Type 1 diabetes in 40% of NOD mice. The study demonstrated that PEI could be used to deliver pIL-10 systemically to suppress autoimmune response in mice.

Polymethacrylates are vinyl-based polymers that have been evaluated for gene delivery. Cationic polymethacrylates are usually engineered with side groups containing tertiary amine, pyridine and imidazole groups which display DNA condensing and proton

buffering abilities similar to PEI but with less toxicity (Dubruel, Christiaens et al. 2003; Dubruel, Christiaens et al. 2004). Basarkar et al. fabricated cationic particles with PLGA and methacrylate copolymer (Eudragit® E100) to deliver mouse pIL-10 i*n vitro* and *in vivo* (Basarkar and Singh 2008). Blended with cationic surfactant cetyl trimethl ammonium bromide (CTAB), the prepared particles displayed a positive zeta potential and were capable of loading DNA on the surface. PLGA/E100 copolymer showed increased buffering ability at endosomal pH in the presence of E100. *In vitro* transfection study indicated that pIL-10 loaded PLGA/E100 particles have greater IL-10 expression in HEK293 cells compared to plain PLGA particles. *In vivo* efficacy study indicated that intramuscular injection of pIL-10 loaded PLGA/E100 particles at a dose of 50 μg/mouse enhanced IL-10 expression in mice tissue, leading to effective protection against insulitis. The study suggested that the cationic polymethacrylates-based particles are feasible for IL-10 gene delivery to prevent autoimmune diabetes.

Other biodegradable cationic polymers, such as poly [α-(4-aminobutyl)-L-glycolic acid] (PAGA) have been synthesized for plasmid DNA delivery. Koh et al. (Koh, Ko et al. 2000) reported that PAGA enhanced plasmid IL-10 transfection efficiency *in vitro*. *In vivo* injection of PAGA/pIL-10 complexes into the tail vein of NOD mice (100 μg/mouse) elevated serum level of IL-10 after 5 days and the development of severe insulitis on these mice was reduced to 15.7% compared with mice injected with naked DNA (34.5%) and non-treated mice (90.9%). The study suggested that PAGA is a suitable vector to deliver plasmid IL-10 to suppress the progression of insulitis in NOD mice.

Delivery of pIL-10 by liposomal delivery systems

Liposomes are vesicles that consist of an aqueous compartment enclosed in a lipid bilayer. They have been used to formulate a wide range of DNA delivery system in recent years. Compared to viral vectors, liposomes are generally less immunogenic and easier to be scaled-up in production. Additionally, their size range, surface charge, and composition can be engineered.

When using liposomes as gene carriers, DNA molecules can be either entrapped inside the aqueous core or be carried in the lipid bilayer of the delivery system. Entrapped DNAs can enter target cells from liposome through fusion with phospholipid bilayer of cell membrane or via endocytic pathway. In the later situation, the mechanism of DNA release from cationic liposome/DNA complexes in endosome is driven by interaction between liposome and endosomal vesicle, and the subsequence destabilization of endocytic membrane that causes disassociation of DNA from the complex and its release to cytoplasm.

Liposome formulations usually contain one cationic lipid such as 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and dioleyloxy propyl trimethylammonium chloride (DOTMA), and one or more neutral helper lipids such as dioleoyl L-phosphatidylethanolamine (DOPE) and cholesterol. Cationic lipids are used to form complexes with negatively charged DNA molecules. The formation of complexes, referred to as lipoplexes, will improve vector association with target cells and offer protection to carried DNA molecules from endonuclease degradation *in vivo*. However,

excess amount of cationic lipids may hamper DNA release from the formulation, leading to low gene expression. Neutral helper lipids function to stabilize cationic bilayers and promote endosomal escape. For example, DOPE undergoes hexagonal phase transition in acidic conditions, which will result in the rupture of endosome membrane and the release of DNA to cytoplasm. Therefore, the ratio and the combination of cationic lipid and helper lipid are considered as important factors for DNA expression as well as liposome stability.

Liposomal delivery systems have been evaluated for plasmid IL-10 delivery in recent years. Fellows et al. used a cationic liposome to deliver IL-10 gene to mice with established collagen induced arthritis (Fellowes, Etheridge et al. 2000). The liposome was formulated from a novel cationic lipid cytofectin (ACHx), a polyamine analogue dimethlaminoethame carbamoyl cholesterol (DC-Chol), and DOPE. Human pIL-10 was constructed with a SV40 promoter and complexed with ACHx/DC-Chol:DOPE liposomes in 1:2.4 (w/w) ratio. Liposomes loaded with pIL-10 were intraperitonealy (I.P.) injected into mice with established arthritis. Experimental data suggested that human IL-10 mRNA was detected in the paws 24 hours after injection and IL-10 protein was also detected in several tissues up to 10 days after injection in mice. Additionally, attenuation of arthritis was achieved in pIL-10 treated mice for 30 days. The results suggested that cationic liposomes can be use to systemic deliver plasmid IL-10.

Kabay et al. used cationic liposomes (DOTAP: Cholesterol [1:1 molar ratio]) to I.P. deliver mouse pIL-10 to suppress inflammatory damage triggered by intestinal

ischemia (Kabay, Aytekin et al. 2005). The authors found that IL-10 protein expression was improved in mice injected with liposome/pIL-10 complexes compared to the one without IL-10 treatment. Data also showed that the subsequent expression of IL-10 resulted in attenuation of leukocyte infiltration locally and reduction of pulmonary tissue injury that is normally induced by intestinal ischemia. The study indicated that cationic liposomes could be used to deliver IL-10 gene *in vivo* to modulate damaged immune responses.

Furukawa et al. used liposome vectors (N-[3-aminopropyl]-N, N-dimethyl-2,3-bis[dodecyloxy]-1-propaniminium bromide/DOPE) to *ex vivo* deliver pIL-10 (50µg) to a rabbit cardiac allograft and examined the efficacy and cardiac adverse effects using a functional cervical heterotrophic transplant model. The study demonstrated that the expression of IL-10 gene was long lasting and localized in the target organ. The mean survival of cardiac allograft was prolonged for greater than 100 days. Expression of IL-10 inhibited the infiltration of T lymphocyte and cytotoxicity in the cardiac allograft and modulated pro-inflammatory cytokine production (Furukawa, Oshima et al. 2005). The study indicated that liposome could be used to *in vivo* deliver pIL-10 to suppress transplant rejection in rabbit allograft heart transplant model.

Limitations of current polymer-based and liposomal delivery systems

Although extensive efforts have been made for the development of non-viral gene delivery vectors, current systems are not without problems. Non-viral vectors generally suffer from low transfection efficiency compared to viral vectors. Additionally, some of

the non-viral delivery systems are associated with cytotoxicity and immunogenicity when administered *in vivo*.

It was shown that chitosan administered at high doses can cause hypocholesterolemia in humans. Branched PEI that has high cationic potential can be extremely cytotoxic due to induction of apoptosis. PLL suffers from toxicity induced by its cationic amino acid backbone, which could disrupt cellular membrane structure. PLL are also potentially immunogenic, making them unfavorable for long-term treatments aiming to suppress immune responses. Additionally, PLL/DNA complexes may undergo nonspecific binding to cell membrane, thus limiting their application in targeting delivery.

Clinical use of liposomes is limited by their cytotoxic effects. It has been confirmed in various studies that cationic lipids cause cytotoxicity and pulmonary toxicity to varying degrees. Those effects are caused by lipid incorporation in the plasma membrane facilitated by lipid fusion or mixing (Stamatatos, Leventis et al. 1988; Zelphati and Szoka 1996), which inhibits protein kinase C activity (Farhood, Bottega et al. 1992).

In addition to the safety and immunogenicity concerns, non-viral vectors including chitosan, PLL-DNA polyplexes and cationic lipoplexes usually have a high degree of polydispersity, making it difficult to characterize and control the physicochemical properties of the drug products, which may lead to less reproducible efficacy.

Characterization of non-viral vectors

In order to achieve efficient gene delivery using non-viral vectors, it is important to understand the relationship between the physicochemical properties of the delivery systems and their transfection activities. Studies have revealed that the physicochemical properties have an impact on stability of non-viral vectors and carried DNAs, as well as biological processes such as association with target cells, intracellular uptake and trafficking.

Since DNA complexes are usually formed by the electrostatic interaction between the cationic charged vectors and anionic charged DNA molecules, the mixing ratio of the two components can be adjusted to produce delivery complexes with different characteristics, such as particles size and zeta potential.

Particle size of non-viral vectors plays a critical role in gene delivery to target cells. For *in vivo* delivery, particle size needs to be well defined to govern critical factors including biodistribution, toxicity, and transfection efficiency based on the target sites. For example, the optimum particle size for pulmonary delivery generally is in the range of 1-5 microns because particles larger than 5 microns deposit primarily in the upper airways. Submicron particles with diameters less than 400 nm are prefer candidates for tumor-targeted delivery because the particles are small enough to extravasate through the leaky capillaries (pore size ~400 nm) in the tumor without excessive distribtion to normal tissues. Additionally, particle size of non-viral vectors should be optimized to achieve effective entries to target cells and avoid being taken up by macrophages from the

reticuloendothelial (RES) system.

Zeta potential is an essential property for gene delivery vectors because positive zeta potential facilitate non-specific uptake of DNA complexes via the interaction of the excess positive charge of the complex to the negative charge cell membrane. Additionally, particles and complexes can be stabilized in storage condition by their excess zeta potential. Therefore, optimization of zeta potential is necessary in order to yield efficient transfection rate in target cells and prevent aggregation of DNA complexes.

Binding between DNA molecules and non-viral vectors stabilizes DNA complexes from denaturation during both extracellular and intracellular transit (Prevette, Lynch et al. 2008). However, excess binding between DNA and cationic vectors may hamper the release of DNA from its carrier, causing low gene expression. Binding strength of DNA complexes can be explored by titrating the complex with anionic molecules such as low molecular weight heparin and dextran sulfate. The released DNA can be quantified using gel electrophoresis or Picogreen® assay. In addition, thermodynamic approaches including UV melting curve measurement, differential scanning calorimetry (DSC), and isothermal titration calorimetry (ITC) are used to examine the stability of DNA/cationic carrier complexes.

DNA UV melting curve analysis has been employed to characterize binding stability of DNA/carrier complexes. Melting of DNA is conveniently monitored by an

increase in UV absorbance at 260 nm that results from the disruption of base stacking in DNA duplex due to the breakage of hydrogen bonds. DNA base pairs complexed with cationic polymers or lipids are more stable than free, unbound base pairs. Therefore, melting of free DNA molecules occurs at a lower temperature, while melting of DNA complexes occurs at a higher temperature. When a stoichiometric stable complex is formed between DNA and cationic condensate, only the transition due to the denaturation of the complexed DNA will be observed at higher temperature. However, when an unstable complex is formed between cationic condensate and DNA, disassociation of the constituent molecules may have occurred in the complex, resulting in shift of transition temperature of the melting curve (Mandel and Fasman 1976).

DSC method has been employed to characterize the interaction between DNA and cationic condensate. The principal of the DSC approach is that denaturation of DNA molecules induces heat adsorption peaks in a thermogram, and any increase in the stability of DNA complex due to interaction with cationic condensate will be directly reflected in the change of temperature and enthalpy of the endotherm in a DSC profile. Compared to free DNAs, the endothermic peaks of DNA complex is presented at a higher temperature range in DSC curves owing to the increase of stability in the system. Additional methods such as ITC have been used to directly quantify thermodynamic parameters of DNA/carrier complexes, including binding affinity (Ka), enthalpy changes (ΔH), and binding stoichiometry (n) for the evaluation of interactions between cationic condensate and DNA.

Polystyrene and PLGA polymeric particulates for DNA delivery to DCs

Recently, nanoparticles (size ≤ 100 nm) or submicron particles (10-1000 nm) have become attractive tools for drug delivery applications due to the reasons shown as follows. First, nano or submicron particles are able to adsorb, bind and stabilize molecules such as DNA and proteins on the surface. Second, the relatively large surface to volume ratio of these particles make them capable of carrying more drugs on the surface compared to other systems. Third, compared with other larger particulates (> 1000 nm), the smaller size of these particles allows them to penetrate deeper tissues and to be taken up relatively easily by different types of cells. DCs pick up particles more efficiently than soluble antigen because their dimensions are comparable to microorganisms. It has been found that particles larger than 1000 nm are internalized through phagocytosis by APCs including both macrophages and DCs, while nano and submicron particles are taken up via endocytosis preferentially by DCs (Vidard, Kovacsovics-Bankowski et al. 1996; O'Hagan, Singh et al. 2004). Therefore, these types of particles are more advantageous in passive targeting delivery of plasmid DNA to DCs.

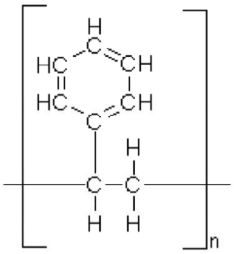
Submicron particles fabricated from polymers including polystyrene and PLGA have been investigated in drug delivery applications. Polystyrene (Figure 2.5A) consists of a long hydrocarbon chain with every other carbon connected to a phenyl group. The material can easily adsorb proteins and peptides owing to its hydrophobic surface. Polystyrene particles can stabilize DNA molecules by providing DNA absorption and protection sites on their surface. Polystyrene is classified as a biocompatible material by the Food and Drug Administration (FDA), and has been approved, including particulate

form, for administration to human via the oral route. It is also a component of specific types of bone cement used in humans. Synthetic polymeric particles fabricated from polystyrene have been tested as vaccine carriers in mice and sheep (Fifis, Gamvrellis et al. 2004; Scheerlinck, Gloster et al. 2006). Minigo et al. have developed formulations made of PLL-coated polystyrene nanoparticles, which condense and deliver DNA vaccines to C57BL/6 mice (Minigo, Scholzen et al. 2007).

Polystyrene is generally safe for *in vivo* use. Thermal and photo-oxidative degradation of polystyrene can occur *in vitro* but produces only carbon dioxide and water residue. An independent study that evaluated the safety profile of polystyrene particles demonstrated that subcutaneous/intradermal administration of the material in the Sprague Dawley rat did not induce pathology changes in the tissue or organs tested (including draining lymph nodes, liver, kidneys, spleen, heart, adrenals, lungs, brain) over 28–56 days (Minigo, Scholzen et al. 2007). Additionally, *in vivo* clearance studies in the mouse after intradermal injection indicated that polystyrene particles are being slowly cleared via the RES system.

Because polystyrene is a non-degradable polymer, it may be suitable for making particles that are for short-term treatments (e.g. *ex vivo* gene delivery). Repeat injection of non-degradable particles may cause accumulation of the polymer material in the cells and tissues, which could lead to potential toxicity. Therefore, a particle delivery system based on a biodegradable polymer is more suitable for applications that require long-term

administration.



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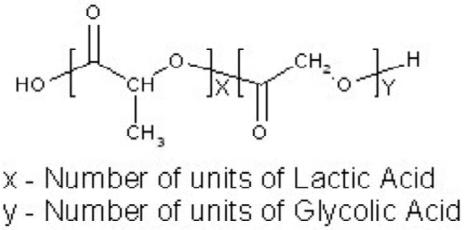


B

Figure 2.5: Structure of polystyrene (A) and poly (lactic-co-glycolic acid) (B).

PLGA (Figure 2.5B) refers to biodegradable copolymers consisting of various ratios of two monomers: glycolic acid and lactic acid. Glycolic acid is achiral and the lactic acid used is a racemic mixture (d and l). PLGA is a commercially available, GMP grade material that has been approved by the FDA for medical applications because of its biocompatibility and biodegradability. The co-polymer is amorphous rather than crystalline and shows a glass transition temperature in the range of 40-60 °C. PLGA degrades by hydrolysis of its ester linkages in the presence of water. It has been demonstrated that the time required for PLGA degradation is related to its monomer ratio: the higher the content of glycolide units, the lower the time required for degradation. An exception to this rule is the co-polymer with 50:50 monomer ratio exhibits a faster degradation (~ 2 months) (Astete and Sabliov 2006). In the body, hydrolysis of ester links of PLGA produces the original monomers, lactic acid and glycolic acid. The two monomers are by-products of various metabolic pathways in the body. Thus, PLGA is biodegradable and there is very minimal systemic toxicity associated with it. PLGA has long been used in cosmetic and orthopedic applications, and in other therapeutic devices (e.g. implants, internal sutures) in humans, as well as a delivery system for peptide and protein drugs (Athanasiou, Niederauer et al. 1996; Temenoff and Mikos 2000). Additionally, it was reported that PLGA facilitates the escape from endosome into cytoplasm due to a change in surface charge from negative to positive at endosome pH (Panyam, Zhou et al. 2002). Therefore, PLGA is attractive to many researcher involved in DNA delivery.

In most of the previous studies, DNA or protein drugs are encapsulated in PLGA

polymer matrices during fabrication processes that use double emulsion (water–oil–water), solvent evaporation technique. An emulsion containing a two-phase system is prepared by combining two immiscible liquids, in which droplets of one phase are dispersed uniformly through the other phase (Troy 2005). During the preparation of emulsions, emulsifying agents such as polyvinyl alcohol (PVA) are added to the system to reduce the surface energy between the hydrophilic phase and hydrophobic phase, therefore stabilizing the emulsion system. In the preparation of double or multiple emulsions (e.g. water-oil-water) system, a two-stage emulsification procedure is employed, including forming the primary emulsion (e.g. water-oil) and adding it to the second phase (e.g. water-oil-water). The preparation of emulsions also requires a certain amount of energy to form the interface between the two phases on the droplet. Certain processes including sonication, homogenizing and milling are required for providing energy to the system.

The advantage of a DNA encapsulation approach in particle preparation is that the polymer matrix should protect embedded DNA from endonuclease degradation. Additionally, sustained expression of plasmid DNA could be achieved by the prolong release of the embedded DNA on the order of weeks (Cohen, Levy et al. 2000). However, there are several drawbacks in this system for DNA delivery. First, because of the large size and hydrophilic character of plasmid DNA, encapsulation of a high amount of DNA molecules in hydrophobic PLGA particles is a challenge. Second, DNA may be inactivated during the encapsulation process by sonication. The shear force in energy-driven particle size reduction (e.g. sonication) during PLGA particle fabrication can

severely affect the tertiary structure of plasmid DNA (Levy, Collins et al. 1999). The interaction of shear with air-liquid interfaces shows the highest potential for damaging plasmid DNA during sonication. The extent of damage is also found to be dependent on plasmid size and ionic strength of the environment. Third, it is well known that hydrolysis of PLGA may substantially decrease the pH environment in PLGA particles, potentially resulting in degradation of DNA (Walter, Moelling et al. 1999; Wang, Robinson et al. 1999). Fourth, due to the fact that plasmid DNA has to diffuse through the polymer matrices, the rate of release is often too slow (release lasts for several days/weeks) (Walter, Moelling et al. 1999; Tinsley-Bown, Fretwell et al. 2000; Zhu, Mallery et al. 2000; Luten, van Steenis et al. 2003). The slow release of DNA molecules may result in less optimal gene expression because of the following reasons: 1) the amount of plasmid DNA immediately available after cellular uptake by APCs is too limited, and 2) plasmid DNA released after days/weeks from the particles may be significantly damaged due to the acid microenvironment in PLGA particles.

Alternatively, plasmid DNAs can be loaded on polymeric particle surface associated with cationic agents, rather than entrapping them in the particle matrices. The cationic surface of particles is capable of binding plasmid DNA through electrostatic interaction, and upon entering cytoplasm intracellular glutathione can facilitate DNA release from bound particles (Zugates, Anderson et al. 2006).

Cationic peptides and polymers such as PLL and PEI can be used as DNA condensing agents to improve delivery efficiency and to protect DNA from enzymatic

degradation (Pack, Hoffman et al. 2005). However, PLL is not considered as an effective polymeric vector presumably because of the lack of an efficient mechanism for endosomal escape. Endosomolytic agents such as chloroquine are required to achieve DNA release to the cytoplasm when using PLL, but this is not applicable for *in vivo* use. PEI can condense DNA with its high cationic charge potential (Boussif, Lezoualc'h et al. 1995). It also provides endosomal escape through the ‘‘proton sponge’’ mechanism (Sonawane, Szoka et al. 2003). However, PEI is not an ideal gene delivery agent by itself because the polymer causes cell necrosis and apoptosis (Moghimi, Symonds et al. 2005).

O10H6, a cationic peptide of 10 ornithine and 6 histidine residues, has been shown in our lab to have more efficient uptake by DCs and is less toxic than other PLL containing cationic peptides (K16 and K10H6) (Chamarthy, Kovacs et al. 2003). Ornithine, a non-natural amino acid with a basic side chain (-CH2-CH2-NH2), provides most of the positive charges at physiological pH. The positive charge of the peptide contributes to DNA condensation that can increase DNA uptake and protect DNA from nucleases (Albert, Jegathesan et al. 2001; Meng and Butterfield 2005). The later is crucial for *in vivo* gene delivery where DNA is susceptible to serum digestion. To achieve successful transfection, DNA must escape from the endosome and enter the nucleus after it is taken up by host cells. Histidine, which has a near-neutral pKa group (pKa = 6) in the imidazole group on the side chain, can buffer the acidic environment of the endosome, causing water uptake by a pH pump in order to maintain lower pH (5-6) inside and induce endosome membrane rupture (Putnam, Gentry et al. 2001). Additionally, physical

properties of particles such as surface potentials can be adjusted by varying the amount of cationic peptides.

CHAPTER 3

PHYSICAL CHARACTERIZATION OF POLYCATION-MODIFIED POLYMERIC PARTICLES LOADED WITH PLASMID IL-10

Introduction

Polymeric submicron particles have become versatile tools for macromolecular drug delivery (Vasir and Labhasetwar 2006). Macromolecules such as DNA, RNA and protein can be either encapsulated within the matrix of polymer particles, or loaded on the particle surface. In the second situation, the submicron size gives the particle large surface area to volume ratio ($4л \times radius^2/1.33л \times radius^3$) (Rotello 2003), making them capable of carrying more drugs compared to micron scale particles (Faraji and Wipf 2009). Moreover, experimental data showed particles smaller than 500 nm are taken up preferentially by DCs via endocytosis (Vidard, Kovacsovics-Bankowski et al. 1996; O'Hagan, Singh et al. 2004). Therefore, surface loaded submicron particles are chosen as vectors for passive targeting delivery to DCs.

Polystyrene is stable *in vivo* and is classified as a biocompatible material by the FDA for diagnostic uses in humans. Particles fabricated from polystyrene have been used for passive targeting delivery of DNA vaccines to DCs (Minigo, Scholzen et al. 2007). A recent study demonstrated that polystyrene particles within optimal size range (100-500

nm) can be taken up efficiently by a large portion of DC population within 4 hours of incubation *in vitro* (Foged, Brodin et al. 2005). When modified with negatively charged carboxylated functional groups, polystyrene particles can adsorb cationic peptides on the surface, making them capable of binding and condensing plasmid DNA molecules via electrostatic interactions. Such types of polycation-modified carboxylated polystyrene particles have been used in our lab to successfully deliver oligonucleotides and DNA to DCs. However, polystyrene is a non-biodegradable polymer that can accumulate slowly in the spleen and liver after repeated injections. Therefore, delivery vectors formulated with biodegradable polymers would be more suitable for clinical applications that require long-term administration.

PLGA has been approved by the FDA for drug and protein delivery due to its good biocompatibility and biodegradability (Panyam and Labhasetwar 2003; Bala, Hariharan et al. 2004). PLGA has been used as a copolymer for the fabrication of nucleic acids carriers because of its capacity to encase macromolecules in matrices (Panyam and Labhasetwar 2003). In this research work, we chose to load plasmid DNA on the polymeric particle surface rather than entrapping in the particle matrice in order to reduce degradation of encapsulated DNA during particle fabrication and drug release (Walter, Moelling et al. 1999; Wang, Robinson et al. 1999). It has been reported in our lab that the cationic peptide O10H6 can be adsorbed on both PS and PLGA particle surfaces, providing a positive surface capable of loading and condensing DNA molecules (Zheng, Kovacs et al. 2006). Compared to other PLL containing cationic peptides (K16 and K10H6), O10H6 has been shown to have more efficient uptake by DCs with less

cytotoxicity (Chamarthy, Kovacs et al. 2003). Another purposed advantage of PS_{O10H6} and $PLGA_{O10H6}$ particles is their physical properties such as surface potential which can be controlled by varying the amount of the cationic peptide O10H6. These properties have an impact on their stability as well as biological processes such as target cell association of these particles.

In the work presented in this chapter, physical properties (e.g. particle size distribution and zeta potential) of PS_{O10H6} and $PLGA_{O10H6}$ particles loaded with plasmid DNA were evaluated. Their ability to condense and stabilize DNA molecules from serum endonuclease was also determined. These characteristics have a great impact on the stability of gene delivery vectors, as well as critical biological processes during gene transfer. The objective is to evaluate the physical characteristics of PS_{O10H6} and $PLGA_{O10H6}$ particles, and determine the suitability of these particles to deliver plasmid DNA to DCs.

Methods

O10H6 peptide and plasmid DNAs

The cationic peptide, O10H6 ((MW = 2250) (Figure 3.1 A) was custom synthesized by Sigma-Genosys (Houston, TX). Crude peptides were purified by HPLC (>90%) and their identities were confirmed by liquid chromatography-mass spectrometry. Peptides were aliquoted in sterile distilled water and stored at -80°C until use. Plasmids encoding murine IL-10 (Figure 3.1B, pUMVC3-mIL10; 4563 bp), Green Fluorescent Protein (pUMVC3-GFP) and Luciferase (pUMVC3-Luc) were obtained from Aldevron (Fargo, ND), aliquot in distilled water, and stored in -80°C until use. The transgenes in these vectors are driven by the cytomegalovirus (CMV) promoter/enhancer with trimmed intron A and a rabbit β-globin polyadenylation (poly A) signal.

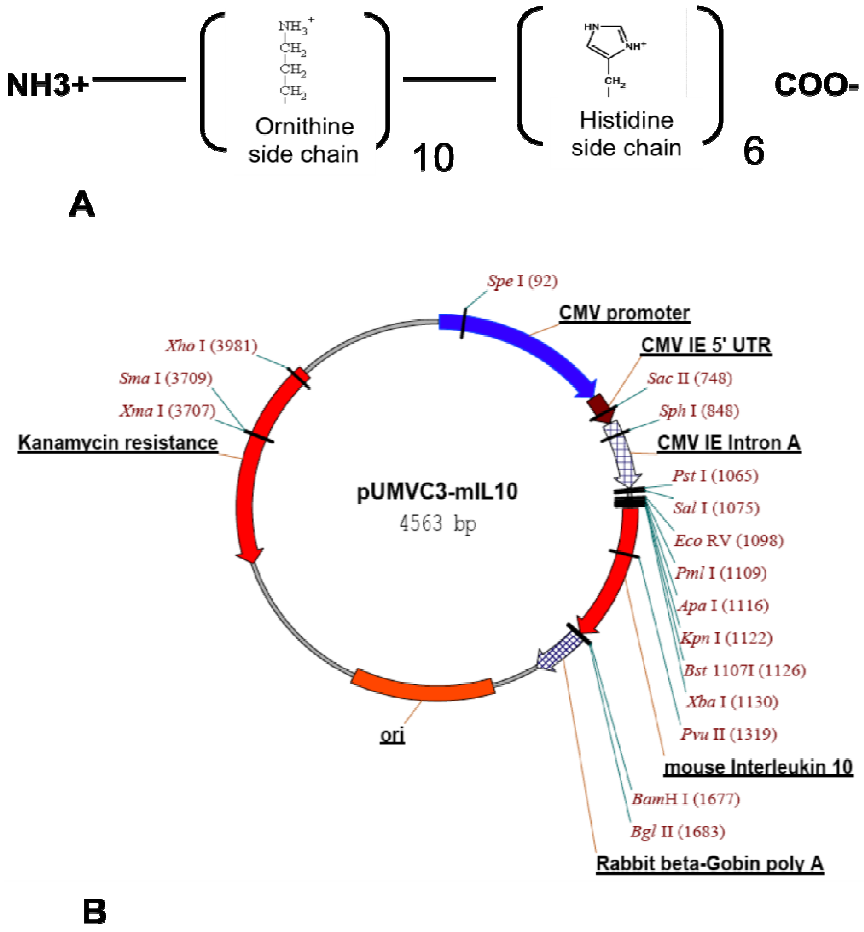


Figure 3.1: Chemical structure of O10H6 cationic peptide (in acidic environment) (A); mammalian expression vector for mouse IL-10 (B).

Preparation of PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particles

The vectors for plasmid IL-10 delivery were carboxylated polystyrene (Polysciences Inc, Warrington, PA) and PLGA (50:50, M.W. 12,000-16,500, Polysciences Inc, Warrington, PA) particles coated with O10H6.

The plain polystyrene particle has an average diameter of 100 nm with less than 10% variance. The polystyrene vector was formulated as follow: 3 μL (75 μg) of PS particles (2.5% w/v; 4.55×10^{11} particles.mL^{-1}) were first coated with 200 μg of O10H6 in 300 μL of deionized-distilled water (ddH_2O). To assure sufficient binding of O10H6 to the particles, the components were equilibrated in a 1.5mL Eppendorf tube with gentle shaking for 2 h at room temperature on a mini shaker at a setting that is equivalent to the second lowest speed. The resulting positively charged particles were then equilibrated with the plasmid DNA (15 μg) for an additional 30 min with gentle shaking at room temperature. The N: P ratio for PS_{O10H6}-pDNA particle was estimated to be 9-18:1. The final particle had plasmid DNA (e.g. pIL-10) condensed by O10H6 associated with polystyrene particle surface and was referred to as PS_{O10H6}–pDNA (e.g. PS_{O10H6}-pIL-10, PS_{O10H6}-pLuc and PS_{O10H6}-pGFP).

PLGA particles were prepared in our lab using a double emulsion (w/o/w) solvent evaporation technique described previously (Wang, Robinson et al. 1999; Jain 2000). 200 μL of aqueous solution and 6 mL of a solution of methylene chloride ($MeCl_2$) and PLGA (3%, w/v) were emulsified by sonication. A Fisher model 100 sonic microprobe was used to introduce 24 W of energy (over 2 min) to form this water-in-oil emulsion mixtures

were sonicated on ice. This primary emulsion was added drop wise into 20 mL of aqueous solution of poly (vinyl alcohol) (0.5%, w/v) to form a water-in-oil-in-water emulsion. PVA, a non-mutagenic polymer with low acute oral toxicity (LD50 = 15–20 g/kg), has been commonly used as an emulsion stabilizer in the preparation of PLGA particles. The resulting double emulsion was stirred for 4 hours in chemical fume hood to allow the $MeCl_2$ to evaporate. Particles were recovered by ultracentrifugation and washed twice with ddH_2O before lyophilization to remove excess PVA and un-trapped DNA.

The PLGA particles used in the experiments were either lyophilized or freshly prepared prior to each analysis. Our lab data showed that freeze-dried $PLGA_{O10H6}$ loaded with oligonucleotide retains 90% of its physical characteristics (particle size and zeta potential) upon reconstitution (Zheng, Kovacs et al. 2006). The PLGA delivery vector presented in this chapter was formulated as follows: 60 μg of lyophilized PLGA was first coated with 200 μg of O10H6 in 300 μL of ddH_2O. To assure sufficient binding of O10H6 to the particles, the components were equilibrated in a 1.5 mL Eppendorf tube with gentle shaking for 2 hours at room temperature on a mini shaker at a setting that is equivalent to the second lowest speed. The resulting positively charged particles (denoted as $PLGA_{O10H6}$) were then equilibrated with the plasmid DNA (15 μg) for an additional 30 min with gentle shaking at room temperature. The N: P ratio for the $PLGA_{O10H6}$-pDNA particle was estimated to be 9-18:1. The final particle had plasmid DNA condensed by O10H6 associated with the PLGA particle surface and was referred to as $PLGA_{O10H6}$–pDNA (e.g. $PLGA_{O10H6}$-pIL-10, $PLGA_{O10H6}$-pLuc and $PLGA_{O10H6}$-pGFP).

Particle size and zeta potential analysis

A Nicomp 380 ZLS analyzer (Particle Sizing Systems, Santa Barbara) was used to determine the zeta potential of PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particles. The instrument operates on the dynamic light scattering (DLS) principle, capable of detecting particles size diameter ranging 1–5000 nm.The mean particle size was measured using suspensions (3 mL) of fresh polystyrene or PLGA particles, and particles modified with O10H6 and pIL-10, diluted 10 times with ddH_2O in 4 mL disposable glass tubes. Radius was calculated using the Stokes–Einstein equation based on diffusion coefficient of the particles in the medium. Data were integrated over 10 min in duplicate experiments and were test fit for Gaussian and non-Gaussian functions.

Zeta potential measurements were performed with samples diluted 30 times with ddH_2O (pH = 7.4) in 3 mL disposable glass tubes. Data were integrated over 10 min in duplicate experiments. The measurement is based on electrophoretic mobility of the sample particles when subjected to an electric field.

Gel electrophoresis

In order to confirm the binding of pDNA onto the surface of $PLGA_{O10H6}$ and PS_{O10H6} particles, the electrophoretic mobility of naked plasmid or the respective DNA complexes (O10H6-pIL-10, $PLGA_{O10H6}$–pDNA, and PS_{O10H6}–pDNA) was evaluated by agarose gel electrophoresis. Samples were analyzed in 1% agarose gel (MP Biomedicals, Solon, Ohio) with or without the addition of low molecular weight heparin sodium (Eikins-Sinn, Inc., Cherry Hill, NJ) (50 U/mL) that can release charge-bound DNA. DNA

was visualized by ethidium bromide staining. The binding of DNA on the particles is determined based on the ability of each complex to prevent the migration of negatively charged pDNA toward the anode of the electric field of the agarose gel.

To assess the stability of pDNA against nuclease, naked plasmid or the respective DNA complexes (O10H6-pIL-10, $PLGA_{O10H6}$–pDNA, and PS_{O10H6}–pDNA) were incubated with 10% fetal bovine serum (FBS) at 37 °C for overnight. Following incubation samples were analyzed in agarose gel. Low molecular weight heparin sodium (50 U/mL) was added to samples to release charge-bound DNA in 1% agarose gel. DNA was visualized by ethidium bromide staining. Percentage of DNA recovery was calculated by dividing the band intensity of DNA recovered with the intensity of DNA loaded (multiple by 100).

Results

Physical characterization of PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particles as colloidal dispersions

Particle sizes and zeta potentials of unmodified particles (PS and PLGA), O10H6 coated particles (PS_{O10H6} and $PLGA_{O10H6}$), and DNA loaded particles (PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10) were measured using Nicomp 380 ZLS analyzer. Particle size data from three independent experiments indicated that addition of O10H6 and pIL-10 slightly increased the size of the complex relative to unmodified particles (Figure 3.2, Table 3.1). Carboxylated polystyrene particles had an average hydrodynamic size of 104 ± 0.8 nm whereas sizes of PS_{O10H6} and PS_{O10H6}-pIL-10 were found to be 121.7± 28.4 nm and 126 ± 8.3 nm, respectively. Modifying the surface of PLGA particles (259 ± 3 nm) with O10H6 and pIL-10 generated slightly larger particles with 277.9± 5.9 nm ($PLGA_{O10H6}$) and 297 ± 14.1 nm ($PLGA_{O10H6}$-pIL-10) in diameter. This change in size is consistent with association of O10H6 and DNA on the particles. The particle size deviation of the complex became slightly larger after addition of O10H6 and pIL-10 (Table 3.1), but the relative standard deviation in PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 remained to be 6.6% and 4.6%, respectively.

Data also showed that both PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 displayed unimodal (Gaussian) distributions (Figure 3.2) in the submicron range (Chi-squared [$\chi 2$] = 0.76). This result indicated that both systems exist as uniform stable colloidal dispersions, which is a prerequisite in parenteral administration. It also implied that the

three-component particle (PS_{O10H6}-pIL-10 or $PLGA_{O10H6}$-pIL-10) is the dominant specie in the dispersion. Because both systems remained below 500 nm in diameters, PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particles are suitable for passive targeting delivery into DCs (Cohen, Levy et al. 2000; Panyam, Dali et al. 2003).

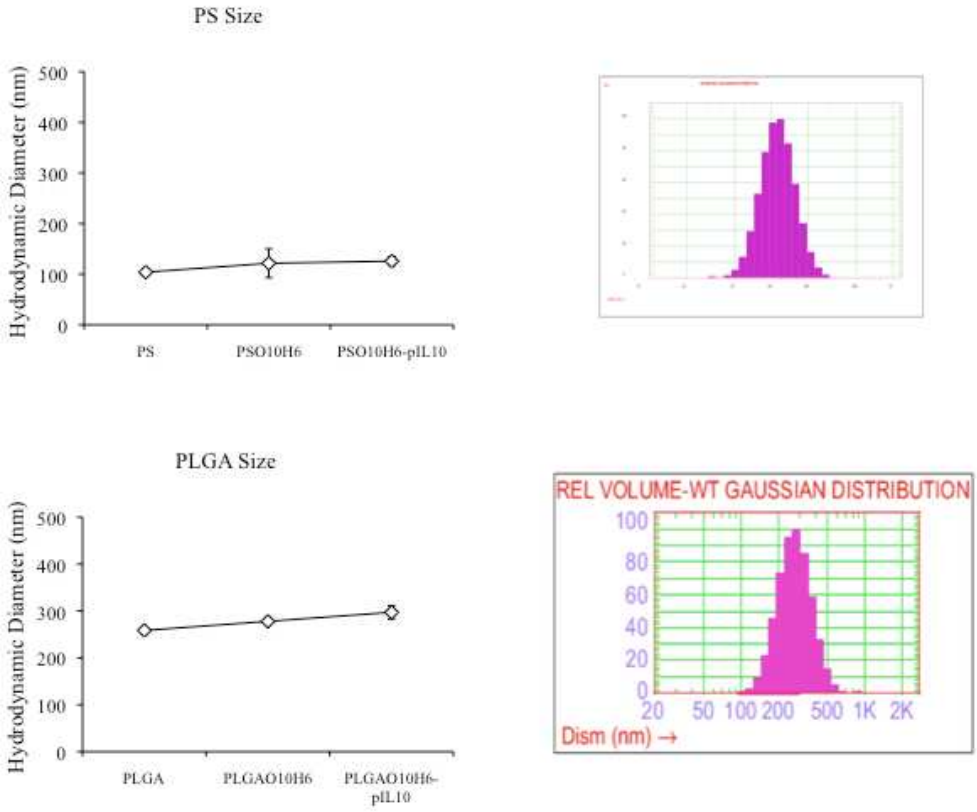


Figure 3.2: Mean diameter (left) of PS and PLGA particles. Histogram (right) represents data fit to Gaussian function ($\chi 2 = 0.76$) and non-Gaussian fit also yielded a single peak.

7

Particle	Mean diameter (nm)	Standard Deviation (nm)
PS	104.0	±0.8
PS_{O10H6}	121.7	±28.4
PS_{O10H6}-pIL-10	126.0	±8.3
PLGA	258.6	±3.4
$PLGA_{O10H6}$	277.9	±5.9
$PLGA_{O10H6}$-pIL-10	297.2	±14.1

Table 3.1: Summary of particle size analysis of PS and PLGA particles.

The surface repulsive force of colloidal particles is associated with the stability of colloidal dispersion. This property can be evaluated by the surface electrostatic potential (zeta potential) of the particle. Figure 3.3 and Table 3.2 are the results of zeta potential analysis from three independent experiments. The average surface potential of unmodified PS particle is -36.31±5.26 mv. This negative potential is due to the presence of carboxylate groups on the surface of PS particles. The average zeta potential of unmodified PLGA particle is slightly negative (-12.3±0.21 mv), which is due to the presence of PVA molecules (mainly hydroxyl groups) on the surface. The negative surface of both unmodified particles is capable of absorbing cationic peptide O10H6 via electrostatic interaction. As shown in Figure 3.3 and Table 3.2, addition of O10H6 reverses the surface potential of both unmodified PS and PLGA particles from negative to positive. Modifying the surface of PS particles with O10H6 changed their zeta potential to 30.61±3.05 mv. Likewise, altering the surface of PLGA particles with O10H6 changed their surface potential to 5.2 mv. The reversal of the zeta potential is the result of absorption of O10H6 peptide on the particle surface and the protonation of excess amount of polycation O10H6 at neutral pH. This positive potential provides a tunable binding surface for plasmid DNA. After addition of pIL-10, the surface potential of both particles was not changed dramatically. As shown in Figure 3.3 and Table 3.2, the zeta potential of PS_{O10H6}-pIL-10 and PS_{O10H6}-pIL-10 particles are 31.26±2.46 mv and 4.7 ±0.5 mv, respectively. This positive surface potential stabilizes PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particle dispersion.

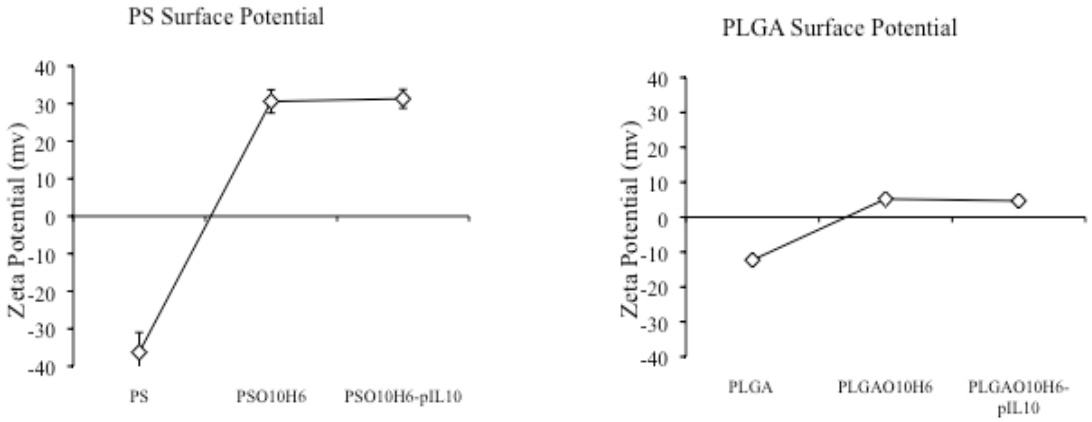


Figure 3.3: Zeta potentials of PS and PLGA particles.

Particle	Zeta potential (milli-volt)	Standard Deviation (milli-volt)
Plain PS	-36.31	±5.26
PS_{O10H6}	30.61	±3.05
PS_{O10H6}-pIL-10	31.26	±2.46
Plain PLGA	-12.30	±0.21
$PLGA_{O10H6}$	5.2	
$PLGA_{O10H6}$-pIL-10	4.65	±0.53

Table 3.2: Summary of zeta potential analysis of PS and PLGA particles.

Complexation of plasmid IL-10 on $PLGA_{O10H6}$ particles

Particle size distribution and zeta potential data suggested that cationic peptide O10H6 was associated on the surface of PS_{O10H6} and $PLGA_{O10H6}$ particles. The ability of polycation O10H6 coated particles to bind and condense DNA molecules was further confirmed using gel electrophoretic analysis. As shown in Figure 3.4b, pIL-10 was immobilized on $PLGA_{O10H6}$ particles after running the gel at 90 volts for 50 min when free pIL-10 was shifted to anode (on the right) in the same electric field. The lack of DNA migration in samples suggested plasmid molecules were associated with $PLGA_{O10H6.}$ Further electrophoretic analysis after the addition of excessive amount of low molecular weight heparin sodium (an anionic polymer that competes the electrostatic binding between cationic peptide and DNA) showed that free pIL-10 was released from $PLGA_{O10H6}$ particles (Figure 3.4a). The immobilization and release of pIL-10 on $PLGA_{O10H6}$ particles before and after heparin addition was consistent with an O10H6-pIL-10 complex, indicating that the binding between $PLGA_{O10H6}$ and plasmid DNA is mainly via strong electrostatic interactions. By comparing the band intensity of DNA released by heparin from $PLGA_{O10H6}$ particles with the control well loaded with the same amount of free pIL-10 (Figure 3.4a) using ImageJ software, we estimated that 87% (percent recovery) of pIL-10 on $PLGA_{O10H6}$ was associated with O10H6 through ionic interactions. Taken together, these data suggested that a PLGA particle presents an electrostatic surface conducive for O10H6 binding and DNA condensation.

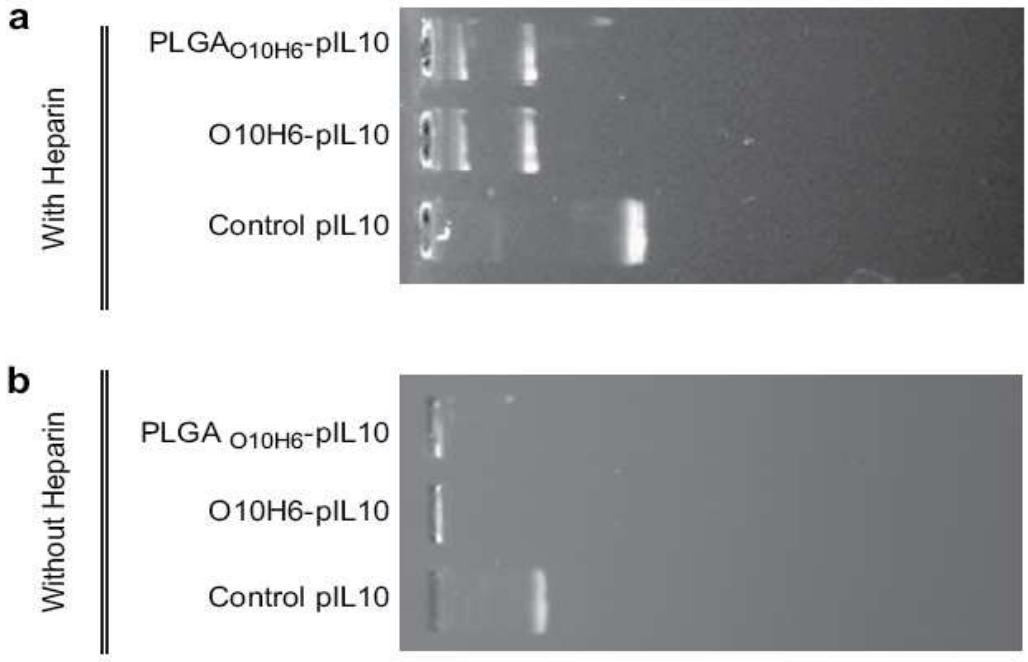


Figure 3.4: Complexation of pIL-10 with PLGA$_{O10H6}$. Samples of free plasmid IL-10, O10H6-pIL-10 complex and PLGA$_{O10H6}$-pIL-10 particle were loaded in 1% agarose gel and mobilized in an electric field (anode on the right, 90 volt) for 50 min (b). Low molecular weight heparin sodium was then added to samples to release charge-bound DNA in 1% agarose gel which was mobilized in the same electrophoresis condition (a). DNA was visualized by ethidium bromide staining.

Serum stability of plasmid DNA carried by PS_{O10H6} and $PLGA_{O10H6}$

At *in vivo* conditions, DNA molecules are susceptible to degradation by serum endonuclease. Cationic peptides including O10H6 are capable of protecting these molecules from nuclease by forming complexes with them. To evaluate the ability of PS_{O10H6} and $PLGA_{O10H6}$ particles to protect plasmid DNA from serum destabilization, free plasmid DNA, O10H6-DNA complexes, PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particles were incubated with 10% FBS at 37 ˚C for up to 48 hours. An excess of low molecular weight heparin sodium was added to samples after incubation to release charge-bound DNA from the complexes prior to electrophoresis in 1% agarose gel. Unbound DNA molecules were mobilized in an electric field (anode on the right, 90 volt) for 50 min and DNA was visualized by ethidium bromide staining. The results (Figure 3.5) demonstrated that DNA molecules carried by PS_{O10H6} particles had highest amount of DNA remaining among all the groups after extended incubation with FBS. In contrast, naked DNA and DNA incubated with PS only (without O10H6) were completely degraded after serum incubation. DNA complexed with O10H6 (without PS) also resulted in some DNA degradation after incubation with FBS, as evidenced by the lower DNA recovery compared to PS_{O10H6}-pIL-10 particles. These data suggested that PS_{O10H6} particles protected DNA molecules from serum degradation. DNA directly incubated with PS particles was not protected in serum because no complex was formed between the two. The association of cationic peptide O10H6 on the particle provides a binding surface for plasmid DNA, improving the stability of O10H6-pIL-10 complexes against serum degradation.

Likewise, $PLGA_{O10H6}$ also confers plasmid DNA protection in serum. Complexed or free plasmids (pLuc) were incubated in the presence or absence of 10% fetal bovine serum at 37 °C for 48 hours and heparin was added to each incubated sample. DNA was analyzed in 1% agarose gel electrophoresis and visualized by staining with ethidium bromide. Percent DNA recovery was calculated using pLuc released without serum incubation as the control. As shown in Figure 3.6, greater than 50% of pLuc loaded onto $PLGA_{O10H6}$ was recovered after incubation. Conversely, free plasmids were completely degraded after serum incubation. This result indicated that condensation of plasmid DNA on $PLGA_{O10H6}$ surface protects DNA from serum digestion. Although O10H6 bound the plasmid molecules, in the absence of PLGA, the DNA was completely degraded by serum incubation over 48 hours, while more than 50% of DNA still bound to the $PLGA_{O10H6}$ particle after the incubation. This result demonstrated that PLGA particle improved stability of O10H6-DNA complexes in serum. Presumably, the polymer backbone may provide an anchorage for O10H6-DNA complexes, rendering extra protection mechanism against serum endonucleases.

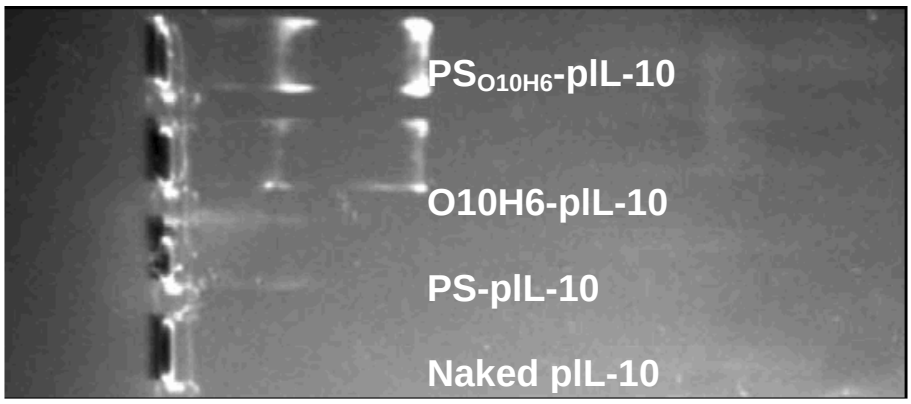


Figure 3.5: PS_{O10H6}-pIL-10 particles protect plasmid IL-10 from serum digestion. Naked plasmid DNA or the respective DNA complexes were incubated with 10% FBS at 37°C for overnight. Low molecular weight heparin sodium was added to samples to release charge-bound DNA in 1% agarose gel. DNA was visualized by ethidium bromide staining. A representative of three experiments is shown.

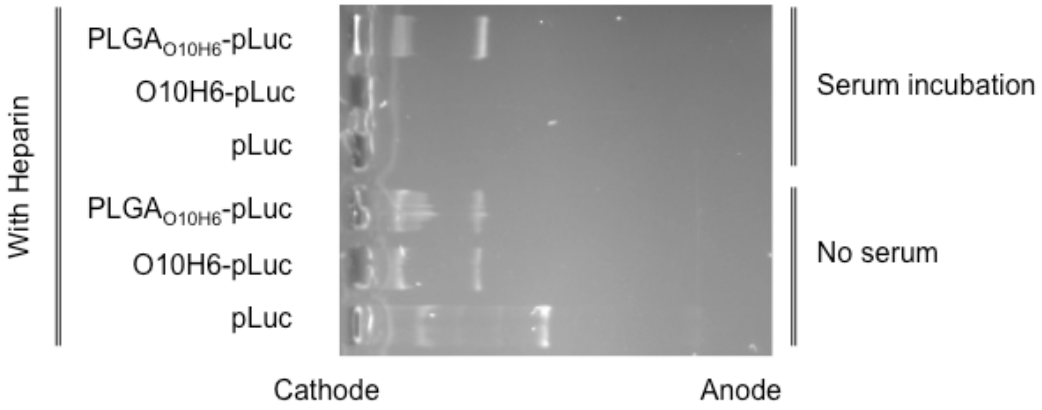


Figure 3.6: PLGA$_{O10H6}$-pIL-10 particles protect plasmid IL-10 from serum digestion. Complexed or free pLuc were analyzed in 1% agarose gel with or without incubation with 10% fetal bovine serum (37 °C, 48 hours). DNA in wells was released by heparin (50 U/mL) and visualized by staining with ethidium bromide. Percent recovery was calculated using pLuc released without serum incubation as the control. DNA in control ‘‘pLuc’’ lanes (with and without serum) was allowed to migrate before the addition of heparin to other groups. A representative of three independent experiments is shown.

Discussion

The results presented in this chapter demonstrated that PS_{O10H6} and $PLGA_{O10H6}$ particles are suitable carriers for DNA delivery to DCs.

The goal of particle formulation is to form a stable, colloidal dispersion that retains both monodispersity and particle size in an effort to achieve consistent efficacy. A colloidal system consists of particles (usually ranging in size from 1nm to 1μm) distributed evenly throughout a dispersion medium. In pharmaceutical applications, colloids such as liposomes, micelles or submicron particles have been used as drug delivery systems for therapeutics in parenteral administration.

Particle size distribution of PS_{O10H6} and $PLGA_{O10H6}$ was measured by dynamic light scattering with a Nicomp 380 particle analyzer. DLS is a technique measuring the particles suspended in a liquid in the submicron region. The principle of this method is briefly described as follows. Particles in a colloidal system are in the state of random movement caused by Brownian motion, and this motion causes the intensity of scattered light to fluctuate as a function of time. The speed of given particles is inversely proportional to its size and smaller particles move faster than their larger counter parts. Diffusion coefficient of the particles can be measured by analyzing the fluctuation rate of scattered laser light in the system, which is obtained from the correlation function of signal decays. Thus, size of spherical particles will be calculated using the Stokes–Einstein equation based on diffusion coefficient of the particles in the medium.

The unimodal distribution of PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particles indicated that both systems exist as uniform stable colloidal dispersions, and the three-component particle (PS_{O10H6}-pIL-10 or $PLGA_{O10H6}$-pIL-10) is the dominant specie in the dispersion, given that their mean diameters are 126 ± 8.3 nm for PS_{O10H6}-pIL-10, and 297 ± 14.1 nm for $PLGA_{O10H6}$-pIL-10 (Chi-squared [$\chi 2$] = 0.76). These results also indicated that particle sizes of PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 remain below 500 nm, which is preferred for DC uptakes.

Zeta potential is a measurement of the electric charge acquired by a particle, which is a good indication of how stable the system is. Colloidal dispersion is governed by both the repulsive force from the electric double layer of particles and the attractive Van Der Waals force that particles experience as they approach one another. When the particles collide as a result of Brownian motion, they can adhere and form aggregates. The electric repulsive force of the particle, on the other hand, presents an energy barrier to overcome the attractive Van Der Waals force, and thereby stabilizes the system.

Particles with positive or negative zeta potential will repulse each other and remain in a stable, monodisperse form. However, excessive high surface potential could increase the toxicity of particles by disrupting the structure of negatively charged cellular membranes. The positive zeta potential stabilizes PS_{O10H6}-pIL-10 (31.3 mv) and PLGA $_{O10H6}$-pIL-10 (4.3 mv) particles as colloidal dispersions without increasing their toxicities.

Our data also showed that both plain PS and PLGA particles have negative zeta potentials, owing to the presence of carboxylate and PVA groups on PS and PLGA particles, respectively. The display of PVA molecules on the exterior of PLGA particles was confirmed in our lab using an IR spectrometer, which is consistent with the result reported previously by Sahoo et al. (Sahoo, Panyam et al. 2002). The carboxylate and PVA groups on PS and PLGA particles serve as conduits for polyelectrolyte-adsorption of polycation O10H6. Successful coating of O10H6 on both particles was confirmed by the reversal of zeta potential upon addition of O10H6 peptide. The binding of the peptide was also indirectly verified by the slight increase of particle size of PS_{O10H6} and $PLGA_{O10H6}$ particles compared with the plain particles.

The principle role of O10H6 is to exert a tunable DNA binding surface so that DNA can be loaded and protected on the particle, considering that the ionic interaction between the DNA and O10H6 peptide is strong enough to bind DNA. Previous data in our lab showed that short oligonucleotides bind to O10H6 on polystyrene particles and that the half-maximal concentration of O10H6 required for DNA immobilization is 0.5 mg/mL. Gel electrophoresis data presented in this chapter demonstrated that pIL-10 was condensed on $PLGA_{O10H6}$ particle via electrostatic interaction, as evidenced by the release behavior after the addition of excessive amount of low molecular weight heparin. The binding of pIL-10 on $PLGA_{O10H6}$ particles was indirectly confirmed by the slightly increase of particle size of PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particles compare with PS_{O10H6} and $PLGA_{O10H6}$ particles.

One of the important features of cationic peptide /DNA complex is its ability to condense and protect DNA against serum endonuclease, which is critical for *in vivo* delivery. Our data confirmed that O10H6 peptide alone protected DNA in serum for up to 24 hours. Moreover, data from gel electrophoretic analysis showed that polycation particles PS_{O10H6}-DNA and $PLGA_{O10H6}$-DNA have greater DNA retention compared to O10H6-DNA complexes after 48 hours incubation in serum. The result indicated that coating of polycation on particles improved its protective effect on DNA against serum endonuclease. Presumably, the polymer structure of polystyrene and PLGA provided anchorages for the cationic peptide/DNA complexes, and thus rendered additional protection mechanism against nuclease in serum.

In summary, PS_{O10H6} and $PLGA_{O10H6}$ particles existed as a colloidal dispersion with a preferential size range for DC uptake. The O10H6 coated surface on these particles is capable of binding and protecting plasmid DNA from serum endonuclease. Thus, PS_{O10H6} and $PLGA_{O10H6}$ particles are suitable for DNA delivery to DCs and these vectors might serve as a non-inflammatory alternative to lipid and viral vectors.

CHAPTER 4

O10H6 MODIFIED POLYSTYRENE AND POLY (LACTIC-*CO*-GLYCOLIC ACID) PARTICLES ENHANCE GENE TRANSFER INTO DENDRITIC CELLS

Introduction

Gene therapy has emerged as a field with the goal to modulate cell functions by introducing gene of interest to target cells. Efficient delivery of DNA-based therapeutics is challenged by physicochemical and biological barriers presented in blood circulation, tissues, and cells. Major delivery barriers include degradation of DNA in the extracellular space by nuclease, internalization of DNA into target cells, escape of DNA from endosome into the cytoplasm, and transfer of DNA into the nucleus. Development of vectors to overcome these barriers is important to achieve efficient gene delivery.

The objective of the work in this chapter is to evaluate the performance of PS_{O10H6} and $PLGA_{O10H6}$ particles as vectors to deliver plasmid DNA to DCs *in vitro*. The hypothesis is that the polycation surface-modified polymeric particles could facilitate *in vitro* gene transfer to DCs for three reasons. First, coating of cationic peptide O10H6 on polymeric particle improves its ability to condense DNAs and stabilize these molecules against endonuclease in serum (Albert, Jegathesan et al. 2001; Meng and Butterfield 2005). Data presented in the previous chapter demonstrated that PS_{O10H6} and $PLGA_{O10H6}$ particles increased the stability of loaded DNA in serum. Second, submicron range

particles have been used in passive targeting DNA delivery to DCs, because these particles can be taken up preferentially by DCs through the endocytic pathway. Third, the histidine cluster in O10H6 peptide functions as an acid-buffering agent to assist DNA escape from the endosomal compartment to the cytosol (Midoux, Kichler et al. 1998; Putnam, Gentry et al. 2001). In this chapter, the *in vitro* transfection efficiency of PS_{O10H6} and $PLGA_{O10H6}$ particles was investigated in pGFP, a reporter gene plasmid and pIL-10, a therapeutic gene plasmid.

Methods

Generation of primary DCs

Six to eight weeks old female certified virus free C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from Hilltop Lab Animals, Inc. (Scottdale, PA) and housed in the Duquesne University Animal Care Facility. All experimentations were handled in accordance with the institutional animal care policy.

The method of generating primary DCs was adapted from the Inaba protocol with modifications (Inaba, Inaba et al. 1992; Meng, Butterfield et al. 2001). Harvested bone marrow progenitors from femurs were cultured overnight in serum free Roswell Park Memorial Institute (RPMI) medium (Life technologies, Gaithersburg, MD). Nonadherent cells were then re-plated at 10^6 cells/mL in 6-well tissue culture plates (Costar, Cambridge, MA, USA) in RPMI containing 10% heat-inactivated fetal bovine serum, 50 μM 2-mercaptoethanol, antibiotics (penicillin/streptomycin/fungizone), all from Life Technologies (Gaithersburg, MD), and cytokines (5 ng/mL of murine GM-CSF and 5 ng/ml of murine IL-4; R&D Systems, Minneapolis, MN) and maintained at 37 °C in 5% CO_2. On day 4, nonadherent cells were removed by aspirating 80–90% of the media and adherent cells were re-fed with culture medium (1 mL/10^6 cells) containing murine GM-CSF and IL-4.

In vitro IL-10 gene transfection

On day 6 of primary DC culture, culture medium was removed and cells were

washed two times with 1 mL of 1× sterile PBS at room temperature, followed by complete removal of PBS from each well and addition of 1.2 mL of sterile serum reduced Opti-MEM media (Invitrogen). DCs were transfected with a plasmid reporter gene encoding green fluorescent protein (pGFP, 5.3 kb, Fargo, ND) and pIL-10 using PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particles. Free plasmids were used as the control. 75 μg of PS or 60 μg of PLGA particles coated with 200 μg of O10H6 and 15 μg 300 μL of DNA in 200 μL of ddH_2O were then added to each well and incubated for 4 h at 37 °C in 5% CO_2. Upon completion Opti-MEM was then removed and DCs were washed with 1 mL of 1× sterile PBS. Fresh RPMI 1640 media containing 10% FBS and GM-CSF/IL-4 was added to each well and DCs were cultured in for an additional 24–48 h at 37 °C in 5% CO_2. Expression of pGFP or pIL-10 after the transfection was analyzed using confocal imaging or reverse transcriptase polymerase chain reaction (RT-PCR) analysis, respectively.

Confocal imaging analysis of pGFP expression

Bone marrow-derived primary DCs were allowed to adhere to coverglasses, washed with PBS, and exposed to 300 μL of $PLGA_{O10H6}$-pGFP particles in 1.2 mL Opti-MEM media for 4 h 37 °C in 5% CO_2. Upon completion of transfection, DCs were washed and cultured in RPMI medium containing 10% FBS and GM-CSF/IL-4 for an additional 48 h. After the incubation period, cells were washed in PBS twice and fixed in 4% paraformaldehyde for 15 min at 4 °C before mounting on slides with MOWIOL4-88 anti-fade media (CalBiochem, Darmstadt, Germany). Images were collected using a

Leica TCS SP-2 spectral microscope (Leica Microsystems AG, Wetzlar, Germany) with an argon/helium laser and FITC filter.

IL-10 RT-PCR analysis of IL-10 gene expression

Total RNA of transfected DCs was isolated using a SV total RNA isolation kit (Promega, Madison, WI). 0.1-0.5 mg of RNA sample was amplified using One-Step Eppendorf Master RTplus PCR kit (Eppendorf, Hamburg, Germany) in an Eppendorf MasterCycler. 2 μL of primer pairs (7.5 pmoles/ μ L) specific for IL-10 and β-actin (R&D Systems, Minneapolis, MN) were added to 0.1-0.5 μg of RNA sample in a reverse transcription (50 °C for 50 min) step, followed by denaturation (94 °C for 4 min). The amplification steps consisted of 35 cycles of template denaturation (94 °C for 45 s), primer annealing (55 °C for 45 s) and primer elongation (68 °C for 45 s). 20 μL of PCR products were loaded in 2% agarose gel and run at 90 volts for 50 minutes and DNA was visualized by 0.5 μg/mL of ethidium bromide in agarose gel.

Results

To evaluate the gene transfer efficiency, bone marrow derived DCs (BMDCs) were transfected *in vitro* with PS_{O10H6} and $PLGA_{O10H6}$ particles carrying a reporter gene pGFP. Free pGFP was used as a control vector to investigate whether the O10H6-modified polymeric particles can facilitate *in vitro* gene transfer to DCs. Reporter genes are common tools for evaluating gene transfer efficiency of a delivery vector. Expression of pGFP will result in the synthesis and accumulation of green fluorescence protein in cytoplasm, which has a major excitation peak at a wavelength of 498 nm and an emission peak at 509 nm in the lower green portion of the visible spectrum. Thus, pGFP expression can be evaluated by confocal imaging analysis using an inverted Leica TCS SP-2 spectral microscope with an argon/helium laser and a FITC filter cube as the excitation source.

The confocal images shown in Figure 4.1 are cells expressing green fluorescence protein after transfection with PS_{O10H6}-pGFP (a), $PLGA_{O10H6}$-pGFP (b), and free pGFP(c) using a 63X oil immersion objective. The green fluorescence in the images was emitted by the green fluorescence protein and the average intensity in each image was quantified using ImageJ software. Analysis of the images showed that DCs transfected with PS_{O10H6}-pGFP resulted in 77% increase in fluorescence intensity compared to those transfected with free pGFP (Figure 4.1a and c), while DCs transfected with $PLGA_{O10H6}$-pGFP showed 72% of enhancement of pGFP intensity over cells treated with free pGFP (Figure. 4.1b and c). The increased intensity of green fluorescence in cells came from the more efficient expression of pGFP, indicating that both PS_{O10H6} and $PLGA_{O10H6}$ particles

can effectively facilitate gene delivery to DCs, leading to increased transgene expression over free plasmid DNA.

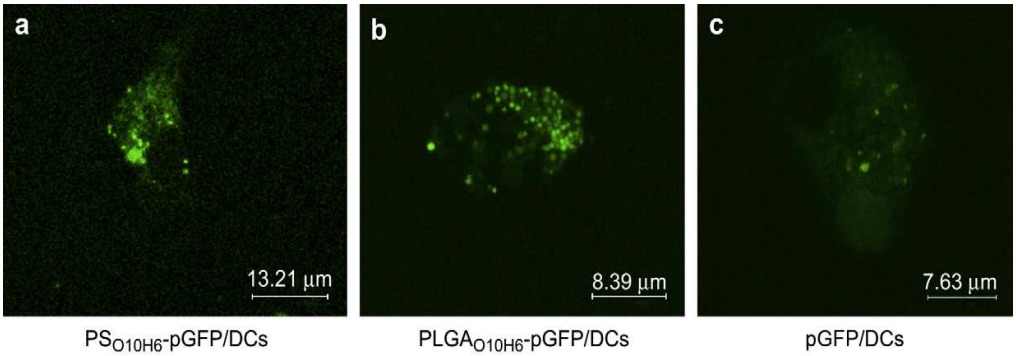


Figure 4.1: Transgene expression in DCs transfected by complexed or free pGFP. Confocal images of cells expressing (GFP) after transfection with PSO10H6-pGFP (a), PLGAO10H6-pGFP (b), and free pGFP (c). Slides were prepared by adhering DCs incubated with the respective complexes for 2 h at 37 °C. Images were captured using a Leica confocal microscope at 63× magnification.

Our results showed that PS_{O10H6} and $PLGA_{O10H6}$ particles can effectively facilitate plasmid reporter gene delivery to DCs. The next step is to investigate whether these particles can increase therapeutic gene (e.g. pIL-10) expression in DCs. To test this, BMDCs from C57/BL6 mice were *in vitro* transfected with pIL-10 loaded on PS_{O10H6} and $PLGA_{O10H6}$ particles. DCs transfected with free pIL-10 alone were designed as a control to evaluate the efficiency of IL-10 gene transfer using PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particles. IL-10 expression in DCs was evaluated by isolating total RNA of transfected DCs in each delivery mode and semi-quantifying levels of mIL-10 using RT-PCR analysis. RT-PCR is a highly sensitive technique that has been used to measure the expression of a defined gene. The specific RNA strand is first reverse transcribed into its DNA complement, followed by amplification of the resulting DNA using PCR. The final PCR products were analyzed in 2% agarose gel and DNA was visualized by ethidium bromide staining. Expression of β-actin, a housekeeping gene in cells, was measured in the same sample for semi-quantification purpose.

Figure 4.2 and table 4.1 are the results of IL-10 gene expression in cells transfected with PS_{O10H6}-pIL-10, $PLGA_{O10H6}$-pIl-10, and free pIL-10. Bands shown in Figure 5.2a matched predicted PCR products of IL-10 (235 bp) and β-actin (302 bp), respectively. The intensity of the band indicates the level of mRNA. Consistent with the result from reporter gene transfer, IL-10 mRNA expressions were enhanced in PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 transfected DCs compared to DCs transfected with free pIL-10, as evidenced by the increased intensity of IL-10 band shown in Figure 4.1a.

The results of IL-10 gene expression from two independent experiments were also semi-quantitatively analyzed by normalizing the intensity of IL-10 against the corresponding β-actin band (Figure 4.2b, Table 4.1). The result demonstrated a 2.8-fold and three-fold increase of IL-10 expression in DCs transfected with PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 ($p<0.05$, student's t-test one-tailed, equal variance) relative to DCs transfected with free pIL-10, respectively. These results indicate that PS_{O10H6} and $PLGA_{O10H6}$ can facilitate pIL-10 entering the nuclear compartment in DCs, resulting in higher expression of the IL-10 gene in target DCs compared to free plasmid IL-10. Therefore, PS_{O10H6} and $PLGA_{O10H6}$ can be used as vectors to achieve increased pIL-10 transfer to DCs *in vitro*.

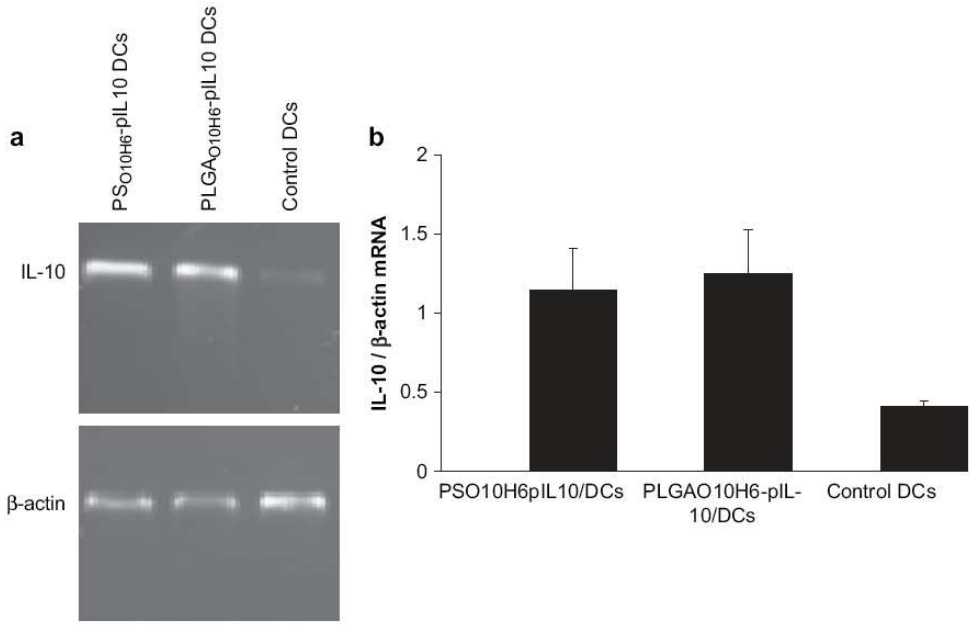


Figure 4.2: Up-regulation of IL-10 in transfected DCs. (a): IL-10 mRNA was determined using RT-PCR in cells transfected with the respective complexes. (b): Expression was normalized against β-actin mRNA. RNA was extracted from bone marrow-derived DCs and amplified using murine IL-10 specific primers. Bands identified were consistent with predicted PCR products of IL-10 and β-actin with 235 and 302 bp, respectively.

IL-10/ β-actin mRNA		
	Mean	**SEM**
PS_{O10H6}pIL-10 Transfected DCs	1.14*	0.27
$PLGA_{O10H6}$-pIL-10 Transfected DCs	1.25**	0.28
Control DCs	0.41	0.04

Table 4.1: IL-10 mRNA expression in transfected and control (untransfected) DCs. Data were normalized against β-actin mRNA from two independent experiments. (*p=0.05; **p<0.05. Student's t-test, one-tailed compared to control.)

Discussion

Previous data from our lab showed that PS_{O10H6} particles are efficient carriers of oligonucleotides, capable of introducing NF-κB decoy molecules into greater than 50% of primary DCs *in vitro.* Results from the research presented in this chapter demonstrated that O10H6-modified polymeric particles PS_{O10H6} and $PLGA_{O10H6}$ can effectively deliver plasmid DNA to DCs. This is confirmed by the successful delivery of both reporter gene pGFP and therapeutic gene pIL-10 *in vitro*.

Delivery of free plasmid DNA is challenged by physicochemical and biological barriers presented in blood circulation, tissues, and cells. Those barriers cause low gene transfection when free plasmid DNA is used. Development of vectors to overcome these barriers is important to achieve efficient gene delivery.

The enhanced DNA transfection by PS_{O10H6} and $PLGA_{O10H6}$ particles over free plasmid DNA was most likely influenced by four factors. First, DCs take up particulates efficiently due to active and responsive cytoskeletal remodeling mechanisms (Mellman 2005), thereby particle systems can facilitate the internalization of carried DNA molecules into DCs. Previous data from our lab demonstrated that PS_{O10H6} particles significantly increase oligonucleotide uptake in DCs by five-fold compared to free oligonucleotide (Kovacs, Zheng et al. 2005). Likewise, oligonucleotide encapsulated in PLGA particles is taken up by DCs efficiently (two-fold increase compared to free DNA transfection) (Zheng, Kovacs et al. 2006).

Second, the amine group (pKa = 6) on the imidazole ring of the histidine cluster in O10H6 functions as an acid-buffering agent to assist DNA escape from endosome. DCs are equipped with robust endo-lysosomal machinery (Wakkach, Fournier et al. 2003), resulting in the accumulation of relatively minor fractions of exogenous materials in the cytosol. Data from our lab show that accumulation of plasmid DNA in cytosol can be mediated by O10H6 coated polymeric particles through an endosome escape mechanism., We used confocal microscopy with a FITC-labeled short oligonucleotide (ODN) as a probe to observe enhanced DNA escape within 30 min and accumulation outside of acidic compartments in DCs (stained with a pH-sensitive LysoTracker dye) exposed to PS_{O10H6}-ODN (Jia, Kovacs et al. 2008). Conversely, in cells exposed to free ODN, few DNA clusters were found outside of lysosomes compartment. It is worth to point out that PLGA also facilitates the escape of therapeutic agents from endosome into cytoplasm, owing to a change in surface charge of the polymer from negative to positive (Panyam, Zhou et al. 2002; Bala, Hariharan et al. 2004). Given that carboxylated polystyrene particles do not confer acid-buffering effect as PLGA does, escape of DNA was most likely mediated by O10H6.

Third, surface loading of nucleic acids allows DNA to be released inside cells without having to diffuse through acidic matrices, thereby increasing DNA transfection efficiency. Upon hydration lactic and glycolic acids (polymer byproducts) accumulate within the matrix of PLGA polymer (Ding and Schwendeman 2004). These byproducts will drop the interior pH value of submicron PLGA particles below 3, which is harmful to the integrity of embedded DNA molecules. Consistent with this theory is that Kasturi et

al. (Kasturi, Sachaphibulkij et al. 2005) have reported superior transfection efficiency when plasmid DNA is carried on the exterior of PLGA particles compared to that encapsulated within.

Fourth, submicron particles have a very large surface-to-volume ratio compared to larger particles. Thus, even small quantities of particles present extremely large surface areas available for DNA binding, allowing a large dose of plasmid DNA to translocate across the plasma membrane with each particle entry, and transfer from cytoplasm to nucleus. Additionally, rapid accumulation of plasmid DNA in the cytosol resulting from large surface-to-volume ratio and the endosomal escape mechanism of PS_{O10H6} and $PLGA_{O10H6}$ particles provides sufficient copies of plasmid DNA to enter the nucleus during mitosis. This is an important factor for gene delivery because primary DCs divide relatively slowly *in vitro.*

In summary, we reported in this chapter that the polycation O10H6 modified polymeric submicron particles (PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10) are capable of facilitating plasmid DNA transfer into dendritic cells. Thus, these vectors should be further investigated as a means of genetically modifying DCs to suppress T cell responses. One of the potential advantages of these particulate vectors is they can be adjusted to achieve different payloads of DNA by changing the amount of O10H6 peptide added to the particles during fabrication. Increasing amounts of O10H6 coated on the particles will likely to increase amount of positive charge available for DNA binding, thereby increasing DNA loading per particle.

CHAPTER 5

SUPPRESSIVE EFFECTS OF PS_{O10H6}-pIL-10 AND $PLGA_{O10H6}$-pIL-10 MODIFIED DENDRITIC CELLS ON T CELL RESPONSES

Introduction

IL-10 is an important cytokine with a central role in preventing damaging immune responses in healthy individuals. Because dendritic cells have exclusive privilege to initiate naïve T cell activation, induction of adaptive immune tolerance through IL-10 is believed to be primarily mediated by DCs (Mahnke, Johnson et al. 2007). Literature data suggested that IL-10 can modulate DCs to trigger T cell suppression by altering the expression of MHC class II and co-stimulatory molecules (Kubin, Kamoun et al. 1994; Willems, Marchant et al. 1994; Tong, Toshiaki et al. 2005) and modifying the release of pro-inflammatory cytokines from DCs (Fortsch, Rollinghoff et al. 2000). Additionally, IL-10 modified DCs can induce regulatory T cell expansion in allogeneic or Ag-activated T lymphocytes (Takayama, Nishioka et al. 1998; Zeller, Panoskaltsis-Mortari et al. 1999; Mahnke and Enk 2005). IL-10 secreted by DCs could also directly act on T cells and other APCs to induce their suppressive functions.

Owing to the above reasons, IL-10 has been used to render DCs into a suppressive phenotype to attenuate damaging immune responses in autoimmunity, inflammation, and transplant rejection. Efforts have been taken to genetically modify DCs with the IL-10

gene to render suppressive signals in local microenvironment.

We have shown in previous chapters that PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particles are effective vectors to deliver plasmid IL-10 to DCs. In this chapter, we evaluated the suppressive effects of PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 modified DCs on T cell responses. An *in vitro* mixed lymphocyte reaction (MLR) model that used two mouse strains (C57BL/6 and BALB/c) with mismatched class II MHC ($H\text{-}2^b$ and $H\text{-}2^d$) was employed to evaluate the ability of these IL-10 gene-modified DCs to stimulate allogeneic T cells. Additionally, an *ex vivo* Matrigel implant system embedded with DCs was used to study the ability of IL-10 gene-modified DCs to alter allogeneic immune responses *in vivo*. The hypothesis is that DCs treated with PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particles will attenuate allogeneic T cell response *in vitro* and *in vivo*. The rationale is that delivery of the IL-10 gene to DCs will increase IL-10 expression in the local microenvironment and alter the functionality of these DCs. Specifically, DC's ability to present Ag or stimulate allogeneic T cells, as well as their characteristics of cytokine production could be affected. Moreover, IL-10 modified-DCs could expand regulatory T cells in the local microenvironment to suppress T cell responses.

Methods

Enrichment of primary DCs

DCs of C57/BL6 mice (H-2^b) were generated from mouse bone marrow progenitors as described in the previous chapter. Cells were harvested from cultures on day 6, washed, and stained with anti-CD11c magnetic beads in PBS containing 2 mM EDTA (Figure 5.1). Stained cells were washed with separation buffer (PBS, 2 mM EDTA, 0.5% bovine serum albumin) and passed through MiniMACS (Miltenyi Biotec) columns or separated by the IMag^{TM} system (BD Bioscience). Greater than 80% of the eluted population were CD11c+ cells.

Mixed leukocytes cultures for T cell proliferation

CD11c+ DCs were transfected with DNA complexes using the method described in the previous chapter, and cultured with fresh RPMI 1640 media containing 10% fetal bovine serum and GM-CSF/IL-4 (R&D Systems, Minneapolis, MN) for an additional 48 hours. Control and transfected DCs (H-2^b) were cultured with splenocytes of mismatched BALB/c mice (H-2^d) in AIM V liquid medium (Invitrogen/Gibco) containing 10% fetal bovine serum and GM-CSF/IL-4 (R&D Systems, Minneapolis, MN) for 5 days at 37°C 5% CO_2. The splenocytes were passed through nylon mesh (70 micron pores) prior to the mixing. T cells were seeded with DC at a stimulator to responder ratio of 1:5 in 24-well or 96-well tissue culture plates (Costar, Cambridge, MA). T cell proliferations were analyzed using bulk T cell proliferation studies, and flow cytometry in CD4 and CD8 T cell subset proliferation studies (Figure 5.2).

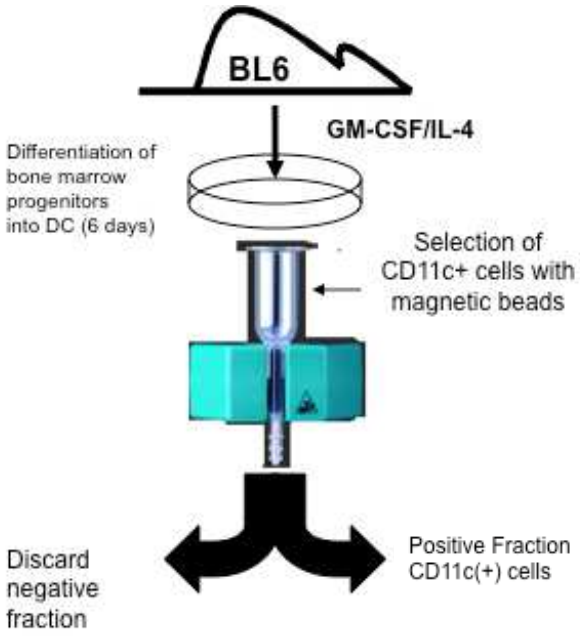


Figure 5.1: Schematic diagram of primary DC enrichment.

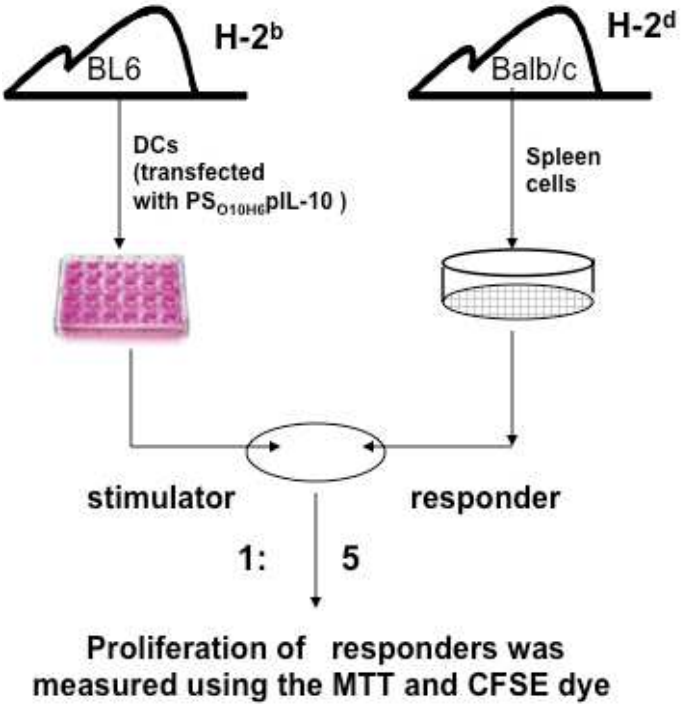


Figure 5.2: Schematic diagram of mixed lymphocyte reaction model.

MTT and flow cytometry assays for T cell proliferation analysis

In bulk T cell proliferation studies, the tetrazolium salt 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Cell Titers 96 kit, Promega, Madison, WI), which is cleaved into formazan in metabolically active cells, was added to each well and equilibrated for 4–6 h at 37 °C in a 5% CO_2 environment. Proliferation was determined by colorimetric changes based on optical density measured at 590 nm using a Perkin Elmer microplate reader. One-way ANOVA was used to calculate statistical significance and pairwise comparison was determined by the Tukey–Karmer test.

To quantify CD4 and CD8 subset proliferation, T cells were first stained with 1.5 μM of carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Invitrogen/Molecular Probes, Eugene, OR) for 8 min at room temperature followed by quenching with 50% fetal bovine serum. Stained cells were washed extensively with PBS containing 2% fetal bovine serum prior to mixing with DC. On day 5, T cells were harvested, pre-treated with purified Fcγ III/II receptor (BD Pharmingen, San Diego, CA) for 10 min at room temperature, and stained with PE-Cy5 conjugated anti-mouse CD8α and PE conjugated anti-mouse CD4 antibody (BD Pharmingen, San Diego, CA) and analyzed by a Beckman Coulter EPICS XL flow cytometer gated on live cells. Student's t-test was used to evaluate statistical significance.

Grafting of Matrigel seeded with allogeneic DCs

Matrigel (BD Bioscience, Bedford, MA) was mixed with IL-10 transfected or

control DCs (0.5-1.5×10^6 cells) derived from BALB/c (H-2^d) mice, and injected (subcutaneous) into C57BL/6 (H-2^b) mice (Figure 5.3). Volume of injection was maintained at 0.4 mL in all experiments. The cell-matrix was kept on ice prior to injection to prevent gel from solidifying at temperature above 10 °C. Solid plugs were formed in the host upon injection. Matrigel plugs were retrieved 7 days post-implantation and cell infiltration was analyzed either by flow cytometry or live-cell confocal microscopy in phase contrast mode. In flow cytometry analysis, cells in Matrigel plugs were recovered in 1 mL recovery solution (BD Bioscience) at 4 °C for 2 hours and washed with PBS to remove matrix components. Single cell suspensions were analyzed using a Beckman Coulter EPIC XL flow cytometer. In microscopy analysis, Matrigel plugs were observed using the phase contrast mode of a confocal microscope with a 10× objective. Photos were taken at the same location of the gel in each sample, and total cell numbers were counted using ImageJ software.

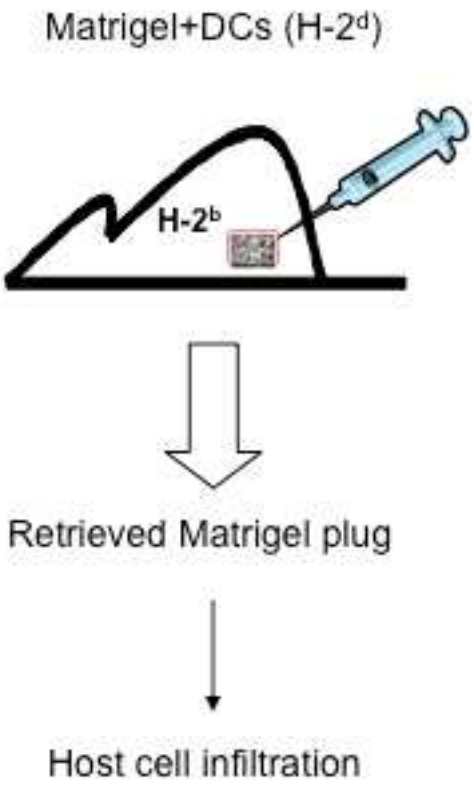


Figure 5.3: Schematic diagram of DC- embedded Matrigel injection allogeneic to host mouse.

Results

DCs modified with PS_{O10H6}-pIL-10 elicit weak allogeneic bulk T cell proliferation

We have shown that PS_{O10H6} and $PLGA_{O10H6}$ particles successfully delivered pIL-10 into DCs. The ability of these IL-10 gene-modified DCs to suppress T cell responses was further evaluated using a MLR model (Schwarz 1968), in which DCs are mixed with allogeneic T cells *in vitro*. The mismatch between MHC class II of donor DCs ($H\text{-}2^b$) and TCR of recipient T cells ($H\text{-}2^d$) will trigger T cell activation and proliferation to induce T cell responses. Herein we hypothesize that IL-10 gene-modified DCs is tolerogenic and can weaken allogeneic T-cell proliferation *in vitro*. In this experiment, CD11c (a marker of DCs)-enriched DCs from C57BL/6 mice were transfected with equal quantity of pIL-10 using different delivery modes including free pIL-10, O10H6-pIL-10 complexes, and PS_{O10H6}-pIL-10 particles. DCs exposed to PS_{O10H6} particles alone and left untreated served as control groups. After the transfection, DCs were mixed with BALB/c ($H\text{-}2^d$) T cells at a stimulator to responder ratio of 1:5 (DC: T cell) and bulk T cell proliferation was analyzed using a tetrazolium salt MTT assay. Proliferation was quantified by measuring colorimetric changes in absorbance at 590 nm.

Data collected from four independent experiments showed that DCs transfected with PS_{O10H6}-pIL-10 particles induced significant weak allogeneic T cell proliferative response (58±6% of control) compared to untreated control DCs (Figure 5.4, Table 5.1), as shown in a one-way analysis of variance data (F = 0:0016) and Tukey–Kramer

pairwise comparison between PS_{O10H6}-pIL-10 and the control group ($p<0.05$). Proliferation of T cells stimulated with DCs exposed to PS_{O10H6} particles alone was neither significantly increased nor reduced compared to untreated control DCs (106±11% vs. 100±8%, respectively, $p>0.05$). This result showed that the delivery system itself does not affect the functionality of DCs, and thereby the suppressive effect of PS_{O10H6}-pIL-10 modified DCs on T cell proliferation was not due to potential toxicities of the PS_{O10H6} particle. DCs transfected with naked plasmid IL-10 and O10H6-pIL-10 showed minor reduction of T cell proliferation (90±6% and 81±7% of control, respectively), and the proliferation of T cells stimulated with O10H6-pIL-10 transfected DCs is slightly lower than those treated with plasmid IL-10 transfected DCs. However no statistically significent difference from the control group was observed in either transfection mode (*p* >0.05). PS_{O10H6}-pIL-10 treated DCs showed lowest bulk T cell proliferation among all the delivery modes and is the only group that was significantly different from control DCs. These results support the notion that PS_{O10H6} particles are more efficient at gene delivery into DCs compared to free plasmid and other vectors such as O10H6-DNA complexes. The delivery of IL-10 genes into DCs by PS_{O10H6} elicits the weakest bulk T cell responses between all the delivery modes. These data suggeste that O10H6 modified polymeric particles were effective in transferring pIL-10 into DCs to induce suppression of immune responses, particularly in allogeneic T cells.

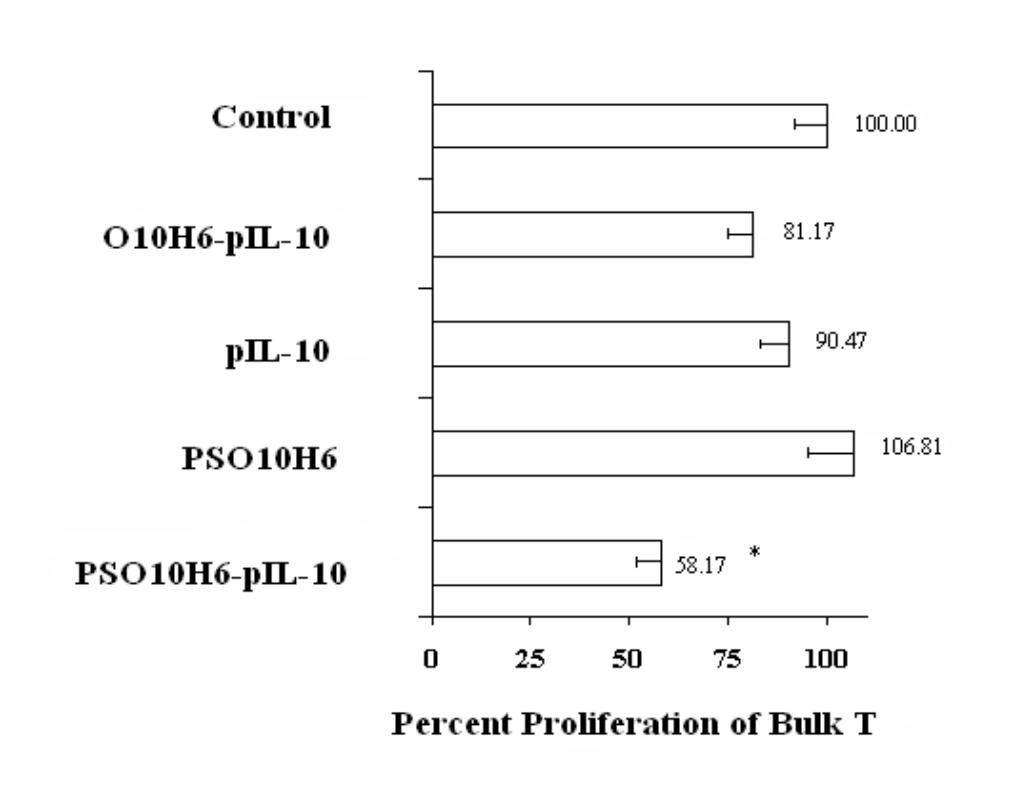


Figure 5.4: Proliferation of bulk T cells in response of allogeneic DCs. DCs transfected with PS_{O10H6}-pIL-10 elicit weak allogeneic T cell response. Data were averaged from four independent replicate experiments (n = 20-30) and error bars indicate standard error of mean. One-way analysis of variance data in groups was significantly different (F = 0:0016). Pairwise comparison was performed using Tukey–Kramer method (* indicates $p<0.05$).

Percentage Proliferation of Bulk T cells		
	Mean	**SEM**
Control	100	8.05
O10H6-pIL-10	81.17	6.27
pIL-10	90.47	7.41
PSO10H6	106.81	11.49
PSO10H6-pIL-10	58.17	6.34

Table 5.1: Data summary of proliferation of bulk T cells in response of allogeneic DCs.

DCs modified with PS_{O10H6}-pIL-10 elicit weak proliferation in CD4 and CD8 T cell subsets

We have demonstrated that PS_{O10H6}-pIL-10 modified DCs can suppress allogeneic bulk T cell proliferation. Effector T cells such as CD8+ and CD4+ T cells can be directly stimulated by allogeneic DCs driven by the interaction between MHC class II on DCs and mismatched TCR on T cells. Upon activation, CD4+ T cells will release pro-inflammatory cytokine INF-γ to trigger CD8+ T cell expansion. To further evaluate the effect of IL-10 gene-modified DCs on these effector T cells, proliferation of CD4+ and CD8+ T cell subsets in the mixed lymphocyte culture was analyzed using flow cytometry with carboxyfluorescein succinimid ester (CFSE) staining. CFSE, an intracellular fluorescent dye, is transported into T cell cytoplasm during incubation and binds covalently to cytoplasm proteins to become membrane impermeable without adversely affecting cellular function. Analysis of cell division can be determined by its intensity when measured by flow cytometry. With each round of cell division, the fluorescent intensity per cell division is reduced by 50%, thus providing a measure of the mitotic activity within a specific population of cells.

In this experiment, allogeneic T cells were mixed with CD11+ DCs that were transfected with an equal quantity of pIL-10 using different delivery modes including PS_{O10H6}pIL-10 particles, O10H6-pIL-10 complexes and free pIL-10. Untreated DCs served as the control group. Results of CD8+ T cell proliferation in mixed lymphocyte culture are shown in Figure 5.5 and Table 5.2. The CD8+ T cell (PE-Cy5 positive) population was selected from the upper-left region of the PE-Cy5 vs PE dot-plot for

analysis (Figure 5.5A). The distribution of CFSE intensity in CD8+ T cells was displayed with a histogram and the population of proliferated CD8+ T cells ($CFSE^{low}$) was determined based on the distribution of unstimulated CFSE-stained T cells in the histogram (Figure 5.5A).

Results from three independent experiments (Figure 5.5, Table 5.2) indicated that DCs transfected with PS_{O10H6}-pIL-10 particles induced significant weak proliferation of allogeneic CD8+ T cells compared to untreated control DCs (percentage of proliferated CD8+ T cells: 18.00±1.20% and 44.17±5.17%, respectively; $p < 0.05$ in Tukey–Kramer pairwise comparison). In a representative experiment (Figure 5.5A), BALB/c ($H\text{-}2^d$) CD8+ T cells proliferation decreased from 41% in control (cells stimulated with untreated DCs) to 15% in PS_{O10H6}-pIL-10 modified DCs. DCs exposed to O10H6-pIL-10 and pIL-10 alone exhibited slightly lower CD8+ T cell proliferation (26% and 35%, respectively) in the representative experiment compared to control DCs. However, data combined from three independent experiments indicated no significant difference of CD8+ T cell proliferation from the control group in both transfection modes (Figure 5.5, Table 5.2, $p<0.05$). PS_{O10H6}-pIL-10 treated DCs showed lowest CD8+ T cell proliferation among all the delivery modes and is the only group that was significantly different from control DCs.

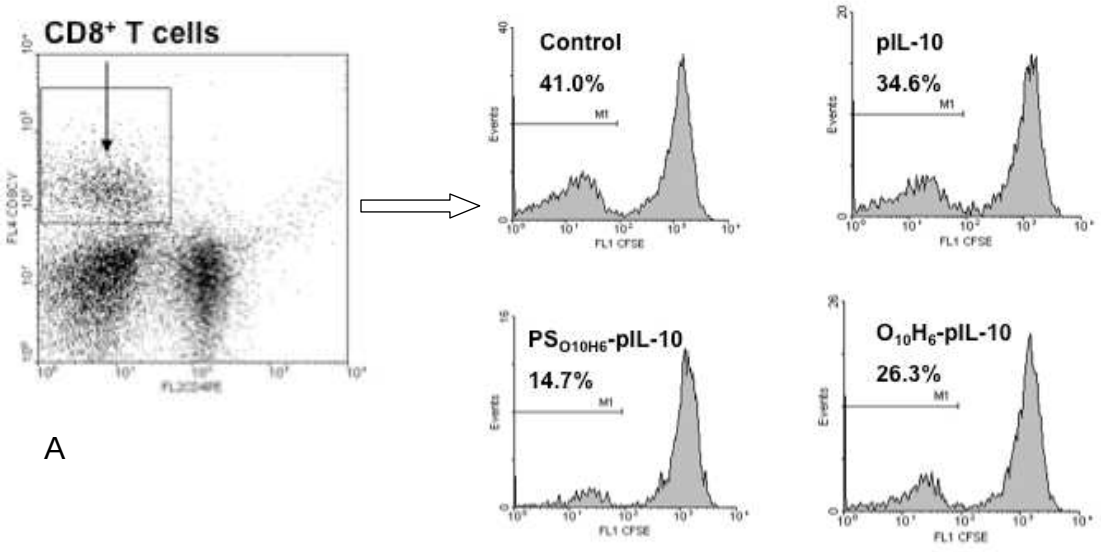
Results of CD4+ T cell proliferation in mixed lymphocyte culture are shown in Figure 5.6 and Table 5.3. The CD4+ T cell (PE positive) population was selected from the upper-right region of the PE-Cy5 vs. PE dot-plot for analysis (Figure 5.6A).

Distribution of CFSE intensity in CD4+ T cells was plotted in a histogram and the population of proliferated CD4+ T cells ($CFSE^{low}$) was determined based on the distribution of unstimulated CFSE-stained T cells in the histogram (Figure 5.6B). Similarly, data from three independent experiments (Figure 5.6, Table 5.3) indicated that DCs transfected with PS_{O10H6}-pIL-10 particles induced a significant weak proliferation of allogeneic CD4+ T cells compared to untreated control DCs (percentage of proliferated CD4+ T cells: 11.83±5.08% and 22.56±4.05%, respectively; $p < 0.05$ in Tukey–Kramer pairwise comparison). In a representative experiment (Figure 6.6), BALB/c ($H\text{-}2^d$) CD4+ T cell proliferation was almost eliminated falling from 13.8% in controls (cells stimulated with untreated DC) to 3.8% in PS_{O10H6}-pIL-10 transfected DCs. DCs exposed to pIL-10 alone and O10H6-pIL-10 exhibited almost the same or slightly lower CD4+ T cell proliferation (13.0% and 6.5%, respectively) in the representative experiment compared to control DCs. However, combined data from three independent experiments showed no significant difference of CD4+ T cell proliferation from the control group in both transfection modes (Figure 5.6, Table 5.3, $p>0.05$). Likewise, PS_{O10H6}-pIL-10 treated DCs showed the lowest CD4+ T cell proliferation among all the delivery modes and is the only group that was significantly different from control DCs.

Again, these results support the notion that PS_{O10H6}-pIL-10 particles are effective vehicles in delivering pIL-10 into DCs. The expression of IL-10 in PS_{O10H6}-pIL-10 modified DCs induced the suppression of allogeneic effector T cells such as CD4+ and CD8+ T cells. These T cell subsets play important roles in triggering T cell activation and

cell-mediated immune response. Thus, PS_{O10H6}-pIL-10 modified DCs are effective in suppress T cell responses.

We have shown that cationic peptide coated polymeric particles are more efficient in gene transfer to BMDCs compared to peptide-DNA complexes (Chamarthy, Jia et al., 2004). The enhanced expression of IL-10 in PS_{O10H6}-pIL-10 transfected DCs contributes to the higher T cell suppression over DCs transfected with O10H6-pIL-10. The submicron polymeric particles are preferentially taken up by DCs, and the backbone of polymeric materials provide an anchorage to further stabilize O10H6-DNA complexes on the particle surface. These factors may contribute to the enhanced IL-10 expression and T cell suppression in polycation O10H6 modified polymeric particles compared to other non-particulate vectors.



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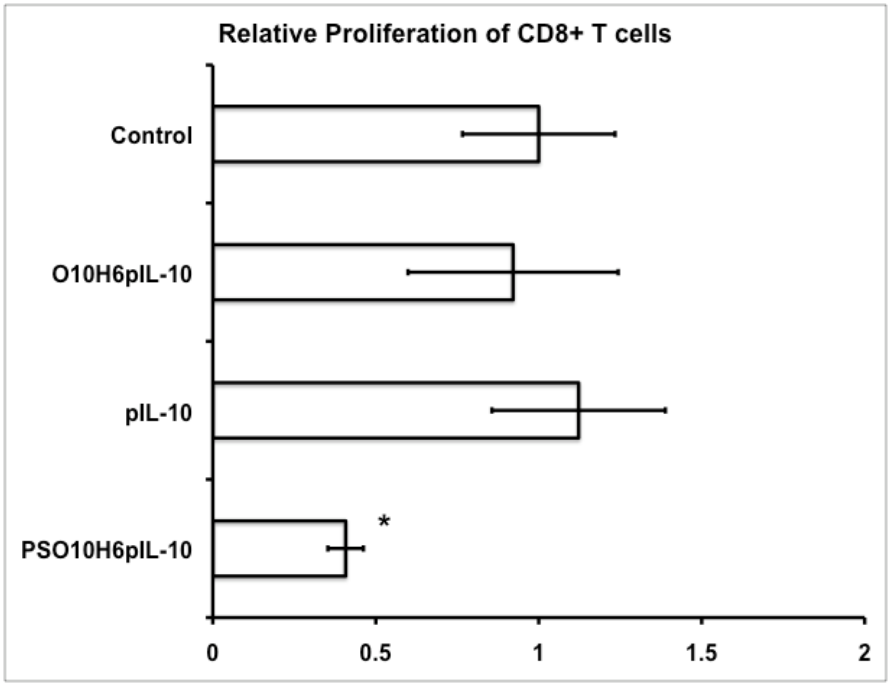
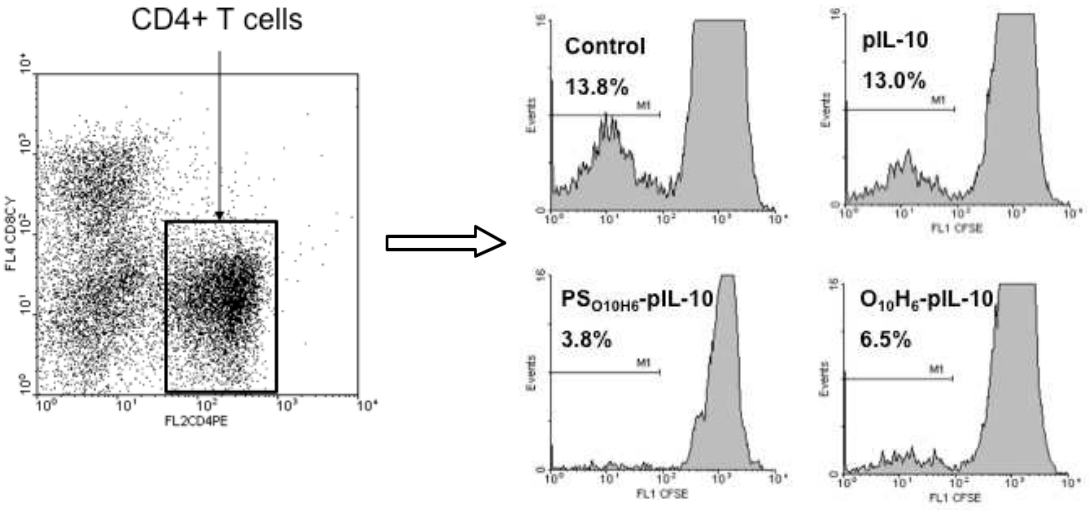


B

Figure 5.5: Response of CD8+ T cell subset to DC. T cells were first stained with CSFE prior to mixing with CD11c-enriched DC. Cells were stained with anti-CD8 and analyzed using flow cytometry. Data shown represent typical proliferation profiles of T cells gated on CD8 positive region (A). Bar graphs depict collective analysis of three independent experiments (B). *$p<0.05$ compared to control, pIL-10, and O10H6-pIL-10 DC.

Experimental Groups	Percentage of proliferated CD8+ T cells		Relative proliferation of CD8+ cells	
	Mean	SEM	Mean	SEM
Control	44.17	5.17	1	0.12
O10H6-pIL-10	40.69	7.12	0.92	0.16
pIL-10	49.57	5.88	1.12	0.13
PSO10H6-pIL-10	18.00*	1.20	0.41*	0.03

Table 5.2: Data summary of the response of the CD8+ T cell subset in response of allogeneic DCs. *p<0.05 compared to control, pIL-10, and O10H6-pIL-10 DCs.



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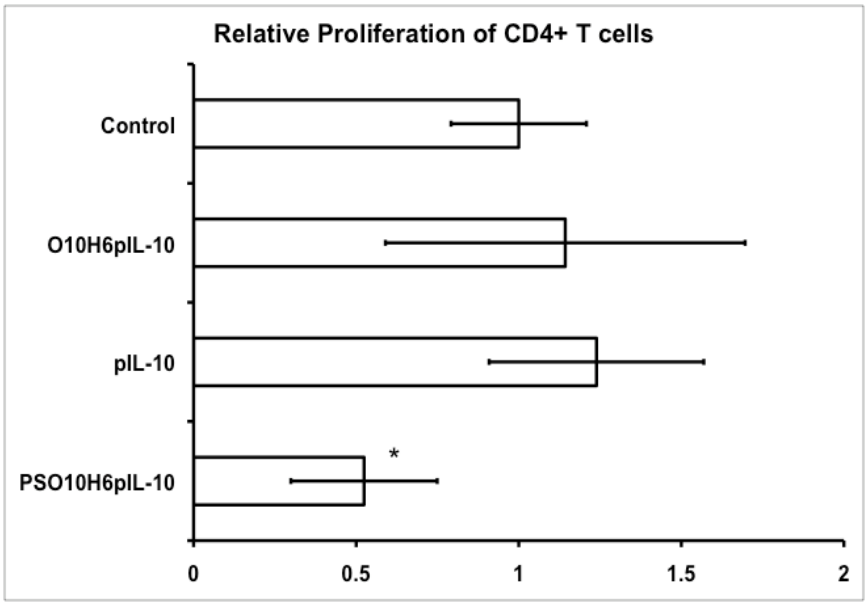


B

Figure 5.6: Response of CD4+ T cell subset to DC. T cells were first stained with CSFE prior to mixing with CD11c-enriched DC. Cells were stained with anti-CD4 and analyzed using flow cytometry. Data shown represent typical proliferation profiles of T cells gated on CD8 positive region (A). Bar graphs depict collective analysis of three independent experiments (B). *p<0.05 compared to control, pIL-10, and O10H6-pIL-10 DC.

Experimental Groups	Percentage of proliferated CD4+ T cells		Relative proliferation of CD4+ cells	
	Mean	**SEM**	**Mean**	**SEM**
Control	22.56	4.50	1	0.21
O10H6-pIL-10	25.79	12.48	1.14	0.55
pIL-10	27.96	7.45	1.24	0.33
PSO10H6-pIL-10	11.83*	5.08	0.52*	0.03

Table 5.3: Data summary of the response of the CD4+ T cell subset in response of allogeneic DCs. *$p < 0.05$ compared to control, pIL-10, and O10H6-pIL-10 DC.

Ex vivo IL-10 gene-modified DCs suppress host cell infiltration

We have shown that PS_{O10H6}-pIL-10 transfected DCs suppressed responses in both bulk allogeneic T cells and CD4+, CD8+ effector T cells *in vitro*. It is important to know whether a correspondent suppression of T cell responses could be observed *in vivo*. To determine if PS_{O10H6}-pIL-10 modified DCs could alter the immune response *in vivo*, we employed an *ex vivo* DC transfection model that utilizes Matrigel (BD Bioscience, Bedford, MA) as a scaffold to establish a surrogate ‘‘graft’’ in allogeneic mice. Matrigel, which solidifies at a temperature above 10 ˚C has been used extensively as a matrix scaffold for implanting tissues *in vivo* (Shih and Towle 1995; Edamura, Ohgawara et al. 2001; Zimmermann, Didie et al. 2002; Zimmermann, Melnychenko et al. 2004; Bharat, Benshoff et al. 2005). Matrigel consists primarily of laminin, collagen IV, and growth factors (Kleinman, McGarvey et al. 1982) that can support cell attachment, infiltration and differentiation in various lineages. In this dendritic cell transplant study, DCs from BALB/c ($H\text{-}2^d$) mice were transfected *ex vivo* with PS_{O10H6}-pIL-10 or $PLGA_{O10H6}$-pIL-10 particles and then mixed with Matrigel and injected (subcutaneous) into C57BL/6 ($H\text{-}2^b$) mice to form a surrogate graft. Seven days after implantation, Matrigel plugs were retrieved and host cell infiltration was analyzed using either flow cytometry or microscope imaging analysis.

Figure 5.7 is representative of four independent flow cytometry experiments from Matrigel plugs embedded with PS_{O10H6}-pIL-10 transfected DCs and untransfected control DCs. Data from the dot-plots (“FF”-forward scan and “SS”-side scan) of recovered cells coming from Matrigel samples embedded with control DCs (Figure 5.7a, b) indicated that

host cells were infiltrated into the plug 7 days after implantation. Approximately 9/10 less cells (Figure 5.7c) were detected using flow cytometry in Matrigel samples seeded with PS_{O10H6}-pIL-10 modified DCs at day 7 compared to samples seeded with control DCs (Figure 5.7b). These data showed that PS_{O10H6}-pIL-10 modified DCs reduced allogeneic host cell infiltration into graft tissue *in vivo* compared to untransfected control DCs.

The same experiment was performed using Matrigel embedded with $PLGA_{O10H6}$-pIL-10 modified DCs as the implant plug. Host cell infiltration was evaluated under a confocal microscope in phase contrast mode. Figures 5.8 are the microscope images taken from the central area of Matrigel implant plugs seeded with untransfected control DCs (Figure 5.8a), and $PLGA_{O10H6}$-pIL-10 modified DCs (Figure 5.8b) under the same magnifying power. Numbers of infiltrated host cells were quantified using ImageJ software. Consistent with the results from PS_{O10H6}-pIL-10 modified DCs, the $PLGA_{O10H6}$-pIL-10 transfected DCs hampered allogeneic host cell infiltration into graft Matrigel *in vivo*: approximately 53% less cells infiltrated in to Matrigel seeded with $PLGA_{O10H6}$-pIL-10 modified DCs compared to Matrigel seeded with control DCs 7 days after implantation (Figure 5.8).

Taken together, data presented in this section demonstrate that both PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 modified DCs can reduce allogeneic host cell infiltrations in an *ex vivo* Matrigel implant model. The results indicated that a suppress phenotype exists in these IL-10 gene-modified DCs with increasing IL-10 expression, which may play a critical role in hampering allogeneic host cell infiltration in the local microenvironment.

This was in consistence with the data from the *in vitro* mixed lymphocyte model, in which suppressive functionality of IL-10 gene- modified DCs was observed.

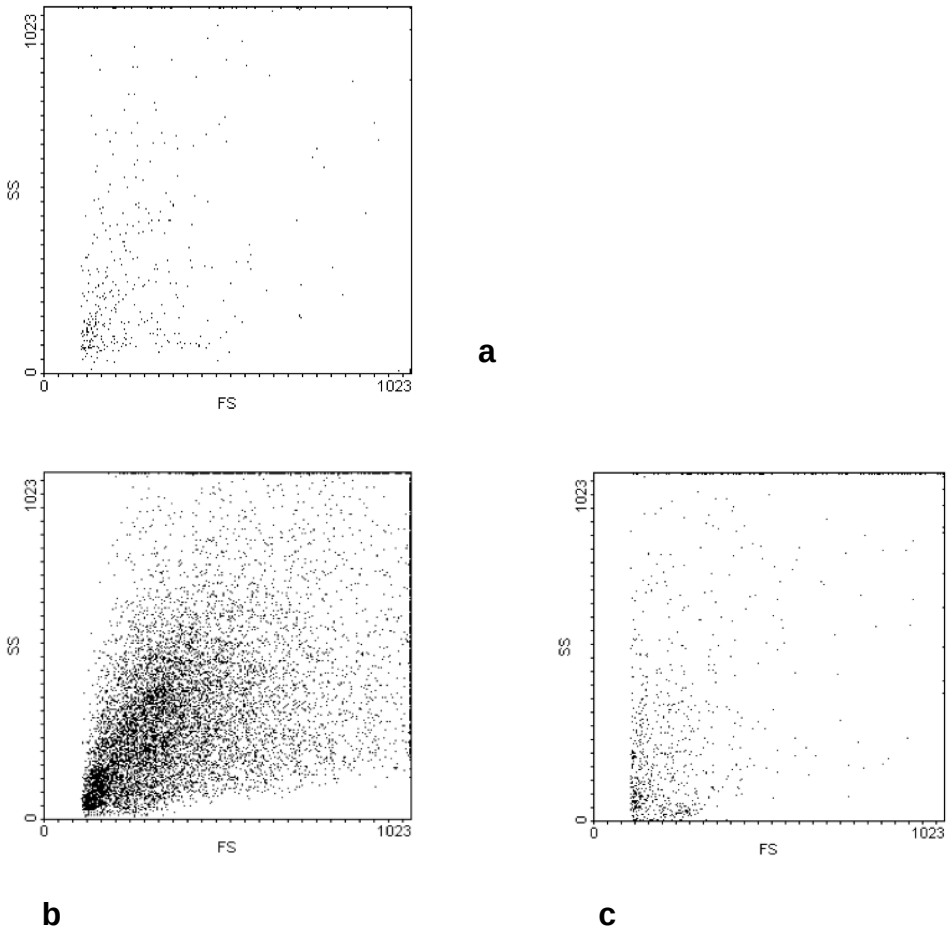
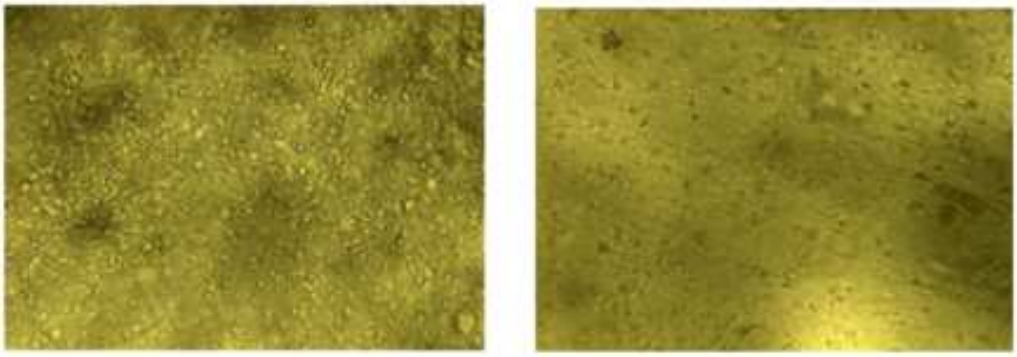


Figure 5.7: Flow analysis of host cell infiltration into Matrigel. Flow plots from Matrigels embedded with untransfected DCs recovered from host 1 day (a) and 7 days (b) after injection; Matrigel embedded with PS_{O10H6} -pIL-10 DCs 7 days after injection. JB6, an epithelial cell line, and bone marrow-derived DC, both generated from BALB/c mice (H-2^d) were implant into C57BL/6 mice to elicit host cell infiltration. JB6 and DC, untreated (a, b) or transfected with PS_{O10H6} -pIL-10 (c), were mixed with Matrigel on ice and injected into flanks (subcutaneous space) of C57BL/6 (H-2^b) host. Matrigel, which solidifies at temperature above 10 ºC, forms plugs once injected in the recipient mice. Plugs were retrieved from mice 7 days after implantation and associated cells were recovered in BD recovery solution on ice with gentle shaking for 2 h. Released cells were then washed and analyzed using flow cytometry. Result is a representative of four independent experiments. FS: forward scan; SS: side scan.



(a) Host cell infiltration in Matrigel with control DCs

(b) Host cell infiltration in Matrigel with $PLGA_{O10H6}$-pIL-10 transfected DCs

Figure 5.8: Confocal images of host cell infiltration into Matrigel embedded with $PLGA_{O10H6}$-pIL-10 transfected and control DCs. Bone marrow-derived DC generated from BALB/c mice ($H\text{-}2^d$) were implant into C57BL/6 mice to elicit host cell infiltration. DC, untreated (a) or transfected with $PLGA_{O10H6}$ -pIL-10 (b), were mixed with Matrigel on ice and injected into flanks (subcutaneous space) of C57BL/6 ($H\text{-}2^b$) host. Matrigel, which solidifies at temperature above 10 ºC, forms plugs once injected in the recipient mice. Plugs were retrieved from mice 7 days after implantation and gel image were taken under confocal microscope in phase contrast mode. Numbers of host cell infiltration were analyzed using ImageJ software (a: 549; b: 259).

Discussion

In the research presented in this chapter, we evaluated the immune suppressive effects of DCs modified with pIL-10 loaded on O10H6 coated polymeric particles (PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10). IL-10 is a pleiotropic cytokine that has been demonstrated to induce adaptive immune tolerance. DCs were chosen as a target for IL-10 delivery because of their key role in activating T cell responses. Data from previous chapters indicated that both PS_{O10H6} and $PLGA_{O10H6}$ particles are efficient carriers of plasmid DNA, capable of increasing IL-10 gene expression in DCs about two-fold over free IL-10 plasmid. Herein we evaluated the ability of PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 modified DCs to suppress T cell responses. An *in vitro* mixed lymphocyte model that uses DCs (C57BL/6) to stimulate T cells from a different strain (BALB/c) was chosen to evaluate the suppressive function of IL-10 gene-modified DCs. This is because the mismatch between MHC class II on DCs ($H\text{-}2^b$) and allogeneic T cell ($H\text{-}2^d$) triggers T cell proliferation and DCs contributes to a large part of this kind of response (Steinman 1991). Proliferation of bulk T cells as well as CD8+ and CD4+ subsets was measured as the quantitative indicator of T cell activation. The results presented in this chapter supported the hypothesis that PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 modified DCs can attenuate allogeneic T lymphocyte response *in vitro* and in *vivo*, indicating that IL-10 gene modification can modulate DCs into suppressive phenotype. These data also suggested that PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 are more effective in modulating DC functions compared to other non-particulate vectors, indicating that polycation O10H6 modified polymeric particles are effective tools for genetically modifying DCs with therapeutic genes.

In addition, we employed a cell transplant model that utilizes Matrigel seeded with *ex vivo* pIL-10 modified DCs from BALB/c (H-2^d) mice as a scaffold to establish surrogate ''graft'' in C57BL/6 (H-2^b) mice. The immune response from the recipient was evaluated by analyzing host cell infiltration into the graft plug 7 days after implantation. Expectedly, Our result confirmed that both PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 modified DCs attenuated allogeneic host cell infiltration into graft plugs *in vivo*, which is in agreement with suppression of T cell proliferation when they are exposed to PS_{O10H6}-pIL-10 modified DCs *in vitro*. The effect may result from the following mechanisms: First, donor DCs without IL-10 modification will trigger host allogeneic T cell activation and secretion of chemokines and pro-inflammatory cytokines including GM-CSF, IL-2, IFN-γ, and TNF-α for the recruitment and activation of other effectors (e.g macrophages, NK cells, and CD8+ T cells), leading to host cell infiltration. However, PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 modified DC present suppressive phenotype *in vivo* and may suppress allogeneic T-cell recognition and response to deter infiltration of host T cells in the local microenvironment (Thomson 2002). Second, IL-10 gene-modified DCs may induce and expand regulatory T cells (Wakkach, Fournier et al. 2003) *in vivo* to suppress T cell activation and trafficking into the plug. Third, IL-10 gene-modified DCs may traffic to draining lymph nodes to induce the expansion of specific Treg cells. Additionally, IL-10 itself has also been shown to prevent APC accumulation (Qin, Noffz et al. 1997), thus expression of IL-10 in the local microenvironment may limit host DCs and macrophages to elicit indirect recognition mechanisms.

The use of polycation O10H6 modified polymeric particles (e.g. PS_{O10H6} and $PLGA_{O10H6}$) for intracellular delivery of oligonucleotide and plasmid DNA has been broadly explored in our lab (Kovacs, Zheng et al. 2005; Jia, Kovacs et al. 2006; Zheng, Kovacs et al. 2006; Jia, Kovacs et al. 2008). Potentially, these particulates could be administered *in vivo* to deliver DNA therapeutics (e.g. NF-κB decoy oligonucleotide, plasmid IL-10) to DCs to modulate immune functions with the following advantages: First, particles can be taken up preferentially by DCs, thereby targeted distribution of delivered oligonucleotide and DNA molecules in those cells can be increased; Second, because of their particulate nature and the submicron size, those polymeric vectors are likely to gain access to draining lymph nodes, where DCs prime Ag-specific T cells. It has been reported that particles from 20 nm to 400 nm in diameter are accumulated in draining lymph nodes upon injection (Manolova, Flace et al. 2008), suggesting that those polymeric particles can directly act on DCs located in these tissues and alter their interaction with effector T cells.

Alternatively, these polymeric particles can be used for *ex vivo* gene transfer to DCs. Specifically, *ex vivo* delivery of plasmid IL-10 loaded on O10H6 modified polymeric particles to donor DCs could induce their suppressive functionality, thereby reducing the risk of graft rejection from host immune system. It was reported that e*x vivo* transfection of suppressive gene to donor tissue could decrease the risk of transplant rejection in a variety of tissue grafts including lung and islet (Yano, Mora et al. 1999; Akamaru, Ito et al. 2003). The triggering of transplant rejection involves the recognition of MHC-derived antigens on the donor DCs by host T cells designated as direct pathway,

and the recognition of donor MHC products by host antigen presenting cells designated as indirect pathway. The former plays a role in acute rejection and the later contribute to chronic rejection. Thus DCs in donor tissues could be an effective target for gene therapy to suppress T cell response in acute transplant rejection. IL-10 has been demonstrated to affect chronic allograft changes and systemically administrated IL-10 was studied as a treatment for prolonging transplant survivals in different animal models (Hong, Mullen et al. 2003; Zhang, Pileggi et al. 2003; Martins, de Perrot et al. 2004; Carter, Ellett et al. 2005; Chen, Kapturczak et al. 2007). Therefore, *ex vivo* targeting delivery of IL-10 gene to donor DCs could reduce recipient T cell response to donor organs, and subsequently reduce the risk of transplant rejection.

In conclusion, the abilities of PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 modified DCs to attenuate immune response *in vitro* and *in vivo* are confirmed in the data presented in this chapter. A Matrigel system embedded with *ex vivo* transfected DCs provides an opportune setting for studying alloreactivity of *ex vivo*-modified DC *in vivo*. An advantage is the number of donor cells in the implant can be controlled, leading to reproducible inflammation. These polymeric particulates could be further investigated in their ability to suppress excessive immune reactions in autoimmune diseases, inflammation, and transplant rejections.

CHAPTER 6

IMMUNOSUPPRESSIVE MECHANISMS OF DENDRITIC CELLS MODIFIED WITH PS_{O10H6}-pIL-10 AND $PLGA_{O10H6}$-pIL-10 PARTICLES

Introduction

Dendritic cells are being considered as a therapeutic means of attenuating damaging immune responses in autoimmune diseases and transplant rejections. Efforts have been taken to genetically render DCs into tolerogenic phenotypes with IL-10 gene to suppress lymphocyte activation and proliferation in locales where these cells are programmed (Kubin, Kamoun et al. 1994; Willems, Marchant et al. 1994; Tong, Toshiaki et al. 2005). It was also demonstrated in our lab that DCs modified with synthetic vectors, PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 suppressed allogeneic T cell response *in vitro* and host cell infiltration in a MHC-mismatched graft model *in vivo*.

The ability of DCs to induce adaptive immune tolerance have been extensively investigated in recent years (Lu and Thomson 2002; Slavin, Tarner et al. 2002; Kubach, Becker et al. 2005) and the following mechanisms have been proposed for the suppressive effect of IL-10 modified DCs. First, it was demonstrated in earlier studies (Kubin, Kamoun et al. 1994; Willems, Marchant et al. 1994; Tong, Toshiaki et al. 2005) that IL-10 suppresses antigen presenting functions of DCs by down-regulating their surface expression of B7 co-stimulatory molecules (e.g. CD80 and CD86). However,

other studies suggested that depending on the model system employed, expression of CD80 and CD86 could also be unchanged or up-regulated in IL-10 modified DCs (Enk, Angeloni et al. 1993; Mitra, Judge et al. 1995; Morel, Quaratino et al. 1997; Sharma, Stolina et al. 1999; Faulkner, Buchan et al. 2000). Therefore, other mechanisms exist for modulating the suppressive functionality of DCs. Second, IL-10 modified DCs could regulate the secretion of Th1 cytokines (e.g. IL-12, IFN-γ, and TNF-α) from DCs and T cells (Fortsch, Rollinghoff et al. 2000). These cytokines stimulate or synergize the activation and proliferation of immune effector T cells. Third, immunosuppression by IL-10 modified DCs often develops concomitantly with an expansion of CD4+ regulatory T cells (Takayama, Nishioka et al. 1998; Zeller, Panoskaltsis-Mortari et al. 1999; Mahnke and Enk 2005), even at their fully matured state ($B7^{high}$). These Treg cells are marked by constitutive expression of CD25 on cellular membrane and intracellular Foxp3, the master transcriptional regulator constitutively and exclusively expressed in these cells (Hanck, Glatzel et al. 2000). Functionally, Treg cells are characterized by the capacity to restrain proliferation and cytotoxic activity of effector T cells. Because of the potential of Treg cells to curtail damaging immune responses toward auto- and alloantigens, the ability to induce or expand Foxp3-expressing Treg cells has emerged as a key measure of the tolerogenic propensity of DCs (Lu, Lee et al. 1999; Thomson 2002).

In the work presented in this chapter, we examined the functional utility of PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 modified DCs ($H\text{-}2^b$) that are immunosuppressive in cultures mixed with lymphocyte from MHC-mismatched mice ($H\text{-}2^d$). The hypothesis is the suppressive functionality of these IL-10 gene-modified DCs is related to the

expansion of allogeneic regulatory T cell populations. Other mechanisms including alteration of co-stimulatory molecules and Th1 cytokine secretion from DCs may also contribute to the suppressive phenotypes of these cells.

Methods

Mixed leukocytes cultures

In order to explore the immunosuppressive mechanism of IL-10 modified DCs, a mixed leukocyte culture between MHC miss matched mice was performed as described in the previous chapter. In brief, CD11c+ DCs from C57BL/6 (H-2^b) mice were transfected with PS_{O10H6}-pIL-10 or $PLGA_{O10H6}$-pIL-10 particles at day 6 of DC culture. After two days, control or pIL-10 transfected DCs were mixed with splenocytes of BALB/c mice (H-2^d) after passing through nylon mesh (70 mm pores) at a stimulator to responder ratio of 5:1. Expansion of regulatory T cells, secretion of cytokines and chemokines in DC-T cell co-cultures were quantified using Foxp3 RT-PCR and ELISA/cytokine microarray analysis, respectively.

Flow cytometry analysis of CD80 and CD86 expression on PS_{O10H6}-pIL-10 or $PLGA_{O10H6}$-pIL-10 transfected DCs

DCs from C57BL/6 (H-2^b) mice were transfected with PS_{O10H6}-pIL-10 or $PLGA_{O10H6}$-pIL-10 particles at day 6 of DC culture using the method described in Chapter 5. Two days after transfection, DCs were harvested and stained with PE-conjugated anti-CD80 or PE-conjugated anti-CD86 antibody (BD Pharmingen) on ice and analyzed by a Beckman Coulter EPICS XL flow cytometer gated on live cells. Student's t-test was used to evaluate statistical significance.

Foxp3 RT-PCR analysis

Two days after mixing IL-10 modified DCs (H-2^b) with splenocytes (H-2^d), cells were harvested and stained with anti-CD4 magnetic beads (BD BioScience, San Jose, California) in PBS. The cell suspension was then washed with separation buffer (PBS, 2 mM EDTA, 0.5% bovine serum albumin) and CD4+ T cells were selected using an IMag^{TM} cell separation system (BD Bioscience, San Jose, CA). Total RNA from the CD4+ T cells was then isolated by SV total RNA isolation kit (Promega, Madison, WI). 0.5 mg of this RNA was amplified using One-Step Eppendorf Master RT plus PCR kit (Eppendorf, Hamburg, Germany) in an Eppendorf MasterCycler. Primer pairs specific for murine Foxp3 (SuperArray Bioscience, Frederick, MD) and β-actin (R&D Systems, Minneapolis, MN) were added to the RNA sample in reverse transcription (50 °C for 50 min) step, followed by denaturation (94 °C for 4 min). The amplification steps consisted of 35 cycles of template denaturation (94 °C for 45 s), primer annealing (55 °C for 45 s) and primer elongation (68 °C for 45 s). PCR products were analyzed in 2% agarose gel and DNA visualized by ethidium bromide staining.

Cytokines array and ELISA

The amount of INF-γ and TGF-β produced in DC-T cell co-cultures were analyzed by DuoSet Enzyme-Linked ImmunoSorbent Assay (ELISA) kits (R&D Systems, Minneapolis, MN) and Emax ImmunoAssay System (Promega, Madison, WI) in a 96 well plate according to manufacturers' protocols, respectively. Expression of other cytokines and chemokines (IL-10, G-CSF, M-CSF, IL-1α, IL-2, IL-3, IL-4, IL-5,

IL-6, IL-12, IL-13, INF-γ, TNF-α, GM-CSF, VEGF, MIG, Rantes and MCP-1α) were analyzed using TranSignal™ mouse cytokine antibody arrays (Panomics, Redwood City, CA). Cell culture supernatant was cleared by centrifugation and stored at -80°C until analysis. Samples were analyzed following manufacture's protocol. ELISA assay samples were read using a microplate reader at 450 nm in a 96-well plate. Data were fitted into a standard curve and concentrations of INF-γ and TGF-β for the samples were then calculated based on the standard curve. In cytokine array analysis, chemiluminescence signals from each spot on the membrane were captured using a Kodak image station and relative signal intensities were quantified using ImageJ software.

Results

PS_{O10H6}-pIL-10 or $PLGA_{O10H6}$-pIL-10 modified DCs induce allogeneic regulatory T cell expansion

To evaluate the effect of IL-10 modified DCs on allogeneic regulatory T cell expansion, BMDCs from C57BL/6 mice (H-2^b), transfected with PS_{O10H6}-pIL-10 or $PLGA_{O10H6}$-pIL-10 were mixed with allogeneic splenocytes containing T cells and NK cells from BALB/c mice (H-2^d). Foxp3 was amplified using RT-PCR method to characterize Treg cells population in the DC-T cell co-culture. β-actin was also amplified as a control gene for semi-quantitative analysis purpose. Untransfected BMDCs were used as a control mode in the mixed lymphocyte culture to evaluate the effect of IL-10 gene-modification of DCs on Treg cell expansion in the co-culture.

Figure 6.1 and Table 6.1-2 are the results of Foxp3 gene expression in allogeneic T cells exposed to PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 modified DCs (Figure 6.1, Table 6.1-2). Bands shown in those figures were consistent with predicted PCR products of Foxp3 (221 bp) and β-actin (302 bp), respectively. The intensity of the band reflected the amount of mRNA expressed. The results demonstrated that Foxp3 expression in allogeneic splenocytes cultured with PS_{O10H6}-pIL-10 modified DCs was up-regulated compared to T cells exposed to control untransfected DCs (Figure 6.1a, Table 6.1). Gene expression was semi-quantified by normalizing the intensity of Foxp3 band to the corresponding β-actin band (Foxp3/ β-actin) and data obtained from two independent

experiments showed a more than one fold increase of Foxp3 expression in allogeneic T cells exposed to PS_{O10H6}-pIL-10 modified DCs.

Similarly, allogeneic T cells exposed to $PLGA_{O10H6}$-pIL-10 modified DCs expressed higher levels of Foxp3 compared to these cells cultured with control untransfected DCs as shown in the gel electrophoresis analysis of the intensity of final RT-PCR products (Figure 6.1b, Table 6.2). Semi-quantitative analysis of Foxp3 gene expression from two independent experiments indicated Foxp3 was up-regulated at least one fold in allogeneic splenocytes exposed to $PLGA_{O10H6}$-pIL-10 DCs compared to the one mixed with control DCs.

Regulatory T cells are capable of suppressing proliferation and cytotoxic activity of effector T cells (CD4 and CD8) through contact dependent or independent manner. These results demonstrated that Foxp3, the master transcriptional gene constitutively and exclusively expressed in mouse Treg cells, were up-regulated in T cells stimulated with PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 modified DCs, indicating that these IL-10 gene-modified DCs can expand allogeneic regulatory T cell population *in vitro*. This mechanism contributes to the immunosuppressive phenotypes of DCs modified with pIL-10 loaded O10H6 modified polymeric particles.

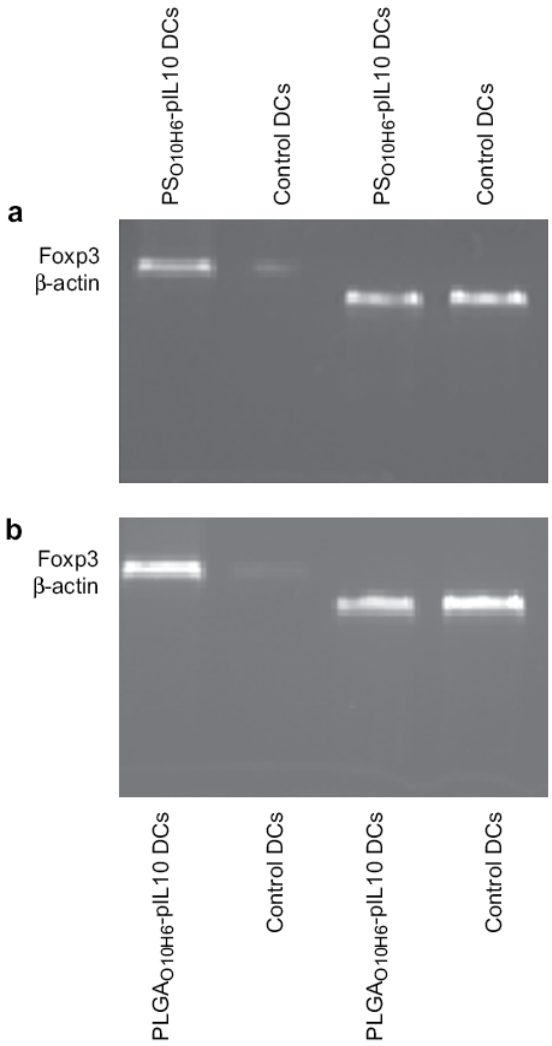


Figure 6.1: Foxp3 mRNA expression in allogeneic T cells stimulated with DCs transfected with PS_{O10H6}-pIL-10 (a) and $PLGA_{O10H6}$-pIL-10 (b). Untransfected DCs were used as the control. Bands identified were consistent with predicted PCR products of Foxp3 (left two lanes) and β-actin at 221 and 302 bp, respectively.

	Relative Foxp3 expression	
	Mean	SEM
PS_{O10H6}-pIL-10 transfected DCs	0.82	0.08
Control DCs	0.46	0.06

Table 6.1: Summary of Foxp3 expression in T cells conditioned by PS_{O10H6}-pIL-10 transfected and control DCs. Data were obtained from two independent experiments

	Relative Foxp3 expression	
	Mean	SEM
$PLGA_{O10H6}$-pIL-10 transfected DCs	1.03	0.09
Control DCs	0.48	0.01

Table 6.2: Summary of Foxp3 expression in T cells conditioned by $PLGA_{O10H6}$-pIL-10 transfected and control DCs. Data were obtained from two independent experiments

Expression of B7 molecules in IL-10 gene-modified DCs

Co-stimulatory molecules (B7, e.g. CD80, and CD86) which are up-regulated on mature DCs trigger the secondary signal between DCs and T cells, which is critical for Ag-specific or allogeneic T cell activation (Zhou and Tedder, 1995; Banchereau and Steinman, 1998). To evaluate the expression of B7 molecules, DCs were stained with PE-conjugated anti-CD80 or PE-conjugated anti-CD86 antibody (BD Pharmingen) two days after IL-10 transfections with PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particles. The frequency of CD80 and CD86 on these cells was analyzed using a Beckman Coulter EPICS XL flow cytometer.

Our data indicated no changes in CD80 and CD86 expression when DCs were transfected with PS_{O10H6}-pIL-10 (Figure 6.2a-b, solid line) compared to untransfected control DCs (Figure 6.2a-b, dashed line) in a representative experiment. Comparison of positive fractions from three independent experiments (Figure 6.2c, Table 6.3) using student's t-test (two-tailed, equal variance) showed no significant difference in PS_{O10H6}-pIL-10 transfected DCs versus control DCs (CD80, p=0.5; CD86, p=0.5). In DC transfected with PS_{O10H6}-pIL-10, 71.6% (±1.4) were stained positive for CD80 and 73.8% (±2.8) for CD86, while approximately the same fractions of control DCs expressed these molecules (CD80 = 70.2 ± 1.5%, CD86 = 72.2 ± 2.9%).

Likewise, DCs transfected with $PLGA_{O10H6}$-pIL-10 (Figure 6.3a-b, solid line) express similar levels of CD80 and CD86 compared to control cells (Figure 6.3a-b, dashed line). Comparison of positive fractions of B7 molecules from three independent

experiments (Figure 6.3c, Table 6.4) using student's t-test (two-tailed, equal variance) showed no significant difference (CD80, p = 0.7; CD86, p=0.2) in $PLGA_{O10H6}$-pIL-10 transfected DCs (CD80 = 54.8 ± 1.4%, CD86 = 54.2 ± 2.0%) versus control DCs (CD80 = 51.5 ± 3.3%, CD86 = 52.0 ± 5.1%).

These results indicate that PS_{O10H6}-pIL-10 and PLGA $_{O10H6}$-pIL-10 particles do not affect CD80 and CD86 expression in DCs. Thus, the immunosuppression mechanism of these IL-10 gene-modified DCs is not related to the modulation of co-stimulatory pathways based on the data presented in this section. Additionally, we believe that those two polymers can be used as carriers for the delivery of immunosuppressive agents, because no up-regulation of B7 was found in DCs exposed to PS_{O10H6}-pIL-10 and PLGA $_{O10H6}$-pIL-10,

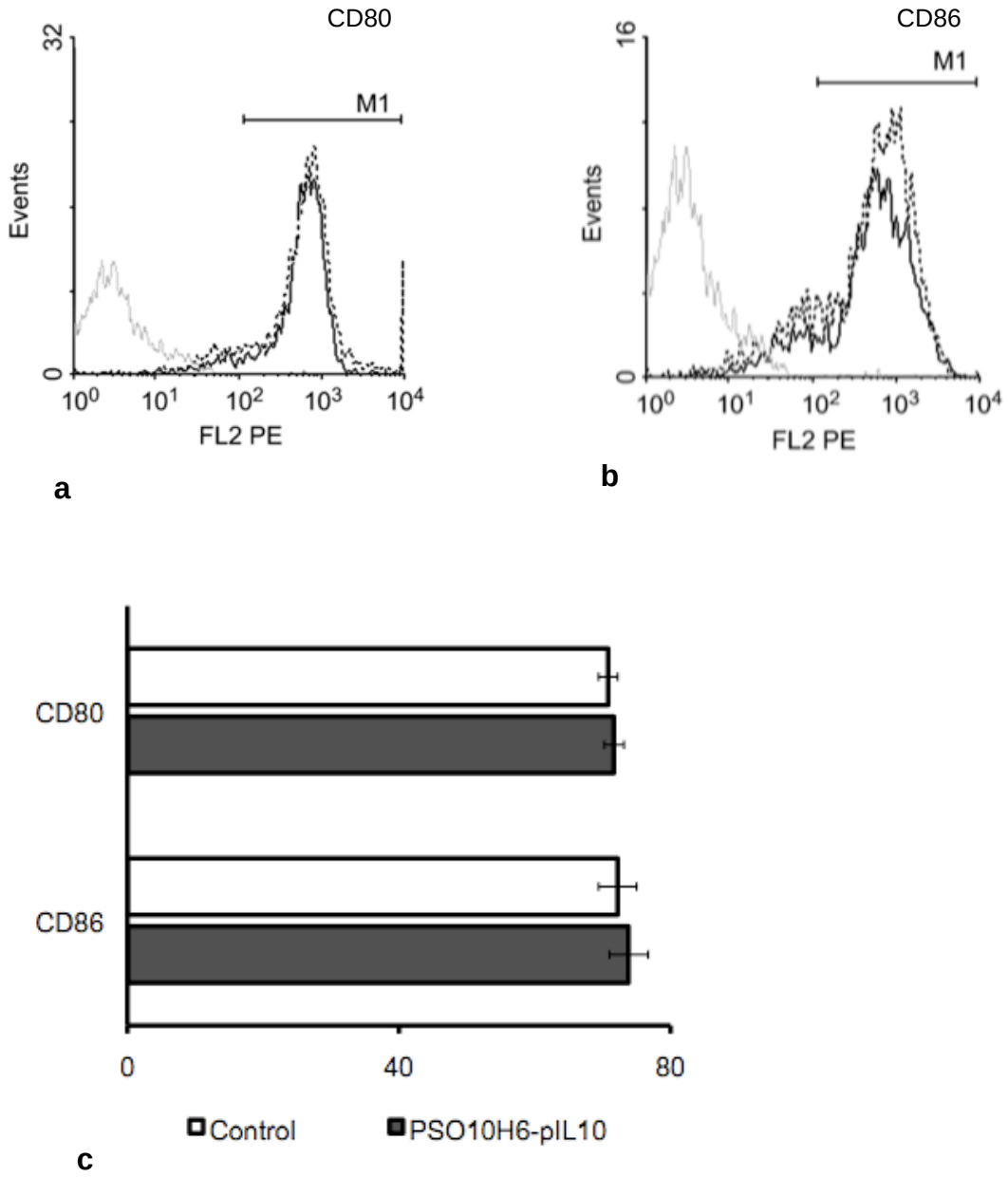


Figure 6.2: Expression of CD80 (a) and CD86 (b) in DCs transfected with PS_{O10H6}-pIL-10. Stained control DCs are indicated as dashed line and transfected DCs are shown as solid black line. Unstained DCs (grey solid line) were included as baseline; (c) and (d) show expression of CD80 and CD86, respectively, in stained control DCs (dashed line) and in DCs transfected with $PLGA_{O10H6}$-pIL-10 (black solid). Histograms shown are representative of analysis of three independent DCs cultures. Student's t-test (two-tailed, equal variance) comparison of positive fractions indicates no significant difference in PS_{O10H6}-pIL-10/DCs versus control DCs (CD80, $p = 0.2$; CD86, $p = 0.5$)

	Percentage of CD80+ DCs		Percentage of CD86+ DCs	
	Mean	SEM	Mean	SEM
Control DCs	70.8	1.5	72.2	2.9
PS_{O10H6}-pIL-10 transfected DCs	71.6	1.4	73.8	2.8

Table 6.3: CD80 and CD86 expression in control DCs and PS_{O10H6}-pIL-10 transfected DCs. Data were obtained from three independent experiments (p=0.2).

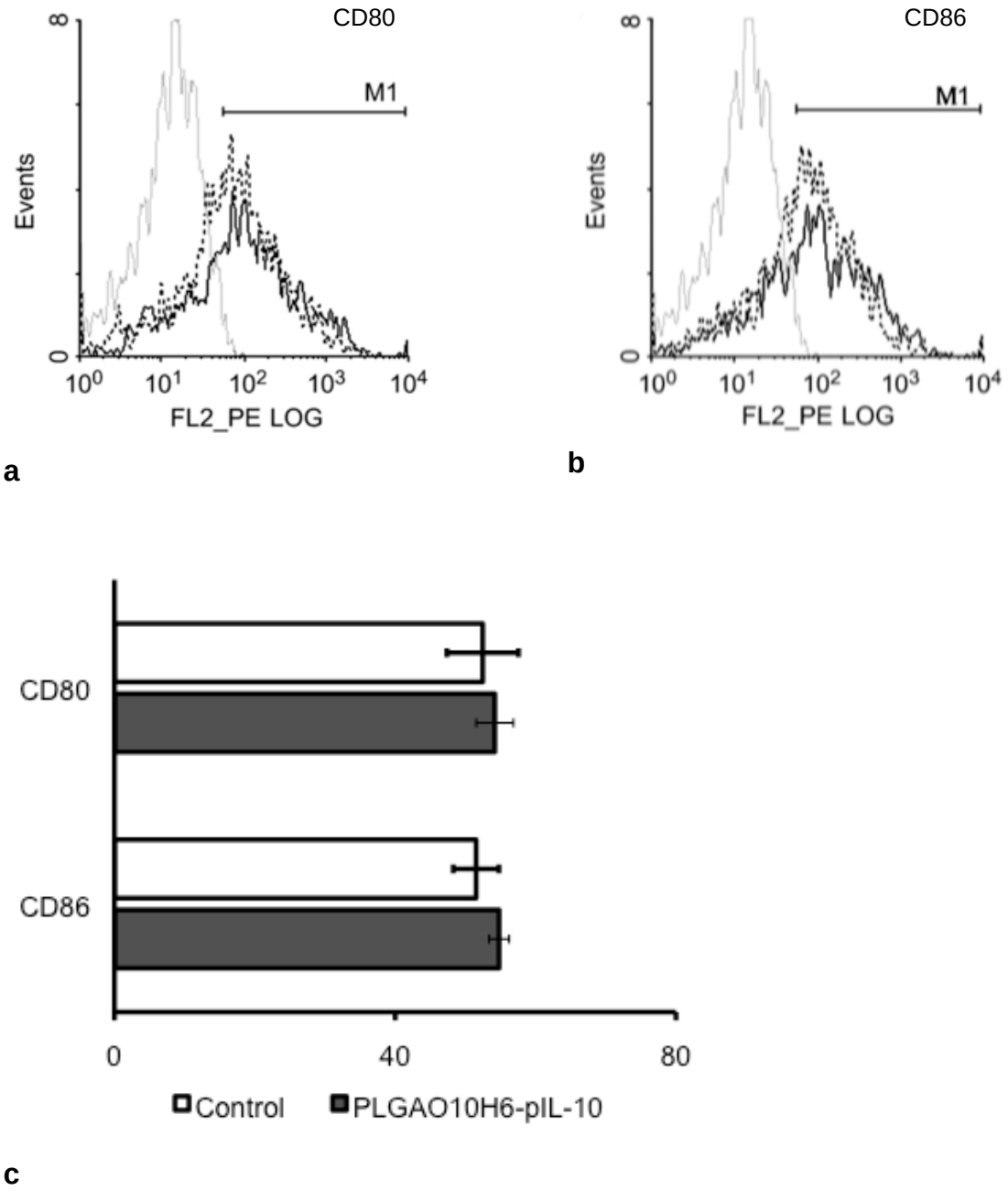


Figure 6.3: CD80 (a) and CD86 (b) in DCs transfected with PS_{O10H6}-pIL-10. Stained control DCs are indicated as dashed line and transfected DCs are shown as solid black line. Unstained DCs (grey solid line) were included as baseline; (c) and (d) show expression of CD80 and CD86, respectively, in stained control DCs (dashed line) and in DCs transfected with $PLGA_{O10H6}$-pIL-10 (black solid). Histograms shown are representative of analysis of three independent DCs cultures. Student's t-test (two-tailed, equal variance) comparison of positive fractions indicates no significant difference in $PLGA_{O10H6}$-pIL-10 versus control DCs (CD80, p = 0.7; CD86, p=0.2)

	CD80		CD86	
	Mean	SEM	Mean	SEM
Control DCs	51.5	3.3	52.0	5.1
PLGAO10H6-pIL-10 transfected DCs	54.8	1.4	54.2	2.7

Table 6.4: CD80 and CD86 expression in control DCs and $PLGA_{O10H6}$-pIL-10 transfected DCs. Data were obtained from three independent experiments (p=0.2).

Altered cytokine production in allogeneic T cells stimulated with IL-10 gene-modified DCs

The cytokine environment in which DCs and T cells interacted reflects the dominant immunological drive. To further evaluate the immune suppressive mechanism of IL-10 gene-modified DCs, we examined the secretion of cytokines and chemokines in the $PLGA_{O10H6}$-pIL-10 transfected DC (C57BL/6) and allogeneic T cell (BALB/C) co-culture in which expansion of Foxp3 expression was noted. To profile the expression of multiple cytokines and chemokines in the co-culture, an array system (Panomics TranSignal^{TM} Arrays, Redwood City, CA) utilizing a membrane spotted with antibodies that capture a variety of protein analytes (IL-10, G-CSF, M-CSF, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12, IL-13, INF-γ, TNF-α, GM-CSF, VEGF, Monokine Induced by Gamma interferon [MIG], Rantes and Macrophage Inflammatory Proteins [MCP-1α]) were used. Those cytokines and chemokines play key roles in the maturation of effector cells and activation of immune response. Compared with the conventional ELISA essay, the array method, which detects the presence of analytes by chemiluminescence, can sensitively and accurately characterize multiple cytokines and chemokines in a single experiment.

Data obtained from cultures containing $PLGA_{O10H6}$-pIL-10 modified DCs were compared with those from untransfected DCs to evaluate the effect of IL-10 gene-modification on DC cytokine secretion. Results from cytokine microarray analysis were obtained from two independent experiments and the relative level of cytokine expression in the DC: T cell co-culture was indicated as the percentage of positive control signal (Figure 6.4a). The result showed no significant changes in the levels of IL-1α, IL-2, IL-3,

IL-4, IL-6, IL-12, IL-13, TNF-α, INF-γ, G-CSF, MIG, and Rantes between the supernatants from cultures containing $PLGA_{O10H6}$-pIL-10 modified DCs and control untransfected DCs. IL-1α, IL-2, IL-3, IL-6, IL-12, TNF-α, INF-γ and G-CSF are pro-inflammatory cytokines whose function includes activating effector T cells, promoting T cell differentiation and proliferation, and inducing inflammation. MIG and Rantes are chemokines that promote leukocyte trafficking and recruitment to inflammatory sites. IL-4 and IL-13 are anti-inflammatory cytokines because of their ability to suppress Th1 response and down-regulate IL-1 and TNF-α. The result showed that IL-10 gene modification in DCs does not decrease major pro-inflammatory cytokine and chemokine production in DC : T cell co-culture. Additionally, anti-inflammatory cytokines were not up-regulated in the co-culture. These results indicated that immunosuppressive function of $PLGA_{O10H6}$-pIL-10 modified DCs was not related to the alteration of the production of these pro- and anti-inflammatory cytokines, which affect Th1 response as well as T cell trafficking.

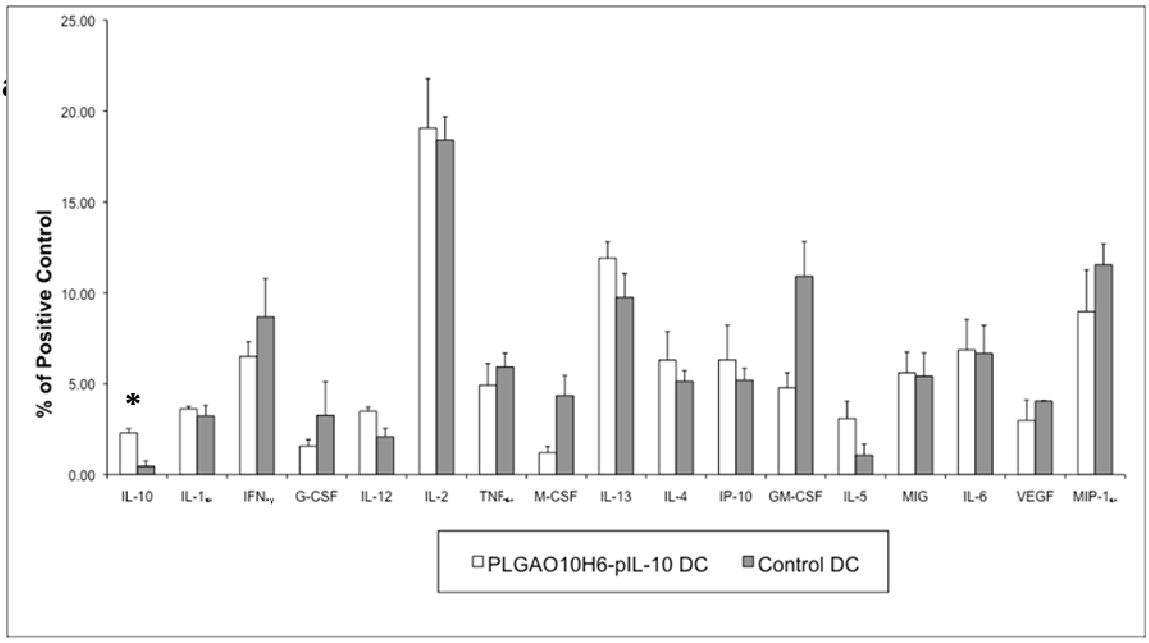
Conversely, we found increase in IL-10 and IL-5 expression, and decrease in macrophage colony-stimulating factor (M-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) when T cells were stimulated with $PLGA_{O10H6}$-pIL-10 modified DCs (n = 2; Figure 6.4 a-b). IL-10 production was increased when DCs were transfected with $PLGA_{O10H6}$-pIL-10 (p = 0.005). The up-regulation of suppressive cytokine IL-10, and down-regulation of pro-inflammatory cytokine M-CSF and GM-CSF indicated that the IL-10 gene-modified DCs display a suppressive phenotype in the co-culture.

Given that TGF-β production is an important characteristic of Treg cells (Izcue, Coombes et al. 2006), we evaluated the expression of this cytokine using ELISA in the DC: T cell co-culture in which expansion of Foxp3 expression was noted. Co-culture with untransfected DCs and T cells were assayed as control group to exam the alteration of TGF-β production. The result showed that TGF-β up-regulated four-fold in supernatants from T cell culture conditioned by $PLGA_{O10H6}$-pIL-10 modified DCs (1598.23±194.22 pg/mL) compared to control DCs (357.95±102.53 pg/mL) ($p = 0.001$; Figure 6.5a). This result is in agreement with the expansion of Treg cells in mixed lymphocyte cultures containing IL-10 gene-modified DCs.

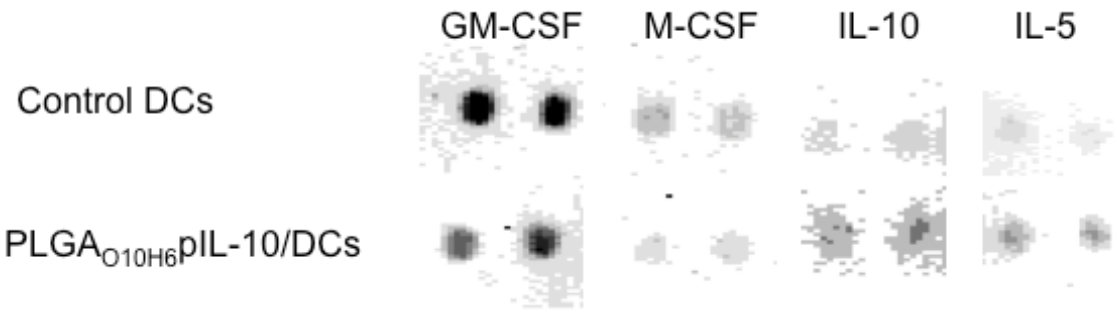
IFN- γ is a major product of Th1 cells and plays a key role in promoting Th1 type immune response. Results from the cytokine array experiment showed that amount of INF-γ remained unchanged in the co-cultures containing $PLGA_{O10H6}$-pIL-10 modified DCs compared to control untransfected DCs. Herein we confirmed the result of IFN- γ secretion in the DC: T cell co-culture using ELISA. The amount of INF-γ remained unchanged ($p = 0.4$; Figure 6.5b) in the co-cultures when $PLGA_{O10H6}$-pIL-10 modified DCs (423.86±41.38 pg/mL) were used as stimulators compared to control untransfected DCs (441.57±37.43 pg/mL). This result is consistent with the one observed in the cytokine array experiment, indicating that the suppressive effect of IL-10 gene-modified DCs was not mediated by altering the secretion of cytokines that promote Th1 response.

Thus, the inhibitory cytokine TGF-β appears to dominate immune activating

cytokines in these cultures. These data confirmed the suppressive phenotype of IL-10 gene-modified DCs, reconfirmed that their immunosuppressive mechanism is based on the expansion of regulatory T cells.

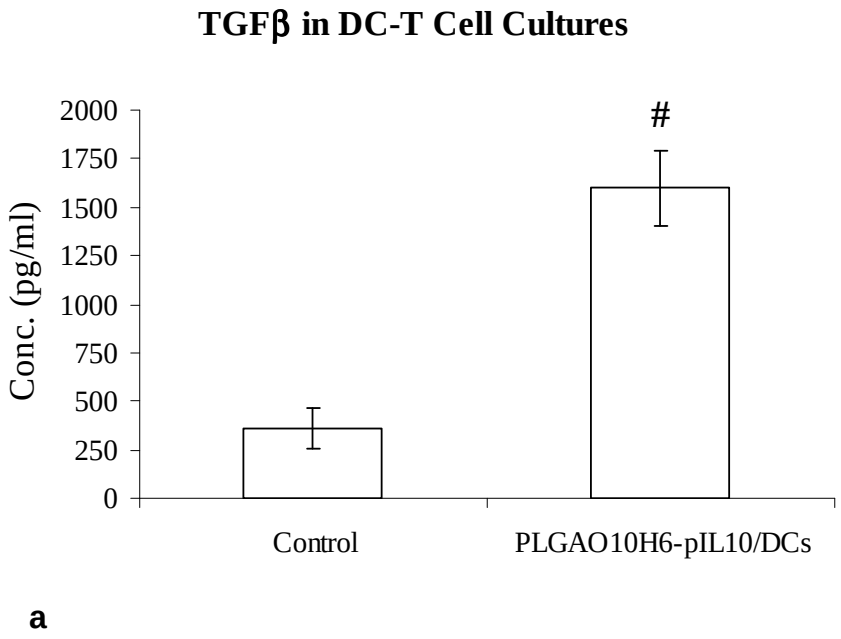


a

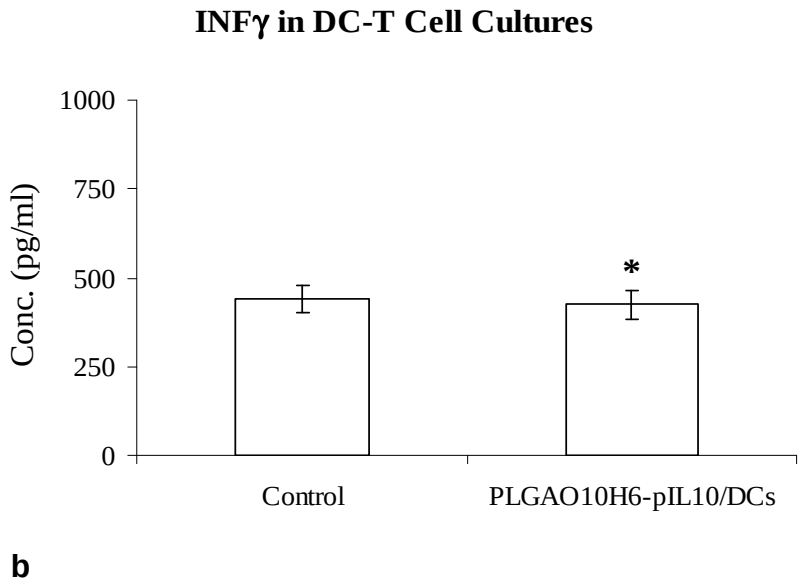


b

Figure 6.4: Cytokine released in DC- allogeneic T cell co-cultures with PLGA$_{O10H6}$-pIL-10 or control DCs using TranSignal™ Arrays (Panomics). a. Cytokine levels in the co-culture were presented as percentages of positive control signals. Images intensity was quantified using the NIH Image J software. Data were averaged from two independent experiments performed. Error bars indicate standard error of mean. b. Images of GM-CSF, M-CSF, IL-10, and IL-5 detected in a cytokine array membrane in one of the representative experiments. Statistical significance was determined using the Student's t-test (two-tailed, paired-samples; *p = 0.005).



a



b

Figure 6.5: TGF-β1 (a) and INF-γ (b) ELISA. The cytokine was assayed in DC-allogeneic T cells co-cultures with $PLGA_{O10H6}$-pIL-10 transfected DCs or control DCs. Data were averaged from three independent experiments performed. Error bars indicate standard error of mean. Sensitivity of the assay was 15.6 pg/ml. Background TGF-β1 signal from sera in culture media was subtracted. Statistical significance was determined using the Student's t-test (two-tailed, paired-samples; #p = 0.001, *p = 0.4).

	TGF-β (pg/ml)		IFN-γ (pg/ml)	
	Mean	SEM	Mean	SEM
Control DCs	357.95	102.53	441.57	37.43
PLGAO10H6-pIL-10 transfected DCs	1598.23#	194.22	423.86*	41.38

Table 6.5: Summary of data from TGF-β1 and INF-γ ELISA assay. Data were averaged from three independent experiments performed. Statistical significance was determined using the Student's t-test (two-tailed, paired-samples; #$p = 0.001$, *$p = 0.4$).

Discussion

Previous data from our lab has shown that the PS_{O10H6} particle is an efficient vector for oligonucleotide delivery to DCs, capable of introducing NF-κB decoy molecules into greater than 50% of primary DC *in vitro*, down-regulating DC maturation and Th1 cytokine IFN-γ production (Kovacs, Zheng et al. 2005). IL-10 gene-modified DCs have promising therapeutic potentials (Mahnke, Johnson et al. 2007). We have shown that DCs transfected with synthetic vector PS_{O10H6}-pIL-10 suppressed allogeneic T cell response *in vitro* and host cell infiltration into MHC-mismatched graft tissue *in vivo*. Potential immunosuppressive mechanisms of IL-10 gene-modified DCs include down-regulation B7 co-stimulatory molecule expression, alteration of cytokine secretions, and up-regulation of allogeneic or Ag-specific Treg cells. In this chapter, we explored the suppressive functional utility of PS_{O10H6}- pIL-10 and $PLGA_{O10H6}$-pIL-10 transfected DCs in a mixed lymphocyte model in which T cell suppression was noted.

We first examined the expansion of regulatory T cells in pIL-10 modified DC: T cell mixed lymphocyte cultures because of the potential of Treg cells to suppress damaging immune response toward auto and alloantigens. Regulatory T cells are capable of restraining proliferation and cytotoxic activity of effector T cells (CD4 and CD8) through contact dependent or independent manner. An important measure of T cell-mediated tolerance is the expansion of Foxp3, the master transcriptional regulator constitutively and exclusively expressed in mouse regulatory cells (Hanck, Glatzel et al. 2000). In our experiments, we observed up-regulation of Foxp3 expression in allogeneic T cells co-cultured with PS_{O10H6}-pIL-10 or $PLGA_{O10H6}$-pIL-10 gene-modified DCs,

indicating that both DCs render their suppressive effects through the expanding allogeneic regulatory T cells.

Co-stimulatory molecule expression is related to the maturation functions of DCs. Interaction between these molecules expressed on the membrane of DCs (CD80 and CD86) and the T cell (CD28) is required as the secondary signal to activate Ag-specific T cell responses. In the literature, conflicting data are found regarding the effects of IL-10 on B7 co-stimulatory molecules in DCs. Depending on the model system employed, CD80 and CD86 could be up-regulated, unchanged, or down-regulated in IL-10-conditioned DCs (Enk, Angeloni et al. 1993; Mitra, Judge et al. 1995; Morel, Quaratino et al. 1997; Sharma, Stolina et al. 1999; Faulkner, Buchan et al. 2000). In this study, the effect of PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 particles on CD80 (B7.1) and CD86 (B7.2) expression on DCs was evaluated using flow cytometry analysis. Results showed no significant changes in CD80 and CD86 expression on DCs transfected with PS_{O10H6}-pIL-10 or $PLGA_{O10H6}$-pIL-10 compared to control DCs, indicating that the suppressive effect of IL-10 gene-modified DCs is not mediated through the changes in DC maturation. Additionally, because no up-regulation of B7 was found in DCs exposed to PS_{O10H6}-pIL-10 and PLGA $_{O10H6}$-pIL-10, we believe that those two polymers can be used as carriers for the delivery of immunosuppressive agents.

To further evaluate the phenotypes of IL-10 gene-modified DCs, cytokine secretion in DC: T cell co-cultured with $PLGA_{O10H6}$-pIL-10 modified or untranfected DCs was analyzed. Cytokines are regulators of host responses to infection, immune

responses, and inflammation. Depending on their role in infection and/or inflammation, some cytokines clearly promote inflammation and are called pro-inflammatory cytokines, whereas other cytokines suppress the activity of pro-inflammatory cytokines and are called anti-inflammatory cytokines. IL-1, up-regulated in mature DCs, are pro-inflammatory cytokines involved in immune defense. IL-1 activates effector T cells, increases the expression of adhesion factors on endothelial cells to enable transmigration of leukocytes. IL-2 promotes the differentiation and proliferation of T cells. In addition, IL-2 is necessary during regulatory T cells development (Thornton and Shevach 1998; Thornton, Donovan et al. 2004). IL-3 is secreted by activated T cells to support growth and differentiation of T cells in immune response. Tumor necrosis factor-alpha (TNF-α), IFN-γ and IL-6 are also classified as pro-inflammatory cytokines because of their role in promoting T cell differentiation, NK cell activation, and neutrophil trafficking. IL-12 is a cytokine produced by mature dendritic cells. Its pro-inflammatory functionalities include promoting the differentiation of naive T cells into Th1 cells and stimulating the production of IFN-γ and TNF-α from T cells. MIP, secreted by macrophages, induces the synthesis and release of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. IL-4 and IL-13 are anti-inflammatory cytokines owing to their ability to suppress Th1 response and the expression of pro-inflammatory cytokines such as IL-1, and TNF-α.

Our results showed no changes in most pro-inflammatory cytokines (less than one fold) including IL-1α, IL-2, IL-3, IL-6, IL-12, TNF-α, INF-γ, G-CSF, MIP-1α, MIG, and Rantes in the supernatants from cultures containing $PLGA_{O10H6}$-pIL-10 modified DCs compared to control DCs. Additionally, expression of IL-4 and IL-13 were also the same

between co-cultures containing IL-10 modified DCs and control DCs.

Generally, IL-10 is considered an anti-inflammatory cytokine capable of suppressing TNF-α and other pro-inflammatory agents. In some experimental models, autocrine IL-10 showed no suppression of pro-inflammatory cytokines, which is in agreement with our data (Connor, Harkin et al. 2005). Strle et al. also found in their model, the suppressive effect of IL-10 is not mediated by the down-regulation of inflammatory cytokines (TNF-α and IL-6), but rather through the interference of their downstream signaling, such as TNF-α-induced phosphorylation of Jun N-terminal kinase (Strle, McCusker et al. 2007).

Among all the chemokines tested, MIG, also known as Chemokine (C-X-C motif) ligand 9 (CXCL9), contributes to T cell trafficking (Liao, Rabin et al. 1995). Rantes (CCL5) promotes the recruitment of leukocytes including T cells, eosinophils, and basophils into inflammatory sites. Our results showed that $PLGA_{O10H6}$-pIL-10 modified DCs does not alter the secretion of MIG and Rantes by T cells 2 days post-treatment, indicating that the suppressive effect of IL-10 gene-modified DCs is not mediated through the interference of these chemokines in our model. Krensky et al. reported that CCL5 was not up-regulated in normal T lymphocytes until 3 to 5 days after their activation (Krensky and Ahn 2007), suggesting that longer observation period may be required in order to observe a change of the level of CCL5 in the co-culture.

Conversely, we detected decreases in M-CSF and GM-CSF when T cells were

stimulated with IL-10 gene-modified DCs compared with control DCs (Figure 6.4). Members of the CSF cytokine family play important roles in stimulating immune cell activation, differentiation, and recruitment. GM-CSF, produced by activated T cells and macrophages, promotes Th1 immune response, allergic inflammation, and autoimmunity (Fleetwood, Cook et al. 2005; Cao 2007; Eksioglu, Mahmood et al. 2007). M-CSF, also named as CSF-1, is known for its role in promoting macrophage and T cell survival and proliferation, as well as enhancing functionalities of these cells (Martinez-Moczygemba and Huston 2003; Barreda, Hanington et al. 2004; Eksioglu, Mahmood et al. 2007). M-CSF also up-regulates MHC class II molecule on DCs by promoting its synthesis and translocation to the plasma membrane. Previous studies showed that IL-10 inhibits the production of GM-CSF and M-CSF by monocytes (de Waal Malefyt, Abrams et al. 1991). In agreement with this study, our result showed that both M-CSF and GM-CSF were suppressed in co-cultures containing $PLGA_{O10H6}$-pIL-10 modified DCs compared to control DCs, indicating that these IL-10-gene modified DCs display a suppressive phenotype in the mixed lymphocyte culture.

Additionally, we observed increases in IL-10 and IL-5 when T cells were stimulated with IL-10 gene-modified DCs compared with control DCs (Figure 6.4). IL-10 production increased four-fold when DCs were transfected with $PLGA_{O10H6}$-pIL-10. $PLGA_{O10H6}$-pIL-10 transfected DCs could contribute to the secretion of IL-10 in the co-culture. However, because only one in 5 cells in the culture was DCs, a major portion of IL-10 could come from Foxp3-expressing Treg cells, as it was demonstrated that Foxp3+ Treg cells secrete IL-10 (Izcue, Coombes et al. 2006; Uhlig, Coombes et al. 2006). We

cannot rule out, however, that other suppressor T cells (type 1 and type 3) in the system which might have contributed to the IL-10 pool (Liston and Rudensky 2007). IL-5 was initially characterized as a Th2 cytokine that promote the growth and differentiation of eosinophils. IL-5 could also contributes to counter-regulating the production of Th1 cytokines, including IFN-γ (Randolph and Fathman 2006). Recent study by Nakagome et al. demonstrated that IL-5 suppresses Ag-specific proliferation of CD4+ T cell *in vivo* (Nakagome, Dohi et al. 2007). Further study revealed that IL-5 plays a role in indirectly promoting immunosuppressive response through up-regulating TGF-β production. Thus, an increase of IL-5 in the co-culture may contribute to the suppressive phenotype of IL-10 gene-modified DCs.

Our data also show that suppressive cytokine TGF-β appears to dominate activating cytokines in these cultures, up-regulated four-fold in T cells conditioned by $PLGA_{O10H6}$-pIL-10 modified DCs compared to control DCs. TGF-β is a pleiotropic cytokine with potent immunoregulatory properties and is essential for the maintenance of immunological self-tolerance in the CD4+ T-cell compartment (Letterio and Roberts 1998). Studies showed that TGF-β contributed to CTLA--4-mediated T-cell inhibition (Shull, Ormsby et al. 1992) to IL-12-induced JAK2 and TYK2 phosphorylation kinases, which is associated with T-cell proliferation and IFN-γ production (Bright and Sriram 1998). TGF-β is also a key feature of Treg cells (Enk 2006), playing an important role in the conversion of naive CD4+ CD25- T cells into CD4+ CD25+ and Foxp3+ Treg cells. Besides, TGF-β signaling is required for the immunosuppressive capacity of CD4+ CD25+ Treg cells (Shull, Ormsby et al. 1992). Thus, our result of TGF-β secretion is

consistent with the expansion of Treg cells.

In summary, suppression of co-stimulatory molecules was not observed on IL-10 modified DCs and among all the pro-inflammatory cytokine tested, only GM-CSF and M-CSF secretion were suppressed in the co-culture containing IL-10 gene-modified DCs. Nevertheless, emergence of Treg cells in the DC: T cell co-culture was evident in the generation of Foxp3-expressing CD4+ cells concomitant with elevated IL-10 and TGF-β production, suggesting that the suppressive effect of these IL-10 gene-modified DCs is mainly mediated through the expansion of allogeneic regulatory T cells. In general, our results establish that PS_{O10H6}-pIL-10 and $PLGA_{O10H6}$-pIL-10 modulates DCs into suppressive phenotypes.

CHAPTER 7

SUMMARY AND ORIGINAL CONTRIBUTIONS

Summary

In this work, we investigated polystyrene and poly (lactic-co-glycolic acid) particles modified with the cationic peptide O10H6 as carriers to introduce a plasmid encoding murine interleukin-10 to dendritic cells to modulate immune functions.

The delivery systems were formed by coating O10H6 on PLGA and polystyrene fabricated particles and their physical characteristics were evaluated. Results from particle size analysis showed that both $PLGA_{O10H6}$-pIL-10 and PS_{O10H6}-pIL-10 particles display unimodal (Gaussian) distributions in the submicron range (mean diameter 297.2±14nm of $PLGA_{O10H6}$-pIL-10 and 126.0±8nm of PS_{O10H6}-pIL-10), indicating that both systems exist as uniform stable colloidal dispersions, which is preferable for DC uptake. Their positive zeta potential (4.7 ±0.5 mv of $PLGA_{O10H6}$-pIL-10 and 31.26±2.46 mv of PS_{O10H6}-pIL-10) stabilize the particles in the dispersion. Gel electrophoresis analysis revealed that pIL-10 was condensed on the surface of $PLGA_{O10H6}$ particles via electrostatic interaction, as evidenced by the release behavior after the addition of excessive amount of negatively charged low molecular weight heparin. Results from the serum stability study demonstrated that the O10H6 coated surface on these particles can bind and protect plasmid DNA molecules from serum degradation.

Using confocal image analysis and reverse transcription polymerase chain

reaction method, we demonstrated that PS_{O10H6} and $PLGA_{O10H6}$ particles are effective in delivering reporter gene pGFP as well as plasmid IL-10 to DCs. Results showed that IL-10 gene expression was enhanced by three-fold in both PS_{O10H6}- pIL-10 and $PLGA_{O10H6}$-pIL-10 transfected DCs compared to control DCs.

The suppression of T cell responses by IL-10 gene-modified DCs was evaluated using an *in vitro* mixed leukocyte model containing DCs and allogeneic T cells. The T cell response was quantified by the proliferation of allogeneic lymphocytes. Our results indicated that DCs transfected with PS_{O10H6}pIL-10 and $PLGA_{O10H6}$pIL-10 particles elicit the weakest proliferation in allogeneic bulk T cells, as well as in CD4+ and CD8+ T cells among other transfected DCs. Furthermore, using a cell-embedded Matrigel as a surrogate graft, we showed that PS_{O10H6}pIL-10 and $PLGA_{O10H6}$pIL-10 transfected DCs suppress allogeneic host cell infiltration *in vivo* compared to untransfected DCs. These data demonstrated that PS_{O10H6}-pIL10 and $PLGA_{O10H6}$pIL-10 transfected DCs display a suppressive phenotype to down-regulate allogeneic T cell responses.

To further evaluate their immunosuppressive mechanisms, co-stimulatory molecule expression, regulatory T cell expansion and cytokine production in co-cultures containing IL-10 gene modified DCs and allogeneic T cells were characterized. Results showed that co-stimulatory molecule expression was not changed in DCs transfected with PS_{O10H6}-pIL10 and $PLGA_{O10H6}$pIL-10 particles. Data also demonstrated that regulatory T cell marker Foxp3 was up-regulated in allogeneic T cells exposed to IL-10 gene-modified

DCs. This result was confirmed with the elevation of TGF-β, a cytokine that plays an important role in regulatory T cell development.

Taken together, data from this work demonstrated that O10H6 surface-modified PS and PLGA particles are effectual vehicles to deliver IL-10 gene to DCs to induce T cell tolerance. The expansion of allogeneic regulatory T cells by IL-10 transfected DCs may lead to the down-regulation of effector T cell responses in local environments. Collectively, these results raise the prospect of using PS_{O10H6} or $PLGA_{O10H6}$ as vectors to deliver immunosuppressive therapeutics to modulate T cell responses *in vivo*. Biodegradable $PLGA_{O10H6}$ particles are the choice for treatments that require long-term administrations.

Original Contributions

IL-10 has been investigated in the past as a therapeutic means of attenuating damaging immune response. However, using polymeric particles as vectors to genetically modified DCs with IL-10 gene to modulate immune functions has not been examined extensively. This approach could potentially overcome the limitations of current IL-10 delivery strategies, namely, short half-life of recombinant IL-10 protein when administrated I.V. (Moore, de Waal Malefyt et al. 2001), immunological responses toward viral vectors (Sen, Hong et al. 2001; Buonocore, Van Meirvenne et al. 2002), and inflammation associated with cationic lipids in liposomal formulations (Stamatatos, Leventis et al. 1988; Zelphati and Szoka 1996).

This work found that the immunosuppressive mechanisms of IL-10 gene applied to DC can be attributed to as alteration of the release of TGF-β (Pestka, Krause et al. 2004; Kopydlowski, Salkowski et al. 1999; Kubin, Kamoun et al. 1994; Willems, Marchant et al. 1994; Tong, Toshiaki et al. 2005) and expansion of regulatory T cells. Recent studies have found that Treg cells play a critical role in inducing immune tolerance via restraining proliferation and cytotoxic activity of effector T cells. Our data emphasize the important role of Treg cells in IL-10 inducing immune tolerance, perhaps by increasing the local concentration of TGF-β .

This work demonstrated that PS and PLGA can be used as a component of carriers to deliver an immunosuppressive gene to modulate immune functions. Synthetic polymeric particles fabricated from polystyrene and PLGA have been tested as vaccine carriers in animal models in the past (Fifis, Gamvrellis et al. 2004; Scheerlinck, Gloster et al. 2006). Minigo et al. have developed formulations made of PLL-coated polystyrene nanoparticles, which condense and deliver DNA vaccines to C57BL/6 mice (Minigo, Scholzen et al. 2007). Our work suggested that PLGA copolymers are suitable materials in constructing platforms to deliver genes that encoding suppressive cytokines to promote immune tolerance. It has been reported in literatures that the adjuvant effect of PLGA may hamper their use in tissue engineering or applications that promote immune tolerance. Some studies have shown that PLGA particles and films elicit human and murine DC maturation (Yoshida and Babensee 2004; Yoshida, Mata et al. 2007), implying that the polymer might be pro-inflammatory. Conversely, Fischer et al. have

demonstrated that DC phenotypes remain unchanged when exposed to PLGA and polyelectrolyte-coated PLGA particles (Fischer, Uetz-von Allmen et al. 2007). Although we did not directly address the effects of PLGA itself on DC maturation status in these experiments, our data do support the notion that $PLGA_{O10H6}$-pIL-10 delivers an overriding suppressive signal to T cells. Consistent with these observations are results reported by Haddadi et al. showing that immunosuppressive effects of rapamycin on DCs are enhanced by encapsulating the drug in PLGA nanoparticles (Haddadi, Elamanchili et al. 2008).

Several studies have shown that PLGA particles and films elicit human and murine DC maturation (Yoshida and Babensee 2004; Yoshida, Mata et al. 2007), implying that the polymer might be pro-inflammatory. This effect of polymers may be desired in vaccine delivery, but may not be acceptable in tissue engineering or in applications that promote immune tolerance. Our results showed no significant changes in CD80 and CD86 expression in DCs transfected with $PLGA_{O10H6}$-pIL-10 as well as PS_{O10H6}-pIL-10 particles. Although we did not directly address the effects of PLGA on DC maturation, the immune suppression data presented in this dissertation support the notion that $PLGA_{O10H6}$-pIL-10 delivers an overriding suppressive signal to T cells, suggesting that PLGA are suitable materials in constructing vectors to deliver immunosuppressive modalities to promote immune tolerance.

This work also demonstrated that the polycation O10H6 is effective in modifying polymeric particles for DNA binding. Conventionally, DNA can be encapsulated in

polymeric particles (Cohen, Levy et al. 2000). However, hydrolysis of PLGA may substantially decrease the pH environment in PLGA particles, potentially resulting in degradation of DNA (Walter, Moelling et al. 1999; Wang, Robinson et al. 1999). Moreover, because plasmid DNA has to diffuse through the polymer matrices, the rate of release is often too slow (release lasts for several days/weeks) (Walter, Moelling et al. 1999; Tinsley-Bown, Fretwell et al. 2000; Zhu, Mallery et al. 2000; Luten, van Steenis et al. 2003), leading to less optimal gene expression**.** Surface loading of nucleic acids with polycation allows DNA to be protected and released inside cells without having to diffuse through acidic matrices, thereby increases DNA transfection efficiency. Additionally, surface loading of nucleic acid allows DNA to be release faster compared to encapsulation methods, which will result in more DNA accumulation in cytoplasm and more efficient gene transfer. In this work, we employed polycation O10H6 modified polymeric particles loaded with pIL-10 as the delivery vector to DCs. O10H6, a cationic peptide that has endosomal escape mechanism, has been shown in our lab to have more efficient uptake by DCs and is less toxic than other PLL containing cationic peptides. The principal role of O10H6 is to exert a tunable DNA binding surface so that DNA can be loaded and protected on the particle, considering that the ionic interaction between the DNA and O10H6 peptide is strong enough to bind DNA. One of the potential advantages of these particulate vectors is they can be adjusted to achieve different payload of DNA by changing the amount of O10H6 peptide added to the particles during fabrication. Excessive amount of O10H6 coated on the particles will likely to increase amount of positive charge available for DNA binding, thereby increasing DNA loading per particle.

A cell transplant model that utilizes Matrigel seeded with *ex vivo* pIL-10 modified DCs from BALB/c (H-2^d) mice as a scaffold to establish surrogate ‘‘graft’’ in C57BL/6 (H-2^b) mice. Matrigel has been used extensively as a matrix scaffold for implanting tissues *in vivo* (Shih and Towle 1995; Edamura, Ohgawara et al. 2001; Zimmermann, Didie et al. 2002; Zimmermann, Melnychenko et al. 2004; Bharat, Benshoff et al. 2005). In this system, the immune response from the recipient can be evaluated by analyzing host cell infiltration to the graft after implantation. This Matrigel system embedded with *ex vivo* transfected DCs provides an opportune setting for studying alloreactivity of *ex vivo*-modified DC *in vivo*. An advantage is the number of donor cells in the implant can be controlled, leading to reproducible inflammation.

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APPENDIX 1

Glossary

Antigen-presenting cell (APC) : a cell that can break down protein antigens into peptides and present the peptides, in conjunction with major histocompatibility complex on the cell surface, where they can interact with T cell receptors. Professional APCs include dendritic cells, Macrophages, and B cells.

Colloidal dispersion : A system in which particles of colloidal size of any nature (e.g. solid, liquid or gas) are dispersed in a continuous phase of a different composition (or state).

Cytotoxic T cell (CTL): a sub-group of T lymphocytes with a CD8 marker that recognizes antigens on the surface of infected cells or tumor cells. It is capable of inducing the death of these infected somatic or tumor cells.

Effector T cell (helper T cell) : a sub-group of lymphocytes that play an important role in establishing and maximizing the capabilities of the immune system by activating and directing other immune cells.

Enzyme-linked immunosorbent assay (ELISA):. A sensitive immunoassay that uses an enzyme linked to an antibody or antigen as a marker for the detection of a specific protein.

Flow cytometry: a technique for counting, examining, and sorting microscopic particles suspended in a stream of fluid. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical and/or electronic detection apparatus.

Liposome : a spherical vesicle composed of a phospholipid and cholesterol bilayer. Liposomes contain a core of aqueous solution.

Major histocompatibility complex (MHC): a group of genes that code for proteins found on the surfaces of cells that help the immune system recognize foreign substances. The proteins encoded by the MHC display both self and nonself antigens to T cell that has the capacity to kill or co-ordinate the killing of pathogens, infected or malfunctioning cells.

Regulatory T cell: a specialized subpopulation of T cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens.

Reverse transcription polymerase chain reaction (RT-PCR): a sensitive method for the detection of mRNA expression levels. Usually, RNA strand is first reverse transcribed into its DNA complement (complementary DNA, or cDNA) using the enzyme reverse transcriptase, and the resulting cDNA is then amplified using primers specific for one or

more genes. RT-PCR can also be carried out as one-step RT-PCR in which all reaction components are mixed in one tube prior to starting the reactions.

Zeta potential: electric potential in the interfacial double layer at the location of the slipping plane versus a point in the bulk fluid away from the interface. In other words, zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle.