

Immune therapy offers exciting prospects for patients with cancer, autoimmunity and transplanted organs. Recently a deluge of studies have investigated the practical utility of dendritic cells (DC) as tools for immune modulation. The ability of DC to act both as augmenters and inhibitors of immune response has prompted investigation into their therapeutic use experimentally and clinically. A shortcoming of DC-therapeutics is the present inability to gene-specifically modify the DC in an effective manner. The emergence of RNA interference (RNAi) as being the most potent, effective and

the periphery, constantly patrolling for foreign antigens. Immature DC are highly phagocytic, but possess low T cell stimulatory activity.

Upon recognition of various foreign entities through pattern recognition receptors, DC mature, upregulate expression of lymph node homing receptors, and migrate into T cell-rich areas for stimulation of immunity (9). Phagocytosed material is stored inside DC endosomes and upon activation, the pre-formed endosomes are rapidly exported to

(Tol-DC) through the inhibition of the IKK- β pathway using LF-15015, an analogue of the immune-suppressive drug deoxyspergualin (11). These Tol-DC are inhibitors of T cell activation, inhibitors of costimulated T cells, and induce production of T regulatory cells. Interestingly, the expression of MHC II, CD40, CD86 and IL-12 was suppressed. Supporting the idea that Tol-DC possess less of the Signals 1, 2 or 3 comes from experiments with KLH-pulsed DC from CD40-knock-out which induced antigen-specific T regulatory (Treg) cells *in vivo* (12). On the other hand, DC transfected to express high levels of one or more of the 3 signals can be used for stimulating potent immune responses against

RNA Interference: A Potent Tool for Dendritic Cell Immune Modulation

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practical method for gene-specific silencing has offered new hope for immunotherapy with DC. Prompted by our first findings of the practicality of siRNA-DC immune modulation, we will review the research and therapeutic possibilities that siRNA offers to immunologists studying this fascinating cell type.

● Dendritic Cells and Immune Modulation

Stimulation and control of T cell (1), B cell (2), NK cell (3,4) and NKT cell (5) function is co-ordinated directly and indirectly by the DC. Acting as the most potent of all antigen presenting cells (APC), the DC is uniquely able to influence the immune response through possessing 3 broadly defined molecular signals: 1) Direct antigenic signals for stimulation of the T cell receptor (TCR) such as MHC I, MHC II, and CD1d (6). 2) Membrane-bound costimulatory signals (ie CD40, CD80/86 and OX-40L) (7). 3) Soluble stimulatory molecules (ie IL-12, LIGHT) (8) that act as a polarizing signal. Additionally, the biology of the DC is uniquely formed for its ability to activate T cell responses. Generally immature DC are found in



transplant rejection and autoimmune diseases.

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the cell surface where the MHC II-Ag complexes activate T cells (9). On the other hand, the ability of the immature DC to constantly phagocytose self-antigens, leads to its ability to generate tolerance to "self" and thus prevent autoimmunity (10).

The ability of DC to control whether a stimulatory or inhibitory response will follow antigenic immunization depends of which of the 3 signals described above are present, and in what concentration they are present. For example, we have previously generated tolerogenic DC

viruses, bacteria, or cancer-antigens (13). Such stimulatory DC are particularly beneficial in clinical circumstances where a predisposition exists for weakened immune response such as cancer, in which DC vaccination upregulates beneficial Th1 immunity (14).

● Stimulatory Manipulation

The fact that DC are potent stimulators of immunity has prompted their use for treatment of conditions that require activation of T cells such as cancer. The inherent immunogenicity of DC can be upregulated by stimulation of

these cells through various receptors, such as the Toll-like receptor (TLR) family. Ligands of these receptors such as long double-stranded RNA (TLR-3) (15), unmethylated CpG motifs (TLR-9) (16), and synthetic drug imiquimod (TLR-7) (17) are potent DC stimulators. Host factors, however, play certain roles that can inhibit ability of DC to stimulate immune response. For example, melanoma is known to secrete high amounts of vascular endothelial growth factor (VEGF) that inhibits DC maturation through blocking NF- κ B activation (18). Alternatively, prostate cancer secretes a soluble DC-apoptosis inducing factor (19). In order to generate more potent DC, investigators have transfected DC with antigenic mRNA (20), or cytokine genes such as IL-12 (21), flt-3L (22), or GM-CSF (23). These manipulations have resulted in the production of DC with increased immune stimulatory activity in a variety of experimental systems, however further work needs to be performed for optimization and entry into the clinic. Consequently, a more detailed understanding of the receptor-ligand interactions associated with inhibition of DC will assist in developing modified DC that are successful for usage in these conditions. A potential modification of the DC for use in cancer would be blocking the receptors associated with immune suppressive signals, such as the IL-10 (24) or VEGF receptors (25).

● **Inhibitory Manipulation**

An immature DC is classified phenotypically as having low expression of Signal 1, 2, and 3, and functionally as a poor stimulatory of mixed lymphocyte reaction (MLR) (26). Typically, immature DC are known to be tolerogenic, inhibiting immune response through providing weak Signal 1, 2 and/or 3 (27). While immature DC are highly phagocytic and can be pulsed with specific antigens for a "tolerogenic vaccine", a concern is that these DC will mature upon *in vivo* administration. Generation of maturation-resistant DC was reported by Lutz et al through culture in low dose GM-CSF in absence of IL-4 (28). Further studies have generated immature DC through culture with inhibitory cytokines such as IL-10 (29) and TGF- β (30), although maturation-resistance was not evaluated. Recent

studies have noted that Tol-DC and immature DC may not be exactly the same. For example Sato et al demonstrated that for maximum tolerogenicity, DC must be raised under conditions that inhibit Signal 2 and 3, while stimulation with LPS/TNF- α is needed for induction of high level of Signal 1 (31). In light of this, it will be important to specifically be able to silence certain immune stimulatory genes, while at the same time not alter the basal immune inhibitory genes found in the DC.

● **Gene Manipulation of DC**

Alteration of DC function can be achieved by transfection with immunomodulatory molecules, or conversely, endogenous signals from the DC can be inhibited by either pharmaceutical, genetic, or cell-culture techniques. There are a multitude of reports describing gene-transfection of DC. We ourselves have used FasL-transfected DC for the induction of donor-reactive apoptosis (32). Others have reported that augmenting the levels of immune inhibitory cytokines through transfection with IL-4 (33), IL-10 (34) or TGF- β (35), allows the DC to inhibit graft rejection, or to protect from autoimmune diseases.

On the other hand, the tolerogenic DC can be modified through blocking immune stimulatory genes. One of the conventional method to block genes in the DC is antisense oligonucleotides (AO). AO are sequences of DNA designed to block target genes by annealing with target mRNA, forming a RNA-DNA duplex, which is recognized and cleaved by the enzyme RNase H (36). AO was the first therapeutic modality to offer the possibility of gene-specific suppression. Disadvantages of the original AO technology included susceptibility of the nucleotides to intracellular and *in vivo* degradation. Overcoming this problem through the use of morpholino and phosphothiorate backbones has led to widespread interest in AO therapies (37,38). Unfortunately, the problems of non-specific suppression and longevity of gene-suppressing effect remain significant drawbacks that impede the wide-spread clinical use of AO as drugs. In fact, a recent Phase III trial of AO for colitis has demonstrated no significant benefit (39).

Despite these drawbacks, the ability of AO to specifically inhibit genes of interest has stimulated the interest of immunologists. Due to the importance of cytokines in controlling immune functions, immunomodulation using AO to cytokines has been proposed (40). An interesting early experiment targeted the T cell stimulatory cytokine IL-2 in the context of allograft rejection. Using osmotic pumps to deliver AO to IL-2, Qu et al have increased allograft survival by blocking IL-2 production in murine cardiac allograft recipients (41). Targeting other genes important for immune function has also been performed. Blocking expression of the LPS co-receptor CD14 using AO, increased survival in murine models of septicemia (42). By targeting expression of intercellular adhesion molecule-1 with AO, Toda et al reduced leukocyte-induced damage to ischemic lungs (43).

As DC are the most potent immune regulatory cell, application of AO to this cell is of particular interest. DC transfection with AO has been reported using either electroporation or liposomal methods. Interestingly, one of the first gene targets using AO on DC was the peptide transporter, *Transport Associated Protein* (TAP). Inhibition of this protein endowed DC with increased antigen presenting function (44). Following this finding, targeting of the MHC invariant chain with AO was also performed. Through facilitating less competition for the MHC binding groove, DC with suppressed invariant chain were able to prime immune responses better than control DC (45). Another AO approach to generating DC with heightened immune stimulating capability was performed by suppressing the inhibitory cytokine IL-10. It is known that during DC differentiation, IL-10 acts in a negative autocrine manner to regulate the maturation, and T cell-stimulating capacity of DC. Taking this into account, Igietseme et al compared the T cell-activating function of wild-type DC, DC from IL-10 knockout mice, and DC treated with AO to IL-10. The knockout and the AO-suppressed IL-10 DC possessed greater T cell stimulating function, and also invoked the generation of a Th1 phenotype (ie high IFN- γ , low IL-4) (46).

Inhibitory DC would have practical applications for treatment of transplant rejection and autoimmunity. To this end, inhibition of the T cell costimulatory molecules CD80 and CD86 was reported by AO (47). Using these CD80/CD86 inhibited DC as stimulators of MLR resulted in suppressed T cell proliferation and a Th1>Th2 cytokine switch. *In vivo* administration of these DC to allogeneic transplant recipients prolonged graft survival and resulted in an increased percentage of apoptotic T cells with a lower number of donor-reactive CD8 CTL (47). Targeting of other DC-bound immune stimulatory molecules was also performed. Gorczynski et al demonstrated that suppressing expression of MD-1 on DC resulted in inhibition of allostimulatory activity, Th1>Th2 cytokine switch and prolongation of allograft survival (48). These effects were dependent on ability of suppressed MD-1 to increase expression of the DC inhibitory molecules OX-2. In addition to this, AO inhibition of the novel costimulatory molecule B7H3 on DC has resulted in DC with similar inhibitory functions as described above (49). The ability to induce immune modulation through suppressing DC genes suggests a novel and practical method of altering immune function. The recent observation that administration of manipulated DC can not only inhibit the generation of immune response, but can also inhibit a T cell response after initiation, suggests the practicality of DC immunotherapy (12). However, the fact that AO possess temporally limited effects provides the concern that DC may start to re-express the immune stimulatory genes after being placed *in vivo*. In such a situation, the administered DC may actually serve the counter-purpose of being immune stimulatory. Although AO are theoretically promising, clinical applications have not been beneficial. Additionally, several problems are intrinsic to AO therapeutics: 1. Large quantities are needed for effects; 2. Lack of specificity in some cases (50,51) and; 3. Poor transfection into target cells (50,51). For example, in the study cited above using IL-2 specific AO to block graft rejection, a very high dose of AO was needed to be administered using continuous intravenous osmotic pump in order to achieve a modest graft survival benefit over untreated controls (41). Similarly, although AO

have entered Phase III clinical trials, there was no significant difference over placebo (52). For these reasons, novel methods therapeutically applicable gene-specific silencing are desired.

● RNA interference (RNAi)

RNAi is a process by which a double-stranded RNA (dsRNA) selectively inactivates homologous mRNA transcripts. The initial suggestion that dsRNA may possess such a gene silencing effect came from work in *Petunias* in which overexpression of the gene responsible for purple pigmentation actually caused the flower to lose their endogenous color (53). This phenomenon was termed co-suppression since both the inserted gene transcript and the endogenous transcript were suppressed. In 1998, Fire et al injected *C. elegans* with RNA in sense, antisense and the combination of both in order to suppress expression of several functional genes. Surprisingly, injection of the combined sense and antisense RNA led to more potent suppression of gene expression than sense or antisense used individually. Inhibition of gene expression was so potent that approximately 1-3 molecules of duplexed RNA per cell were effective at knocking down gene expression. Interestingly, suppression of gene expression would migrate from cell to cell and would even be passed from one generation of cells to another. This seminal paper was the first to describe RNAi (54). One problem present at the initial description of RNAi, and subsequent papers following, was that in order to induce RNAi, long pieces 200-800 base pairs, of dsRNA had to be used. This is impractical for therapeutic uses due to the sensitivity of long RNA to cleavage by RNases found in the plasma and intracellularly. In addition, long pieces of dsRNA induce a panic response in eukaryotic cells, part of which includes nonspecific inhibition of gene transcription but production of interferon- α (55). In 2001, it was demonstrated that subsequent to entry of long dsRNA duplex into the cytoplasm, a ribonuclease III type enzymatic activity cleaves the duplex into smaller, 21-23 base-pairs which are active in blocking endogenous gene expression. These small pieces of RNA, termed small interfering RNA (siRNA) are capable of blocking gene expression in mammalian cells without triggering the nonspecific panic response (56).

Therefore, there are 2 methods of inducing RNAi, the naturally occurring method that takes place when viral or long double-stranded RNA enters the cell. Upon crossing the membrane, the dsRNA is recognized by: 1) (2',5')oligoadenylate synthesis, an enzyme that turns on an enzymatic cascade leading to inhibition of protein synthesis (57), 2) Activation of the protein kinase R (PKR) which also results in non-specific shut-down of cellular activity (58), and 3) DICER, a nuclease cuts the dsRNA into 21-23 base-pairs that are active in blocking endogenous gene expression (59) (Figure 1A). This method of gene-silencing is not advantageous for research or experimental purposes due to the non-specificity of effects. However, theoretically it is conceivable that administration of long dsRNA targeting cancer immune suppressive genes would have the two-fold effect of non-specifically blocking tumor proliferation, as well as silencing the immune suppressive genes. The other method of inducing RNAi is through administration of pre-formed, synthetic siRNA of 21-23 nucleotide base-pairs (60). This approach only targets the endogenous RNA transcript and does not possess indiscriminate inhibitory effects (Figure 1B).

Several recent studies have demonstrated the utility and practicality of siRNA mediated gene silencing for blocking expression of disease-associated genes *in vitro*. Novina et al demonstrated inhibition of HIV entry and replication using siRNA specific for CD4 and gag, respectively (61). Suppression of human papilloma virus gene expression in tissue biopsies from women with cervical carcinoma was reported using siRNA specific for the E6 and E7 genes (62). Furthermore, induction of leukemic cell line apoptosis and complete inhibition of bcr-abl expression was achieved using siRNA (63). The first report of siRNA used in animal models is from McCaffrey et al who suppressed expression of luciferase in mice by administration of siRNA using a hydrodynamic transfection method (64). A subsequent study using HeLa cells xenografted on nude mice compared efficacy of gene suppression between AO and siRNA. Consistent

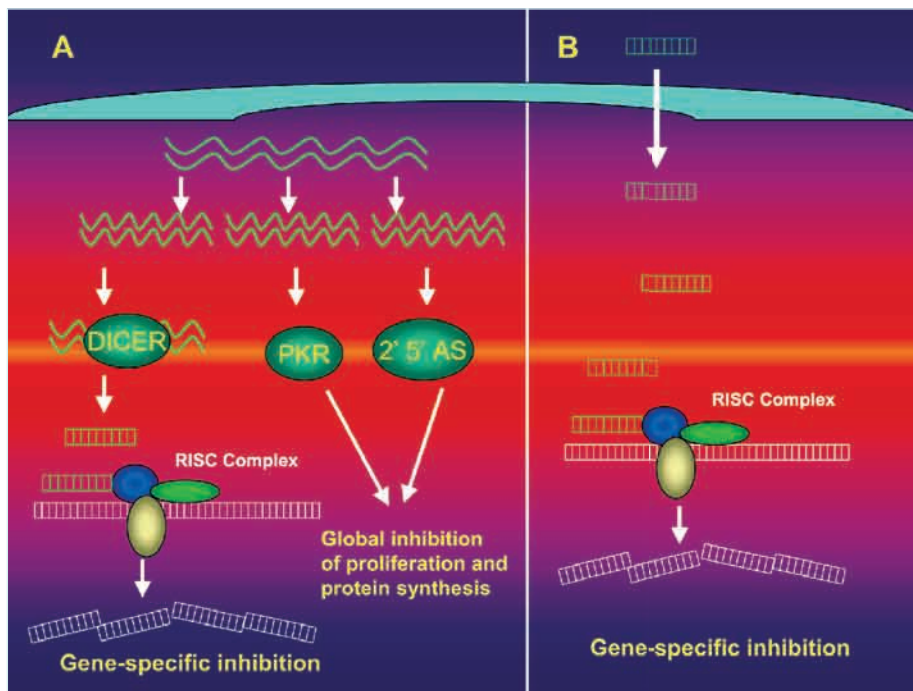


Figure 1: Induction of RNA Interference. 2 Methods of inducing RNAi exist. **A) Long double stranded RNA.** In the naturally occurring method, long double stranded RNA enters the cell and activates 2'5' oligosynthetase, which induces interferon response and blockade of cellular activities. Extracellular double stranded RNA binds TLR-3 in dendritic cells and induces activation. Once the long double stranded RNA is recognized by the type III endonuclease DICER, there is a sequential cutting of the DNA that results in 21 nucleotide double-stranded fragments. These fragments then form complexes with the RISC complex that goes on to induce cleavage of endogenous transcripts with strict homology. **B) Short interfering RNA (siRNA).** In order to obtain gene-silencing in absence of other cellular responses, synthetic double stranded RNA of 21 nucleotides is added to the cell. This siRNA is not recognized by DICER or 2'5' oligosynthetase, but instead directly binds the RISC complex, that then induces selective silencing of endogenous transcripts.

with *in vitro* suggestions, *in vivo* siRNA administration resulted in a more potent and longer lasting suppression of gene expression than obtained with AO (65).

Silencing gene expression through siRNA is superior to conventional gene or antibody blocking approaches due to the following: 1) Blocking efficacy is more potent (65); 2) Targeting gene expression is more specific (66); 3) Inhibitory effects can be pass for multiple generations (67); 4) *In vitro* transfection efficacy is higher and can be expressed in a stable manner (68); 5) *In vivo* use is more practical and safer due to lower concentration needed and no neutralizing antibody; 6) Tissue or cell specific gene targeting is possible using specific promoter vector (69, 70) or specific antibody conjugated liposome; 7) Simultaneously targeting multiple genes or multiple exons silencing is possible for increasing efficacy (71).

● Methods of inducing RNAi

There are several methods of inducing

RNAi available today. The simplest one is using presynthesized siRNA oligonucleotides that can be readily purchased from many manufacturers (ie Ambion Inc, Austin, TX). At present there are even manufacturers that will suggest specific sequences of siRNA for silencing genes based on proprietary screening systems (Dharmacon Inc, Lafayette, CO). The advantages of this approach are that sequences can be made in a relatively short time frame and with strong backbone composition so to avoid degradation by intracellular enzymes. Disadvantages include cost, lack of constitutive expression, and the fact that they cannot be grown up in a bacterial expression system. A simple, although more time consuming, method of generating siRNA for DC is using a hairpin-loop expression vector (72). Since siRNA is double stranded, the transcription of a hairpin loop should yield a double stranded portion of RNA, which is recognized by cellular processing machinery and cleaved into siRNA. Due to the early termination associated with conventional RNA

pol II promoters, it was discovered that driving hairpin loop expression by RNA pol III promoters is ideal for hairpin loop production (73). Using the commercially available pSilencer system (Ambion Inc) we have successfully cloned several immune stimulatory genes. Transfection of DC using pSilencer was effective and the rates of gene-silencing was more potent that administration of siRNA oligos.

Since cloning into pSilencer is time-consuming and requires verification of the cloned sequence, an easier approach to screening sequences was developed by Castanotto et al. This method termed siRNA-expression cassettes (SEC) (74). Basically, a PCR product is made that once transcribed forms a RNA hairpin loop that intracellularly is cleaved into siRNA. Gene-specific SEC are generated by 3 PCR reactions. The first reaction produces the promoter and half of the hairpin siRNA, the second reaction makes the precursor SEC, and the last reaction adds the terminator sequence (74). In our laboratory, once gene-specific primers are available, SEC can be generated in several hours. Since SEC can be designed with restriction sites, it is possible to clone effective SEC sequences into expression plasmids in order to raise large quantities of SEC. The current explosion of interested in siRNA has made it affordable for laboratories to design and test siRNA for gene silencing for a small fraction of the cost of AO.

● Gene Silencing in DC by siRNA

The above-mentioned advantages of siRNA for gene manipulation have prompted our group to investigate this methodology for application into DC for the purposes of immune modulation. Although it is possible to generate Tol-DC *in vitro* and *in vivo*, the current methodology available does not allow for potent *ex vivo* silencing. While we considered using AO initially, the problems associated with non-specific suppressive effects, and the high concentrations needed, were concerns in regards to DC, which are very sensitive to manipulation.

Initially we synthesized siRNA oligonucleotides from Dharmacon Inc to target the potent Th-1 promoting, DC-expressed gene IL-12. Since IL-12 is comprised of 2 subunits (p35 and p40), it was important to determine

the optimum one to silence (75). It is reported that the IL-12p35 is constitutively expressed by DC in absence of activation. The activation inducible p40 heterodimerizing with p35 to produce the bioactive IL-12 p70, in contrast, the constitutively produced p40 homodimerizes to form a biologically active antagonist to the p70 (76). The fact that IL-12 p40 can also heterodimerize with p19 to form IL-23, prompted us to choose p35 as our silencing target (77). Our initial investigations into silencing efficacy demonstrated >95% suppression of bioactive p70 production by as little as 60 picomolar concentration of siRNA as detected by ELISA (78). Subsequent experiments demonstrated that 1) siRNA can be effectively transfected into DC; 2) silencing of p35 did not effect p40 transcription and vice versa; 3) silencing IL-12 led to increased production of IL-10; 4) IL-12 silenced DC promoted Th1>Th2 switch *in vitro* and *in vivo* (79). Our present work has focussed on the longevity of the siRNA-silencing effect on the DC (5 days), investigation of simultaneous multi-gene silencing (so far we have concurrently silenced 3 genes), and the design of DC-specific systems to deliver siRNA *in vivo* (data not shown).

Subsequently, Laderach et al utilized siRNA to silence the p50 component of NF- κ B in human monocyte-derived DC (80). Using presynthesized siRNA oligonucleotides, transfected through electroporation, the paper demonstrated gene-specific inhibition at the transcript and protein level. Furthermore, silencing of p50 was associated with suppressed IL-12 production, but did not alter expression of costimulatory molecules or MHC II (80). Since suppression of NF- κ B using chemical inhibitors in known to block IL-12, costimulatory signals and MHC II expression, the findings of Laderach et al suggest that individual components of NF- κ B complex possess distinct biological activities in DC. Since siRNA is more specific than chemical inhibitors, it is a potent tool for dissecting the cell-specific signalling events that until know were difficult to study.

The utility of siRNA for DC signalling studies was also demonstrated by Wong et al who blocked expression of the neuronal

protein plexin-A1 on to demonstrate dependency of DC:T cell interactions on this membrane-bound protein (81). Targeting of the plexin-A1 transcript in bone marrow-derived DC resulted in suppression of T cell activation. Interestingly the suppression was not mediated by inhibited peptide loading or costimulatory molecules. This novel finding used siRNA to demonstrate that DC-T cell interactions use similar molecular mechanisms as those used by neuron-neuron interactions.

● Future directions

RNA has been of key interest to immunologists back from three decades ago when it was believed that RNA is the critical signal through which APC activate T cells (82). The discovery of siRNA as a potent and specific method of silencing immunological genes will most certainly restimulate the interest of immunologists in the future decades. The vast quantity of genomic and proteomic information being accumulated at a breakneck speed provides an immense number of targets for gene-silencing. Besides individual genes, the upstream controllers of such genes are exciting potential targets for siRNA. The fact that cytokines are controlled by transcription factors, and that certain transcription factors (STAT-6, GATA-3) control Th2 (83), while others (STAT-4) control Th1 (84) cytokine production makes these attractive targets for siRNA immune modulation. The inflammatory-associated transcription factor NF- κ B was previously silenced by siRNA, resulting in reduced IL-12 production (80). Unfortunately, the authors only silenced p50, which is more restricted in activity compared to Rel-B. Reports that Rel-B KO mice possess Tol-DC that are active in inducing tolerance when transferred makes Rel-B an interesting target for siRNA-mediated gene silencing (12).

Problems to be overcome include targeting of the siRNA to DC specifically *in vivo* through specialized delivery mechanisms, or alternatively, driving siRNA expression through DC-specific promoters. Nevertheless, the demonstrated potency, specificity and *in vivo* activity of siRNA will definitely make this a powerful tool for the immunologist in the upcoming years.

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