4-15-2013 12:00 AM

Targeted siRNA Delivery Methods for RNAi-Based Therapies

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Pathology

TARGETED siRNA DELIVERY METHODS FOR RNAi-BASED THERAPIES

Thesis format: Integrated Article

by

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Graduate Program in Pathology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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Abstract

RNAi has great potential in future therapeutics as it has the ability to regulating many disease-related genes. However, many barriers prevent practical applications. To overcome the barriers, the specific targeting, efficient delivery system, the validated gene and the potent siRNA sequence are all vital important. The studies throughout this thesis have been focued on examining the validation of three RNAi therapies for two different disease models: allergic contact dermatitis and melanoma. For allergic contact dermatitis, I developed and tested a novel topical delivery system for siRNAs targeting TNFα (siTNFα) and MyD88 siRNA (siMyD88). While siRNAs applied without the transdermal enhancer are ineffective, treatment with combined siMyD88 and siTNFα significantly attenuated contact hypersensitivity (CHS) symptoms in mice, and did so better than treatment with siMyD88 or siTNFα as single agents. This is the first demonstration of topical gene silencing of TNFα and MyD88 to treat allergic reactions, highlighting a potential clinical use of RNAi therapy for skin and allergic diseases. For melanoma, I examined two RNAi therapies using liposomes attached to mannose and folate ligand, respectively. We developed a novel DC (dendritic cell)-targeted siRNA delivery system using mannosed liposomes (Man-lipo) with encapsulated IDO siRNA (Man-lipo-siIDO), which preferentially knocks down IDO in DCs. Mice treated with Man-lipo-siIDO had a delayed time of onset of implanted murine melanomas, increased survival time, reduced tumour size, and increased reactivity of T cells from spleen and lymph nodes against melanoma antigens. This study supports the concept that Man-lipo-siIDO has potential for development as an immune-targeting therapeutic anticancer agent. We constructed a liposomal folate receptor-targeting siRNA delivery system. Compared to controls (non-treatment mice, mice treated with Folate-lipo-siScramble, or with non-folate-lipo-siBRAF), mice treated with Folate-lipo-siBRAF had reduced tumour volume, lower tumour weight, and reduced expression of PCNA and vascular networks in tumour tissue. This study highlights the potential of Folate-lipo-siBRAF for development as an anticancer therapeutic agent. In conclusion, the therapies for allergic dermatitis that use topical delivery of siTNFα and siMyD88, for melanoma using the Man-lipo-siIDO or the Folate-lipo-siBRAF, have the potential to be effective RNAi therapies to treat allergic dermatitis and melanoma.

Keywords

RNAi therapy, siRNA delivery, Allergic contact dermatitis; siRNA; MyD88; TNF-α, melanoma, IDO, BRAF, mannose, folate, tumour siRNA delivery, topical siRNA delivery.

Acknowledgments

This thesis could not have been accomplished by alone. Lots of supports around me make it possible to accomplishment.

I would like to thank my supervisor Dr. Weiping Min with gratitude for his continued support, guidance and advice throughout my project. He is always supportive and giving plenty of trust to me, which offers plenty of space and freedom to my research. He is also high demanded in independence ability and research quality. His attitude towards research and his supportive help will serve me greatly in the future. It has been an honor and pleasure to be his graduate student.

I would like to express my deep thanks and gratitude to the member of Min lab. With their help, I overcome the most stressful time through the research and thesis writing process. Special thanks to Xusheng Zhang, who gave lots of support and helpful advice during my research.

Further thanks to my advisory committee members. To Dr.Koropatnick, I could not thank you enough for offering lots of cooperation opportunity as well as offering helpful advice and suggestion on both of my experiments, papers and thesis writing. Your enthusiasm inspired me and will continue to inspire me in the future. To Dr. Xiufen Zheng, I would like to thank you for your advice on the project design and problem shooting, epically at the very beginning of my project, your suggestion helps me overcomes lots of difficulties. To Dr. Chandan Chakraborty, I would like to thank you for the scientific presentation skill you taught me and the interested you showed on my study, which encourage me on my way of the project.

I feel thankful to the department of pathology. Special thanks to Tracey, who has been very supportive and helpful in arranging all the events during my graduate study, including advisory committee meeting, comprehensive examination, thesis defense etc. I wish to express my thanks to Dr. Mark Darling, who has shown great interest on my study and very kindly in helping revising my thesis.

Finally, I must express my deepest appreciation and thanks to my family. The unconditional love and support of my mother, Wanyun Chen, My father, Xiang Chen, my mother in law, Yuezhen Su, my father in law, Yuanhan Lin, and my husband, Jian Lin. You have always enough me to strive for the best, to follow my dreams and to be happy. I love you all very much and had it not been for your help, the journey to this point would have been longer and less joyful. Also, I would like to thank my daughter, who is in her six monthes development when I defense. She brings me lots of greatful experience and feelings as a future mother. You all played a pivotal role in helping me accomplish my goals.

Table of Contents

List of Tables

List of Figures

LIST OF ABBREVIATIONS

3-HK	3-hydroxyky-nurenine
5-HT	5-hydroxytryptamine
ACD	Allergic contact dermatitis
AGO2	Argonaute 2
ALM	Acral lentiginous melanoma
AML	acute myelogenous leukemias
APCs	antigen presenting cells
Bin1	bridging integrator
(B-RAF);	v-Raf murine sarcoma viral oncogene homolog B1
D-1MT	dextro-1-methyltryptophan
DAB	diaminobenzidene
DCs	dendritic cells
CHS	animal contact hypersensitivity
CML	chronic myelogenous leukemia
DMSO	Dimethyl sulfoxide
DNFB	1-fluoro-2, 4-dinitrobenzene
DOTAP	1,3-Dioleoyl-3-trimethylammonium propane
DSPE-PEG2000	1,2-distearoyl-sn-glycero-3-

	phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]
DSPE-PEG2000- carboxylic acid	glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000]
dsRNA	double-stranded RNA
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EPR	enhanced permeability and retention
ERBB2	epidermal growth factor receptor 2
ERK	extracellular signal-regulated kinase
FBP	folate-binding proteins
FBS	fetal bovine serum
Folate-DSPE-PEG	folate-distearoylphosphatidylethanolamine-N-poly(ethyleneglycol)
Folate-lipo-Cy3-siBRAF	Folate liposome encapsulated Cy3-labelled B-RAF siRNA
Folate-lipo-siRNA	folate liposome encapsulated siRNA
Folate-lipo-siScramble	folate liposomes encapsulated scramble siRNA

FR	folate receptor
GCN2	general control non-depressible 2
GPR	G protein-couple receptor
GSL I	Griffonia Simplicifolia Lectin I
GTP	guanosine triphosphate
H&E	Hematoxylin and eosin
HER2	epidermal growth factor receptor 2
IB4	Isolectin B4
IDO	indoleamine 2, 3-dioxygenase
IDO-2	indoleamine2,3-dioxygenase-2
IFP	interstitial fluid pressure
IFN	interferons
IκB	inhibitory κB
IKK	inhibitory κB kinase
IL-2	Interleukin 2
IRAK-4	IL-1 receptor associated kinase 4
JNK	c-Jun N-terminal kinases
KYN	kynurenine
KYNA	kynurenic acid
L2K	lipofectamine 200

LCs	langerhans cells
LN	lymph node
M1	macrophages
Man-lipo	mannosed liposomes
Man-lipo-Cy3-siRNA	mannosed liposomes encapsulated Cy3-labelled siRNA
Man-lipo-siIDO	mannosed liposomes encapsulated IDO siRNA
Man-lipo-siScramble	mannosed liposomes encapsulated scramble siRNA
MAPK	mitogen-activated protein kinase
MHC	Major histocompatibility complex
MET	hepatocyte growth factor receptor
MEK	Mitogen-activated protein kinases kinase
mRNA	messenger RNA
miRNAs	microRNAs
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation primary response gene 88

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	nature killer
NMDA	N-methyl-D-aspartate
Non-folate-siBRAF	Non folate liposome encapsulated B-RAF siRNA
Non-folate-lipo-Cy3-siBRAF	Non folated liposome encapsulated Cy3-labelled B-RAF siRNA
Non-mannosed-lipo-siIDO	Non mannosed liposomes encapsulated IDO siRNA
Non-mannosed-lipo-siRNA	Non mannosed liposomes encapsulated IDO siRNA
PAZ domain	PIWI–Argonaute–Zwille domain
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD	programmed death
pDCs	plasmacytoid dendritic cells
PDGFR	platelet-derived growth factor receptor
PEG	polyethyleneglycol
PKN3	proteins kinase N3

PTGS	post-transcriptional gene silencing
qPCR	quantitive polymerase reaction
QUIN	quinolinic acid
RES	reticuloendothelial system
RGP	radial-growth-phase
RISC	RNA Induced Silencing Complex
RNAi	RNA interference
SC	Stratum corneum
shRNAs	short-hairpin RNAs
siMyD88	siRNAs targeting MyD88 siRNA
siScramble	non-targeting scrambled siRNA
siRNAs	small interference RNA
siTNFα	siRNAs targeting TNFα
SNALP	stable nucleic acid lipid particle
SNPs	single nucleotide polymorphisms
SSM	Superficial spreading melanoma
STAT1	signal transducer and activator of transcription 1
TAB-1	binding protein-1
TAK-1	transforming growth factor-activated kinase-1

Tc1	CD8+ cytotoxic T-cells
TDLNs	tumor-draining lymph nodes
TDO	tryptophan2,3-dioxygenase
TGF-β	transforming growth factor beta
TGS	transcriptional gene silencing
Th1	T-helper 1
TIR	Toll/interleukin-1 receptor
TLRs	Toll like receptors
TNF-α	Tumor necrosis factor alpha
TRAF-6	TNF receptor-associated factor-6
Tregs	regulatory T cells
TRY	L-Tryptophan
VEGFR	vascular endothelial growth factor receptor
VGP	vertical-growth-phase

Chapter 1

1 Introduction

1.1 RNAi and siRNA

1.1.1 History of RNAi

Before 1980, RNA was viewed as an inert nucleic acid intermediate for protein production. This view has changed in the 1980s when Sidney Altman and Thomas Cech were recognized with a Nobel Prize for their discoveries describing catalytic RNAs [1, 2] . This development inspired many researchers to shift their attention towards these nucleic acids.

In the late 1980s and early 1990s, many studies demonstrated that expression of sections of a viral genome in plants protected them from infection by the subsequent virus and related viruses [3]. It was also found that transgenes led to the silencing of endogenous genes in a homology-dependent manner and the transgene-mediated gene silencing or viral protection showed sequence specificity [4, 5]. However, in transgene technology, researchers observed two unexpected complications. First, most plants with an antisense transgene did not exhibit suppression of the corresponding endogenous gene [6]. Second, some of the control lines with the sense construct exhibited coordinate suppression (co-suppression) of the transgene and the homologous endogenous gene [4, 7, 8]. Although both the antisense and co-suppression mechanism were known to function at the post-transcriptional level, the mechanisms were actually not well understood and so were not viewed as connected [9].

In 1998, an inspired analysis involving the nematode *Caenorhabditis elegans* revealed that injected double-stranded (ds) RNA containing both sense and antisense RNA preparations could specifically target endogenous mRNA before translation and induce genetic interference. This phenomenon was named RNA *i*nterference (RNAi) by Fire and colleagues [10-12]. Subsequently, (during 1998 and 2000) it was established in plants that if dsRNA was produced, suppression of endogenous gene expression was more efficient than in response to sense or antisense transgenes applied alone. Inverted repeat

transgenes were particularly efficient, but simultaneous expression of sense and antisense RNA was also effective [13, 14].

In 1999, the presumably antisense RNA that guided the degradation of the co-suppressed RNA was discovered when the size was redirected from >100 nucleotides to up to 25 nucleotides [15]. This second important discovery followed from the search for the specific determinant of co-suppression in plants. This link between the short RNAs and RNAi was subsequently confirmed in *Drosophila melanogaster*. The same work also confirmed the prediction that the short RNAs guide a ribonuclease complex called *R*NA *I*nduced *S*ilencing *C*omplex (RISC) to its target RNA in RNAi [16].

The discovery that short RNAs play a key role in RNAi-mediated suppression of gene expressions precipitated an avalanche of discoveries that are relevant to the application of RNAi and co-suppression, as well as understanding of the natural roles of these processes. For example, short RNAs have been characterized in detail and are now known to exist in a double-stranded form, with two-nucleotide overhangs at each 3′ end, and are known as *s*mall *i*nterference RNA (siRNAs) [17].

During and around 2002, several different proteins were identified as associates with siRNAs in ribonucleo-proteins [18, 19]. The mechanism for how dsRNA is processed into siRNAs has also been established. The processing enzyme is known as Dicer, a member of the RNase III family with dsRNA binding regions and a conserved *PIWI – Argonaute – Zwille* (PAZ) domain shared with members of the piwi, argonaute and zwille enzyme family[20], from which it takes its name. ATP is required at several stages in the processing of dsRNA and assembly of RISC [21]. In some instances an RNA-dependent RNA polymerase is also involved in converting a single-stranded RNA into a double-stranded siRNA precursor [22-24].

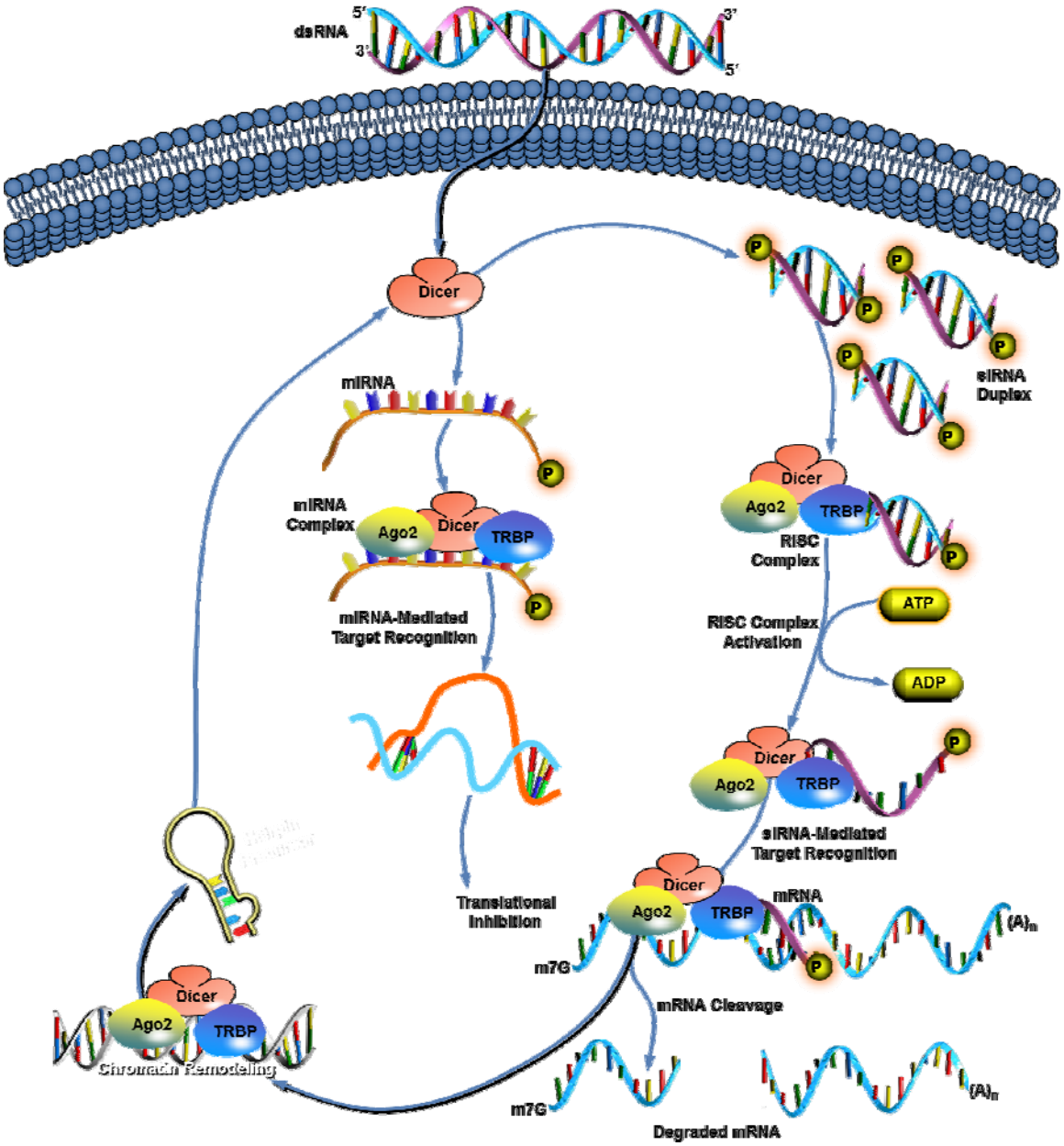
The discovery of RNA-mediated gene silencing, changed our view of gene regulation and led to the development of new genetic tools and methods for selective gene silencing, and have opened a window for developmemt of novel therapeutics against various diseases[25].

1.1.2 Mechanism of RNAi action

RNAi is a naturally evolved mechanism in nematodes, insects and plants, as a result of a developed intrinsic defense against RNA virus and transposons [26]. This characteristic makes it ideal as the basis for a physiologic approach for both *in vitro* and *in vivo* gene silencing [20, 27]. This mechanism has been described in several eukaryotic organisms such as Neurospora [28], *Drosophila spp* [29-31], and mammals [32, 33], including human cell lines and primary cells [34].

RNAi is induced by endogenous and exogenous small RNAs that include siRNAs and microRNAs (miRNAs) [32, 35] (Fig 1-1). The process is initiated through cleavage of dsRNA by a two-subunit RNAse III-family enzyme called Dicer located in the cytoplasm. This results in smaller, 21-23 nucleotide-long dsRNA fragments termed siRNA molecules. Each of these siRNA molecules consists of both a sense and an antisense strand to the homologous RNA target messenger RNA (mRNA) sequence. These siRNA molecules serve as a trigger for the RISC cellular machinery, composed of associated proteins and RNA nucleases, which selectively degrade the target complementary mRNA [20, 36, 37].The endonuclease Argonaute 2 (AGO2) is responsible for the cleavage mechanism of RISC, and AGO2 is the only member of the Argonaute subfamily of proteins with observed catalytic activity in mammalian cells [38]. AGO2 is the critical enzymatic component of RISC: it carries two characteristic domains, named PAZ and PIWI. RISC activation is thought to initially involve AGO2-mediated cleavage of the sense or passenger strand of the double-stranded siRNA [39, 40], yielding the single-stranded antisense strand that serves to guide RISC to complementary sequences in target mRNAs. This guide strand is bound within the catalytic, RNase H-like PIWI domain of AGO2 at the 5′ end [41] and a PAZ domain that recognizes the siRNA 3′ end [25, 42]. The cleavage of targeted mRNA takes place between bases 10 and 11 relative to the 5′ end of the siRNA guide strand [43], resulting in subsequent degradation of the cleaved endogenous mRNA [44]. On activation by the siRNA guide strand, RISC can undergo multiple rounds of mRNA cleavage to mediate a robust *p*ost-*t*ranscriptional *g*ene *s*ilencing (PTGS) response against the target gene [45].

Figure 1-1 RNAi Pathway. Adapted from QIAGEN: http://www.qiagen.com/products/genes%20and%20pathways/Pathway%20Details.aspx?pwid=398


dsRNA
5'
3'
3'
5'
Dicer
siRNA Duplex
miRNA
miRNA Complex
Ago2
Dicer
TRBP
miRNA-Mediated Target Recognition
RISC Complex
RISC Complex Activation
ATP
ADP
siRNA-Mediated Target Recognition
Translational Inhibition
mRNA
m7G
(A)n
mRNA Cleavage
Degraded mRNA
Chromatin Remodeling

PTGS by mRNA cleavage has been exploited as the method of choice for potential therapeutic applications of RNAi because of the potency of this catalytic gene-silencing pathway [46]. In addition to cleavage of perfectly complementary mRNAs, translational repression and transcript degradation (for imperfectly complementary targets), RNAi pathways can also direct *t*ranscriptional *g*ene *s*ilencing (TGS) in the nucleus [47, 48].

1.1.2.1 Maximizing Gene Silencing by RNAi

Maximizing the activity of the effector sequence could be vitally important as it influences conditions for cellular introduction and/or expression and subsequence processing [49, 50]. For example, it is been shown that the stable 5'end of antisense RNA are preferentially incorporated into RISC [49, 51]. Thus, it implies that there are biases in designing RNAi effectors as better guide strands. There are additional biases in other positions; for example, high thermodynamic stability is preferred between nucleotides 5-10 of the guide strand. Furthermore, by comparing multiple sets of active and inactive siRNA sequences, much experience has been accumulated and is being applied to the design of optimally active siRNA sequences. Many design tools have been developed, and have been applied in commercially-available RNAi molecules. To maximize RNAi activity, it is also important to record and organize the increasing number of validated sequences developed by the scientific community and commercial sources. RNAi effectors are designed to correspond to reported active endogenous RNA sequences. Targeted sequences that are similar to unreported sequences in the genome (including, for example, *s*ingle *n*ucleotide *p*olymorphisms or SNPs), might lead to RNAi effectors being unable to efficiently induce RNAi [52]. However, the influence might be less than predicted, since RISC is able to tolerate some mismatches, especially those far from the cleavage site [53, 54]. Despite aberrations in target RNA that could influence RNAi, they also have the potential to be used to more specifically target mutated transcripts that relate to disease. This approach has been applied in several contexts, such as targeting of cancer-specific mutations, targeting of a single-base mutation associated with the dominant genetic disorder spinocerebellar ataxia, and the silencing of mutant β globin as an approach to treat sickle cell anemia [54-56]. In addition to potential

sequence discrepancies, transcript variants of genes targeted for downregulation using RNAi must also be considered when designing RNAi effectors. With increased understanding of RNAi, use of more than one effector against target RNA is recommended when testing the specificity and activity of RNAi-mediated reduction of each target [57].

1.1.2.2 Off-target effects associated with RNAi

To achieve the best silencing efficiency for RNAi effectors, minimization of off-target effects is critical. Off-target effects can result from several events, including both sequence-independent and sequence-dependent processes.

Sequence-independent effects (so-called nonspecific effects) are related to transfection conditions, inhibition of endogenous miRNA activity, and stimulation of pathways associated with the immune response. Examples of sequence-independent nonspecific effects include RNAi machinery saturation [58-60] and immune response against RNAi effector [61-65]. The studies revealing these effects strongly suggest that the use of the lowest effective RNAi effectors, and incorporation of negative control RNAi effectors as comparitors into design of experiments, is essential when planning studies that use RNAi to explore the function of RNAs.

Sequence dependent effects are associated mainly with associations between RNAi effector molecules and non-targeted RNAs that are partially complementary to those RNAi effector molecules: in this case, those non-targeted nucleic acids are silenced by miRNA-like interactions [65]. Sequence-dependent effects also include receptor-mediated immune stimulation where certain short nucleotide sequences are recognized by receptors and are activated by interaction with them [65].

Furthermore, where RNAi is applied *in vivo*, off-target delivery could induce specific or non-specific silencing effects in non-targeted organs, tissues or cells. This possibility is described below (section 1.4, page15).

1.2 Application of RNAi

1.2.1 In vitro RNAi application

When RNAi is used to target gene expression, gene-specific reagents are introduced into cells to trigger 'knockdown' or reduction of gene function via sequence-specific degradation and translational interference of mRNA transcripts. This method has truly revolutionized the field of functional genomics because, unlike other traditional genetic screening methods, RNAi provides a powerful reverse genetic approach, especially for organisms with complex genomes a large number of genes (including mammals). Therefore, RNAi can be used as a powerful tool for genomic studies [66-72]. RNAi screening provides a powerful reverse-genetic approach for large-scale functional analysis in cells grown and studied in culture. Cell-based RNAi screening builds upon established instrumentation and assays, and other methods previously developed for chemical screening that provide a relatively rapid and accessible platform for genome-scale functional studies [66, 68, 69, 71]. A large number of RNAi screens have been performed in cultured insect (*Drosophila*) and mammalian cells [66].

Most RNAi-based experiments are designed to achieve sequence-dependent cleavage and reduction of protein-encoding mRNAs. Although most studies focus on the RNAi analysis of these targets, any RNA species can be targeted (for example, noncoding RNA transcripts or viral RNAs). Only a small number of mammalian cell types can tolerate RNAi induced by large, exogenous dsRNAs (e.g., embryonic stem cells). Thus, in mammalian systems, it is necessary to select from a variety of RNAi effector molecules to achieve specific target reduction without unacceptable toxicity. Effector molecules include siRNA duplexes formed by annealing of two independent RNA strands, or single-stranded RNA molecules that contain a dsRNA domain, termed short hairpin RNAs (shRNAs) may be employed. ShRNAs are synthesized from siRNA duplexes that formed through the annealing of two independent RNA strands, or single-stranded RNA molecules that contain a dsRNA domain. Both are designed to have full complementarity with target transcript mRNA without regions of non-complementarity, resulting in target cleavage and degradation [66].

SiRNAs used to target RNAs in cultured cells can be generated by annealing single-stranded synthetic oligonucleotides. Most synthetic siRNAs consist of 19 perfectly matched complementary ribonucleotides with unmatched, single-stranded 3'dinucleotide overhangs. For ease of synthesis, the synthetic oligonucleotides annealed to generate siRNAs are often deoxyribonucleotides. SiRNAs can also be generated by a number of other methods including *in vitro* transcription, plasmid-based tandem or convergent expression cassettes, polymerase chain reaction (PCR) [57], but siRNAs generated by annealing synthetic oligodeoxynucleotids are most common. Transfected siRNAs can yield a substantial decrease in the steady-state levels of target mRNAs for approximately 24 - 120 h [57].

ShRNAs are designed to mimic miRNA precursors, which are then processed by the endogenous RNAi machinery and loaded into RISC complexes. A number of different shRNA expression systems, where shRNA expression vectors are transiently or stably incorporated into target cells, have been described. Variations in the structure of those vectors include differences in promoter-terminator combinations, linker sequences, flanking sequences, duplex length, and regulatory elements that can be used for spatial and/or temporal specific expression. Additionally, selection markers, used to generate stable cell lines, and unique sequence elements, used to identify active shRNAs among larger populations, have been employed. As opposed to siRNAs, the stable expression of shRNAs allows for a non-transient reduction of targeted mRNAs. Thus, the choice of RNAi effector (siRNA or shRNA) depends on the situation under investigation [57].

In this study, we focus on the transient siRNA RNAi effector over shRNA in treating allergic contact dermatitis and melanoma for several reasons: First, allergic contact dermatitis, a disease induced by the immune systems response to repeated contact with antigens, does not warrant the long-term silencing of anti-immune-response genes, such as Tumour necrosis factor alpha (TNF-α) and Myeloid differentiation primary response gene 88 (MyD88). Consequently, prolonged downregulation of these genes once the antigen has been removed could increase the risk of future pathogen invasion. Second, in targeting the host endogenous immune-tolerance gene indoleamine 2, 3-dioxygenase (IDO) for silencing in melanoma using RNAi, it is understood that this is a normal gene

that is abnormally expressed in host during the course of the disease. Because IDO is not normally expressed in adult tissues, it is expected that the normal immune balance will not be influenced by the silencing effect in the therapy. Third, in targeting the B-RAF in melanoma, we used the RNAi effector (siRNA) that was fully complementary with the normal B-RAF mRNA and transiently transfected rather than stably incorporated into target cells. Thus, rather than long-term silencing, transient silencing of the gene in tumour tissue was preferred. Here, the transient silencing effects of siRNA offer the benefit of repeated application of the effectors without long-term effect. Stable silencing, on the other hand, is mediated by shRNAs that are transported to the cytoplasm via the miRNA export pathway and processed into siRNAs by Dicer. Direct use of synthetic siRNA effectors is simpler, does not involve intermediate processer by Dicer, and usually results in potent gene silencing [73]. Fifth, the method of antisense delivery needs to be considered. ShRNAs are most efficiently delivered by virus while siRNAs can be delivered through many of non-viral vectors, such as cationic lipids or polymers [74]. In our study, siRNAs, but not shRNAs, could be efficiently delivered into cells by a topical delivery system. We also used a lipid based system to achieve tissue or cell specific delivery, which performing well in delivering reagents to targeted tissues and cells in circulation. Moreover, in our lipid based delivery system, we had difficulty in achieving a shRNA-lipid complex size less than 200 nm in diameter, while 100nm has been described as the optimum for nano-particles to achieve tissue penetration and cell entry.

Regardless of the RNAi effector used, it is important to include negative control effector molecules in any RNAi-based experiment. The siRNA controls which are usually commercially available incorporate sequences with minimal complementarity to any endogenous transcript.

1.2.2 In vivo RNAi applications

RNAi has great potential to be used in many genetic or acquired diseases. It can be used to reduce the levels of toxic gain-of-function proteins, trigger cytotoxicity within tumours, and block viral replication [75, 76]. RNAi can be used to regulate the expression of

proteins that are not easily accessed by traditional pharmacological approaches, such as molecules lacking ligand-binding domains or proteins that share high degree of structural homology. Thus, RNAi based therapeutic approaches are especially appealing to achieve a high degree of specificity and to target molecules that are considered to be "undruggable". *In vivo* RNAi-mediated knockdown in mouse liver has been reported as a consequence of high-pressure tail vein injection of both siRNAs and shRNAs [75, 76]. Subsequent in vivo studies have focused on the improved delivery and efficacy of RNAi effectors. These efforts have used the experience gained through two decades of developing ribozyme and antisense-based therapeutics and the gene therapy field as a whole [57, 66].

Several proof-of-principle studies have shown the delivery of fluorophor-labeled siRNA molecules into various organs [77-79]. Beyond that, the specific in vivo knockdown of artificially introduced reporter genes like green fluorescent protein (GFP) or luciferase, or various endogenous target genes, has been described. Due to the body's natural tendency to process injected delivery vehicles in the liver, it has become the common target for antisense therapy, but gene targeting in other organs or in tumour xenografts has been reported as well. Taken together, these studies provide valuable insights into the delivery and efficacy of siRNAs for the induction of RNAi [57, 80, 81]. RNAi-based methods are now a common tool for gene perturbation in mammalian tissue culture cells (mouse, rat, monkey, and human). Beyond cell culture screens, RNAi is being used in a number of in vivo studies where the RNAi reagents are delivered topically and directed to specific tissues or organs, such as the retina, brain, or muscles; used ex vivo (for example in hematopoietic cells); or delivered as transgenes [67, 82].s

1.3 RNAi therapies

RNAi has great potential in future therapeutics as it has the potential to regulate disease-related genes. In essence, all any human disease caused by inappropriately increased activity of one or more genes could, in theory be regulated by RNAi-based therapies [73]. The numbers of RNAi-based preclinical and clinical trials have grown over the past

several years and have included studies in retinal degeneration, dominant-negative inherited brain and skin diseases, viral infections, respiratory disorders, cancer and metabolic diseases [83]. To date, RNAi therapies in clinical trials have targeted approximately 14 different diseases [83]. Numerous siRNA therapies are at preclinical stage. The delivery methods for siRNA drugs are continually improved to maximize the specificity of siRNA delivery while minimizing toxicity and degradation effects that compromise drug efficacy [74]. Strategies exist that use synthetic materials or natural carriers (viruses and bacteria), and the preferred method typically depends on the application, the required duration of the therapeutic, and the targeted tissue.

Three clinical trials have used ex vivo delivery of the siRNA/shRNA therapeutics, in which cells were collected from patients, treated with siRNAs/shRNAs and re-implanted/re-infused back into the patient [81]. This procedure is known as autologous cell therapy. One of the three clinical trials involves use of an anti-tumour bifunctional siRNA (bi-siRNA) for treatment of metastatic melanoma. A phase I clinical trial at Duke University (Durham, North Carolina, USA) is focusing on an siRNA drug to treat metastatic melanoma, a form of cancer that originates in melanocytes. Patients in this study are treated with autologous DCs (dendritic cells) transfected with siRNA in combination with mRNAs that encode for various tumour antigens. The purpose of this therapy is to install an anti-melanoma immune response by alteration of proteasome-mediated processing in combination with increased immune recognition of melanoma-associated antigens [84, 85]. The siRNA-induced knockdown of the proteasome is thought to enhance the presentation of melanoma antigen by the DCs with the aim of provoking a strong immune response against the melanoma cells in these patients. Ideally, cancer cells will be killed through the actions of the patient's own immune system. Part of this thesis (on pages 104 to 133) describes an APC-targeting siRNA therapy for treatment of melanoma. In this approach, I used the strategy of silencing IDO expression in *a*ntigen *p*resenting *c*ells (APCs) in order to reinstall the capacity of endogenous immune cells to recognize tumour antigens and activate anti-tumour immune response against cancer cells.

To date, there are nine siRNA therapies at various stages of clinical testing, all involving intravenous injection as the route of administration. Among these are five that use a cationic liposome delivery technology, four that use the *s*table *n*ucleic *a*cid *l*ipid *p*article (SNALP) carrier developed by Tekmira, and one that uses an siRNA-lipoplex known as AtuPLEXTM [86-90]. A Phase I clinical test that is currently being conducted by Silence Therapeutics (London, UK) that employs an siRNA lipoplex that targets *p*roteins *k*inase *N3* (PKN3) in advanced solid cancers [91, 92]. In two studies described in this thesis (on pages 104 to 133 and 140 to 170), we also employ cationic liposomes as the delivery system and use siRNA lipoplexes as a potential therapy for treatment of melanoma. In our studies, we tested the use of targeting ligands to guide siRNA lipoplexes to specific cell types and achieve specific gene downregulation preferentially in target cells that express molecules on their surfaces capable of binding those ligands.

Systemic delivery methods that often require injection of the siRNA into the bloodstream are being tested for siRNA delivery to the kidney, liver, and some solid tumours [90]. Similarly, RNAi drug candidates can be injected directly into the desired tissue or tumour to release the drug with the degradation of a biodegradable carrier. Tissues such as the eye, lung and skin are suitable for topical delivery siRNA [93].

1.3.1 The potential of RNAi therapy for cancer

Cancer is a disease characterized by self-sufficiency in growth signalling, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion [94-98]. Malignancy is a result of long-term accumulation of genetic and epigenetic alterations. While various cancers and/or different patients commonly share some of those alterations, heterogeneity is usually unique in different tumours and in different patients. As a result, tumour molecular profiles differ from patient to patient and personalized therapy is needed. RNAi has the advantages of specificity, adaptability and breadth of targeting capability, thus giving it great potential to serve as a method for personalized gene therapy for cancer. Antisense therapy in particular has advantages in knocking down RNA targets

that are up-regulated during, and/or are causally related to, cancer progression. In addition, upregulated proteins that mediate malignant characteristics and are, for a variety of reasons, difficult to target with small molecules or antibodies (*e.g.*, poor affinity for target, cross-reactivity with other essential molecules in normal cells leading to unacceptable toxicity) may be good choices for suppression using RNAi targeting the mRNAs that encode them.

1.3.2 Challenges in tumour siRNA delivery

There are many challenges to applying siRNA in cancer therapy, including the major issue of selective delivery of intact siRNA to target cells and cellular compartments. The common barriers for siRNA delivery to both non-solid cancers (*e.g.*, leukemia) and solid tumours are degradation in the blood stream and extracellular fluids (including both the stability of the delivery system and the siRNA itself), surface opsonization and subsequent entrapment by the mononuclear phagocytic system and reticuloendothelial system, and rapid renal clearance [99, 100]. For solid tumours, which make up 85% of human cancers, the delivery of siRNA from injection site to tumour involves transport by the circulation system to tumours, extravasation from tumour vasculature, and transport in tumour interstitium. In this process, diffusion and convection is more important than transcytosis [101]. There are barriers in each of the processes.

Tumours have vasculature that is poorly organized, tortuous, defective and displays location-dependent heterogeneity [102]. Compared to normal tissue, tumours have lower blood flow, higher flow resistance and, as a result, lower exposure to siRNA injected intravenously. On the other hand, tumour blood vessels have discontinuous endothelium which makes them leaky and permeable to larger size molecules (100–780 nm). This property facilitates diffusion of larger siRNA-containing nanoparticles (*e.g.*, 100 nm diameter or more) from blood vessels into the interstitial space to allow passive tumour targeting [102].

In solid tumours, the lymphatic system is impaired, preventing particulates and interstitial fluid from rejoining circulating blood. This enhances retention of macromolecules and siRNA-containing nanoparticles in tumour interstitium which, combined with the effect of leaky blood vessels in tumours, results in *e*nhanced *p*ermeability and *r*etention (EPR) of administered therapeutic nucleic acids in tumours[103]. The absence of lymphatic drainage also increases the *i*nterstitial *f*luid *p*ressure (IFP) which, in turn, reduces the hydraulic conductivity and fluid flow, and results in reduced of convective extravasation and interstitial transport.

Interstitial transportation of siRNA-containing nanoparticles into tumour cells occurs mainly through convection, which depends on hydraulic conductivity and pressure gradient. The structure of the *e*xtra*c*ellular *m*atrix (ECM) or stroma creates barriers for siRNA transport. Proteins found in the ECM such as collagens, glycosaminoglycans, preoteoglycans, fibrous proteins and glycoproteins, pose barriers to siRNA-containing nanoparticle delivery by presenting physical obstacles or by binding to the complex [102].

1.4 Introduction to siRNA delivery

Over the past two decades, altering or manipulating gene expression has shown great potential in a wide variety of diseases. Since the idea of gene delivery illustrates treatment of disorders at the genetic level, the principal of gene delivery has been well discussed and established [104-106]. Classical gene delivery mainly includes two systems: viral vectors and non-viral delivery systems. Viral delivery systems are far more efficient than other gene delivery systems. However, many obstacles such as innate immunity issues, and the potential of oncogenicity and mutation insertion risk limits its wide application [104]. Non-viral delivery systems are considered to be safer and immunologically protective [104].

The choice of delivery system also depends on the differences of RNAi effectors, which includes long dsRNAs, shRNA and siRNA.

Long dsRNAs (200-500 nucleotides) can be injected into animals or (in most cases) into blastocysts to achieve widespread stable incorporation and expression in multiple cells and tissues, delivered by bacteria, expressed as transgenes, or delivered into cultured cells by transfection or bathing cells in dsRNA-containing medium. In endoribonuclease-prepared siRNA methods, long dsRNA are used to produce a pool of small siRNAs that are then transfected into cells [107]. However, long dsRNAs are usually not used in mammalian systems since they trigger an unwanted interferon response that masks gene-specific effects [67].

ShRNAs are usually expressed from plasmids or virus based expression vectors. The first studies applying shRNAs in vivo used plasmid DNA [108]; Most subsequent studies, however, have focused on the use of viral vectors. The choice of viral delivery system usually depends on the cell type under investigation and on the need for short- or long-term shRNA expression. Until now, most shRNA-based studies have employed the standard viral vector expression systems used in traditional gene therapy [86].

siRNAs play an entirely different role in the transcription and expression process for short-term non-viral nucleic acid delivery [109]. siRNAs are potent and specific inhibitors of gene expression and are being used as a new technology for drug target validation, study of functional genomics, design of transgenes and as promising therapeutic agents for diseases with a genetic etiology [110].

In vitro, siRNAs are usually transfected into cultured mammalian cells by standard physico-chemical methods, such as methods based on cationic lipids, cationic polymers or electroporation. Different cell systems might have different efficacious transfection methods [111].

In vivo delivery of siRNA has many challenges. First, naked siRNAs are not stable in serum. They are either easily degraded by RNAase or easily removed by renal clearance, which results it a short half-life in serum. Therefore, some investigators tried to chemically modify the sugars, the backbone or the base of oligoribonucleotides to stabilize the siRNA molecule without impairing capacity to specifically and efficiently knock down the target [112, 113]. However, the cell membrane is hydrophobic and

negatively charged, which creates a barrier by repelling the hydrophilic and negatively charged polymers. Additionally, siRNAs only transiently silence the intended gene product when they enter the cell since the concentration decreases with each cell division [46]. Nanoparticle carriers have the ability to prevent siRNAs from intravascular degradation and to deliver them into cells safely and efficiently and with lowered risk of degradation and/or interaction with non-target molecules *en route* to target cells and tissues. However, in the bloodstream, nanoparticles will encounter many non-target molecules and non-target cells, including monocytes, leukocytes, platelets and dendritic cells. Nanoparticle uptake by immune cells can lead to subsequent (and potentially undesirable) immune responses. Moreover, the physical and chemical properties of the nanoparticle surface, such as size and surface charge, may also lead to haemolysis, thrombogenicity and complement activation, resulting in altered biodistribution and potential toxicity [114].

Second, to achieve effective RNAi application in vivo, barriers to entry into tissues and targeting of intracellular compartments must to be overcome. Nanoparticles for siRNA are ranged from 1nm to 1000 nm in diameter. However, for safe and effective delivery of RNAi to targeted mRNAs, it is now clear that nanoparticles >100 nm in diameter are likely to be trapped by cells of the *r*eticulo*e*ndothelial *s*ystem (RES) in the liver, spleen, lung, and bone marrow when intravenously injected *in* vivo, leading to RNAi effector molecules by activated monocytes and macrophages. Thus, nanoparticles of around 100 nm in diameter are thought to be optimal for avoiding RES clearance [115].

Third, cell entrance and endosomal escape are crucial issues for effective siRNA delivery. Many nanoparticles enter cells by endocytosis [116]. As soon as the nanoparticle is endocytosed by cells, it begins travel in early endosomal vesicles. The early endosome will then fuse with sorting endosomes, which transfer their contents to late endosomes. The late endosomes maintain a low pH through the activity of membrane-bound proton-pump ATPases. Late endosomes will relocate to lysosomes, which are acidic (~pH4.5) and contain nucleases that degrade RNA [117]. Thus, if the nanoparticle is not able to escape from the endosome, siRNAs will not be released into the cell cytoplasm and function as RNAi effectors. Mechanistic approaches designed to promote endosome

escape involve the use of fusogenic lipids, fusogenic peptides, photosensitive molecules, pH-sensitive lipoplexes, and pH-sensitive polyplexes. Fusogenic lipids promote endosomal release by increasing the interactions between liposomal and endosomal membranes. pH-sensitive materials use proton "sponge" effects, in which the delivery material induces an influx of chloride ions and subsequent osmosis, resulting in endosomal rupture and the release of endosomal contents [117].

Fourth, the intracellular factors have drawn the majority of research efforts; less commonly studied extracellular factors in the tumour microenvironment could be more important. For example, in the targeted delivery of cancer cells, nanoparticle must exravasate and move through the ECM to reach the cancer cells. This complex tumour microenvironment poses a challenge for accumulating therapeutic drug concentrations in target cells [118]. The biological and physical properties of the ECM could result in the unpackaging of nanoparticles and the release of their contents prior to delivery to target cells [119]. These dynamic factors *in vivo* stress a limitation of in vitro testing that are inherently static conditions that do not reflect dynamic forces *in vivo* and, therefore, may inaccurately predict *in vivo* effectiveness. To minimize this disparity three dimensional models could be useful in imitating the ECM barriers for nanoparticles delivery [120]. The immune system is also an important extracellular barrier. Particles deviating from an overall neutral electrical charge (i.e. cationic or anionic) are more easily phagocytosed by macrophages like Kupffer cells in liver and dendritic cells in lymph nodes than those in neutral nanoparticles [114]. Besides macrophages, neutrophils can also function as extracellular traps for nanoparticles [115, 121, 122].

1.4.1 Topical siRNA delivery

1.4.1.1 Skin structure

Skin has a surface area that approximately spans over 2 m^2 in the average adult. It shields the body from the environment and protects the body from toxic chemicals, ultraviolet radiation, microorganisms, and mechanical trauma. It is of vital importance in preventing water and electrolyte loss. Skin is also an important organ for excretion of certain

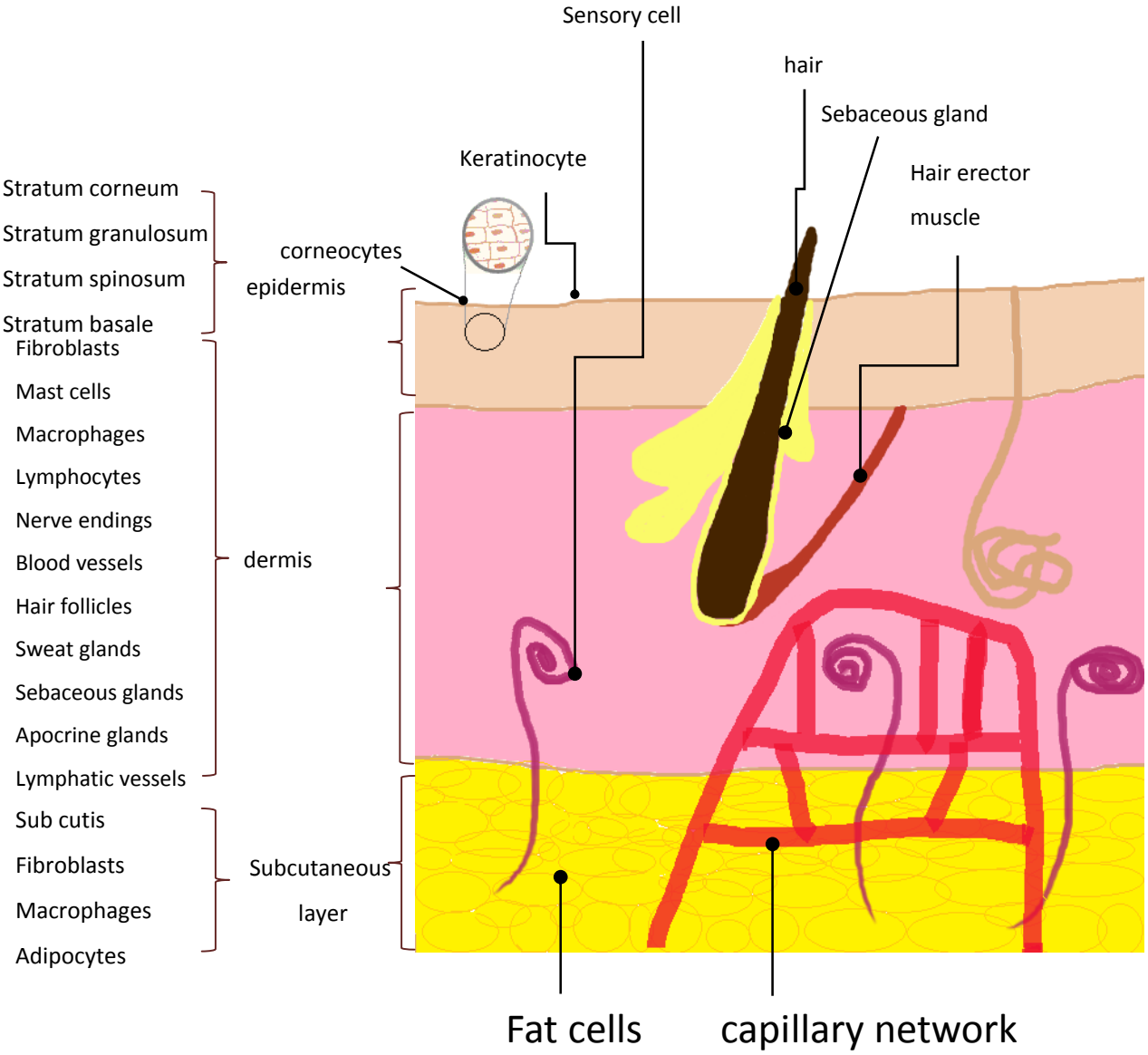
substances, immunological sensing, and in regulating body temperature by direct radiation and secretion of water [123, 124] .

There are three major layers of skin: epidermis, dermis, and underlying subcutaneous tissue (Figure 1-2). The barrier function of skin relies mainly on the epidermis. The epidermis is composed of keratinocytes, including the stratum basale (basal cell layer), stratum spinosum, stratum granulosum, and stratum corneum (from body to surface, in that order). Immediately under the epidermis is the basal lamina, which supports the epidermis. The dermis contains capillary and lymphatic vessels, and skin appendages such as nerve endings, hair follicles, sebaceous glands, and sweat glands. Hair follicles and sweat ducts open directly into the environment at the skin surface and provide the so-called appendageal route of skin permeation, which is very important for topical drug delivery. Below the dermis is the subcutaneous layer which provides insulation, protective padding, and energy storage [125, 126].

1.4.1.2 Advantages and challenges of topical delivery of siRNA

Increased understanding of the components of signaling pathways, coupled with generation of small molecules and antibodies capable of interacting with those components, has profoundly influenced treatment of inflammatory diseases, cancers, genetic disorders, and diseases in skin [127-129]. However, most protein-based therapeutics are costly, have limited targeting ability, and can lead to systemic toxicity when applied systemically [130, 131]. Topical delivery of siRNA is particular attractive for treatment of skin disorders, because proteins larger than a few hundred daltons are not easily delivered into skin, and high concentrations of proteins are necessary for cutaneous effect [132]. SiRNA has great potential for treatment of skin disease by specifically inhibiting expression of one or several proteins. Topical delivery of siRNA allows preferential, direct targeting of skin cells [94]. Cutaneous delivery of siRNA is locally restricted to the affected skin area, the systemic dose of topically applied siRNA is significantly less than for intravenous administration, and topical delivery can lead to

Figure 1-2 Skin structure.


Sensory cell
hair
Sebaceous gland
Keratinocyte
Hair erector
muscle
corneocytes
Stratum corneum
Stratum granulosum
Stratum spinosum
Stratum basale
epidermis
Fibroblasts
Mast cells
Macrophages
Lymphocytes
Nerve endings
Blood vessels
Hair follicles
Sweat glands
Sebaceous glands
Apocrine glands
Lymphatic vessels
dermis
Sub cutis
Fibroblasts
Macrophages
Adipocytes
Subcutaneous
layer
Fat cells
capillary network

high local cutaneous dose (assuming good skin penetration). Generally speaking, molecules less than 500 Da have high penetrating capacity [133], As result, systemic toxicity is reduced [134]. Moreover, the region to which siRNA is applied can be visually monitored or easily biopsied if monitoring of therapeutic effects and/or pathological analysis is required. Also, topically siRNA delivery is user-friendly. Three siRNA topically-delivered drugs are undergoing clinical trial, including siRNA eye drops for the treatment of ocular hypertension and glaucoma (from Sylentis, Inc.), an inhaled siRNA drug for asthma (from ZaBeCor, Inc.), and an siRNA drug for *r*espiratory *s*yncytial *v*irus (RSV, from Alnylam, Inc.)ccz delivered to human patients by nebulizer and nasal spray [126]. However, the cutaneous delivery of siRNA still faces many obstacles. The penetration of oligonucleotides through the epidermis barrier becomes the predominant major technical challenge in cutaneous delivery of siRNA [135].

In vivo delivery of siRNA is challenging, since siRNA itself has difficulty being absorbed by skin and it is relatively unstable and subject to degradation prior to interaction with target RNA [136]. Moreover, siRNA is negatively charged and relatively large: this leads to repulsion by cell membrane (also negatively charged) and difficulty in being taken up by cells through pores in plasma membranes. Regardless of that, keratinocytes have the ability to take up unmodified DNA or siRNA in a mouse skin model [123, 125, 137-139]. This is important in that it suggests that cutaneous gene therapy is feasible. However, keratinocyte uptake of nucleic acids without modification (or encapsulation in delivery vehicle) requires intradermal injection, which is not user-friendly and is painful when repeated injection is necessary. Moreover, injection can applied to only a small area of skin for each injection site, which makes it limited for application to larger skin areas or whole body skin diseases. Thus, penetration-enhancing techniques are needed for cutaneous topical siRNA delivery.

Stratum corneum (SC) in epidermis is the major barrier to cutaneous siRNA delivery. The SC is composed of terminally differentiated nonnucleated keratinocytes (also called corneocytes). These cells run perpendicular to the skin surface and are packed in tight columns. The cellular matrix between corneocytes is composed of hydrophobic non-polar lipids. Those lipids are assembled into lamellar stacks to form a very densely packed

structure [126], that is resistant to permeation by topically-applied siRNA. Moreover, the dense network of polysaccharides and fibrous proteins also creates a barrier for siRNA penetration. Since the basal membrane is an electron-dense adhesion structure, even topically-delivered siRNA that passes successfully through the barrier of the stratum corneum has difficulty in entering cells and reaching reaching target RNA [127].

In my study, I generated a chemical siRNA transdermal enhancer composed of three chemical reagents selected to assist in moving siRNA through multiple barriers in skin and skin cells to reach target mRNA. We applied the new cutaneous delivery reagent and siRNA complex to treatment of allergic contact dermatitis (ACD).

1.4.2 Lipid based siRNA delivery

Currently, most *in vivo* studies use synthetic lipid-based siRNA carriers with or without modifications to the carriers themselves. The beneficial properties of liposomes as drug delivery vectors were first recognized in the 1970s, spurring an increase in biopharmaceutical research on this area that has steadily increased since then [140-142]. Liposomes are spherical soft matter particles consisting of one or more bilayer membrane(s), and are most commonly composed of phospholipids encapsulating a volume of aqueous medium. Liposomes boast many advantages as a drug carrier such as good biocompatibility, biodegradability, low toxicity and structural variability [143].

Cationic liposomes in particular serve as good candidates for the delivery of siRNA. First, properly designed cationic liposomes have demonstrated potent transfection efficiencies both *in vitro* and *in vivo* [144]. Cationic liposomes can tightly compact and encapsulate siRNA through electrostatic interactions, thus protecting the siRNA from circulation dynamics and nuclease degradation while traveling in the vasculature [145, 146]. Second, the size of cationic liposomes can be manipulated through extrusion [147], preventing excretion in the urine when too small or blocking of the microcirculation when too large. Particle size is also of vital importance in optimizing ability to overcome the tissue barrier, transfecting cells efficiently, and regulating surface properties. Third, the

structural variability of liposomes allows a variety of modifications, such as conjugation of ligands or polymers onto the surface for targeting delivery, and addition of other modulator molecules to the interior compartment of liposomes to improve transfection [144]. The cationic surface of the liposome helps to shield the electrostatic repulsion between the cell membrane and naked nucleic acid, aiding in uptake. The cationic liposome is at least 1000 fold more efficient in delivering encapsulated nucleic acids than delivery of naked nucleic acids [148].

1.4.2.1 Mannose liposome delivery

Mannose receptors are highly expressed in cells of the immune system (*i.e.*, macrophages and DCs, especially in professional APCs [149-153]. Transmembrane mannose receptors mediate endocytosis, function as antigen capture receptors and are involved in antigen capture and presentation [154-156]. The preferential expression of mannose receptors in the cells of the immune system makes it a promising target for various therapies, including chemotherapeutic treatments targeting macrophages, vaccines against DCs [157], and gene delivery to APCs. Mannosylation has been successfully applied to vaccination and, in fact, several constructs are currently undergoing clinical trials [158].

Mannosylated particulates are formed by the association of the mannose derivative to a carrier (liposome, nanoparticle, *etc.*). The mannose derivative acts as a targeting agent whereas the particulate or carrier acts as a reservoir for the therapeutic agent. Their particulate nature allows these particles to be engulfed by phagocytic APCs and mannosylation will further improve their phagocytosis mediated by specific receptors.

Compared to other carrier delivery systems, liposomes offer multiple advantages for drug delivery, including their biocompatibility, biodegradability and safety. Mannosylated liposomes are considered to be promising non-live vectors for targeted delivery purposes. In fact, mannosed liposomes have been successfully applied in APCs targeting DNA vaccine against cancer [159] and protein vaccine delivery against *N. meningitides* [160].

1.4.2.2 Folate liposome delivery

Folic acid is a water-soluble B vitamin essential for the *de novo* synthesis of DNA and one carbon transfer reactions in cells [161]. *In vivo*, folate receptor (FR) has high affinity for folate.

FRs, also known as folate-binding proteins (FBPs), are *N*-glycosylated proteins with high binding affinity to folate. FRs include at least four isoforms: α, β, γ / γ ′ and δ. The affinities of folic acid for the FRs are: FR-α , Kd~ 0.1 nM [162]; FR-β , Kd ~1 nM [163]; and FR-γ , Kd ~ 0.4 nM [164]. Functional FR expression is low or absent in most normal tissues, with FR-α expression limited to the luminal surface of certain epithelial cells and inaccessible to blood circulation [165]. In contrast, many malignant cancers such as those arising in brain, kidney, lung, and breast and, in particular, in epithelial carcinomas such as ovarian cancers, consistently and uniformly express high levels of FR-α, which is accessible to circulating blood [161, 166].

FR-β is a differentiation marker in the myelomonocytic lineage during neutrophil maturation [167] and is amplified in activated monocytes and macrophages [168]. FR-β is functionally expressed in certain cancers, such as chronic myelogenous leukemia (CML) and in 70% of acute myelogenous leukemias (AML) [111, 169].

The soluble FR-γ/γ′ is expressed mainly at low levels in certain haematopoietic cells, and has a relatively insignificant role in FR-targeted drug delivery.

FR-δ is expressed on regulatory T cells and has recently been discussed as a potential therapeutic target [170].

The frequent overexpression within tumours and highly restricted distribution in normal tissues suggest that both FR-α and -β can potentially be exploited as a tumour-specific cell surface marker that can be used in the targeted delivery of cancer therapeutics [161, 171].

Folate-based targeting systems have already been successfully applied as a therapeutic agents and imaging agents [172, 173]. Folate liposomes have good biocompatibility,

biodegradability, and safety. Further, liposomes are able to accumulate within tumour tissue due to the passive accumulation of colloidal macromolecules of approximately 40 kDa and above, which contributes to the EPR [174]. The accumulation of liposome in tumour tissue could be further improved through the use of receptor targeting moieties (*i.e.*, folate) that are either conjugated to the surface of liposomes after encapsulation or siRNA, or are attached to lipids that become incorporated within the liposomal bilayer during liposome assembly [175].

Conjugation of folate to a lipid-PEG linker through the γ -carboxyl [171, 176] of folate serves to both anchor the folate molecule to liposomes and maintain its affinity to the FR. Therefore, liposomes that included folate-conjugated lipids are considered to be promising non-live vectors for targeted delivery of therapeutic molecules to tumours.

Two different disease models (ACD and melanoma) were used to examine the three targeted RNAi-based therapies described in this thesis.

1.5 Introduction to allergic contact dermatitis

Contact dermatitis is an eczematous skin reaction caused by direct and usually repeated exposure to harmful objects or chemicals. It is one of the most common skin diseases and may vary from slight hyperkeratosis and small fissures to extensive redness, swelling and scaling [177]. There are three types of contact dermatitis: ACD; irritant contact dermatitis, and photo-contact dermatitis. ACD is known the most prevalent form of immuno-toxicity in humans.

ACD is the consequence of an undesired immune reaction to hapten contacting the skin. It is a type IV delayed hypersensitivity [178, 179]. Its initiation needs the development of the effector T cell response against the hapten, which results in the skin inflammation. There are two phases during this process: the sensitization phase and the elicitation phase.

The sensitization phase begins with contact between skin and hapten and leads to generation of hapten-specific T cells in lymph node (LN). Those hapten-specific T cells

then return to skin. Haptens induce sensitization through two distinct properties: intrinsic pro-inflammatory properties, and by binding to amino acid residues in self-proteins to generate new antigenic determinants in skin [180]. When haptens induce sensitization through their pro-inflammatory properties, they activate innate immune activity in skin tissue and deliver signals which induce the migration and maturation of cutaneous DCs. When haptens induce sensitization by binding to amino acid residues to modify self proteins, the haptens or haptenated proteins are loaded into cutaneous DCs and are expressed as haptenated peptides in the groove of *m*ajor *h*istocompatibility *c*omplex class I (MHC I) and class II molecules of those cells. Those DCs will migrate from the skin to regional lymph nodes (LNs) where they are capable of priming $CD8^+$ and $CD4^+$T cells for recognition of those antigens. T cells will then proliferate and migrate out of LNs and circulate between the lymphoid organs and the skin. The sensitization step lasts 10 to 15 days in man, and 5 to 7 days in the mouse.

In the elicitation phase, the challenge with the same hapten in the sensitized individuals leads in the apparition of ACD. When haptens diffuse into the skin and are taken up by skin cells that express MHC I and II/haptenated peptide complexes, hapten-specific T cells are then activated in the dermis and epidermis and trigger the inflammatory process responsible for the cutaneous lesions. Understanding of ACD derives from experiments undertaken in animal *c*ontact *h*yper*s*ensitivity (CHS) model. $CD8^+$ cytotoxic T lymphocytes are the main effector cells of CHS and they are recruited early after challenge. The elicitation phase of CHS takes 72 hours in man, and 24 to 48 hours in mouse. The inflammatory reaction persists for only a few days and then rapidly decreases following down regulatory events [178].

1.5.1 The role of TNFα and MyD88 in contact dermatities

TNFα is a cytokine known to be involved in systemic inflammation. The primary role of TNFα is in the regulation of immune cells [181]. It activates neutrophils, up-regulates expression of the endothelial adhesion molecules, increases capillary permeability and has a direct cellular toxic effect. In the CHS model, the release of TNFα can activate

local endothelial cells and facilitate T cell recruitment [181, 182]. Among the mediators of inflammation, TNFα is one of the most important factors and plays a major role in the elicitation of CHS [183]. TNFα that released by mast cells and platelets is important in initial recruitment of T cells [184, 185].

The MyD88 is critical for signaling from all *T*oll *l*ike *r*eceptors (TLRs) except TLR3 [186], and is expressed in a variety of human and murine tissue albeit at varying levels [187]. Its involvement in the *T*oll/*i*nterleukin-1 *r*eceptor (TIR) signaling pathway, results in NF-κB activation, cytokine secretion and and inflammation. MyD88-deficient mice are unable to mount a CHS response [188].

Most information about ACD has been acquired using a mouse model of induced CHS [183]. In this study, we use this model and 1-fluoro-2, 4-dinitrobenzene (DNFB)-induced CHS in mice, which is well-established as a model inducer of CHS to model ACD [189].

1.6 Introduction to melanoma

Melanoma is the sixth most common cancer and the human malignancy with the highest recent rise in incidence (a rise that continues) [86]. Melanomas can arise at any anatomical site occupied by monocytes. However, cutaneous melanoma, which develops from epidermal skin melanocytes, represents the most common site of origination. In this study, I focus on cutaneous melanoma. Cutaneous melanoma is the deadliest form of skin cancer and it is arguably one of the most aggressive malignancies overall. Classically, the pathological workup for melanoma includes description of the growth pattern and measurement of vertical thickness (also called Breslow) [131]. Superficial spreading (nodular, acral lentiginous, lentigo maligna, lentiginous and desmoplastic) are all included in histological growth patterns. Although prognosis depends on lots of data, such as ulceration and penetration through cutaneous layers or mitotic rate, Breslow thickness is the critical prognostic parameter.

In 2009, the American Joint Committee on Cancer (AJCC) updated melanoma TNM classification categories [132] (Table 1-1, 1-2) . Mitotic rate and ulceration become

Table 1-1TNM classification AJCC 2009

TNM classification AJCC 2009 [132]		
T	**Thickness**	**Ulceration, Mitotic rate**
T1	Up to 1 mm	a. No ulceration and <1 mitosis/mm^2 b. Ulceration or >1 mitosis/mm^2
T2	01 - 2 mm	a. No ulceration b. Ulceration
T3	1-4 mm	a. No ulceration b. Ulceration
T4	>4 mm	a. No ulceration b. Ulceration
N	**Number of nodes**	**Tumour burden**
N1	1	a. Micrometastasis b. Macrometastasis
N2	2-3	a. Micrometastasis b. Macrometastasis c. In transit satellites but no distant nodes
N3	>3 or in transit satellites with metastatic nodes	
M		
M1a	Distant soft tissues, normal LDH	
M1b	Lung, normal LDH	
M1c	Other viscera or elevated LDH	

Table 1-2AJCC 2009 staging classification

AJCC 2009 staging classification [132]		
IA	**T1a**	**N0M0**
IB	**T1b**	
IIA	**T2bT3a**	
IIB	**T3bT4a**	
IIC	**T4b**	
IIIA	**T1a-T4a N1a-N2a**	**N+M0**
IIIB	**T1b-T4b N1a-N2a** **T1a-T4a N1b-N2b-N2c**	
IIIC	**T1b-T4b N1b-N2b-N2c** **Any N3**	
IV	**Any M1**	**M1**

important with tumour thickness in patients with localized melanoma. The N category depends on the number of positive lymph nodes, tumour burden, and ulceration in patients with metastatic lymph nodes. Among patients with localized disease, it is important to define a subgroup of patients with a greater rate of relapse. This population receives adjuvant therapy [131].

Patients with stage IIB, IIC or III disease, such as those with ulcerated tumours with Breslow thickness 2-4 mm, tumours with Breslow thickness >4mm, or tumours with positive lymph nodes are considered to be at high risk [131]. Among those at high risk, differences in prognosis can be marked. For example, while patients with stage IIIA disease have a ten-year overall survival rate of over 70%, stage IIIB and IIIC have only 40% and 25% ten year survival rates, respectively. Stage IV survival rate depends on tumour burden and the location of metastases. While average survival in this stage is 6-7 months, less than 10% of the patients survive longer than one year [131]. There is promise that this survival rate will be increased by application of inhibitors of v-Raf murine sarcoma viral oncogene homolog B1 (B-RAF) in the future [132]. For several decades progress in melanoma treatment has been slow. However, new staging classification, insights into the patterns of relapse at early stage, better understanding of the molecular biology underlying the malignant characteristics of melanoma, and development of new drugs for treatment of advanced diseases, have all increased progress. Effort has now focused on identification of altered gene pathways (knowledge of which is known to be helpful in diagnosis) and the development of new therapeutics [131]. The identification of mutant B-RAF as a major driver of melanoma progression highlighted targeting of B-RAF as a therapeutic opportunity in treatment of melanoma [131].

Several years following discovery of B-RAF mutations in melanoma, the first selective inhibitor (vemurafenib) entered clinical trials. However, although tumour regression during and after the early course of the therapy was observed, resistance to therapy emerged during subsequent treatments. The response duration ranged from 2 to >18 months. Current research focuses on mechanisms underlying resistance to B-RAF [190]. Because siRNA therapy targets a major mechanism underlying resistance to targeted

agents (*i.e.,* target overexpression), it's use may be desirable to help avoid resistance that can develop following treatment with small molecule inhibitors that target specific proteins without reducing mRNAs available to mediate increased synthesis of additional target molecules. Moreover, targeting mRNA sequences has increased potential to enhance target specificity and reduce off-target effects.

Melanoma is characterized as one of the most immunogenic tumours due to the presence of tumour infiltrating lymphocytes in resected melanoma, occasional spontaneous regressions, and clinical responses to immune stimulation. The immunogenicity of melanoma has led investigators to study novel immune strategies to overcome tumour immune evasion [191]. One mechanism relates to indoleamine 2, 3-dioxygenase (IDO), a molecular express on APCs could block T cell activation and induce T cell apoptosis, generating more regulatory T cells and thereby create a tolerance immune environment for tumour progress. In 2007, dextro-1-methyltryptophan (D-1MT), a classical IDO inhibitor, entered clinical trials. It is able to influence complex culture systems, in which many proteins take place in immunoregulatory role. However, it observed that D-1MT did not inhibit purified IDO [130]. A paradox has emerged that the immunoregulatory role of D-1MT needs IDO expression to function whereas its ability of inhibiting purified IDO is weak. The mechanism of why D-1MT needs IDO expression in mice to function and its effect in clinical trials still need to be further studied. As the inhibitory activity for both of the isomers (D-1MT and L-1MT) of 1MT are weak, it is questionable whether they will ever be useful in the clinic as IDO inhibitors. Gene silencing by therapeutic nucleic acids, particularly siRNA, is a promising way of specifically inhibiting the expression of a protein with potential in cancer therapy [192].

Two difference approaches, in which two molecules that have great potential of being used as therapeutic molecular in the future have been targeted in our study: IDO and B-RAF.

1.6.1 Introduction to IDO

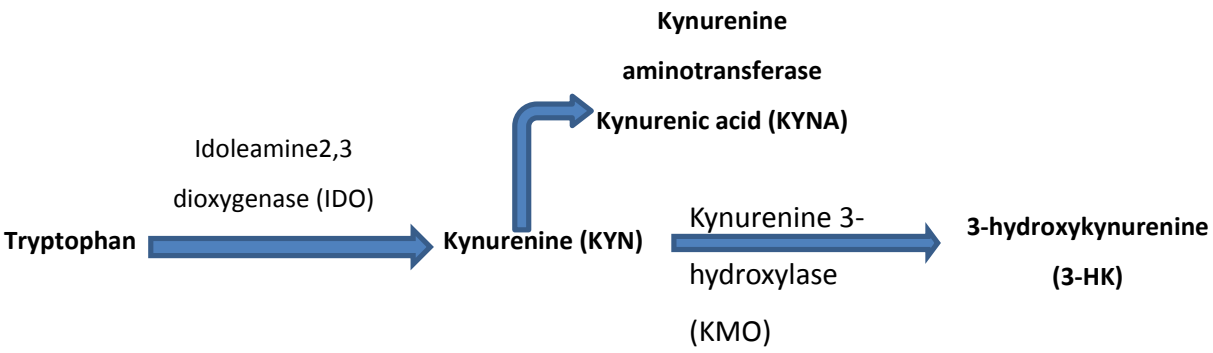
Cutaneous melanoma arises from the largest organ with immune function, the skin. Neoplastic cells can bypass immune surveillance by influencing and inhibiting immune cell function at virtually all stages of tumour development, including initiation, promotion/progression, and tumour invasion and metastases. IDO expression in DCs has been identified as a property of those cells that decreases DC function and contributes to immune tolerance of tumours. IDO is the first and rate-limiting enzyme of tryptophan catabolism in the kynurenine pathway (Figure 1-3), thus depleting tryptophan and reducing T cell growth and activity [193].

1.6.1.1 Tryptophan and kynurenine pathway

L-Tryptophan (L-Trp) is an essential metabolite in mammals and the least abundant of all essential amino acids (Figure 1-3). It must be metabolized through several strictly controlled pathways to form biologically active compounds [194]. These compounds include: aminergic neurotransmitter serotonin (5-hydroxytryptamine, 5-HT), neurohormone melatonin, several neuroactive metabolites of melatonin, products of the kynurenine (KYN) pathway, and tryptamine [195]. Among the several metabolic pathways for tryptophan, the kynurenine pathway is the main route of non-protein metabolism (Figure 1-3). Under normal circumstances, protein catabolism is balanced by protein synthesis so that the protein-related pool of tryptophan remains fairly constant. While the protein related pool of L-Trp is constantly balanced, approximately 99% of dietary intake L-Trp is processed by the kynurenine pathway of catabolism [196] making its importance vital in L-Trp metabolism.

In the kynurenine pathway, IDO and tryptophan 2,3-dioxygenase (TDO) are cytosolic heme dioxygenases that catalyze the oxidative cleavage of the C2-C3 bond of the indolic ring of L-Trp. This reaction is the first and rate-limiting step of the kynurenine pathway of tryptophan catabolism [197-199].

Figure 1-3 Schematic representation of kynurenine pathway


Kynurenine
aminotransferase
Kynurenic acid (KYNA)
Idoleamine2,3
dioxygenase (IDO)
Tryptophan
Kynurenine (KYN)
Kynurenine 3-
hydroxylase
(KMO)
3-hydroxykynurenine
(3-HK)

After the transformation of tryptophan into kynurenine, the action of enzymes along the kynurenine pathway will then convert kynurenine into other catabolites [200, 201]. Kynurenine 3-hydroxylase (kynurenine 3-monooxygenase) converts KYN to toxic3-hydroxyky-nurenine (3-HK) [198]. One of the end products is quinolinic acid (QUIN), an agonist of the *N-m*ethyl-*D-a*spartate (NMDA)-sensitive glutamate receptors [202]. Another arm of the kynurenine pathway produces kynurenic acid (KYNA), which is an endogenous antagonist of NMDA receptors. Moreover, KYNA can noncompetitively block the activity of α7-nicotinic acethylcholine (α7-nACh) receptors [203-205].

Expression analysis indicates that the *G p*rotein-*c*oupled *r*eceptor (GPCR) GPR35 is selectively present in immune and intestinal tissues and is also a receptor of KYNA [206]. The KYN pathway has been widely studied in relation to neurodegenerative or excitotoxic insults [207]. KYNA protects the central nervous system in excitotoxic neurological diseases [198, 208] while quinolinic acid can act as a neurotoxin [209-213]. Various physiological and pathological processes are related to ‘‘kynurenines’’, the collectively term of metabolites of kynurenine pathway [214].

1.6.1.2 IDO in the immune system and tumour immunity

Interest in the role of kynurenines in immune function has grown. Stone and coworkers [209, 215] demonstrated that QUIN might play an important role in normal immunosurveillance. In recent years, the role of kynurenine pathway in both the innate and adaptive immune responses has been widely studied, and tryptophan metabolism and kynurenine production reflect a crucial interface between the immune and nervous systems [216].

In mammals, it is noteworthy that excess dietary tryptophan is not catabolized by IDO through the kynurenine pathway. Tryptophan dioxygenase and nicotinamide adenine dinucleotide levels are not maintained by synthesis, but by salvage. It implies that tryptophan levels are maintained for purposes other than simply satisfying requirements for protein synthesis. This was revealed by Munn and his colleagues in 1998, who also

showed that IDO suppresses maternal T cell activity against embryonic tissues by catabolizing tryptophan [217].

Since then, the role of IDO as a crucial enzyme in kynurenine pathway, and in immunoregulation during infection, pregnancy, autoimmunity, transplantation and neoplasia has been widely investigated [218, 219]. Recently, a novel enzymatic isoform of IDO was described and termed indoleamine 2,3-dioxygenase-2 (IDO-2) (Ball *et al.* 2007, 2008). IDO and IDO-2 have a high degree of sequence homology (43% identity). IDO-2 is predominantly expressed in murine kidney, liver, and in the male and female reproductive system where, similar to the activity of IDO, it catalyzes oxidative cleavage of a broad range of indole-bearing substrates [195, 220, 221].

In fact, the details of how IDO modulates the immune system are still not completely clear. Two main theories have been proposed: the first pertains to starving cells of tryptophan, while the other revolves around tryptophan metabolites. In the tryptophan starvation theory, IDO has an effect on mammalian T cells similar to its effect in bacteria [222, 223]. That is, tryptophan depletion induces cell cycle arrest in mammalian T lymphocytes [220, 224] and makes these cells more vulnerable to apoptosis [225]. An IDO-induced decrease in tryptophan concentration results in cell cycle arrest and anergy (a state of unresponsiveness in peripheral T cells)[226, 227].

The tryptophan metabolite theory, on the other hand, is based on the observation that certain downstream tryptophan-derived metabolites, mainly kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and QUIN are toxic to lymphocytes [228]. These molecules cause cell cycle arrest and apoptosis [229, 230], and can induce differentiation of naive $CD4^+$ T cells into immunosuppressive regulatory T cells (T_{regs})[231].

Much work has gone into understanding the relationship between IDO and tumour immunity. IDO^+ plasmacytoid dendritic cells (pDCs) are present in tumour lesions and in *t*umour-*d*raining *l*ymph *n*odes (TDLNs) at sites of tumour growth in mice and humans. Melanoma patients with abnormal accumulations of IDO^+ cells with DC markers in human sentinel LN draining sites had poorer clinical outcomes [98, 232].

IDO also promotes tumour development through its effect on T cells. Cytotoxic T-Lymphocyte Antigen 4 ($CTLA4^+$) T_{regs} ligate B7 on pDCs, maintaining IDO activity in pDCs [233-235]. Blockade of CTLA4/B7 interactions had a significant impact on the reduction of IDO enzymatic activity and T_{reg} activation. IDO-activated T_{regs} mediated suppression via a unique and distinctive mechanism that depended on intact programmed death (PD)-1/PDL-1 signaling, which was not dependent on Interleukin 2 (IL-2), IL-10 or transforming growth factor beta (TGF-β) [236, 237].

Increased expression of IDO through a pathway involving *s*ignal *t*ransducer and *a*ctivator of *t*ranscription *1* (STAT1) and *n*uclear *f*actor *kappa*-light-chain-enhancer of activated *B* cells (NF-κB) caused the loss of *b*ridging *i*ntegrator (Bin1)[238]. Bin1 loss is evident in various cancers [219, 239], including those arising in breast [240], prostate [241], colon, and lung [242]. Therefore, there is a link between loss of Bin1 and increased levels of IDO. This link is tentative, however, and requires further investigation to be confirmed.

We used mannosed-liposome and IDO siRNA complexes to silence IDO expression in DCs in melanoma-bearing mice. We hypothesized that this strategy would reinstall anti-tumour immunity and restrict melanoma xenograft growth through host mouse immune activity.

1.6.2 Introduction to B-RAF

The RAS gene family harbours some of the most frequently-detected genetic alternations in epithelial cancers and plays a central role in both normal and malignant cell growth [243]. The first identified downstream effectors of RAS were the RAF serine/threonine kinases [244-246]. There are three members in RAF family; A-RAF, B-RAF and C-RAF (or RAF1), which have a high degree of homology in three conserved regions. Each kinase has a RAS-binding domain and cysteine-rich domain that mediates interaction with guanosine triphosphate (GTP)-bound RAS [247, 248]. In association with RAS, RAF is recruited to the plasma membrane and the inhibitory intramolecular interaction of the RAF catalytic domain with the NH_2 terminus is diminished, leading to

phosphorylation of sites located in the catalytic loop of the activation domain (Thr599 and Ser602 of B-RAF) [249]. Although all RAF isoforms are similar in these initiating events, additional activation outside the catalytic domain (Tyr340, Tyr341, and Ser338 in C-RAF) is required for full activation of A-RAF and C-RAF.

Since oncogenic B-RAF mutation was discovered in 2002, it stands as one of the most powerful affirmations of the transformative potential of systematic cancer genome characterization [250, 251]. Amino acid residues corresponding to sites of oncogenic mutation in the B-RAF gene are either substituted with aspartic acids (Asp448 and Asp449) or seem to be constitutively phosphorylated (Ser446) in tumour cells [252]. These changes highlight key events that reveal that B-RAF is the most important physiologic *m*itogen-*a*ctivated *p*rotein *k*inase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) activator. It is reported that MEK/ERK activation was significantly disrupted in B-RAF $^{-/-}$ embryonic fibroblasts [253] but not in cells lacking A-RAF or C-RAF [254], which further supports a predominant role for B-RAF as an important MEK activator. Taken together, these data provide a mechanistic explanation for the observation that B-RAF mutations, but not comparable mutations in the other RAF proteins, are thought to underly the propensity of B-RAF for MEK/ERK activation.

B-RAF mutations have been reported to be present in over 70% of melanomas [255]. The mutations are also found in premalignant atypical or dysplastic nevi, implicating B-RAF activation as an initiating event in tumourigenesis [248, 256]. While there was a lower frequency of B-RAF mutations in liver, pancreas, nonsmall cell lung cancer, glioma, and acute myelogenous leukemia, a high frequency of B-RAF mutations were detected in papillary thyroid carcinomas, serous ovarian cancers, and colorectal serrated adenocarcinomas. Nearly 90% of B-RAF mutations are a T1799A transversion in exon 15 that results in Val600Glu (V600E) amino acid substitution, which leads to constitutive kinase activation [257]. Interestingly, less than 1% of the melanomas carry both B-RAF and RAS mutations (the latter occurring in more than 10% of melanomas), which provides strong genetic evidence of direct RAS/B-RAF signaling pathway in this tumour type. Moreover, even in cancers without RAS or B-RAF mutations, increased RAF/MEK/ERK signaling can arise from other mechanisms [258, 259] including

increased signaling from *e*pidermal *g*rowth *f*actor *r*eceptor (EGFR), *h*uman *e*pidermal growth factor *r*eceptor 2 (ERBB2 or HER2), platelet-derived growth factor receptor (PDGFR), hepatocyte growth factor receptor (MET), and *v*ascular *e*ndothelial *g*rowth *f*actor *r*eceptor (VEGFR), and loss of downstream negative regulators such as MAPK phosphatase 3 (DUSP6) and RAF kinase inhibitor protein. Therefore, it is thought that the majority if human tumours, not only those with RAS or B-RAF mutations, engage the RAF/MEK/ERK signaling cascade. In general, B-RAF is an attractive therapeutic target [260].

In our study, we used a lipid-based tumour-targeting siRNA delivery system and a local topical siRNA delivery system to delivery B-RAF siRNA to cutaneous melanoma. We hypothesized that these delivery systems can preferentially target tumour cells and achieve B-RAF silencing in cutaneous melanoma, thereby restricting tumour growth.

1.7 References

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Chapter 2

2 A novel topical delivery reagent for siRNA-based contact dermatitis therapy: preventing allergic dermatitis by topical administration of MyD88 and TNFα siRNAs

2.1 Summary

Topical delivery of siRNA is an attractive strategy to treat skin disorders that are resistant to drug treatment, including allergic contact dermatitis (ACD). Topical siRNA has the potential to specifically target critical disease-mediating RNAs while reducing the risk of systemic toxicity. However, transdermal delivery of siRNA is challenging because of the dense, occluding structure of the dermis and the chemical characteristics of siRNA. To achieve cutaneous siRNA delivery for treatment of ACD and other skin diseases, a potent transdermal enhancer is needed. ACD is a type IV delayed hypersensitivity initiated by skin sensitization to an allergen. Understanding of the pathophysiology of ACD is derived primarily from elucidation of events underlying contact hypersensitivity (CHS), where skin inflammation is induced skin contact by haptens. TNFα and MyD88 are inflammatory cytokines, involved in CHS progression, that are potential CHS treatment targets. In this study, we develop and tested a novel topical delivery system for siRNAs targeting TNFα (siTNFα) and MyD88 siRNA (siMyD88). The topical delivery system resulted in *in vivo* mouse dermis cell gene silencing efficiency of 62% for siMyD88 and 54% for siTNFα siRNA, while siRNAs applied without the transdermal enhancer are ineffective. Treatment with combined siMyD88 and siTNFα significantly attenuated CHS symptoms in mice, better than treatment with siMyD88 or siTNFα as single agents. Combined siMyD88 and siTNFα reduced inflammatory cell infiltration into skin and local accumulation of $CD8^+$ and $CD4^+$ T cells. In summary, we have developed a novel delivery system for effective delivery of siRNA into skin cells for relief of CHS symptoms in mice. This is the first demonstration of topical gene silencing of TNFα and MyD88 to treat allergic reactions, highlighting a potential clinical use of RNAi therapy for skin and allergic diseases.

2.2 Introduction

Topical delivery of therapeutic drugs is a particularly attractive strategy to treat skin disorders [1]. It maximizes direct drug contact with target skin cells while minimizing systemic exposure [2] as a method to increase treatment potency and reduce off-target toxicity through high local (but low systemic) dosage, and to maximize ease of use [3]. However, cutaneous delivery of therapeutic agents is challenging due to tight packing of corneocytes within a dense hydrophobic cellular matrix, and the dense network of polysaccharides and fibrous proteins in the stratum corneum [4] all of which constitute a physical barrier to drug entry. Potent transdermal delivery enhancers are essential to facilitate drug uptake.

Contact dermatitis is an eczematous skin reaction caused by direct and usually repeated exposure to harmful objects or chemicals. It is one of the most common skin diseases and may vary from slight hyperkeratosis and small fissures to extensive redness, swelling and scaling [5]. There are three types of contact dermatitis: allergic contact dermatitis (ACD), irritant contact dermatitis, and photo-contact dermatitis. ACD is the most prevalent form of immunotoxicity in humans, and is a Type IV delayed hypersensitivity reaction mediated by hapten-specific T cells that generate inflammatory signals and induces apoptosis in epidermal cells. Inflammation and apoptosis result in skin inflammatory infiltrate and adverse clinical symptoms [6]. There are two phases during this process: the the sensitization phase and the elicitation phase. The sensitization phase occurs after the first contact of the hapten and the skin. The elicitation phase is also known as challenge phase, which occurs when the haptens challenge the sensitized individuals [7]. Our understanding of ACD derives from experiments undertaken in an animal contact hypersensitivity (CHS) model. In this study we use 1-fluoro-2, 4-dinitrobenzene (DNFB)-induced CHS (a well-established animal CHS disease model) to explore the efficacy of a novel siRNA topical delivery vehicle, and targeting of TNFα and MyD88, to treat ACD.

RNAi is an endogenous mechanism in which double-stranded RNA knocks down the expression of homologous genes to induce gene silencing. Since it was first observed in late 1980s, it had been used as a powerful laboratory technique to explore gene function [8]. Recent research on animal disease models of cancer, viral infection, age-related

macular degeneration, and other pathological conditions has resulted in development of RNAi molecules for treatment of human diseases. However, the lack of specific and effective *in vivo* delivery methods is a major impediment to clinical application [9, 10].

*T*umor *N*ecrosis *F*actor alpha (TNFα) is a systemic cytokine mediating inflammation. TNFα activates neutrophils, up-regulates endothelial adhesion molecules, increases capillary permeability, and exerts direct cellular toxic effects [11]. In the CHS model, release of TNFα can activate local endothelial cells and facilitate T cell recruitment [11, 12].

The *My*eloid *D*ifferentiation primary response gene *88* (MyD88) is critical for signaling from all Toll-like receptors (TLRs) except TLR3 [13]. MyD88 is expressed in a variety of human and murine tissue albeit at varying levels [14]. It involves Toll/interleukin-1 receptor (TIR) signaling pathway, which results in NF-κB activation, cytokine secretion and inflammatory response. Recently, Paul A. Klekotka's study shows that MyD88 deficient mice were unable to mount an CHS model response [15].

In this study, we describe a novel topical RNAi delivery therapy for CHS. We report that topical administration of siRNA encapsulated in the new delivery vehicle silences expression of MyD88 and TNFα in dermal cells and inhibits progression of CHS by reducing local accumulation of inflammatory $CD4^+$ T cells and $CD8^+$ T cells.

2.3 Materials and Methods

2.3.1 Chemicals

1,2,3-Propanetriol (glycerol) and 1-fluoro-2,4-dinitribenzene (DNFB) were purchased from Sigma-Aldrich, Inc. (Oakville, Ontario, CA). Dimethyl sulfoxide (DMSO) was purchased from Caledon Laboratory Chemicals, Inc. (Georgetown, Ontario, CA) and lipofectamine 200 (L2K) was purchased from Invitrogen Life Technologies, Inc. (Burlington, Ontario, CA).Glycerol was purchased from VWR International, Inc. (Edmonton, Alberta, CA)

2.3.2 Mice

BALB/c mice (male, 6-8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained under pathogen-free conditions in the barrier facility at The University of Western Ontario. All experiments were performed in accordance with the *Guide for the Care and Use on Animals Committee Guidelines* and standard operating procedures as required by the Animal Use Subcommittee of The University of Western Ontario.

2.3.3 siRNAs

Double-stranded siRNAs targeting murine TNFα siRNA (siTNFα) and MyD88 siRNA (siMyD88), and control non-targeting scrambled siRNA (siScramble) were obtained from Dharmacon, Inc. (Lafayette, CO, USA). The sequences were:

siTNFα: sense, 5'-GACAACCAACUAGUGGUGC-3'

antisense, 5'-GCACCACUAGUUGGUUGUC-3'

siMyD88: sense, 5'-CAGCGAGCUAAUUGAGAAAUU-3'

antisense, 5'-UUUCUCAAUUAGCUCGCUGUU-3'

siScramble: sense, 5'-CGUACGCGGAAUACUUCGA-3'

antisense, 5'-CGTACGCGGAATACTTCGA-3'

2.3.4 In vitro silencing of TNFα and MyD88

B16F10 cells were transfected with siTNFα and siMyD88 using L2K. Untreated cells and cells transfected with siScramble were used as controls. Briefly, cells were plated in 24-well plates (1.5×10^5 cells per well) and allowed to grow for approximately 16 h (80% confluence), transfected with siTNFα (1 μg/well) and/or siMyD88 (1 μg/well) in 500 μl

serum-free medium for 4 h, and then incubated in complete medium for 24 h. Cells were then harvested for RNA isolation.

2.3.5 *TNFα and MyD88* mRNA measurement

After *in vitro* transfection with siTNFα or siMyD88, total tissue or cellular RNA was isolated using Trizol (Invitrogen, Burlington, Ontario, CA) and used as a template for cDNA synthesis and non-quantitative PCR and quantitative PCR (qPCR). qPCR reactions were performed as described previously [16] using gene-specific forward and reverse primers (100 nM each) and SYBR Green PCR Master mix (Stratagene, La Jolla, CA) in a Stratagene MX 4000 Multiplex qPCR System. Primers used for the amplification of murine TNFα, MyD88 and GAPDH (control to assess relative levels of TNFα and MyD88 mRNA) were:

TNFα: forward, 5'-CTCCCTCCAGAAAAGACACCAT-3'

reverse, 5'-ATCACCCCGAAGTTCAGTAGACAG-3'

MyD88: forward, 5'-TAGACCGTGAGGATATACTGAAGG-3'

reverse, 5'-TTAGCTCGCTGGCAATG-3'

GAPDH: forward, 5'-TGATGACATCAAGAAGGTGGTGAA-3'

reverse, 5' -TGGGATGGAAATTGTGAGGGAGAT-3'

2.3.6 Development of topical siRNA delivery reagent

Fluorescently-labeled siRNAs with or without candidate topical transdermal delivery vehicle, and candidate transdermal delivery vehicles alone, were prepared as follows:

A: L2K (5 μl+ 15 μl nuclease-free water (NFW))

B: Glycerol (5 µl+15 µl NFW)

C: L2K (1 µl)+Cy3 siRNA (2 µg/8 µl NFW)+NFW (11 µl)

D: Glycerol (5 µl)+Cy3 siRNA (2 µg/8 µl NFW)+NFW (7 µl)

E: L2K (1 µl)+Glycerol (5 µl)+Cy3 siRNA (2 µg/8 µl NFW)+NFW (6 µl)

F: DMSO (5 µl)+Cy3 siRNA (2 µg/8 µl NFW)+NFW (7 µl)

G: L2K (1 µl)+Glycerol (5 µl)+DMSO (5 µl)+NFW (9 µl)

H: L2K (1 µl)+DMSO (5 µl)+Cy3 siRNA (2 µg/8 µl)+NFW (6 µl)

I: Glycerol (5 µl)+DMSO (5 µl)+Cy3 siRNA (2 µg/8 µl)+NFW (2 µl)

J: L2K (1 µl)+Glycerol (5 µl)+DMSO (5 µl)+Cy3 siRNA(2 µg/8 µl)+NFW (1 µl)

Each was applied onto the shaved dorsal skin of CD1 mice in a 0.5 mm^2 area. Mice were sacrificed and dorsal skin resected and fixed in OCT for cryosectioning. Fluorescent was observed microscopically to assess cell and tissue uptake of a model fluorescently-labeled but non-targeting siRNA.

2.3.7 Preparation and application of a topical delivery reagent for CHS

Glycerol (8 µl), DMSO (1 µl), L2K (1 µl) and 8 µl NFW water Glycerol (8 µl), DMSO (1 µl), L2K (1 µl) and 2 µg siTNFα diluted in 8 µl NFW water or Glycerol (8 µl), DMSO (1 µl), L2K (1 µl) and 2 µg siMyD88 diluted in 8 µl NFW water or Glycerol (8 µl), DMSO (1 µl), L2K (1 µl), 1 µg siMyD88 and 1 µg siTNFα diluted in 8 µl NFW water were mixed and applied in mouse right ear in each treatment group. Reagents were applied from day1 and applied once/day for 4 days.

2.3.8 Assessment of siRNA stability in serum

SiRNA (0.3 μg) was mixed with novel reagents and incubated in fetal bovine serum (FBS) 20μl (Gibco, Burlington, ON, CA) for 1, 2, 3, and 4 h. The mixture was collected at each time point and siRNA degradation was assessed by loss of intensity of bands visualized by gel electrophoresis.

2.3.9 Mouse CHS model and siRNA administration

DNFB (25 μl of a 0.5% solution in acetone/olive oil [4:1]) was applied to both right and left mouse ears on day 0 and day 1 (the second application was to the same area as the first) to generate CHS model conditions. DNFB (10 μl of 0.2% in 4:1 actone/olive oil) was applied to the same region on the right ear only on day 5 as an immunogenic re-challenge (the immunogen application area on the left ear was re-challenged with vehicle only). SiRNAs in each candidate topical application vehicle was applied after the DNFB application (4 hours post DNFB application) on day 1. SiRNAs in each candidate topical application vehicle was applied to the immunogen application on the right ear only on days 1, 2, 3, and 4. Experimental groups were:

1. Untreated control

2. DNFB alone without siRNA treatment

3. DNFB with topical delivery reagent alone

4. DNFB with siTNFα (2 μg) in candidate topical delivery reagent

5. DNFB with siMyD88 (2 μg) in candidate topical delivery reagent

6. DNFB with both siTNFα (2 μg) and siMyD88 (2 μg) in candidate topical delivery reagent.

2.3.10 Assessment of the degree of DNFB-induced CHS

Ear swelling: Mouse ear thickness was measured 48 h after the last DNFB hapten treatment. Net ear swelling was the thickness of the DNFB-treated ear (measured 48 h after the final DNFB treatment) minus the thickness of the vehicle-treated ear measured at the same time.

Redness and scaling: Redness and scaling in each ear were subjectively assessed by 2 independent observers on a scale of 1 to 9 (9 being the greatest amount of redness or scaling and 1 the least) daily from day 1 to 8. The first measurement was finished 24 h after the first DNFB application and before the second DNFB application. Each category has maximally three scores; therefore the highest score will be 9 in this scoring system. The scores for each of the three symptom categories (redness, swelling, and scaling) were added to provide an overall estimate of CHS symptom intensity. Differences between and among treatment and control groups were analyzed for significant differences indicative of the capacity of siRNAs targeting TNFα and/or MyD88 to alleviate CHS symptoms.

2.3.11 Assessment of neutrophil infiltration into dermis

24 h post challenge, skin from treated ear regions were dissected from mice, fixed in 10% formalin, embedded in paraffin, sectioned (5 μm) and Hematoxylin and eosin (H&E) stained, in which basophilic white blood cells stain dark blue and eosinophilic white blood cells stain bright red, neutrophils stain a neutral pink.

2.3.12 Immunohistochemical visualization of $CD4^+$ $CD8^+$ T cells in skin

Skin tissue was fixed in OCT to obtain frozen sections. Sections were incubated with primary αCD4 and αCD8 MAbs (Abcam, Cambridge, USA, diluted 1:100), incubated with biotinylated secondary Ab (45 min), and then with horseradish peroxidase-labeled streptavidin (37°C, 45 min). Slides were diaminobenzidene (DAB)-stained and counterstained with hematoxylin.

2.3.13 Statistical analysis

All data are presented as mean of *n* measurements ± SD. Statistical comparisons between groups were performed using Student's *t*-test. Statistical significance was determined *a priori* to be achieved when $p<0.05$.

2.4 Results

2.4.1 Development of a new topical reagent for siRNA delivery

Transdermal delivery was, at one time, sufficiently problematic that intact skin was considered to be completely impermeable to all substances [17]. The greatest barrier to transdermal delivery is the tight structure of the stratum corneum (SC) in epidermis. The SC is composed of both keratin-rich corneocyte and the hydrophobic lipid-rich matrix which serve as the "bricks" and "mortar" of SC [1]. To test the capacity of candidate delivery agents, we compared the ability of those agents to deliver siRNA into and through skin. Candidate reagents were mixed with Cy3-labelled siRNA and applied to a fixed area (0.5 cm^2) of dorsal skin on live mice.

L2K is a well-established and widely used reagent for gene transfection *in vitro* [7]. Glycerol is a common ingredient in many skin medicines [8]. Firstly, it has to be confirmed that L2K and glycerol alone did not display auto-fluorescence (Fig. 2-1A&B). L2K alone did not deliver siRNA through the stratum corneum and into dermis (Fig. 2-

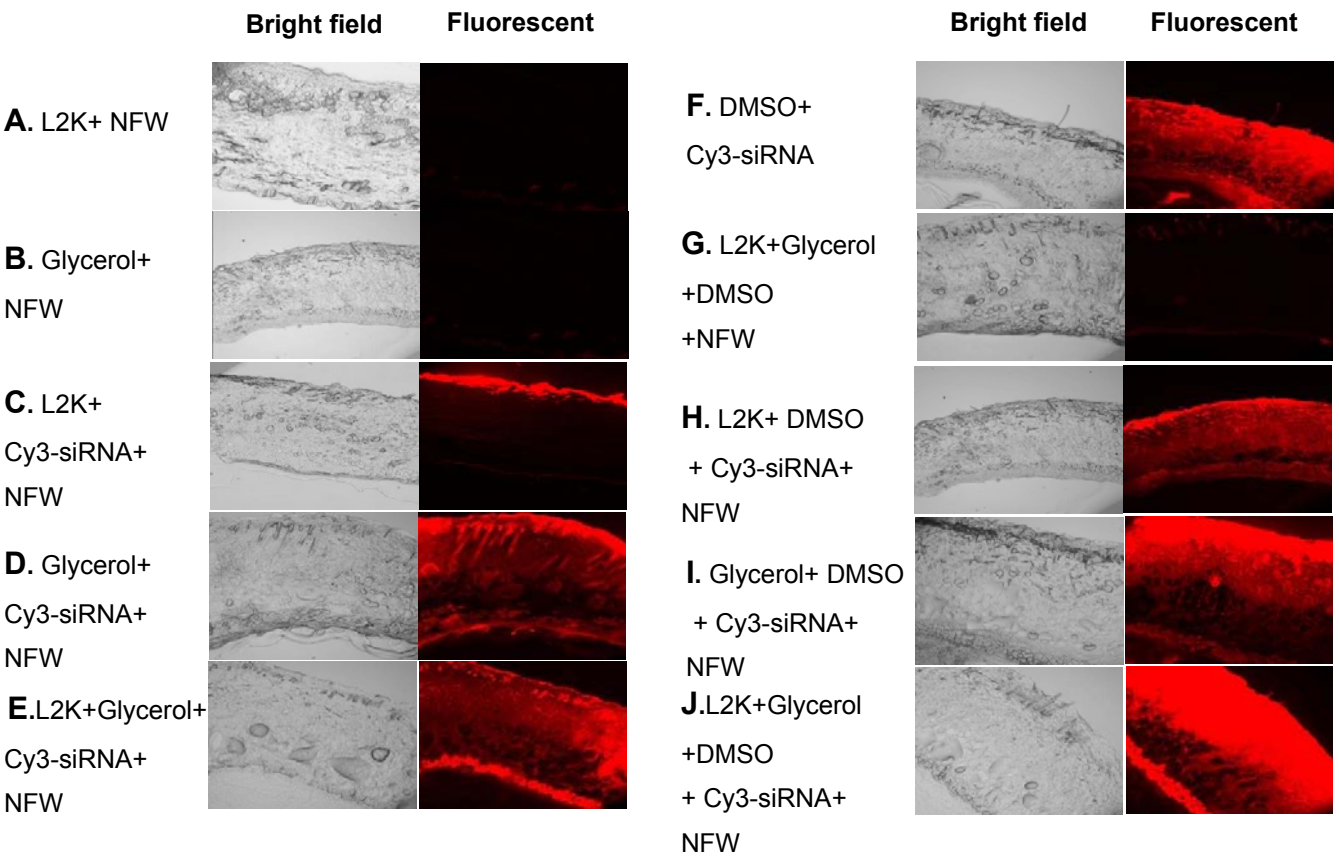
1C). Glycerol as a single agent delivered siRNA into dermis (Fig. 2-1D). A combination of glycerol and L2K increased the amount of siRNA delivered into dermis, and did so to a greater degree than glycerol or L2K alone (*i.e.*, the degree of fluorescence due to the presence of Cy3-labeled siRNA was greater) (Fig. 2-1E). These data support the hypothesis that glycerol combined with L2K is the most effective facilitator of siRNA entry into dermis compared to either reagent alone.

DMSO is a universal drug delivery reagent successfully used in many *in vivo* drug delivery systems [18, 19]. It is comfirmed that DMSO combine with L2K and glycerol has no auto-fluorescent (Fig1 2-1G). DMSO alone delivered Cy3-labeled siRNA into skin, to a degree similar to that observed with glycerol alone (Fig. 2-1F). However, the pattern of delivery suggested that delivery efficiency was uneven and irregular compared to glycerol (*i.e.*, some dermis regions received high levels of siRNA while others received little). DMSO combined with L2K facilitated transfer of Cy3-labeled siRNA into dermis, although a large fraction of applied siRNA remained on the skin surface without penetrating deeply (Fig. 2-1H). A combination of DMSO and glycerol facilitated siRNA transfer into dermis better than other single transfer reagents or two reagent combinations (Fig. 2-1I). However, the most effective Cy3 siRNA transfer (*i.e.*, fluorescent staining in dermis) was mediated by combining glycerol, DMSO, and L2K (Fig. 2-1J). These data support the hypothesis that a combination of glycerol, DMSO, and L2K is the most efficient combination of delivery agents among those tested for transfer of siRNA across the stratum corneum and into dermis.

2.4.2 Optimization of glycerol:L2K:DMSO siRNA delivery reagent

We further optimized the glycerol:L2K:DMSO topical siRNA delivery reagent combination by testing the relative capacity of different glycerol:DMSO ratios (v/v) to deliver Cy3-labeled siRNA to dorsal mouse skin dermis. Lower glycerol concentrations were relatively ineffective while increased glycerol with decreased DMSO enhanced

Figure 2-1 Development of a novel topical delivery adjuvant for siRNA. The indicated reagents (combinations A-J, as described in the text) were mixed and 20 μl applied to mouse skin, as described in *Materials and Methods*. Mice were euthanized at 4 h post-treatment and skin collected for bright field and fluorescent microscopy. Fluorescence indicates the presence of Cy3-labeled siRNA.


Bright field
Fluorescent
A. L2K+ NFW
B. Glycerol+ NFW
C. L2K+ Cy3-siRNA+ NFW
D. Glycerol+ Cy3-siRNA+ NFW
E.L2K+Glycerol+ Cy3-siRNA+ NFW
Bright field
Fluorescent
F. DMSO+ Cy3-siRNA
G. L2K+Glycerol +DMSO +NFW
H. L2K+ DMSO + Cy3-siRNA+ NFW
I. Glycerol+ DMSO + Cy3-siRNA+ NFW
J.L2K+Glycerol +DMSO + Cy3-siRNA+ NFW

siRNA delivery. Considering that DMSO may cause skin irritation if absorbed through skin, and increased viscosity could facilitate penetration by prolonging residency of the mixture on the skin surface, we tested additional glycerol:DMSO ratios and observed that ratios of 9:1 retained a high level of penetration without inducing skin rash. We concluded that the optimal combination for glycerol:DMSO:L2K:siRNA solution was 9:1:1:8 in a volume ratio(Fig. 2-2). In this experiment, 2 μg siRNA dissolved in 8 μl NFW was used as siRNA solution.

2.4.3 TNFα and MyD88 knockdown in vitro and in vivo

To validate the efficacy of siRNAs selected to knock down MyD88 (siMyD88) and TNFα (siTNFα), we transfected mouse melanoma cells (B16) *in vitro* which each siRNA. MyD88 and TNFα mRNA levels were largely decreased 24 h post-transfection in B16 cells (Fig. 2-3A). Thus, the siRNAs selected to reduce each of these mRNA targets are effective.

To evaluate *in vivo* gene silencing after topical delivery of siMyD88 and siTNFα on mice dorsal skin, we prepared glycerol:DMSO:L2K:siRNA (9:1:1:8) and apply it topically to dorsal mouse skin. The mice were sacrificed 24 h after application and skin was collected for determination of target mRNA levels. MyD88 and TNFα mRNA were reduced by 62% and 54% respectively (Fig. 2-3B, C).

Another consideration is that siRNA might be easily degraded in the topical application. To clarify whether the reagent may facilitate protection of RNA degradation, we mixed siRNA prepared with topical reagent or without topical reagent with freshly-prepared mouse plasma (rich in RNases). Undegraded siRNA was visualized by electrophoretic mobility at various incubation times. Compared to naked siRNA, the topical delivery reagent successfully protected siRNA from degradation (Fig. 2-3D).

Figure 2-2 Optimization of topical siRNA delivery using a combined glycerol:DMSO cocktail. L2K (1 μl) and Cy3-labeled siRNA (8 μl) were mixed with glycerol and DMSO at the indicated volume ratios and applied to mouse skin as described in *Materials and Methods*. Mice were sacrificed 4 h post-treatment and skin was collected for bright field and fluorescent microscopy. Fluorescence indicates the presence of Cy3-labeled siRNA.

Glycerol: DMSO

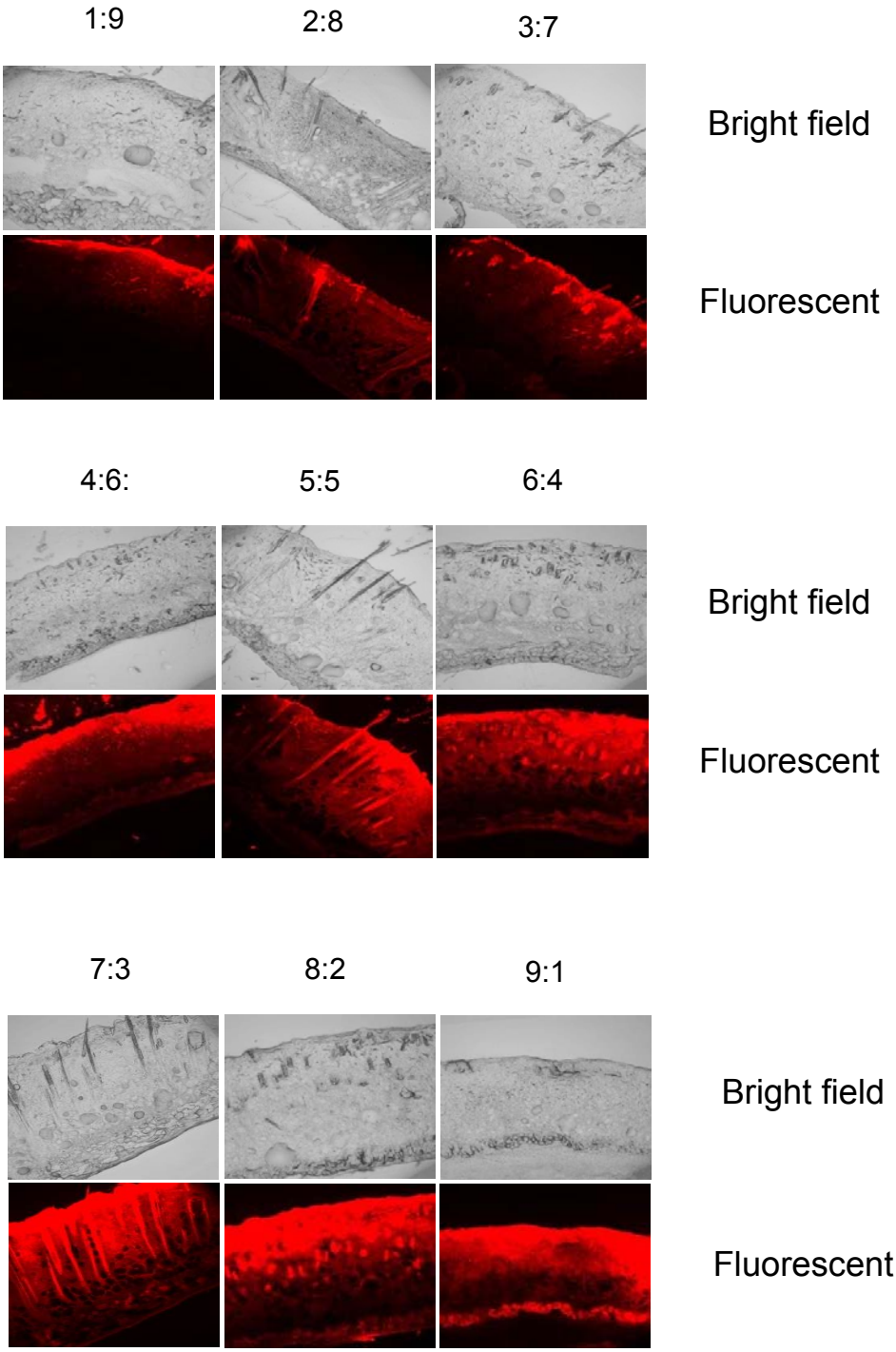
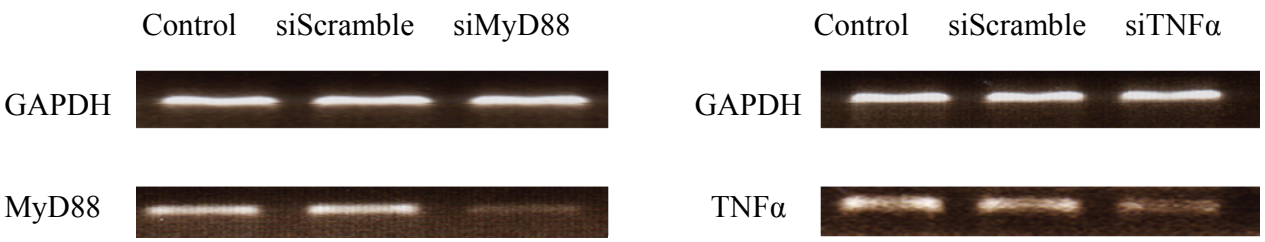
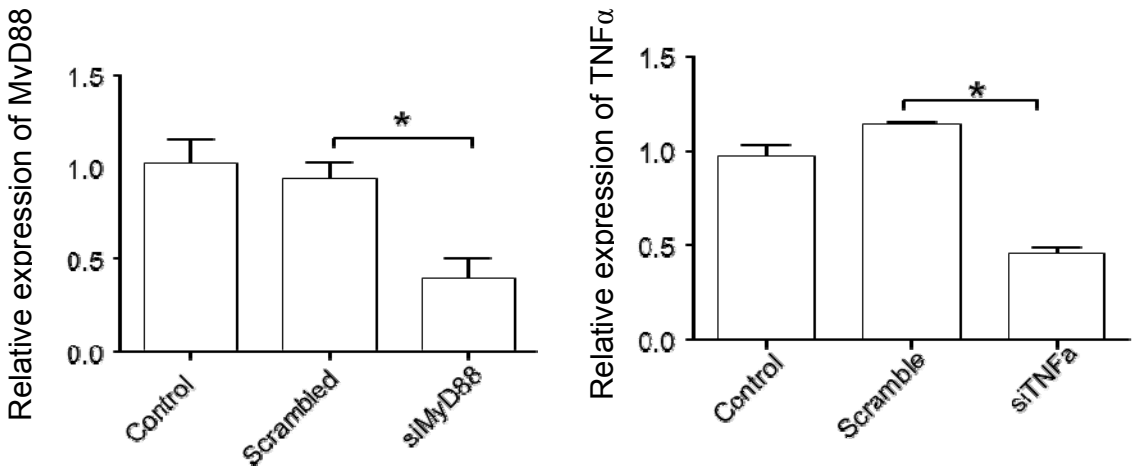


Figure 2-3 siRNA-mediated gene silencing of MyD88 and TNFα *in vitro* and *in vivo*. (A) *In vitro* silencing efficacy of siMyD88 and siTNFα siRNA: B16F10 cells were cultured and transfected with siRNAs targeting MyD88 and/or TNFα, or control, non-targeting siRNA (siScramble). MyD88 and TNFα mRNAs were measured relative to GAPDH mRNA at 24 h post-transfection by RT-PCR and gel electrophoresis as described in *Materials and Methods*. (B, C) *In vivo* silencing of MyD88 and TNFα in skin: siMyD88, siTNFα, or siScramble were mixed with the novel delivery reagent (glycerol:DMSO:L2K:siRNA = 9:1:1:8) and applied to mouse skin. Mice were euthanized and skin collected at 24 h post-treatment. The levels of MyD88 and TNFα mRNA were determined by qPCR as described in *Materials and Methods*. Relative quantity of MyD88 and TNFα mRNA was expressed as mean ± SD. *Student's *t*-test, $p<0.05$. (D) Delivery reagent protection of siRNA from serum-mediated degradation: The novel reagent and 0.3 μg siRNA were mixed with 20μl FBS and incubated in 37^0C for 1, 2, 3, or 4 h. Integrity of siRNA was detected by gel electrophoresis as described in *Materials and Methods*.

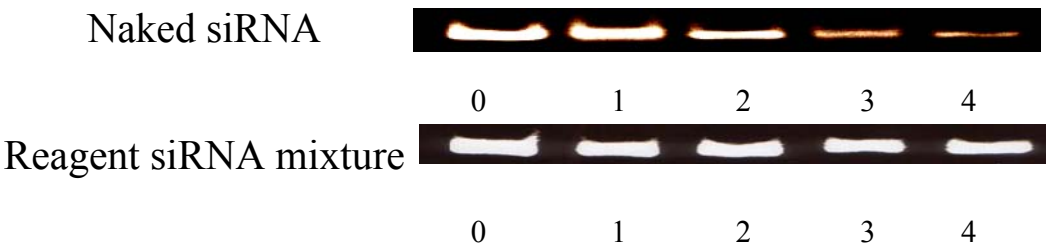
A


Control
siScramble
siMyD88
GAPDH
MyD88
Control
siScramble
siTNFα
GAPDH
TNFα

B


Relative expression of MvD88
1.5
1.0
0.5
0.0
*
Control
Scrambled
siMyD88
Relative expression of TNFα
1.5
1.0
0.5
0.0
*
Control
Scramble
siTNFa

C


Naked siRNA
0
1
2
3
4
Reagent siRNA mixture
0
1
2
3
4

2.4.4 Attenuation of CHS by topical treatment with siMyD88 and siTNFα in delivery reagent

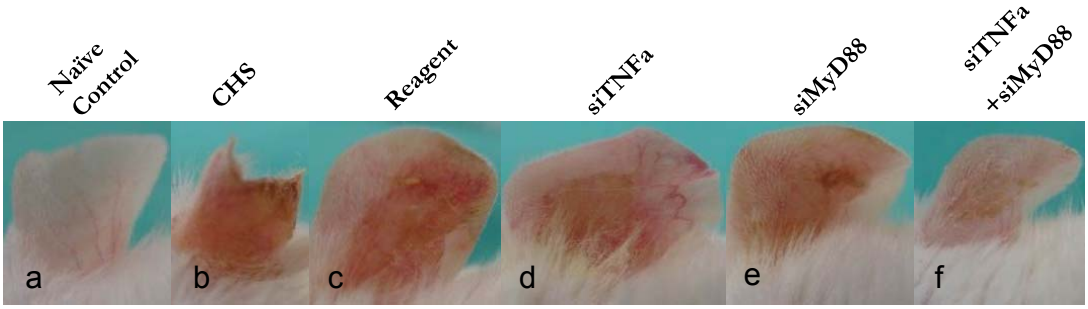
DNFB is commonly used to induce model contact dermatitis in mice [20, 21], characterized by swelling, redness and scaling [5]. We hypothesized that silencing of MyD88 and TNFα in local skin tissue would reduce both swelling [22] and the severity of other symptoms due to reduction in cell-mediated responses mediated by MyD88 and TNFα during CHS sensitization phase [15]. To this end we applied freshly-prepared topical delivery agent with siTNFα and/or siMyD88 daily to the skin of mice. After 4 d treatment with siMyD88 or siTNFα alone, or siMyD88 combined with siTNFα, the overall clinical symptoms of redness, swelling and scaling (as assessed by subjective inspection of skin sections) were decreased compared to CHS treated with topical delivery reagent alone or untreated CHS (Fig. 2-4A).

Ear swelling was reduced in groups treated with either single siRNA, implying that siTNFα and siMyD88 alone have protective and/or ameliorative capacity. Silencing of TNFα to reduce swelling of CHS has been reported and our result is consistent with that report [22]. On the other hand, use of siMyD88 to reduce swelling is reported by us for the first time. However, redness and scaling were reduced to only a minor degree by treatment with either siRNA alone. Combined siTNFα and siMyD88 treatment reduced swelling 48 h after re-challenge, compared to treatment with siTNFα or siMyD88 alone (Fig 2-4B).

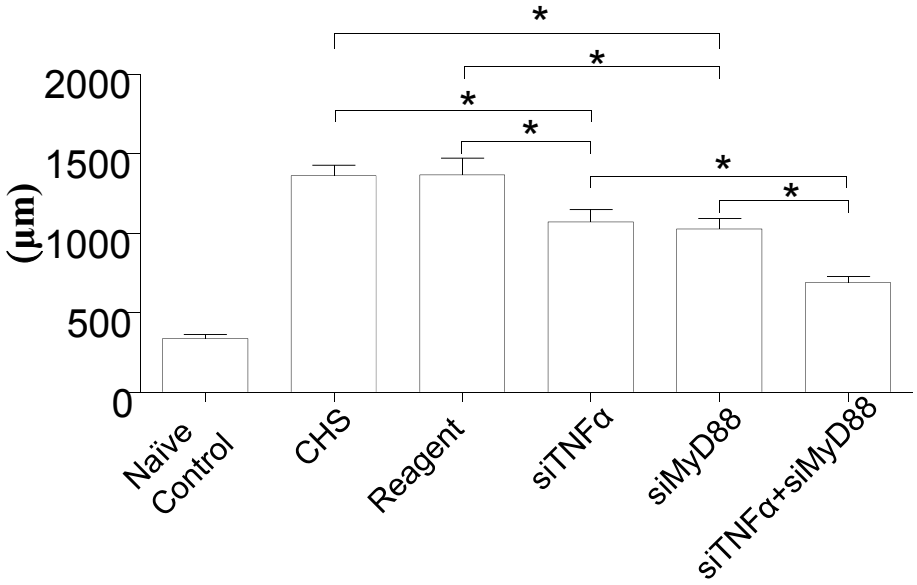
To have an overall and real time assessment of symptom including swelling, redness and scaling in different groups from day1 to day 8, we created a scoring system to assess the severity of CHS and applied that system to quantitate siRNA-mediated CHS symptom changes (after transfer of siRNAs, alone or in combination, in the glycerol:L2K:DMSO topical delivery reagent). Score from day1 is assessed 24 h after first DNFB application and before first treatment. Quantitative assessment revealed that reagent alone exerts a capacity to reduce CHS symptoms, implying that topical delivery reagent was both non-toxic and therapeutic. Both single siRNA treatments (siTNFα alone or siMyD88 alone) have significantly attenuated the symptoms 24 h after the re-challenge. However, siTNFα alone could also significantly release the overall symptom 48 h after the second

Figure 2-4 Attenuation of CHS by topical delivery of siMyD88 and siTNFα: (A) *CHS symptom severity in experimental and control groups:* CHS was induced with DNFB as described in *Materials and Methods*. CHS in mouse ears was treated by topical application of glycerol:DMSO:L2K:siRNA (9:1:1:8) alone (*Reagent*) or containing siMyD88 + siTNFα, siMyD88 alone, or siTNFα alone. Control mice were left untreated with DNFB and did not suffer from CHS (*Naïve reagent*). All reagent and siRNA treatments were once per day for 4 days. Severity of symptoms was assessed 48 h after re-challenge with DNFB on day 4. (B) *Quantification of swelling during CHS:* Ear thickness was measured 48 h after re-challenge with DNFB on day 4. Net swelling thickness = (thickness, hapten re-challenged ear, μm) - (thickness, vehicle-treated ear, μm). *Student's *t*-test, $p<0.05$.

A


Naïve Control
CHS
Reagent
siTNFa
siMyD88
siTNFa +siMyD88
a
b
c
d
e
f

B


2000
1500
1000
500
0
(μm)
*
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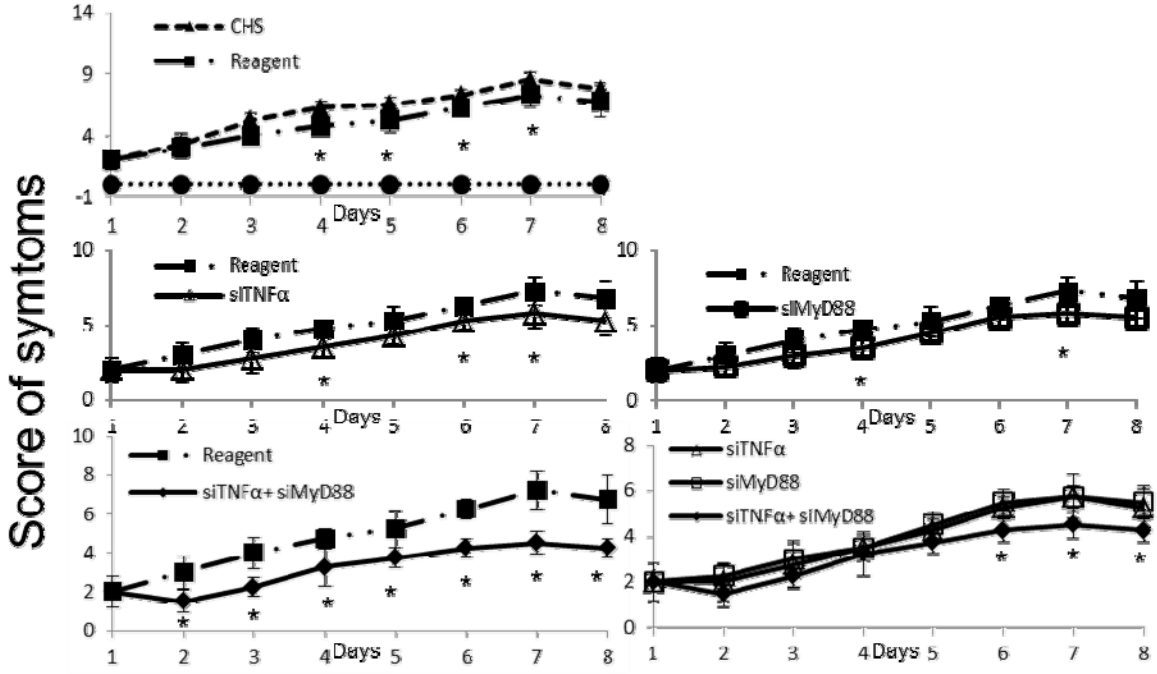
*
*
*
*
Naïve Control
CHS
Reagent
siTNFα
siMyD88
siTNFα+siMyD88

application of DNFB. Therefore, single siTNFα treatment can reduce the symptoms both in sensitization phase and elicitation phase while single siMyD88 treatment only reduce symptoms in elicitation phase. Combining siTNFα and siMyD88 resulting in alleviation of CHS symptoms to a greater degree than single siRNA treatment groups. The combination significantly attenuated the symptoms since 48 h after the first DNFB application and last for over 72 h after re-challenge, which covers nearly the whole sensitization phase and elicitation phase. When compare to single siRNA treatment, the severity of symptoms in combination treatment group significantly less from 24 h post re-challenge to 72 h post re-challenge. These data suggest that simultaneous suppression of both TNFα and MyD88 provides a therapeutic advantage in preventing development of CHS symptoms and/or reducing their severity, over reduction of either target alone and particularly after repeated exposure to hapten (Fig. 2-5C).

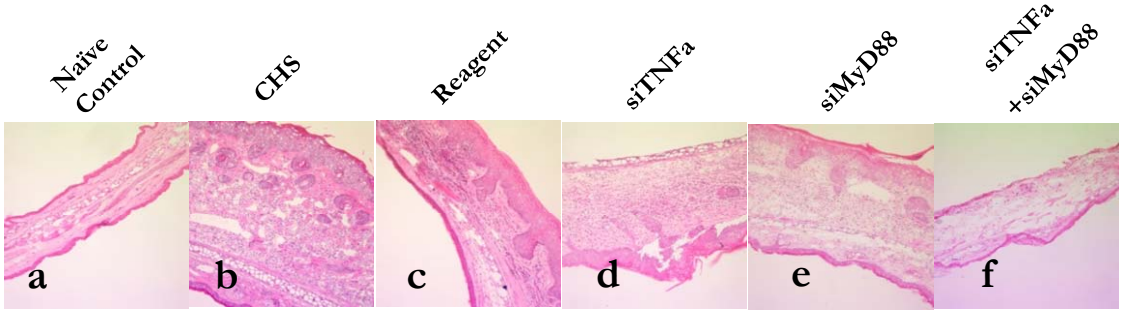
The number of infiltrating neutrophils in tissues affected by CHS is closely related to the severity of symptoms [22]. The H&E staining for the part in each group that carries most severe symdrom is shown in (Fig. 2-5D). Neutrophils are among the most important initiators and effectors of CHS [9]. To determine whether treatment with siMyD88 and siTNFα can reduce neutrophil infiltration in CHS, we treated ears of mice with siRNA in topical delivery reagent and quantitatively scored CHS symptoms (Fig. 2-5C) and subjectively observed neutrophil infiltration (Fig. 2-5D) at 48 h after DNFB rechallenge. Treatment with topical delivery reagent alone reduced swelling slightly (Fig. 2-5C) but did not reduce neutrophil infiltration (Fig. 2-5D). Treatment with single siRNAs reduced neutrophil infiltration in CHS compared to untreated CHS or CHS treated with topical delivery reagent alone (Fig. 2-5D). Combined treatment with both siTNFα and siMyD88 were subjectively more effective at reducing neutrophil infiltration than treatment with single siRNAs (Fig. 2-5D). These data reveal that neutrophil infiltration in CHS can be reduced or prevented by down-regulation of MyD88 and TNFα through topical delivery of siRNAs. Moreover, the epidermis thickness obviously increased and Eosinophils cell clusters can clearly be seen. While this change is lighter in single treatment group, the combination treatment is mostly similar to naïve group (Fig. 2-5D).

Figure 2-5 Attenuation of CHS by topical delivery of siMyD88 and siTNFα. (C) *CHS symptom severity in control and treated skin:* Summary scores of CHS symptoms (redness, swelling, scarring) were evaluated from day 1 to day 8 as described in *Materials and Methods*. The data presented are from one of three independent experiments (n=6 mice/group). *Student's *t*-test, $p<0.05$. (D) *Neutrophil infiltration*: CHS was induced in mouse skin and treated with siTNFα and siMyD88 as described in *(C)*. Mice were euthanized 48 h post-rechallenge and skin collected for microscopic analysis of neutrophil infiltration, as described in *Materials and Methods*.

C


Score of symtoms
CHS
Reagent
siTNFα
siMyD88
siTNFα+ siMyD88
Days

D

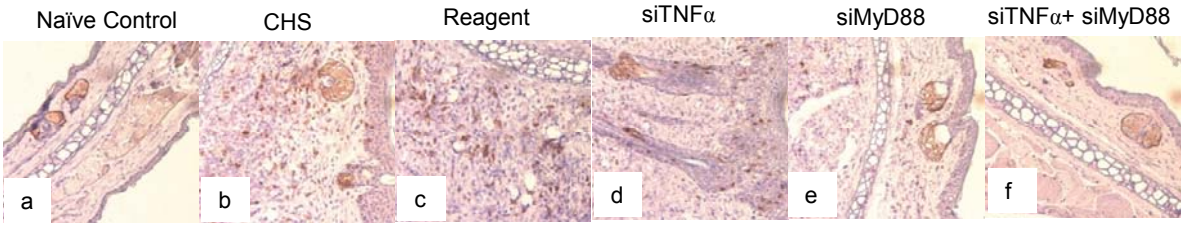

Naïve Control
CHS
Reagent
siTNFa
siMyD88
siTNFa +siMyD88
a
b
c
d
e
f

2.4.5 Immune suppression by topical delivery of siMyD88 and siTNFα

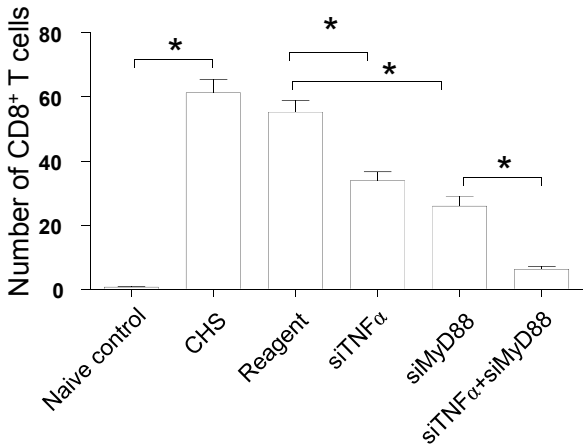
It is believed that $CD4^+$ cells and $CD8^+$ cytotoxic T cells are important factors in CHS [20, 21, 23]. They accumulate locally in regions in direct contact with hapten and play a major role in triggering CHS. To assess whether treatment with siRNAs targeting TNFα and/or MyD88 modulated accumulation of these cells in CHS, mouse ear tissue was obtained after induction of CHS and 48 h after hapten rechallenge, and $CD8^+$ and $CD4^+$ T cell accumulation assessed by immunostaining. Obvious accumulation of $CD8^+$ and $CD4^+$ T cells was observed in untreated CHS. Treatment with single siRNAs (either siTNFα for siMyD88) reduced $CD8^+$ (Fig. 2-6A) and $CD4^+$ (Fig. 2-6B) T infiltration, but incompletely. The combination treatment with both siRNAs almost completely prevented infiltration by $CD8^+$ and $CD4^+$ T cells (Fig. 2-6A,f; Fig. 2-6B,x). These data suggest that topical delivery of siMyD88 and siTNFα can prevent accumulation of $CD4^+$ and $CD8^+$ T cells that contribute to CHS severity.

Figure 2-6 Immune suppression by topical delivery siMyD88 and siTNFα. (A) *$CD8^+$ T cell accumulation*: Mice were treated with DNFB to induce CHS and re-challenged 4 d later with DNFB. At 48 h after re-challenge, mice were euthanized and skin was collected and stained with antibodies to detect $CD8^+$ T cells: (a) Non treatment naïve mice; (b) mice with CHS without any treatment; (c) mice with CHS treated with reagent alone; (d) mice with CHS treated with reagent with siTNFα alone; (e) mice with CHS treated with reagent and siMyD88 alone and (f) mice with CHS treated with reagent mixed with siMyD88+siTNFα show the $CD8^+$ cells. (B) $CD4^+$ T cell accumulation. Skin sections were made as decribed above, and stained with CD4+ cells: T (g) Non treatment mice; (h) mice with CHS without any treatment; (i) mice with CHS treated with reagent alone; (j) mice with CHS treated with reagent with siTNFα alone; (k) mice with CHS treated with reagent and siMyD88 alone; (l) mice with CHS treated with reagent mixed with siMyD88+siTNFα represent the $CD4^+$ positive cells. Pictures were taken under the microscope in 100 time magnification. The data presented complied results from 3 experiments.

A **CD8⁺ T cells**

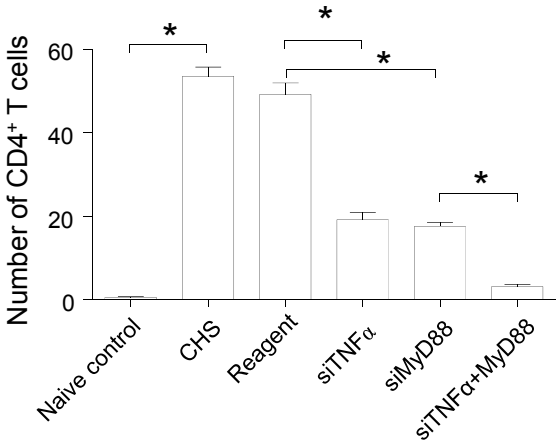


B



C **CD4⁺ T cells**

D



2.5 Discussion

Topical delivery of siRNA has important advantages: First, it could directly target the cells in the disease site. Second, there is minimum worry about the risk of systemic side effects. Third, it is easy for administration since it is noninvasive, painless and readily accessible nature.

However, the resilient barrier of the epidermal layers of the skin, primarily the stratum corneum, prevents the diffusion of exogenous chemical agents, which makes skin graft a significant obstacle to dermal and transdermal drug delivery [24]. Also, siRNA itself is difficult to be absorbed by skin and it is not stable enough [25]. Moreover, siRNA is negative charge which makes it being repelled by the cell membrane (also negative charge), and its size is relatively large makes it difficult to penetrate the cell. Even the siRNA successfully enters into cells, it might also face challenges as endosomal escape [1]. Various physical and chemical methods have been applied in previous study for overcoming the skin barrier while limiting the damage to the skin. Several studies has report the intradermal injection of DNA or siRNA in mouse skin model [1, 26-29]. However, transdermal injection can only be applied in small area of the skin and it is actually not user friendly and painful if multiple injections are necessary. To reduce the pain, needless injector which could generate high pressure beam of injection for physical penetration [30], or low frequency sonophoresis have been applied for transdermal delivery [31]. However, needless injector carries the sample problem of intradermal injection as they only able to cover small area of the skin, which limits their application. The sonophoresis technic needs the carrier of special equipment which could be costly while compare to chemical transdermal methods. Other study reported chemicals as acetone to weaken the stratum corneum, thus to facility the drug delivery. However, it is potentially harmful to skin since it is difficult to control the intensity of the weaken process [32].

In this study, we create a novel reagent which could not only efficiently carry siRNA penetrating the stratum corneum, but also could help siRNA transfecting the cells in skin and achieve silencing.

We optimize the ratio between the components of the novel reagent to minimize the irritation to skin while maximally keeping the ability of penetration (Fig2). Base on the thickness measurement data and the score of syndromes in various experimental groups, the reagent control has lighter syndromes when compare to non-treatment group, which implies our reagent has certain level of protection function while apply on CHS mouse skin. It is reasonable since our reagent contains majority of glycerol, which plays a role in skin as hydration, cutaneous elasticity and epidermal barrier repair. As a lubricant, it has been applied in lots of commercial available skin care products [33]. The majority of glycerol in the combination makes the novel reagent retains a certain level of viscosity, which prevents the fast evaporation of the delivery combination and provides longer time for penetration. And solvent viscosity results in friction against proteins in solution, and this should result in decreased motion, inhibiting catalysis in motile enzymes [34], which might contribute to the stability of the siRNA in skin. DMSO is an important polar aprotic solvent that dissolves both polar and nonpolar compounds and is miscible in a wide range of organic solvents as well as water. It has a distinctive property of penetrating the skin very readily. However, topically apply DMSO alone can arose skin irritation, which has been observed in our study. L2K is well established as a reagent for in vitro siRNA delivery. It can condense with siRNA and brings them into cells efficiently. The novel reagent adopts the advantages of each component and minimized their weaknesses, which makes itself a powerful reagent to deliver various siRNA into skin layers and against various kinds of dermatologic diseases. Since RNAi owns the characteristic of specifically knock down the target genes, our therapy also displays a potential application in treating diseases with strong drug resistant.

The DNFB induced CHS mouse model has been extensively studied and termed a Type IV delayed hypersensitivity reaction involving a cell-mediated allergic response. CHS arises as a result of two essential stages: An induction phase, which primes and sensitizes the immune system for an allergic response, and an elicitation phase, in which this response is triggered. In induction phase, contact allergens are able to cross the stratum corneum of the skin then the haptens will associate with epidermal proteins forming hapten-protein conjugates. The conjugate formed is then recognized as a foreign body by the MHC class I- expressing langerhans cells (LCs) (or Dendritic cells) [21]. Then the

LCs will internalize the protein; transport it via the lymphatic system to the regional lymph nodes; and present the antigen to T-lymphocytes. In the lymph glands, the differentiated DCs present the allergenic epitope, associated with the allergen, to T lymphocytes. These T cells then divide and differentiate, clonally multiplying so that in the elicitation phase when the allergen is experienced again by the individual, these T cells will respond more quickly and more aggressively. It is showed that both $CD4^+$ T-helper 1 (Th1) and $CD8^+$ cytotoxic T-cells (Tc1) cells are crucial effectors in the CHS response to DNFB [23]. The $CD8^+$ effector T cells migrate to the challenged site and initiate the skin inflammation via Fas and/or perforin-mediated cytolytic function [20, 35, 36]. $CD4^+$ T lymphocytes secreting IL-2 and IFN-γ play a preeminent role in the evolution of CHS. In this study, we treat mice after induction phase and before the elicitation phase. Our result agrees with these reports: compare to control group, there are less $CD4^+$ $CD8^+$ T cell accumulation in siTNFα and siMyD88 treatment group 48 h post challenge in the place where the reaction was triggered.

TNFα is a very important mediator, which activates local endothelia to facilitate T cell recruitment[37, 38]. TNFα can also activate NF-kappa B which leads to the production and secretion of more inflammatory cytokines (IL-1, IL-6). Some study reported that TNFα level correlates with swelling, which is the classical manifestation of contact hypersensitivity. TNFα also closely related to the density of dermal infiltration [39].

Contact hypersensitivity initiates with innate immune reaction and results in an adaptive immune response. Toll like receptors constructs a vital important signal pathway for cells of innate immune system. MyD88 is the adaptor protein for all TLR members except TLR3. MyD88 associates with the Toll/interleukin-1 receptor (TIR) domain of TLRs, the interleukin-1 receptor-associated kinases IRAK1 and IRAK4, and tumor necrosis factor receptor-associated factor 6 (TRAF6) [40]. This interaction results in NF-κB activation, cytokine secretion and inflammatory response. Recently study report that IL-1R and IL-18R/MyD88 pathways are required in distinctly different cells during the sensitization phase of CHS [41].

In current view, human ACD is a disease that once established, and then it lasts a lifelong. The only available etiologic treatment of ACD is elimination of the contact allergen [6]. Locally application of corticosteroids that targets lymphocyte activation remains the main approaches for ACD. However, corticosteroids has lots of side effects, such as cutaneous addiction, hyperglycemia, insulin resistance, diabetes mellitus, osteoporosis, cataract, anxiety, depression, colitis, hypertension, ictus, erectile dysfunction, hypogonadism, hypothyroidism, amenorrhoea, and retinopathy [42]. Our study provides a possibility of a new therapy for ACD, which is protective, potent and user-friendly.

In conclusion, we report here a novel reagent that composed of glycerol, DMSO and L2K can effectively delivery siRNA into deep skin layer and achieve gene silencing, which leads to the syndrome attenuation of DNFB induced contact hypersensitivity, reduced inflammation cells infiltration and $CD4^+$ $CD8^+$ accumulation.

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Chapter 3

3 Targeted siRNA silencing of IDO in dendritic cells using mannose-conjugated liposomes: a novel strategy for treatment of melanoma

3.1 Summary

Indoleamine 2, 3-dioxygenase (IDO) expressed in antigen presenting cells (DCs) blocks T cell activation, induces T cell apoptosis, and promotes T cell differentiation into regulatory T cells ($Treg_s$) that help tumor cells escape immune surveillance. Mannose receptors are highly expressed in antigen-presenting cells (APCs). In this study, we developed a novel APC-targeted siRNA delivery system using mannosed liposomes (Man-lipo) with encapsulated IDO siRNA (Man-lipo-siIDO), which preferentially knocks down IDO in melanoma bearing mice draining lymphnode and spleen. Mice treated with Man-lipo-siIDO had a delayed time of onset of implanted murine melanomas, increased survival time, reduced tumor size, and increased reactivity of T cells from spleen and lymph nodes against melanoma antigens. The enhanced anti-tumor immunity may be causally associated with inhibition of apoptosis in $CD8^+$ and $CD4^+$ T cells as well as Treg cells in spleen and lymph nodes. This study is the first to demonstrate that Man-lipo-siIDO can preferentially target DCs and efficiently silence IDO expression *in vitro* and *in vivo*, events expected to enhance anti-tumor immune reactions against melanoma xenografts. This study supports the hypothesis that Man-lipo-siIDO may have potential for development as an immune-targeting therapeutic anticancer agent.

3.2 Introduction

Immune evasion and T cell tolerance induction have an important role in neoplastic growth, and recently, increasing evidence has demonstrated that tryptophan catabolism has a part in immune evasion. Indoleamine 2, 3-dioxygenase (IDO) is the first and rate limited enzyme that degrades tryptophan alone in kynurenine metabolic pathways. IDO plays an important role in immuno-regulation during infection, pregnancy, induction of

autoimmune events, transplantation, and neoplasia [1-3]. By depleting tryptophan, IDO induces cell cycle arrest in T lymphocytes and makes these cells more vulnerable to apoptosis, which subsequently leads to the attenuation of the immune system [1, 4, 5]. In addition, metabolic products generated by IDO, including kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and quinolinic acid, can cause cell cycle arrest and apoptosis [6]. Moreover, IDO can also induce differentiation of naive $CD4^+$ T cells into regulatory T cells (Tregs) that further suppress anti-tumor immune responses [7, 8]. A recent clinical study reported strong expression of IDO in malignant melanoma metastases that correlated with a high density of Foxp3-positive Treg cells and was associated with decreased survival times [9].

RNAi is an endogenous mechanism in which double-stranded RNA specifically knocks down the expression of target genes to induce partial or complete gene silencing. Small interfering RNA (siRNA), a mediator of RNAi, has been used as a powerful laboratory tool to reduce or ablate gene expression [10]. Recently, investigation of RNAi applications in animal models of human diseases including cancer, viral infection, and age-related macular degeneration has moved RNAi forward into clinical applications. However, the lack of specific and effective *in vivo* delivery methods reduces the potential effectiveness of siRNA-based therapies [11, 12].

Mannose receptors are highly expressed in cells of the immune system, especially in professional APCs including DCs and macrophages [13-15]. Transmembrane mannose receptors mediate endocytosis, function as antigen capture receptors, and are involved in antigen presentation [16, 17]. The preferential expression of mannose receptors in cells of the immune system makes them promising targets for various therapies, including macrophage-targeted chemotherapy and DC-targeted vaccines [18]. Mannose has been successfully applied to gene delivery to APCs and development of therapeutic vaccines, and some of them are currently undergoing clinical trials [19].

In this study, we constructed mannosed liposomes (Man-lipo), as APC-targeted carriers for IDO siRNA (siIDO) delivery. We show that Man-lipo-siIDO can efficiently and preferentially silence IDO expression in DCs *in vitro* and *in vivo*; and that treatment with

Man-lipo-siIDO inhibited formation of melanoma tumour xenografts in recipient mice, restrained melanoma tumor xenograft growth, and enhanced anti-tumor immunity in xenograft host mice.

3.3 Materials and Methods

3.3.1 Chemicals

1,3-Dioleoyl-3-trimethylammonium propane (DOTAP), cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}- carboxylic acid) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG_{2000}) were purchased from Avanti Lipids, Inc. (Alabaster, AL). D-(+)-mannose and protamine sulfate were purchased from Sigma-Aldrich Canada, Ltd. (Ontario，Canada). All other chemicals were reagent grade.

3.3.2 Mice

C57BL/6 mice (6-8 weeks old males) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained under pathogen-free conditions in the Western University barrier rodent facility. All experiments were performed in accordance with guidelines and standard operating procedures set out by the Canadian Council on Animal Care and the Western University Animal Use Subcommittee.

3.3.3 IDO siRNA design

A 21 nt, double-stranded siRNA targeting murine IDO, as described in our previous report [20] and synthesized by Dharmacon, Inc. (Lafayette, CO, USA). The sequences were: IDO sequence: sense, 5’-GGGCUUCUUCCUCGUCUCUTT-3’; antisense, 5’-

AGAGAC GAGGAAGAAGCCCTT-3'. The sequence for scrambled siRNA was: sense, 5'-CGUACGCGGAAUACUUCGA-3'; antisense, 5'-CGTACGCGGAATACTTCGA-3'.

3.3.4 Cell culture

Bone marrow-derived DCs were generated as previously described [21]. Briefly, bone marrow cells were flushed from the femurs, washed in ice-cold PBS, and cultured in 12-well tissue culture plates (Corning, MA, USA; 2 X 10^6 seeded cells/well). Each well contained 2 ml of RPMI1640 medium (Invitrogen Life Technologies, CA, USA) supplemented with 10% FBS (Invitrogen Life Technologies) and penicillin (100 U/ml), streptomycin (100 μg/ml), 2-ME (50 μM; Invitrogen Life Technologies), recombinant murine GM-CSF (10 ng/ml; PeproTech, Quebec, Canada), and IL-4 (10 ng/ml; PeproTech). Non-adherent cells were removed after 48 h of culture, and medium was changed after every 48 h of culture. DCs were used for *in vitro* experiments after 7 days. B16-F10 mouse melanoma tumor cells were purchased from the American Type Culture Collection (ATCC, VA, USA) and grown and maintained in RPMI1640 tissue culture medium supplemented with 10% FBS.

B16F10 murine melanoma cells were cultured in 75-mm culture dishes in Gibco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Burlington, ON, CA) and antibiotics (100 U/ml of penicillin and 100 lg/ml of streptomycin).

3.3.5 Synthesis of DSPE-PEG_{2000}-mannose lipid

DSPE-PEG_{2000}-amine (0.12 mmol) was lyophilized, dissolved in DMSO (2 mL), and mixed with mannose (1 mg) at 25^{o} C for 16 h. The reaction mixture was then evaporated in a vacuum and the resulting dried material resuspended in water. The water suspension was dialyzed against distilled water for 48 h using a 3 kDa cut-off membrane (Spectrum, TX, USA). After the dialysis, the water suspension of DSPE-PEG_{2000}-mannose was filter-centrifuged using Amicon Ultra-15 centrifugal filter units with a 3 kDa MW cut-off (Millipore, MA, USA). The resulting suspension was lyophilized.

3.3.6 Preparation of Man-lipo-siIDO

DOTAP, DPPC, cholesterols, and mannose-DSPE-PEG were mixed (3:10:10:4 molar ratio) to construct a mannose receptor-targeting liposome. The same reagents without the addition of mannose were used to construct the control, non-mannosed liposome. The lipids were dissolved in chloroform in a glass tube and evaporated to complete dryness under vacuum. The lipid film was hydrated by siRNA and protamine mixture. siRNA (targeting IDO in distilled water was added to an equal volume of liposomes and protamine at a ratio of 600 nmol DOTAP:30 μg protamine:50 μg siRNA and incubated at room temperature for 10 min to form multilamellar liposomes and siRNA mixture. The multilamellar liposome and siRNA mixture suspension was vortexed for 5 min followed by sonication for 2 min at 50°C in a bath sonicator, and the sonicated suspension was extruded sequentially through polycarbonate membranes of decreasing pore size (0.4, 0.2 and 0.1 mm diameter pores). Finally, a post-insert technique [22] was used to construct non-mannosed and mannosed liposomes. The liposome nanoparticle solution was incubated (50°C, 10 min; followed by cooling to 25^{o}C) with DSPE-PEG2000 or mannose-DSPE-PEG2000 to generate non-mannosed and mannosed liposomes, respectively.

3.3.7 Mannosed liposome particle diameter and zeta potential

Freshly prepared non-mannosed and mannosed liposomes were diluted with phosphate-buffered saline (PBS) and the mean particle diameter and surface charge (zeta potential) measured using a Zetasizer Nano Series (Malvern Instruments Ltd., UK) according to the manufacturer's protocol.

3.3.8 *In vitro* uptake of liposomes loaded with Cy3 siRNA (Man-lipo-Cy3 siRNA)

Mouse dendritic cells were cultured for 6 days and then transfected with Man-lipo-Cy3 siRNA, non-mannosed-lipo-Cy3 siRNA, empty Man-lipo, empty non-mannosed-lipo, or naked Cy3 siRNA. Cells were harvested 4 h after transfection and Cy3 fluoresence visualized by fluorescent microscopy. Untransfected DCs and DCs transfected with Man-lipo-Cy3 siRNA or non-mannosed-lipo-Cy3 siRNA were similarly collected 4 h post-transfection and analyzed for Cy3 siRNA uptake by flow cytometry.

3.3.9 *In vitro* silencing of IDO in DCs using Man-lipo-siIDO

Mouse DCs were cultured for 6 days and then transfected with Man-lipo-siIDO. Mannosed liposomes loaded with control siRNA (non-complementary to any known mouse RNA; Man-lipo-siScramble) were used as negative controls. Total cellular RNA was isolated from DCs 24 h after transfection for quantitative PCR analysis.

3.3.10 *In vivo* distribution of Man-lipo-Cy3-siRNA

Non-mannosed-lipo-Cy3 siRNA or Man-lipo-Cy3 siRNA were injected intravenously into mouse tail veins (50 μg/mouse). Mice were euthanized 6 h post-injection and organs (heart, liver, spleen, lung and kidney) collected, rinsed with saline, snap-frozen in liquid nitrogen, mounted for cryostat sectioning, and viewed using fluorescent microscopy.

3.3.11 *In vivo* silencing of IDO using Man-lipo-siIDO

Man-lipo-siIDO and Man-lipo-siScramble were injected intravenously into mouse tail veins (50 μg/mouse). Mice were euthanized 24 h later and splenocytes and lymphocytes were collected for RNA isolation. All RNAs were prepared for quantitative PCR analysis.

Man-lipo-siIDO and Man-lipo-siScramble were injected intravenously into mouse tail veins (50 μg/mouse). Mice were euthanized 24 h later and splenocytes and lymphocytes were collected and $CD11C^+$ DCs were isolated using CD11C mouse Microbeads (Miltenyi Biotec, Auburn, CA) for RNA isolation. All RNAs were prepared for quantitative PCR analysis.

3.3.12 Quantitative PCR analysis of IDO mRNA

After *in vivo* treatment of mice or *in vitro* treatment of DCs with liposomes loaded with control or IDO siRNA, total cellular or tissue RNA was isolated using Trizol (Invitrogen) and then used as a template for cDNA synthesis and quantitative PCR (qPCR). qPCR reactions were carried out using gene-specific forward and reverse primers (100 nM each) and SYBR Green PCR Master mix (Stratagene, La Jolla, CA,) in a Stratagene MX 4000 Multiplex qPCR System. Primers used for the amplification of murine IDO and GAPDH were: IDO, 5'-GAAGGATCCTTGAAGACCAC-3' (forward) and 5'-GAAGCTGCGATTTCCACCAA-3'(reverse); GAPDH, 5'-TGATGACATCAAGAAGGTGGTGAA-3' (forward) and 5' -TGGGATGGAAATTGTGAGGGAGAT-3' (reverse).

3.3.13 Effect of Man-lipo-siIDO on *in vivo* mouse melanoma tumor growth

B16-F10 mouse melanoma cells (2×10^5 cells) were suspended in 20 μl of PBS and injected s.c. and dorsally into each C57BL/6 mouse. Man-lipo-siIDO or control liposomes encapsulating 100ug siRNA in a volume of 300ul were injected i.v. into mice 2 days prior to injection with tumor cells and once per week thereafter. Control mice were identically injected with tumor cells followed, at the same time as injection of mice with Man-lipo-siIDO, injection with vehicle control, Man-lipo-siScrambled, or non-mannosed-lipo-siIDO. Mice were checked for tumor formation on alternate days. The day of tumor appearance was noted and thereafter each tumor was measured by caliper and volumes estimated using the following formula: tumor volume = $0.5 \times (\text{width}^2) \times (\text{length})$.

3.3.14 Flow cytometry

Estimation of Cy3 siRNA incorporation by freshly-isolated and cultured DCs was performed by flow cytometry (BD Biosciences, CA). All monoclonal antibodies (mAbs) were from BD Pharmingen, NJ). T cell subsets were analyzed by staining with PE-conjugated or Cy5-conjugated CD4 mAbs, Cy5-conjugated CD8 or CD25 mAbs, and FITC-conjugated FoxP3 antibodies. Apoptosis of T cells was determined by staining with the above mAbs, FITC-conjugated annexinV and Propidium iodide (PI). All flow cytometric analyses were performed using appropriate isotype controls.

3.3.15 Tumor antigen response

Mice were euthanized 28 days after tumor inoculation, T cell suspensions prepared from spleens and lymph nodes [23]. Spleenocytes from melanoma bearing mice were isolated by gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Montreal, Quebec, Canada) and cultured in 96-well plates ($4x10^5$ seeded cells/well) in the presence or absence of B16-F10 tumor Ag in a gradient concentration of 5ug/ml, 25 μg/ml and 50 μg/ml for 72h. Tumor antigens were prepared as previous description [24]. Briefly, B16-F10 cells were trypsinized and resuspended at a density of $5x10^6$/ml in serium frees RPMI. Then the suspension was freeze-thawed by -80^{o}C and 4°C cycles for at least 5 times. The protein (from both cell cytoplasma and cell membrane) concentration of the lysate was determined by a commercial assay (Bio-Rad, Munich, Germany).The mixed lymphocytes and Ag were cultured at 37°C for 72 h in 200 μl of RPMI 1640 supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin and pulsed with 1 μCi/well of 3H-labelled thymidine (Amersham Pharmacia Biotech) for the last 18 h of culture. Results were expressed as mean counts per minute of triplicate cultures ± SEM.

3.3.16 Statistical analysis

Data are presented as means ± SD. Control and experimental data were compared using one-way ANOVA followed by Newman-Keuls testing. p-values less than 0.05 were assumed *a priori* to indicate significant differences.

3.4 Results

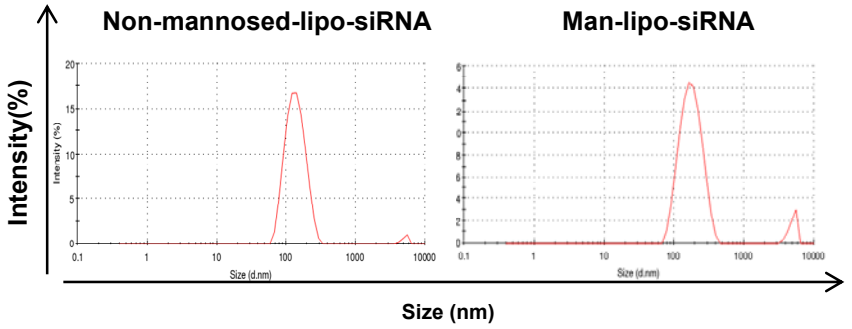
3.4.1 Preparation and characterization of Man-lipo-siRNA

The size and surface charge of liposomes are of vital importance to transfection efficiency and biodistribution [25]. Small size avoids preferential uptake by Kupffer and other reticuloendothelial cells, and a slight positive charge facilitates association with negatively-charged RNA and target cell plasma membrane components [26, 27]. To estimate whether Man-lipo-siRNAs prepared by us had the appropriate size and surface charge for effective *in vivo* delivery, we measured final liposomal particle diameter and zeta potential (Fig. 3-1, A and B). Particle size was approximately 180 nm (Fig.3-1A) and surface charge of Man-lipo-siRNA (represented by zeta potential) was slightly positive and approximately 20 mV (Fig. 3-1B). The Man-lipo and the Non-man-lipo has a similar size and zeta potential. The addition of mannose does not influence the size and zeta potential of the liposome. These data suggest that, according to previously described parameters, the size and surface charge of the prepared liposomes were suitable for potential *in vivo* siRNA delivery [28]. This experiment was repeated three times. In each experiment, over 300 liposome particles were tested.

To determine whether IDO siRNA was encapsulated inside the liposome and protected from RNase degradation, the stability of liposomal siRNA was assessed. Fresh mouse plasma was mixed with Man-lipo-siIDO, non-mannosed-lipo-siIDO, or naked siIDO. Compared to naked siIDO, siIDO encapsulated within mannosed liposomes or non-mannosed-liposomes were protected for at least 48 h (Fig. 3-1C) suggesting that liposomes provide protection against systemic degradation of siRNA by nucleophiles and nucleolytic enzymes, and that addition of mannose to liposomes does not diminish that protective capacity.

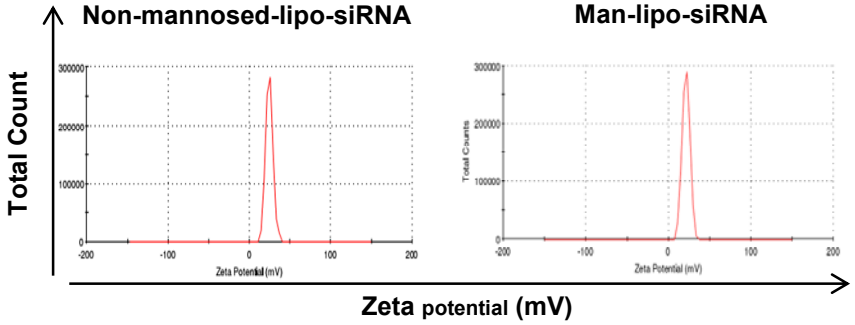
Figure 3-1 Preparation and characterization of Man-lipo-siRNA. (A & B) Determination of the size and zeta potential of Man-lipo-siRNA and non-mannosed-lipo-siRNA. Man-lipo-siRNA or non-mannosed-lipo-siRNA were prepare as described in materials and methods. The size (A) and the zeta potencial (B) of Man-lipo-siRNA and non-mannosed-lipo-siRNA were measured by zetasizer. (C) Serum protection assay. Naked siRNA, Man-lipo-siRNA, or non-mannosed-lipo-siRNA were incubated with fresh mouse plasma at 37 ^{o}C at various time points. 1, 4, 8, 24 and 48 h post incubation, liposomes that encapsulated siRNA were lysised by Tris-Triton 100. siRNA was visualized by agarose electrophoresis. The data shows the compiled data from all three experiments.

A



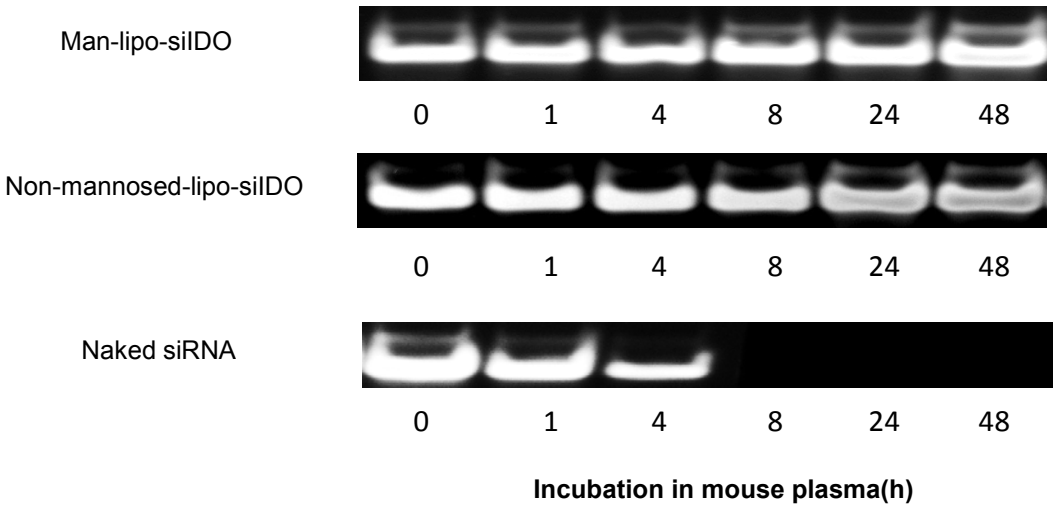
	Average size (nm)	±SD
Non-mannosed-lipo-siRNA	178	7.071068
Man-lipo-siRNA	183	9.291573

B



	Average Zeta potential (mV)	±SD
Non-mannosed-lipo-siRNA	23.375	0.953502
Man-lipo-siRNA	21.95	0.43589

C



3.4.2 *In vitro* targeting by Man-lipo-Cy3 siRNA and gene silencing of IDO

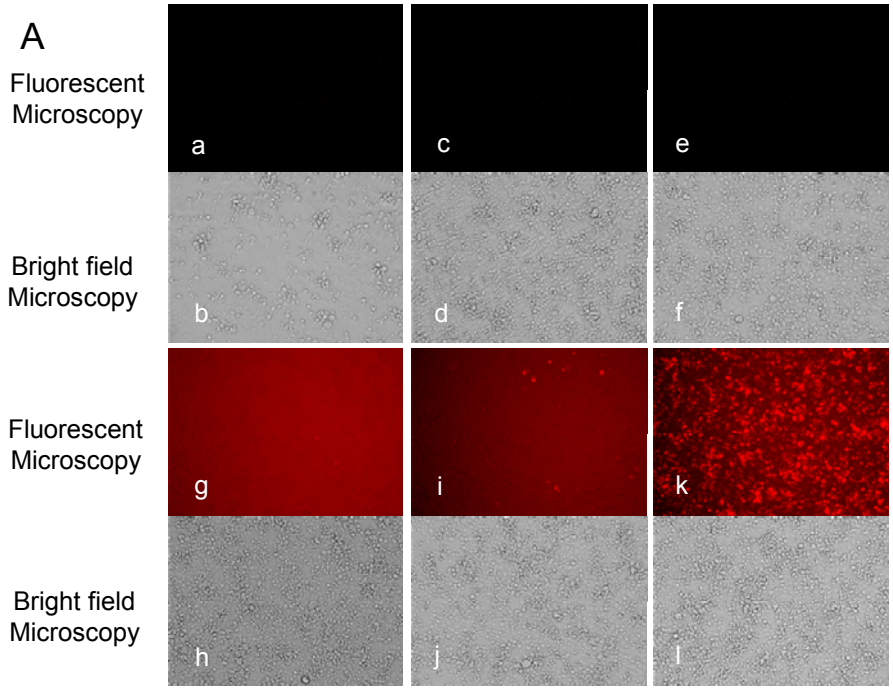
DCs express mannose receptors [16] and are among the most important antigen-presenting cells *in vivo*. To evaluate the targeting efficiency of Man-lipo-Cy3 siRNA to mannose receptor, DCs isolated from mouse bone marrow and cultured *in vitro* for 6 days were used to assess capacity of Man-lipo-Cy3 siRNA to deliver siIDO and reduce IDO mRNA levels. Compared to non-mannosed-lipo-Cy3 siRNA, Man-lipo-Cy3 siRNA has a significantly higher targeting efficiency and cellular uptake (Fig. 3-2, A and B).

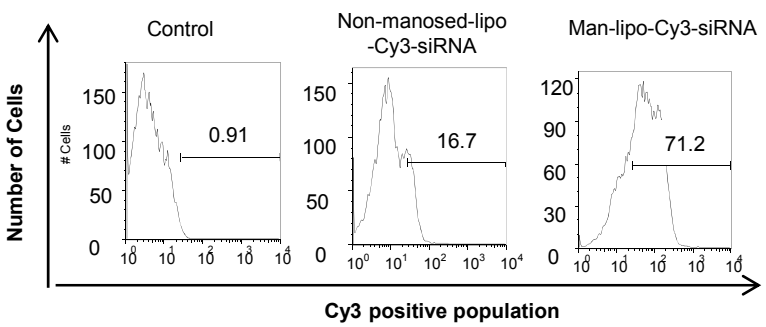
To determine the silencing efficiency of the Man-lipo-siIDO in vitro, Man-lipo-siIDO was used to deliver IDO siRNA to identically-prepared DCs *in vitro*. Compared to Man-lipo-siScrambled, Man-lipo-siIDO significantly reduced the level of IDO mRNA (Fig. 3-2C). The differences of IDO expression between the Non treatment control and Man-lipo-siScrambled are not significant. This data demonstrates that Man-lipo-siIDO is capable of delivering siRNA to DCs and to reduce IDO expression.

3.4.3 Bio-distribution of Man-lipo-Cy3-siRNA and *in vivo* IDO gene silencing

To test targeting efficacy of Man-lipo-siRNA *in vivo*, we investigated the biodistribution of Man-lipo-Cy3 siRNA in mice. Man-lipo-Cy3 siRNA or non-mannosed-lipo-Cy3 siRNA was injected into mice and the distribution of Cy3 siRNA (red fluorescent signal) to heart, liver, spleen, lung and kidney were compared between the two and with organs from mice untreated with liposomes (Fig. 3-3A). Compared to non-mannosed-lipo-Cy3 siRNA controls or non-treatment controls, Man-lipo-Cy3 siRNA accumulated to a significantly higher in lymph node, spleen and liver, consistent with the reported location of mannose receptors in normal, non-tumor mouse tissue [19]. The fluorescence that represents the distribution of Man-lipo-Cy3 siRNA is consistant over 24 h and 48 h (data not shown), but the intensity of fluorescence is weaker as time goes by. Therefore, 6 h bio-distribution was chosen. This data indicates that Man-lipo-siRNA can preferentially target mannose

Figure 3-2 In vitro targeting assay of Man-lipo-Cy3-siRNA and gene silencing of IDO. (A) Transfecting DCs with Man-lipo-Cy3-siRNA. Six-day-cultured DCs were transfected with Man-lipo-Cy3-siRNA (k, l) and non-mannosed-lipo-Cy3-siRNA (i,j). Non treatment DCs (a,b), DCs treated with empty Man-lipo (c,d) or empty non-mannosed-lipo (e,f) and DCs treated with naked Cy3 labeled siRNA (g,h) served as controls. The red fluorescence of Cy3 was detected by fluorescent microscope 4h post transfection. The magnification is 10x. (B) Transfection efficacy of Man-lipo-Cy3-siRNA detected by flow cytometry. Six-day-cultured DCs were transfected by Man-lipo-Cy3-siRNA and non-mannosed-lipo-Cy3-siRNA. 4 h after transfection, the cells were harvested and the fluorescence of encapsulated Cy3-labeled siRNA was detected by flow cytometry. Experiment were repeated three times. (C) Gene silencing of IDO using Man-lipo-siIDO. Six-day-cultured DCs were transfected by Man-lipo-siIDO and non-mannosed-lipo-siIDO. 24 h after transfection, DCs were harvested and message RNA was isolated. The IDO expression of DCs was detected by real time PCR. *$p<0.05$.





	Transfection rate (%)	±SD
Non-mannosed-lipoCy3--siRNA	17	4.163332
Man-lipo-Cy3-siRNA	68	7.505553

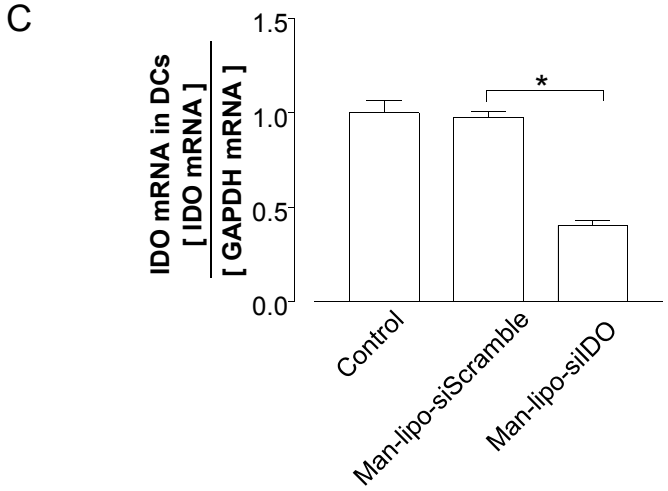
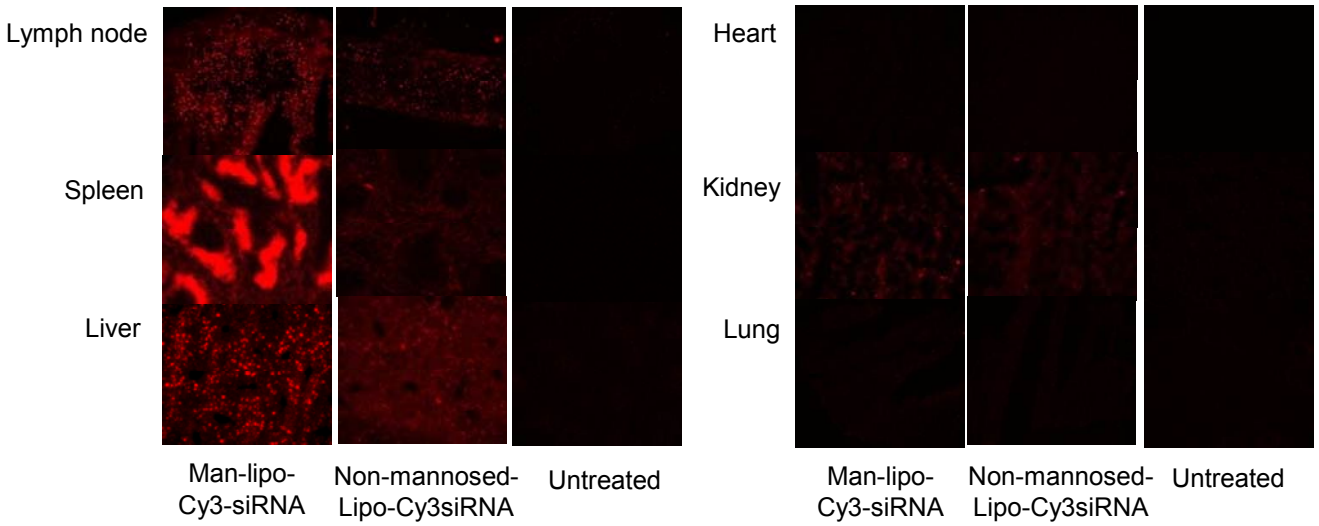


Figure 3-3 Bio-distribution of Man-lipo-Cy3-siRNA. (A) Bio-distribution of Man-lipo-siRNA. Mice were i.v injected with Man-lipo-Cy3-siRNA and non-mannosed-lipo-Cy3-siRNA. 6 h post administration, mice were sacrificed and lymph node, spleen, liver, heart, kidney, lung were extracted for frozen sections. The red fluorescence of Cy3-labeled siRNA in the various tissue sections was observed under fluorescent microscope. The data presented one of three independent experiments. The magnification is 10x.

A


Lymph node
Spleen
Liver
Heart
Kidney
Lung
Man-lipo-Cy3-siRNA
Non-mannosed-Lipo-Cy3siRNA
Untreated
Man-lipo-Cy3-siRNA
Non-mannosed-Lipo-Cy3siRNA
Untreated

receptor- expressing tissues and efficiently deliver siRNA into liver and important immune organs such as spleen and lymph node.

In order to assess *in vivo* gene silencing efficacy in addition to siRNA delivery, Man-lipo-siIDO was injected i.v. into mice. Expression of the IDO gene in lymph node and spleen was significantly reduced compared to organs in animals treated with Man-lipo-siScrambled control (Fig. 3-4 A&B).

To further confirm that DCs in vivo have been targeted and the IDO expression in DCs has been knocked down, 24 hours post administration, $CD11C^+$ cells of non-treatment mice, mice injected with Man-lipo-siScramble and Man-lipo-siIDO were isolated from splenocytes and lymphocytes of Balb/C mice. Total RNA was isolated from the $CD11C^+$ cells in different experiment groups and the IDO expression was determined by qPCR. The IDO expression in Man-lipo-siIDO treatment group was significantly decreased when compare to Man-lipo-siScramble, and no significantly different could be found between non treatment control and Man-lipo-siScramble treatment (Fig. 3-4 C).

3.4.4 Therapeutic effects of Man-lipo-siIDO

It has been reported that more aggressive tumor grow is observed in the cancer patients with more IDO^+ DC expression in DCs [9]. In order to determine whether knock down of IDO may restrain the tumor growth, we treated melanoma-bearing mice with Man-lipo-siIDO. As shown in Fig. 3-5 A&B&C, compare to non-mannosed-lipo-siIDO, Man-lipo-siScrambled control and non-treatment control, melanoma bearing mice that were injected with Man-lipo-siIDO has lengthened time between tumor cell inoculation and the appearance of palpable tumors; reduced tumor size at equal times after tumor cell inoculation; lower tumor weight (28 days after tumor inoculation). The tumor weight is different between the Man-lipo-siIDO and non-mannosed-lipo-siIDO treatment groups when compared to tumor size. Compared to melanoma in early stage, advanced melanoma contains more liquid inside the tumor since advanced vascularization is formed. The early stage melanoma contains more connective tissue,

Figure 3-4 In vivo gene silencing of IDO. (A & B) In vivo gene silencing by Man-lipo-siIDO. Man-lipo-siIDO or Man-lipo-siScrambled were i.v injected into melanoma bearing C57/BL6 mice. 24 h after injection, mice were sacrificed and the IDO expression in spleen (A) and lymph node (B) were detected by real time qPCR.*$p<0.05$. (C) In vivo silencing of IDO on $CD11C^+$ DCs by Man-lipo-siIDO. Man-lipo-siIDO or Man-lipo-siScrambled were i.v injected into C57/BL6 mice. 24 h after injection, mice were sacrificed and $CD11C^+$ DCs were isolated for IDO expression determination. IDO expression was detected by qPCR. *$p<0.05$. The data presented complied results from 3 experiments. 24 mice were randomly distributed in each group in each experiment.

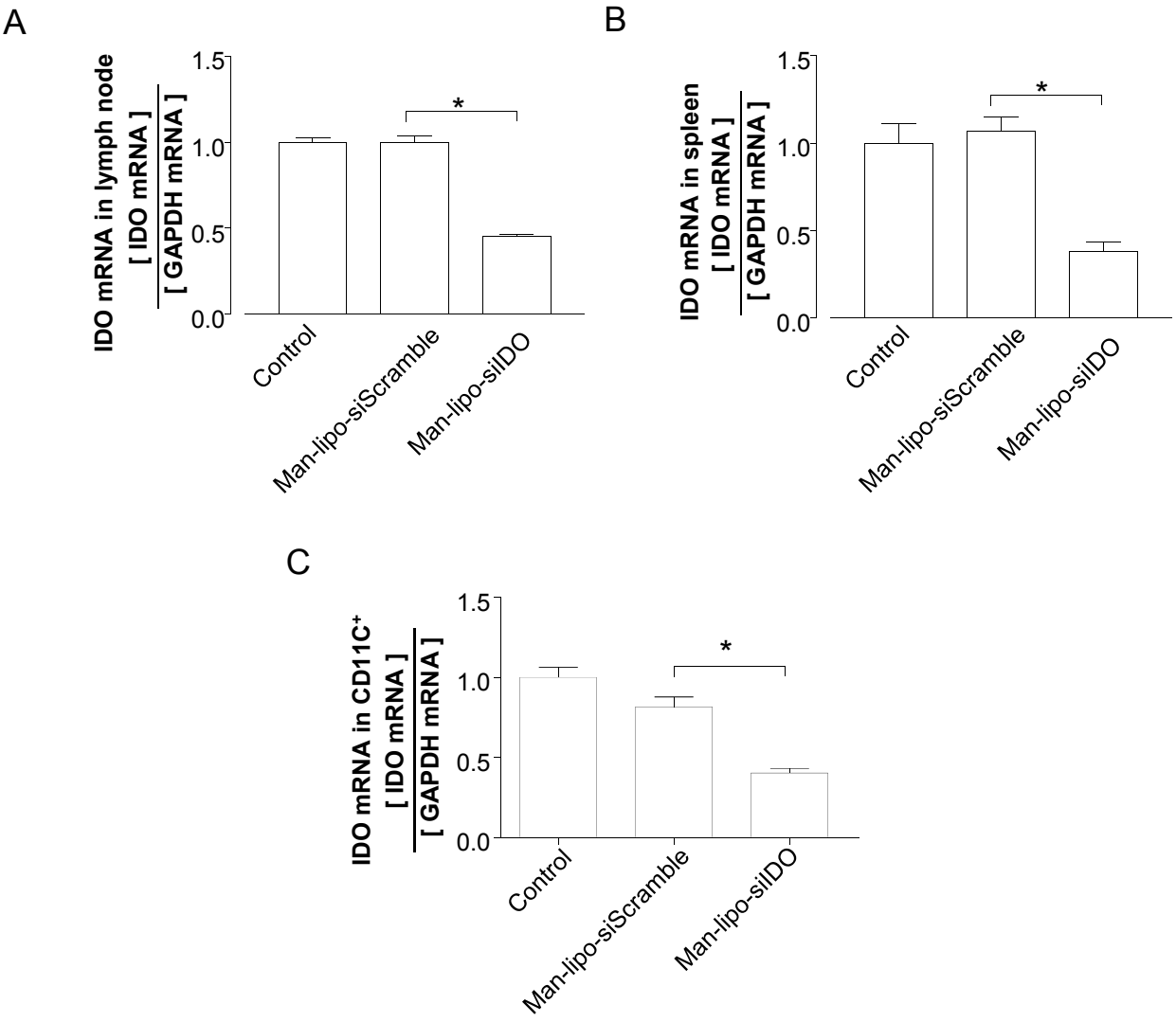
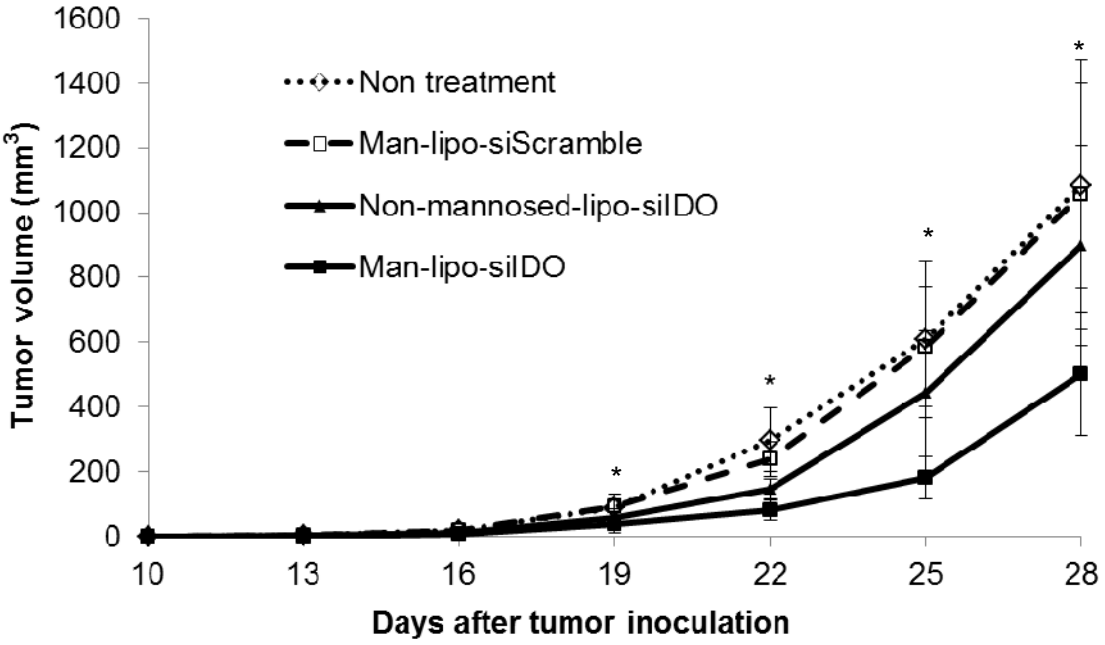
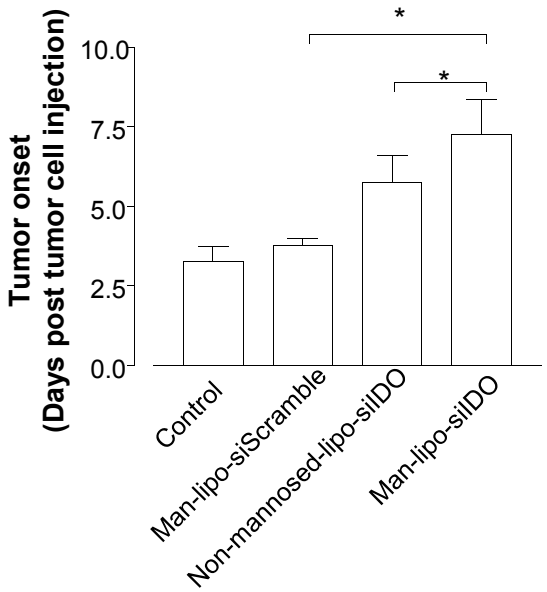

A
IDO mRNA in lymph node
[IDO mRNA]
[GAPDH mRNA]
1.5
1.0
0.5
0.0
*
Control
Man-lipo-siScramble
Man-lipo-siIDO
B
IDO mRNA in spleen
[IDO mRNA]
[GAPDH mRNA]
1.5
1.0
0.5
0.0
*
Control
Man-lipo-siScramble
Man-lipo-siIDO
C
IDO mRNA in CD11C+
[IDO mRNA]
[GAPDH mRNA]
1.5
1.0
0.5
0.0
*
Control
Man-lipo-siScramble
Man-lipo-siIDO

Figure 3-5 Anti-tumor effects of Man-lipo-siIDO. (A & B) Experimental treatment of murine melanoma using Man-lipo-siIDO. Man-lipo-siIDO, or non-mannosed-lipo-siIDO, or Man-lipo-siScramble was i.v. injected into C57/BL6 mice two days before tumor inoculation. Treatments were repeated once a week thereafter. Tumor size (A) in treatment and control mice was monitored every three days. Onset time (B) in treatment and control mice was monitored every day after tumor inoculation. (C) Tumours in different experimental groups or control groups were extracted and weighted in day 28 after mice were sacrificed. Results represent compiled data from all three experiments. Each point represents the weight of 6 mice in each group. Results represent 1 of 3 experiments.*$p<0.05$ (Man-lipo-siIDO vs non-mannosed-lipo-siIDO)

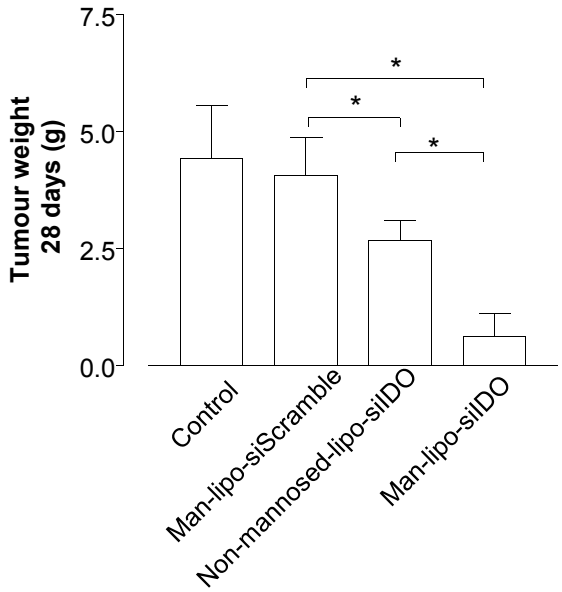
A


1600
1400
1200
1000
800
600
400
200
0
Tumor volume (mm3)
Non treatment
Man-lipo-siScramble
Non-mannosed-lipo-siIDO
Man-lipo-siIDO
10
13
16
19
22
25
28
Days after tumor inoculation
*

B


Tumor onset
(Days post tumor cell injection)
10.0
7.5
5.0
2.5
0.0
*
*
Control
Man-lipo-siScramble
Non-mannosed-lipo-siIDO
Man-lipo-siIDO

C


Tumour weight
28 days (g)
7.5
5.0
2.5
0.0
*
*
*
Control
Man-lipo-siScramble
Non-mannosed-lipo-siIDO
Man-lipo-siIDO

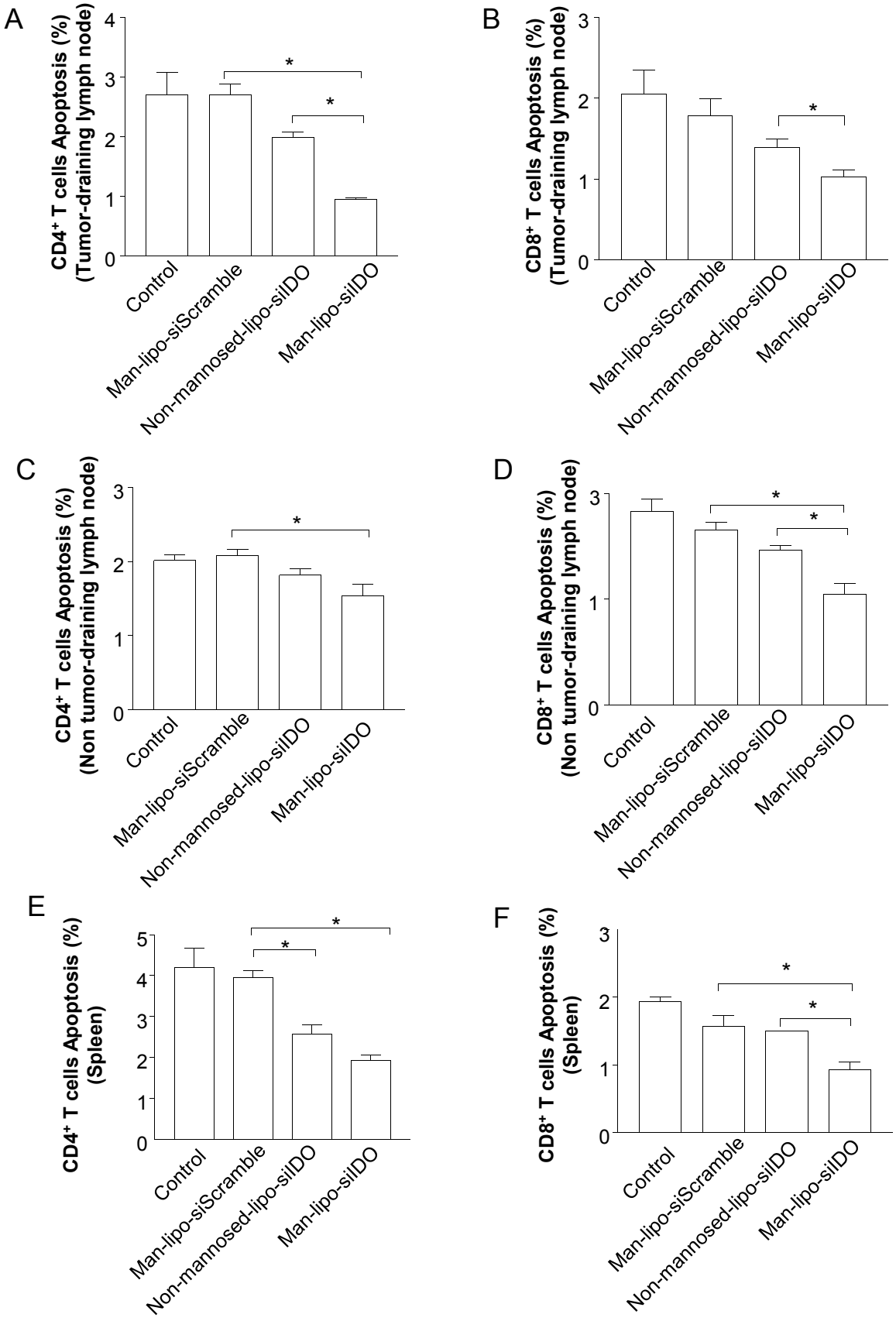
which usually located between the malignant tissue and normal tissue, includes skin and muscle. Since the size measurement includes the connective tissue but the weight measurement does not include the connective tissue, which may explain the inconsistence between the tumor size and tumor weight measurement.

Accumulation of IDO^+ DC in lymph node correlates the poor prognosis of cancers by inducing $CD4^+$ and $CD8^+$ T cells apoptosis, which subsequently leads to the slowing of tumor growth [6, 9, 29]. In order to identify that silencing IDO expression in spleen and lymph node can reduce $CD4^+$ and $CD8^+$ T cell apoptosis, splencoytes and lymphocytes isolated by Ficoll were stained with Cy5-conjugated CD4 mAbs, Cy5-conjugated CD8, FITC-conjugated annexin V and PI were detected by flow cytometer. Dead cells were excluded by PI staining in analysis. Compare various control groups, the $CD4^+$ and $CD8^+$ apoptosis T cells population in drain lymph node, lymph node and spleen were significantly less in Man-lipo-siIDO injected mice (Fig. 3-6 A-F). When compared to Man-lipo-siScramble, non-mannosed-lipo-siIDO has significantly less apoptosis $CD4^+$ cells in draining lymph node and spleen, also less apoptosis $CD8^+$ T cells in non-draining lymph node and spleen. However, the apoptosis was further reduced by the addition of mannose into the delivery system. This suggests that Man-lipo-siIDO arouses anti-tumor effects might partially attribute to the reducing $CD4^+$ and $CD8^+$ T cell apoptosis population in spleen, lymph node and draining lymph node.

3.4.5 Enhancement of anti-tumor immunity by Man-lipo-siIDO

The expression of IDO induced tumor escape is mainly caused by the IDO induced T cell apoptosis and immune suppression[7]. Thus, we detected the anti-tumor immunity by evaluating the anti-tumor antigen ability of the splenocyte and lymphocyte. When compare to Man-lipo-siScramble and Non-treatment control, non-mannosed-lipo-siIDO treatment mice has relatively stronger reaction against to the B16-F10 melanoma antigen. The differences between Non-treatment control, Man-lipo-siScramble and non-mannosed-lipo-siIDO are not statistically significant. Mice treated with Man-lipo-siIDO

Figure 3-6 Suppression of T cells apoptosis by Man-lipo-siIDO. C57/BL6 mice were treated with Man-lipo-siIDO, or non-mannosed-lipo-siIDO, or Man-lipo-siScramble, as described in the legend to Fig. 4. The mice were sacrificed in day 28, and the $CD4^+$ and $CD8^+$T cells in drain lymph node (A, B), lymph node (C, D) and spleen(E, F) were isolated. The apoptosis in $CD4^+$ and $CD8^+$ T cells were detected by flow cytometry after Annexin V staining. Results represent complied results from 3 experiments (n = 6 per group/experiment). *$p<0.05$.


A
CD4+ T cells Apoptosis (%)
(Tumor-draining lymph node)
0
1
2
3
4
*
*
Control
Man-lipo-siScramble
Non-mannosed-lipo-siIDO
Man-lipo-siIDO
B
CD8+ T cells Apoptosis (%)
(Tumor-draining lymph node)
0
1
2
3
*
Control
Man-lipo-siScramble
Non-mannosed-lipo-siIDO
Man-lipo-siIDO
C
CD4+ T cells Apoptosis (%)
(Non tumor-draining lymph node)
0
1
2
3
*
Control
Man-lipo-siScramble
Non-mannosed-lipo-siIDO
Man-lipo-siIDO
D
CD8+ T cells Apoptosis (%)
(Non tumor-draining lymph node)
0
1
3
*
*
Control
Man-lipo-siScramble
Non-mannosed-lipo-siIDO
Man-lipo-siIDO
E
CD4+ T cells Apoptosis (%)
(Spleen)
0
1
2
3
4
5
*
*
Control
Man-lipo-siScramble
Non-mannosed-lipo-siIDO
Man-lipo-siIDO
F
CD8+ T cells Apoptosis (%)
(Spleen)
0
1
2
3
*
*
Control
Man-lipo-siScramble
Non-mannosed-lipo-siIDO
Man-lipo-siIDO

have significantly stronger response against to tumor antigen when compared to mice treated with non-mannosed-lipo-siIDO (Fig. 3-7 A&B).

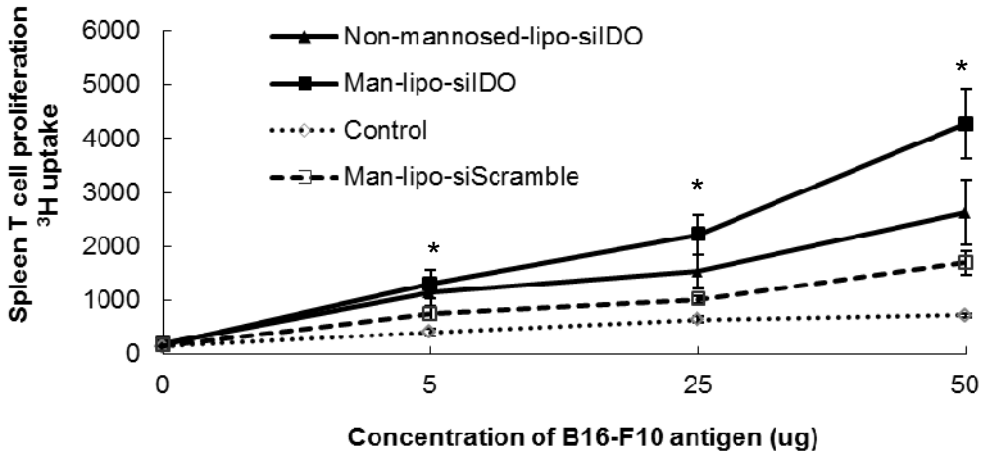
Recent evidence suggests that IDO promotes the generation of induced Tregs[30]. Clinical studies reveal that there is a positive correlation between the levels of IDO induced Tregs and poor clinical outcomes [31]. We speculated that by down regulation of IDO expression, the expression of Tregs in spleen, especially in drain lymph node will be decreased. As shown in Fig.3-7 C&D, mice injected with Man-lipo-siIDO had lower Treg expression when compare to non-treatment control and Man-lipo-siScrambled control. These results highlight that the Man-lipo-siIDO was able to inhibit Treg cells and enhance the overall anti-tumor immunity of T cells.

3.5 Discussion

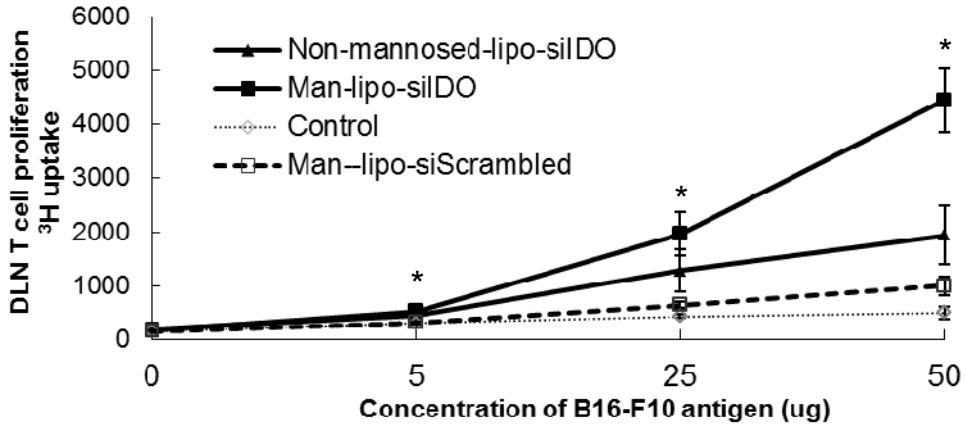
Increasingly accumulating data from clinical and basic research demonstrates that IDO plays an important role in tumor immune evasion[32]. The tumor escape induced by IDO is mostly attributed to IDO^+ DC induced T cell apoptosis and Treg accumulation [9, 33, 34], which leads to the immune dysfunction of the immune organ, especially the sentinel lymph node [35, 36]. However, effective therapy by knocking down the expression of IDO through specifically targeting APCs in lymph node and spleen has not yet been reported. In this study, we developed a APC-targeting delivery system based on specific affinity of mannosed-liposome to mannose receptor expressing APCs. We observed that Man-lipo-siIDO can efficiently target DCs and repress IDO expression on DCs in vitro and in vivo. By silencing IDO expression in spleen and lymph node of tumor bearing mice, tumor growth could be restricted, which might attribute to the significantly decreased of Treg cells populations as well as significantly decreased apoptotic $CD4^+$ and $CD8^+$ effector T cells in the draining lymph node and spleen.

Figure 3-7 Manipulation of anti-tumor immunity by Man-lipo-siIDO. (A & B) Tumor-Ag-specific T cell response. T cells isolated from lymph node (A) and spleen (B) of various experimental and control groups were mixed with indicated concentration of tumor antigen respectively. Cells were pulsed with 1 μCi of [^{3}H] thymidine for the last 18 h. T cells proliferation level was determined by radioactivity detection. Results were expressed as the mean counts per min of triplicate cultures ± SEM (n = 6 per group/experiment) *= $p<0.05$ (Man-lipo-siIDO vs non-mannosed-lipo-siIDO). (C&D) Treg cells in Man-lipo-siIDO treated tumor-bearing mice. C57/BL6 mice were treated with Man-lipo-siIDO, or non-mannosed-lipo-siIDO, or Man-lipo-siScramble, as described in the legend to Fig. 4. After mice were sacrificed, $CD4^+$ T cells from drain lymph node (C) and spleen (D) were isolated. $CD4^+CD25^+FoxP3^+$ Treg cells were detected by flow cytometry. Results represent 1 of 3 experiments (n = 6 per group/experiment). *$p<0.05$.

A


Spleen T cell proliferation
^{3}H uptake
6000
5000
4000
3000
2000
1000
0
Non-mannosed-lipo-siIDO
Man-lipo-siIDO
Control
Man-lipo-siScramble
*
*
*
0
5
25
50
Concentration of B16-F10 antigen (ug)

B


DLN T cell proliferation
^{3}H uptake
6000
5000
4000
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Non-mannosed-lipo-siIDO
Man-lipo-siIDO
Control
Man--lipo-siScrambled
*
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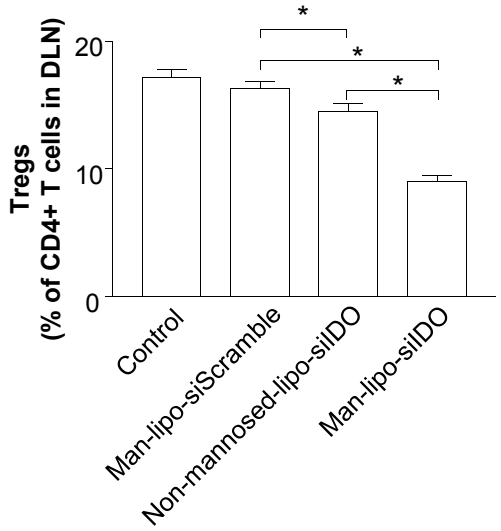
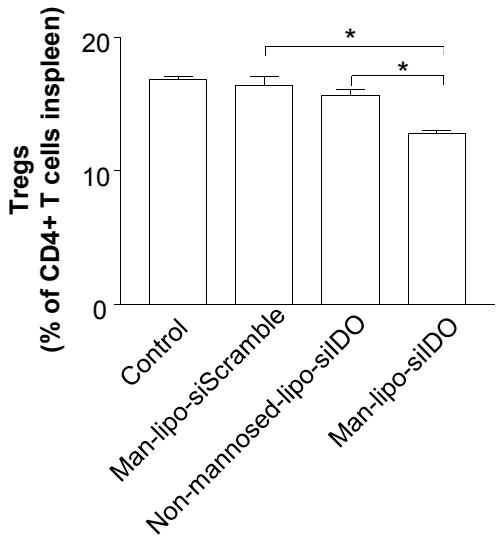
Concentration of B16-F10 antigen (ug)

C


Tregs
(% of CD4+ T cells in DLN)
20
10
0
*
*
*
Control
Man-lipo-siScramble
Non-mannosed-lipo-siIDO
Man-lipo-siIDO

D


Tregs
(% of CD4+ T cells inspleen)
20
10
0
*
*
Control
Man-lipo-siScramble
Non-mannosed-lipo-siIDO
Man-lipo-siIDO

RNAi is an endogenous mechanism in which double-stranded RNA knocks down the expression of homologous genes, thus induces gene silencing. Since it was first observed in late 1980, it had been used as a powerful laboratory technique for gene silencing [10]. Recently, some research on animal disease model, such as cancer, viral infection, and age-related macular degernation has moved RNAi forward in clinical application. However, the lack of specific and effective in vivo delivery methods is the major barrier for its application [11, 12]. While “flooding” the system with siRNA in the form of hydrodynamic administration is effective in animal models, this approach is not practical for clinical intervention [37]. The ability to selectively deliver siRNA to specific cells would allow for lower concentrations of siRNA to be used, as well as possibly the alleviating need for systemic delivery. We have been developed several tissue/cell-specific targeting methods for siRNA delivery [38, 39]

Comparing liposomes with other carrier delivery systems, liposomes offer multiple advantages for drug delivery, including their biocompatibility, biodegradability and safety. Mannosed liposomes have been considered as promising non-live vectors for targeted delivery purposes [40]. In order to achieve DCs-targeted delivery of IDO siRNA, we conjugated D-(+)-mannose to the liposome, which specifically binds to the Lectins expressed in mice/humans that have shown mannose-binding activity include transmembrane proteins such as the classical Macrophage mannose receptor (MMR group VI), Endo180, dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), DC-SIGN related (L-SIGN, group II, type 2 receptors) or their mouse homologues (SIGNR), Langerin or secreted/soluble proteins such as the collectins mannose binding protein (MBP), surfactant protein A and surfactant protein D (group III) [19].

Mannosed particulates are formed by the association of the mannose derivative to a carrier (liposome, nanoparticle, etc.). The mannose derivative acts as a targeting agent whereas the particulate or carrier acts as a reservoir for the therapeutic agent. Because of their particulate nature, these particles will be engulfed by phagocytic APCs and mannosylation will further improve their phagocytosis mediated by specific receptors. Transmembrane mannose receptors mediate endocytosis, function as antigen capture

receptors and are involved in antigen capture and presentation[17, 41]. After recognition, MMR delivers the antigen to the early endosomes and recycles to the surface [42]. The content of endosomes is subsequently targeted to lysosomes in which the degradation produces antigen fragments that, after presentation in MHC molecules, can stimulate the adaptive immune responses[41]. For DC-SIGN, this receptor delivers the bound components to late endosomes or lysosomes where they are degraded. The generated antigens are processed for MHC class II presentation to T cells [43]. Under specific conditions, both MMR and DC-SIGN appear to deliver the antigens in MHC class I molecules [44, 45]. Mannosed liposome has been used in various vaccine studies targeting DCs in vivo. For example, the peptide-based mannosed lipoarabinomannan (ManLAM) is reported to be able to target the host DCs and arouse antibodies against Mycobacterium tuberculosis [46]. In our study, we observed a strong targeting ability of our Man-lipo-siRNA towards DCs. Further, We also observed that the Man-lipo-siIDO can efficiently target macrophage in vivo, which is also an important antigen presenting cell that influence T cell proliferation [5]. When compared to non-mannosed-lipo-siIDO, which could only deliver siRNA into 15% of $CD11b^+$ cells in splenocytes and lymphocytes, the Man-lipo-siIDO could transfected up to 70% of $CD11b^+$ cells in splenocytes and lymphocytes. The IDO expression can be significantly knocked down in macrophage (data not shown). In our study, although a much less targeting efficiency in Non-mannosed-lipo-siIDO groups when compared to Man-lipo-siIDO groups is observed, the restriction of tumor growth, reduction apoptotic $CD4^+$ and $CD8^+$ T cells and the up-regulation of antigen response is significant. It could attribute to the characteristic of the liposomes; they would finally be engulfed by APCs when circulate in the circulation system. By being engulfed by the APCs, they silenced the IDO expression and exerted the anti-tumor effect. However, compared to non-mannosed-lipo-siRNA, the Man-lipo-siRNA locates much more in spleen and lymph node and less located in liver. This result demonstrates that the addition of mannose to the liposome can enhance the cellular uptake of siRNA by guiding the whole lipoplex to organs that are rich of APCs and significantly increased the anti-tumor ability.

DCs and macrophages are the most important APCs, which are closely related to cancer immunity and clinical outcome. Accumulating reports have extended on the immune-

repressive role of IDO in cancer diseases [47]. IDO^+ DCs in humans often show a morphology of plasmacytoid and express CD123 and CCR6 chemokine receptor simultaneously [48, 49]. It is possible that IDO expression of IDO in these cells was stimulated by Toll-like receptor (TLR)-9 ligands activation, and them facilitates the generation of adaptive Treg cells from naïve allogeneic $CD4^+CD25^-$ T cells [7]. In mice, the IDO^+ DCs occupies a proximally 0.3-0.5% of total lymph node cells, these cells accumulates in tumor-draining lymph nodes can results in local T cell repression[9]. In our study, we observed a significantly decreased apoptosis of $CD4^+$ and $CD8^+$ T cell in Man-lipo-siIDO groups, which demonstrates that by specifically silencing IDO, local T cell can be protected from apoptosis. We also successfully reduced the Treg population in both tumor draining lymph nodes and spleen, which plays an important role in limiting tumor growth and metastasis [50]. The splenocytes and lymphocytes are reinstalled with anti-tumor ability through our therapy, when compare to other controls, such as non-mannosed-lipo-siIDO control, Man-lipo-siScrambled control and non-treatment control, Man-lipo-siIDO treated mice has significantly anti-tumor immunity, which further demonstrates that our therapy is preferentially targeting and potent.

In summary, we report here a mannose expressing APCs targeting systems to delivery IDO siRNA can efficiently block the IDO expression in splenocytes and lymphocytes. Treatment of Man-lipo-siIDO results in significantly lengthened tumor onset time, reduced tumor size and tumor weight; decreased apoptosis of $CD8^+$ and $CD4^+$ T cells isolated from spleen and lymph nodes; increased reactivity of T cells isolated from spleen and lymphe nodes against B16 melanoma cell antigens and decreased $CD4^+CD25^+FoxP3^+$ Treg cells isolated from spleen and draining lymph nodes.

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Chapter 4

4 Targeted siRNA silencing of BRAF in melanoma using folate receptor-conjugated liposomes

4.1 Summary

The high frequency of mutation (60-80%) in melanoma and its central role in regulation of cell proliferation and survival makes v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) a heat key target for melanoma therapy. However, the off target and drug resistance effects create many difficulties in practical use. Targeting gene silencing by siRNA could be a promising alternative, since it is highly specific to the mRNA sequence of the intended gene. B16-F10 murine melanoma cell line retains high levels of BRAF expression while consistently expressing folate receptor. In this study, we constructed a liposomal folate receptors targeting siRNA delivery system. The B16-F10 targeting efficiency of folate liposome encapsulated siRNA (Folate-lipo-siRNA) was detected by flow cytometry *in vitro* and fluorescent microscopy *in vivo*. Cells that were transfected with BRAF siRNA had significantly less proliferation in vitro. In a mouse melanoma model, compared to controls (Non-treatment mice, mice treated with Folate-lipo-siScramble, or with non-folate-lipo-siBRAF), mice treated with Folate-lipo-siBRAF had a reduced tumor volume, tumor weight and reduced expression of PCNA and vascular networks in tumor tissue. This study demonstrates that using Folate-lipo-siBRAF can preferentially silence BRAF expression in folate receptor expression cells *in vitro* and *in vivo* and that silencing BRAF by siRNA can prevent melanoma growth. This study highlights that Folate-lipo-siBRAF has potential for development as an anticancer therapeutic agent.

4.2 Introduction

Melanoma is the sixth most common cancer and the malignancy with the highest continuing rise in incidence [1]. For several decades the progress in the melanoma field is slow. Systemic therapies have made little or no contribution to overall survival rate [2-4].

However, the identification of mutant BRAF, which is now known to be a major driver of melanoma progression, provided great progress in treatment of melanoma [5].

RAF is the first identified RAS downstream effectors. The RAF family is composed of three members, A-RAF, B-RAF and RAF1 (or C-RAF), within which, B-RAF is considerably stronger in kinase activity than the other two isotypes [6, 7]. B-RAF is serine threonine kinase, which is part of the RAF/MEK/ERK serine threonine kinase cascade. This cascade , also called 'classical' MAPK pathway, regulates cell growth, survival and differentiation [8].The discovery of oncogenic BRAF mutations in melanoma was in 2002, since then, B-RAF stands as one of the most powerful affirmations of the transformative potential of systemic cancer genome characterization [7, 9]. Recently, B-RAF mutations have been reported at high frequencies of over 70% in melanoma [10]. The mutations are also found in premalignant atypical or dysplastic nevi, which suggest that B-RAF activation could be an initiating event in tumorigenesis[11, 12]. Lots of B-RAF inhibitors have emerged and been tested in clinical trials. However, resistance is found in the later or follow-up treatment on patients. siRNA therapy could be a potential alternative since siRNA can knock down the expression of any proteins or non-coding RNAs and can be easily synthesized or cloned into expression vectors (shRNA) with minimal toxicity. In contrast, chemical inhibitors against certain proteins are limited and constructing dominant negative mutants is time-consuming. Moreover, the chemical inhibitors and dominant negative mutants are more toxic to the cells compared with siRNAs [13].

Folic acid is a water-soluble B vitamin, which is essential for de novo synthesis and one carbon transfer reactions[14].Folate receptors (FRs), also known as folate-binding proteins (FBP), are *N* -glycosylated proteins with high binding affinity to folate in vivo. FRs include at least four isoforms, α , β , γ / γ ′and δ.The affinities of folic acid for the FRs are: FR- α , Kd~ 0.1 nM[15]; FR- β , Kd ~1 nM[16]; and FR- γ , Kd ~ 0.4 nM[17]. Functional FR expression is low or absent in most normal tissues, only FR- α expression in the luminal surface of certain epithelial cells [18], which are inaccessible to blood circulation. In contrast, many malignant cancers, especially epithelial carcinomas, consistently and uniformly express high levels of FR-α, which is accessible to blood

circulation[14, 19].The frequent overexpression within tumors and highly restricted distribution in normal tissues suggest that both FR-α and -β can potentially be exploited as a tumor-specific cell surface marker that can be used in the targeted delivery of cancer therapeutics[14, 20]. Since B16-F10 melanoma over-express B-RAF while consistently expressing folate receptors [21], this makes it an ideal modal for investigating the potential therapeutic application of Folate-lipo-siBRAF.

In this study, we constructed a liposomal folate receptors targeting siRNA delivery system which can preferentially knock down B-RAF in B16-F10 cells in vitro. Administration of folate receptor targeted liposomes encapsulated B-RAF siRNA (Folate-lipo-siBRAF) resulted in the knockdown of B-RAF expressing in tumor tissue, which leads to the reduced proliferation and vascularization of tumor cells in vivo.

4.3 Materials and Methods

4.3.1 Chemicals

1, 3-Dioleoyl-3-trimethylammonium propane (DOTAP), cholesterol, folate-distearoylphosphatidylethanolamine-N-poly(ethyleneglycol) (Folate-DSPE-PEG) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG_{2000}) were purchased from Avanti Lipids, Inc. (Alabaster, AL). All chemicals were reagent grade.

4.3.2 Mice

C57BL/6 mice (6-8 weeks old males) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained under pathogen-free conditions in the Western University barrier rodent facility. All experiments were performed in accord with

guidelines and standard operating procedures set out by the Canadian Council on Animal Care and the Western University Animal Use Subcommittee.

4.3.3 BRAF siRNA design

A double-stranded siRNA targeting murine B-RAF, as described in previous report[22] and was synthesized by Dharmacon, Inc. (Lafayette, CO, USA). The sequences were: B-RAF sequence: sense, 5'-GCUUACUGGAGAGGAGUUACA-3'; antisense, 5'-UGUAACUCCUCUCCAGUAAGC-3'. The sequence for scramble siRNA was: sence, 5'-CGUACGCGGAAUACUUCGA-3'; antisense, 5'-CGTACGCGGAATACTTCGA-3'.

4.3.4 Cell culture

B16F10 murine melanoma cells were cultured in 75-mm culture dishes in Gibco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Burlington, ON, CA) and antibiotics (100 U/ml of penicillin and 100 lg/ml of streptomycin).

4.3.5 In vitro silencing using siBRAF and evaluation of anti-proliferation effect of siBRAF

B16F10 cells were transfected with B-RAF siRNA using L2K (Invitrogen, Carlsbad, CA, USA). The non-treatment cells were used as negative controls. Briefly, cells were plated into 24-well plates (1.5×10^5 cells per well) and allowed to grow overnight, to reach 80% confluence. Cells were transfected with 0.5μgB-RAF siRNA in serum-reduced medium for 4 h, and then incubated in complete medium for 24 h. All RNAs were prepared for quantitative PCR (qPCR) analysis.

24 h post siRNA transfection, B16-F10 cells were re-plated in 96 well/plate in a number of 500/well. Cells were then subculture for 68 h then (3-(4,5-Dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) was added and co-incubate for 4 h. After co-incubation, cells were centrifuged and supernatant was removed and 150µl of DMSO were added and mixed. Absorbence was read by microplate reader (bench mark microplate reader, Bio Rad, Mississauga, Ontario, CA) at a wavelength of 695nm as a reference and 590nm as the absorbance.

24 h post siRNA transfection, B16-F10 cells were re-plated in 96 well/plate in a number of 500/well. Cells were then subcultured in the indicated time point. 24 h, 48 h, 72 h, 96 h and 120 h after re-plating, cells were suspended and trypan blue was used for cell counting.

4.3.6 Western Blot

B16-F10 cells were plated into 24-well plates (1.5×10^5 cells per well) and allowed to grow overnight, to reach 80% confluence. Then cells were transfected with siBRAF or siScramble using lipofectamine for 72 h. Cells were washed and lysised by RIPA buffer (cells signaling technology, Danvers, USA) and total cellular protein was isolated from B16-F10 cells 72 h after transfection for western blot analysis using anti-mouse B-RAF (Abcam, Toronto, ON, CA) antibody.

4.3.7 Preparation of Folate-lipo-siBRAF

DOTAP, DPPC, cholesterols, and Folate-DSPE-PEG were mixed (3.75:13:13:1 molar ratio) to construct a folate receptor-targeting liposome. DSPE-PEG2000 (molar ratio to DOTAP was 3.75:4) was added after extrusion. The same reagents without the addition of folate were used to construct the control, non-folate liposome. The lipids were dissolved in chloroform in a glass tube, evaporated to complete dryness under vacuum. The lipid film was hydrated by the siRNA and protamine mixture. siRNA (targeting B-RAF in distilled water was added to an equal volume of liposomes and protamine at a ratio of 600 nmol DOTAP:30 µg protamine:50 µg siRNA and incubated at room temperature for 10 min to form multilamellar liposomes and siRNA mixture. The

multilamellar liposome and siRNA mixture suspension was vortexed for 5 min followed by sonication for 2 min at 50°C in a bath sonicator, and the sonicated suspension extruded sequentially through polycarbonate membranes of decreasing pore size (0.4, 0.2 and 0.1 mm diameter pores). The liposome nanoparticle solution was incubated (50°C, 10 min; followed by cooling to 25^{o}C) with DSPE-PEG2000 in a molar ratio to DOTAP of 3.75:4 and 3.75: 5 to generate Folate and Non-folate liposomes, respectively.

4.3.8 Folate liposome particle diameter and zeta potential

Freshly prepared Non-folate and Folate liposomes were diluted with phosphate-buffered saline (PBS) and the mean particle diameter and surface charge (zeta potential) measured using a Zetasizer Nano Series (Malvern Instruments Ltd., UK) according to the manufacturer's protocol.

4.3.9 *In vitro* uptake of liposomes loaded with Cy3 siRNA (Folate-lipo-Cy3 siRNA)

Freshly prepared mouse bone marrow cells ($2x10^5$) and B16-F10 cells ($2x10^5$/well) were plated in 12 well/plate and transfected with Folate-lipo-Cy3 siRNA, Non-folate-lipo-Cy3 siRNA. Cells were harvested 4 h after transfection and Cy3 fluoresence were analyzed by flow cytometry.

4.3.10 *In vitro* silencing of B-RAF in B16-F10 using Folate-lipo-siBRAF

B16-F10 cells were plated into 24-well plates (1.5×10^5 cells per well) and allowed to grow overnight, to reach 80% confluence. Then cells were transfected with Folate-lipo-siBRAF or Folate-lipo-siScramble for 24 h. Total cellular RNA was isolated from B16-F10 cells 24 h after transfection for qPCR analysis.

4.3.11 *In vivo* distribution of Folate-lipo-Cy3-siRNA

C57/BL6 mice were used for melanoma inoculation using B16-F10 cells ($2x10^5$/ mice). Mice bearing melanoma were ready to be used when tumors reached the size of $500mm^3$. Non-folate-lipo-Cy3siRNA or Folate-lipo-Cy3 siRNA were injected intravenously into mouse tail veins (50 μg/ mouse). Mice were euthanized 6 h post-injection and organs (tumor, heart, liver, spleen, lung and kidney) were collected, rinsed with saline, snap-frozen in liquid nitrogen, mounted for cryostat sectioning, and viewed using fluorescence microscopy.

4.3.12 *In vivo* silencing of BRAF using Folate-lipo-siBRAF

Folate-lipo-siBRAF and Non-folate-lipo-siBRAF were injected intravenously into mouse tail veins (50 μg/ mouse). Mice were euthanized 24 h later and tumors were collected for RNA isolation. All RNAs were prepared for qPCR analysis.

4.3.13 Quantitative PCR analysis of B-RAF mRNA

After *in vivo* treatment of mice or *in vitro* treatment of B16-F10 cells with liposomes loaded with control or B-RAF siRNA, total cellular or tissue RNA was isolated using Trizol (Invitrogen) and then used as a template for cDNA synthesis and quantitative PCR (qPCR). qPCR reactions using gene-specific forward and reverse primers (100 nM each) and SYBR Green PCR Master mix (Stratagene, La Jolla, CA,) in a Stratagene MX 4000 Multiplex qPCR System. Primers used for the amplification of murine B-RAF and GAPDH were: B-RAF, 5'-CAATTGGCTGGGACACGGACAT-3' (forward) and 5'-TTGACAACGGAAACCCTGGAAAAG-3'(reverse); GAPDH, 5'-TGATGACATCAAGAAGGTGGTGAA-3' (forward) and 5' -TGGGATGGAAATTGTGAGGGAGAT-3' (reverse).

4.3.14 Effect of Folate-lipo-siBRAF on *in vivo* mouse melanoma tumor growth

B16-F10 mouse melanoma cells (2 x 10^5 cells) were suspended in 20 μl of PBS and injected s.c. and dorsally into each C57BL/6 mouse. Folate-lipo-siIDO or control liposomes encapsulates 100 μg siRNA in a volume of 300 μl were injected i.v. into mice 2 days after injection with tumor cells and once per week thereafter. Control mice were identically injected with tumor cells followed, at the same time as injection of mice with Folate-lipo-siIDO, Folate-lipo-siScrambled, or non-folate-lipo-siIDO were also injected. Mice were checked for tumor formation on alternate days. Each tumor was measured by caliper and volumes estimated using the following formula: tumor volume = 0.5 x (width^2) x (length).Mice were sacrificed 21 days after tumor inoculation and tumor was extracted for weighting.

4.3.15 Immunohistochemistry staining of PCNA and Isolectin B4 in tumor tissue

The melanoma tissue was fixed in 4% paraformaldehyde. Sections were incubated with primary antibody of antiPCNA and Griffonia Simplicifolia Lectin I (GSL I) – Isolectin B4 (IB4) (Vector, Burlington, Ontario, CA) mAb at 1:100 dilution. Then the sections were incubated with biotinylation secondary antibody for 45 min, followed by horseradish peroxidase-labeled streptoavidin at 37◦C for 45 min. Slices were finally developed with diaminobenzidene (DAB) and counterstained with hematoxylin. Slices were observed under microscopy in a magnification of 400x. Tunel staining was using TdT FragEL TM DNA Fragment Detect Kit (Billerica, MA, USA). Slices were observed under microscopy in a magnification of 200x.

4.3.16 Statistical analysis

Data are presented as means ± SEM. Control and experimental data were compared using one-way ANOVA followed by Newman-Keuls testing. P-values less than 0.5 were assumed *a priori* to indicate significant differences.

4.4 Results

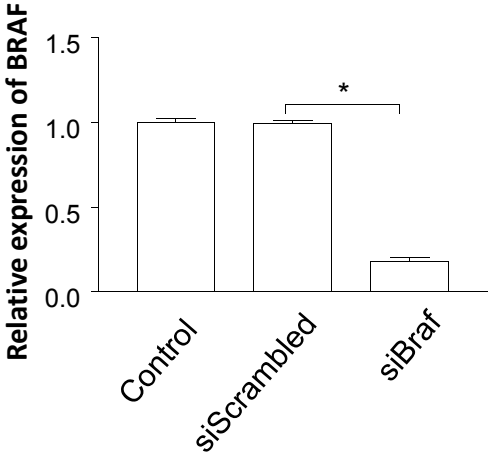
4.4.1 In vitro silencing and anti-proliferation assay on B16-F10 cells by B-RAF siRNA

B-RAF is an important cascade kinase in the classical MAPK pathway, which plays a crucial role in cell proliferation and survival. The mutation induced overexpression of B-RAF and stimulates proliferation and survival, thus providing essential tumor growth and maintenance functions [24].Suppression of B-RAF mutations using chemical inhibitors are known to restrict tumor cell growth[25, 26]. In this study, we proposed to knock down B-RAF using siRNA, which could be more specific, fewer potential side effects and potent [27-30]. In order to detect the silencing efficacy and the inhibition ability of the B-RAF siRNA, in vitro siRNA transfection was applied on B16-F10 cells. siBRAF and siScramblewas were transfected in to B16-F10 cells and the B-RAF mRNA and protein expression levels were detected by qPCR and western blot respectively (Fig. 4-1, A and B). Based on the result, the mRNA expression level of B-RAF was knocked down by more than 80% (Fig. 4-1A) and the protein expression is also largely reduced in the siBRAF treated cells (Fig. 4-1.B). It demonstrates that the siBRAF could efficiently repress the B-RAF expression level in B16-F10 cells.

To evaluate the inhibition efficacy of cell growth by siBRAF, a cell proliferation assay was used. After siBRAF and siScramble transfection, cells were subcultured and harvested at indicated time points, proliferation rate was determined by MTT assay and Trypan blue assay (Fig 4-1, C and D). According to the result, cell proliferation rate decreased up to 65% in 96 h (Fig. 4-1C) and the proliferation rate started to decrease 48 h after

Figure 4-1 In vitro gene silencing and anti-proliferation assay on B16-F10 cells by B-RAF siRNA. (A&B) Determination of the silencing efficiency of B-RAF siRNA. siBRAF and control siScrambled were transfected by lipofectamine 2000 into B16-F10 cells. (A) 24 h post transfection, cells were harvested and the total mRNA were isolated. The B-RAF expression level was determined by qPCR. (B) 72 h post transfection, cells were harvested and the B-RAF protein expression level was determined by western blot. (C&D) Determination of the anti-proliferation effect of B-RAF siRNA on B16-F10 cell. siBRAF and control siScrambled were transfected into B16-F10 cells by lipofectamine 2000. (C) 92 h after transfection, MTT assay was conducted as described in Materials and Methods. 4 h post addition of MTT, absorbance was detected by microreader. (D) After transfection, cells were relocated from 12well plate into 96 well plate in a number of 1000/well. Cells were harvested, stained with trypan blue and cell numbers were counted under microscope in 24 h, 48 h, 96 h and 120 h post transfection.

A


Relative expression of BRAF
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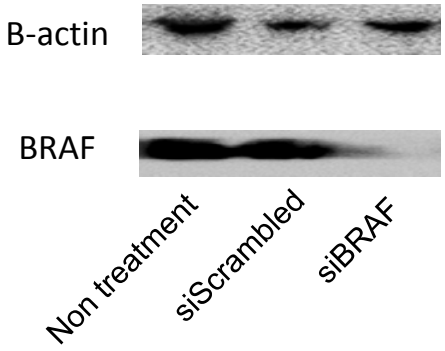
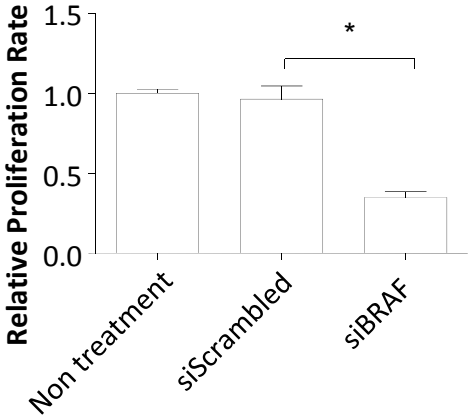
1.0
0.5
0.0
*
Control
siScrambled
siBraf

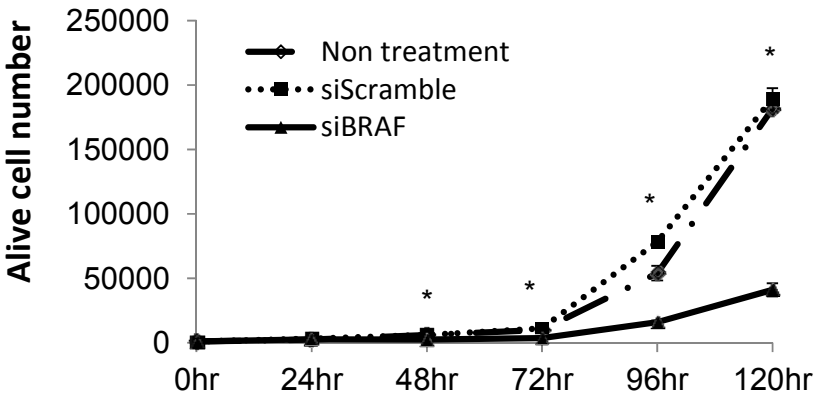
B


B-actin
BRAF
Non treatment
siScrambled
siBRAF

C


Relative Proliferation Rate
1.5
1.0
0.5
0.0
*
Non treatment
siScrambled
siBRAF

D


Alive cell number
250000
200000
150000
100000
50000
0
Non treatment
siScramble
siBRAF
*
*
*
*
0hr
24hr
48hr
72hr
96hr
120hr

transfection. Thereafter, the difference between siScamble control and Non treatment control increased continually till 120 h, which implies that the silencing effect could be maintained within siBRAF transfected cells for at least 120 h (Fig. 4-1D).

4.4.2 Characterization of Folate-lipo-siRNA

Size and surface charge of liposomal nanopartticles are important for in vivo delivery particles, since larger size particle might ends up eaten by Kupffer and other reticuloendothelial cells rich organs, while negatively charged particles are repelled by siRNA and cell plasma membrane components [1, 18, 31]. To estimate the size and surface charge of the Folate-lipo-siBRAF, the diameter and the zeta potential (represents the surface charge) was detected (Fig. 4-2 A and B). Based on the result, the diameter of the folate-lipo-siBRAF was around 160nm (Fig. 4-2A) and the zeta potential is around 37 mV (Fig. 4-2B). According to the previous report [32], the size and the surface charge of the prepared liposomes were suitable for potential *in vivo* siRNA delivery.

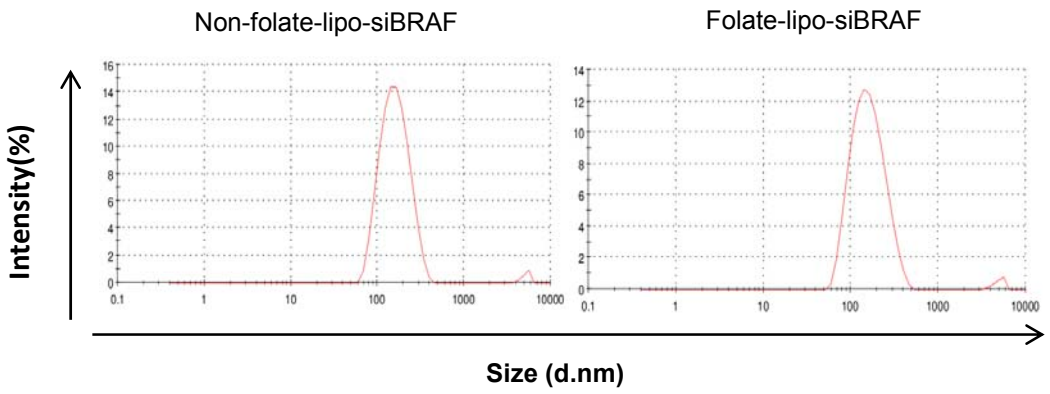
The rapid degradation of siRNA is another major hurdle of siRNA in vivo delivery [33, 34]. To determine whether siRBAF were encapsulated and protected by the Folate liposomest, a degradation assay was applied using 90% FBS mixing with folate-lipo-siBRAF, Non-folate-siBRAF or naked siBRAF. Compare to the naked siBRAF, Folate-lipo-siBRAF and Non-folate-siBRAF could protect siBRAF from being degraded at least 48 h (Fig. 4-2 C), which implies that both the Folate and Non-folate liposome provide protection against systemic degradation of siRNA by nucleophiles and nucleolytic enzymes, and that addition of folate to liposomes does not influence the protective capacity.

4.4.3 In vitro targeting assay of Folate-lipo-Cy3-siRNA and gene silencing of B-RAF

FRs are known to express in many malignant tumor cells but not normal cells [18]. To determine the preferentially targeting efficacy of the Folate-lipo-siRNA towards malignant cells, an in vitro cell targeting assay was applied. Freshly cultured bone

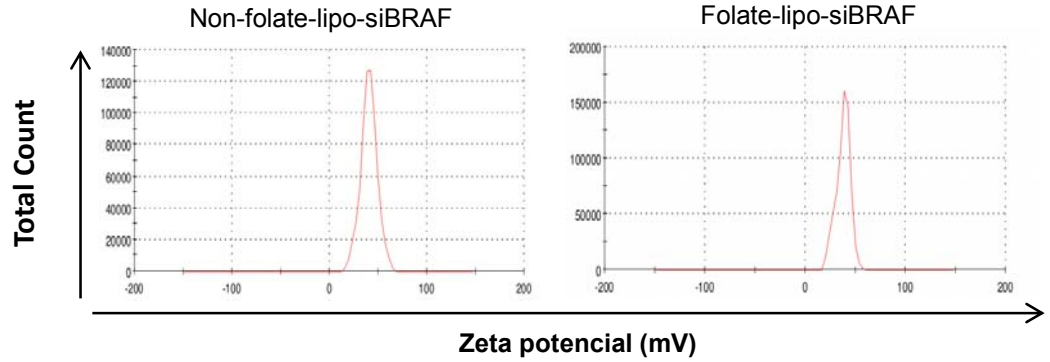
Figure 4-2 Preparation and characterization of Folate-lipo-siRNA. (A & B) Determination of the size and zeta potential of Folate-lipo-siRNA.Folate-lipo-siRNA and non-folate-lipo-siRNA were prepare as described in the Materials and Methods. The size (A) and the zeta potencial (B) of Folate-lipo-siRNA and non-folate-lipo-siRNA were measured by zetasizer. (C) Serum protection assay. Naked siRNA, Folate-lipo-siRNA, or non-folate-lipo-siRNA were incubated with 90% FBS at 37 ^{o}C at various time points. 1, 4, 8, 24 and 48 h post incubation, liposomes that encapsulated siRNA were harvested by Trizol, siRNA was isolated by chloroform and isopropanol method, then visualized by 15% page gel electrophoresis. The data presented one of three independent experiments.

A



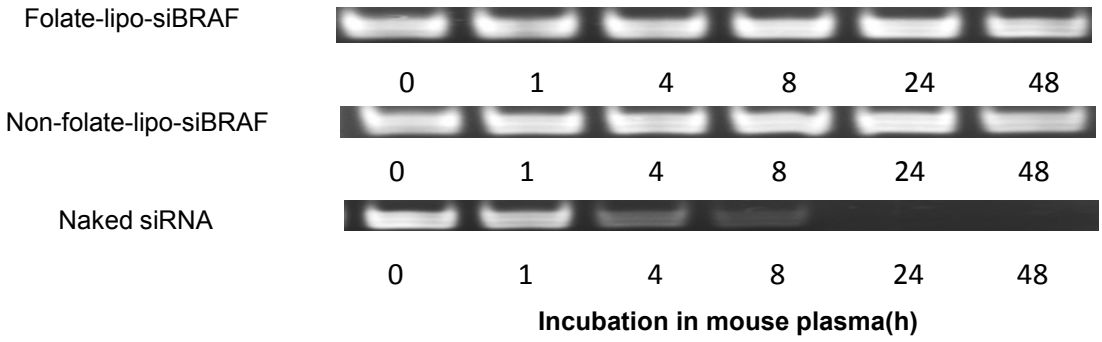
	Average size (nm)	±SD
Non-folate-lipo-siRNA	155 .05	20.8
Folate-lipo-siRNA	152.67	18.9

B



	Average (mV)	±SD
Non-folate-lipo-siRNA	33.45	5.7
Folate-lipo-siRNA	31.83	8.6

C



marrow cells (non-FR-expressing controls) and consistently FR-expressing B16-F10 cells were transfected with both Folate-lipo-Cy3-siRNA and Non-folate-Cy3-siRNA.The intensity of the Cy3 fluorescent in cells were detected by Flow cytometry (Fig. 4-3 A). From the result, we observed much higher uptake of Folate-lipo-Cy3-siRNA in tumor cell B16-F10 when compare to normal bone marrow cells, while similar uptake of Non-targeting-Cy3-siRNA was observed in both cells. While there are large differences between Folate-lipo-Cy3-siRNA and Non-folate-Cy3-siRNA in FRs expressing cells, the difference is trivial in non FRs expressing cells. It illustrated that, most of the uptake of Folate-lipo-Cy3-siRNA were FRs expression associated. While the characteristics of the Folate-lipo-Cy3-siRNA and the Non-folate-lipo-Cy3-siRNA do not influence much of the transfection rate, the addition of folate to liposomes makes large difference in targeted delivery capability.

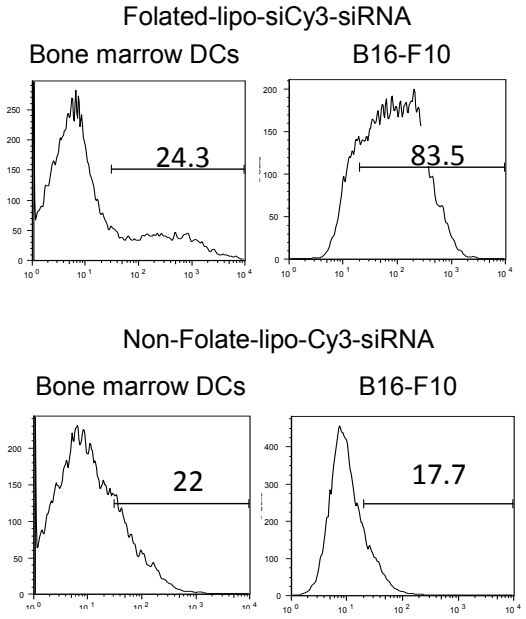
To evaluate the in vitro silencing ability of Folate-lipo-siBRAF, B16 cells were used and transfected with Folate-lipo-siScramble and Folate-lipo-siBRAF respectively. 24 h later, cells were harvested and the BRAF expression was determined by qPCR. Compared to Folate-lipo-siScramble treatment group, BRAF expression in cells treated with Folate-lipo-siBRAF was significantly knocked down to 70% (Fig. 4-3B), which demonstrates that Folate-lipo-siBRAF is potent for in vitro silencing.

4.4.4 Bio-distribution of Folate-lipo-Cy3-siRNA and in vivo gene silencing of B-RAF

Cell surface receptors for folic acid are generally overexpressed in human cancer cells [35]. In mouse melanoma, B16-F10 consistently expresses FRs. To determine the in vivo targeting efficacy of Folat-lipo-siBRAF, melanoma bearing mice were used for the in vivo targeting assay. Mice inoculated with B16-F10 melanoma and bearing melanoma tumors were intravenously injected with Folate-lipo-Cy3-siBRAF or Non-lipo-Cy3-siBRAF. Based on the intensity of the fluorescent, an overall stronger fluorescent in tumor tissues that administrated with Folate-lipo-Cy3-siBRAF than the tumor tissues that

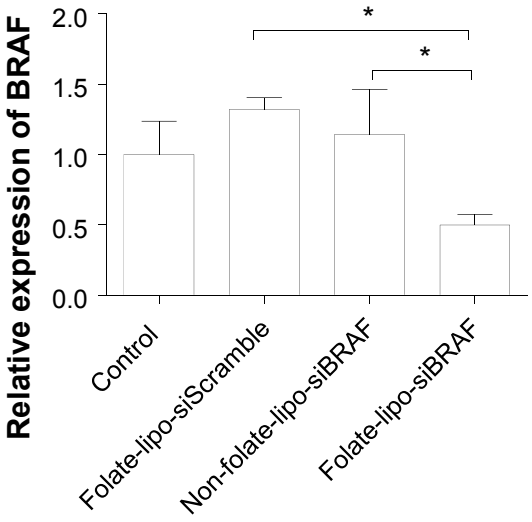
Figure 4-3 In vitro targeting assay of Folate-lipo-Cy3-siRNA and gene silencing of BRAF. (A) Transfecting efficacy of Folate-lipo-Cy3-siRNA detected by flow cytometry. B16-F10 cells and freshly prepared bone marrow cells were transfected by Folate-lipo-Cy3-siRNA and non-folate-lipo-Cy3-siRNA. 4 h after transfection, the cells were harvested and the fluorescence of encapsulated Cy3-labeled siRNA was detected by flow cytometry. (B) Gene silencing of BRAF using Folate-lipo-siBRAF. B16-F10 cells were transfected by Folate-lipo-siBRAF and Folate-lipo-siScramble. 24 h after transfection, cells were harvested and the mRNA were isolated. The BRAF expression of B16-F10 was detected by qPCR. **$p<0.01$

A



	B16-F10 Transfection rate (%)	Bone marrow Transfection rate (%)
Non-folate-lipo-Cy3-siRNA	79.8 ± 13.8	21.6 ± 3.81
Folate-lipo-Cy3-siRNA	13.8 ± 3.8	19.9 ± 3.04

B



administrated with Non-lipo-Cy3-siBRAF can be observed. Strong fluorescence was observed in liver while little was seen in spleen. No fluorescence was detectable in other vital organs. Moerover, the fluorescence in tumor tissues are more collective than those in liver and spleen, which might attribute to the disorganized vascularization in tumor tissue (Fig. 4-4A). Although the strongest fluorescence can be observed in liver, strong fluorescence could be seen in tumor tissue in Folate-lipo-Cy3-siBRAF administrated mice. Remarkably, the flourecence in tumor tissue of the Folate-lipo-Cy3-siBRAF administrated mice is much stronger than the tumor tissue of Non-lipo-Cy3-siBRAF administrated mice, which illustrated that Folate-lipo-siBRAF is able to enhance the delivery of the siRNA into tumor tissue and the addition of folate to the liposomes could increase the tumor delivery efficacy.

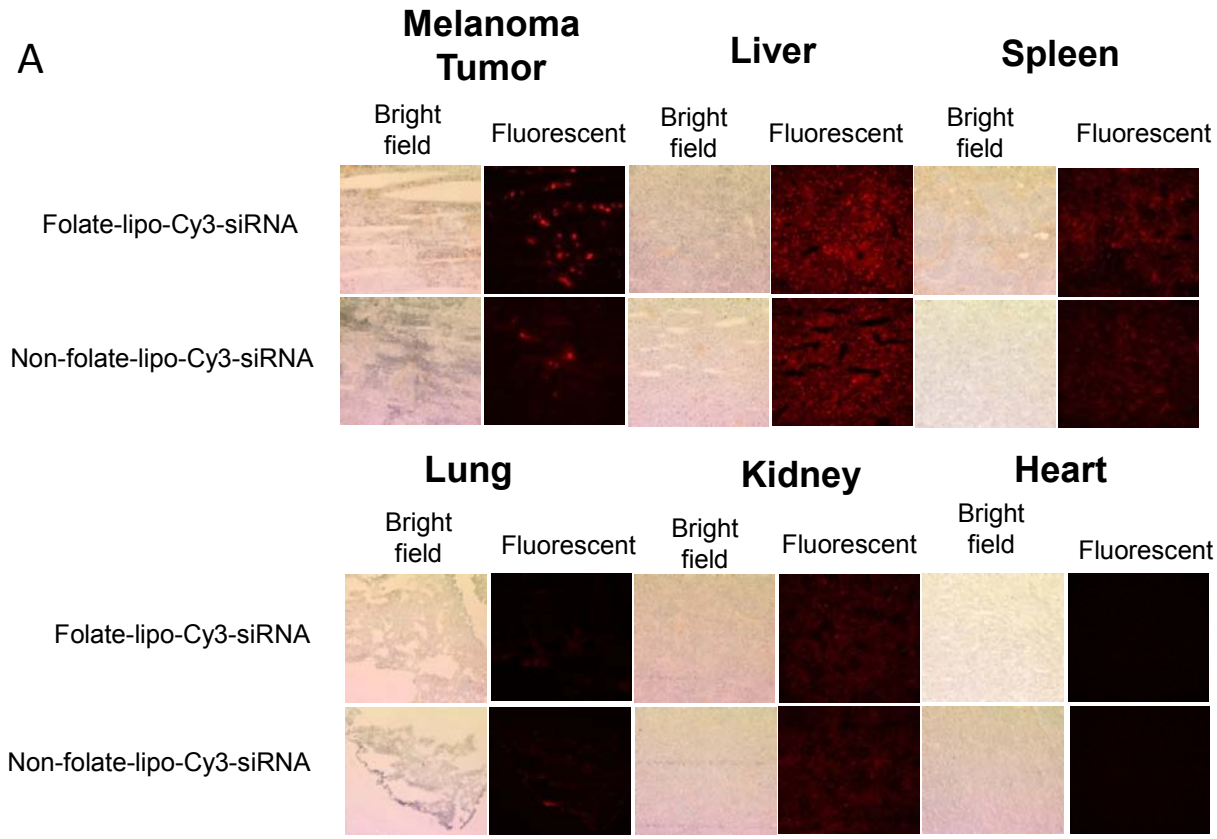
For efficient drug delivery, a carrier system should facilitate not only tumor localization but also intracellular access [35]. To estimate the ability of the Folate-lipo-siBRAF in helping the encapsulated siRNA reaching the targeted cells and therefore induce silencing of the intended gene, in vivo tumor silencing assay was applied. Folate-lipo-siBRAF and Folate-lipo-siScramble were intravenously injected into melanoma bearing mice. 24 h post administration, mice were sacrificed and tumor tissues were extracted for qPCR. Based on the qPCR result, the BRAF mRNA expression level has been largely knocked down when compare to Non treatment control and scramble control (Fig. 4-4B), which proves that Folate-lipo-siBRAF is capable in repressing the BRAF expression level in vivo in tumor tissue.

4.4.5 Anti-tumor growth of melanoma by Folate-lipo-siBRAF.

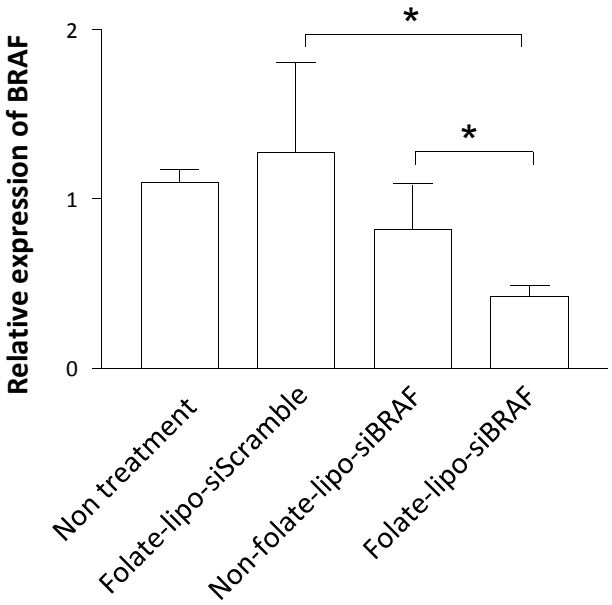
The inhibition of B-RAF in B-RAF overexpressing malignant cells would restrict the growth of the tumor [26]. To determinate whether the Folate-lipo-siBRAF is able to restrict tumor growth in vivo, Folate-lipo-siBRAF was applied as a therapy for melanoma bearing mice. Folate-lipo-siBRAF, Non-folate-lipo-siBRAF and Folate-lipo-siScramble was intravenously injected to the tumor bearing mice two days after tumor inoculation

Figure 4-4 Bio-distribution of Folate-lipo-Cy3-siRNA and in vivo gene silencing of B-RAF. (A) Bio-distribution of Folate-lipo-siRNA. Mice were i.v injected with Folate-lipo-Cy3-siRNA and non-folate-lipo-Cy3-siRNA. 6 h post administration, mice were sacrificed and tumor tissues, liver, spleen, lung, kidney and heart were extracted for frozen section. The red fluorescence of Cy3-labeled siRNA in the various tissue sections were observed under a fluorescent microscope. The data presented one of three independent experiments. The magnification was 10x. (B) In vivo gene silencing by Folate-lipo-siBRAF. Folate-lipo-siBRAF or Folate-lipo-siScrambled were i.v injected into C57/BL6 mice. 24 h after injection, mice were sacrificed and the B-RAF expression in tumor tissue was detected by qPCR. **$p<0.01$.

A


Melanoma Tumor
Liver
Spleen
Bright field
Fluorescent
Bright field
Fluorescent
Bright field
Fluorescent
Folate-lipo-Cy3-siRNA
Non-folate-lipo-Cy3-siRNA
Lung
Kidney
Heart
Bright field
Fluorescent
Bright field
Fluorescent
Bright field
Fluorescent
Folate-lipo-Cy3-siRNA
Non-folate-lipo-Cy3-siRNA

B


Relative expression of BRAF
2
1
0
*
*
Non treatment
Folate-lipo-siScramble
Non-folate-lipo-siBRAF
Folate-lipo-siBRAF

and once/week thereafter. Base on the tumor size measurement data, mice treated with Folate-lipo-siBRAF have significantly smaller tumor size in the same day after tumor inoculation when compare to Non treatment control, Non-folate-lipo-siBRAF and Folate-lipo-siScramble (Fig. 4-5 A). Mice treated with Non-folate-siBRAF also had a relatively smaller tumor size when compare to Non treatment control and Folate-lipo-siScramble, however, the difference was not significant.

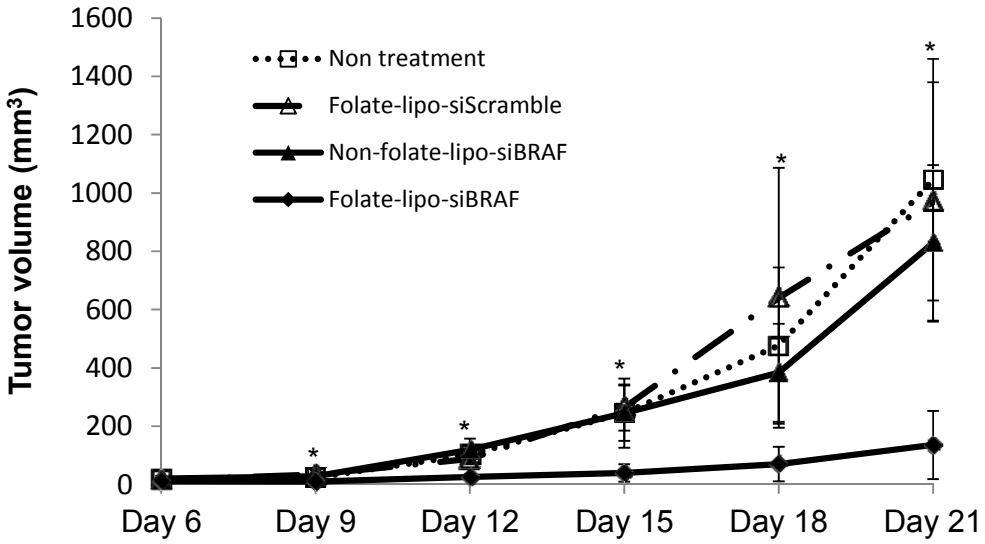
21 d after tumor inoculation, mice were sacrificed and tumors were extracted for weight evaluation. Mice in Folate-lipo-siBRAF treatment groups have significantly small tumor weight as comparing to Non treatment control, Non-folate-lipo-siBRAF and Folate-lipo-siScramble treatment groups (Fig. 4-5 B). It demonstrated that Folate-lipo-siBRAF is able to restrict tumor growth and the therapeutic effect is much better when there folate is added into the delivery system.

4.4.6 Anti-proliferation, anti-vascularization in melanoma by Folate-lipo-siBRAF

The inhibition of B-RAF would induce less proliferation and more apoptosis, which serves as the major drive of tumor growth restriction [29]. Proliferating cell nuclear antigen (PCNA) was identified as the processivity factor of DNA polymerase delta (PFDPδ) [36, 37], which is part of a donut-shaped multi-protein complex playing a role in DNA repair and replication [38, 39]. It is a well-conserved protein in all eukaryotic species which is a processivity factor of DNA polymerased that required for DNA synthesis [40-43]. To determine whether Folate-lipo-siBRAF treated tumor tissue were restricted in proliferation, immunochemistry staining was applied. Based on the result, Folate-lipo-siBRAF treated tumor tissue has much less PCNA (Fig. 4-6 A) expression than Non treatment control, Non-folate-lipo-siBRAF and Folate-lipo-siScramble treatment groups, which illustrates that the Folate-lipo-siBRAF is able to induce in vivo anti-proliferation in tumor, and therefore restrict tumor growth. There is trivial difference between the Non treatment control, Non-folate-lipo-siBRAF and Folate-lipo-siScramble treatment groups, which implies that although the fluorescence in tumors can be seen in

Figure 4-5 Anti-tumor effects of Folate-lipo-siBRAF. (A & B) Experimental treatment of murine melanoma using Folate-lipo-siBRAF. Folate-lipo-siBRAF, or non-folate-lipo-siBRAF, or Folate-lipo-siScramble was i.v. injected into C57/BL6 mice two days after tumor inoculation. Treatments were repeated once a week thereafter. Tumor size (A). Tumors in treatment and control mice were monitored every three days. Tumor weight (B). Tumours in different experimental groups or control groups were extracted and weighted in day 21 after mice were sacrificed. Each point represents the weight of 6 mice in each group. Results represent 1 of 3 experiments. **$p<0.05$.

A


Tumor volume (mm³)
1600
1400
1200
1000
800
600
400
200
0
Day 6
Day 9
Day 12
Day 15
Day 18
Day 21
Non treatment
Folate-lipo-siScramble
Non-folate-lipo-siBRAF
Folate-lipo-siBRAF
*

B

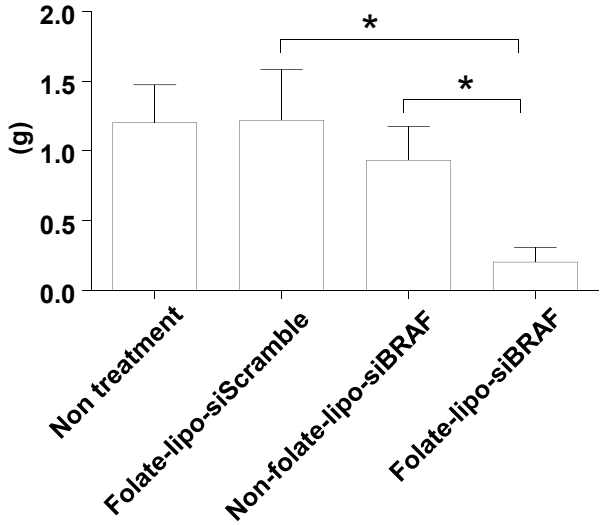
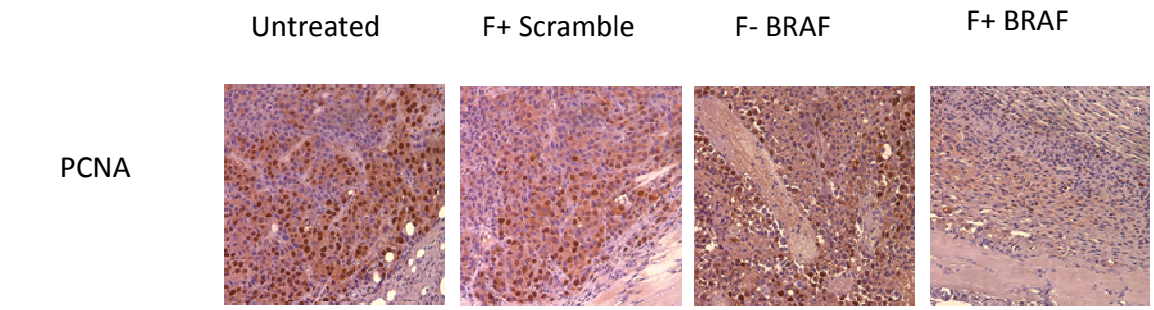

(g)
2.0
1.5
1.0
0.5
0.0
*
*
Non treatment
Folate-lipo-siScramble
Non-folate-lipo-siBRAF
Folate-lipo-siBRAF

Figure 4-6 Anti-proliferation effects by Folate-lipo-siBRAF in melanoma. Immune histochemistry staining of PCNA in tumor tissues. 21 days after tumor inoculation, mice in different experimental groups were sacrificed and the tumor tissues were harvested for immune histopathology staining. (A) PCNA positive cells in Non-treatment, Folate-lipo-siBRAF, non-folate-lipo-siBRAF, or Folate-lipo-siScramble treated tumor tissue were displayed.

A



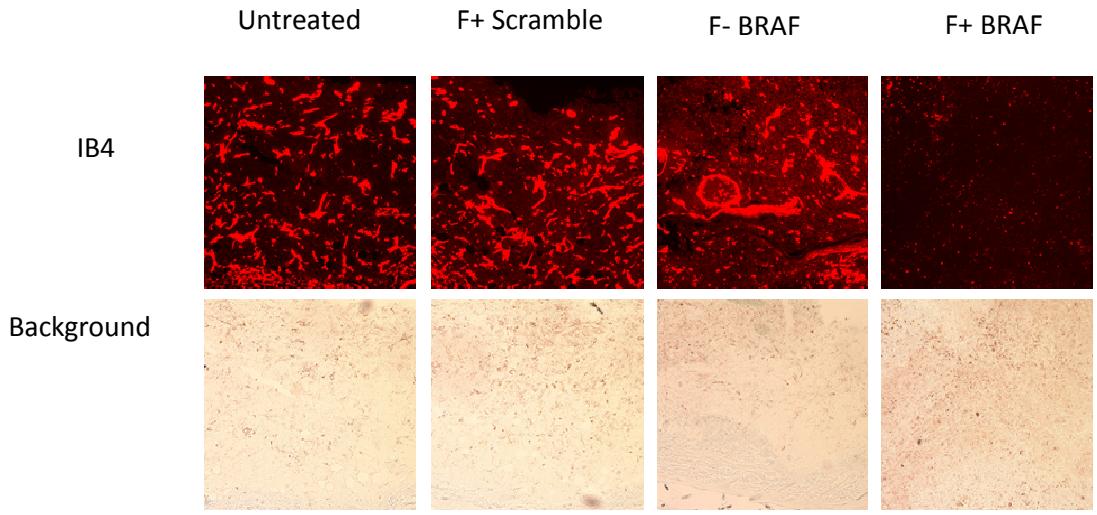
Non-folate-lipo-siBRAF, the uptake of the liposome is not efficient enough to restrict the tumor growth. Lectins are specific carbohydrate-binding proteins that have been applied in identifying the glycosylation status specifically in a variety of tissues [44-46]. Within the lectins family, IB4 has been shown to be useful for analysis of microvascular structure and function. IB4 binds on various cells that express α-galactosyl residues, including endothelial cells [45-48] in normal mouse, rabbit, rat and human tissues [49]. IB4 also has been used to identified neovascular structures specifically within tumor vascular networks [50]. To determine whether Folate-lipo-siBRAF treated tumor tissue were restricted in vascular network or not, IB4 was used to stain the melanoma tissues in various experimental groups (Fig. 4-7B). According to the result, mice treated with Folate-lipo-siBRAF have much less microvasculature than the Non treatment control, Folate-lipo-siScramble and Non-folate-lipo-siBRAF treatment group, which implies that the in vivo silencing of BRAF is able to restrict the vascular network. Although the melanoma tissues treated with Non-folate-lipo-siBRAF have slightly less endothelial cells, the difference between the Non treatment group and Folate-lipo-siScramble control group is trivial, which illustrates that with the folate ligand, the liposomal system provides better therapeutic effect.

4.5 Disscussion

Melanocytes originate from highly motile cells with enhanced survival properties, which attribute to the high aggressiveness, high metastasis and high resistance to cytotoxic agents. When compare to other malignancies, melanoma is more resistance to chemotherapy, radiotherapy and immunotherapy, which is a major barrier for successful treatment [52]. Targeted therapies that focus on those which cancer cells have come to depend for progression are most attractive[53]. B-RAF is implicated in several aspects of melanoma induction and progression. Several years after B-RAF mutation was discovered, the first selective inhibitors, vemurafenib, has entered clinical trials. However, after the regression in the early course of the therapy was recorded, it is observed that resistance to therapy emerged in the subsequent and follow-up treatment. The response duration ranged from 2 to >18 months. Now, a lot of research groups have focus on the

Figure 4-7 Anti-vascularization effects by Folate-lipo-siBRAF in melanoma. (B) Immuno histochemistry staining of IB4. 21 days after tumor inoculation, mice in different experimental groups were sacrificed and the tumor tissues were harvested for immuno histopathology staining. IB4 positive endothelial cells in Non-treatment, Folate-lipo-siBRAF, non-folate-lipo-siBRAF, or Folate-lipo-siScramble treated tumor tissue were displayed.

B



resistance mechanism in B-RAF therapy [53]. siRNA could be an alternative choice: it is potent, highly specific to the targeting mRNA sequence and less toxic or size effect. Moreover, siRNA has more potential in controlling more than one gene's expression, which offers great potential for "personal" cancer therapy [54]. In our study, we observe a potent silencing of B-RAF expression by siRNA and a significantly decrease of cell proliferation in vitro and tumor growth in vivo.

However, in vivo application of siRNA faces many challenges. First, siRNA is not stable in circulation. Second, to achieve effective in vivo RNAi application, tissue barrier and intracellular delivery have to be overcome. Third, cell entrance and endsomal escape are the crucial issues for siRNA delivery. Fourth, siRNA in tumor delivery is especially difficult since tumor vasculature is poorly organized, tortuous, defective and location-dependent heterogeneity. Therefore, when compare to normal tissue, tumors have lower blood flow, higher flow resistance and as a result, lower presentation of siRNA to tumors. Meanwhile, tumor blood vessels have discontinuous endothelium, which makes it leaky and permeable to larger size molecules (100 – 780nm). These properties facilitate larger siRNA combined nanoparticles (e.g., 100nm) extravasation through diffusion and convection in tumors and realize passive tumor targeting [55-59]. Thus, an effective delivery system is needed.

Folate based targeting systems already be successfully applied as a therapeutic agents or imaging agents[60, 61]. Comparing liposomes with other carrier delivery systems, liposomes offer multiple advantages for drug delivery, including their biocompatibility, biodegradability and safety. Further, liposomes are able to accumulate within tumor tissue due to the passive accumulation of colloidal macromolecules of approximately 40kDa and above in tumors, which cause the enhanced permeation and retention effect (EPR) [62]. The accumulation of liposomes in tumor tissue could be improved through the use of receptor targeting moieties that are either postconjugated to the surface of liposomes or attached to lipids that become incorporated within the liposomal bilayer[31]. Since FRs are generally overexpressed on human cancer cells [21, 43, 63, 64] and the affinity of FRs will not be affected when PEG covalently connected the γ-carboxyl [20,

65] of folate. Therefore, liposomes that included folate conjugated lipids have been considered as promising non-live vectors for tumor targeted delivery purposes.

In our study, we observed a protective effect of the folate liposome to the encapsulated siRNA and a significantly preferential targeting effect in Folat-lipo-siBRAF, which offer a promising delivery system for siRNA tumor delivery.

However, excessive cationic charge would induced the the first-pass clearance by lung and liver, which offer strong reticuloendothelial system (RES) that allows strong interaction between the cationic surface of the liposome-siRNA complex (lipoplex) and the plasma proteins [2, 3, 66]. In our study, we observed an intense fluorescence in liver, however, no fluorescence in lung. What's more, a relative strong fluorescence could be seen in tumors, which implies that the liposome we generated is not too "cationic" that enable it better escaping the RES and further being delivery into tumor. One of the methods to shield the charge of lipoplexes is to add additional neutral membrane bilayer outside the lipoplex[40, 67]. In the same purpose, in our procedure of generating the lipoplex, we added DSPE-PEG lipids, which is a neutral lipids, after extrusion and before it could be used. In fact, the in vivo study results show that the liposome was able to deliver siBRAF into B16-F10 melanoma and silenced the B-RAF expression in tumor, with the silencing efficacy potent enough to restrict the tumor growth.

Human ^{v600E} B-RAF, which is a mutation of B-RAF that leads to the overexpressiong of B-RAF, stimulates constitutive ERK signalling, stimulating proliferation and survival and providing essential tumour growth and maintenance functions [68]. ^{v600E}BRAF also contributes to neoangiogenesis by stimulating autocrine vascular endothelial growth factor (VEGF) secretion. It has been shown that, the blockade of RAF/MEK/ERK pathway could induce apoptosis, especially in B-RAF V600E melanoma cells [69-71]. Apoptosis is a frequent sign of the response to B-RAF inhibitor, such as PLX4032/RG7204, an analogue to PLX4720, which suggesting that it is the major biological consequence of inhibition of mutant B-RAF [51]. Based on the immune-histopathology staining, we observed a similar anti-tumor effect with human that by reducing the BRAF expression in tumor cells, the proliferation of the tumor cells as

decreased and less endothelial cells were observed, which implies the anti-proliferation effects, anti-vascularization and increase apoptosis effects of the Folate-lipo-siBRAF.

In summary, we report here a folate receptor expressing malignant cells targeting systems to delivery B-RAF siRNA can efficiently block the B-RAF expression in tumor cells in vitro and tumor tissues in vivo. Treatment of Folate-lipo-siBRAF results in significantly reduced tumor size and tumor weight; decreased tumor cells proliferation, reduced tumor tissue capillary and increase tumor cells apoptosis in vivo.

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Chapter 5

5 General Discussion

5.1 General Discussion

The studies throughout this thesis focus on examining the validation of different RNAi therapies for different diseases. To study the validation of RNAi as a potential therapy, two disease models and four specific siRNA sequences were applied in Chapters 2-4. In each chapter, a specific RNAi therapy was examined:

- Validation of transdermal delivery by a novel topical delivery reagent of siTNFα and siMyD88 in contact hypersensitivity therapy.
- Determination of whether mannosed liposomes encapsulating siIDO is a good immune therapy for melanoma.
- Determination of whether folated liposomes encapsulating siBRAF is a good therapy for melanoma.

In Chapter 2, we describe development of a topical delivery reagent for efficient delivery of siRNA into dermis as a potential therapy for ACD. The current standard for ACD treatment involves local application of corticosteroids that target lymphocyte activation. However, this method results in many unwanted side effects. These side effects can become amplified when addiction to cutaneous application requires increasing dosages. We propose that topical application of siRNA could alleviate some of these problems since it directly contacts the disease site, restricts toxicity to the application area, and is user-friendly. However, as previously discussed, effective topical siRNA delivery requires penetration-enhancing techniques to infiltrate the SC and reach target cells. There are two routes of passive diffusion for permeation of the SC: the trans-epidermal route and the trans-appendageal route. The trans-epidermal route can be further broken down into two pathways: polar or hydrophilic compounds prefer passing through the stacked layers of corneocytes, while lipophilic compounds favour travelling through the intercellular spaces [1, 2]. Although the intercellular space route only occupies 1% of the

available area [3], it is thought to be the predominant route of entry through the stratum corneum [4]. In 1997, Menon and Elias suggested the “aqueous pore pathway” [5], which states that the discontinuous lacunar domains in the lipid matrix will expand and become a continuous pathway when the SC is being hydrated. With the removal of the permeabilizing agent, the continuous lacunar domains will recover to a fragmented state [6].

The trans-appendageal penetration route is through skin appendages such as sweat glands, sebaceous glands, and hair follicles. However, these pathways only occupy a small area of the total cutaneous surface, so they play a limited role in drug diffusion [7, 8]. Of the possible appendages in skin, the hair follicle is likely to play the largest role due to its relatively large size, and the presence of sebum in the duct of the pilosebaceous gland could facilitate the diffusion of lipophilic drugs [3]. Many studies using various methods, such as confocal laser scanning microscopy, biopsies, and tape stripping techniques have been used to study the penetration of drug molecules through the hair follicles [7]. Under the stratum corneum are the layers of the epidermis, which is 50-100 μm in thickness in total. These layers contain live, nucleated cells that have much less resistance to diffusing compounds [3]. Extremely hydrophilic or lipophilic molecules might have difficulty in penetrating this portion of the epidermis since the cells form tight linkages, limiting diffusion through the cell membranes and cytosol [9]. Based on the data in Chapter 2, it is possible that the novel topical reagent penetrates the SC via both trans-epidermal and trans-appendageal routes. Under the fluorescent microscope, fluorescence could be seen distributed evenly throughout both the SC layer and other layers of cells in the dermis, which implicates the trans-epidermal route. Stronger fluorescence could also be seen in hair follicles illustrating the trans-appendageal route. By utilizing both routes, the novel reagent could reach higher transdermal ability. The topical reagent is neither too hydrophilic nor lipophilic, which facilitates its penetration through the living cell layers of the dermis. Helping siRNA penetrate the living cells of the dermis, including Langerhans cells in the stratum spinosum, could alter their biologic function through silencing.

Tumor necrosis factor (TNF) is a systemic inflammation-induced cytokine and a member of a group of cytokines that stimulate the acute phase reaction. It can be produced by $CD4^+$ lymphocytes and NK cells, but is mainly produced by activated macrophages (M1). The primary role of TNF is in regulating immune cells. It can induce fever, apoptotic cell death, sepsis (by inducing IL1 and IL6 production), cachexia, inflammation, and it can inhibit tumorigenesis and viral replication. TNFα is one member of the TNF family of receptor ligands. It has multiple biological activities, including immunomodulation and oncostatic, osteolytic, pirogenic, proinflammatory, and other activities [10].

The first TLR adaptor molecule discovered was MyD88. It is induced by IL-6 and was isolated from M1 myeloleukaemic cells differentiated into macrophages by IL-6 treatment, and characterized as a myeloid differentiation response gene [9]. MyD88 is a TIR domain-containing cytoplasmic protein. The pathway activated by the TIR domain of TLRs that recruits MyD88 has been widely studied [11]. With the exception of TLR3, MyD88 mediates signaling by all TLRs [1]. MyD88 interacts with IL-1 receptor associated kinase 4 (IRAK-4), causing IRAK-4 and IRAK-1 to form a complex which leads to its phosphorylation and activation [2]. IRAK-4 and IRAK-1 then dissociate from the complex and interact with tumour necrosis factor (TNF) receptor-associated factor-6 (TRAF-6), which then recruits transforming growth factor-activated kinase-1 (TAK-1)-binding protein-1 (TAB-1) and TAB-2 to the complex. This leads to the phosphorylation and activation of the kinase TAK-1 [4]. Next, TAK-1 activates kinases upstream of p38 and JNK and the inhibitory κB (IκB) kinase (IKK) complex leading to NF-κB activation [3].

Once established, ACD is a lifelong disease. Prevention and avoidance of the causative agent (hapten) is critically important. Prevention and avoidance are of even greater importance for patients exposed to more than one sensitizer or extremely diffuse sensitizers. Methods that limit hapten penetration may help to reduce hapten-peptide complex formation, thus limiting the allergic reaction. However, the efficacy of these methods is limited. Most of the therapeutic approaches against ACD aim at blocking T cell transmigration to skin, reducing leukocyte activation, or preventing keratinocyte-dependent amplification of the inflammatory response.

With understanding of the mechanisms that govern T cell recruitment, many compounds that could inhibit the leukocyte-endothelial cell interactions have been developed and implemented in clinical trials. This approach has advantages in selectively targeting T cell subpopulations while maintaining immunoprotection in other situations. However, it is limited by the redundancy of the mechanisms involved in T cell extravasation and positioning into the skin.

Other proposed targets in treatment of ACD include selectins (which mediate initial rolling of leukocytes over the endothelium), adhesion molecules, and chemokine-chemokine receptor pairs [12-14]. However, strategies that interfere either with selectin functions or the modulation of the post-trascriptional glycosylation of selectin ligands have all had disappointing results when tested in humans [15].

Another attractive strategy is to target chemokine receptors in selective leukocyte subpopulations. Unfortunately, approaches to block chemokine-receptor axes have provided inconsistent results [16].

Most of the therapies for ACD are currently based on systemically or locally administrated drugs that target lymphocyte activation. Topical application of corticosteorids remains the mainstay treatment of ACD. More severe ACD reactions often require a systemic immunosuppressive treatment, typically based on a systemic corticosteroid regimen or cyclosporine administration. Therapeutic effectiveness of cyclosporine in ACD results from its dramatic effects on T cell functions, as well as on cytokine release [6].

My study focuses on one of the most important cytokines in ACD (TNFα) as well as a vitally important molecule in mediating transduction of innate immune signaling (MyD88). In accord with reported data [7, 8], my study shows that a single treatment of TNFα alone is not sufficiently potent to alleviate symptoms of ACD, with the exception of a reduction in swelling.

In Chapters 3 and 4, I discuss two potential RNAi immune therapy for cutaneous melanoma. Melanoma is the most deadly form of skin cancer. If diagnosed early,

melanoma can be cured by surgical resection and approximately 80% of cases are dealt with in this manner. Nevertheless, the metastatic nature of melanoma malignancies results in high resistance to therapy and poor prognosis. Malignant melanoma patients with metastatic disease have a median survival of 6 months and a 5 year survival of less than 5%, illustrating the pressing need for new and better treatment.

Many factors could contribute to melanocyte oncogenesis, including mutations in critical growth regulatory genes, the production of autocrine growth factors, and the loss of adhesion receptors [12]. As melanocytes progress toward malignancy they proliferate and spread, forming a naevus or common mole. The proliferation of melanocytes can be restricted to the epidermis (junctional naevus), dermis (dermal naevus), or overlapping components of both (compound naevus). Naevi are benign but have the potential to progress to radial-growth-phase (RGP) of melanoma, which is an intra-epidermal lesion that can involve some local micro-invasion of the dermis. RGP cells can further progress into vertical-growth-phase (VGP), which is a more dangerous stage where cells tend to be more invasive and metastatic and have nodules or nests of cells invading the dermis. Melanocytes or naevi can directly develop to RGP or VGP, and cells in RGP or VGP can progress into metastatic malignant melanoma.

There are four main clinical subtypes of melanoma [12]. Nodular melanoma consists of raised nodules without a significant flat portion. Acral lentiginous melanoma (ALM) is not associated with UV exposure since it is usually found on the palms of the hands, soles of the feet, and in the bed of fingernails or toenails. In non-Caucasian populations, ALM accounts for 50% of melanomas [13]. Lentigo maligna tends to correlate with chronic sun exposure since it occurs in the elderly on sun-exposed skin regions. It is generally flat in appearance. Superficial spreading melanoma (SSM) is the most common form of melanoma. It linked to severe sunburn, especially at an early age. It is usually flat with an intra-epidermal component. SSM accounts for the remaining 50% of melanomas in non-Caucasians. It is the third most common cancer in young people in the UK and USA [14, 15].

Melanoma is recognized as an immuno-responsive type of tumour since T lymphocyte infiltration into the tumour correlates with improved patient survival [17]. A longstanding conventional therapy for melanoma involves administration of interleuklin-2 (IL-2). IL-2 is thought to exert antitumour effects by enhancing the host's antitumour response and by inducing T cell activation. IL-2 treatment alone results in a response in only approximately 15% of cases. Moreover, the use of IL-2 in high doses is associated with considerable mortality, attributed to the development of gastrointestinal, cardiovascular, renal and neurological toxicities including vascular leakage syndrome where fluid accumulates within the lung and liver. Another therapeutic approach is to use type I interferons (IFN-α, IFN-α2β). In some studies, IFN-α and IFN-α2β treatment has proven beneficial in prolonging disease-free survival when applied in an adjuvant setting. A modified IFN-α2β treatmment has entered a phase III study where its use as a single agent versus combination with other treatments in an adjuvant approach [18].

In spite of the demonstrated capacity of the immune system to mount an antitumour attack, tumours are still able to evade that attack, a process known as immune escape. IDO is one important factor aiding escape of tumours from immune attack. In Chapter 3, I described application of man-lipo-IDO, which targets a broad range of mannose receptor-expressing APCs, to silence IDO expression in those APCs to achieve a therapeutic effect. This preferential targeting strategy has both advantages and disadvantages. One disadvantage is the case of non-specific binding to non-mannose-receptor-expressing cells and tissues: this increases the required dose of siRNA to be delivered due to intended cells and tissues, where silencing of the unintended gene would prove inconsequential or, perhaps, deleterious. However, an advantage of targeting not only DCs but all mannose receptor expressing APCs is that it might offer broader therapeutic effects that contribute more than expected. This notion stems from the fact that DCs and IDO-expressing DCs represent only a very small subpopulation of immune cells. Overall, a broader APC targeting approach could be more effective than extremely specific targeting strategies.

Melanoma is an aggressive tumour that is resistant to all standard anticancer therapies. Resistances mechanisms in melanoma are complex since a wide range of antineoplastic

treatments are ineffective at killing melanoma cells. In this tumour type, it is suggested that drug resistance is due to the inherent malignant behavior of melanoma cells rather than acquired genetic alternations selected for over therapy [19]. The success of systemic therapy for metastatic melanoma has been minimal [20]. The identification of mutant B-RAF as a major driver of melanoma progression appeared as a breakthrough in melanoma therapy [21]. The development of vemurafenib, a B-RAF kinase inhibitor with high specificity for mutant B-RAF was a turning point [22, 23]. Vemurafenib progressed rapidly through early phase trials and significant levels of tumor shrinkage were seen in patients whose melanomas harbored B-RAF V600E mutations [24, 25]. The overall response rate for vemurafenib was 48% compared with 5% for dacarbazine [26]. However, almost all patients treated eventually developed resistance and most tumours progressed on therapy [25-27].

In Chapter 4, a lipid-based delivery system that targets oncogenic gene B-RAF was applied as a strategy for treatment of melanoma. We found that using cationic lipid delivering siRNA and preferentially targeting tumor cells is an effective way to treat melanoma in the xenograft model we employed. It restricted tumor growth by inhibiting tumor cell proliferation, reducing angiogenesis, and promoting tumour cell apoptosis. In my study, however, there are still many areas that can be improved upon. First, it was observed that the fate of most injected liposomes was the liver, and this accumulation of therapeutic agent in non-target tissue remains a major barrier to improving the efficacy of this therapy. Although most intravenously-administered drugs share this limitation, we believe our system could be improved by further optimizing a novel combination of lipids within the liposome or adjusting the ratio of already-used components. Second, using a human xenograft model in immune-deficient mice could more realistically mimic a clinical setting. Regardless of these limitations, we have established that RNAi therapy could be a potential alternative for melanoma treatment.

Compared to other methods such as use of chemical inhibitors as therapeutic agents and use of dominant-negative mutants to explore gene function, siRNA provides many advantages. First, siRNA can be designed to target any class of genes (including both protein-coding and non-coding RNAs) and can potentially silence the expression of any

gene. Second, while constructing dominant negative mutants is time-consuming, siRNA can be easily synthesized for direct administration or cloned into expression vectors for expression in cultured cells *in vitro* or xenografts or induced tumours *in vivo*[28]. Third, compared to both chemical inhibitors and dominant-negative mutants, siRNA-mediated silencing is more specific and less toxic. Fourth, human tumours display different gene expression profiles not only compared to normal tissue but also compared to each other. Such heterogeneity would suggest the benefit of using a "personal" therapy approach. The ability of detecting pathways that malignant tissue depends on and specifically knocking them reveals the potential of RNAi to be a "personal" cancer therapy with capacity to be specifically tailored to therapy of each individual patient, depending on the molecular profile of their tumour [29, 30]. Finally, while most chemical inhibitors and dominant-negative mutants are designed to inhibit only specifically-identified enzymatic or other activities of over-expressed proteins in tumors (*i.e.*, those attributable to specific sub-regions of the protein, without affecting other, unidentified activities that could be mediated by regions unaffected by targeted mutations or association with chemical inhibitors), siRNA prevents production of the protein entirely, thus eliminating all activities, both known and unknown. This makes RNAi a potent tool to inhibit function.

For the reasons outlined above, RNAi therapy is, overall, an attractive alternative to knock down overexpressed genes involved in cancer progression and metastasis, particularly where conventional treatment fails [31]. SiRNA has the potential to be more effective than some current treatments of cancer because it targets the genetic material at the core of the problem, while many other treatments aim only at reducing clinical symptoms [32].

A major barrier to clinical application of siRNA is sub-optimal *in vivo* delivery. To overcome this difficulty, many efforts have been exerted on developing a specific, efficient and safe delivery method. At the same time, to validate it as a therapy, the choice of target and the specific siRNA sequence itself are also vitally important.

Non-virus *in vivo* delivery strategies can be classified into two groups based on the route of administration: local and systemic [33]. Local administration could be applied on

easily accessible tissues. Local application of siRNA could directly deliver siRNA into target tissues, which allows the accumulation of the proper siRNA amount and substantially targeted release in the intended tissue. In our studies, we also used local application of siRNA on skin for the treatment of ACD. Later on, we also discuss the validation of using cationic lipids to achieve the systemic delivery of siRNA in treating of melanoma [34].

Preformed cationic liposomes are among the most efficient *in vitro* transfection reagents for nucleic acids/siRNA. The negatively charged phosphate backbone of siRNA can be complexed electrostatically with cationic lipid, which contributes to its high efficacy [35, 36]. In addition, the net positive charge complexes can avidly bind to anionic proteoglycans on cell surfaces, facilitating their uptake [36-38].

However, most of the commonly used cationic lipids have failed in clinical trials due to poor *in vivo* performance because of excessive cationic charge, which results in low *in vivo* transfection efficiency, toxicity, immune activation, and unfavorable biodistribution. The lipoplexes are rapidly cleared when systemically administered because they accumulate in the capillary bed of first-pass organs such as the lungs [39, 40]. After this first clearance, hepatic elimination becomes the major factor for the lipoplexes 60 minutes after injection, just after the lipolexes redistribute from lung to liver [41]. In many cases, lipoplexes would strongly interact with plasma proteins, leading to entrapment and clearance by the RES system [42, 43]. Moreover, some studies found that the siRNA lipoplexes could be easily dissociated in the presence of complete serum [44, 45].

In our study, all of the cationic liposomes generated by us contain polyethylene glycol (PEG), which has been reported to be helpful in avoiding fast clearance and delaying uptake of lipid-based nanoparticles into RES. PEG could shield and stabilize the lipid bilayer as it is a hydrophilic polymer [46, 47]. However, the addition of negatively charged PEG molecules could reduce liposome association with cell membranes, reduce cell uptake, and reduce the potential for escape of siRNA from the endosomal compartment (essential for antisense activity), thus lowering the overall transfection

efficiency [46-48]. To address these issues, we further added a targeting ligand, which aided the capacity of liposomes to be taken up into target cells through receptor-mediated endocytosis. This led to significant increased in transfection efficiency for the targeting liposome compared to our non-targeting liposomes.

In conclusion, the topical delivery reagent that delivers siTNFα and siMyD88 has the potential for development into an effective RNAi therapy for ACD. The targeting liposomes, Man-lipo-siIDO and Folate-lipo-siBRAF, could serve as potential anti-melanoma RNAi therapies.

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