Chapter 14

Perspectives on RNA Interference in Immunopharmacology and Immunotherapy

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Additional information is available at the end of the chapter

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Abstract

RNA interference (RNAi), mediated by short interfering RNA (siRNA), vector-derived short hairpin RNA (shRNA) and microRNA (miRNA), brings about revolutionary features to basic biomedical research and clinical application. New drugs based on RNAi have been developed for therapeutic applications. The family of RNAi molecules are efficient agents to modulate mammalian immune system, and many studies reported that these molecules could manipulate immune defence, surveillance and homeostasis. Both perfect match of siRNA/shRNA and non-perfect match of miRNA could be beneficial for designing RNAi-based drugs for treatment of tumour and viral infection. This chapter provides a view to control or utilize the immune regulation of various small RNAs that should help researchers to understand the successful clinical application of RNAi.

Keywords: RNA interference, siRNA, miRNA, immunopharmacology, immunotherapy

1. Introduction

RNA interference (RNAi) is a conserved mechanism against exogenous nucleic acid and transposon transcripts in plants and lower animals. No matter of transfected siRNA, vector-delivered shRNA or pre-miRNA (transcribed mainly by Pol II) , Dicer (DCLs) and Agronaute (AGO) family proteins efficiently process small RNAs into short double-stranded RNA(dsRNA). Further, dsRNAs assemble into the RNAi-induced silencing (protein) complex (RISC) to guide and cleave target mRNA, promote mRNA degradation or inhibit mRNA translation. The great potential of RNAi is to specifically repress the expression of disease-causing genes while avoiding undesirable effects.

It is well accepted that siRNA can be recognized by endosomal pathways, Toll-like receptor 3 (TLR3), TLR7, TLR8 and cytoplasmic pathways, retinoic acid-inducible gene I (RIG-I),

melanoma differentiation-associated antigen 5 (MDA-5) and RNA-activated protein kinase (PKR), resulting in immune activation [1–5]. For example, it has been demonstrated that siRNA can cause activation of at least three key transcription factors, including NF-κB, interferon regulatory factor 3 (IRF-3) and IRF-7, and stimulate interferon (IFN) secretion. This activates T cells and dendritic cells (DCs) in the spleen in a TLR7-dependent manner [2,6]. Furthermore, 5′-triphosphate siRNA was demonstrated to activate RIG-I signal pathway, and then natural killer (NK) cells and DCs were activated [7,8]. In most circumstances, immune system stimulation is regarded as an unwanted side effect; therefore, siRNA-induced immune response should be controlled by using proper delivery system or chemical modification, although immune stimulation has been proved to be essential in cancer treatment and viral infection.

miRNAs are critical in regulating the development, differentiation, function and destiny of immune cells, including DCs, granulocytes, monocytes/macrophages, NK and natural killer T (NKT) cells and B and T lymphocytes. miRNAs influence both innate and adaptive immune defence and individual miRNAs may contribute their implications to various immune-mediated diseases. Furthermore, pattern recognition receptors (PRRs), kinases, adaptors, inflammatory factors and IFN could all be targets of miRNAs. Extra effort has been made to develop miRNA-based oligos or vectors for anti-infection purpose by manipulating corresponding immune genes.

In addition to silencing of targeted genes in a sequence-specific manner, components of RNAi technology often induce immune response. Several strategies were reported to design RNAi molecules with gene silencing and immune regulatory properties. Bifunctional molecules rely on the activation of PRRs such as TLR7/8, TLR9 or RIG-I, or just rely on down-regulation of target gene. This chapter summarizes RNAi-involved immune responses in the past 10 years and discusses the anticipated therapeutic application.

2. Chemically synthesized siRNA and vector-derived shRNA

2.1. RNAi drugs based on targeting specific immune genes

Immune disorders, both autoimmune diseases and immune defective or deficiency, are always caused by high-level overexpression of certain immune genes. A variety of immune inhibitory genes can serve as targets for RNAi-mediated gene silencing. Targeting specific immune suppressor could re-balance immune network and subsets.

Elevated activity of signal transducer and activator of transcription 3 (STAT3) has been found in several kinds of human tumours. Use of RNAi to knockdown STAT3 expression and inhibit its activation would reduce the tumour cell growth such as pancreatic cancer, colorectal cancer, melanoma and hepatocarcinoma cells. STAT 3 knockdown could induce bystanders immune response in vitro and in vivo, where CD4+, CD8+ and NKT cells were activated as well as the secretion of interferon-γ (IFN-γ), interleukin-12 (IL-12) and tumour necrosis factor alpha (TNF-α) was increased significantly [9–11]. siRNA-STAT3, synthetical-

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ly linked to CpG (agonist of TLR9), was demonstrated to silence immune suppressor STAT3 gene in TLR9+ myeloid cells and B cells. This strategy of therapy leads to activation of various populations of immune cells, including DCs and macrophages, that ultimately induce potent anti-tumour immune responses [10,12]. Hossain DM et al. recently reported that CpG-siRNA-STAT3 conjugates could efficiently silence the target expression, and abrogate inhibition of CD8+ T cells in patients who received myeloid-derived suppressor cells (MDSCs) [13]. Researchers proved that immune-stimulation-inducing CpG(A)-STAT3-siRNA was non-toxic for normal human leukocytes [14]. In another experiment, Luo Z et al. [15] generated a nano-vaccine loaded with poly I:C (a TLR3 agonist) and STAT3 siRNA. Researchers found this kind of siRNA could promote the maturation of DC and reverse immunosuppression in the tumour micro-environment; the function of inhibitory cells in tumour-draining lymph nodes were inhibited; thus, anti-tumour immune responses were potently induced; and the survival were prolonged [15]. Therefore, STAT3 siRNAs are expected to be a promising immunomodulatory drugs to improve the treatment efficacy of cancer vaccines by abrogating tumour immunosuppression.

Suppressor of cytokine signalling 1 (SOCS1) is a negative regulator of antigen-presenting cell (APC)-based immune response. Silencing of SOCS1 gene expression by RNAi is essential for DCs to enhance Ag-specific anti-tumour immunity [16]. SOCS1-silenced bone marrow dendritic cells (BMDCs) were more potent in suppressing tumour growth [17]. When SOCS1 was silenced, maturation of DCs (i.e. expressions of CD80, CD40, CD86 and major histocompatibility complex II [MHC II]) was significantly accelerated. As a result, SOCS1 inhibition up-regulated the expression of IFN-γ and IL-12, and decreased IL-4 secretions, which induced Th1 cell differentiation and thereby affected the development of Th2 cell. The combined nanoparticle (NP) delivery, which can render both tumour antigen and siRNA-SOCS1 to BMDCs, simultaneously could enhance immunotherapeutic effects in BMDC-based cancer therapy [16,18]. DC-targeted delivery of SOCS1 siRNA has been shown to enhance antifungal immunity in response to *Candida albicans* in vitro and HIV-specific cytotoxic T cell in mice [16, 19]. This evidence suggests the use of SOCS1–siRNA, as a potent adjuvant to improve immune response.

A20 is usually regarded as an attractive target for siRNA-mediated gene knockdown in DCs because it is a negative feedback regulator of multiple pro-inflammatory signal transduction. Several reports demonstrated that RNAi-mediated A20 silencing in DCs enhanced expression of co-stimulatory molecules (CD80, CD86, CD40 and MHC class II) and pro-inflammatory cytokines (IL-6 and TNF-α). Tumour-infiltrating cytotoxic T lymphocytes (CTLs), T helper cells that produced IL-6 and TNF-α were also activated by siA20-DC. A20 silencing in DCs can enhance the immune response against self-tumour-associated antigens [20,21]. Furthermore, A20-silenced DCs were proved to overcome CD4+CD25+regulatory T (Treg) cell suppression [21,22]. A20-silenced DCs could skew naive CD4+ T cells towards Th1 cell, but not Treg, Th2 or Th17 cells. Because a high amount of IL-10 was produced in A20-silenced DCs, simultaneous down-regulation of IL-10 and A20 resulted in enhanced T cell stimulatory capacity in DCs. A20 down-regulation resulted in enhanced CTLs immune response by the NF-κB and AP-1 pathways [20,23]. RNAi of A20

has enabled DCs to gain a potent ability to activate CTLs and Th cells, and inhibit Treg, providing a novel strategy to promote a tumour immune response.

Programmed death ligand (PD-L) is another exciting target on the surface of antigen-presenting cells (APCs); PD-L/PD-1 interactions were related to functional impairment and exhaustion of tumour antigen-specific CD8+ T cells. Although PD-L antibody exerts a potent anti-tumour effect, previous reports [24] have demonstrated that PD-L1-siRNA-PEI were preferentially and avidly engulfed by tumour-associated CD11c+PD-L1+ tolerogenic DCs at ovarian cancer locations. This kind of nanoparticle uptake stimulated multiple TLRs signalling, mainly via myeloid differentiation factor 88 (MyD88). Then, regulatory DCs activated into potent stimulators of CTLs that led to significant anti-tumour immunity in mouse models of ovarian cancer. Most importantly, PD-L knockdown DCs showed superior potential to expand minor histocompatibility antigen (MiHA)-specific CD8+ effector and memory T cells from leukaemia patients early after donor lymphocyte infusion and later during relapse. Combined PD-L1 and PD-L2 knockdown resulted in improved proliferation of CD4+ T cells and enhanced cytokine production [25,26]. In addition, another report demonstrated the improved effector functions of tumour-specific CD4+ and CD8+ human T cells by siRNA-mediated silencing of PD-1 ligands, PD-L1 or PD-L2 [27]. These results suggest that siRNA-mediated knockdown of PD-L is a fascinating strategy to inhibit a negative regulatory mechanism of tumour-specific T cells.

siRNA-CD40, delivered by a novel delivery system with a poly-dA extension at the 5'-end of the siRNA sense strand that was stably incorporated into 1,3-β-glucan, was captured and incorporated into DCs through its receptor, Dectin [28]. This strategy could induce antigen-specific Tregs, resulting in the permanent acceptance of mouse cardiac allografts. CD40 knockdown significantly suppressed Th1-type cytokines and induced Th2-type cytokines in rats with myocarditis. Knockdown of CD40 in experimental autoimmune myocarditis (EAM) rats promoted Foxp3 gene expression and increased Treg cells [29].

In addition, when silencing of CD40 or CD80/CD86, DCs exhibited suppressed allostimulatory activity with impaired APC function. In the well-established collagen-induced arthritis (CIA) model, multigene-silenced DCs were capable of delaying onset of joint pathology. Therapeutic effects of gene-silenced DCs were mediated by the inhibition of collagen II-specific Ab production and suppression of T cell recall responses. Also, multigene-silenced DCs inhibited Th1 and Th17 response, demonstrating IFN-γ and IL-2 inhibition [30]. Thus, inhibition of specific co-stimulatory molecules of DCs reveals a promising approach of suppressing immune responses in autoimmunity. These findings highlight the potential of immunomodulation of siRNA-CD40, and have important implications for developing RNAi-based clinical therapy in the transplantation field.

It is well documented that tumours could secrete immunosuppressive molecules, including the cytokines transforming growth factor β (TGF-β) and IL-10. This creates an immunosuppressive environment, which inhibits anti-tumour immunity. The suppression of Treg cell, induced by targeting TGF-β1 using siRNA, can enhance the efficacy of a DC vaccine against a poorly immunogenic tumour in mice [31]. Nanoparticle-delivered TGF-β siRNA enhances

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vaccination against advanced melanoma, and the tumour micro-environment was modified with increased levels of tumour-infiltrating CD8+ T cells and decreased level of regulatory T cells [32]. siRNA targeting IL-10 receptor α (siIL-10RA) initiated the significant antigen-specific CD8+ T cell immune responses. Concordantly, the combination of knockdown of IL-10RA and TGF-βR in DCs showed significant up-regulation of MHC I, enhancing co-stimulatory molecules CD40, CD80, CD86 and chemokine CCR7 after lipopolysaccharide (LPS) stimulation. It induced the strongest anti-tumour effects in the TC-1 P0 (a cervical cancer model expressing the human papillomavirus [HPV]-16 E7 antigen) tumour model, and even in the immune-resistant TC-1 (P3) ones [33]. These data revealed that siRNA co-targeting immunosuppressive molecules enhance the potency of DC-based immunotherapeutics.

High-mobility group box 1 (HMGB1) is highly expressed in tumour cells and increased levels of HMGB1 in tumour cells are usually associated with a greater tumour angiogenesis, growth, invasion and metastasis. Knockdown of tumour cell-derived HMGB1 by shRNA did not affect tumour cell growth, while naturally acquired long-lasting tumour-specific IFN-γ- or TNF-α-producing CD8+ T cell responses were induced, and ability to induce Treg was attenuated. This led to naturally acquired CD8 T cell-dependent anti-tumour immunity [34].

Foxp3, a master gene that controls the development and function of Treg cells, contributes to pathogenesis of several different tumours. Owing to the intracellular localization of Foxp3, RNAi technology was employed to knockdown its activation to suppress Treg activity in vivo. Tsai et al. [35] performed a study targeting silencing Foxp3 gene expression by shRNAs-mediated RNAi using a lentivirus vector in a murine model of leukaemia. The lentiviral vector was used to overcome poor transfection efficiency. Lentiviral-mediated Foxp3 RNAi showed suppressive effects on tumour growth and prolonged the survival of tumour-transplanted mice. Furthermore, Foxp3 knockdown mediated by siRNA increased the ratio of Th1/Th2 in chronic hepatitis B patients; transcription factors T-bet and GATA-3 may be partly involved in this progress [36]. This strategy provides a novel view about how to decrease the number of Treg cells and weaken its function.

Selective knockdown of CCL22 and CCL17 expression in monocyte-derived DC (MoDC) by siRNA decreased the ratios of CD4+ to CD8+ as well as lowered the frequency of Tregs recruited by MoDC. Furthermore, intratumoural injection of MoDC, which was transfected with siCCL22 and siCCL17, significantly reduced the number of Tregs while inducing CD8+ T cells infiltration in thymic nude mice with human tumour xenografts [37]. Using siRNA to selectively silence chemokines may lead to a new strategy for DC vaccine development to improve cancer biotherapy.

High expression of indoleamine 2, 3-dioxygenase (IDO) in DCs leads to the suppression of T cell responses. Gene silencing by siRNA or shRNA of IDO in DC would up-regulate IL-12 and IFN-γ and inhibit apoptosis in CD8 and CD4 T cells as well as Treg cells; IL-10 expression was significantly down-regulated, thus finally restraining tumour growth. DC-based vaccine with IDO silence was demonstrated to augment and enhance the anti-tumour response against breast cancer, melanoma, bladder tumour and liver cancer [38–40]. A novel APC-targeted siRNA delivery system using mannosed liposomes (Man-lipo) with encapsulated siRNA-IDO

(Man-lipo-siIDO) was demonstrated to preferentially silence IDO in APCs and efficiently enhance anti-tumour immune response [41–43].

It was reported that natural killer group 2, member D (NKG2D) activation was involved in NK cell and CD8+ cell-mediated liver inflammation, and blockade of NKG2D by silencing of multiple NKG2D ligands on hepatocytes was considered efficient in liver disease intervention. Huang et al. [44] constructed a plasmid containing the three shRNA sequences (shRae1-shMult1-shH60). After hydrodynamic injection into mice, they found that the expression of all three NKG2D ligands on hepatocytes was down-regulated, and fulminant hepatitis mediated through NKG2D in NK cell was attenuated. Furthermore, simultaneous knockdown of multiple human NKG2D ligands (MHC class I polypeptide-related sequence B/A(MICA/B), UL16-binding protein 2 [ULBP2] and ULBP3) also significantly attenuated NK cell cytolysis. Simultaneous knockdown of multiple ligands of NKG2D is a potential therapeutic approach to treat liver diseases induced by NKG2D-expressing NK cells and CD8+ cells. Furthermore, inhibition of human leukocyte antigen-G (HLA-G) by siRNA boosted NK cell lytic function [45,46].

Among several molecules involved in immune response, the choice of targets should be carefully reviewed and validated comprehensively according to the emerging knowledge about their function.

2.2. Advantage of non-target immune effect of siRNA/shRNA drugs

siRNA/shRNA have the potential to recruit immune receptors specialized in RNA sensor, such as TLR3, TLR7/8 [2,3]. 5′-triphosphate siRNA (3p-siRNA) was demonstrated to be detected by RNA sensors RIG-I. These immunostimulatory siRNA or shRNA can non-specifically induce innate immune response, the so-called 'off-target' effects that have considerable implications for clinical application to cure cancer and infection disease.

IFN response is a common side effect of siRNAs and siRNAs with GU-rich sequences, which are very potent in inducing IFN-α response. A newly published report demonstrated that siRNA could induce IFN-α responses, and then induced the analgesic effects in the spinal cord. This off-target analgesia is dose- and sequence-dependent while non-GU-rich sequences also produced off-target analgesia at high doses, where pain relief by a designed siRNA may not be attributable to target gene knockdown but IFN response [47].

Early in 2004, Karikó et al. [48] demonstrated that siRNAs and shRNAs induce immune activation by signalling through TLR3 and activate sequence-independent inhibition of gene expression. Kleinman et al. [4] showed that non-targeted (against non-mammalian genes) and targeted (against vascular endothelial growth factor [VEGF] or VEGFR1) siRNAs suppressed choroidal neovascularization (CNV) via cell-surface TLR3 and its adaptor TIR-domain-containing adaptor-inducing interferon-β (TRIF), leading to the induction of IFN-α and IL-12. The effect of non-targeted siRNA to suppress dermal neovascularization in mice was as effective as vascular endothelial growth factor (VEGF) siRNA. This finding showed that two investigational siRNAs in clinical trials owe their anti-angiogenic effect in mice, which was not due to target knockdown but due to TLR3 activation. The efficiency of RNAi by siRNA is

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believed to be comparable with anti-VEGF antibodies. Kleinman's group then concluded that a 21-nucleotide (nt) non-targeted siRNA suppresses both hemangiogenesis and lymphangiogenesis in mouse models of neovascularization, induced by corneal sutures or hindlimb ischemia, as efficiently as a 21-nt siRNA targeting VEGF-A [1].

Among 15 siRNAs, Khairuddin et al. [49] identified an extremely immunostimulatory siRNAs, targeting the HPV, which exerted potent anti-tumoural function. This bifunctional siRNA could reduce growth of established TC-1 tumours in C57BL/6 mice, and its effect was TLR7 dependent, where ablation of TLR7 recruitment via 2'O-methyl modification of the oligo backbone reduced these anti-tumour effects. Flatekval et al. [40] designed either monofunctional siRNAs devoid of immunostimulation or bifunctional siRNAs with IDO silencing and immunostimulatory activities. They showed that bifunctional siRNAs were able to knockdown IDO expression and induce cytokine production through either endosomal TLR7/8 or RIG-I.

In the past 10 years, several studies reported that bifunctional 3p-siRNA (Exp:targeting Bcl2/TGF-β/Survivin/Glutaminase/IDO) with target silencing and an innate immunity stimulation via RIG-I activation could confer potent anti-tumour efficacy. This is illustrated for the first time by the work of Poeck et al. [8], who reported that bifunctional siRNAs, with 5'-triphosphate targeting Bcl2 (3p-siRNA), led to better melanoma tumour reduction than OH-siRNA or 5'-triphosphate siRNAs containing target mismatches. Poeck and his colleagues revealed that siRNA with 5'-triphosphate ends could be recognized by RIG-I and activate an innate immune cells such as DC; then, expression of IFNs was directly induced, leading to apoptosis in tumour cells. These bifunctional 3p-siRNAs with RIG-I activation and RNAi-mediated Bcl2 silencing could provoke massive apoptosis of tumour cells in lung metastases in vivo. This was the first report demonstrating that 3p-siRNA represents a single molecule-based approach in which RIG-I function activates immune cell and gene silencing, leading to a key molecular event. Researchers subsequently found that 3p-TGFβ1-siRNA combining RIG-I activation with gene silencing of TGF-β1 induced profound tumour cell apoptosis and revealed potent anti-tumour efficacy in pancreatic cancer. This kind of 3p-siRNA induces a Th1 cytokine profile, demonstrating IFN-γ induction and IL-4 inhibition. High level of IFN and CXCL10 recruited more activated CD8+ T cells to the tumour. Frequency of immunosuppressive CD11b+ Gr-1+ myeloid cells was reduced after 3p-TGFβ1-siRNA treatment [50].

In addition, 3p-siRNA against survivin gene was designed and generated. This finding demonstrated that 3p-survivin-siRNA inhibited lung cancer cell proliferation and induced a RIG-I-dependent type-I interferon response [7]. Recently, 5'-triphosphate siRNA combining glutaminase (GLS) silencing with RIG-I activation was demonstrated to induce more prominent anti-tumour responses than RIG-I ligand or GLS silencing capability alone. 3p-siRNA-GLS effectively induced intrinsic proapoptotic signalling, and GLS silence sensitized malignant cells to apoptosis induced by RIG-I activation. Moreover, cytotoxicity was enhanced, resulting from disturbed glutaminolysis induced by GLS silencing. Finally, RIG-I activation by 3p-siRNA-GLS blocked autophagic degradation, leading to dysfunction of mitochondria, whereas GLS silencing severely impaired reactive oxygen species (ROS) scavenging systems, leading to a vicious circle of ROS-mediated cytotoxicity [51]. Immature

monocyte-derived DCs had been transfected with siRNA-bearing 5′-triphosphate-activated T cells [40].

In addition, 3p-siRNA can inhibit hepatitis B virus (HBV), Influenza A Virus and Coxsackievirus, by gene silencing and RIG-I activation. RNAi provides a promising approach for the specific treatment of HBV infection. Our laboratory has previously demonstrated that 3p-HBx-siRNA and shRNA-HBx not only directly inhibit HBV replication but also stimulate innate immunity against HBV, which are both beneficial for the inversion of HBV-induced immune tolerance [52]. In HBV-positive hepatoma HepG2.2.15 cells, 3p-HBx-siRNA combining RIG-I activation with HBx gene silencing induce stronger type I IFN response than non-target 3p-scramble-siRNA, indicating that a potent immunostimulatory effect may partly contribute to the reversal of immune tolerance through decreasing HBV load; 3p-HBx-siRNA more strongly inhibited HBV replication and promoted IFN production than HBx-siRNA in primary HBV(+) hepatocytes, and this effect was mediated by RIG-I activation [52]. This was consistent of the other two reports [53,54]. Our dually functional vector containing both an immunostimulatory single-stranded RNA (ssRNA) and an HBx-silencing shRNA could reverse HBV-induced hepatocyte-intrinsic immune tolerance; TLR7 signalling pathway was attributed to this progress [55].

Lin et al. [56] designed and tested a 3p-mNP1496-siRNA against influenza virus. They found that 3p-mNP1496-siRNA could activate the RIG-I-mediated IFN-β pathway and significantly reduce virus load and virus-induced pathogenesis. The inhibition effect was in an siRNA- and RIG-I-dependent manner, demonstrating siRNA playing dual antiviral roles: viral gene-specific silencing and non-gene-specific RIG-I activation. This strategy was also proved to elicit potent antiviral effects in coxsackievirus myocarditis, and virus-specific 3p-siRNA was superior to both conventional virus-specific siRNA and non-target 3p-siRNA in inhibiting viral replication and subsequent cytotoxicity [57].

In the attempt to inhibit the expression of woodchuck hepatitis virus (WHV), Meng et al. [58] found that innate immune responses could be enhanced by RNAi through the PKR- and TLR-dependent signalling pathways in primary hepatocytes. The immunostimulation by RNAi may contribute to the antiviral activity of siRNAs in vivo.

Furthermore, siRNA can also synergistically enhance DNA-mediated type III IFN (a newly characterized antiviral interferon) response in non-immune or primary immune cells. This enhancement is mediated by crosstalk signalling pathway between RIG-I (RNA sensor) and IFI16 (DNA sensor) [59].

Designing with GU sequences, addition of triphosphate motifs to siRNA, co-treatment with CpG oligos are believed to activate innate immunity when siRNA was applied in vitro and in vivo. Accumulating evidence suggests these bifunctional siRNAs could activate NK cells and CD8+ T cells in different models. Thus, specific clinical applications of RNAi can benefit from a concurrent activation of the immune system.

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3. miRNA

It has been well discussed how miRNAs regulate signalling pathways, and the dynamics of the immune response, tolerance and homeostasis. Here we summarize and explore updated achievements of special miRNAs in immunopharmacology.

3.1. miRNAs as intrinsic targets in antiviral immunity

In addition to the conventional innate and adaptive immune responses, even in the earlier phase after virus invasion, the host cell suppresses viral replication by evolving the profile of special and constitutively expressed genes. These cell-intrinsic antiviral approaches based on host restriction factors may be no less important than in considerations of conventional immunity. At the same time, viruses also gain some countermeasures or adapt the unique phenotype of their hosts substantially to survive. Moreover, miRNAs may also be involved in the inextricably intertwined relationship between viruses and their hosts.

In 2005, a liver-specific miRNA, miR122, which is involved in cholesterol and lipid metabolism [60], was illustrated to be necessary for hepatitis C virus (HCV) accumulation in cultured liver cells [61]. Researchers found that miR122 directly binds to two close sites in the 5′ non-coding region of the HCV genome and promote HCV translation [62–64]. This miRNA kept conserved among all HCV subtypes [65,66]. Even in non-hepatic cell, miR122 could boost HCV replication [67]. Moreover, miR122 was further proved to be significantly reduced after IFN-β treatment, and miR122 mimics neutralized IFN-induced anti-HCV effect [68]. Epidemiological and genomic researches further suggested that the level of miR122 in individuals with HCV might be an 'indicator' for IFN therapy, and only those patients with high levels of miR122 responded well to IFN therapy [69,70]. Therefore, miR122 antagonist would also be called as IFN 'sensitizer' in HCV immune treatment.

Santaris Pharma designed and synthesized an LNA-based miR122 inhibitor, named Miravirsen (or SPC3649), to eradicate HCV. The product was first evaluated in preclinical studies in mice [71], cynomolgus monkeys [72], green African monkeys and chimpanzees [73,74]. Here the key concern is that whether miR122 inhibitor can effectively lower the level of free miR122 and inhibit HCV replication without disturbing normal cholesterol and lipid metabolism or without any potential chemical toxicity. Interestingly, although there was a reduction of cholesterol levels in plasma by nearly 40%, Miravirsen caused a dose-dependent reduction of miR122 and maintained ~5-week-long half-life in the liver of monkeys and chimpanzees [73, 74]. Moreover, in the high-dose treatment group, Miravirsen decreased HCV subtype 1a or 1b more than 2 orders of magnitude compared to control group. In all animal species, Miravirsen was reported to be safe, without serious adverse effects or dose-related toxicities in rats, monkeys and human [75,76].

In May 2008, Miravirsen was put into human clinical trials as the first miRNA-based drug (https://clinicaltrials.gov/ct2/show/NCT00979927). There was a significant, dose-dependent reduction and sustained decrease of HCV viremia after drug administration in human subjects, and several patients became even HCV undetectable during the study. At the same time, only

infrequent and moderately adverse effects were caused to some volunteers and did not influence the trial process [77]. Because miR122 is only liver enriched in physiological conditions and there is high amount of miR122 in adult human liver, it may be an ideal target to design highly specific anti-HCV drugs with good resistance to HCV infected person, particularly to those who have no tolerance to traditional treatments. In the following years, miR196 [78], let-7b family [79] and some other miRNAs were then proved to influence HCV life cycle, providing new target to restrict hepatitis C infection and avoid chronic infection.

Besides HCV, some other kind of viruses also encode miRNAs or regulate the miRNAs expression in host cells to disturb the expression of many immune-associated genes directly and/or indirectly, so that they can be critical regulators for viral life cycle. For example, in HEK293T cell, prototype foamy virus I (PFV-1) encodes Tas protein to counteract cell-encoded miR32, which could inhibit PFV-1 gene expression and accumulation [80]. Kaposi's sarcoma-associated herpesvirus (KSHV)-induced miR132 could silence p300 expression, which is critical for the transcription initialization of many antiviral genes, to help themselves maintain long-time latency [81]. The hematopoietic-cell-specific miR142-3p potently restricts the replication of eastern equine encephalitis virus in myeloid-lineage cells by binding to the 3′-untranslated region (UTR) of viral genome [82]. Even Drosha, the enzyme that processes miRNA biogenesis and maturation, was an independent factor for limiting RNA virus replication along with canonical type I IFN system in particular cell type [83]. Above of all, it is much likely that miRNA mimics (for viral inhibitory miRNAs) or antagonists (for viral beneficial miRNAs) can be effective antiviral strategies as intrinsic immune drugs.

3.2. miRNA regulation antimicrobial and anti-tumour immunity

3.2.1. miRNA in antimicrobial innate immunity

Of the known PRRs, TLRs and RIG-like receptors (RLRs) have been well studied in mediating antimicrobial and inflammatory responses during infections, which may be targets of pathogens or host-encoded miRNAs.

The first PRR targeting miRNA let7i was reported in 2007 [84], which targeted TLR4 mRNA in a MyD88/NF-κB-dependent way during *Cryptosporidium parvum* infection, controlling the production of inflammatory factors. During *Bacillus Calmette-Guérin* (BCG) infection, miR124 exerts its function by targeting multiple components of the TLR signalling pathway, including TLR6, MyD88, TNF receptor-associated factor 6 (TRAF6) and TNF-α in mouse lung cell [85]. After HCV infection, miR373 was induced and negatively regulated the type I IFN signalling pathway by suppressing Janus kinase 1(JAK1) and IRF9 in hepatocytes [86]. Experimental evaluation using miR124 inhibitors or miR373 knockout up-regulated BCG-induced pro-inflammatory factors or type I IFN and so as to inhibit BCG or HCV more efficiently. Besides using host miRNAs, human cytomegalovirus (HCMV) targeted TLR2 by encoding its own miRNA, miR-UL112-3p, and reduced the expression of IL-1β, IL-6 and IL-8 upon stimulation with a TLR2 agonist [87]. Neutralizing this miRNA might recover normal cytokines production.

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Besides immune inhibitory miRNAs, dengue virus (DENV)-induced miR30e* up-regulated IFN-β and the downstream IFN-stimulated genes (ISGs) by suppressing IkBα and promoting NF-κB-dependent IFN production [88]. The transfection of miR30e* would increase the expression of 2′-5′-oligoadenylate synthetase 1(OAS-1), myxovirus resistance A (MxA) and interferon-induced transmembrane protein (IFITM). In 2014, miR526 [89] was proved to enhance RIG-I-induced viral replication by suppression of the expression of cylindromatosis (CYLD), which suppresses RIG-I K-63-linked polyubiquitin. Moreover, Enterovirus 71(EV71) inhibited miR526 transcription in an IRF-dependent way and so as to attenuate virus-triggered type I interferon production. These studies suggested that recruitment or increase of miR30e* or miR526 would stimulate type I IFN expression and inhibit virus more quickly.

3.2.2. 'Immune miRs' as immunopharmaceutic agents

With the general knowledge of immunologically relevant miRNAs established in the past 10 years, many miRNAs have been intensively investigated using gain- and loss-of-function methods, showing how this novel class of small non-coding RNA participates in mammalian immunity. And individual immune miRNA might contribute its implications to various immune-mediated diseases.

The role of miR125b in immune signalling may be paradoxical. After stimulation with LPS, miR125b was down-regulated and TNF-α, one of miR125b targets, was overexpressed in RAW264.7, which is essential for antimicrobial activity. Moreover, during *M. tuberculosis* infection, the overexpression of miR125a significantly attenuated the antimicrobial effects in macrophages through targeting UV radiation resistance–associated gene (UVRAG) [90]. Nevertheless, in diffuse large B cell lymphoma (DLBCL), miR125a and miR125b directly target a negative NF-κB regulator tumour necrosis factor alpha-induced protein 3(TNFAIP3) and present a positive self-regulatory property to maintain prolonged NF-κB activity. Taken together, whether overexpression or inhibition of miR125b in an anti-infection therapeutic study depends on concrete circumstances.

miR146a also acts as a negative regulator in immune sensing. Both in mouse and human, miR146a was always exploited by virus to attenuate innate and adaptive antiviral immunity mainly in DC [91], lymphocyte [92] and hepatocytes by inhibiting interleukin-1 receptor-associated kinase 1(IRAK1), TRAF6 [93], son of sevenless homolog 1 (SOS1) [94] and STAT1 [95]. Silencing of miR146a via the delivery of sponge or antagomiR could restore the expression of inflammatory factors, augment type I IFN production and promote clearance of vesicular stomatitis virus (VSV) [96], dengue virus [97], enterovirus 71 (EV71) [94,98] and HBV [95]. Because miR146a was also abnormally expressed in hepatocellular carcinoma (HCC) and exerted negative anti-tumour effects by up-regulation of immunoinhibitory cytokines such as TGF-β, IL-17, VEGF, miR146a may also be a novel immunotherapeutic target for HCC [99].

Unlike miR146a, miR155 always promotes immune signal transduction, enhances immune function or speeds lymphocyte proliferation. Mice lacking miR155 have impaired CTL cell responses to infections with lymphocytic choriomeningitis virus and the intracellular bacteria *Listeria monocytogenes* because of insufficient activation of Akt pathway after TCR cross-linking [100]. miR155 knockout mice died soon after Erdman (a variant from severe acute respiratory

syndrome [SARS]) infection and held higher level of colony-forming units (CFU) in lungs than wild-type mice [101]. During HIV infection, miR155 inhibited the HIV-activating effects of tripartite motif-containing protein 32 (TRIM32), and therefore, it might promote a return to latency in CD4+ reservoir cells [102]. In addition, in NK cells, miR155 might regulate T cell immunoglobulin-3 (Tim-3)/T-bet/STAT-5-signalling axis, and following cytokine expression that balanced antiviral response and immune injury during chronic HCV infection [103]. A remarkably ectopic up-expression of miR155 can be observed by delivering hepatotropic adeno-associated virus 8 (AAV8) vectors to the liver of mice, and then high level of miR155-enhanced GAP's protective capacity against parasite [104]. These studies imply miR155 as an immune-augmenting adjuvant in improving the antigenicity of vaccination.

miR223 was already proved to be of importance in myeloid progenitor cells proliferation and responsiveness to pathogenic stimuli in neutrophils by targeting myocyte-specific enhancer factor 2C (MEF2C), acting as a fine-tune regulator both in normal granulocytes generation and in preventing aberrant expansion and over-activated inflammatory responses. In recent years, miR223 was involved in inflammasome response by targeting NLR family pyrin domain containing 3 (NLRP3) in human [105]. Moreover, Epstein–Barr virus (EBV) encoded a mimic of hsa-miR223, called miR-BART15, targeting the same site within the NLRP3 3′-UTR to repress inflammasome activation. Furthermore, this miRNA can be secreted from EBV-infected B cells into exosomes to rheostat NLRP3 inflammasome activity in non-infected cells [106]. miR223 sponge would balance the amount of NLRP3 and 'absorb' EBV-miR-BART15 in macrophages and DCs.

It is noteworthy that two groups of miRNAs, which shaped NK-mediated cytotoxicity, have potent value for developing antiviral and anti-tumour biodrugs. First, NKG2D–NKG2D-L interaction plays a predominant role in 'NK cell–abnormal cell' recognition. MICB/A, ULBPs targeting miRNAs, are not only encoded by human genome as stress regulators but also synthesized by some virus (e.g. HCMV-miR-UL112 [107], EBV-miRBART2 [108], KSHV-miR-K12-7 [108] and BK virus (BKV)-miR-B1-3p, JC virus (JCV)-miR-J1-3p[109]), to escape from NK cell killing. Meanwhile, viral infected cell and tumour cell always express low MICA/B because of up-regulated MICB/MICA, targeting miRNAs such as miR20a, 93, 103, 106b [110] to maintain a compromised micro-environment. Furthermore, non-classical human leukocyte antigen G (HLA-G) is known as an inhibitory ligand, which suppresses the cytotoxic activity of T and NK cells. Studies demonstrated a strong post-transcriptional gene regulation of the HLA-G by miR148a, miR148b and miR152, and lower expression of these miRNAs in renal carcinoma [111] and placental choriocarcinoma cells [112]. Stable manipulation of these activating and inhibitory miRNAs may enhance NK and LAK cell-mediated cytotoxicity against infected and tumour cells. Therefore, it could be concluded that modulating the expression or inhibition of specific miRNAs could boost immune response during viral infections or against cancers.

3.3. miRNA in maintaining immune homeostasis

Because several miRNAs participate in immune cell development and differentiation, abnormal expression of miRNA may cause a disturbance of homeostasis by changing the ratio of helper and regulatory cell subsets, or perturb the functionality and survival of effect-

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memory cells that lead to lymphoproliferative disease. Utilization of miRNA interference techniques may recover regular immune balance.

3.3.1. miR17-92, miR146a and miR155 in Systemic Lupus Erythematosus (SLE)

In 2007, a unique mouse strain, 'sanroque', presented a pattern of lupus pathology, revealing the core role of T follicular helper (Tfh) in systemic autoimmunity [113]. miR17-92 was found to regulate Tfh cell differentiation, which is essential for maintenance of the germinal centre formation and sustained antibody responses. Overexpression of this miRNA in T cells would enhance Tfh cell proliferation and survive an autoantibody production [112]. Similarly, miR155 increased IL-21-mediated STAT3 signalling in T cell [114], which might accelerate Tfh differentiation and maturation as well. Moreover, miR155 deficiency ameliorates autoimmune inflammation of SLE by targeting s1pr1 in mice [115]. Therefore, miR17-92 and miR155 might be a new target to restrain aggressive autoimmune response in SLE.

3.3.2. miR29 and miR146 in type 1 diabetes

Type 1 diabetes (T1D) is a chronic autoimmune disease that results from the persisting destruction of pancreatic β-cells by autoreactive CD8+ T cell and Th1 cytokines. Dicer 1 deletion in β-cell would disrupt normal β-cell development and survival, lead to impairment of insulin secretion and diabetes development [116], apparently suggesting that miRNAs network is necessary for normal glycometabolism. Recently, endogenous miR29b released from pancreatic β-cells within exosomes stimulated TNF-α secretion in spleen cells isolated from diabetes-prone non-obese diabetic (NOD) mice. Delivery of miR29b to mice activated myeloid cell and pDCs to induce IFN-α, TNF-α and IL-6 production [117]. Abnormal expression of miR146 is associated with high serum titers of glutamic acid decarboxylase antibody in T1D patients, indicating the involvement of miR146 in the sustained immune imbalance during T1D progress [118]. These findings raised the possibility of developing a new clue for T1D immunotherapy using miRNA-based agents.

3.3.3. miR146a and miR155 in rheumatoid arthritis

The role of miR146a in controlling Treg-mediated decrease of Th1 responses has been demonstrated [119]. In contrast, miR155 promoted Th1 and Th17 differentiation and cell formation and lowered T cell sensitivity to IFN-γ-driven proliferation by targeting C-MAF and IFNγRα [120]. Therefore, imbalance of miR146a and miR155 may be an epigenetic phenotype for autoimmune response. In rheumatoid arthritis (RA), decreased expression of miR146a contributes to an abnormal Treg phenotype and allows Th1/Th17 skewing while low level of miR155 failed to support effective Th2 immunity [121]. Systemic administration of miR146a has potential therapeutic intervention for preventing bone destruction by inhibited Th1 and Th17 cells, as well as IL-1β, IL-6 and TNF-α [122].

3.3.4. miR15 and miR326 in multiple sclerosis

Multiple sclerosis (MS) is manifested by chronic and progressive inflammatory demyelination of the central nervous system and is one of the main causes of regressive neurological diseases.

Study on MS animal model illustrated that mice with fewer Th17 cells were less susceptible to experimental autoimmune encephalomyelitis (EAE) [123]. Therefore, Th17-targeting biotherapeutic approaches may be a promising way to cure multiple sclerosis. Gang Pei' s laboratory [124] found that miR326 promoted Th17 differentiation by targeting Ets-1 (a negative regulator of Th17 polarization) and antagonizing miR326 by sponge vector that resulted in fewer Th17 cells and Th17 cytokines and remitting EAE symptom. Inversely, increased miR155 in primary human microglia up-regulated pro-inflammatory cytokine secretion and co-stimulatory surface marker expression suggested that miR155 inhibition in myeloid cell might be useful to suppress allogeneic T cell responses [125]. In conclusion, reverse pathological expressed miRNAs and re-balance dysregulated immune genes are of consideration to treat multiple sclerosis.

4. Conclusion

RNAi technology holds promise for treating various human diseases. It is becoming apparent that clinical outcome of cancer immunotherapy and infectious diseases can be improved by targeted strategies to abrogate tumour-induced immunosuppression. Anti-tumour strategies using siRNA/shRNA/miRNA for both silencing of oncogenes and recruiting of innate receptors were designed. The present researches highlighted the potential therapeutic applications of this new generation of siRNAs in immunotherapy.

Additionally, but importantly, siRNA/shRNA or miRNA drugs with regard to pharmacodynamic difficulties and unwanted side effects are even more complicated compared to low molecular weight drugs and hard to be delivered into immune cells. This requires more extensive procedure than any other traditional drugs. Considering clinical challenges for RNA-based nucleic acid drugs, including barriers and RNases, the advanced tissue-directed delivery systems with safety, high efficiency and specificity, long-term function and controllability are required. Although the exploration of such tiny regulators causally bring pharmacists a considerable effort to draw up individualized and tailor-made strategies, we believe that immunoregulation triggered by siRNA/shRNA/miRNA can be used to regulate the host immunity against cancers or viruses. The development of multifunctional RNAi molecules will greatly contribute to the future arsenal of tools to combat not only microbial pathogens but also hard-to-treat cancer.

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