Functional Studies of Lymphocytes Using RNAi Technology

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Key Words

RNAi · siRNA · Lymphocyte · Adaptive immunity · Immunotherapy

Summary

RNA interference (RNAi) has become an important gene knockdown technique which has been widely used in recent years. This technique has been extensively employed in studying the genetics, molecular biology and physiology of cancer cells or normal cells. RNAi technology has also been used to explore the biology of the cells involved in the immune response. Among those, lymphocytes have been the most thoroughly studied. RNAi has been used to study lymphocyte development, activation and effector mechanisms. In addition, it has been applied to modify the properties of lymphocytes to influence the outcomes of immune responses and to create opportunities for potential therapeutic applications in vivo. While the application of RNAi technology to influence immune responses in vivo is quite promising, significant obstacles, such as in vivo delivery, nonspecific immune responses and 'off-target effects', need to be overcome before this technology can be successfully translated for clinical application. In this review, we have summarized the current use of RNAi in the study of lymphocyte biology and the potential clinical translations associated with this technique.

Schlüsselwörter

RNAi · siRNA · Lymphozyt · Adaptive Immunität · Immuntherapie

Zusammenfassung

RNA-Interferenz (RNAi) ist zu einer wichtigen Gen-Knockdown-Technik geworden, die in den letzten Jahren breit angewandt worden ist. Insbesondere zur Untersuchung von Genetik, Molekularbiologie und Physiologie maligne entarteter und normaler Zellen ist RNA-Interferenz ausgiebig eingesetzt worden. Die RNAi-Technologie wurde auch zum Verständnis der Biologie von Zellen, die in die Immunantwort involviert sind, genutzt. Am intensivsten wurden dabei Lymphozyten untersucht. Schwerpunkt dieser Studien waren die Entwicklung, Aktivierung und Effektormechanismen von Lymphozyten. Zudem wurde die RNAi-Technologie eingesetzt, um das durch Lymphozyten vermittelte Outcome der Immunantwort zu modifizieren und Möglichkeiten für einen therapeutischen In-vivo-Einsatz der Technologie zu eruieren. Zwar verspricht RNAi mittlerweile einige Chancen, die Immunantwort in vivo zu modifizieren, doch sind noch wesentliche Hindernisse zu überwinden, bevor die Technologie erfolgreich unter klinischen Bedingungen eingesetzt werden kann. Zu den Schwierigkeiten gehören derzeit die Entwicklung einer effizienten Delivery-Technologie, die Induktion nichtspezifischer Immunantworten und die so genannten «Off-Target-Effekte». Die vorliegende Übersichtsarbeit fasst den aktuellen Stand des Einsatzes der RNAi-Technologie zum Verständnis der Lymphozytenbiologie zusammen und beschreibt mögliche klinische Einsatzgebiete der RNAi-Technologie.

Introduction

RNA interference (RNAi) is an evolutionarily conserved process of post-transcriptional gene silencing. This naturally occurring and highly specific gene silencing phenomenon was first noticed in the late 1980s, but its mechanism was not clear until the end of the last century [1–3]. Figure 1 shows an overview of the RNAi mechanism. Briefly, double-stranded RNA (dsRNA) present in the cytoplasm, such as the ones produced by viruses or transposons, is chopped into 21–28 nucleotide duplexes called small interfering RNA (siRNA) by the RNase III-like enzyme Dicer. 1 of the 2 strands of the siRNA will then be incorporated into a protein complex named RNA-induced silencing complex (RISC). The complex can use the siRNA as a template, and if it finds an mRNA that is complementary to the siRNA, the enzyme subunit in the complex (Argonaute 2) will cleave the target RNA in the center of the region homologous to the siRNA, thus resulting in mRNA degradation and gene silencing. Alternatively, the binding of the complex can lead to translational arrest. Therefore, RNAi can serve as a cellular mechanism to defend against viruses or transposons, as well as a gene control mechanism in developmental processes. Because the siRNA template is protected within the RISC complex, reactions can last for a long time, being even passed on to the progeny of the silenced cells. Various kinds of artificial small dsRNA have been inserted into different kinds of cells and organisms to demonstrate that the RNA interference machinery can be used as a tool to knock down specific genes and study the gene function. Understanding of these mechanisms allowed RNAi to become a very popular biotechnology only a decade after its discovery.

Significant progress has been made regarding the understanding of immune responses. In general, immune response can be conceptually classified into innate immunity and adaptive immunity. Lymphocytes are the most important type of immune cells for the adaptive immune response. B lymphocytes are involved in the production of antigen-specific antibodies, while T lymphocytes can be directly involved in the killing of cells infected with specific pathogens or tumor cells, or can facilitate the generation of immune response against pathogens or tumors. A better understanding of the immunobiology of the lymphocytes may allow us to improve the efficacy of adaptive immune responses.

RNAi technology has been extensively used to illustrate the immunobiology of lymphocytes, including the development of lymphocytes, lymphocyte activation, T cell effector functions, senescence and apoptosis of lymphocytes, chemotaxis, and the interactions between lymphocytes and lymphotropic pathogens. Furthermore, RNAi technology has been used to modify the properties of lymphocytes to influence the outcome of adaptive immune responses. In this review, we describe the current progress of RNAi technology in illustrating the immunobiology of lymphocytes and review the examples

Fig. 1. Introduction of the basic mechanisms of RNAi. Different forms of dsRNA, including siRNA, small hairpin RNA (shRNA) or long dsRNA, can be processed by the enzyme Dicer into 19–21 nucleotide double-strand fragments. One of the fragment will be incorporated into the RISC complex and serve as a template. This complex can bind to a target mRNA, which has a complementary sequence to the template, and the enzymatic subunit Argonaute 2, cleave the target mRNA and cause it to be degraded. A single RISC can cleave mRNA repeatedly so the process is highly efficient.

of improving adaptive immune response by RNAi technology in vivo. The limitations of applying RNAi technology to the modification of lymphocytes in vivo are also discussed.

Modification of the Properties of Lymphocytes by RNAi Technology

In the following section, we organize the studies of lymphocyte biology using RNAi technology based on the sequential events involved in the role of lymphocytes in the adaptive immune response, namely the development, activation, effector mechanism, chemotaxis, senescence and apoptosis of lymphocytes. In addition, we discuss the application of RNAi technology to analysis of the interactions between lymphocytes and lymphotropic pathogens. These studies show the wide range of RNAi applications in illustrating the biology of lymphocytes. Figure 2 is an overview of lymphocyte biology that has been studied by RNAi, and table 1 summarizes the molecules involved in lymphocytes biology, which have been targeted by RNAi technology.

Lymphocyte Development

Lymphocytes develop from stem cells in the bone marrow. Their developmental process is distinguished from that of other leukocytes, such as neutrophils or macrophages, and unique genetic recombination in the lymphocyte precursors affords the immune system its remarkable plasticity. The

Fig. 2. Lymphocyte biology categories that have been studied by RNAi technology. Many biological events related to lymphocytes have been studied, including lymphocyte development, activation, effector function, apoptosis, chemotaxis and interaction with pathogens. Examples of molecules within each category that have been targeted are listed in this figure. The formation of immunological memory is an additional category of lymphocyte biology that is potentially a good research topic for the RNAi technology.

process has been extensively studied, and RNAi has proven a particularly useful tool in this kind of research. One example is the employment of RNAi technology in studying the phenomenon of immunoglobulin gene expression exclusively by B lymphocytes. It is now known that a special regulatory sequence called downstream immunoglobulin control element (DICE) can control B cell activity. The protein that interacts with this special sequence is called BEN (general transcription factor II I repeat domain-containing 1). Silencing BEN by siRNA led to a decrease of Ig heavy chain expression [4]. Another example is the regulation of differentiation of T lymphocytes by siRNA. When a T lymphocyte approaches maturation, 1 of 2 surface markers, CD4 or CD8, will be maintained, and the other will be lost. The presence of CD4 or CD8 on lymphocytes will determine whether the T lymphocyte will become a helper T (Th) cell or a cytotoxic T lymphocyte (CTL). The transcription factor GATA-3 (GATA-binding protein 3) plays an important role in driving CD4⁺ T cell differentiation while inhibiting CD8⁺ T cell. Silencing GATA-3 by siRNA has been shown to inhibit CD4⁺ T cell differentiation [5]. The developmental process of lymphocytes is a complicated phenomenon. RNAi technology represents an important tool for dissecting the process.

Lymphocyte Activation

Activated Th lymphocyte responses can be categorized into Th1 and Th2. The Th1 response will augment cellular immunity and activate macrophages to kill pathogens, while the Th2

response will promote humoral immunity by stimulating B cells to produce antibodies. Usually, only 1 of the 2 responses will become dominant, and weakening or enhancing one of them will cause an opposite change in the other type of Th cell response. Direct modification of the molecules important for determining Th1 versus Th2 immune responses by siRNA can result in a particular type of Th lymphocyte response. For example, GATA-3 is an important transcription factor, as mentioned above, for driving CD4⁺ T cell differentiation. If CD4⁺ T cells continue to express GATA-3, the CD4⁺ T cells will become Th2 lymphocytes. Silencing GATA-3 has been shown to block the Th2 response [6]. Another example is the silencing of T-bet by siRNA. The continuous expression of T-bet causes CD4⁺ T cells to become Th1 lymphocytes. Silencing T-bet has been shown to impair the Th1 response [7]. These examples indicate that the modification of the key molecules important for immune responses by siRNA technology may be utilized to change the type of immune responses. The ability to select for a particular type of immune response has a potential clinical implication. The siRNA technology may potentially be used to decrease a harmful type of Th cell response, such as the Th2 response in allergy, or strengthen a protective type of response, such as the Th1 response in antileprosy immunity.

T cell activation involves several signal transduction pathways, including PLC- γ activation [8]. The activation of the PLC- γ pathway leads to an increase in the cytosolic calcium concentration. The increased cytoplasmic calcium concentration can result in the activation of protein kinase C which in turn activates a series of signaling molecules, leading to the prolifera-

Table 1. Molecules important for lymphocyte biology, which have been targeted with siRNA

Functional categories	Molecules targeted by siRNA
Lymphocyte development	GATA-3 [5], BEN [47], NF-kappaB [48], ETS1 [48]
Lymphocyte activation	T-bet [7], Csk [29], CD28 [49], Egr-2 [50], MTA3 [51], GATA-3 [6] [52], 3BP2 [53] [54], CD20 [55], PKC-theta [56] [57] [58], PKC-alpha and beta1 [58], Itk [59], dynamin 2 [60], TRPM4 [9], HIP-55 [61], PDK-1 [62], Deltex [63], CaMKIV [64], Btk [65] [66], G3BP [67], RasGRP1 [68], Ro52 [69], Dlg1 [70], mTOR [71], CD227 [72], MEF2 [73], PRMT [74], CREB [75], RUNX3 [76], MALT1, TAK1, TRAF6, and TRAF2 (IKK activation pathway) [77], galectin-2 [78]
Lymphocyte effector mechanism	NKG2D [11], granulysin [10],
Chemotaxis	Rac and Vav1 [15], G2A [17], Beta-arrestin 2 [18], Jak2 [14]
Lymphocyte senescence and apoptosis	Cat D, Bax, and AIF (apoptotic pathway) [19], Fas [21], p16 [79], p53 [4], Syk [20], OSCP [80], Ian4 [81], NF-kappaB [82]
Lymphocyte-pathogen interaction	ATR and GADD45alpha [83], CCR5 [84] [25] [26], CXCR4 [26], deoxyhypusine synthase [24], β 2m [85], histone H1B [86], Tsg 101 [87], chk2 [27], APOBEC3G [22], Murr1 [23]

3BP2 = SH3-domain binding protein 2; AIF = apoptosis-inducing factor; APOBEC3G = apolipoprotein B mRNA editing enzyme = catalytic polypeptide-like 3G; ATR = Rad3-related protein; BAX = BCL2-associated $\times$ protein; BEN = general transcription factor II I repeat domain-containing 1; Btk = Bruton's tyrosine kinase; CaMKIV = calmodulin-dependent kinase IV; Cat D = cathepsin D; CCR5 = chemokine (C-C motif) receptor 5; Chk2 = check point kinase 2; CREB = cAMP response element binding protein; Csk = C-terminal Src kinase; CXCR4 = chemokine (C-X-C motif) receptor 4; Dlg1 = discs = large homolog 1 (*Drosophila*); Egr-2 = early growth response gene-2; ETS1 = v-ets erythroblastosis virus E26 oncogene homolog 1 (avian); Fas = Fas (TNF receptor superfamily = member 6); G2A = G protein-coupled receptor 132; G3BP = Ras-GTPase-activating protein SH3-domain-binding protein; GADD45alpha = growth arrest and DNA damage-45 protein alpha; GATA-3 = GATA binding protein 3; HIP-55 = drebrin-like; Ian4 = GTPase = IMAP family member 3; MALT1 = mucosa associated lymphoid tissue lymphoma translocation gene 1; TAK1 = TGF- activated kinase 1; TRAF6 = TNF receptor-associated factor 6; TRAF2 = TNF receptor-associated factor 2; MEF2 = myocyte enhancer factor 2; MTA3 = metastasis associated 3; mTOR = mammalian target of rapamycin; Murr1 = copper metabolism (Murr1) domain containing 1; NF-kappaB = nuclear factor kappa B; NKG2D = killer cell lectin-like receptor subfamily K = member 1; OSCP = ATP synthase = H^+ transporting = mitochondrial F1 complex = O subunit (oligomycin sensitivity conferring protein); PDK-1 = 3-phosphoinositide-dependent kinase 1; PKC-theta = protein kinase C-theta; PRMT = protein arginine methyltransferases; Rac = v-akt murine thymoma viral oncogene homolog 1; RasGRP1 = RAS guanyl releasing protein 1; Ro52 = tripartite motif-containing 21; RUNX3 = runt-related transcription factor 3; Syk = spleen tyrosine kinase; TRPM4 = transient receptor potential cation channel = subfamily M = member 4; Tsg 101 = tumor susceptibility gene 101; Vav1 = vav 1 oncogene.

tion and differentiation of the T cell. Silencing the molecules important for T cell activation by siRNA may change the outcome. For example, TRPM4 (transient receptor potential cation channel, subfamily M, member 4), a calcium-activated nonselective cation channel protein, has been found to be important in the control of T cell calcium concentration. Knockdown of the TRPM4 by siRNA increased the cytoplasmic calcium concentration and IL-2 production in vitro, leading to enhanced T cell activation. Thus, TRPM4 may be an endogenous regulator of T cell activation and effector response [9]. Other molecules important for T cell activation which are targeted by siRNA are summarized in table 1.

Effector Function of Lymphocytes

The effector mechanisms of lymphocytes represent another interesting area of research using RNAi technology. As mentioned above, Th cells enhance the macrophage or B lymphocyte response and coordinate immune responses via cytokines, while CTLs perform direct killing using several weapons. One of these is granulysin. Silencing of granulysin

by siRNA technology has been shown to lead to loss of CTL killing function. For example, Ma et al. [10] showed that granulysin is the main molecule that CTLs use to attack the fungus *Cryptococcus neoformans*. siRNA knockdown of this molecule will largely abrogate the anti-fungal effect of CTLs. Similar strategies can be used to study other important molecules for CTL effector function, such as NKG2D (killer cell lectin-like receptor subfamily K, member 1). It has been shown that CTLs can attack multiple kinds of tumors, and the surface molecule NKG2D is an important tumor recognition mechanism of these lymphocytes. RNAi silencing of this molecule will decrease the T cell-mediated anti-tumor effect in vitro [11]. TGF- β is another important molecule that can regulate CTL function and immune response. Previous research pointed out that TGF- β secreted by tumor cells can decrease the CTL killing of the cells, and siRNA knockdown of TGF- β in the tumor cells can reverse this inhibition of T cells [12]. Down-regulation of the TGF- β receptors on CTLs may also overcome this inhibition [13]. These reports suggest that silencing of TGF- β receptors on the CTLs by siRNA may be another plausible approach to enhance the T cell-mediated immune response against tumors.

Fig. 3. Overview of apoptotic pathways. There are 2 major apoptotic pathways, the intrinsic pathway and the extrinsic pathway. When cells are exposed to radiation or chemotherapy, DNA damage occurs, and the intrinsic pathway will be activated. Proteins on the mitochondria surface, such as BAX and BAK, will facilitate cytochrome C release from the mitochondria and activate caspase 9. Caspase 9 will activate caspase 3 which will activate a series of enzymes, leading to cellular macromolecule degradation and cell death. On the other hand, extracellular death signals can activate the extrinsic apoptosis pathway through receptors such as Fas and TNF. The receptor signal will activate caspase 8 which can also activate caspase 3, and this will also lead to cellular apoptosis.

Chemotaxis of Lymphocytes

Chemotaxis of lymphocytes is an important means by which our immune system tackles infections or neoplasia. T lymphocytes have different migration patterns under different conditions. When they are resting, they usually circulate the whole body and pass through lymphoid organs, such as the lymph nodes and spleen. The route is controlled by a series of molecules, including the cell adhesion molecules and chemokines. When infection occurs, tissue cells will produce new signals to attract lymphocytes and other leukocytes, and activated lymphocytes will change their surface molecules and receptors to respond to these new signals. The lymphocytes may migrate to the infected tissue, leave the blood vessels and enter the interstitial space. This process is called chemotaxis and extravasation. RNAi technology provides opportunities to further dissect the molecular mechanisms of lymphocyte/chemokine interaction. For example, RNAi has been used to study a chemokine called CXCL12 [14, 15]. Binding of this chemokine to the chemokine receptor on T lymphocytes can activate a signal transduction pathway. This signal will lead to an affinity increase of a surface adhesion molecule called integrin $\alpha 4 \beta 1$ and promote T lymphocyte extravasation through capillary walls. Two molecules, GTPase Rac (v-akt murine thymoma viral oncogene homolog 1) and guanine-nucleotide exchange factor Vav1, have been found to be important components of the CXCL12-induced chemotactic activity in T cells. Silencing of either of these molecules by siRNA abolished the chemotactic function of CXCL12 [15]. Another example of the employment of RNAi technology for the study of chemotaxis is the study of lysophosphatidylcholine (LPC), found to be important for T cell chemotaxis [16]. The receptor for LPC located

on the T cell surface is called G protein-coupled receptor 132 (G2A). Knockdown of the expression of G2A by siRNA has been shown to decrease T cell chemotaxis toward LPC in vitro [17]. RNAi technology has also been used to study other important chemotaxis molecules, such as CXCR4 (chemokine (C-X-C motif) receptor 4) [18]. However, most of the current RNAi research into chemotaxis dissects the chemokine-induced responses in lymphocytes using cell culture systems. Translating these studies into in vivo models would be an important research highlight in the future.

Senescence and Apoptosis of Lymphocytes

The study of lymphocyte apoptosis (programmed cell death) by RNAi technology is an active research field. For example, RNAi technology has been applied to target several molecules involved in cellular apoptotic pathways, such as the extrinsic and intrinsic apoptotic pathways. The molecules Cat D (cathepsin D), Bax (BCL2-associated X protein) and AIF (apoptosis-inducing factor) are important for the early phase of lymphocyte commitment to the intrinsic apoptotic pathway. Silencing of any of these molecules has been shown to increase lymphocyte survival [19]. Molecules that are important for the extrinsic apoptotic pathway have also been explored using siRNA technology. For example, spleen tyrosine kinase (Syk) is an important molecule involved in the tumor necrosis factor (TNF) signal pathway. The activation of Syk by TNF signaling in the lymphocytes can lead to an increased level of nuclear factor(NF)- κ B activity and balance the pro-apoptotic effect of TNF stimulation, thus resulting in resistance to apoptotic cell death. Silencing the Syk molecule by siRNA de-

creased NF-kappaB signal pathway activity and resulted in increased apoptotic cell death evoked by the TNF signal [20]. siRNA can also be used to overcome immune evasion mechanisms of the pathogens or tumor cells related to lymphocyte apoptosis. Some tumor cells can express Fas ligand (TNF receptor superfamily, member 6) which can activate the extrinsic apoptosis pathways in CTLs, thus evading CTL killing. Silencing Fas, the receptor for Fas ligand in CTLs, by siRNA has been shown to render the CTLs resistant to apoptosis induced by Fas ligand-expressing cancer cells [21]. This manipulation may be able to break the immune evasion of tumor cells and enhance the effect of the CTL-mediated anti-tumor effect. In general, it is believed that, if we can utilize RNAi technology to prolong the life of lymphocytes or increase the number of memory lymphocytes, we may be able to enhance vaccination potency or immunity against infection and cancers. Several key pro-apoptotic molecules involved in the extrinsic as well as the intrinsic apoptotic pathway can potentially serve as targets for siRNA intervention. Figure 3 summarizes these molecules within the apoptotic pathways.

Interaction of Lymphocytes with Lymphotropic Pathogens

siRNA has been used to study the interaction of lymphocytes with lymphotropic pathogens. Lymphocytes possess natural mechanisms to protect themselves from attack by pathogens, which are especially important in certain disease processes, such as HIV infection. For example, a molecule called APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G) is present in CD4+ T lymphocytes and can function as a deoxycytidine deaminase. It has been shown that APOBEC3G can inhibit HIV replication by inducing extensive mutations in the reverse transcription process of the virus. siRNA knockdown of this molecule will relieve the blockage of HIV replication in T lymphocytes, leading to enhanced HIV replication. This data confirmed the molecule's role as a natural anti-HIV protection mechanism [22]. Another molecule called Murr1 (copper metabolism domain containing 1), which is also expressed in the CD4+ T lymphocytes, has been shown to be able to inhibit NF-kappaB activity, which is important for HIV replication. Silencing this molecule with siRNA will lead to increased HIV replication [23]. These RNAi studies indicated the importance of the natural defense mechanisms of T lymphocytes against HIV infection.

There are molecules in the T lymphocytes which have been shown to be utilized by HIV and human T-lymphotropic virus 1 (HTLV-1) to facilitate their replication. Silencing of these host molecules by siRNA has been shown to limit virus replication and alleviate the concern of viral resistance. For example, deoxyhypusine synthase (DHS) may serve as a good target for RNAi therapy. Inhibition of DHS by siRNA has been shown to suppress hypusine formation, resulting in decreased activation of eukaryotic initiation factor 5A (eIF-5A). Be-

cause eIF-5A is a cellular cofactor of the HIV-1 Rev regulatory protein which is important for HIV replication, DHS silencing by RNAi could reduce virus replication [24]. Silencing of the HIV receptor by siRNA in T lymphocytes, such as CXCR4 or CCR5, may also help to control the spread of HIV among T cells and other immune cells, such as macrophages and dendritic cells (DCs). Knockdown of CXCR4 or CCR5 on T cells has generated significant protection against HIV infection in vitro [25, 26].

Interaction of T cells with HTLV-1 has also been studied using siRNA technology. The HTLV-1 viral protein Tax has been shown to form a complex with cellular proteins, such as check point kinase 2 (Chk2), in the nucleus of the infected cell, and this Tax/Chk2-associated complex has been speculated to be related to suppressed DNA damage repair and cell cycle abnormality, thus contributing to the carcinogenic ability of HTLV-1. siRNA silencing of Chk2 decreased the cell cycle abnormality (accumulation in G2/M). This study confirmed the significant role of Chk2 in the T cell interaction with HTLV-1 [27].

Thus, the employment of RNAi technology has revealed many important mechanisms related to interactions of lymphocytes with lymphotropic pathogens. New understanding of these molecular mechanisms provides opportunities for therapeutic intervention.

Limitations for RNAi Technology

Although RNAi technology has been widely used for the study of cellular function, several limitations must be addressed. These limitations include delivery of siRNA to specific types of cells and tissues in vivo, nonspecific suppression responses and off-target effects.

Delivery of siRNA to Specific Types of Cells and Tissues in vivo

In order to illustrate the function of cells in vivo using RNAi technology, it is important to specifically target siRNA to specific types of cells and/or tissues. Several methods have been developed for siRNA delivery to cells in vitro, such as DNA vector, dsRNA or viral vector. However, mature lymphocytes are among the hardest targets for efficient RNA interference [28]. A viral vector using the lentivirus has been applied to deliver siRNA to lymphocytes [21], and the efficiency of transfection of the siRNA can be improved with the electroporation method [29]. These methods are performed using an in vitro culture system. It is clear that extensive research is needed before we can deliver siRNA efficiently, specifically and safely into animals or humans.

On their way to the target cells/tissues in vivo, the siRNAs or the vectors carrying them will encounter a series of challenges. First, they need to be delivered specifically to the targeted cells/tissues without being degraded extracellularly. Second,

the siRNA or the vector needs to cross the plasma membrane of the targeted cells/tissues. Third, the RNA material must be processed correctly to become active siRNA to achieve gene silencing effects. Fourth, the duration of the silencing should be optimized. Significant progress has been made to address some of these issues, particular in other fields (non-lymphocyte) of RNAi research. For example, chemical modification of the siRNAs can enhance their in vivo stability and prevent serum degradation [30]. siRNAs can be coupled with fusogenic peptides or linked to antibodies against cell surface receptor ligands in order to target specific cell types/tissues [3]. Co-immunization of animals with both DNA vaccines and siRNA has been successfully tested using the gene gun method targeting the epidermal DCs [31, 32]. Using a viral vector, such as the lentiviral vector mentioned above, may achieve persistent siRNA production and lengthen the duration of RNAi silencing [33–36].

Specifically targeting the immune system, particularly lymphocytes, in vivo is particularly challenging. Currently, there have been only very few in vivo lymphocyte RNAi studies. One way of solving this problem is to do ex vivo engineering of the lymphocytes and transfer them into the organism. For example, Gimeno et al. [4] performed ex vivo silencing of p53 in CD34+ cell and observed their in vivo differentiation into T cells and other leukocytes. In another example, Lovett-Racke et al. [7] silenced T-bet in autoreactive T cells in vivo. These T cells originally caused experimental autoimmune encephalitis (EAE) when transferred into mice, because of strong Th1-mediated autoimmune responses. As mentioned above, T-bet is important for Th1 phenotype development. Silencing of T-bet in autoreactive T lymphocytes generated a decrease of Th1 phenotype (reduced INF- γ secretion). After adoptive transfer of the autoreactive T cells into mice, they showed that tail vein injection of siRNA targeting T-bet were able to prevent the induction of EAE in these mice. Continuous development of more effective delivery method for siRNA is important for future clinical translation.

Nonspecific Effects

Mammalian cells are capable of inducing an interferon response against dsRNA. The interferon response is a natural defense mechanism against viruses. The response includes nonspecific RNA degradation and translation inhibition, and it promotes very significant changes in the physiology of the cell and the organism, as well as changes in the immune system. siRNAs can also induce this kind of response, which is a serious concern when applying this technology for studying cell biology [37]. Another related nonspecific response is the activation of the Toll-like receptor (TLR)-3 or TLR-7 signal pathways by siRNA. This may cause sequence-independent gene suppression and lead to immune activation, especially in DCs [38, 39]. Nonspecifically activated DCs may potentially

affect lymphocyte function. Preventing these responses is of particular importance to researchers studying the immune system. siRNA length is an important consideration when trying to avoid the interferon response [3], and the siRNA sequence may also be an important factor to consider [40].

Usage of viral vectors to deliver siRNA into the cell raises additional concerns. Their introduction may alter the target cell's physiology, especially in cells of the immune system. For example, lentiviruses are a common gene transduction tool and are widely used in RNAi studies; however, an example of DC research pointed out that lentiviruses can affect the immunological function of transduced DCs [41]. Interestingly, this alteration may also be corrected using RNAi [41].

In summary, controlling nonspecific responses is important for the accurate interpretation of the results derived from RNAi experiments. Sensitive detection and effective prevention of these nonspecific immune responses are very important for RNAi to be widely applied in the future, especially in the clinical arena.

Off-Target Effects

Although RNAi is a highly specific phenomenon, expression of genes other than the target gene may still be affected due to sequence similarity [42]. This unintended outcome has been termed off-target effects. It has been proposed that in some cases, non-target genes with up to 3–4 base mismatches can still be silenced. GU wobble base pairing is even more likely to be tolerated by the cellular RNAi machinery [43], which may not be as easily appreciable as the IFN response or other nonspecific responses, except when a critical gene is affected and immediate results are produced [44].

Therefore, RNAi drugs or vaccines should be carefully designed in order to assure that their effect is specific. Usage of modified nucleic acids to synthesize siRNA has been shown to be able to enhance specificity [30]. Computer algorithms and software has also been developed to predict possible off-target effects and to optimize siRNA design [45, 46]. Despite these efforts, possible off-target effects should still be considered as a potential confounding factor when interpreting the results of an RNAi experiment.

Conclusion

RNA interference is gradually evolving from a powerful laboratory tool utilized to elucidate the function of novel genes, to a potential therapeutic modality in the diverse armamentarium of immune therapy. Currently, most of the studies of lymphocytes using RNAi technology are mainly employing in vitro culture systems. Confirmation of the modification of lymphocyte function by RNAi in animal models is an important next step. This will require efficient delivery and targeting

and avoidance of nonspecific or off-target effects. As we begin to more clearly understand the mechanisms by which RNAi regulates gene expression, we can hope to overcome the many obstacles that currently exist in this system and exploit this powerful tool as an adjunct in future multi-modality immunotherapies.

Acknowledgements

We thank Mr. Bruno Macaes for the preparation of the manuscript and Dr. Richard Roden for his critical review.

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