REVIEW ARTICLE

Advances in siRNA delivery to T-cells: potential clinical applications for inflammatory disease, cancer and infection

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The specificity of RNAi and its ability to silence ‘undruggable’ targets has made inhibition of gene expression in T-cells with siRNAs an attractive potential therapeutic strategy for the treatment of inflammatory disease, cancer and infection. However, delivery of siRNAs into primary T-cells represents a major hurdle to their use as potential therapeutic agents. Recent advances in siRNA delivery through the use of electroporation/nucleofection, viral vectors, peptides/proteins, nanoparticles, aptamers and other agents have now enabled efficient gene silencing in primary T-cells both *in vitro* and *in vivo*. Overcoming such barriers in siRNA delivery offers exciting new prospects for directly targeting T-cells systemically with siRNAs, or adoptively transferring T-cells back

into patients following *ex vivo* manipulation with siRNAs. In the present review, we outline the challenges in delivering siRNAs into primary T-cells and discuss the mechanism and therapeutic opportunities of each delivery method. We emphasize studies that have exploited RNAi-mediated gene silencing in T-cells for the treatment of inflammatory disease, cancer and infection using mouse models. We also discuss the potential therapeutic benefits of manipulating T-cells using siRNAs for the treatment of human diseases.

Key words: gene silencing, knockdown, RNAi, siRNA, T-cell, therapeutic.

INTRODUCTION

The discovery of RNAi- and siRNA-mediated silencing of gene expression has revolutionized basic biomedical science and translational medicine, ranging from loss-of-function studies in the laboratory setting to the exciting prospect of therapeutically silencing disease-causing or disease-associated genes in the clinic [1]. RNAi-mediated gene silencing also holds great promise for the manipulation of T-cells to address basic immunological questions and for potential therapeutic applications. T-cells are an essential component of the adaptive immune response and mediate continual surveillance against pathogens, micro-organisms and tumours [2]. At the same time, research over the last few decades has clearly implicated T-cells in the pathogenesis of many autoinflammatory/autoimmune diseases, allergies, graft-versus-host disease and organ transplant rejection [3–6]. Hence designing strategies that block pro-inflammatory T-cells and/or activate Tregs (regulatory T-cells) in the context of inflammatory disease is an active area of basic and clinical research. Indeed, many of the therapies that are currently used in the clinic for the treatment of immunological disorders directly inhibit T-cell activation or trafficking [7,8]. In contrast with inflammatory diseases and organ transplantation where suppression of pro-inflammatory T-cells is desired, harnessing of the pro-inflammatory T-cell immune response has been shown to be efficacious for the treatment of many types of cancer, including metastatic melanoma and chronic lymphocytic leukaemia [9–11]. Furthermore, directly targeting pro-survival pathways in malignant T-cells will have therapeutic benefits for the treatment of T-cell leukaemias and lymphomas

[12,13]. T-cells are also susceptible to viral infections such as HIV, and blocking viral entry and replication are current targets of anti-HIV therapy in the clinic [14].

Although manipulating T-cell function has been shown to be efficacious for inflammatory disorders, cancers and viral infections, many of these therapies have significant side effects and toxicities. It is well known, for example, that susceptibility to opportunistic infections is common in patients receiving immunosuppressive therapies, including those that target T-cells [7,8,15]. With regard to T-cell-based cancer immunotherapies, the number of patients that respond to such treatments needs to be improved greatly [10], yet clinical trials have reported that some patients who do respond to therapy experience autoimmunity or severe inflammatory reactions [9,10,15,16]. Furthermore, while anti-retroviral drugs effectively control HIV progression, such treatments require strict adherence to life-long medications that have significant side effects. Most importantly, a cure is still not available [14,17]. It is clear, therefore, that new and more refined therapies that modulate T-cell function are urgently needed.

Silencing of gene expression in T-cells using siRNAs is considered an attractive therapeutic strategy for the treatment of inflammatory disease, cancer and infection, because in principle it is now possible to silence any gene product implicated in such conditions. For example, silencing the expression of a key component in a signalling pathway that blocks the activation and/or trafficking of pro-inflammatory T-cells could be exploited as a treatment for inflammatory diseases. In contrast, silencing of an inhibitory signal could be used to augment the activity of inflammatory T-cells for the treatment of cancer. Finally, inhibiting viral entry or

Abbreviations used: CAR, chimaeric antigen receptor; EAE, experimental autoimmune encephalomyelitis; HJV-E, haemagglutinating virus of Japan envelope protein; IL, interleukin; I-tsNP, integrin-targeted stabilized nanoparticle; JAK, Janus kinase; LFA-1, lymphocyte function-associated antigen 1; PAMAM, poly(amidoamine); PBMC, peripheral blood mononuclear cell; PKC, protein kinase C; PKR, protein kinase R; PTD-DRBD, peptide transduction domain-dsRNA-binding domain; RIG-1, retinoic acid-inducible gene-1; SOCS, suppressor of cytokine signalling; SWNT, single-walled carbon nanotube; T-ALL, T-cell lymphoblastic leukaemia; TAR, TAT-activated RNA; TAT, transactivator of transcription; TCR, T-cell receptor; TLR, Toll-like receptor; Treg, regulatory T-cell; VSV-G, vesicular stomatitis virus glycoprotein; ZAP-70, ζ -chain (TCR)-associated protein kinase of 70 kDa.

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Table 1 The challenges of delivering siRNAs into T-cells using *in vivo* or *in vitro/ex vivo* targeting methods

<i>In vivo</i> siRNA delivery	<i>In vitro/ex vivo</i> siRNA delivery
Requirement for a T-cell-targeting moiety	–
Degradation of siRNAs in the blood by nucleases	–
Filtration by the kidneys	–
Delivery of siRNA across the cell membrane (size/charge of siRNA)	Delivery of siRNA across the plasma membrane (size/charge of siRNA): T-cells are 'hard to transfect' <i>in vitro</i>
siRNA stability inside the cell and duration of gene silencing	siRNA stability inside the cell and duration of gene silencing
Off-target effects of siRNAs	Off-target effects of siRNAs
Stimulation of the innate immune system and T-cells	Stimulation of the innate immune system and T-cells
–	Methods used to deliver siRNAs into T-cells <i>in vitro</i> may lead to excessive cell death, may require pre-activation of T-cells and may result in low transfection efficiency

replication in T-cells using siRNAs could also be used as a possible treatment for HIV infection. A potential advantage of siRNAs is that the specificity of RNAi-mediated gene silencing may avoid many of the side effects and toxicities that have been reported with conventional therapies. RNAi also offers the prospect of silencing so-called 'undruggable' targets, i.e. those that do not have a ligand-binding domain or enzymatic function. Despite these exciting prospects, the exploitation of RNAi for therapeutic purposes has been hampered by the fact that: (i) siRNAs are not readily taken up into cells because of their large size (~14 kDa) and negative charge; (iii) siRNAs have poor bioavailability and are generally unstable *in vivo*; (iii) localized or targeted delivery requires that siRNAs are complexed with moieties that permit selective uptake into certain cells or tissues; and (iv) siRNAs may have off-target effects and/or stimulate the innate immune system [18–20]. Off-target effects are defined as silencing of genes other than the intended target and usually occur because of homology between nucleotides located in the 'seed region' of the siRNAs with 3' UTRs of mRNA [20]. Recognition of siRNAs by cells of the innate immune system through TLRs (Toll-like receptors) 3, 7 and 8 [and possible recognition by RIG-1 (retinoic acid-inducible gene-1) and PKR (protein kinase R; or dsRNA-dependent protein kinase)], can result in production of type 1 interferons and cytokines, leading to systemic inflammation and/or non-specific down-regulation of gene expression [21,22]. Very recent studies have also shown that entrapment of siRNAs within endosomes is a limiting factor for efficient gene silencing [23,24], which also emphasizes the challenges of exploiting cell-surface receptors and receptor-mediated endocytosis for siRNA delivery.

THE CHALLENGES OF DELIVERING siRNAs INTO T-CELLS

An obvious route for targeting T-cells with siRNAs is via injection into the bloodstream. This route of systemic administration has a number of challenges (Table 1), many of which have already been outlined above. These include the need to conjugate the siRNAs with a T-cell-targeting moiety that selectively delivers them to this cell type *in vivo* and transports them across the plasma membrane. Whereas the majority of T-cell immunotherapies currently on the market are monoclonal antibodies that bind cell-surface receptors and therefore do not need to be cell-permeant to exert their therapeutic effects [7,8], siRNAs must be delivered into the cytoplasm of the cell for gene silencing to occur (or to the nucleus in the case of shRNAs) [19]. As mentioned already, siRNAs do not readily cross the plasma membrane of cells because of their size and negative charge. The requirement to protect siRNAs from degradation in the bloodstream by nucleases and to minimize filtration by the kidneys is also a significant challenge for targeting T-cells with siRNAs *in vivo* [25]. It is important to note that T-cells,

like their innate immune cell counterparts, express TLRs 3, 7 and 8 (and other RNA-sensing machinery such as RIG-1 and PKR) [26,27] and therefore have the potential to trigger inflammatory responses following siRNA delivery.

An alternative method that could potentially be used for siRNA delivery into T-cells for therapeutic purposes is an *ex vivo* route, whereby T-cells could be isolated from a patient, manipulated with siRNAs and infused back into the same patient. Re-infusion of autologous human T-cells that have been genetically modified *ex vivo* to redirect them towards tumour antigens [by expression of tumour-specific TCRs (T-cell receptors) or CARs (chimaeric antigen receptors)] has shown very promising results in clinical trials for the treatment of cancer [10]. In addition, the isolation of tumour-infiltrating T-cells, followed by their expansion *ex vivo* and re-infusion back into patients is clinically approved for the treatment of metastatic melanoma and is known as adoptive T-cell therapy [9]. Hence manipulation of T-cells *ex vivo* and their infusion back into patients is well characterized and may provide a potential therapeutic strategy for the treatment of immunological disorders, cancer and infectious disease with siRNAs.

Delivering siRNAs into purified T-cell populations using an *ex vivo* route mitigates the need for conjugation with a T-cell-targeting moiety. In addition, degradation of siRNAs by nucleases in the bloodstream and filtration by the kidneys is not a concern. Nonetheless, silencing of gene expression in T-cells with siRNAs in the laboratory has also presented a number of significant challenges, therefore making *ex vivo* delivery challenging (Table 1). It has long been known that primary T-cells isolated from blood are 'hard to transfect' *in vitro* [28–31]; conventional lipid/polymer-based transfection methods that effectively deliver nucleic acids into adherent cells are generally ineffective for primary T-cells [32–34]. Alternative methods of siRNA delivery for *in vitro* and *ex vivo* applications, such as electroporation or viral vectors, may also have drawbacks, including low transfection efficiency, loss of cell viability, the requirement for T-cell activation, transient gene silencing and safety concerns. Complicating this issue further is that the RNAi machinery in T-cells appears to be inefficient in comparison with other cell types [35].

New and exciting research over the last 10 years, however, has resulted in a number of highly innovative strategies for the delivery of siRNAs into primary T-cells using *in vivo* or *in vitro* targeting strategies. In the present paper we review these technological advances in siRNA delivery to T-cells and outline the mechanism and therapeutic opportunities of each method. We emphasize studies that have exploited RNAi-mediated gene silencing in T-cells for the treatment of inflammatory disease, cancer and infection using mouse models. We also highlight a pilot clinical trial where autologous human T-cells were manipulated *ex vivo* with siRNAs in combination with other RNA-based

inhibitory strategies and infused back into HIV-infected patients as a potential therapeutic strategy. Finally, we discuss the possible benefits of manipulating human T-cells with siRNAs for the treatment of human disease.

METHODS FOR siRNA DELIVERY INTO T-CELLS

Electroporation and nucleofection

Mechanism

Electroporation is a process whereby high voltages applied to cells induce transient permeabilization of the plasma membrane, allowing delivery of small molecules such as plasmids, siRNAs or antibodies into the cells [36]. A common problem with electroporation, however, is loss of cell viability, as the high voltages required for transfection and the longer resealing time of the pores can result in cell stress and/or cell death [36]. The first report of siRNA-mediated gene silencing in primary T-cells using electroporation in 2002 demonstrated effective gene silencing of CD4 or CD8, albeit that silencing was observed in stimulated T-cells only, and significant losses in cell viability were observed after 72 h [37]. Since that time, electroporation conditions have now been optimized for delivery of siRNAs into primary T-cells (including non-stimulated cells) that result in efficient gene silencing [38–40]. Nucleofection® is an electroporation-based procedure that was launched by the company Amaxa (now Lonza) in 2002. Nucleofection consists of a proprietary device, cell-type-specific solutions and pre-set programmes for the delivery of siRNAs and plasmids into cells, including hard-to-transfect cell types [41]. Nucleofection is now one of the most commonly used methods for delivery of siRNAs into primary T-cells and has led the way in basic research in helping to elucidate the signalling pathways that regulate T-cell function.

Electroporation and nucleofection are most commonly used to deliver ~ 21-mer synthetic siRNAs into primary T-cells for gene silencing studies. Because synthetic siRNAs do not integrate into the host genome, gene silencing is transient and will be lost upon cell division due to dilution of the siRNAs between daughter cells or because of degradation in the cell. The transient nature of gene silencing with synthetic siRNAs may be overcome by expressing siRNAs/shRNAs in T-cells using plasmids or siRNA expression cassettes, which are assumed to lead to constitutive expression of these nucleotides when delivered by electroporation/nucleofection [42–48]. To the best of our knowledge, however, there have been no studies that have directly compared the longevity of gene silencing in T-cells using synthetic siRNAs with those that are expressed from plasmids/expression cassettes. Expression of siRNAs/shRNAs from plasmids and expression cassettes have the added advantage of permitting enrichment of the transfected cell population by incorporation of selectable markers on the plasmids [44] or via co-expression of cell-surface markers that can be purified with antibody-coated beads [43].

Conditions for effective gene silencing in primary T-cells using electroporation and nucleofection have been investigated by a number of groups using synthetic siRNAs. Gene silencing appears to be more efficient if T-cells are activated through the TCR either alone or in combination with CD28 before electroporation/nucleofection [37,42,49,50], which correlates with studies that have reported higher levels of gene expression in activated primary T-cells using plasmids compared with non-activated T-cells [28,30,32]. The reason why RNAi-mediated gene silencing is more efficient in activated T-cells is not fully understood and may not be simply explained by increased siRNA

uptake into these cells. In this regard, Gust et al. [49] reported that nucleofection of non-activated and activated primary murine T-cells with siRNAs targeting the tyrosine kinase ZAP-70 [ζ -chain (TCR)-associated protein kinase of 70 kDa] resulted in mRNA silencing in both T-cell populations, whereas depletion of ZAP-70 protein was only observed in the activated T-cell population. This suggests that gene silencing may be more efficient in activated T-cells because of increased protein turnover in these cells, although this will need to be investigated further. Elsewhere, Mantei et al. [50] reported that nucleofection was highly effective for delivery of siRNAs into primary T-cells, but that gene silencing was short-lived, particularly when the cells were stimulated post-nucleofection. Interestingly, the loss of siRNA-mediated gene silencing was not due to dilution of the siRNA upon stimulation and cell division, but was attributed to the altered metabolic state of the activated T-cells. The authors also reported that chemical modification of the siRNAs, including 2'-deoxy or 2'-methoxy modifications, enhanced their stability and resulted in prolonged gene silencing in comparison with conventional siRNAs following nucleofection into T-cells [50].

Therapeutic opportunities

The use of nucleofection for gene silencing in T-cells now extends beyond basic experimental research, as very recent studies have shown that silencing of signalling components in murine T-cells, followed by adoptive transfer of the T-cells into recipient mice attenuates inflammatory disease and allergy. For example, knockdown of the tyrosine kinase ZAP-70 in murine T-cells via nucleofection with siRNAs, followed by adoptive transfer of these cells into recipient mice suppresses delayed type hypersensitivity (a Th1-mediated inflammatory response) [49]. ZAP-70 is a key component of TCR-induced signalling cascades and thus its silencing is expected to perturb T-cell activation. In other studies, Moriawaki et al. [51] demonstrated that silencing of SOCS3 (suppressor of cytokine signalling 3) expression in murine T-cells via nucleofection attenuated allergic airway responses when these cells were adoptively transferred into recipient mice. Increasing the potency of T-cells via silencing of inhibitory pathways using nucleofection may also hold promise for cancer immunotherapies. Nucleofection of siRNAs targeting the adenosine receptors A_{2A}R and A_{2B}R into anti-tumour murine CD8⁺ T-cells reduced lung metastasis and improved overall survival when these modified T-cells were adoptively transferred into tumour-bearing mice [52]. In another study, silencing of Cbl-b expression in primary murine CD8⁺ T-cells with siRNAs via nucleofection, followed by adoptive transfer of the cells into recipient mice, was shown to potentiate the effects of an anti-cancer vaccine [53]. These studies in mice suggest that silencing of gene expression in autologous primary human T-cells via electroporation/nucleofection and their adoptive transfer back into patients may have therapeutic potential for the treatment of inflammatory disease or cancer. Furthermore, because chemically modified siRNAs have been shown to prolong gene silencing in murine T-cells when adoptively transferred into recipient mice [50], such siRNAs may be more suitable than conventional siRNAs for adoptive T-cell therapy regimes in humans where prolonged gene silencing is desired.

Challenges

Challenges of electroporation and nucleofection include the aforementioned loss of cell viability as a result of the voltages required to transport the siRNAs into the cell. In relation to this point, Ward and colleagues reported that nucleofection of naïve primary human T-cells with siRNAs followed by

cellular stimulation resulted in high levels of cell death [54]. Furthermore, we and others have found that the recommended nucleofection programmes for activated primary human T-cells resulted in significant losses in cell viability and therefore required re-optimization of new programmes and protocols for nucleic acid delivery into these cells [54a,55]. In terms of the nucleofection system, both the nucleofection solutions and pre-set delivery programmes are proprietary knowledge, which limits the users' ability to develop alternative delivery conditions. Nucleofection and electroporation of primary T-cells also generally require >10-fold higher concentrations of siRNA for effective gene silencing compared with lipid-based silencing protocols for adherent cells [37,41,49,51,53,54], which in addition to the nucleofection reagents makes this delivery method quite expensive when performed on a routine basis or when performing siRNA screens [56]. The high concentrations of siRNA required for gene silencing in primary T-cells are probably due to the fact that: (i) the RNAi machinery works inefficiently in T-cells in comparison with other cell types [35]; (ii) the transient nature of gene silencing using synthetic siRNAs in combination with nucleofection/electroporation; and (iii) limitations of the nucleofection/electroporation process itself.

Accell siRNAs

Mechanism

Accell siRNAs are sold by Thermo Scientific Dharmacon as chemically modified synthetic siRNAs that enter cells without the need for a transfection or delivery agent. The chemical modification that promotes the uptake of these siRNAs is proprietary knowledge. Accell siRNAs are marketed for gene silencing studies in 'hard-to-transfect' cells, including primary T-cells and neurons. Several research groups have now used Accell siRNAs for silencing of gene expression in primary T-cells *in vitro* and demonstrated satisfactory levels of gene knockdown [57–61].

Therapeutic opportunities

An interesting study by Mascaro and colleagues [62] recently demonstrated the efficiency and duration of gene silencing in primary human T-cells using Accell siRNAs and their potential clinical applications for the manipulation of T-cell function. In this study, the authors reported that a fluorescently labelled Accell siRNA was efficiently delivered into 90% of primary human CD3⁺ T-cell blasts, while Accell siRNAs targeting JAK (Janus kinase) 1 or JAK3 resulted in efficient gene silencing for up to 10 days *in vitro* and inhibited T-cell activation. Silencing JAK3 expression in antigen-specific murine T-cells using Accell siRNAs followed by adoptive transfer of the cells into recipient mice also suppressed Th1-mediated inflammatory responses [62].

Challenges

The use of Accell siRNAs for gene silencing in T-cells does have a number of limitations, such as the relatively small numbers of cells [e.g. $(0.2\text{--}0.4) \times 10^6$ T-cells] and the high concentration of siRNAs (e.g. 1 μM) required for gene silencing. Since Accell siRNAs are synthetic in nature, their use will also result in transient gene silencing. Gene silencing with Accell siRNAs requires that cells must be incubated with these agents under serum-free or low-serum conditions for at least 48 h, as serum inhibits their delivery. In addition, because Accell siRNAs do not have a cell-targeting moiety, this precludes their use for directly targeting T-cells *in vivo*.

Viral vectors

Mechanism

The ability of viruses to infect (transduce) and replicate in mammalian cell types has been exploited for the manipulation of T-cell function and immune responses. These include clinical trials where autologous T-cells have been infused back into cancer patients following transduction with genes encoding tumour-specific TCRs/CARs *ex vivo* [9–11,63]. Viral vectors have also been used to deliver siRNAs into T-cells in the form of expression cassettes that produce shRNAs and induce silencing of gene expression. Advantages of using virus-based delivery methods, particularly those derived from retroviruses and lentiviruses, are that these vectors integrate into the host genome and thus the gene/shRNA is stably expressed for the lifetime of the cell [64–66]. Continual advances have been made in viral packaging and viral vector design to ensure that infectious viral particles are not produced after the virus enters the cell and integrates into the host genome. Improvements have also been made to alter the natural cell tropism of the viruses by substituting the viral envelope protein with envelope protein from a different virus (a process known as pseudotyping), thereby expanding the range of cell types that can be transduced [64–66]. Viral vectors often encode a reporter gene such as GFP or a selectable marker so that the transduced cell population can be tracked or enriched.

Retroviral vectors

Therapeutic opportunities. Retroviruses have been widely used for expression of shRNAs in primary T-cells to address basic immunological questions and, more recently, for potential clinical applications. In terms of the latter, silencing of signalling components in murine T-cells using retroviral shRNAs, followed by adoptive cell transfer into recipient mice, suppresses inflammatory disease [67,68] and allergy [69,70]. For infectious disease, Epstein–Barr virus-specific murine T-cells expressing an shRNA that renders the cells resistant to the actions of an immunosuppressive drug maintain their cellular functionality and provide effective immunity when adoptively transferred into recipient mice [71]. This strategy may have therapeutic potential for patients who have undergone organ transplantation, where suppression of the patient's immune system is required to prevent graft rejection yet specific anti-viral immunity is desired. For potential cancer immunotherapies, depletion of the tyrosine phosphatase SHP-1 (Src homology 2 domain-containing protein tyrosine phosphatase 1), a negative regulator of T-cell signalling, in tumour antigen-specific murine T-cells and their adoptive transfer into tumour-bearing mice results in increased T-cell expansion *in vivo* [72]. In other studies, a retroviral vector encoding an shRNA targeting the endogenous TCR in combination with a codon-optimized shRNA-resistant TCR specific for tumour antigens may also have potential for cancer immunotherapy [73–76]. Transduction of primary T-cells with these retroviral vectors resulted in expression of the tumour-specific TCR, reduced expression of the endogenous TCR, and enhanced antigen-specific lysis of target/tumour cells both *in vitro* and when adoptively transferred into tumour-bearing mice [73–76]. Silencing endogenous TCR expression may be important for cancer immunotherapies where T-cells have been redirected to express tumour-specific TCRs, because of the possibility that the introduced tumour-specific TCR α/β chains may mis-pair with the endogenous TCR α/β chains, resulting in new TCRs and possible autoimmunity. In this regard, a previous study reported lethal autoimmunity in mice as a result of mis-pairing of the endogenous

and introduced TCR chains following adoptive transfer of the transduced T-cells into recipient mice [77]. It should be noted, however, that there have been no reports of TCR mis-pairing in human clinical trials where patients received autologous T-cells transduced with tumour-specific TCRs [78], so whether silencing endogenous TCRs with retroviral vectors will be beneficial for cancer immunotherapy awaits future studies.

Challenges. A limitation of using retroviral vectors for delivery of siRNAs or other genetic material into primary T-cells is that integration of the virus into the host genome is dependent on T-cell activation and proliferation, as retroviral entry into the nucleus requires breakdown of the nuclear envelope during mitosis [31,63]. Because *in vitro* T-cell activation results in a host of phenotypic and genomic alterations, this may limit their use for certain therapeutic applications [79]. Immune responses to retroviral vector epitopes have also been reported in some human patients receiving autologous T-cells transduced with CARs for cancer immunotherapy [80]. The use of retroviral vectors for therapeutic purposes also has serious safety concerns, as demonstrated by the incidences of haematological malignancies, including T-ALL (T-cell lymphoblastic leukaemia), after patients enrolled in clinical trials received autologous haemopoietic progenitor cells transduced with retroviral vectors to restore expression of a defective gene (reviewed in [64]). Analysis of patients who developed T-ALL in two of these clinical trials revealed that the retroviral vector integrated near proto-oncogenes (enhancing its transcription), in addition to chromosomal translocations, copy number changes and deletion of tumour suppressor genes being found [81–83]. Hence, the normal process of T-cell development was perturbed as a consequence of retroviral integration into the genome of the haemopoietic progenitor cells and was a major setback to the gene therapy field. Transduction of mature T-cells with retroviral vectors, however, appears to be safer in comparison with haemopoietic progenitor cells, as studies in mice [84–86] and in human subjects (including a very recent report of three decade-long clinical trials incorporating >500 years of patient follow-up) [87–89] found no evidence that retroviral vector-mediated gene transfer into mature T-cells resulted in integration-induced immortalization and malignant transformation. A mechanistic basis for why retroviral vector-mediated gene transfer into T-cells appears to be safer in comparison with haemopoietic progenitor cells was proposed by Biasco et al. [90], who reported that the integration profile of the vector is distinct in these two cell types and is influenced by the genetic/chromatin state of the cell. These studies demonstrating safety of retroviral vectors may help allay fears over the use of these vectors for therapeutic purposes in T-cells, including gene-silencing approaches.

Lentiviral vectors

Lentiviral vectors have also been exploited for delivery of shRNAs and genes into T-cells. Lentiviruses are a subclass of the retrovirus family, with the most widely used vectors derived from HIV [65]. Historically, lentiviral vectors have been pseudotyped with VSV-G (vesicular stomatitis virus glycoprotein), due to the restricted tropism of the HIV envelope protein for the CD4 molecule (which is only expressed on helper T-cells, monocytes/macrophages and some cells of the central nervous system [91]) and because of greater stability [65]. Until recently, primary T-cells required cellular activation for efficient transduction with VSV-G-pseudotyped lentiviral vectors [79], but it is now known that cytokines such as IL (interleukin)-7, IL-15 or IL-21 (or specific combinations thereof) promote VSV-G-pseudotyped lentiviral

uptake into resting T-cells without the need for T-cell stimulation, thus preserving the functionality of naïve T-cells [79,92,93]. IL-7 has therefore been used for transduction of primary T-cells with lentiviral vectors for expression of shRNAs [93,94].

Therapeutic opportunities. The clinical potential of exploiting lentivirus-mediated gene silencing in T-cells for the treatment of inflammatory diseases or cancer has been demonstrated by adoptive transfer studies in mice. Knockdown of the CD28 receptor, a key co-stimulatory molecule for T-cell activation, in murine splenocytes suppressed graft-versus-host disease following adoptive transfer into recipient mice [95]. In contrast, silencing the expression of an inhibitory receptor on Tregs using a lentiviral vector, to increase their immunosuppressive function, has been demonstrated to delay the onset of Type 1 diabetes when adoptively transferred into non-obese diabetic mice [96]. In cancer immunotherapy studies performed in tumour-bearing mice, knockdown of SOCS1 in anti-tumour CD8⁺ T-cells [97] or STAT3 in CD4⁺ T-cells [98], using lentiviral vectors promotes tumour regression following adoptive transfer. Another area where lentiviral vectors have been widely used for silencing of gene expression in T-cells is in the context of HIV infection. HIV encodes nine viral genes, infects T-cells and is the causative factor of AIDS [91]. HIV entry is mediated via the viral envelope protein which binds the CD4 molecule expressed on host cells, while host chemokine receptors such as CXCR4 and CCR5 also serve as co-receptors for viral entry at later stages. Once inside the cell, replication and integration of HIV into the host genome is dependent on both viral and host cellular factors. Silencing of these viral and host cell factors in T-cells using siRNAs or shRNAs has been shown to inhibit HIV infection and replication *in vitro* [91]. Because HIV is prone to viral escape when a single siRNA/shRNA is used to target the virus, lentiviral vectors have been developed that multiplex shRNAs in various formats in a single cassette [99,100]. Lentiviral vectors that express an shRNA in combination with other RNA-based inhibitors such as a ribozyme and a decoy for TAR [TAT (transactivator of transcription)-activated RNA] on the same vector have also been developed as a potential therapy for HIV [99,101]. The use of a single shRNA in combination with other RNA-based inhibitors may avoid toxicity associated with oversaturation of the RNAi machinery [102]. The clinical potential of exploiting siRNA-mediated gene silencing in T-cells for therapeutic purposes was recently assessed in a Phase 0 human clinical trial (<http://ClinicalTrials.gov> Identifier NCT01153646). This study was carried out to assess the safety and study feasibility of infusing autologous CD4⁺ T-cells transduced with a lentiviral vector encoding three RNA-based inhibitors (including an shRNA targeting the viral *tat/rev* genes in combination with a TAR decoy and ribozyme) back into HIV-infected patients who had failed highly active anti-retroviral therapy. The study was initiated in June 2010 but was terminated recently for reasons that have yet to be disclosed.

Advances in lentiviral vector design have led to significant improvements for expression of shRNAs in T-cells, including the choice of promoter employed to drive the expression of the shRNA [103,104]. In addition, lentiviral vectors that inducibly express an shRNA in response to a drug or following infection with HIV have also been used in T-cells [105–107]. Other improvements include conditions that enhance lentiviral transduction of T-cells, including resting T-cells. For example, inhibition of the proteasome with the small-molecule inhibitor MG132 increases lentiviral transduction of T-cells when stimulated with anti-CD3 and IL-2 [108], whereas a lentiviral vector pseudotyped with a modified RD114 envelope glycoprotein results in enhanced

transduction of primary human T-cells in comparison with VSV-G-pseudotyped lentiviral vectors [109]. Finally, pseudotyping lentiviral vectors with measles virus glycoproteins [110] or the wild-type HIV envelope protein [111] efficiently transduces primary T-cells without the requirement for any exogenous stimulation such as cytokines. It should be noted though that these advances described above have only been used for lentiviral gene delivery to primary T-cells and have not yet been exploited for delivery of shRNAs. Such new methods may permit more efficient gene silencing in T-cells and/or maintain the cells in their native state.

In vivo delivery of lentiviral vectors encoding shRNAs may also have clinical applications for the manipulation of T-cell immune responses. For example, Lee et al. [112] demonstrated that the intra-tracheal application of a lentiviral vector encoding an shRNA targeting GATA-3, a key transcription factor for Th2 differentiation, blocked ovalbumin-induced airway hyperresponsiveness, inflammation and Th2 cytokine release in a mouse model of asthma. Exploitation of this *in vivo* shRNA delivery strategy for therapeutic purposes, however, would have major safety concerns, because of the absence of a cell-targeting moiety on the virus. Lentiviral vectors that display antibodies on their surface have shown efficacy for targeting specific cell types *in vivo* (including an OKT-3 antibody that targets the TCR) and may therefore have potential for *in vivo* delivery of shRNAs to T-cells [113,114].

Challenges. Like retroviral vectors, a key issue is whether the use of lentiviral vectors for therapeutic purposes in T-cells will lead to malignant transformation. Although no safety data were disclosed for the Phase 0 clinical trial using autologous CD4⁺ T-cells transduced with lentiviral vectors discussed above, a long-term follow-up study of another clinical trial was very recently published and reported no evidence of clonal selection or integration near oncogenes when autologous CD4⁺ T-cells were re-infused back into HIV patients following transduction with a lentiviral vector encoding a long antisense molecule to the HIV envelope protein [115]. This study, in addition to the fact that HIV infection has not been associated with T-cell leukaemias in patients [63], suggests that the use of lentiviral vectors for the manipulation of T-cell immune responses is safe. However, as discussed elsewhere [63], the safety profile of every viral vector that encodes a gene or shRNA will need to be assessed on a case-by-case basis.

Peptides, proteins and protein transduction domains

Mechanism

Peptides and proteins that penetrate the plasma membrane of cells have been exploited for effective siRNA delivery into primary T-cells. These include the TAT peptide derived from HIV, and cell-penetrating peptides such as poly-arginine which efficiently crosses the plasma membrane despite its large size [116].

Therapeutic opportunities

Dowdy and colleagues took advantage of the cell-penetrating properties of the TAT peptide to generate a PTD–DRBD (peptide transduction domain–dsRNA-binding domain) fusion protein that binds siRNAs with high affinity, masks the negative charge of the siRNA, and efficiently transports these siRNAs into a variety of cell types, including primary T-cells [33]. Incubation of activated primary murine T-cells with PTD–DRBDs and siRNAs targeting CD4 or CD8 *in vitro* resulted in gene silencing of these receptors [33]. Other studies have reported gene silencing in

the Jurkat leukaemic T-cell line and PBMCs (peripheral blood mononuclear cells) *in vitro* using mimics of PTDs based on oligo-9-arginine and Pep cell-permeant peptides [117] or the TP10 cell-penetrating peptide [118]. Because PTD–DRBDs and cell-penetrating peptides lack a cell-targeting moiety, their use will be restricted for *ex vivo* delivery of siRNAs to T-cells. To the best of our knowledge, these peptide transporters have not yet been used for adoptive transfer of T-cells into recipient mice.

Other nucleic-acid-binding proteins and peptides have been conjugated with antibodies specific for cell-surface molecules, enabling targeted siRNA delivery to leucocytes and T-cells both *in vitro* and *in vivo*. Song et al. [119] exploited the nucleic-acid-binding properties of protamine in combination with a cell-targeting antibody fragment for delivery of siRNAs specific for the HIV *gag* gene into infected cells. Because the antibody recognizes the HIV envelope protein on the surface of infected cells, siRNA delivery was restricted to infected cells only. This antibody–protamine complex inhibited HIV replication in primary human CD4⁺ T-cells *in vitro* and efficiently targeted cells that expressed the HIV envelope protein *in vivo* when injected into mice [119]. Kumar et al. [120] utilized a similar strategy for *in vivo* delivery of siRNAs into T-cells for the treatment of HIV infection in mice. In their study, a CD7-specific single-chain antibody that selectively targets T-cells was conjugated to the cell-permeable oligo-9-arginine peptide and used to deliver siRNAs specific for the CCR5 chemokine receptor (required for HIV entry) in a humanized mouse model. Systemic delivery of this complex via intravenous injection resulted in silencing of CCR5 in the T-cell population and blocking of HIV infection. Interestingly, it was also demonstrated that a cocktail of siRNAs targeting both the CCR5 receptor and HIV genes was more potent as an anti-viral therapy compared with the single siRNA targeting CCR5 alone [120]. Similar antibody-conjugated peptide delivery systems have been employed to deliver siRNAs into tumour T-cells used to inoculate mice, albeit that the percentage of tumour T-cells that were targeted *in vivo* was low [121].

Antibody-conjugated proteins may also have therapeutic potential for targeted delivery of siRNAs to activated T-cells and leucocytes *in vivo* for the treatment of inflammatory disease. Peer et al. [122] used this system to selectively deliver siRNAs into activated leucocytes, including T-cells, by conjugating protamine with a single-chain antibody that recognizes the high-affinity conformation of the leucocyte-specific LFA-1 (lymphocyte function-associated antigen 1) integrin. In T-cells, LFA-1 is converted into the high-affinity conformation following TCR or chemokine stimulation, and regulates interaction with antigen-presenting cells and cellular migration [123,124]. Intravenous injection of the antibody–protamine complex into mice resulted in siRNA delivery to cells expressing high-affinity LFA-1, whereas non-activated leucocytes expressing the low-affinity form of LFA-1 were not targeted [122]. Specifically targeting activated leucocyte populations may be beneficial in the context of chronic inflammatory diseases and could avoid global immunosuppression that is commonly observed with other therapies. These studies demonstrate that conjugation of peptide and protein transporters with cell-targeting antibodies as vehicles for siRNA delivery may have therapeutic applications for combating HIV infection [119,120], cancer [121] and inflammatory disease [122].

Certain cell-surface receptors are differentially expressed on distinct T-cell populations, which may facilitate delivery of siRNAs into particular subsets of T-cells using antibodies. For example, gene microarrays have demonstrated that surface receptors, such as CD103 and CD81, are more highly expressed on Tregs compared with pro-inflammatory T-cells [125,126],

whereas the expression of CCR1 and CXCR4 chemokine receptors are higher on Th2 cells compared with Th1 cells [127]. Gene silencing may therefore be feasible in a particular subset of Tregs or pro-inflammatory T-cells. Peptide and protein transporters could also be coated with a pan-T-cell-targeting antibody (e.g. CD7) for silencing of a gene target that is differentially required for effector T-cells and Tregs. For example, PKC (protein kinase C) θ is a serine/threonine kinase that positively regulates the production of cytokines in pro-inflammatory T-cells, whereas it negatively regulates the suppressive activity of Tregs [128]. Silencing of PKC θ is therefore considered an attractive therapeutic target for the treatment of inflammatory disorders and autoimmune disease, because inhibiting this kinase should block the pro-inflammatory actions of T-cells and at the same time enhance the suppressive activity of Tregs. Peptide and protein transporters conjugated with a CD7-specific antibody may be an ideal delivery system for silencing of PKC θ expression in this context.

Challenges

A drawback of the peptide and protein delivery systems described above, however, is that, on a per molar ratio, the siRNA-binding capacity of these agents is relatively low and thus the 'payload' of siRNA delivered to cells is limited [129]. PTD-DRBDs bind siRNAs at a 4:1 ratio only [33], whereas the protamine-antibody fusion molecule binds five or six siRNA molecules [119,122]. Furthermore, it was reported that oligo-9-arginine peptides conjugated with an antibody against CD7 bound siRNA at a ratio of 1:2 [120], while optimum siRNA delivery into tumour T-cells using antibody-conjugated oligo-9-arginine peptides was observed at an 8:1 ratio of antibody/peptide relative to siRNA [121]. A potential concern of using antibody-conjugated systems for siRNA delivery to T-cells is that antibody engagement of the surface receptor may lead to cellular activation, which would not be beneficial in the context of an inflammatory disease. There have been no reports to date, however, that antibody-siRNA complexes cause T-cell activation [34,122]. The use of single-chain antibodies for siRNA delivery will probably be advantageous in this context, as these antibodies are not expected to cross-link (and thus activate) surface receptors [122]. Antibody-coated delivery systems that target integrins on T-cells and leucocytes will also have to be carefully considered in light of the well-documented cases of progressive multifocal leukoencephalopathy in patients receiving monoclonal antibody immunotherapies that block LFA-1 and other integrins [130]. The judicious use of single-chain antibodies and/or antibodies that recognize only the high-affinity conformation of integrins may be advantageous in this setting. Furthermore, although these peptide and protein transporters have demonstrated therapeutic potential in murine studies, the applicability of these delivery systems for the treatment of human diseases will require 'humanization' of the mouse antibodies on the surface of the particles to avoid recognition by the host and the generation of an immune response.

Nanoparticles

Mechanism

Nanoparticles are typically defined as having a dimension of 100 nm or less, although larger molecules are often referred to as nanoparticles. Nanoparticles are effective vehicles for siRNA delivery, as their small size enables them to pass through the plasma membrane without the use of carrier agents [131].

Therapeutic opportunities

Liu et al. [132] investigated SWNTs (single-walled carbon nanotubes) for delivery of siRNAs into human T-cell lines and PBMCs *in vitro*. These SWNTs were 1–3 nm in diameter and 200 nm in length, functionalized to make them water-soluble and modified with cleavable disulfide bonds for binding to siRNAs [132]. Silencing of the CXCR4 chemokine receptor and CD4 was reported when functionalized SWNTs were mixed with siRNAs specific to these gene targets and incubated with the cells [132]. Elsewhere, Yosef et al. [133] very recently used vertical silicon nanowires for delivery of siRNAs into primary naïve T-cells and demonstrated effective gene silencing of 34 gene targets at the mRNA level. In this system, silicon nanowires are first complexed with siRNAs, and impaling the cells on the nanowires results in siRNA delivery into the cytosol [134]. Chitosan nanoparticles that have a diameter of 320 nm have also been evaluated for siRNA delivery to T-cells [135]. Chitosan is a derivative of the natural polysaccharide chitin and was chemically conjugated to a T-cell-targeting CD7-specific single-chain antibody. Silencing of CD4 expression was observed when the Jurkat leukaemic T-cell line was incubated with these particles *in vitro*, although no results were reported with primary T-cells [135]. SWNTs, vertical silicon nanowires and chitosan nanoparticles have only been used to address basic biological questions in the laboratory setting to date and so their potential therapeutic applications in T-cells have not yet been evaluated.

Dendrimers are highly branched synthetic polymers that bind siRNAs via positively charged functional groups and are cell-permeant [131]. Carbosilane dendrimers (300–370 nm diameter) or PAMAM [poly(amidoamine)] dendrimers (100 nm diameter) have been complexed with siRNAs targeting HIV genes and shown to inhibit viral replication in T-cell lines and PBMCs *in vitro* [136,137]. Interestingly, intravenous injection of PAMAM dendrimer-siRNA complexes into a humanized mouse model of HIV also suppressed viral loads and protected against HIV-mediated T-cell depletion [137]. Although the PAMAM dendrimer-siRNA complexes in this study did not have a cell-targeting moiety on their surface, they primarily accumulated in PBMCs and the livers of mice following intravenous injection, with little or no accumulation in the spleen, lung or kidney. Incorporation of a T-cell and/or macrophage-targeting moiety (e.g. with an antibody or antibody fragment) may refine the delivery of siRNAs specifically to these cell types and avoid accumulation in the liver. Antibody-conjugated PAMAM dendrimers have recently been exploited for targeted siRNA delivery and shown to result in enhanced uptake and more potent gene silencing in a prostate cancer cell line [138], which suggests that antibody-conjugated dendrimers should be explored for T-cell-specific siRNA delivery.

The most innovative and effective siRNA delivery system described to date for targeting leucocytes and T-cells *in vivo* are I-tsNPs (integrin-targeted stabilized nanoparticles) [34,139]. In this system, siRNAs are condensed with the nucleic-acid-binding protein protamine and subsequently encapsulated within 100 nm diameter neutral liposome nanoparticles, resulting in a ~4000:1 ratio of siRNAs to nanoparticles. Attached to the surface of the liposome nanoparticle is the glycosaminoglycan hyaluronan, which stabilizes the particle, protects the siRNAs from degradation and serves as a scaffold on to which cell-targeting antibodies can be attached. Shimaoka and colleagues coated I-tsNPs with an antibody specific for the $\beta 7$ integrin for delivery of siRNAs targeting cyclin D1 into gut-infiltrating leucocytes in a mouse model of colitis [139]. The expression of the $\beta 7$ integrin is up-regulated on activated leucocytes and is

required for the trafficking of these cells into the gastrointestinal tract [140]. Remarkably, intravenous injection of a single dose of $\beta 7$ -targeted I-tsNPs into mice resulted in silencing of cyclin D1 expression in leucocytes from the gut and spleen, while leucocyte proliferation and inflammatory Th1 cytokines were also inhibited. Furthermore, these I-tsNPs suppressed leucocyte infiltration into the colon and led to a drastic reduction in intestinal damage [139]. I-tsNPs conjugated with an antibody targeting the LFA-1 integrin were also previously used to deliver siRNAs specific for CCR5 into leucocytes *in vivo*, resulting in silencing of CCR5 expression and prevention of HIV infection in mice [34]. I-tsNPs may have great potential for the manipulation of human T-cell function *in vivo* due to their capacity to encapsulate large amounts of siRNAs and thereby increase the payload of siRNAs into cells.

Challenges

The use of nanoparticles for delivery of siRNAs into T-cells for therapeutic purposes will need to be evaluated for toxicity and effects on cellular function, particularly nanoparticles that accumulate inside cells such as SWCNTs. The safety profile of dendrimers will also need to be assessed in terms of their potential toxicity and the possibility of generating an immune response if used for systemic delivery of siRNAs into T-cells. As we have already highlighted above, delivery agents that are conjugated to integrin-targeting antibodies such as I-tsNPs will also need to be carefully assessed for progressive multifocal leukoencephalopathy. Issues of whether I-tsNPs can be 'scaled-up' for therapeutic purposes in humans have been discussed elsewhere [141].

Aptamers

Mechanism

Aptamers are single-stranded RNA or DNA oligonucleotides selected from random sequence pools that demonstrate high affinity and specificity towards target molecules, including surface receptors [142]. Some receptor-targeting aptamers are internalized into cells, which has resulted in these molecules being exploited for siRNA delivery by producing aptamer–siRNA chimaeras via *in vitro* transcription. In 2006 it was first reported that an aptamer–siRNA chimaera (in which the aptamer portion of the chimaera recognizes a cell-surface receptor) was internalized into prostate cancer cells and resulted in gene silencing [143,144]. Since that time, aptamer–siRNA chimaeras have also been exploited for siRNA delivery into T-cells, primarily in the context of HIV infection and replication.

Therapeutic opportunities

Zhou et al. [145] demonstrated that aptamers specific for the HIV envelope protein (expressed on the surface of infected T-cells) delivered siRNAs targeting viral and/or host genes into a T-cell line and PBMCs *in vitro* and blocked viral replication. The same group demonstrated in a separate study that intravenous injection of these aptamer–siRNA chimaeras into a humanized mouse model of HIV infection silenced viral gene expression in the PBMC population, protected against HIV-mediated CD4⁺ T-cell depletion and suppressed viral loads [146]. Interestingly, the aptamer portion of the chimaera that targets the HIV envelope protein also demonstrated anti-viral activity, albeit that it was not as potent as that of the aptamer–siRNA chimaera [145,146]. This

aptamer therefore serves a dual role: delivery of siRNA into cells by binding a cell-surface molecule and acting as a neutralizing agent against the virus by blocking the interaction of the envelope protein with CD4 or chemokine receptors. Aptamers targeting the CD4 receptor were also recently used to deliver siRNAs into T-cells and prevent HIV infection in human cervicovaginal explants and humanized mice [147]. Delivery of a triple cocktail of siRNAs specific for *vav/gag* viral genes and the host chemokine receptor CCR5 inhibited HIV infection more potently compared with siRNAs targeting CCR5 alone. Again, the aptamer specific for CD4 exhibited some anti-viral activity on its own, but was less effective in comparison with the aptamer–siRNA chimaeras [147].

Developments in aptamer technology have refined their function. For example, aptamers have been synthesized with a bridge sequence that facilitates the non-covalent binding and interchange of various siRNAs with the same aptamer, permitting delivery of siRNA cocktails into T-cells [148]. Rossi and colleagues also investigated the use of chimaeric RNA molecules consisting of pRNA (packaging RNA) derived from the bacteriophage $\phi 29$ DNA-packaging motor in tandem with aptamers for delivery of siRNAs targeting HIV into PBMCs [149]. These chimaeric molecules provided cell-type-specific delivery in combination with HIV inhibitory activity and were modified to prolong RNA stability in serum [149]. Other developments in aptamer function include the use of DNA aptamers that specifically target CD4⁺ or CD8⁺ T-cells for siRNA delivery, albeit these have only been evaluated *in vitro* to date [150,151]. DNA aptamers that target T-cells appear to be more stable in serum than their RNA counterparts and result in enhanced uptake into T-cell lines *in vitro* [150].

Aptamers appear to have many advantages for siRNA delivery into T-cells, including their small size and applications for targeted delivery *in vivo*. In addition, whereas aptamer–siRNA chimaeras are mainly produced by transcription, they can now also be chemically synthesized. Aptamer–siRNA chimaeras are less likely to be immunogenic and are cheaper to produce than other cell-targeting delivery agents such as proteins or antibodies [147]. Wheeler et al. [147] also reported that an aptamer targeting the CD4 molecule on T-cells did not affect the surface levels of CD4, suggesting that uptake of the aptamer–siRNA chimaera into T-cells could be mediated as part of the continuous endocytosis of the CD4 receptor. Furthermore, the chimaera did not affect the expression of other cell-surface markers that are indicative of T-cell activation, which demonstrates that this particular aptamer is not stimulatory [147]. There have been no reports to date of aptamers being used for siRNA delivery into T-cells for the treatment of inflammatory diseases or cancer in animal models so it will be interesting to see whether this delivery system can also be exploited for these diseases. In principle, because aptamers can be generated against any molecule, this opens up the possibility of delivering siRNAs into specific T-cell subsets by targeting cell-surface receptors, including integrins.

Challenges

The selection and characterization of aptamers that target a specific molecule or cell-surface receptor requires time and effort. As the majority of aptamers that are currently used for delivery of siRNAs are composed of RNA, their stability *in vivo* remains a key issue, although steps to increase their stability have been addressed [142]. The use of aptamers for gene-silencing approaches is still in its infancy and their toxicity, immunogenicity and safety profile needs to be further evaluated.

Other delivery agents

A variety of other delivery agents have been used for RNAi-mediated gene silencing in T-cells, with some of these demonstrating potent functional effects following adoptive transfer of the T-cells into recipient mice, or when administered systemically. Yamamura and colleagues [152] utilized the commercially available HJV-E (haemagglutinating virus of Japan envelope protein) for delivery of siRNAs targeting the NR4A2 nuclear orphan receptor into primary T-cells *in vitro*. NR4A2 expression is up-regulated in peripheral blood T-cells of multiple sclerosis patients and in T-cells infiltrating the central nervous system of mice with active disease, thereby making it an attractive therapeutic target for silencing. Adoptive transfer of T-cells that were treated with siRNAs targeting NR4A2 into recipient mice inhibited EAE (experimental autoimmune encephalomyelitis; a mouse model of multiple sclerosis), whereas active disease occurred following adoptive transfer of T-cells treated with control siRNAs [152]. The same group recently reported that incorporation of NR4A2-specific siRNAs into a commercially available AteloGene™ collagen complex that protects siRNAs from degradation *in vivo* reduced Th17-mediated inflammatory responses and delayed the onset of EAE in mice when administered systemically [153].

Biragyn et al. [154] recently generated a novel siRNA delivery system for silencing of gene expression in Tregs both *in vitro* and when administered systemically to mice *in vivo*. Here, the authors created a chimaeric molecule composed of the CCL17 chemokine linked to the 15-amino-acid capsid antigen fragment of hepatitis B virus that binds siRNAs and delivers the complex into cells via the CCR4 receptor (the cognate receptor of CCL17). Silencing of IL-10 or FoxP3 (forkhead box P3) expression in CCR4-expressing Tregs was observed *in vitro* and also *in vivo* when administered systemically to mice [154]. Furthermore, silencing Tregs in this context inhibited metastasis of breast cancer cells to the lung and was associated with increased IFN (interferon)- γ production by CD8⁺ cytotoxic T-cells in the mice. This therapeutic strategy of augmenting the pro-inflammatory response for the treatment of cancer by silencing Treg activity complements alternative siRNA-based strategies of silencing inhibitory signalling pathways in pro-inflammatory T-cells [53,72,98]. Other agents that have been used to deliver siRNAs into primary T-cells *in vitro* include chemically synthesized polymers [155,156], whereas antibody-coated cyclodextran polymers have been used for *in vivo* delivery of siRNAs into primary T-cells and B-cells in mice [157]. Elsewhere, exosomes are 40–100 nm vesicles derived from cells that can be loaded with siRNAs and used for delivery into cells, including primary T- and B-cells *in vitro* [158].

Although T-cells are well known as being 'hard to transfect', a search of the literature reveals that many commercially available lipid-based transfection reagents have been successfully used for siRNA delivery and gene silencing in primary T-cells. For example, Lipofectamine™ [159], HiPerFect™ [160], DharmaFECT™ [161], TransIT-TKO [162], siPORT™ [163], siTran™ [164], Metafectene™ [165] and lipid siRNA transfection reagent from Santa Cruz Biotechnology [166] have all been shown to result in gene silencing in primary T-cells. Furthermore, transfection of T-cells with siRNAs using lipid-based reagents followed by adoptive transfer of the cells into recipient mice inhibits inflammatory/autoimmune disease [167–169], graft rejection [170,171] and infectious disease [172]. Perhaps this 'hard-to-transfect' reputation has been misappropriated to primary T-cells because of the difficulty in delivering plasmids/DNA into the nucleus of these cells. In addition, although it is generally accepted that siRNAs are unstable *in vivo* due to degradation by nucleases, intravenous

delivery of 'naked' siRNAs targeting T-bet (a Th1-specific transcription factor) via tail vein injection of mice results in silencing of T-bet expression and suppresses EAE [167,173]. A similar strategy of intraperitoneal injection of naked siRNAs targeting GATA-3 (a Th2 transcription factor) in mice has been reported to inhibit the proliferation of cancer cells *in vivo* [174]. These studies highlight novel methods for siRNA delivery into T-cells and also challenge the long-held belief that T-cells are hard to transfect.

MANIPULATION OF T-CELL FUNCTION WITH siRNAs: TRANSLATION TO THE CLINIC?

Silencing of gene expression using siRNAs is now one of the most commonly used approaches for characterization of genes in T-cells to address basic biological questions. Such approaches have also permitted analysis of gene function in mature T-cells where generation of gene-deficient mice results in embryonic lethality [175] or where T-cells from gene-deficient mice have failed to reveal a clear phenotype, possibly because of compensatory mechanisms [176]. RNAi-mediated gene silencing has also permitted 'knockdown' mice to be generated that are depleted of proteins exclusively expressed in T-cells such as CTLA-4 (cytotoxic T-lymphocyte antigen 4) [177,178]. A major question in the RNAi and immunology fields, however, is whether siRNA technology can be exploited for therapeutic purposes in T-cells for the treatment of human diseases. Without doubt, the major obstacle that has faced immunologists, clinicians and the RNAi field alike has been the delivery of siRNAs to T-cells both *in vitro* and *in vivo*. As we have outlined in the present review, great progress has now been made in enabling siRNA delivery into primary T-cells both *in vitro* and *in vivo*. Advances in siRNA delivery to T-cells using a variety of methods, including electroporation/nucleofection, Accell siRNAs, viral vectors, peptides/proteins, nanoparticles, aptamers and others, has equipped researchers with a valuable array of agents to modulate gene expression (summarized in Figure 1). The specificity of RNAi-mediated gene silencing, in addition to the realistic possibility of targeting particular subsets of T-cells (i.e. with ligands that target T-cells such as antibodies, aptamers or chemokines), holds great promise for the development of more refined therapies for modulating T-cell function in the context of disease. The applicability of RNAi-mediated gene silencing for the manipulation of T-cells has also taken a step nearer to the clinic by studies which have shown that targeting murine T-cells with siRNAs *in vivo*, or adoptive transfer of RNAi-modified murine T-cells into recipient mice, is efficacious in models of inflammation, cancer and infectious disease. Although a human clinical trial using an RNAi-based strategy for blocking HIV infection in CD4⁺ T-cells was recently terminated for reasons that have not been disclosed, the applicability of RNAi-mediated gene silencing is perhaps finally realizing its therapeutic potential for the manipulation of T-cell and other immune cell-related diseases. A similar clinical trial has been completed where five patients with HIV were infused with autologous haemopoietic progenitor cells transduced with a lentiviral vector encoding three RNA-based inhibitors, including an shRNA targeting the *tat/rev* viral genes [179]. Successful engraftment of the transduced haemopoietic cell population was observed in four out of five patients and two of these patients demonstrated detectable expression of the shRNA and the ribozyme for up to 24 months post-infusion [179]. Now that the barrier of siRNA delivery to T-cells appears to be surmounted, it is anticipated that more clinical trials involving the use of siRNAs for the manipulation of T-cell function will be evaluated in the future. Although there are numerous clinical

Figure 1 Summary of the methods used for delivering siRNAs into T-cells

The applicability of these agents for targeting T-cells *in vivo* or *ex vivo* with siRNAs is indicated. The diagram also indicates whether these delivery agents have been used in mouse models and the relevant references are indicated in square brackets. Not depicted in the diagram are other delivery agents including HIV-E, AteloGene collagen complexes, chimaeric molecules of chemokines linked to the capsid antigen fragment of hepatitis B virus, chemically synthesized polymers, antibody-coated cyclodextran polymers, exosomes, conventional lipid-based transfection methods and 'naked' siRNA delivery into mice.

trials currently evaluating the use of siRNAs for therapeutic purposes, progress in this area has been mixed and some of these trials have been terminated due to lack of a therapeutic benefit [180]. Furthermore, an siRNA has yet to be clinically approved for the treatment of any human disease. Although RNAi holds great potential for the manipulation of T-cell function, many questions remain to be addressed. For example, targeting T-cells systemically or *ex vivo* with siRNAs will dictate the type of delivery agent that is used for gene silencing. Unless viral vectors are used, these delivery agents will result in transient gene silencing, and this poses the question of how often the siRNAs will need to be administered to achieve therapeutic gene silencing. As discussed above, increasing the stability of siRNAs may be beneficial for achieving prolonged gene silencing in this regard. The biological safety of the delivery agent used to transport the siRNA into T-cells, particularly viral vectors, will carry an inherent risk because of their ability to integrate into the host genome, as will antibody-conjugated delivery systems that target integrins and other receptors on the surface of T-cells. Other questions that need to be addressed are whether targeting T-cells with siRNAs for the treatment of inflammatory or autoimmune disease will place patients at increased risk of opportunistic infections like many of the current therapies. On the other hand, whether targeting T-cells with siRNAs to silence inhibitory signals for cancer immunotherapy will lead to inflammatory or autoimmune-like diseases is unknown at present. Although adoptive transfer of murine T-cells treated with siRNAs

into recipient mice has been shown to be efficacious for many inflammatory and autoimmune diseases, the vast majority of these studies have adoptively transferred the modified T-cells before the induction or onset of inflammatory disease. Whether adoptively transferring the T-cells in the context of an active inflammatory disease will need to be investigated and will be more reflective of the human clinical situation.

Other questions that will need to be evaluated will be whether the siRNAs cause silencing of non-intended targets (off-target silencing) and/or whether siRNAs and their delivery agents non-specifically stimulate the immune system. To date, there is limited information regarding both of these fundamental issues. Off-target effects may have unpredictable and potentially delirious effects on T-cell function, whereas recognition of siRNAs by TLRs (and possibly by RIG-I and PKR) in innate immune cells and in T-cells themselves may trigger an inflammatory response in the host [21,22,26,27]. Off-target effects of siRNAs have rarely been addressed by any of the delivery methods that we have highlighted in the present review, and this will be a crucial factor that must be investigated further if this technology is to move nearer towards the clinic. It is important to note, however, that many of these studies have investigated whether the various siRNA delivery systems for targeting T-cells are immunostimulatory in nature. To date, no adverse inflammatory events have been reported when siRNAs complexed with PTD-DRBDs [33], antibody-conjugated peptide/proteins [120,122], nanoparticles [34,137] or aptamers [145-148] were incubated with PBMCs *in vitro* or when

delivered systemically into mice for the manipulation of T-cell function. Determining the potential immunostimulatory potential of siRNAs and their delivery agent is critical, as it is known that the method of delivery influences whether siRNAs are detected by the RNA-sensing machinery [181]. For example, delivery of siRNAs into PBMCs using lipid-based transfection methods (a pathway that exploits endosome-mediated uptake of siRNAs) resulted in TLR activation and inflammatory cytokine production, whereas siRNA delivery to the cytosol using electroporation did not trigger the same inflammatory reaction [181]. This is consistent with the localization of the RNA-sensing TLRs to endosomal structures of cells.

An area that will also have important applications for therapeutic gene silencing in T-cells (and for the clinical applications of siRNAs in all cells in general) is chemical modification of siRNAs to increase their stability and potency and at the same time reduce their off-target effects and their immunostimulatory potential [182]. A plethora of such modifications have now been made to the backbone, ribose sugar and base of the nucleotides in one or both siRNA strands to improve their function [182]. Chemical modifications of siRNA to improve their function have only been investigated in limited detail in T-cells, as demonstrated by Mantei et al. [50] who showed a clear benefit for chemically modifying siRNAs to increase their stability and prolong gene silencing in primary T-cells. Whether similar modifications help to reduce off-target effects or the immunostimulatory potential of the siRNAs in primary T-cells should be investigated. Nonetheless, it is of note that the vast majority of studies have employed commercially available single siRNAs or siRNA pools for silencing of gene expression in primary T-cells, and such siRNAs are now generally modified with proprietary modifications that reportedly minimize undesirable side effects, including off-targeting.

While manipulation of T-cell function with siRNAs alone may have clinical benefits for the treatment of human disease and infection, another outstanding issue is whether an enhanced therapeutic benefit will be seen with combination therapies of siRNAs with other immunomodulatory agents, e.g. siRNA-mediated gene silencing in T-cells in combination with a vaccine for cancer immunotherapy [53] or silencing of inhibitory signalling pathways in inflammatory T-cells in combination with tumour-specific TCRs/CARs to increase the potency of the cells. While combination therapies have already been evaluated in a human clinical trial for HIV infection using genetically modified T-cells, other possible combination therapies could include siRNA-modified T-cells with anti-retroviral drugs [183]. The application of siRNA screening in T-cells will also help define additional gene products that could potentially be targeted with siRNAs for therapeutic purposes. In summary, overcoming the hurdles of siRNA delivery to T-cells now offers the exciting prospect of silencing gene expression in these cells for a variety of potential clinical applications.

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REFERENCES

- Davidson, B. L. and McCray, Jr, P. B. (2011) Current prospects for RNA interference-based therapies. *Nat. Rev. Genet.* **12**, 329–340
- Larosa, D. F. and Orange, J. S. (2008) Lymphocytes. *J. Allergy Clin. Immunol.* **121** (Suppl. 2), S364–S369
- Bolon, B. (2012) Cellular and molecular mechanisms of autoimmune disease. *Toxicol. Pathol.* **40**, 216–229
- Islam, S. A. and Luster, A. D. (2012) T cell homing to epithelial barriers in allergic disease. *Nat. Med.* **18**, 705–715
- Blazar, B. R., Murphy, W. J. and Abedi, M. (2012) Advances in graft-versus-host disease biology and therapy. *Nat. Rev. Immunol.* **12**, 443–458
- Wood, K. J. and Goto, R. (2012) Mechanisms of rejection: current perspectives. *Transplantation* **93**, 1–10
- Steward-Tharp, S. M., Song, Y. J., Siegel, R. M. and O'Shea, J. J. (2010) New insights into T cell biology and T cell-directed therapy for autoimmunity, inflammation, and immunosuppression. *Ann. N. Y. Acad. Sci.* **1183**, 123–148
- Getts, D. R., Shankar, S., Chastain, E. M., Martin, A., Getts, M. T., Wood, K. and Miller, S. D. (2011) Current landscape for T-cell targeting in autoimmunity and transplantation. *Immunotherapy* **3**, 853–870
- Restifo, N. P., Dudley, M. E. and Rosenberg, S. A. (2012) Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat. Rev. Immunol.* **12**, 269–281
- Park, T. S., Rosenberg, S. A. and Morgan, R. A. (2011) Treating cancer with genetically engineered T cells. *Trends Biotechnol.* **29**, 550–557
- Lizee, G., Overwijk, W. W., Radvanyi, L., Gao, J., Sharma, P. and Hwu, P. (2013) Harnessing the power of the immune system to target cancer. *Annu. Rev. Med.* **64**, 71–90
- Liu, H., Chiang, M. Y. and Pear, W. S. (2011) Critical roles of NOTCH1 in acute T-cell lymphoblastic leukemia. *Int. J. Hematol.* **94**, 118–125
- Nasr, R., El Hajj, H., Kfoury, Y., de The, H., Hermine, O. and Bazarbachi, A. (2011) Controversies in targeted therapy of adult T cell leukemia/lymphoma: ON target or OFF target effects? *Viruses* **3**, 750–769
- Levy, J. A. (2011) Virus-host interactions in HIV pathogenesis: directions for therapy. *Adv. Dent. Res.* **23**, 13–18
- Caspi, R. R. (2008) Immunotherapy of autoimmunity and cancer: the penalty for success. *Nat. Rev. Immunol.* **8**, 970–976
- Amos, S. M., Duong, C. P., Westwood, J. A., Ritchie, D. S., Junghans, R. P., Darcy, P. K. and Kershaw, M. H. (2011) Autoimmunity associated with immunotherapy of cancer. *Blood* **118**, 499–509
- Palmer, S., Josefsson, L. and Coffin, J. M. (2011) HIV reservoirs and the possibility of a cure for HIV infection. *J. Intern. Med.* **270**, 550–560
- Peer, D. and Lieberman, J. (2011) Special delivery: targeted therapy with small RNAs. *Gene Ther.* **18**, 1127–1133
- Singh, S., Narang, A. S. and Mahato, R. I. (2011) Subcellular fate and off-target effects of siRNA, shRNA, and miRNA. *Pharm. Res.* **28**, 2996–3015
- Gavrilov, K. and Saltzman, W. M. (2012) Therapeutic siRNA: principles, challenges, and strategies. *Yale J. Biol. Med.* **85**, 187–200
- Samuel-Abraham, S. and Leonard, J. N. (2010) Staying on message: design principles for controlling nonspecific responses to siRNA. *FEBS J.* **277**, 4828–4836
- Sioud, M. (2010) Recent advances in small interfering RNA sensing by the immune system. *Nat. Biotechnol.* **27**, 236–242
- Gilleron, J., Querbes, W., Zeigerer, A., Borodovsky, A., Marsico, G., Schubert, U., Manygoats, K., Seifert, S., Andree, C., Stoter, M. et al. (2013) Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. *Nat. Biotechnol.* **31**, 638–646
- Sahay, G., Querbes, W., Alabi, C., Eltoukhy, A., Sarkar, S., Zurenko, C., Karagiannis, E., Love, K., Chen, D., Zoncu, R. et al. (2013) Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling. *Nat. Biotechnol.* **31**, 653–658
- Goldsmith, M., Mizrahy, S. and Peer, D. (2011) Grand challenges in modulating the immune response with RNAi nanomedicines. *Nanomedicine* **6**, 1771–1785
- Kabelitz, D. (2007) Expression and function of Toll-like receptors in T lymphocytes. *Curr. Opin. Immunol.* **19**, 39–45
- Pettersson, T., Mansson, A., Riesbeck, K. and Cardell, L. O. (2011) Nucleotide-binding and oligomerization domain-like receptors and retinoic acid inducible gene-like receptors in human tonsillar T lymphocytes. *Immunology* **133**, 84–93
- Cron, R. Q., Schubert, L. A., Lewis, D. B. and Hughes, C. C. (1997) Consistent transient transfection of DNA into non-transformed human and murine T-lymphocytes. *J. Immunol. Methods* **205**, 145–150
- Goffinet, C. and Keppler, O. T. (2006) Efficient nonviral gene delivery into primary lymphocytes from rats and mice. *FASEB J.* **20**, 500–502
- Lai, W., Chang, C. H. and Farber, D. L. (2003) Gene transfection and expression in resting and activated murine CD4 T cell subsets. *J. Immunol. Methods* **282**, 93–102
- Hurez, V., Haulton, R. D., Oliver, J., Matthews, R. J. and Weaver, C. K. (2002) Gene delivery into primary T cells: overview and characterization of a transgenic model for efficient adenoviral transduction. *Immunol. Res.* **26**, 131–141
- Van Tendeloo, V. F., Willems, R., Ponsaerts, P., Lenjou, M., Nijs, G., Vanhove, M., Muylaert, P., Van Cauwelaert, P., Van Broeckhoven, C., Van Bockstaele, D. R. and Berneman, Z. N. (2000) High-level transgene expression in primary human T lymphocytes and adult bone marrow CD34⁺ cells via electroporation-mediated gene delivery. *Gene Ther.* **7**, 1431–1437

- 33 Eguchi, A., Meade, B. R., Chang, Y. C., Fredrickson, C. T., Willert, K., Puri, N. and Dowdy, S. F. (2009) Efficient siRNA delivery into primary cells by a peptide transduction domain-dsRNA binding domain fusion protein. *Nat. Biotechnol.* **27**, 567–571
- 34 Kim, S. S., Peer, D., Kumar, P., Subramanya, S., Wu, H., Asthana, D., Habiro, K., Yang, Y. G., Manjunath, N., Shimaoka, M. and Shankar, P. (2010) RNAi-mediated CCR5 silencing by LFA-1-targeted nanoparticles prevents HIV infection in BLT mice. *Mol. Ther.* **18**, 370–376
- 35 Oberdoerffer, P., Kanellopoulou, C., Heissmeyer, V., Paeper, C., Borowski, C., Aifantis, I., Rao, A. and Rajewsky, K. (2005) Efficiency of RNA interference in the mouse hematopoietic system varies between cell types and developmental stages. *Mol. Cell. Biol.* **25**, 3896–3905
- 36 Gehl, J. (2003) Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research. *Acta Physiol. Scand.* **177**, 437–447
- 37 McManus, M. T., Haines, B. B., Dillon, C. P., Whitehurst, C. E., van Parijs, L., Chen, J. and Sharp, P. A. (2002) Small interfering RNA-mediated gene silencing in T lymphocytes. *J. Immunol.* **169**, 5754–5760
- 38 Ovcharenko, D., Jarvis, R., Hunnicke-Smith, S., Kelnar, K. and Brown, D. (2005) High-throughput RNAi screening *in vitro*: from cell lines to primary cells. *RNA* **11**, 985–993
- 39 Kunii, N., Zhao, Y., Jiang, S., Liu, X., Scholler, J., Balagopalan, L., Samelson, L. E., Milone, M. C. and June, C. H. (2013) Enhanced function of redirected human T cells expressing linker for activation of T cells that is resistant to ubiquitylation. *Hum. Gene Ther.* **24**, 27–37
- 40 Saldanha-Araujo, F., Haddad, R., Farias, K. C., Souza Ade, P., Palma, P. V., Araujo, A. G., Orellana, M. D., Voltarelli, J. C., Covas, D. T., Zago, M. A. and Panepucci, R. A. (2012) Mesenchymal stem cells promote the sustained expression of CD69 on activated T lymphocytes: roles of canonical and non-canonical NF- κ B signalling. *J. Cell. Mol. Med.* **16**, 1232–1244
- 41 Gresch, O., Engel, F. B., Nesic, D., Tran, T. T., England, H. M., Hickman, E. S., Korner, I., Gan, L., Chen, S., Castro-Oregon, S. et al. (2004) New non-viral method for gene transfer into primary cells. *Methods* **33**, 151–163
- 42 Yin, J., Ma, Z., Selliah, N., Shivers, D. K., Cron, R. Q. and Finkel, T. H. (2006) Effective gene suppression using small interfering RNA in hard-to-transfect human T cells. *J. Immunol. Methods* **312**, 1–11
- 43 Tahvanainen, J., Pykalainen, M., Kallonen, T., Lahteenmaki, H., Rasool, O. and Lahesmaa, R. (2006) Enrichment of nucleofected primary human CD4⁺ T cells: a novel and efficient method for studying gene function and role in human primary T helper cell differentiation. *J. Immunol. Methods* **310**, 30–39
- 44 Gonzalez, S., Castanotto, D., Li, H., Olivares, S., Jensen, M. C., Forman, S. J., Rossi, J. J. and Cooper, L. J. (2005) Amplification of RNAi-targeting HLA mRNAs. *Mol. Ther.* **11**, 811–818
- 45 Zhao, M., Sun, Y., Gao, F., Wu, X., Tang, J., Yin, H., Luo, Y., Richardson, B. and Lu, Q. (2010) Epigenetics and SLE: RFX1 downregulation causes CD11a and CD70 overexpression by altering epigenetic modifications in lupus CD4⁺ T cells. *J. Autoimmun.* **35**, 58–69
- 46 Ahlfors, H., Limaye, A., Elo, L. L., Tuomela, S., Burute, M., Gottimukkala, K. V., Notani, D., Rasool, O., Galande, S. and Lahesmaa, R. (2010) SATB1 dictates expression of multiple genes including IL-5 involved in human T helper cell differentiation. *Blood* **116**, 1443–1453
- 47 Jacque, J. M., Triques, K. and Stevenson, M. (2002) Modulation of HIV-1 replication by RNA interference. *Nature* **418**, 435–438
- 48 Li, C. Y., Peng, J., Ren, L. P., Gan, L. X., Lu, X. J., Liu, Q., Gu, W. and Guo, X. J. (2013) Roles of histone hypoacetylation in LAT expression on T cells and Th2 polarization in allergic asthma. *J. Transl. Med.* **11**, 26
- 49 Gust, T. C., Neubrandt, L., Merz, C., Asadullah, K., Zugel, U. and von Bonin, A. (2008) RNA interference-mediated gene silencing in murine T cells: *in vitro* and *in vivo* validation of proinflammatory target genes. *Cell Commun. Signaling* **6**, 3
- 50 Mantei, A., Rutz, S., Janke, M., Kirchhoff, D., Jung, U., Patzel, V., Vogel, U., Rudel, T., Andreou, I., Weber, M. and Scheffold, A. (2008) siRNA stabilization prolongs gene knockdown in primary T lymphocytes. *Eur. J. Immunol.* **38**, 2616–2625
- 51 Moriwaki, A., Inoue, H., Nakano, T., Matsunaga, Y., Matsuno, Y., Matsumoto, T., Fukuyama, S., Kan, O. K., Matsumoto, K., Tsuda-Eguchi, M. et al. (2011) T cell treatment with small interfering RNA for suppressor of cytokine signaling 3 modulates allergic airway responses in a murine model of asthma. *Am. J. Respir. Cell Mol. Biol.* **44**, 448–455
- 52 Ohta, A., Gorelik, E., Prasad, S. J., Ronchese, F., Lukashev, D., Wong, M. K., Huang, X., Caldwell, S., Liu, K., Smith, P. et al. (2006) A_{2A} adenosine receptor protects tumors from antitumor T cells. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 13132–13137
- 53 Hinterleitner, R., Gruber, T., Pfeifhofer-Obermair, C., Lutz-Nicoladoni, C., Tzankov, A., Schuster, M., Penninger, J. M., Loibner, H., Lametschwandner, G., Wolf, D. and Baier, G. (2012) Adoptive transfer of siRNA Cblb-silenced CD8⁺ T lymphocytes augments tumor vaccine efficacy in a B16 melanoma model. *PLoS ONE* **7**, e44295
- 54 Smith, L. D., Hickman, E. S., Parry, R. V., Westwick, J. and Ward, S. G. (2007) PI3K γ is the dominant isoform involved in migratory responses of human T lymphocytes: effects of *ex vivo* maintenance and limitations of non-viral delivery of siRNA. *Cell. Signalling* **19**, 2528–2539
- 54a Freeley, M. and Long, A. (2013) The two-hit hypothesis: an improved method for siRNA-mediated gene silencing in stimulated primary human T cells. *J. Immunol. Methods*, in the press
- 55 Rabinovich, P. M., Komarovskaya, M. E., Ye, Z. J., Imai, C., Campana, D., Bahceci, E. and Weissman, S. M. (2006) Synthetic messenger RNA as a tool for gene therapy. *Hum. Gene Ther.* **17**, 1027–1035
- 56 Sharma, S. and Rao, A. (2009) RNAi screening: tips and techniques. *Nat. Immunol.* **10**, 799–804
- 57 Fausther-Bovendo, H., Vieillard, V., Sagan, S., Bismuth, G. and Debre, P. (2010) HIV gp41 engages gC1qR on CD4⁺ T cells to induce the expression of an NK ligand through the PIP₃/H₂O₂ pathway. *PLoS Pathog.* **6**, e1000975
- 58 Chehtane, M. and Khaled, A. R. (2010) Interleukin-7 mediates glucose utilization in lymphocytes through transcriptional regulation of the hexokinase II gene. *Am. J. Physiol. Cell Physiol.* **298**, C1560–C1571
- 59 Alvarez, R. A., Thorborn, G., Reading, J. L., Reddy, S. K. and Vyakarnam, A. (2011) WFC1 expression identifies memory CD4 T-lymphocytes rendered vulnerable to cell-cell HIV-1 transfer by promoting intercellular adhesive junctions. *Retrovirology* **8**, 29
- 60 Yang, L., Boldin, M. P., Yu, Y., Liu, C. S., Ea, C. K., Ramakrishnan, P., Taganov, K. D., Zhao, J. L. and Baltimore, D. (2012) miR-146a controls the resolution of T cell responses in mice. *J. Exp. Med.* **209**, 1655–1670
- 61 Zhang, J., Zhu, N., Wang, Q., Wang, J., Ma, Y., Qiao, C., Li, Y., Li, X., Su, B. and Shen, B. (2010) MEKK3 overexpression contributes to the hyperresponsiveness of IL-12-overproducing cells and CD4⁺ T conventional cells in nonobese diabetic mice. *J. Immunol.* **185**, 3554–3563
- 62 Gomez-Valades, A. G., Llamas, M., Blanch, S., Perales, J. C., Roman, J., Gomez-Casajus, L. and Mascaro, C. (2012) Specific Jak3 downregulation in lymphocytes impairs γ c cytokine signal transduction and alleviates antigen-driven inflammation *in vivo*. *Mol. Ther. Nucleic Acids* **1**, e42
- 63 June, C. H., Blazar, B. R. and Riley, J. L. (2009) Engineering lymphocyte subsets: tools, trials and tribulations. *Nat. Rev. Immunol.* **9**, 704–716
- 64 Maetzig, T., Galla, M., Baum, C. and Schambach, A. (2011) Gammaretroviral vectors: biology, technology and application. *Viruses* **3**, 677–713
- 65 Sakuma, T., Barry, M. A. and Ikeda, Y. (2012) Lentiviral vectors: basic to translational. *Biochem. J.* **443**, 603–618
- 66 Lambeth, L. S. and Smith, C. A. (2013) Short hairpin RNA-mediated gene silencing. *Methods Mol. Biol.* **942**, 205–232
- 67 Rangachari, M., Zhu, C., Sakuishi, K., Xiao, S., Karman, J., Chen, A., Angin, M., Wakeham, A., Greenfield, E. A., Sobel, R. A. et al. (2012) Bat3 promotes T cell responses and autoimmunity by repressing Tim-3-mediated cell death and exhaustion. *Nat. Med.* **18**, 1394–1400
- 68 Zhao, X., Zheng, B., Huang, Y., Yang, D., Katzman, S., Chang, C., Fowell, D. and Zeng, W. P. (2007) Interaction between GATA-3 and the transcriptional coregulator Pias1 is important for the regulation of Th2 immune responses. *J. Immunol.* **179**, 8297–8304
- 69 Jin, H. S., Liao, L., Park, Y. and Liu, Y. C. (2013) Neddylation pathway regulates T-cell function by targeting an adaptor protein Shc and a protein kinase Erk signaling. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 624–629
- 70 Gorska, M. M., Goplen, N., Liang, Q. and Alam, R. (2010) Uncoordinated 119 preferentially induces Th2 differentiation and promotes the development of asthma. *J. Immunol.* **184**, 4488–4496
- 71 De Angelis, B., Dotti, G., Quintarelli, C., Huye, L. E., Zhang, L., Zhang, M., Pane, F., Heslop, H. E., Brenner, M. K., Rooney, C. M. and Savoldo, B. (2009) Generation of Epstein-Barr virus-specific cytotoxic T lymphocytes resistant to the immunosuppressive drug tacrolimus (FK506). *Blood* **114**, 4784–4791
- 72 Stromnes, I. M., Fowler, C., Casamina, C. C., Georgopoulos, C. M., McAfee, M. S., Schmitt, T. M., Tan, X., Kim, T. D., Choi, I., Blattman, J. N. and Greenberg, P. D. (2012) Abrogation of SRC homology region 2 domain-containing phosphatase 1 in tumor-specific T cells improves efficacy of adoptive immunotherapy by enhancing the effector function and accumulation of short-lived effector T cells *in vivo*. *J. Immunol.* **189**, 1812–1825
- 73 Okamoto, S., Mineno, J., Ikeda, H., Fujiwara, H., Yasukawa, M., Shiku, H. and Kato, I. (2009) Improved expression and reactivity of transduced tumor-specific TCRs in human lymphocytes by specific silencing of endogenous TCR. *Cancer Res.* **69**, 9003–9011
- 74 Ochi, T., Fujiwara, H., Okamoto, S., An, J., Nagai, K., Shirakata, T., Mineno, J., Kuzushima, K., Shiku, H. and Yasukawa, M. (2011) Novel adoptive T-cell immunotherapy using a WT1-specific TCR vector encoding silencers for endogenous TCRs shows marked antileukemia reactivity and safety. *Blood* **118**, 1495–1503

- 75 Iwami, K., Natsume, A., Ohno, M., Ikeda, H., Mineno, J., Nukaya, I., Okamoto, S., Fujiwara, H., Yasukawa, M., Shiku, H. and Wakabayashi, T. (2013) Adoptive transfer of genetically modified Wilms' tumor 1-specific T cells in a novel malignant skull base meningioma model. *Neuro-Oncology* **15**, 747–758
- 76 Asai, H., Fujiwara, H., An, J., Ochi, T., Miyazaki, Y., Nagai, K., Okamoto, S., Mineno, J., Kuzushima, K., Shiku, H. et al. (2013) Co-introduced functional CCR2 potentiates *in vivo* anti-lung cancer functionality mediated by T cells double gene-modified to express WT1-specific T-cell receptor. *PLoS ONE* **8**, e56820
- 77 Bendle, G. M., Linnemann, C., Hooijkaas, A. I., Bies, L., de Witte, M. A., Jorritsma, A., Kaiser, A. D., Pouw, N., Debets, R., Kieback, E. et al. (2010) Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. *Nat. Med.* **16**, 565–570
- 78 Rosenberg, S. A. (2010) Of mice, not men: no evidence for graft-versus-host disease in humans receiving T-cell receptor-transduced autologous T cells. *Mol. Ther.* **18**, 1744–1745
- 79 Frecha, C., Levy, C., Cosset, F. L. and Verhoeven, E. (2010) Advances in the field of lentivector-based transduction of T and B lymphocytes for gene therapy. *Mol. Ther.* **18**, 1748–1757
- 80 Lamers, C. H., Willemsen, R., van Elzakker, P., van Steenbergen-Langeveld, S., Broertjes, M., Oosterwijk-Wakka, J., Oosterwijk, E., Sleijfer, S., Debets, R. and Gratama, J. W. (2011) Immune responses to transgene and retroviral vector in patients treated with *ex vivo*-engineered T cells. *Blood* **117**, 72–82
- 81 Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M. P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C. S., Pawliuk, R., Morillon, E. et al. (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**, 415–419
- 82 Hacein-Bey-Abina, S., Garrigue, A., Wang, G. P., Soulier, J., Lim, A., Morillon, E., Clappier, E., Caccavelli, L., Delabesse, E., Beldjord, K. et al. (2008) Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J. Clin. Invest.* **118**, 3132–3142
- 83 Howe, S. J., Mansour, M. R., Schwarzwaelder, K., Bartholomae, C., Hubank, M., Kempinski, H., Brugman, M. H., Pike-Overzet, K., Chatters, S. J., de Ridder, D. et al. (2008) Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J. Clin. Invest.* **118**, 3143–3150
- 84 Newrzela, S., Cornils, K., Li, Z., Baum, C., Brugman, M. H., Hartmann, M., Meyer, J., Hartmann, S., Hansmann, M. L., Fehse, B. and von Laer, D. (2008) Resistance of mature T cells to oncogene transformation. *Blood* **112**, 2278–2286
- 85 Newrzela, S., Cornils, K., Heinrich, T., Schlager, J., Yi, J. H., Lysenko, O., Kimpel, J., Fehse, B. and von Laer, D. (2011) Retroviral insertional mutagenesis can contribute to immortalization of mature T lymphocytes. *Mol. Med.* **17**, 1223–1232
- 86 Westwood, J. A., Murray, W. K., Trivett, M., Shin, A., Neeson, P., MacGregor, D. P., Haynes, N. M., Trapani, J. A., Mayura-Guru, P., Fox, S. et al. (2008) Absence of retroviral vector-mediated transformation of gene-modified T cells after long-term engraftment in mice. *Gene Ther.* **15**, 1056–1066
- 87 Scholler, J., Brady, T. L., Binder-Scholl, G., Hwang, W. T., Plesa, G., Hege, K. M., Vogel, A. N., Kalos, M., Riley, J. L., Deeks, S. G. et al. (2012) Decade-long safety and function of retroviral-modified chimeric antigen receptor T cells. *Sci. Transl. Med.* **4**, 132ra153
- 88 Recchia, A., Bonini, C., Magnani, Z., Urbini, F., Sartori, D., Muraro, S., Tagliafico, E., Bondanza, A., Stanghellini, M. T., Bernardi, M. et al. (2006) Retroviral vector integration deregulates gene expression but has no consequence on the biology and function of transplanted T cells. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 1457–1462
- 89 Cattoglio, C., Maruggi, G., Bartholomae, C., Malani, N., Pellin, D., Cocchiarella, F., Magnani, Z., Ciceri, F., Ambrosi, A., von Kalle, C. et al. (2010) High-definition mapping of retroviral integration sites defines the fate of allogeneic T cells after donor lymphocyte infusion. *PLoS ONE* **5**, e15688
- 90 Biasco, L., Ambrosi, A., Pellin, D., Bartholomae, C., Brigida, I., Roncarolo, M. G., Di Serio, C., von Kalle, C., Schmidt, M. and Aiuti, A. (2011) Integration profile of retroviral vector in gene therapy treated patients is cell-specific according to gene expression and chromatin conformation of target cell. *EMBO Mol. Med.* **3**, 89–101
- 91 Singh, S. K. (2008) RNA interference and its therapeutic potential against HIV infection. *Expert Opin. Biol. Ther.* **8**, 449–461
- 92 Perro, M., Tsang, J., Xue, S. A., Escors, D., Cesco-Gaspere, M., Pospori, C., Gao, L., Hart, D., Collins, M., Stauss, H. and Morris, E. C. (2010) Generation of multi-functional antigen-specific human T-cells by lentiviral TCR gene transfer. *Gene Ther.* **17**, 721–732
- 93 Guo, L., Martens, C., Bruno, D., Porcella, S. F., Yamane, H., Caucheteux, S. M., Zhu, J. and Paul, W. E. (2013) Lipid phosphatases identified by screening a mouse phosphatase shRNA library regulate T-cell differentiation and protein kinase B AKT signaling. *Proc. Natl. Acad. Sci. U.S.A.* **110**, E1849–E1856
- 94 Bancos, S., Cao, Q., Bowers, W. J. and Crispe, I. N. (2009) Dysfunctional memory CD8⁺ T cells after priming in the absence of the cell cycle regulator E2F4. *Cell. Immunol.* **257**, 44–54
- 95 Sang, W., Zhou, C., Cheng, N., Li, Z., Zeng, L. and Xu, K. (2011) Control of mouse graft-versus-host disease following allogeneic bone marrow transplantation by blocking the CD28/B7 signaling pathway with lentiviral vector-mediated RNA interference. *Immunol. Lett.* **136**, 194–202
- 96 Qin, H., Wang, Z., Du, W., Lee, W. H., Wu, X., Riggs, A. D. and Liu, C. P. (2011) Killer cell Ig-like receptor (KIR) 3DL1 down-regulation enhances inhibition of type 1 diabetes by autoantigen-specific regulatory T cells. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 2016–2021
- 97 Dudda, J. C., Salaun, B., Ji, Y., Palmer, D. C., Monnot, G. C., Merck, E., Boudousquie, C., Utzschneider, D. T., Escobar, T. M., Perret, R. et al. (2013) MicroRNA-155 is required for effector CD8⁺ T cell responses to virus infection and cancer. *Immunity* **38**, 742–753
- 98 Pallandre, J. R., Brillard, E., Crehange, G., Radlovic, A., Remy-Martin, J. P., Saas, P., Rohrich, P. S., Pivot, X., Ling, X., Tiberghien, P. and Borg, C. (2007) Role of STAT3 in CD4⁺ CD25⁺ FOXP3⁺ regulatory lymphocyte generation: implications in graft-versus-host disease and antitumor immunity. *J. Immunol.* **179**, 7593–7604
- 99 Manjunath, N., Wu, H., Subramanya, S. and Shankar, P. (2009) Lentiviral delivery of short hairpin RNAs. *Adv. Drug Delivery Rev.* **61**, 732–745
- 100 Berkhout, B. and Sanders, R. W. (2011) Molecular strategies to design an escape-proof antiviral therapy. *Antiviral Res.* **92**, 7–14
- 101 Burnett, J. C., Zaia, J. A. and Rossi, J. J. (2012) Creating genetic resistance to HIV. *Curr. Opin. Immunol.* **24**, 625–632
- 102 Grimm, D., Streetz, K. L., Jopling, C. L., Storm, T. A., Pandey, K., Davis, C. R., Marion, P., Salazar, F. and Kay, M. A. (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **441**, 537–541
- 103 An, D. S., Qin, F. X., Auyeung, V. C., Mao, S. H., Kung, S. K., Baltimore, D. and Chen, I. S. (2006) Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Mol. Ther.* **14**, 494–504
- 104 Lebbink, R. J., Lowe, M., Chan, T., Khine, H., Wang, X. and McManus, M. T. (2011) Polymerase II promoter strength determines efficacy of microRNA adapted shRNAs. *PLoS ONE* **6**, e26213
- 105 Unwalla, H. J., Li, M. J., Kim, J. D., Li, H. T., Ehsani, A., Alluin, J. and Rossi, J. J. (2004) Negative feedback inhibition of HIV-1 by TAT-inducible expression of siRNA. *Nat. Biotechnol.* **22**, 1573–1578
- 106 Browne, C. Y., Sadlon, T., Gargett, T., Melville, E., Zhang, R., Drabsch, Y., Ling, M., Strathdee, C. A., Gonda, T. J. and Barry, S. C. (2010) Robust, reversible gene knockdown using a single lentiviral short hairpin RNA vector. *Hum. Gene Ther.* **21**, 1005–1017
- 107 Wakim, L. M., Gupta, N., Mintern, J. D. and Villadangos, J. A. (2013) Enhanced survival of lung tissue-resident memory CD8⁺ T cells during infection with influenza virus due to selective expression of IFITM3. *Nat. Immunol.* **14**, 238–245
- 108 Leuci, V., Mesiano, G., Gammaitoni, L., Cammarata, C., Capellero, S., Todorovic, M., Jordaney, N., Circosta, P., Elia, A., Lesnikova, M. et al. (2011) Transient proteasome inhibition as a strategy to enhance lentiviral transduction of hematopoietic CD34⁺ cells and T lymphocytes: implications for the use of low viral doses and large-size vectors. *J. Biotechnol.* **156**, 218–226
- 109 Sandrin, V., Boson, B., Salmon, P., Gay, W., Negre, D., Le Grand, R., Trono, D. and Cosset, F. L. (2002) Lentiviral vectors pseudotyped with a modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary lymphocytes and CD34⁺ cells derived from human and nonhuman primates. *Blood* **100**, 823–832
- 110 Frecha, C., Costa, C., Negre, D., Gauthier, E., Russell, S. J., Cosset, F. L. and Verhoeven, E. (2008) Stable transduction of quiescent T cells without induction of cycle progression by a novel lentiviral vector pseudotyped with measles virus glycoproteins. *Blood* **112**, 4843–4852
- 111 Agosto, L. M., Yu, J. J., Liszewski, M. K., Baytop, C., Korokhov, N., Humeau, L. M. and O'Doherty, U. (2009) The CXCR4-tropic human immunodeficiency virus envelope promotes more-efficient gene delivery to resting CD4⁺ T cells than the vesicular stomatitis virus glycoprotein G envelope. *J. Virol.* **83**, 8153–8162
- 112 Lee, C. C., Huang, H. Y. and Chiang, B. L. (2008) Lentiviral-mediated GATA-3 RNAi decreases allergic airway inflammation and hyperresponsiveness. *Mol. Ther.* **16**, 60–65
- 113 Yang, H., Joo, K. I., Ziegler, L. and Wang, P. (2009) Cell type-specific targeting with surface-engineered lentiviral vectors co-displaying OKT3 antibody and fusogenic molecule. *Pharm. Res.* **26**, 1432–1445
- 114 Yang, L., Bailey, L., Baltimore, D. and Wang, P. (2006) Targeting lentiviral vectors to specific cell types *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 11479–11484
- 115 Tebas, P., Stein, D., Binder-Scholl, G., Mukherjee, R., Brady, T., Rebello, T., Humeau, L., Kalos, M., Papasavvas, E., Montaner, L. J. et al. (2013) Antiviral effects of autologous CD4 T cells genetically modified with a conditionally replicating lentiviral vector expressing long antisense to HIV. *Blood* **121**, 1524–1533
- 116 Eguchi, A. and Dowdy, S. F. (2009) siRNA delivery using peptide transduction domains. *Trends Pharmacol. Sci.* **30**, 341–345
- 117 Tezgel, A. O., Gonzalez-Perez, G., Telfer, J. C., Osborne, B. A., Minter, L. M. and Tew, G. N. (2013) Novel protein transduction domain mimics as nonviral delivery vectors for siRNA targeting NOTCH1 in primary human T cells. *Mol. Ther.* **21**, 201–209

- 118 Andaloussi, S. E., Lehto, T., Mager, I., Rosenthal-Aizman, K., Oprea, I. I., Simonson, O. E., Sork, H., Ezzat, K., Copolovici, D. M., Kurrikoff, K. et al. (2011) Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically *in vivo*. *Nucleic Acids Res.* **39**, 3972–3987
- 119 Song, E., Zhu, P., Lee, S. K., Chowdhury, D., Kussman, S., Dykxhoorn, D. M., Feng, Y., Palliser, D., Weiner, D. B., Shankar, P. et al. (2005) Antibody mediated *in vivo* delivery of small interfering RNAs via cell-surface receptors. *Nat. Biotechnol.* **23**, 709–717
- 120 Kumar, P., Ban, H. S., Kim, S. S., Wu, H., Pearson, T., Greiner, D. L., Laouar, A., Yao, J., Haridas, V., Habiro, K. et al. (2008) T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. *Cell* **134**, 577–586
- 121 Lee, Y. K., Kim, K. S., Kim, J. S., Baek, J. E., Park, S. I., Jeong, H. Y., Yoon, S. S., Jung, K. C., Song, H. G. and Park, Y. S. (2010) Leukemia-specific siRNA delivery by immunonanoplexes consisting of anti-JL1 minibody conjugated to oligo-9 Arg-peptides. *Mol. Cells* **29**, 457–462
- 122 Peer, D., Zhu, P., Carman, C. V., Lieberman, J. and Shimaoka, M. (2007) Selective gene silencing in activated leukocytes by targeting siRNAs to the integrin lymphocyte function-associated antigen-1. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 4095–4100
- 123 Hogg, N., Patzak, I. and Willenbrock, F. (2011) The insider's guide to leukocyte integrin signalling and function. *Nat. Rev. Immunol.* **11**, 416–426
- 124 Alon, R. and Shulman, Z. (2011) Chemokine triggered integrin activation and actin remodeling events guiding lymphocyte migration across vascular barriers. *Exp. Cell Res.* **317**, 632–641
- 125 McHugh, R. S., Whitters, M. J., Piccirillo, C. A., Young, D. A., Shevach, E. M., Collins, M. and Byrne, M. C. (2002) CD4⁺ CD25⁺ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* **16**, 311–323
- 126 Pfoertner, S., Jeron, A., Probst-Kepper, M., Guzman, C. A., Hansen, W., Westendorf, A. M., Toepper, T., Schrader, A. J., Franzke, A., Buer, J. and Geffers, R. (2006) Signatures of human regulatory T cells: an encounter with old friends and new players. *Genome Biol.* **7**, R54
- 127 Chtanova, T., Kemp, R. A., Sutherland, A. P., Ronchese, F. and Mackay, C. R. (2001) Gene microarrays reveal extensive differential gene expression in both CD4⁺ and CD8⁺ type 1 and type 2 T cells. *J. Immunol.* **167**, 3057–3063
- 128 Zanin-Zhorov, A., Dustin, M. L. and Blazar, B. R. (2011) PKC- θ function at the immunological synapse: prospects for therapeutic targeting. *Trends Immunol.* **32**, 358–363
- 129 Peer, D. and Shimaoka, M. (2009) Systemic siRNA delivery to leukocyte-implicated diseases. *Cell Cycle* **8**, 853–859
- 130 Berger, J. R., Houff, S. A. and Major, E. O. (2009) Monoclonal antibodies and progressive multifocal leukoencephalopathy. *mAbs* **1**, 583–589
- 131 Kesharwani, P., Gajbhiye, V. and Jain, N. K. (2012) A review of nanocarriers for the delivery of small interfering RNA. *Biomaterials* **33**, 7138–7150
- 132 Liu, Z., Winters, M., Holodniy, M. and Dai, H. (2007) siRNA delivery into human T cells and primary cells with carbon-nanotube transporters. *Angew. Chem., Int. Ed.* **46**, 2023–2027
- 133 Yosef, N., Shalek, A. K., Gaublomme, J. T., Jin, H., Lee, Y., Awasthi, A., Wu, C., Karwacz, K., Xiao, S., Jorgolli, M. et al. (2013) Dynamic regulatory network controlling TH17 cell differentiation. *Nature* **496**, 461–468
- 134 Shalek, A. K., Robinson, J. T., Karp, E. S., Lee, J. S., Ahn, D. R., Yoon, M. H., Sutton, A., Jorgolli, M., Gertner, R. S., Gujral, T. S. et al. (2010) Vertical silicon nanowires as a universal platform for delivering biomolecules into living cells. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 1870–1875
- 135 Lee, J., Yun, K. S., Choi, C. S., Shin, S. H., Ban, H. S., Rhim, T., Lee, S. K. and Lee, K. Y. (2012) T cell-specific siRNA delivery using antibody-conjugated chitosan nanoparticles. *Bioconjug. Chem.* **23**, 1174–1180
- 136 Weber, N., Ortega, P., Clemente, M. I., Shcharbin, D., Bryszewska, M., de la Mata, F. J., Gomez, R. and Munoz-Fernandez, M. A. (2008) Characterization of carbosilane dendrimers as effective carriers of siRNA to HIV-infected lymphocytes. *J. Controlled Release* **132**, 55–64
- 137 Zhou, J., Neff, C. P., Liu, X., Zhang, J., Li, H., Smith, D. D., Swiderski, P., Aboellail, T., Huang, Y., Du, Q. et al. (2011) Systemic administration of combinatorial dsRNAs via nanoparticles efficiently suppresses HIV-1 infection in humanized mice. *Mol. Ther.* **19**, 2228–2238
- 138 Felber, A. E., Castagner, B., Elsbahy, M., Deleavey, G. F., Damha, M. J. and Leroux, J. C. (2011) siRNA nanocarriers based on methacrylic acid copolymers. *J. Controlled Release* **152**, 159–167
- 139 Peer, D., Park, E. J., Morishita, Y., Carman, C. V. and Shimaoka, M. (2008) Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target. *Science* **319**, 627–630
- 140 Villablanca, E. J., Cassani, B., von Andrian, U. H. and Mora, J. R. (2011) Blocking lymphocyte localization to the gastrointestinal mucosa as a therapeutic strategy for inflammatory bowel diseases. *Gastroenterology* **140**, 1776–1784
- 141 MacLachlan, I. (2008) siRNAs with guts. *Nat. Biotechnol.* **26**, 403–405
- 142 Zhou, J., Bobbin, M. L., Burnett, J. C. and Rossi, J. J. (2012) Current progress of RNA aptamer-based therapeutics. *Front. Genet.* **3**, 234
- 143 McNamara, 2nd, J. O., Andrechek, E. R., Wang, Y., Viles, K. D., Rempel, R. E., Gilboa, E., Sullenger, B. A. and Giangrande, P. H. (2006) Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat. Biotechnol.* **24**, 1005–1015
- 144 Chu, T. C., Twu, K. Y., Ellington, A. D. and Levy, M. (2006) Aptamer mediated siRNA delivery. *Nucleic Acids Res.* **34**, e73
- 145 Zhou, J., Swiderski, P., Li, H., Zhang, J., Neff, C. P., Akkina, R. and Rossi, J. J. (2009) Selection, characterization and application of new RNA HIV gp 120 aptamers for facile delivery of Dicer substrate siRNAs into HIV infected cells. *Nucleic Acids Res.* **37**, 3094–3109
- 146 Neff, C. P., Zhou, J., Remling, L., Kuruvilla, J., Zhang, J., Li, H., Smith, D. D., Swiderski, P., Rossi, J. J. and Akkina, R. (2011) An aptamer-siRNA chimera suppresses HIV-1 viral loads and protects from helper CD4⁺ T cell decline in humanized mice. *Sci. Transl. Med.* **3**, 66ra66
- 147 Wheeler, L. A., Trifonova, R., Vrbancic, V., Basar, E., McKernan, S., Xu, Z., Seung, E., Deruaz, M., Dudek, T., Einarsson, J. I. et al. (2011) Inhibition of HIV transmission in human cervicovaginal explants and humanized mice using CD4 aptamer-siRNA chimeras. *J. Clin. Invest.* **121**, 2401–2412
- 148 Zhou, J., Neff, C. P., Swiderski, P., Li, H., Smith, D. D., Aboellail, T., Remling-Mulder, L., Akkina, R. and Rossi, J. J. (2013) Functional *in vivo* delivery of multiplexed anti-HIV-1 siRNAs via a chemically synthesized aptamer with a sticky bridge. *Mol. Ther.* **21**, 192–200
- 149 Zhou, J., Shu, Y., Guo, P., Smith, D. D. and Rossi, J. J. (2011) Dual functional RNA nanoparticles containing phi29 motor pRNA and anti-gp120 aptamer for cell-type specific delivery and HIV-1 inhibition. *Methods* **54**, 284–294
- 150 Zhu, Q., Shibata, T., Kabashima, T. and Kai, M. (2012) Inhibition of HIV-1 protease expression in T cells owing to DNA aptamer-mediated specific delivery of siRNA. *Eur. J. Med. Chem.* **56**, 396–399
- 151 Wang, C. W., Chung, W. H., Cheng, Y. F., Ying, N. W., Peck, K., Chen, Y. T. and Hung, S. I. (2013) A new nucleic acid-based agent inhibits cytotoxic T lymphocyte-mediated immune disorders. *J. Allergy Clin. Immunol.*, doi:10.1016/j.jaci.2013.04.036
- 152 Doi, Y., Oki, S., Ozawa, T., Hohjoh, H., Miyake, S. and Yamamura, T. (2008) Orphan nuclear receptor NR4A2 expressed in T cells from multiple sclerosis mediates production of inflammatory cytokines. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 8381–8386
- 153 Raveney, B. J., Oki, S. and Yamamura, T. (2013) Nuclear receptor NR4A2 orchestrates Th17 cell-mediated autoimmune inflammation via IL-21 signalling. *PLoS ONE* **8**, e56595
- 154 Biragyn, A., Bodogai, M., Olkhanud, P. B., Denny-Brown, S. R., Puri, N., Ayukawa, K., Kanegasaki, S., Hagoabam, C. M., Wejksza, K. and Lee-Chang, C. (2013) Inhibition of lung metastasis by chemokine CCL17-mediated *in vivo* silencing of genes in CCR4⁺ Tregs. *J. Immunother.* **36**, 258–267
- 155 Weber, N. D., Merkel, O. M., Kissel, T. and Munoz-Fernandez, M. A. (2012) PEGylated poly(ethylene imine) copolymer-delivered siRNA inhibits HIV replication *in vitro*. *J. Controlled Release* **157**, 55–63
- 156 Schallon, A., Synatschke, C. V., Jerome, V., Muller, A. H. and Freitag, R. (2012) Nanoparticulate nonviral agent for the effective delivery of pDNA and siRNA to differentiated cells and primary human T lymphocytes. *Biomacromolecules* **13**, 3463–3474
- 157 Brahmandam, P., Watanabe, E., Unsinger, J., Chang, K. C., Schierding, W., Hoekzema, A. S., Zhou, T. T., McDonough, J. S., Holemon, H., Heide, J. D. et al. (2009) Targeted delivery of siRNA to cell death proteins in sepsis. *Shock* **32**, 131–139
- 158 Wahlgren, J., De, L., Karstom, T., Brisslert, M., Vaziri Sani, F., Teleme, E., Sunnerhagen, P. and Valadi, H. (2012) Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes. *Nucleic Acids Res.* **40**, e130
- 159 Sun, L., Wu, J. and Yi, S. (2012) Foxp3 is critical for human natural CD4⁺ CD25⁺ regulatory T cells to suppress alloimmune response. *Transplant Immunol.* **26**, 71–80
- 160 Ketrrousi, F., Giuliani, M., Bahri, R., Azzarone, B., Charpentier, B. and Durrbach, A. (2011) Lymphocyte cell-cycle inhibition by HLA-G is mediated by phosphatase SHP-2 and acts on the mTOR pathway. *PLoS ONE* **6**, e22776
- 161 Milstein, O., Hagin, D., Lask, A., Reich-Zeliger, S., Shezen, E., Ophir, E., Eidelstein, Y., Afik, R., Antebi, Y. E., Dustin, M. L. and Reisner, Y. (2011) CTLs respond with activation and granule secretion when serving as targets for T-cell recognition. *Blood* **117**, 1042–1052
- 162 Correale, J. and Villa, A. (2010) Role of CD8⁺ CD25⁺ Foxp3⁺ regulatory T cells in multiple sclerosis. *Ann. Neurol.* **67**, 625–638
- 163 Dinesh, R., Hahn, B. H., La Cava, A. and Singh, R. P. (2011) Interferon-inducible gene 202b controls CD8⁺ T cell-mediated suppression in anti-DNA Ig peptide-treated (NZB $\times$ NZW) F1 lupus mice. *Genes Immun.* **12**, 360–369

- 164 Kanda, N., Kamata, M., Tada, Y., Ishikawa, T., Sato, S. and Watanabe, S. (2011) Human β -defensin-2 enhances IFN- γ and IL-10 production and suppresses IL-17 production in T cells. *J. Leukocyte Biol.* **89**, 935–944
- 165 Chung, J. S., Cruz, Jr, P. D. and Ariizumi, K. (2011) Inhibition of T-cell activation by syndecan-4 is mediated by CD148 through protein tyrosine phosphatase activity. *Eur. J. Immunol.* **41**, 1794–1799
- 166 Elahi, S., Dinges, W. L., Lejarcegui, N., Laing, K. J., Collier, A. C., Koelle, D. M., McElrath, M. J. and Horton, H. (2011) Protective HIV-specific CD8⁺ T cells evade Treg cell suppression. *Nat. Med.* **17**, 989–995
- 167 Lovett-Racke, A. E., Rocchini, A. E., Choy, J., Northrop, S. C., Hussain, R. Z., Ratts, R. B., Sikder, D. and Racke, M. K. (2004) Silencing T-bet defines a critical role in the differentiation of autoreactive T lymphocytes. *Immunity* **21**, 719–731
- 168 Saxena, A., Desbois, S., Carrie, N., Lawand, M., Mars, L. T. and Liblau, R. S. (2012) Tc17 CD8⁺ T cells potentiate Th1-mediated autoimmune diabetes in a mouse model. *J. Immunol.* **189**, 3140–3149
- 169 Yang, Y., Weiner, J., Liu, Y., Smith, A. J., Huss, D. J., Winger, R., Peng, H., Cravens, P. D., Racke, M. K. and Lovett-Racke, A. E. (2009) T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. *J. Exp. Med.* **206**, 1549–1564
- 170 Chiyo, M., Iwata, T., Webb, T. J., Vasko, M. R., Thompson, E. L., Heidler, K. M., Cummings, O. W., Yoshida, S., Fujisawa, T., Brand, D. D. and Wilkes, D. S. (2008) Silencing S1P1 receptors regulates collagen-V reactive lymphocyte-mediated immunobiology in the transplanted lung. *Am. J. Transplant.* **8**, 537–546
- 171 Chen, Y., Yan, T., Shi, L. J., Liu, Z., Liang, S. Y., Luan, X. F., Long, F. W., Chen, J., Peng, Y., Yan, L. N. and Gong, J. P. (2010) Knockdown of interleukin-2 by shRNA-mediated RNA interference prolongs liver allograft survival. *J. Surg. Res.* **159**, 582–587
- 172 Srivastava, V., Vashishta, M., Gupta, S., Singla, R., Singla, N., Behera, D. and Natarajan, K. (2011) Suppressors of cytokine signaling inhibit effector T cell responses during *Mycobacterium tuberculosis* infection. *Immunol. Cell Biol.* **89**, 786–791
- 173 Gocke, A. R., Cravens, P. D., Ben, L. H., Hussain, R. Z., Northrop, S. C., Racke, M. K. and Lovett-Racke, A. E. (2007) T-bet regulates the fate of Th1 and Th17 lymphocytes in autoimmunity. *J. Immunol.* **178**, 1341–1348
- 174 Tesniere, A., Abermil, N., Schlemmer, F., Casares, N., Kepp, O., Pequignot, M., Michaud, M., Martins, I., Senovilla, L., Zitvogel, L. and Kroemer, G. (2010) *In vivo* depletion of T lymphocyte-specific transcription factors by RNA interference. *Cell Cycle* **9**, 2830–2835
- 175 Perez-Rivero, G., Cascio, G., Soriano, S. F., Sanz, A. G., de Guinoa, J. S., Rodriguez-Frade, J. M., Gomariz, R. P., Holgado, B. L., Cabanas, C., Carrasco, Y. R. et al. (2013) Janus kinases 1 and 2 regulate chemokine-mediated integrin activation and naive T-cell homing. *Eur. J. Immunol.* **43**, 1745–1757
- 176 Humphries, L. A., Shaffer, M. H., Sacirbegovic, F., Tomassian, T., McMahon, K. A., Humbert, P. O., Silva, O., Round, J. L., Takamiya, K., Haganir, R. L. et al. (2012) Characterization of *in vivo* Dlg1 deletion on T cell development and function. *PLoS ONE* **7**, e45276
- 177 Chen, Z., Stockton, J., Mathis, D. and Benoist, C. (2006) Modeling CTLA4-linked autoimmunity with RNA interference in mice. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 16400–16405
- 178 Gerold, K. D., Zheng, P., Rainbow, D. B., Zernecke, A., Wicker, L. S. and Kissler, S. (2011) The soluble CTLA-4 splice variant protects from type 1 diabetes and potentiates regulatory T-cell function. *Diabetes* **60**, 1955–1963
- 179 DiGiusto, D. L., Krishnan, A., Li, L., Li, H., Li, S., Rao, A., Mi, S., Yam, P., Stinson, S., Kalos, M. et al. (2010) RNA-based gene therapy for HIV with lentiviral vector-modified CD34⁺ cells in patients undergoing transplantation for AIDS-related lymphoma. *Sci. Transl. Med.* **2**, 36ra43
- 180 Burnett, J. C. and Rossi, J. J. (2012) RNA-based therapeutics: current progress and future prospects. *Chem. Biol.* **19**, 60–71
- 181 Sioud, M. (2005) Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. *J. Mol. Biol.* **348**, 1079–1090
- 182 Bramsen, J. B. and Kjems, J. (2012) Development of therapeutic-grade small interfering RNAs by chemical engineering. *Front. Genet.* **3**, 154
- 183 Boutimah, F., Eekels, J. J., Liu, Y. P. and Berkhout, B. (2013) Antiviral strategies combining antiretroviral drugs with RNAi-mediated attack on HIV-1 and cellular co-factors. *Antiviral Res.* **98**, 121–129