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STRATEGIES OF GENE AND IMMUNE THERAPY FOR TUMORS AND VIRAL DISEASES

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SUMMARY

In the treatment of tumors and viral diseases such as HIV-1 infection, immunotherapeutic strategies do not seem to have enough capacity to eradicate an already established disease. However, these strategies could play a unique role as adjuvant treatments of therapies that eliminate the main tumor or viral load in order to avoid the relapse of the disease. Consequently, efforts aimed to improve the current steady state of imunogene-therapy and to find novel therapeutic strategies should be continued.

Cytokines are very efficient immunomodulators. Nevertheless, their application is frequently hampered by high toxicity specially, when applied systemically. Cytokines work in a paracrine form and for that reason a highly regulated release of these substances could be desirable for most therapeutic applications. To avoid systemic effects, the target cells can be autoactivated by cytokines produced by the same cells after cytokine gene transfer. However, the uncontrolled cellular secretion of cytokines could even exert adverse bystander effects. Therefore, the development of additional methods for a more restricted administration is still desirable.

With the aim of improving the administration and release of cytokines in a strictly localized area, we have designed vectors that express the murine granulocytic macrophage colony stimulating factor (mGM-CSF) with different subcellular localization signals. Using this strategy, we have shown that cytokines can be expressed and targeted to different subcellular compartments as stable and active proteins.

We also demonstrated that the mGM-CSF-dependent DA-3 cell line acquires autonomous growth following gene modification with the plasmids encoding either extracellular or intracellular forms of mGM-CSF. In addition, we showed that the autonomous growth induced by the intracellular forms of mGM-CSF in these cells, is mediated by a mechanism of restricted autocrine stimulation without release of detectable cytokine to the extracellular medium. Our findings support the concept that *intracellular cytokines* may be used to provide the desired effect of these substances on the target cells, while avoiding the collateral effects of their uncontrolled secretion.

When our plasmids were tested as adjuvants of genetic immunizations with HIV-1 genes, it was demonstrated that the Th1 pattern of a primary immune response could be switched toward an enhanced Th2 response, by plasmids expressing the secreted form of mGM-CSF or by recombinant mGM-CSF. On the other hand, plasmids expressing an intracellular form of the mGM-CSF, induced consistently an enhanced primary Th1 response.

Currently, chemotherapy is being used with good results for the treatment of some tumors as well as for HIV-1 infection. Nevertheless, resistance and severe side effects have hampered the use of this approach. A new technology with a potential for treatment of tumors and viral diseases is post transcriptional gene silencing induced by RNA interference (RNAi). By using plasmid-mediated expression of small interfering RNAs (siRNA) against either endogenous transcripts for the primary HIV-1 coreceptor, CCR5, or viral transcripts for the Rev gene of HIV-1, we were able to inhibit efficiently both viral infection and replication, respectively. The possibility of inducing a considerable reduction of the viral load or tumor burden using siRNA technology could have a significant impact on the treatment of these diseases in the future.

LIST OF PUBLICATIONS

I. HJ. Arteaga, AM. Jama, B. Christensson, G. Gahrton, CIE. Smith, MS. Dilber Expression and release of stable and active forms of murine Granulocytic Colony Stimulating Factor (mGM-CSF) targeted to different sub-cellular compartments. Cytokine. 2001 May 7;14(3):136-42.

II. H.J. Arteaga, A.J. Mohamed, M.S. Dilber, B. Christensson, G. Gahrton, E. Rollman, C.I.E. Smith, B. Wahren, J. Hinkula. Plasmid encoded variants of murine granulocyte-macrophage colony stimulating factor (mGM-CSF) as Th1 or Th2 adjuvants for HIV-1 DNA immunogens. 2002. Submitted.

III. H. José Arteaga, Abdalla J. Mohamed, Birger Christensson, Ensaf Mahdy, Gösta Gahrton, C.I. Edvard Smith, M. Sirac Dilber. Generation of genetically-modified auto activated cell lines expressing intracellular forms of GM-CSF: an approach for a highly regulated administration of cytokines. 2002. Submitted.

IV. H. Jose Arteaga, Jorma Hinkula, Iris van Dijk-Härd, M. Sirac Dilber, Britta Wahren, Birger Christensson, Abdalla J. Mohamed and C. I. Edvard Smith. Choosing CCR5 or Rev siRNA in HIV-1. Nature Biotechnology 2003 March 21 (3): 230-231.

CONTENTS

INTRODUCTION

Most tumors and some viral infections lead to untreatable and overwhelming diseases that severely undermine the general conditions of the organism, overcome the immunological mechanisms of defense and lead to exhaustion of the immune system. Recent advances in the chemotherapy of cancer as well as of the HIV-1 infection, have allowed to achieve induction of states of almost complete remission of the disease for both HIV-1 and tumors. However, in both cases complete and definite curative treatments do not exist yet and early or late relapses are the rule. In addition, the most successful chemotherapeutic approaches for cancer are hampered by strong side effects and induction of tumor resistance. A similar scenario is valid for HIV-1 chemotherapy.

Although there are uncontroversial evidences regarding the importance of the immune system in protecting against tumor development (evidences from immune compromised individuals), already established tumors seems not to respond to therapeutic immunizations. In a similar way, in the long term, the immune system fails to combat the HIV-1 infection and attempts to induce either a preventive or therapeutic immunization against the virus, have failed so far. Therefore, it may be possible to say that established tumors and AIDS "are two already lost battles for the immune system".

Additionally, the conventional immunization protocols that have been successful for induction of a preventive immune response against several infectious diseases do not seem to be the appropriate ones for immunization against HIV-1 and tumors. In order to induce a complete remission with the current conventional therapeutic methods, unacceptable levels of toxicity have to be reached. However, a combination of the most efficient current conventional treatments with alternative non-toxic approaches could induce partial remission, while preserving the general conditions of the organism and a competent immune system. Such alternative methods may relay on the strategy of selective molecular or genetic targeting of specific components of the virus or of the tumor cells. However, the current methods of gene transfer technology are far from reaching a hundred percent targeting of the transformed or infected cells and this might be an elusive goal for a long time. Conversely, the immune system is naturally endowed with properties for highly selective and specific targeting in this respect superior to any other strategy.

With a host in good general conditions, low pathogenic burden and a competent immune system, the possibility for a successful immunization against tumors or HIV-1 infection would be higher. Therefore, finding new strategies for gene or molecular targeting and improving immunization strategies are very important goals. From the gene transfer technology perspective, two new approaches have shown promising results: post transcriptional gene silencing and DNA vaccination.

The aim and the perspective of this thesis were to develop and improve appropriate immunization strategies for preventive and or therapeutic immunization against tumors or viral diseases. We also aimed to identify and apply new molecular therapeutic strategies.

In the present work we have focused on these two aspects: development of new strategies of cytokine delivery for application in immunotherapeutic methods, and application of post transcriptional specific gene targeting using the RNA interference (RNAi) technology, to inhibit HIV-1 infection.

CONCEPTUAL FRAME

1 ADJUVANTS FOR IMMUNIZATION

1.1 Cytokines in immune therapy for tumors and infectious diseases

Cytokines have been extensively used for different forms of immunomodulation, many of which work mainly by pre-activation of immunocompetent cells. An approach for cancer immunotherapy based on this concept, is the systemic or localized administration of cytokines with the aim of breaking down the state of immunotolerance of the organism to tumors. The general conclusion drawn from several experiments of this kind, is that therapeutic levels of systemically applied cytokines are frequently toxic and that cytokines alone have low capacity to break down the conditions that allow tumors to escape from the antitumor immunological mechanisms (reviewed in [1, 2]). These effects may be explained by the fact that (i) under physiological conditions, the release of cytokines is strictly regulated by signals that take place, usually after cell-to-cell interaction, at specific time points, and (ii) that in the majority of the cases, cytokines work in an autocrine or paracrine fashion. Therefore, the same requirements, i.e. time-controlled and localized release of cytokines, are important for most of their therapeutic applications [3, 4]. In the same line of thinking, peripheral lymphocytes or tumor infiltrating lymphocytes (TILs) have been preactivated *ex vivo* with cytokines and used in experiments of adoptive transfer as antitumor agents with certain, but not consistent success for tumor rejection.

Cytokines have been used as adjuvants in immunization against tumors. One of the best-studied models of this kind of applications is the immunization with cytokine gene-modified irradiated tumor cells. The model was designed in order to achieve a localized release of cytokines. Nevertheless, until now, experiments of immunization using this kind of cells have not shown consistent, good results. A final approach to achieve the requirements of time and site restriction for cytokine stimulation of immunocompetent cells has been the generation of autostimulated cells expressing the cytokine of interest. Cytokine gene transduced TILs or peripheral blood lymphocytes have been used as an approach to mimic the paracrine action of cytokines, again with better, but inconsistent results [5-8]. In the same way, other experimental models such as natural killer (NK) cells constitutively expressing the IL-2 gene [9] or dendritic cells (DC) expressing granulocytic macrophage colony stimulating factor (GM-CSF) are still in the basic or preclinical phase of development [10, 11].

From these experiments, it appears that intratumor localized release of cytokines is a better way of using these substances for immunotherapy against tumors. In the same way, a localized release of the appropriate cytokine in the presence of a good source of antigen (probably the whole tumor cell) may be a better approach for immunization against tumors.

A wide variety of cytokines in different set ups and combinations has been tested as possible antitumoral agents and as adjuvant in antitumor immunizations. Theoretically, for immunizations the best adjuvant could be a cytokine or a combination of cytokines with the ability (i) to recruit the adequate immunocompetent cells at the tumor site (cells from the innate immune system, APC and lymphocytes), (ii) to activate properly

the antigen presenting cells (APC) for the right promoting function during antigen presentation and (iii) to prime and bias naive T lymphocytes toward the correct profile of cytokine production.

The presence of these cytokines during the process of "antigen recognition" and T cell activation should enhance the priming of lymphocytes with the appropriate epitopes from the available source of antigen. These conditions should drive the resulting immune response toward a protective profile, probably a kind of cellular immune response under control of Th1 lymphocytes. The most optioned cytokine candidates for this task, from a theoretical point of view, could be a set of them, such as IL-2, INFγ, IL-12 and GM-CSF, applied in the right combination and probably in a proper sequential manner. However, from the previous experiments of vaccination with cytokine gene-modified tumor cells, GM-CSF alone has shown the best results.

1.2 GM-CSF

GM-CSF is produced by a variety of cells from the immune system and other tissues such as fibroblasts, endothelial cells, epithelial cells and macrophages. GM-CSF is also produced by T lymphocytes of both the Th1 and the Th2 profile. The main known function of GM-CSF is its effect on the development, maturation and activation of granulocytes, monocytes and dendritic cells. In addition, GM-CSF has recently emerged as a proinflammatory cytokine [12, 13]. It induces IL-1 and TNF production from granulocytes and monocytes [14, 15]. It is also a chemotactic factor for eosinophiles and nuetrophiles and increases the oxidative activity of granulocytes. Additionally, in the effector phase of the specific immune response, it increases the function of antibodies-dependent cellular-cytotoxicity (ADCC).

The efficacy of GM-CSF as adjuvant has been associated with its activity in the maturation and recruitment of bone marrow derived dendritic cells. However, it could be also related to its ability to activate some mechanisms of the innate (antitumoral) immune response [16].

Currently, GM-CSF is considered as one of the most promising cytokine candidates for cancer immunotherapy [17, 18] and as adjuvant for DNA vaccines. However, the appropriate protocols optimizing release, concentration and timing schedules are still under development. It would be also important to explore whether the combinations with other immunogens or adjuvants have a synergistic effect. Finally, in the process of designing and selecting the right schedules and combinations for application of GM-CSF, the disease model and the kind of required immune response, should always be considered.

1.3 Cytokine gene-modified cells and *intracellular cytokines*

The interest in preactivation of immunocompetent cells for immunotherapy, together with the fact that systemically applied cytokines are highly toxic [1, 2] as a reflection of the requirement of a paracrine delivery, strengthen the need for a method of localized release of cytokines [3, 4]. Additionally, it has been suggested that methods of highly controlled administration of cytokines may also improve their performance as adjuvant in immunization protocols, especially in the context of DNA vaccines [2, 19]. As mentioned before, an approach to achieve this goal might be the generation of autoactivated cytokine dependent cells expressing the cytokine of interest. Several

examples of this approach using different cell types and cytokines [9, 20], among them GM-CSF, have been reported [10, 11, 21, 22].

Nevertheless, this way of cytokine delivery may still induce certain side effects on the neighboring tissues, such as the inflammatory effects of the soluble forms of GM-CSF [23-26] or Il-2. Therefore, the development of additional methods for a more localized administration of cytokines is still required.

The concept of intracellular cytokines for autoativation of cytokine dependent cells has not been yet exploited, in spite of previous works showing that this is a feasible strategy [21, 27, 28]. Several growth factor dependent hematopoietic cell lines have been rendered autonomous from exogenous factors by induction of endogenous expression of cytoplasmic forms of interleukin-3 (IL-3) [27]. Additionally, it has been demonstrated, using a CCR5 positive cell line and a modified CC-chemokine targeted to the endoplasmic reticulum, that the intracellular chemokine (intrakine) binds to the receptor in the ER and blocks the expression of the receptor to the surface of the cells [29].

The development of plasmids expressing intracellular forms of different proteins has been facilitated by the strategy of protein subcellular targeting through modification with localization signal peptides [28, 29]. The model was originally used for expression of intracellular antibodies [22]. We have extended this technology to the intracellular expression of stable and active forms of cytokines as a method for a restricted cytokine delivery into the target cells [30].

Some potential applications of this approach may be, for instance, the manipulation of the adjuvant activity of GM-CSF, a cytokine with the capacity to enhance either the cellular or the humoral branches of the immune response [17, 18]. However, the enhancement of the proper type of the immune response with this cytokine requires a tightly regulated release [19]. Relocated forms of this cytokine may provide means to achieve this kind of regulation. Another possible application of intracellular cytokines might be the generation of autoactivated NK cells expressing intracellular forms of IL-2 for the treatment of tumors. In this case, the genetic modification of NK cells with genes encoding intracellular forms of IL-2 may avoid the well known undesired collateral effects of the secreted form of this cytokine, especially in the treatment of relapsing hematological tumors following bone marrow transplantation [31].

An additional application of cytokines is the use of genes encoding these proteins as adjuvant for DNA vaccines. Some observations suggest that during these procedures it is possible that bystander APC could be transfected with the plasmids harboring the transgenes [32]. If one of these plasmids harbors a cytokine such as GM-CSF, it may result in the *in vivo* generation of constitutively activated APC by the cytokine.

Based on these ideas, we proposed the concept of “intracellular cytokines” and developed an experimental strategy to demonstrate that this is a novel and feasible method for highly restricted cytokine delivery.

1.4 GM-CSF as adjuvant of DNA vaccines

Immunization by injection of plasmid DNA encoding a specific protein antigen [33, 34] is perhaps the closest approach to the classic way of vaccination with attenuated live microorganisms. DNA vaccines allow the presentation of antigens as endogenous

proteins in the context of both the MHC class I and class II molecules; therefore, they have almost all the potential for induction of cellular immune response and activation of cytotoxic T lymphocytes [35, 36]. These characteristics make DNA vaccination an attractive approach for immunization against intracellular viral infectious agents and tumors [37].

Nevertheless, DNA vaccines are still in the process of improvement and a robust, fully protective DNA vaccine has not been developed yet. DNA vaccines against HIV-1 have shown a modest performance [36, 38-41] and no successful DNA vaccine against tumors has been reported.

In spite of the above mentioned characteristics, two limitations of DNA vaccines can explain their low performance: (i) DNA vaccines do not display a strong danger signal and (ii) the genetic or antigenic material is expressed in low amounts and does not spread widely out of the cells. The success in preclinical trials reported recently by immunization with DNA and modified vaccinia virus Ankara (MVA) vaccines expressing a common immunogen is probably related to these two characteristics.

DNA vaccination by intramuscular (i.m.) route, as it was described in the early stages of development of this technology, generally induces a weak Th1 type primary immune response [42-45]. GM-CSF has been extensively studied and it is considered in general a good adjuvant and perhaps the most effective cytokine as adjuvant of DNA vaccines [46]. Conversely, in most of the reports GM-CSF has shown a moderate adjuvant effect in HIV-1 DNA vaccines [19, 47-49]. It was recently reported that GM-CSF enhanced a high CD4+ T cell response when expressed from a bicistronic vector together with the antigen coding genes [50]. This special behavior could be explained as the effect of expression of the genes encoding for adjuvant and antigen probably in the same antigen-presenting cell. That corroborates the need for a restricted paracrine delivery of cytokines [1, 2]. One characteristic not always considered when using GM-CSF as adjuvant is its proinflammatory activity, which may influence the outcome of an immunization protocol [51].

GM-CSF is involved in the process of migration and accumulation of eosinophils at the allergic inflammation sites. It also induces the release of PGE_2 by alveolar macrophages [23, 26]. PGE_2 is a common inflammatory product, which acts as a modulatory factor that induces a Th2-promoting function in DCs, leading finally to a bias toward Th2 cytokine production in naive Th cells. PGE_2 has also been involved in a cytokine cascade inducing a Th2 profile of lymphocytes. It has been suggested that UV irradiated keratinocytes release PGE_2 which in turn induces an increase of serum IL-4 thereby increasing the levels of serum IL-10 [24, 25]. Hence, targeting the cytokine gene delivery only to the specific subset of cytokine-dependent cells, could allow a tightly controlled effect of these substances. In experiments of DNA immunization against HIV-1 antigens, we have demonstrated that plasmids expressing the secreted form of murine GM-CSF (mGM-CSF) induce a strong humoral immune response of the Th2 type, while plasmids encoding an intracellular form of mGM-CSF consistently enhanced a Th1 like immune response. Taken together, these observations suggest that mGM-CSF, when available as a diffusible substance in the extracellular milieu, before the antigen expression and probably at a specific concentration level, could induce a Th2 response in DNA vaccines. This property of the plasmids expressing the intracellular forms of mGM-CSF may be explained if targeting and

transfection of some kind of cytokine-dependent professional APC, such as DC, take place in the *in vivo* situation by i.m. injection. Such a strategy, would avoid the collateral inflammatory effect of the secreted cytokines, and might be useful when a potent and polarized cellular immune response is required.

The possibility of inducing either a good humoral or cellular immune response with plasmids expressing the secreted or the intracellular forms of GM-CSF increases the flexibility of DNA vaccines. This strategy opens the possibility for the generation of a kind of "diversified immunization" by combining different protocols of immunization in a single individual but at different local sites. This procedure could potentially induce high titers of neutralizing antibodies and good CTL activity at the same time. DNA vaccines offer a good possibility to carry out this kind of experiments. Using properly designed plasmids it may be possible to induce two different kinds of immune response against different antigens from the same organism. There is a possibility that by injecting two DNA vaccines at different topographic regions with different "immunological environments" each procedure could work independently and the outcome could be two different responses against the same organism.

2 RNA INTERFERENCE (RNAI) FOR VIRAL INFECTIONS

2.1 What is RNAi?

RNA interference (RNAi) is a natural biological mechanism of response to double stranded RNA (dsRNA), which mediates specific degradation of homologous mRNA. This is a conserved mechanism in most living organisms, from plants to mammals [52, 53]. In lower organisms, RNAi works as a system of specific post transcriptional gene silencing (PTGS) with different biological functions ranging from regulation of gene expression [54-56] to protection against parasitic nucleic acids [57-61].

2.2 How does it work?

The development of RNAi technology derived from observations of antisense interfering-RNA experiments, which showed that the combination of sense and antisense RNA was ten times more efficient than antisense RNA alone in the induction of gene silencing [54]. The molecular mechanisms of RNAi have been characterized, to a certain extent, in plants and in some eukaryotic organisms, including mammals, as follows. Intracellular dsRNA is recognized by a RNAse III-like enzyme called Dicer [62, 63] and it is cleaved afterwards into 21-23 bp segments [64] named small or short interfering RNA (siRNA). These small segments are then, recognized by another enzymatic complex, termed dsRNA induced silencing complex (RISC) [65-67]. After recognition, one of the components of RISC, a nuclease enzyme, binds to the antisense strand of the siRNA and is driven to the matching sequence of the target mRNA, which in turn is cleaved at the middle of the 22-23 bp of the complementary region (reviewed in [52, 53]).

RNAi can be induced artificially in different kinds of cells by direct delivery of dsRNA synthetic duplexes. However, in mammalian cells dsRNA longer than 30 bp, triggers the interferon (INF) antiviral pathway [68], leading to an unspecific and generalized down regulation of protein expression [69-72]. For that reason, the application of conventional RNAi technology was not feasible in this kind of cells. An important step for the development of the siRNA technology in mammals was the finding that siRNAs

in mammalian cells bypass the INF response and induce, without toxicity, specific mRNA degradation [73-76]. This development allowed for the use of siRNAs in mammalian cells for different purposes in which specific mRNA degradation is desired.

2.3 Is RNAi a natural function of mammalian cells and can be used safely?

The questions about whether RNAi is a natural function and what could be its main biological role in mammalian cells, remains to be answered. In spite of this, RNAi has a number of characteristics that make this technology an attractive strategy for induction of gene silencing in mammalian cells. The short length of siRNAs and their exceptionally high specificity, requiring a hundred per cent matching with the target sequence, ensure that almost every gene can be targeted specifically without cross reactivity with related genes.

In addition, no toxic effects have been reported until now and there is some evidence suggesting that siRNAs may work in mammalian cells through a natural physiological pathway. Recently, a related natural mechanism of gene expression regulation has been discovered in several organisms, including mammals [77-79]. This mechanism is mediated by small segments of a special kind of dsRNA, named micro RNA (miRNA). The molecular machinery of this mechanism shares great similarities with that mediating RNAi in mammals. The link and similarities of these two molecular mechanisms suggest the existence of a common natural pathway of action. This pathway may have evolved for similar purposes and could be exploited safely for therapeutic applications of siRNAs.

2.4 Is RNAi a natural antiviral mechanism?

Several lines of evidence support the concept that in plants dsRNA mediated PTGS is a natural antiviral mechanism [61, 80] and recent observations suggest that RNAi could have a similar function in animals [60]. Considering these observations and the characteristics of the molecular mechanisms mediating the RNAi activity, it was reasonable to suggest that siRNAs could be used to inhibit efficiently viral infections, particularly those caused by RNA viruses.

Preliminary results from *in vitro* and *in vivo* experiments using different models of viral infections, suggest that RNAi actually works as a highly efficient antiviral system in mammals [81-83]. Although HIV-1 has evolved mechanisms to block the antiviral INF pathway [68, 84], which is a response induced by dsRNA, these mechanisms do not undermine the efficiency of the molecular machinery that mediates RNAi in HIV-1 infected cells.

A number of recent reports (discussed below) confirm that siRNAs can be used to inhibit efficiently the HIV-1 infection in different types of cell lines as well as in primary $CD4^+$ T cells.

2.5 Targeting HIV-1 with siRNAs

In theory, siRNAs may affect several stages of the viral life cycle blocking both infection and replication. Besides targetting viral genes, siRNAs can be used to down regulate host cellular receptors and block viral entry, as it will be discussed below.

The current available data from the different studies suggest the following outline of mechanisms: siRNAs can degrade different types of viral RNA at both pre and post integration stages, blocking in this way new infections and viral replication in ongoing infections, respectively. siRNAs can degrade genomic viral RNA before integration, thus inhibiting the insertion of the provirus [85-88]. They could also block the selective transcription of the *nef* and *tat* genes, which are transcribed from the proviral DNA before integration. In this way, an important step for increased viral replication is inhibited [85, 87, 89]. Finally, siRNAs can inhibit HIV-1 replication by degrading mRNA transcribed from the integrated provirus [87, 88, 90]. The overall effect of these events is a reduction in the production of viral mRNA, proteins and full length RNA templates leading to a decreased formation and release of free viral particles. In turn, these effects will be reflected in a reduction of the reinfection cycle and cytopatic viral effects. Taken together, these observations suggest that RNAi technology holds a great potential for therapeutic applications against HIV-1 and probably for other viral infections.

Cotransfection of anti HIV-1 siRNA-producing constructs with plasmids expressing the HIV-1 proviral DNA has shown inhibition of more than 90% of the HIV-1 DNA expression. The effect in transient transfections lasts from 4-9 days [87, 90, 91]. siRNAs targeting the *gag* gene transcripts have been designed to inhibit HIV-1 replication at the post transcriptional level. This was considered a good strategy because the gene encodes a poliprotein precursor, which produces several structural proteins after cleavage [87]. However, it has been demonstrated that siRNAs against *tat* and *rev* genes degrade viral RNA both at pre and post integration stages. Moreover, siRNAs against *tat, rev* or *gag* were able to reduce the expression of all three size classes of HIV-1 mRNA (full length, single spliced, and multiple spliced), suggesting that the mechanism for blocking gene expression with siRNA involves the degradation of all the species of HIV-1 mRNA, thereby inducing a general down regulation of HIV-1 proteins expression [88]. Some reports have suggested that genomic viral RNA, which is tightly associated with nucleocapside proteins, is resistant to siRNA activity [92]. However, it has been demonstrated that genomic HIV-1 RNA, as it exists within a nucleoprotein reverse-transcription complex, is accessible to degradation mediated by siRNA against regions of Nef, Vif and TAR. This experiment confirms that HIV-1 RNA degradation is feasible at pre and post transcriptional level [86].

From the above mentioned data, it seems that almost every functional siRNA sequence against different sites of the HIV-1 genome may have the potential to degrade efficiently genomic RNA at pre and post integration stages and therefore reduce viral infection and replication. However, one of the most obvious limitations of this technology is the appearance of viral escape mutants bearing silent mutations in the sequences encoding conserved domains of the viral proteins. These mutations, while keeping the structural and functional characteristics of the encoded proteins, eliminate completely the effectiveness of siRNAs, since they require a hundred percent sequence matching. There may be possibilities to find sequences with particular characteristics to avoid such events and this is perhaps, one of the main aspects to investigate in a prospective work. It will probably require computerized analysis of the HIV genome, modeling and experimental evaluations.

2.6 Targeting host cell receptors with siRNA

To avoid escape mutants, we and other groups suggested targeting mRNA encoding selected host cellular proteins [87, 93-95]. The CCR5 co-receptor may be the preferred target since its homozygous mutations confer protection against HIV-1 infection and there are a large number of individuals (1% of Europeans) carrying these kinds of mutations in the absence of any known adverse effect on the immune system [96-98].

CCR5 expression has been blocked using synthetic siRNA duplexes with reasonable efficiency (48%). Crossreactivity with the CXCR4 coreceptor, a major related chemokine receptor, has not been reported so far [94]. Using a lentiviral vector expressing another siRNA against CCR5, peripheral blood lymphocytes have been transfected with 40% efficiency. With this method, the CCR5 expression on transduced T lymphocytes was downregulated by 40% and the HIV-1 infection was reduced 30-70%. The protective effect lasted for two weeks without selection. This experiment also showed that the protection against infection of the siRNA targeted cells resulted in an overall reduction of virus load and decreased infection of the non transduced cells [95]. We generated a population of stably transfected U937 cells (a monocytic cell line), expressing a siRNA against CCR5, which displays more than 80% of sustained resistance to HIV-1 infection.

Taken together, these observations suggest that combined approaches using several siRNA sequences to target different and strategic regions of the HIV genome as well as cell coreceptors, may be an important novel means of inhibiting HIV-1 infection.

2.7 Limitations

From the studies carried out until now by several groups as well as from theoretical considerations, some limitations of RNAi technology against HIV-1 may be predicted. So far, there have been no reports on selection of escape of mutants by the anti HIV-1 siRNAs selective pressure. However, in theory, there is a high probability that these variants will appear in long-term experiments. It is also possible that siRNA against CCR5 could speed up the appearance of CXCR4 dependent HIV-1 strains. Reports from two independent studies using two kinds of animal viruses, have shown a lack of siRNA antiviral activity associated with viral stocks containing genetic variants with a single mutation in the target region [83, 86].

Another possible limitation when using siRNA to inhibit viral infections is that the virus may be endowed with proteins that can inhibit the PTGS mechanisms. One example of this kind of virus has been already reported [60]. However, it seems that this is not the case when HIV-1 has been targeted with siRNA in different cell lines or in fresh peripheral blood lymphocytes.

Other possible limitations such as toxicity or alterations in the functionality of the target cells, especially in T lymphocytes, remain to be evaluated in long-term experiments. Efficient gene delivery and targeting are common limitations to all procedures of gene therapy involving genetic modification of T cells.

2.8 The challenge is the delivery

siRNAs can be delivered as synthetic dsRNA duplexes with high efficiency into cell lines as well as primary cells. For some specific purposes this kind of delivery may be

preferred. For such kind of applications a special RNAse H resistant siRNA, has been proposed. These molecules are efficiently transfected complexed to lipofectin or even as naked RNA in presence or absence of serum [85]. siRNA can be also delivered from different kinds of vectors expressing hairpin shaped RNA transcripts or sense and antisense complementary transcripts, which are trimmed by cellular enzymes to produce siRNAs with the correct size and configuration. Most of these systems have used mammalian Pol III promoters which express efficiently stable small RNA transcripts, surprisingly, with the required molecular characteristics of siRNA [90, 99]. Using a plasmid-mediated approach, we have demonstrated that stable expression of anti HIV-1 siRNA is a feasible strategy.

A key problem of gene therapy for AIDS namely, specific and efficient molecular targeting, seems to be reasonably solved using siRNA. However, the lack of an efficient and safe method of gene delivery into CD4 lymphocytes is currently the main limitation for a possible clinical application of this approach.

2.9 Choosing the right siRNA target sequence

To date, there is not a reliable method that can be applied to choose an affective siRNA sequence. Target sequence selection is a difficult and time consuming procedure; it is essentially a trial and error process. A method of choosing target sequences based on the accessibility of RNAse H to the target RNA, has been described. The method while efficient in detecting specific targets for a gene in one genomic context, failed to predict the accessibility to the same target in a different genetic context. The results suggest that the same siRNA sequence may have different levels of efficiency on the same target in different genetic and or cellular contexts [90].

It is generally assumed that combinations of different siRNA sequences may have a synergistic effect. Experiments intended to address this assumption have shown that the combination of two effective sequences have indeed a synergistic effect, while a combination of an effective with a non effective sequence may give a lower average effect than the effective sequence [90]. A new strategy based on the *in vitro* production of siRNAs by digestion of long dsRNA segments with recombinant human Dicer, seems to generate a mixture of siRNA segments which always produce nearly to hundred percent of silencing [100]. It might be of interest to determine the probability of inducing HIV-1 escape mutants against this kind of siRNAs cocktail.

There are some elements that might be considered when choosing a suitable siRNA sequence from the genes encoding the different HIV-1 proteins. siRNAs against different HIV-1 genes reduce the concentration of all three types of viral mRNA. Additionally, siRNA degrades efficiently at the same time, both pre and post integration genomic RNA. These observations suggest that targeting virtually any accessible site at the HIV-1 genome may induce some kind of viral genome destabilization. Therefore, it seems that there are not definitive criteria for a preferential selection among the possible target genes. However, because the regulatory proteins are expressed both at pre and post integration stages, selecting these genes might have some advantage. Thus, when choosing a siRNA sequence against HIV, probably the main effort should be centered on avoiding the induction of escape mutants. One option is to combine several siRNA sequences, choosing among the most conserved protein coding segments of the genome. Another possibility is to search for conserved regions

among DNA/RNA domains needed for essential nucleic acid-protein interactions. An additional option could be targeting a single region coding for more than one protein through different overlapping open reading frames.

AIMS OF THIS THESIS

A. The main aim of this thesis was to explore and develop new strategies of immuno-gene therapy and molecular targeting for the treatment of tumors and viral diseases.

B. The specific aims of this thesis were:

   1. To demonstrate that cytokines can be expressed and targeted as stable and active proteins in the different subcellular compartments.

   2. To demonstrate that *intracellular cytokines* can induce autocrine activation of cytokine dependent cells.

   3. To evaluate the activity of plasmids expressing intracellular or secreted forms of mGM-CSF as adjuvants of DNA vaccines.

   4. To evaluate the antiviral activity of siRNA in *in vitro* models of HIV-1 infection.

COMMENTS ON THE METHODS

1 CONSTRUCTION AND EVALUATION OF PLASMIDS EXPRESSING mGM-CSF TARGETED TO DIFFERENT SUBCELLULAR COMPARTMENTS

Four vectors expressing the mGM-CSF fused to targeting sequences for protein localization into specific subcellular organelles –the plasma membrane (PM), the cytoplasm, the endoplasmic reticulum (ER) and the nucleus–, were assembled using expression plasmids from the pShooter™ family as shown in Figure 1. Details concerning the construction of these vectors are described in Paper I.

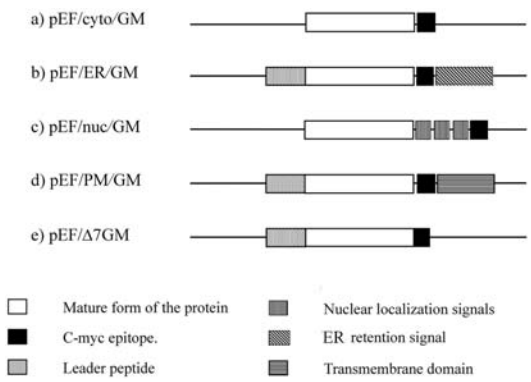

Figure 1. *Schematic representation of plasmids expressing modified forms of mGM-CSF*
The original mouse cDNA encoding the secreted form of mGM-CSF was modified by addition and/or deletion of segments of DNA encoding for different subcellular localization signal peptides as indicated in the graph. The vectors are named as in the text and each one expresses a different form of the cytokine: (a) cytoplasmic, (b) ER, (c) nuclear, (d) PM and (e) secreted form of mGM-CSF.

The biological activity of the mGM-CSF expressed by these vectors was determined by a cell proliferation assay, using the DA-3 cell line line (a murine myeloid mGM-CSF dependent cell line [101]) as an indicator. The specificity of the assay was corroborated by the blocking activity of anti mGM-CSF monoclonal antibodies. The amount of mGM-CSF produced by the different plasmids was quantified by ELISA, and the expression and localization of the protein were visualized by fluorescence and or confocal microscopy. Cos-7 cells were chosen for most of these experiments because of the easiness offered by these cells to perform morphological analyses.

In order to demonstrate the stability and biological activity of the plasma membrane anchored mGM-CSF, DA-3 cells were co-cultivated with feeder monolayers of transiently transfected Cos-7 cells with plasmids expressing different forms of mGM-CSF (see materials and methods Paper I). This experiment showed that the use of fixed monolayers of feeder cells is a suitable method for determination of the biological activity of plasma membrane anchored proteins.

2 GENERATION AND CHARACTERIZATION OF GENETICALLY-MODIFIED AUTO ACTIVATED CELL LINES EXPRESSING INTRACELLULAR FORMS OF mGM-CSF

To demonstrate that cytokine dependent cells can be autoactivated by intracellular cytokines, the DA-3 cell was stably transfected with plasmids encoding either extracellular or intracellular forms of mGM-CSF. The degree of autonomous growth

acquired by these cells after gene modification was determined by conventional cell proliferation assays.

The following set of experiments was designed in order to detect the presence of minimal amounts mGM-CSF in the extracellular medium of the cells expressing the intracellular forms of the cytokine; at the same time, some of the experiments were aimed to estimate the proportion of autonomous growth mediated by intracellular activation:

1. Neutralization of the mGM-CSF biological activity by antibodies added to cultures of autoactivated cell lines was used to determine the amount of intracellular activation, as the proportion of non inhibited growth.
2. Co-cultivation experiments of cells expressing mGM-CSF with unmodified cells, separated by a semi permeable membrane, were used to determine small amounts of mGM-CSF released from the intracellular compartments avoiding any growth signal induced by cell-to-cell contact.
3. Co-cultivation of DA-3 cells expressing different forms of mGM-CSF with DA-3 cells expressing GFP was used to exclude the possibility that cell-to-cell contact may be required for possible paracrine stimulation.

3 DNA IMMUNIZATION AGAINST HIV-1 ANTIGENS USING PLASMID OR RECOMBINANT mGM-CSF AS ADJUVANT

In this part of the work we performed a preliminary test of the adjuvant capacity of our vectors expressing mGM-CSF. It has been demonstrated that GM-CSF as adjuvant of DNA vaccines can induce either a Th1 or a Th2 type of immune response [19]. A Th2 type of immune response is not desired for immunization against tumors, because it can exert deleterious effects on the development of the disease, but it could be of interest for preventive vaccines against HIV-1. The induction of a Th2 profile might be related to the GM-CSF inflammatory activity in the neighboring tissues [19]. We hypothesized that coinjection of plasmids expressing intracellular forms of mGM-CSF together with DNA immunogens is a method of appropriate cytokine delivery to induce an adjuvant effect on the Th1 immune response. To test this hypothesis we used a previously established mouse model of DNA immunization with the *gp160* and *rev* HIV-1 genes. We compared the adjuvant effect of plasmids encoding the secreted wild type, the ER and the PM forms of mGM-CSF and the recombinant mGM-CSF protein. In order to obtain a profile of a primary immune response under different conditions of adjuvant activity, mice were immunized with a single i.m. injection of *gp160* and *rev* genes, together with plasmids expressing the different forms of mGM-CSF or with the recombinant protein. Several kinetics of administration and different locations of mGM-CSF delivery (intracellular, PM, or secreted) were combined with the aim to generate variable immunological environments for the antigen presentation and to induce different profiles of immune response (Th1 or Th2). The protocols of immunization are described in the materials methods and depicted in Figure 1 of Paper III.

4 GENERATION OF PLASMIDS EXPRESSING siRNAs AGAINST CCR5 AND REV TRANSCRIPTS AND RNAi MEDIATED INHIBITION OF HIV-1 INFECTION

Recently, a system for plasmid-mediated expression of siRNA, has been developed [99, 102]. Using the pSUPER (conventional) and pRETRO-SUPER (retroviral) plasmids (expressing hairpin shaped RNA transcripts under control of the U6 promoter), we generated two plasmids expressing siRNAs against the Rev and CCR5 gene transcripts (siCCR5 and siRev). The efficacy of these plasmids to down regulate the expression of the respective targets was initially evaluated in experiments of transient transfections. The siRev plasmids were evaluated by cotransfection with a reporter plasmid expressing a Rev-GFP fusion protein. The siCCR5 plasmid was initially evaluated, in transiently transfected U937 cells (a monocytic cell line constitutively expressing the CCR5 coreceptor) by determination of the down regulation of the CCR5 expression by FACS analysis and western blot (data not shown).

The antiviral effect of these plasmids was then evaluated in experimental infections of susceptible cell lines with the appropriate HIV-1 strains. HeLa CD4 cells (a stable transfectant expressing the CD4 molecule) were transfected with the siRev vector followed by infection with the CXCR4-dependent, T-tropic HIV-1 LAI strain. U937 cells were infected with a primary CCR5-dependent, M-tropic, HIV-1 isolate, after transfection with the vector siCCR5. The percentage of protection was evaluated by determination of HIV-1 p24 antigen after 3 days of infection. Stable transfectants expressing the respective siRNAs were developed in order to evaluate the inhibitory activity of the siRNA expressing plasmids in long-term infections.

RESULTS

1 EVALUATION OF THE BIOLOGICAL ACTIVITY OF PLASMIDS EXPRESSING DIFFERENT FORMS OF mGM-CSF TARGETED TO DIFFERENT SUBCELLULAR COMPARTMENTS

The four plasmids described in the section Comments on the Methods and Paper I, expressed different amounts of biologically active mGM-CSF relocated at the different subcellular compartments. The localization of the protein was determined by immunostaining followed by fluorescence and/or confocal microscopy in different types of cell lines as illustrated in Papers I, II and III (Figures 2-3, 2 and 3 respectively). The targeting was initially corroborated by observation of a consistent characteristic morphological expression pattern in a series of experiments. Then, a detailed analysis by multiple staining was performed. A representative example is shown in Figure 2.

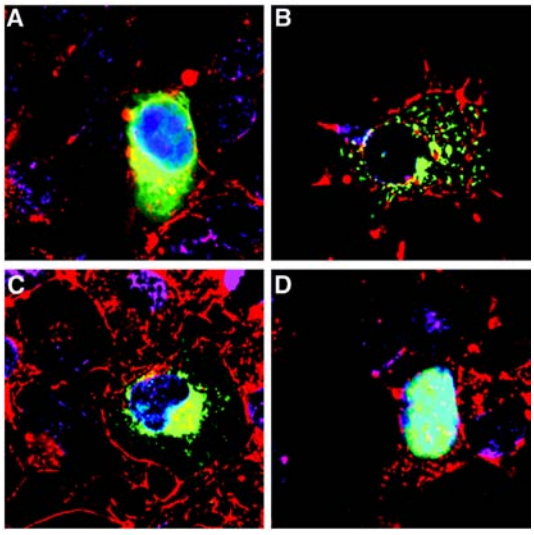

Fig 2. *Expression and localization of mGM-CSF in different subcellular compartments*
Cos-7 cells were transiently transfected with plasmids expressing different forms of mGM-CSF and processed as in Fig. 2. a: cells transfected with the plasmid pEF/cyto/Δ7GM which expresses the secreted form of mGM-CSF tagged with the c-myc epitope. b: cells transfected with the plasmid pEF/cyto/GM which express the mGM-CSF targeted to the cytoplasm. c: cells transfected with the plasmid pEF/ER/GM which express the mGM-CSF targeted to the endoplasmic reticulum. d: cells transfected with the plasmid pEF/nuc/GM which express the mGM-CSF targeted to the nucleus

Determination of biological activity of mGM-CSF showed that, when the protein was targeted to the cytoplasm, nucleus or ER, the majority of the mGM-CSF activity was found in the cell extracts and only a minimal amount was detected in the supernatants of the cell cultures. Similar amounts of mGM-CSF activity were found in the cell extracts of cells transfected with vectors expressing the PM-targeted or the secreted form of mGM-CSF. However, a considerably higher activity was found in the supernatants. In addition, we demonstrated that the PM form of mGM-CSF preserves its biological activity and has the capacity to activate cytokine dependent cells after cell-to-cell interaction (Figures 3 and 4).

2 GENERATION OF AUTO ACTIVATED CELL LINES EXPRESSING INTRACELLULAR FORMS OF mGM-CSF

After characterization of the functionality of plasmids expressing the different forms of mGM-CSF, we demonstrated that the mGM-CSF-dependent DA-3 cell line

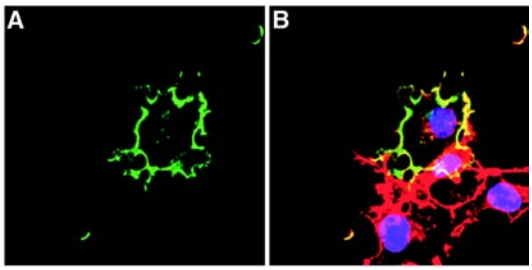

Fig 3. *Expression and localization of the mGM-CSF in the plasma membrane of Cos-7 cells*
Cos-7 cells were transiently transfected with the plasmid pEF/PM/GM and immunostained with a mouse monoclonal antibody against the c-myc epitope and a FICT conjugated goat anti mouse IgG secondary antibody. Plasma membranes and nuclei were counterstained with wheat germ agglutinin (WGA) conjugated with tetramenthylrodhamine and Hoechst, respectively. Cells were visualized by fluorescence microscopy. a: immune-staining with anti c-myc mouse monoclonal antibody; b: colocalization on the plasma membrane of the anti c-myc immune-staining (green) and plasma membrane staining with rodhamine conjugated WGA (red). The nuclei are stained in blue.

acquires autonomous growth after transfection with plasmids encoding either extracellular or intracellular forms of mGM-CSF (Figure 3, Paper II). Cell lines expressing secreted forms of mGM-CSF displayed the highest rates of autonomous growth and released substantial amounts of mGM-CSF to the extracellular medium. However, cell lines expressing intracellular forms of the cytokine also acquired autonomous growth induced by a mechanism of restricted autocrine stimulation and did not release detectable mGM-CSF to the medium (Figures 4 and 6). By adding anti mGM-CSF neutralizing antibodies to the extracellular medium, it was demonstrated that just a minimal proportion of the growth was inhibited in these cells. In contrast, most of the growth was inhibited in the cells expressing the secreted form (Figure 5, Paper II). These results indicate that only a minimal proportion of the autonomous growth observed in the cells expressing the intracellular forms of mGM-CSF may be associated with exogenous release of the cytokine. In addition, we were unable to detect mGM-CSF activity in the extracellular compartment in any of the additional experiments specifically designed to detect minimal amounts of paracrine mGM-CSF activity (Figures 6 and 7, PaperII).

3 EVALUATION OF PLASMIDS EXPRESSING DIFFERENT FORMS OF mGM-CSF AS ADJUVANTS OF DNA VACCINES

We performed a preliminary test of the adjuvant capacity of our vectors using a previously established mouse model of DNA immunization with HIV-1 genes. Here, we compared the adjuvant effect of plasmids expressing the ER, the PM and the secreted forms of mGM-CSF and the recombinant mGM-CSF protein. After immunization with a single i.m. injection of *gp160* and *rev* genes, together with plasmids expressing the different forms of mGM-CSF, a pronounced enhancement of a Th1 type immune response was induced. On the other hand, immunization with recombinant mGM-CSF always induced a Th2 humoral immune response. The plasmid expressing the ER form of mGM-CSF, showed the most consistent and one of the best adjuvant activities for a Th1 response even when injected for three consecutive days before the DNA vaccine (a procedure previously reported as associated with induction of a Th2 response [19]. Immunization with the plasmid expressing the secreted form of mGM-CSF three days prior to the antigen inoculation, showed a good adjuvant effect of a Th2 humoral immune response, in agreement with previous observations [19].

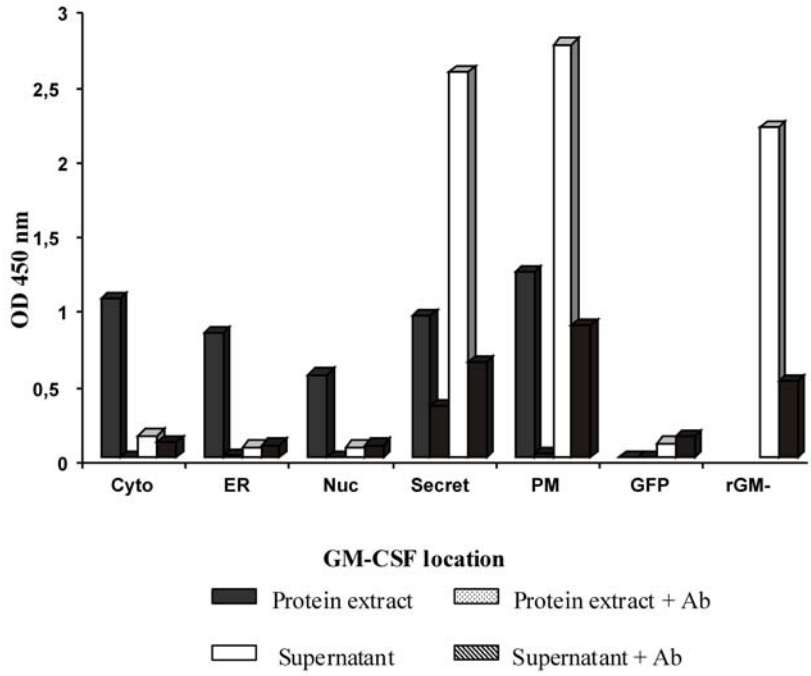

Fig 4. *Biological activity of mGM-CSF obtained from different subcellular compartments.*
Cos-7 cells were transiently transfected with plasmids expressing different forms of mGM-CSF or GFP. After 48 h of transfection the culture supernatants were collected and cells were harvested and washed. Protein extracts were obtained by freezing and thawing in 200 µl of PBS. The biological activity of mGM-CSF in the samples was quantified using the DA-3 cell proliferation assay (see materials and methods). 10^4 DA-3 cells per well were seeded in 96 wells microtiter plates in a 200 µl final volume of 40 µl of cell protein extract plus 160 µl of medium, 100 µl of supernatants plus 100 µl of medium or medium plus 0.25ng/ml of recombinant mGM-CSF (ED50). Parallel samples were prepared with a 1:100 dilution of monoclonal antibody against mGM-CSF. The values are the raw OD at 450 nm obtained after blanking with medium plus WST-1 reagent and come from one of more than two similar experiments. The X bar represents the different mGM-CSF localization. Cyto: cytoplasmic localization, ER: endoplasmic reticulum localization, Nuc: nuclear localization, Secret: secreted formand and PM: plasma membrane localization. GFP: cells transfected with a similar vector expressing GFP. rGM: recombinat mGM-CSF. Ab: monoclonal anti mGM-CSF antibody

These results suggest that mGM-CSF is a good adjuvant for immunization, used either as a recombinant protein or in the context of plasmids, but the kinetics, the localization and perhaps the concentration of the protein can induce either a Th1 or a Th2 response. However, intracellular forms of this cytokine seem to be suitable adjuvants for enhancing consistently a cellular immune response.

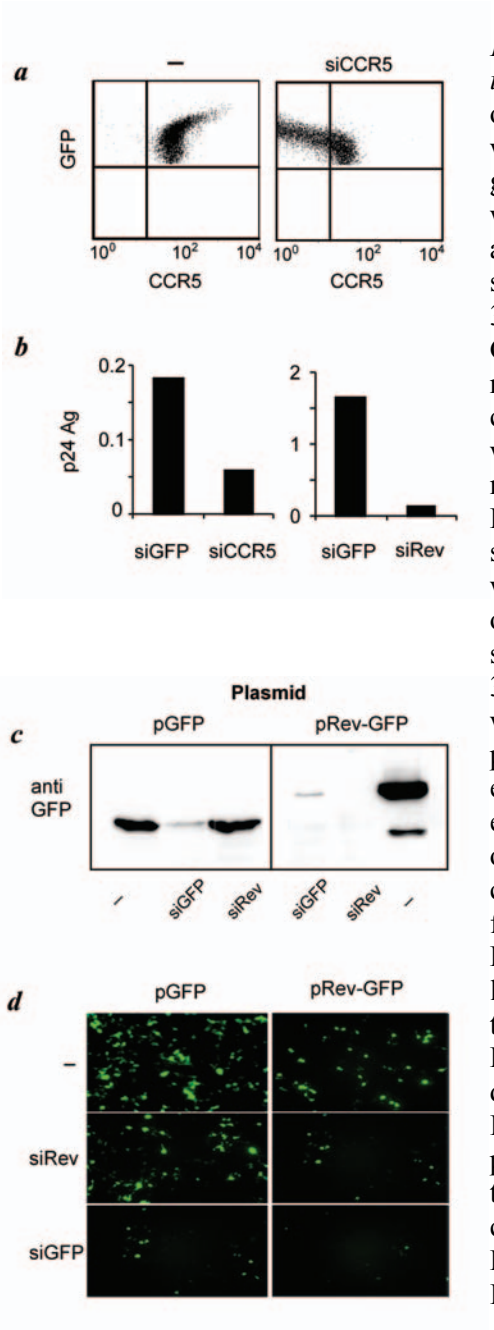

Fig. 5 *Analysis of siRNA-mediated inhibition of HIV-1*. ***a,*** Down-regulation of chemokine receptor CCR5 expression with siRNA. FACS analysis of GFP-gated U937 cells, following transfection with (right panel) or without (left panel) a plasmid encoding a CCR5-specific siRNA (siCCR5; 5'-caggttggaccaagctatg-3') together with a plasmid encoding GFP**.** ***b***, Inhibiton of HIV-1 infection and replication in tissue culture cells. U937 cells expressing a CCR5-specific siRNA were challenged with a primary macrophage tropic isolate of HIV-1. HeLa-CD4 cells expressing Rev-specific siRNA (siRev; 5'-acttactcttgattgtaac-3') were infected with a T-cell tropic strain of HIV-1. In both cases, a GFP-specific siRNA (siGFP; 5'-gaacggcatcaaggtgaac-3') was used as control. Virus replication was monitored by detection of the HIV-1 p24 antigen. ***c,*** siRNA blocks the expression of HIV-1 Rev. Plasmids expressing siRNAs directed against GFP or Rev were co-transfected with constructs encoding GFP or Rev-GFP fusion protein into 293 cells. Determination of protein steady-state levels by western blot analysis 48h post-transfection shows that expression of the Rev-GFP fusion protein was significantly down-regulated by both siRNAs. d, Imaging of cells treated as described in panel c 72h post-transfection. Mock transfection, upper panel. The siRNA cloning vector6 was kindly provided by Dr. R. Agami, The Netherlands Cancer Institute.

4 INHIBITION OF HIV-1 INFECTION BY PLASMID MEDIATED EXPRESSION OF siRNAs AGAINST CCR5 AND REV TRANSCRIPTS

U937 cells transiently transfected with the plasmid siCCR5, showed approximately 40% reduction of CCR5 expression 48 h post-transfection. Recently we have generated a population of stable transfectants, which expresses approximately 50% reduction of CCR5 expression in comparison with the unmodified cells (unpublished data). This treatment inhibited the infection with a primary CCR5-dependent, M-tropic, HIV-1

isolate, by 68% after 3 days of culture in the transient transfectants, and by more than 85% after 14 days in stable transfectants (unpublished data). In addition, the plasmid siRev blocked more than 90% expression of a reporter plasmid expressing a Rev-GFP fusion protein in transiently co-transfected 293 cells. After transient transfection of HeLa CD4 cells followed by infection with the CXCR4-dependent, T-tropic HIV-1 LAI strain viral replication was inhibited by more than 90%, 3 days post-infection. This high efficiency of inhibition was corroborated using cultures with different proportions of stable transfectants. Cell lines stably expressing the siRev plasmids were protected by more than 90% for 10 days and a slow decline was observed afterwards (unpublished data). Figure 5

CONCLUDING REMARKS

Using the protein targeting strategy we have shown that cytokines can be expressed and targeted to different sub-cellular compartments, as stable active proteins.

The mGM-CSF-dependent DA-3 cell line was transformed into a cytokine independent cell line by stable transfection with plasmids encoding either the secreted or intracellular forms of mGM-CSF. The cell lines expressing the intracellular forms of mGM-CSF acquire a pattern of very restricted autocrine stimulation by a mechanism that may take place at intracellular level. We propose this model as a proof of concept that a highly localized form of cytokine delivery is the production of autoactivated cells expressing *intracellular cytokines*. By using this approach, it might be possible to achieve the desired effects of the cytokines on the target cells, in protocols of immunotherapy involving the preactivation of immunocompetent cells and at the same time, it could be possible to avoid the collateral effects of the uncontrolled secretion of these proteins. We suggest that a similar approach could be used for the production of autoactivated cells in other models of interest such as DC expressing GM-CSF for immunization, or autoactivated NK cells expressing intracellular forms of IL-2 for the treatment of tumors. In the latter case, the genetic modification of NK cells with genes encoding intracellular forms of IL-2 may avoid the well known undesired collateral effects of the secreted form of this cytokine, especially in the treatment of relapse of hematological tumors after bone marrow transplantation.

mGM-CSF showed a good adjuvant activity for DNA immunizations against HIV-1, used either as a recombinant protein or in the context of plasmids. These results suggest that intracellular forms of GM-CSF could be used to induce a consistent Th1 response, while soluble forms are suitable for induction of a good quality humoral immune response.

The siRNA technology was evaluated as an alternative strategy for therapy against tumor or viral diseases. We have successfully inhibited both HIV-1 infection and replication by using plasmids expressing siRNA against Rev and CCR5 transcripts Although both approaches seem worthwhile, we believe that treatments limited to the inhibition of viral products by single siRNA species will not be clinically feasible if the possibility of appearance of escape mutants is not tackled successfully [83]. Thus, the use of siRNA directed against suitable cellular products such as CCR5 combined with various siRNAs against strategic regions of the HIV-1 genome, would be preferred. It is likely that the combined targeting of cellular and viral sequences could have a synergistic effect.

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REFERENCES

1. Jaffee, E.M. and D.M. Pardoll, *Considerations for the clinical development of cytokine gene-transduced tumor cell vaccines.* Methods, 1997. **12**(2): p. 143-53.
2. Pardoll, D.M., *Paracrine cytokine adjuvants in cancer immunotherapy.* Annu Rev Immunol, 1995. **13**: p. 399-415.
3. Svanholm, C., B. Lowenadler, and H. Wigzell, *Amplification of T-cell and antibody responses in DNA-based immunization with HIV-1 Nef by co-injection with a GM-CSF expression vector.* Scand J Immunol, 1997. **46**(3): p. 298-303.
4. Samanci, A., et al., *Pharmacological administration of granulocyte/macrophage-colony-stimulating factor is of significant importance for the induction of a strong humoral and cellular response in patients immunized with recombinant carcinoembryonic antigen.* Cancer Immunol Immunother, 1998. **47**(3): p. 131-42.
5. Kawakami, Y., et al., *T-cell recognition of human melanoma antigens.* J Immunother, 1993. **14**(2): p. 88-93.
6. Bergmann, L., *The clinical significance of interleukin-2.* Onkologie, 1990. **13**(6): p. 416-22.
7. Parkinson, D.R., *The role of interleukin-2 in the biotherapy of cancer.* Oncol Nurs Forum, 1989. **16**(6 Suppl): p. 16-20.
8. Parmiani, G., et al., *Cytokine gene transduction in the immunotherapy of cancer.* Adv Pharmacol, 1997. **40**: p. 259-307.
9. Tam, Y.K., et al., *Characterization of genetically altered, interleukin 2-independent natural killer cell lines suitable for adoptive cellular immunotherapy.* Hum Gene Ther, 1999. **10**(8): p. 1359-73.
10. Curiel-Lewandrowski, C., et al., *Transfection of immature murine bone marrow-derived dendritic cells with the granulocyte-macrophage colony-stimulating factor gene potently enhances their in vivo antigen-presenting capacity.* J Immunol, 1999. **163**(1): p. 174-83.
11. Nakamura, M., et al., *Dendritic Cells Genetically Engineered to Simultaneously Express Endogenous Tumor Antigen and Granulocyte Macrophage Colony-stimulating Factor Elicit Potent Therapeutic Antitumor Immunity.* Clin Cancer Res, 2002. **8**(8): p. 2742-9.
12. Leizer, T., et al., *Cytokine regulation of colony-stimulating factor production in cultured human synovial fibroblasts: I. Induction of GM-CSF and G-CSF production by interleukin-1 and tumor necrosis factor.* Blood, 1990. **76**(10): p. 1989-96.
13. Hamilton, J.A., *GM-CSF in inflammation and autoimmunity.* Trends Immunol, 2002. **23**(8): p. 403-8.
14. Filonzi, E.L., et al., *Cytokine regulation of granulocyte-macrophage colony stimulating factor and macrophage colony-stimulating factor production in human arterial smooth muscle cells.* Atherosclerosis, 1993. **99**(2): p. 241-52.
15. Hamilton, J.A., *Colony stimulating factors, cytokines and monocyte-macrophages--some controversies.* Immunol Today, 1993. **14**(1): p. 18-24.
16. Bozinovski, S., et al., *Granulocyte/macrophage-colony-stimulating factor (GM-CSF) regulates lung innate immunity to lipopolysaccharide through Akt/Erk activation of NFkappa B and AP-1 in vivo.* J Biol Chem, 2002. **277**(45): p. 42808-14.
17. Dranoff, G., et al., *Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity.* Proc Natl Acad Sci U S A, 1993. **90**(8): p. 3539-43.
18. Dunussi-Joannopoulos, K., et al., *Gene immunotherapy in murine acute myeloid leukemia: granulocyte-macrophage colony-stimulating factor tumor cell vaccines elicit more potent antitumor immunity compared with B7 family and other cytokine vaccines.* Blood, 1998. **91**(1): p. 222-30.
19. Kusakabe, K., et al., *The timing of GM-CSF expression plasmid administration influences the Th1/Th2 response induced by an HIV-1-specific DNA vaccine.* J Immunol, 2000. **164**(6): p. 3102-11.
20. Liu, K. and S.A. Rosenberg, *Transduction of an IL-2 gene into human melanoma-reactive lymphocytes results in their continued growth in the absence of exogenous IL-2 and maintenance of specific antitumor activity.* J Immunol, 2001. **167**(11): p. 6356-65.
21. Laker, C., et al., *Autocrine stimulation after transfer of the granulocyte/macrophage colony-stimulating factor gene and autonomous growth are distinct but interdependent steps in the oncogenic pathway.* Proc Natl Acad Sci U S A, 1987. **84**(23): p. 8458-62.
22. Persic, L., et al., *Targeting vectors for intracellular immunisation.* Gene, 1997. **187**(1): p. 1-8.
23. Moore, B.B., et al., *GM-CSF regulates bleomycin-induced pulmonary fibrosis via a prostaglandin-dependent mechanism.* J Immunol, 2000. **165**(7): p. 4032-9.
24. Kalinski, P., et al., *T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal.* Immunol Today, 1999. **20**(12): p. 561-7.
25. Shreedhar, V., et al., *A cytokine cascade including prostaglandin E2, IL-4, and IL-10 is responsible for UV-induced systemic immune suppression.* J Immunol, 1998. **160**(8): p. 3783-9.
26. Nagata, M., et al., *Eosinophil-adhesion-inducing activity produced by antigen-stimulated mononuclear cells involves GM-CSF.* Int Arch Allergy Immunol, 2000. **122 Suppl 1**: p. 15-9.

27. Browder, T.M., et al., *Mechanism of autocrine stimulation in hematopoietic cells producing interleukin-3 after retrovirus-mediated gene transfer.* Mol Cell Biol, 1989. **9**(1): p. 204-13.
28. Toby, G., S.F. Law, and E.A. Golemis, *Vectors to target protein domains to different cellular compartments.* Biotechniques, 1998. **24**(4): p. 637-40.
29. Yang, A.-G., et al., *Phenotypic knockout of HIV type 1 chemokine coreceptor CCR-5 by intrakines as potential therapeutic approach for HIV-1 infection.* PNAS, 1997. **94**(21): p. 11567-11572.
30. Arteaga, H.J., et al., *Expression and release of stable and active forms of murine granulocyte-macrophage colony-stimulating factor (mGM-CSF) targeted to different subcellular compartments.* Cytokine, 2001. **14**(3): p. 136-42.
31. Ruggeri, L., et al., *Cellular therapy: exploiting NK cell alloreactivity in transplantation.* Curr Opin Hematol, 2001. **8**(6): p. 355-9.
32. Ou-Yang, P., et al., *Co-delivery of GM-CSF gene enhances the immune responses of hepatitis C viral core protein-expressing DNA vaccine: role of dendritic cells.* J Med Virol, 2002. **66**(3): p. 320-8.
33. Wolff, J.A., et al., *Direct gene transfer into mouse muscle in vivo.* Science, 1990. **247**(4949 Pt 1): p. 1465-8.
34. Tang, D.C., M. DeVit, and S.A. Johnston, *Genetic immunization is a simple method for eliciting an immune response.* Nature, 1992. **356**(6365): p. 152-4.
35. Ulmer, J.B., et al., *Generation of MHC class I-restricted cytotoxic T lymphocytes by expression of a viral protein in muscle cells: antigen presentation by non-muscle cells.* Immunology, 1996. **89**(1): p. 59-67.
36. Boyer, J.D., et al., *HIV-1 DNA based vaccine induces a CD8 mediated cross-clade CTL response.* Dev Biol Stand, 1998. **95**: p. 147-53.
37. Gurunathan, S., D.M. Klinman, and R.A. Seder, *DNA vaccines: immunology, application, and optimization*.* Annu Rev Immunol, 2000. **18**: p. 927-74.
38. MacGregor, R.R., et al., *First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response.* J Infect Dis, 1998. **178**(1): p. 92-100.
39. Boyer, J.D., et al., *Vaccination of seronegative volunteers with a human immunodeficiency virus type 1 env/rev DNA vaccine induces antigen-specific proliferation and lymphocyte production of beta-chemokines.* J Infect Dis, 2000. **181**(2): p. 476-83.
40. MacGregor, R.R., et al., *Safety and immune responses to a DNA-based human immunodeficiency virus (HIV) type I env/rev vaccine in HIV-infected recipients: follow-up data.* J Infect Dis, 2000. **181**(1): p. 406.
41. Calarota, S., et al., *Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients.* Lancet, 1998. **351**(9112): p. 1320-5.
42. Shiver, J.W., et al., *Anti-HIV env immunities elicited by nucleic acid vaccines.* Vaccine, 1997. **15**(8): p. 884-7.
43. Tsuji, T., et al., *Enhancement of cell-mediated immunity against HIV-1 induced by coinnoculation of plasmid-encoded HIV-1 antigen with plasmid expressing IL-12.* J Immunol, 1997. **158**(8): p. 4008-13.
44. Okuda, K., et al., *Induction of potent humoral and cell-mediated immune responses following direct injection of DNA encoding the HIV type 1 env and rev gene products.* AIDS Res Hum Retroviruses, 1995. **11**(8): p. 933-43.
45. Wang, B., et al., *Gene inoculation generates immune responses against human immunodeficiency virus type 1.* Proc Natl Acad Sci U S A, 1993. **90**(9): p. 4156-60.
46. Warren, T.L. and G.J. Weiner, *Uses of granulocyte-macrophage colony-stimulating factor in vaccine development.* Curr Opin Hematol, 2000. **7**(3): p. 168-73.
47. Kim, J.J., et al., *Cytokine molecular adjuvants modulate immune responses induced by DNA vaccine constructs for HIV-1 and SIV.* J Interferon Cytokine Res, 1999. **19**(1): p. 77-84.
48. Lee, A.H., Y.S. Suh, and Y.C. Sung, *DNA inoculations with HIV-1 recombinant genomes that express cytokine genes enhance HIV-1 specific immune responses.* Vaccine, 1999. **17**(5): p. 473-9.
49. Okada, E., et al., *Intranasal immunization of a DNA vaccine with IL-12- and granulocyte-macrophage colony-stimulating factor (GM-CSF)-expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens.* J Immunol, 1997. **159**(7): p. 3638-47.
50. Barouch, D.H., et al., *Potent CD4+ T cell responses elicited by a bicistronic HIV-1 DNA vaccine expressing gp120 and GM-CSF.* J Immunol, 2002. **168**(2): p. 562-8.
51. Jones, T., A. Stern, and R. Lin, *Potential role of granulocyte-macrophage colony-stimulating factor as vaccine adjuvant.* Eur J Clin Microbiol Infect Dis, 1994. **13 Suppl 2**: p. S47-53.
52. Hannon, G.J., *RNA interference.* Nature, 2002. **418**(6894): p. 244-51.
53. Sharp, P.A., *RNA interference--2001.* Genes Dev, 2001. **15**(5): p. 485-90.
54. Fire, A., et al., *Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans.* Nature, 1998. **391**(6669): p. 806-11.
55. Hutvagner, G. and P.D. Zamore, *RNAi: nature abhors a double-strand.* Curr Opin Genet Dev, 2002. **12**(2): p. 225-32.
56. Mette, M.F., et al., *Transcriptional silencing and promoter methylation triggered by double-stranded RNA.* Embo J, 2000. **19**(19): p. 5194-201.
57. Bosher, J.M. and M. Labouesse, *RNA interference: genetic wand and genetic watchdog.* Nat Cell Biol, 2000. **2**(2): p. E31-6.

58. Tenllado, F. and J.R. Diaz-Ruiz, *Double-stranded RNA-mediated interference with plant virus infection.* J Virol, 2001. **75**(24): p. 12288-97.
59. Ruiz, M.T., O. Voinnet, and D.C. Baulcombe, *Initiation and maintenance of virus-induced gene silencing.* Plant Cell, 1998. **10**(6): p. 937-46.
60. Li, H., W.X. Li, and S.W. Ding, *Induction and Suppression of RNA Silencing by an Animal Virus.* Science, 2002. **296**(5571): p. 1319-1321.
61. Dougherty, W.G., et al., *RNA-mediated virus resistance in transgenic plants: exploitation of a cellular pathway possibly involved in RNA degradation.* Mol Plant Microbe Interact, 1994. **7**(5): p. 544-52.
62. Knight, S.W. and B.L. Bass, *A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans.* Science, 2001. **293**(5538): p. 2269-71.
63. Ketting, R.F., et al., *Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans.* Genes Dev, 2001. **15**(20): p. 2654-9.
64. Zamore, P.D., et al., *RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals.* Cell, 2000. **101**(1): p. 25-33.
65. Bernstein, E., et al., *Role for a bidentate ribonuclease in the initiation step of RNA interference.* Nature, 2001. **409**(6818): p. 363-6.
66. Boutla, A., et al., *Short 5'-phosphorylated double-stranded RNAs induce RNA interference in Drosophila.* Curr Biol, 2001. **11**(22): p. 1776-80.
67. Hammond, S.M., et al., *Argonaute2, a link between genetic and biochemical analyses of RNAi.* Science, 2001. **293**(5532): p. 1146-50.
68. Samuel, C.E., *Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities.* Virology, 1991. **183**(1): p. 1-11.
69. O'Malley, R.P., et al., *A mechanism for the control of protein synthesis by adenovirus VA RNAI.* Cell, 1986. **44**(3): p. 391-400.
70. Mathews, M.B., *Control of translation in adenovirus-infected cells.* Enzyme, 1990. **44**(1-4): p. 250-64.
71. Maran, A. and M.B. Mathews, *Characterization of the double-stranded RNA implicated in the inhibition of protein synthesis in cells infected with a mutant adenovirus defective for VA RNA.* Virology, 1988. **164**(1): p. 106-13.
72. Davis, S. and J.C. Watson, *In vitro activation of the interferon-induced, double-stranded RNA-dependent protein kinase PKR by RNA from the 3' untranslated regions of human alpha-tropomyosin.* Proc Natl Acad Sci U S A, 1996. **93**(1): p. 508-13.
73. Wianny, F. and M. Zernicka-Goetz, *Specific interference with gene function by double-stranded RNA in early mouse development.* Nat Cell Biol, 2000. **2**(2): p. 70-5.
74. Elbashir, S.M., et al., *Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells.* Nature, 2001. **411**(6836): p. 494-8.
75. Elbashir, S.M., W. Lendeckel, and T. Tuschl, *RNA interference is mediated by 21- and 22-nucleotide RNAs.* Genes Dev, 2001. **15**(2): p. 188-200.
76. Paddison, P.J., et al., *Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells.* Genes Dev, 2002. **16**(8): p. 948-58.
77. Grosshans, H. and F.J. Slack, *Micro-RNAs: small is plentiful.* J Cell Biol, 2002. **156**(1): p. 17-21.
78. Lee, R.C. and V. Ambros, *An extensive class of small RNAs in Caenorhabditis elegans.* Science, 2001. **294**(5543): p. 862-4.
79. Lagos-Quintana, M., et al., *Identification of tissue-specific microRNAs from mouse.* Curr Biol, 2002. **12**(9): p. 735-9.
80. Waterhouse, P.M., M.B. Wang, and T. Lough, *Gene silencing as an adaptive defence against viruses.* Nature, 2001. **411**(6839): p. 834-42.
81. Hu, W.Y., et al., *Inhibition of retroviral pathogenesis by RNA interference.* Curr Biol, 2002. **12**(15): p. 1301-11.
82. Kapadia, S.B., A. Brideau-Andersen, and F.V. Chisari, *Interference of hepatitis C virus RNA replication by short interfering RNAs.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 2014-8.
83. Gitlin, L., S. Karelsky, and R. Andino, *Short interfering RNA confers intracellular antiviral immunity in human cells.* Nature, 2002. **418**(6896): p. 430-4.
84. Gunnery, S., et al., *Tat-responsive region RNA of human immunodeficiency virus 1 can prevent activation of the double-stranded-RNA-activated protein kinase.* Proc Natl Acad Sci U S A, 1990. **87**(22): p. 8687-91.
85. Capodici, J., K. Kariko, and D. Weissman, *Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference.* J Immunol, 2002. **169**(9): p. 5196-201.
86. Jacque, J.M., K. Triques, and M. Stevenson, *Modulation of HIV-1 replication by RNA interference.* Nature, 2002. **418**(6896): p. 435-8.
87. Novina, C.D., et al., *siRNA-directed inhibition of HIV-1 infection.* Nat Med, 2002. **8**(7): p. 681-6.
88. Coburn, G.A. and B.R. Cullen, *Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference.* J Virol, 2002. **76**(18): p. 9225-31.
89. Wu, Y. and J.W. Marsh, *Selective Transcription and Modulation of Resting T Cell Activity by Preintegrated HIV DNA.* Science, 2001. **293**(5534): p. 1503-1506.

90. Lee, N.S., et al., *Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells.* Nat Biotechnol, 2002. **20**(5): p. 500-5.
91. Paul, C.P., et al., *Localized expression of small RNA inhibitors in human cells.* Mol Ther, 2003. **7**(2): p. 237-47.
92. Bitko, V. and S. Barik, *Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses.* BMC Microbiol, 2001. **1**(1): p. 34.
93. Arteaga, H.J., et al., *Choosing CCR5 or Rev siRNA in HIV-1.* Nat Biotechnol, 2003. **21**(3): p. 230-1.
94. Martinez, M.A., et al., *Suppression of chemokine receptor expression by RNA interference allows for inhibition of HIV-1 replication.* Aids, 2002. **16**(18): p. 2385-90.
95. Qin, X.F., et al., *Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5.* Proc Natl Acad Sci U S A, 2003. **100**(1): p. 183-8.
96. Martinson, J.J., et al., *Global distribution of the CCR5 gene 32-basepair deletion.* Nat Genet, 1997. **16**(1): p. 100-3.
97. Zuber, B., et al., *Induction of immune responses and break of tolerance by DNA against the HIV-1 coreceptor CCR5 but no protection from SIVsm challenge.* Virology, 2000. **278**(2): p. 400-11.
98. Huang, Y., et al., *The role of a mutant CCR5 allele in HIV-1 transmission and disease progression.* Nat Med, 1996. **2**(11): p. 1240-3.
99. Brummelkamp, T.R., R. Bernards, and R. Agami, *A system for stable expression of short interfering RNAs in mammalian cells.* Science, 2002. **296**(5567): p. 550-3.
100. Kawasaki, H., et al., *siRNAs generated by recombinant human Dicer induce specific and significant but target site-independent gene silencing in human cells.* Nucleic Acids Res, 2003. **31**(3): p. 981-7.
101. Ihle, J., *Advances in viral oncology*, G. Klein, Editor. 1984, Raven Press: New York. p. 95/137.
102. Brummelkamp, T., R. Bernards, and R. Agami, *Stable suppression of tumorigenicity by virus-mediated RNA interference.* Cancer Cell, 2002. **2**(3): p. 243.