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REVIEW

Genetically modified immunocompetent cells in HIV infection

G Palù[1], G Li Pira[2], F Gennari[1], D Fenoglio[2], C Parolin[1] and F Manca[2]
[1]*Department of Histology, Microbiology and Medical Biotechnologies, University of Padua, Padua, Italy; and* [2]*Unit of Viral Immunology, Advanced Biotechnology Center, Genoa, Italy*

Even in the era of highly active antiretroviral therapy (HAART), gene therapy (GT) can remain a promising approach for suppressing HIV infection, especially if complemented with other forms of pharmacological and immunological intervention. A large number of vectors and targets have been studied. Here we discuss the potential of genetically treated, antigen-specific immunocompetent cells for adoptive autologous immunotherapy of HIV infection. Cellular therapies with gene-modified CD8 and CD4 lymphocytes are aimed at reconstituting the antigen-specific repertoires that may be deranged as a consequence of HIV infection. Even if complete eradication of HIV from the reservoirs cannot be achieved, reconstitution of cellular immunity specific for opportunistic pathogens and for HIV itself is a desirable option to control progression of HIV infection and AIDS pathogenesis better. Gene Therapy (2001) **8,** 1593–1600.

Keywords: *gene therapy; cell therapy; T cell repertoire; opportunistic pathogens; HIV; immunoreconstitution*

Introduction

HIV infection can be viewed as an acquired genetic disorder. Following infection of a host cell, the viral RNA genome is retrotranscribed into DNA to undergo integration into the host cell genome. This confers to the somatic cell a new genetic trait that becomes inheritable to its progeny. Cells critical for the immune response, such as CD4 lymphocytes and antigen presenting cells (APC), are the preferential targets of HIV infection and cytopathicity. Therefore, severe immune dysfunction is the consequence of this infection. Highly active antiretroviral therapy (HAART) has been successfully adopted to prevent viral replication and infection, with numerical and functional recovery of CD4 cells in many patients.[1,2] The integration of the HIV genome into a latent proviral state, however, renders HIV eradication virtually impossible by antiviral drugs. In fact, whereas HAART can successfully control productive viral replication and plasma viremia in most chronically infected individuals, the presence of residual latently infected cell reservoirs can serve as a potential source of viral reactivation.[3] The presence of latently infected, resting $CD4^+$ T cells carrying replication-competent HIV-1 has been demonstrated in chronically infected individuals who are both antiretroviral therapy-naive or receiving HAART. It has been estimated that viral eradication from the latent reservoir could take as long as 30 to 60 years of antiretroviral therapy.[4–6] Because HIV-1 latency in resting $CD4^+$ T cells is likely established early in the course of infection, when viral loads are high, as well as levels of activated $CD4^+$ T cells, it is critical to assess the possibility of blocking establishment of latency by early treatment. However, the influence of antiviral therapy on the immunological control of virus replication must be considered. In this regard, early HAART for acute HIV-1 infection has been recently reported both to preserve function of HIV-1-specific $CD8^+$ and $CD4^+$ lymphocytes[7] and to inhibit the anti-HIV non cytotoxic $CD8^+$ cell response.[8] These opposite effects could have a quite different impact in the control of retroviral infection. Further studies are therefore needed to elucidate the benefit of early HAART on immune function and disease progression. This conflicting biological evidence notwithstanding, side-effects of HAART, such as toxicity[9] and emergence of resistant strains[10,11] may render an early adopted, life-long treatment quite unacceptable to patients. For these reasons international guidance for clinicians include recommendations to postpone antiretroviral chemotherapy until immunological or virological signs of progressing HIV infection appear.[12,13] Additional therapeutic intervention must therefore be conceived.

In this context gene therapy (GT) appears as an extremely attractive option that could complement HAART early after primary infection. Intracellular expression of a therapeutic gene can prevent *de novo* viral infection, suppress viral replication in infected cells or endow the cells with novel properties that may play an adjuvant role in inhibiting HIV infection/replication. Since the vectors, the gene products and the strategies that can be deployed for GT of AIDS have been the subject of recent reviews,[14–16] we focus here on the therapeutic use of genetically treated cells and propose a new combined modality of gene and cell therapy. This is based on the adoptive transfer of HIV-genetically resistant CD4 cells that recognize specific antigens of opportunistic pathogens or of HIV itself.

Correspondence: G Palù, Department of Histology, Microbiology and Medical Biotechnologies, University of Padua, Via A Gabelli 63, 35121 Padua, Italy

Cellular targets for GT of HIV infection

Different cell types have been tested for GT of HIV infection *in vitro* and *in vivo*. These include cells that are naturally susceptible to HIV infection, as well as progenitors thereof (stem cells) giving rise, through differentiation, to CD4-lymphocytic and monocyte/dendritic lineages carrying the therapeutic genes.[17] CD4 cells can be obtained as proliferating cells by mitogenic activation[18,19] or by antigen stimulation.[20,21] Mitogenically activated cells mirror the polyclonal repertoire, but their activation is not physiological. Antigen-stimulated cells display a more limited repertoire of T cell receptors, selected according to antigen specificity, but their stimulation, involving antigen and antigen presenting cells (APC), is similar to the physiological process of activation.[22] Monocyte/dendritic cells have been studied as a differentiated progeny of stem cells[23] or as cells derived from peripheral blood mononuclear cells (PBMC).[24]

Stem cells

The rationale for targeting hemopoietic progenitor stem cells (HSC) is that they are not susceptible to HIV infection,[25] whilst CD4 lymphocytes and antigen-presenting cells deriving from these precursors are infectable. HSC have been obtained for GT applications from bone marrow,[26] or from peripheral blood in granulocyte colony-stimulating factor (G-CSF)-treated donors.[27] Ribozymes,[28] antisense constructs[23] and a transdominant mutant of the Rev regulatory protein[29] have been introduced as anti-HIV therapeutic gene.

If engineered to become resistant to HIV, HSC can give rise, after *in vitro* culture, to an HIV-free/HIV-resistant progeny ideally suited for the treatment of AIDS. However, a long time may elapse before HSC differentiate along the CD4 T cell lineage, rearrange T cell receptor (TCR) genes to generate antigen specificity[30] and expand upon antigen stimulation to an adequate population size.

The use of HIV-free/HIV-resistant CD4 cells differentiated from HSC *in vitro*[31] may have an important therapeutic impact especially for patients with progressing AIDS or with high viral load because of drug-resistant strains or inability to comply with HAART.[32–34] One should however be conscious that these *ex vivo* differentiated CD4 cells, when reinfused into patients, may carry the risk of generating autoreactive responses for having skipped thymic selection.

Polyclonal CD4 lymphocytes

Polyclonal CD4 lymphoblasts have long been a target for HIV gene therapy.[35] Activated CD4 lymphocytes are naturally susceptible to vectors that transduce replicating cells. These lymphocytes can be easily selected according to drug resistance[29] or cell sorting[36,37] before being challenged with HIV-1. Genetically transduced CD4 lymphocytes have been used in clinical trials to test life span and homing, as well as resistance to HIV.[38,39] Even though increased CD4 counts have been shown following genetic intervention, recovery of immune competence has not been fully demonstrated.[38,39]

An approach for a direct *in vivo* delivery of oncolentiviral vectors able to selectively target CD4 positive cells has been reported.[40,41] This approach, however, is unlikely to give rise to sufficient numbers of transduced cells.

Antigen-specific CD4 lymphocytes and their use in a new combined approach of cell and gene therapy of AIDS

The events leading to CD4 depletion in HIV infection are still poorly understood.[42,43] Selective depletion of CD4 lymphocytes specific for opportunistic pathogens and for HIV is likely to occur following HIV infection, as depicted in Figure 1. Since CD4 cells specific for the most common opportunistic pathogens are in a chronic state of activation, they are ideally suited to sustain HIV replication and cytopathicity and therefore prone to be lost from the repertoire.[44,45] It has also been recently reported that HIV infection of CD4 cells results in downmodulation of the TCR/CD3 complex, resulting in defective antigen recognition.[46] Figure 1 illustrates an additional proposed mechanism by which HIV-infected CD4 cells can be eliminated. This is mediated by HIV-specific CTL that kill target cells expressing viral antigens.[47] Such an event, in principle can be bypassed if HIV-infected cells revert to a viral latency state as during thymopoiesis,[48] or if viral genome expression is inhibited by genetic treatment, as described later.

Outbreak of opportunistic infections is the consequence of the loss of specific CD4 cells.[49] Intracellular bacteria, such as *Mycobacteria*, figure among opportunistic pathogens that prevail in HIV-infected patients. The cause is an inefficient macrophage activation due to decreased production of IFNγ by Th1 CD4 cells.[50] Similar mechanisms may be at work in fungal infections, specific CD4 cells being essential to control *Candida* and *Pneumo-*

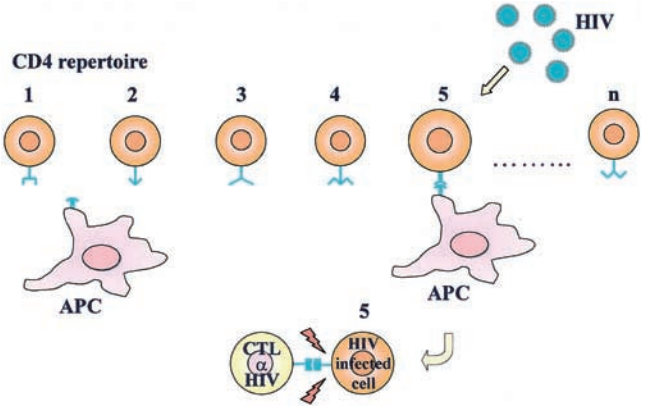

Figure 1 *Consequences of selective infection of activated CD4 cells by HIV on T cell repertoire. The repertoire of CD4 T helper lymphocytes is represented by cells 1 to n, each one exhibiting a distinct specificity. The CD4 Th lymphocyte indicated as 5 is specific for an antigen of an opportunistic pathogen (or for HIV itself) that is presented by the antigen presenting cell (APC) as an antigenic peptide in the context of an HLA class II molecule. CD4 Th cell 5 is activated to undergo clonal expansion. The activated cell (and its proliferating progeny) is susceptible to HIV infection, whereas the other Th cells in a quiescent state are more resistant to infection. This may account for selective depletion of antigen-specific CD4 lymphocytes. Depletion of these cells expressing HIV antigens after de novo infection may also occur through HIV-specific CTL, as shown in the lower part of the figure. Different outcomes may result from HIV infection of CD4 T cells specific for opportunistic pathogens or specific for HIV itself. In the former case, in fact, the virus impacts on a well-developed memory repertoire that contains largely expanded clones and T cells that may be in an activated state or in a resting state in different anatomical compartments. In the latter case, HIV impacts on a naive HIV-specific repertoire that is being primed at the same time of peak viraemia. This may account for severe damage of the HIV-specific CD4 repertoire that persists over time.*

cystis in *in vivo* models.[51,52] Other opportunistic agents most frequently responsible for symptomatic infections, accompanying disappearance of the CD4 defence, include viruses such as EBV and CMV. In this case, the immune defect likely resides in the loss of CD4 helper cells that fail to activate and to expand virus-specific cytotoxic CD8 lymphocytes.[53,54] Loss of HIV-specific CD4 cells may result in defective help not only for activation and expansion of HIV-specific CTL, but also for production of neutralizing antibodies. Both effects may contribute to failure to control HIV spreading and to clear infection.[55–58]

Immune reconstitution with specific lymphocytes has been investigated in pioneering work with CD8 cells recognizing CMV[59] or EBV[60] antigens in an attempt to control viral pneumonia and B lymphomas in bone marrow graft recipients. These studies demonstrated the efficacy of reinfused cells and suggested that addition of specific CD4 cells may extend CTL survival, increasing the therapeutic potential of CD8 cell adoptive immunotherapy.[61] While anti-HIV drug treatment can restore immune functions and reduce the incidence of opportunistic infections in AIDS patients, some defects in the repertoire can not be replenished,[62–64] even if expansion of mature T cells can contribute to CD4 T cell regeneration.[65] Therefore, for a new genetic treatment of AIDS, we advocate the adoptive transfer of genetically treated, HIV-resistant, *ex vivo* selected and expanded autologous CD4 cells specific for opportunistic pathogens and for HIV. These cells, while being refractory to *de novo* HIV infection after inoculation *in vivo*, should indeed provide the natural help for production of neutralizing antibodies and for activation and expansion of specific CTLs. If collected early before disease development (primary infection) and administered to pre-symptomatic patients, antigen-specific CD4 cells may replace the holes caused by HIV cytopathicity in the CD4 repertoire and contribute to displacing HIV reservoirs.

Antigen-specific CD4 T cell lines can be produced from HIV-infected donors, if precursors are spared.[66,67] If the specific precursors are infected, exposure to single antiviral drugs,[68] or to cocktails that mimic HAART, may prevent viral replication and infection spreading in culture.

As far as the anti-HIV genetic treatment of antigen-specific CD4 lymphocytes is concerned, we have shown that a retrovirally transduced tRNA anti-tat antisense gene[69] effectively inhibited HIV replication. Antigen-specific CD4 lines were obtained from normal and from HIV-infected donors and these lines maintained functional immune properties after transduction.[70,71] Irrespective of the gene being transferred,[72–74] GT should protect antigen-specific CD4 lymphocytes *in vivo* more efficiently than HAART, when drug-resistant HIV strains are present.[32–34] For a more effective treatment, however, this immune-genetic approach should be adopted in combination with antiretroviral chemotherapy and/or with IL-2 administration to favor expansion of activated CD4 cells.[75]

Antigen presenting cells (APC)

Genetic resistance of APC can be achieved by gene therapy of stem cells, from which APC lineages (monocytes and dendritic cells) derive or by targeting APC directly with vectors suitable for non-replicating cells. HIV-resistant monocytes derived from gene-modified stem cells have been described.[23,76,77] Dendritic cells can also be targets for viral and nonviral vectors,[78] but use of anti-HIV genes in these cells has not been reported so far.

HIV-specific CD8 CTL

HIV-specific CTL have been reinfused in several patients[79–82] based on correlates of CTL function and disease control.[83] The inverse correlation between HIV-specific CTL expansion and viremia has been challenged in a recent report that attributed the phenomenon to impaired CTL effector function.[84] In one case, reinfusion of HIV-specific CTL resulted in selection of CTL-resistant viral mutants.[81] A negative role of CTL as killers of HIV-infected CD4 T helper lymphocytes (thus contributing to CD4 depletion) has also been advocated and should be kept in mind as a possible drawback.[47] HIV-specific CTL were genetically modified with drug-resistance genes and with suicidal genes to either trace the reinfused cells or to kill them, had harmful reactions arisen.[82] Even though a long-lasting beneficial effect was not observed in these trials, important pieces of information were attained. Modified cells were indeed capable of homing in selected areas of the lymph nodes, but underwent immunoclearance for expressing antigens encoded by the foreign genes.[61]

Even if HIV-specific CTL are a key feature of the antiviral response,[83] production of HIV-specific CTL cell lines from each individual is cumbersome and the CTL activity may be poor, possibly because of a Th defect. Therefore, non-specific CD8 T cell lines were produced, having the zeta chain of the CD3–TCR complex replaced with a CD4 molecule. Thus, a non-specific CD8 cell[85–87] could be directed to HIV-infected gp120 expressing target cells by the newly acquired CD4 receptor to allow killing in an MHC-independent fashion.

The possibility of making CD8 cells independent of CD4 help by GT has been tested *in vitro*. Since CD8 cells produce large amounts of GM-CSF and can be activated via the IL-2 receptor transduction pathway,[88] the intracytoplasmic and transmembrane regions of the IL-2 receptor were engineered to express the extracellular domain of the GM-CSF receptor. The practical feasibility of this approach is still to be demonstrated.

Drawbacks of GT of AIDS using genetically modified antigen-specific CD4 cells

Several drawbacks impinge on GT of AIDS when using genetically modified antigen-specific CD4 cells: (1) the immune response to vector encoded antigens; (2) the antigenic complexity of the pathogens; (3) the clonal complexity of the antigen-specific CD4 cell lines. While (1) is common to all GT approaches dealing with expression of a foreign gene, (2) and (3) particularly apply to our immune cell therapy approach.

Immune response to vector encoded antigens

Vectors introduce genes encoding for selection markers such as the hygromycin resistance gene (Hy) or products, such as the thymidine kinase (TK) gene that makes cells sensitive to gancyclovir. Being exogenous proteins, Hy and TK behave as new intracellular antigens and can be processed and presented as peptides for recognition by

CTL in the context of MHC class I.[82,89] To bypass this difficulty, improved transduction protocols to enhance efficiency[90] or non-antigenic markers can be adopted. For instance, expression of the human nerve growth factor receptor (NGFR) has been used to select transduced cells.[36]

In the case of a therapeutic protein encoded by a transgene (eg the transdominant RevM10), the transduced cells persisted for months after reinfusion.[38] Lack of immune clearance was attributed to low level protein expression or to poor antigenicity.[38] Intrabodies (recombinant variable antibody regions) have also been investigated for therapeutic potential.[91] Depending on the intrabody specificity, different functional or structural HIV proteins or cellular proteins involved in the virus life cycle (eg HIV co-receptors) can be targeted.[92] Although responses to idiotypic determinants could be predicted, they have not been reported.[93] On the other hand, vector-encoded proteins of human origin[73,74] should be fully tolerated.

Antigen complexity

It is not yet established whether individual proteins or whole pathogens would be more efficient to produce antigen-specific CD4 lines that maintain repertoire breadth and protective efficacy. In the case of mycobacteria, defined antigens confer resistance if used as immunogens.[94] For pathogens like toxoplasma, candida, cryptococcus, aspergillus, pneumocystis, antigens responsible for protection have not been well identified. In this case, inactivated bodies can be used that, for having a complex antigenic structure, should recruit most of the specific CD4 clones. The same may hold true for a number of purified virion particles (eg CMV or EBV). Individual or pooled recombinant proteins should be used in the case of HIV, since inactivated virions cannot be proposed for *in vitro* stimulation. In case proteins are identified, synthetic peptides can also be proposed for stimulating specific CD4 cells.[95]

Clonal heterogeneity of antigen-specific CD4 T cells

Maintenance of CD4 clonal heterogeneity *in vitro* is a requisite to reconstitute a specific T cell repertoire close to the normal condition. CD4 cell depletion and modes of clonal reconstitution are illustrated in Figure 2. In the upper panel, a hypothetical repertoire is depicted, each antigen-specific CD4 clone being represented by one peak. The elevation of the peaks refers to the number of cells that actually account for the clonal progeny. Two modes of depletion are proposed. Horizontal depletion in the left panel, in which each clone loses a fraction of its progeny, but residual cells are spared. Vertical depletion in the right panel, in which the majority of the clones are lost as a whole, but few clones are preserved intact. Even though the total number of residual specific cells may be similar in the two instances, in case of immunoreconstitution (ie recovery of CD4 counts) horizontal depletion is compatible with restoration of a clonally heterogeneous repertoire, whereas vertical depletion is compatible with a skewed repertoire recovery only. Skewing may occurr as a consequence of *in vitro* culture[96,97] or when therapeutic genes are artificially introduced.[70] Therefore, monitoring of clonal heterogeneity of antigen-specific T cell lines should be performed according to TCR BV gene family usage and spectrotyping.[98]

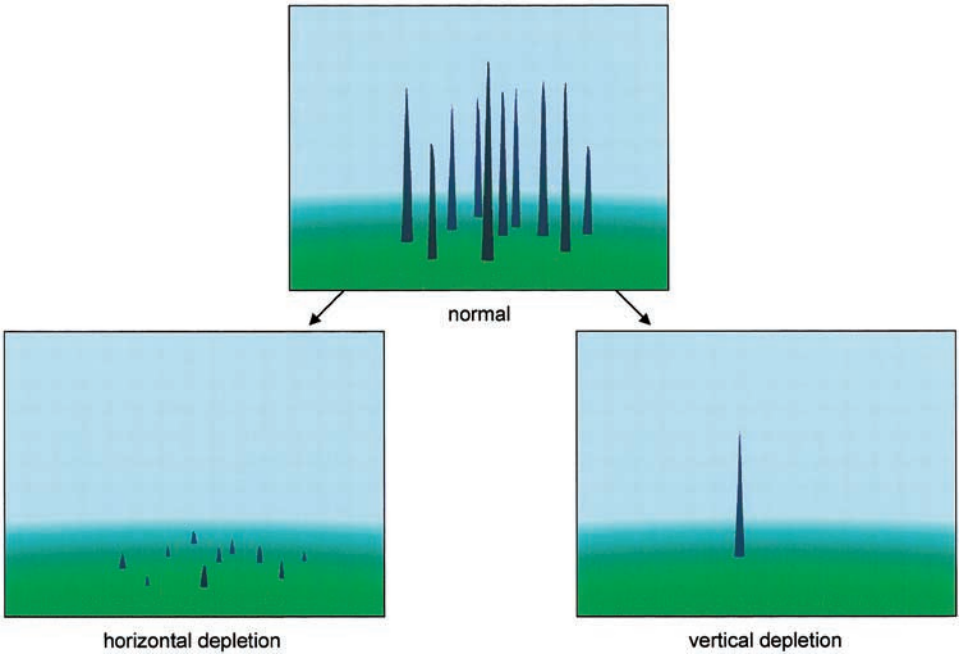

Figure 2 *Models for depletion of an antigen-specific CD4 repertoire. A hypothetical CD4 repertoire specific for a given antigen is depicted in the upper panel. Different clones are represented as peaks, their height indicating the size of the clonal progeny. Upon HIV infection, the number of CD4 cells decreases with two possible modes. In the right panel most of the clones are lost as a whole resulting in holes in the repertoire (vertical depletion). In the left panel all clones are preserved, but the number of cells in each clone is reduced (horizontal depletion). A recovery of CD4 counts does not restore the clonal holes in vertical depletion, whereas all preserved clones in horizontal depletion may increase in size and contribute to reconstitute a clonally heterogeneous repertoire.*

In vivo tracing of reinfused cells

It is important to trace and monitor the genetically modified reinfused cells in order to determine homing and survival after *in vivo* transfer. This could be achieved by marker gene tracing and clonotype tracing.

Marker gene tracing has been mostly performed by PCR and *in situ* hybridization. By using fluorescent primers, reinfused CTL were identified as neo resistance gene-expressing cells in peripheral blood and in lymph nodes.[61] Alternatively, HIV-specific CD8 cells transduced with the hygromycin resistance-thymidine kinase genes (Hy-TK) were quantitated by an Hy-specific PCR in the peripheral compartments.[61]

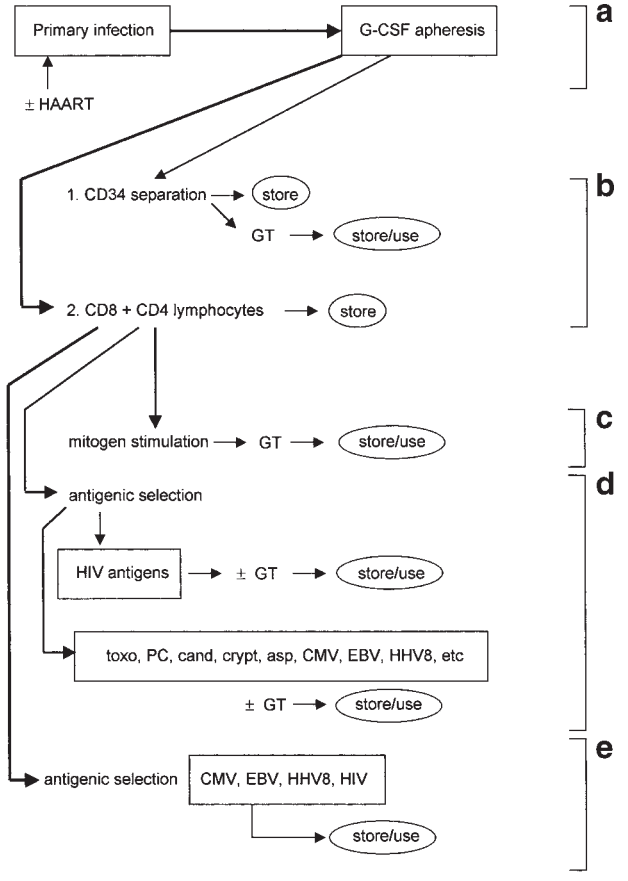

Figure 3 *Possible modes of adoptive cellular therapy complemented by GT. At an early stage of primary infection, irrespective of HAART, the patient undergoes leukapheresis following mobilization of CD34 stem cells with G-CSF (a). CD34 cells are removed and frozen for future use (+/- GT) (b). CD4 lymphocytes can be stimulated polyclonally (+/- GT) and frozen for future use (c). CD4 lymphocytes can be stimulated in vitro with different antigens (HIV and antigens of opportunistic pathogens, eg candida, pneumocystis, toxoplasma, cryptococcus, aspergillus, mycobacteria, CMV, EBV, etc), +/- GT and frozen for future use (d). CD8 cells can be selected for specificity for HIV, CMV, EBV, HHV8, etc. and frozen for future use (e). All these cells can be thawed and further expanded before reinfusion in different combinations. In case of recurrent infections with bacteria or fungi, specific CD4 cells should provide protection. In case of viruses (CMV and EBV), the corresponding CD8 cells should be reinfused along with CD4 T helper cells with the same specificity, to provide adequate helper function for prolonged survival of CTL. HIV-specific, genetically resistant CD4 lymphocytes can be utilized in combination with HAART, vaccination or IL-2 to extend the length of virological remission. This combined treatment can be administered early in the course of HIV infection or when virological and immunological parameters warrant adoption of antiviral chemotherapy. This complex protocol is meant to illustrate the concept that reinfusion of specific CD4 cells may help control opportunistic infections and HIV infection, in synergy with CD8 cells. In a simplified protocol, expansion of specific CD4 cells with a cocktail of antigens from the most relevant opportunistic pathogens can be foreseen.*

Clonotypic tracing

Clonal heterogeneity is a desirable feature for a therapeutic T cell line, although molecular identification of reinfused cells may be hampered. Therefore, one could consider focusing on few representative clones, and sequencing the corresponding hypervariable region of the T cell receptor (TCR). A PCR making use of clonotypic primers, designed on the basis of the deduced TCR nucleotide sequence, can trace the reinfused cells. This method can identify *in vitro* fewer than 40 clonotype positive cells out of one million negative cells.[96] TCR BV gene usage with sequencing of the PCR products was reported as a way to monitor CD8 clones specific for CMV.[99] A similar approach can be adopted to trace antigen-specific CD4 cells independently of the marker gene utilized. Labeling methods with radioactive or non-radioactive isotopes[100,101] could be used with specific CD4 cells as well.

Conclusions and perspectives

Monitoring the immune function of CD4 cells vis-à-vis specific infectious pathogens is particularly critical in HIV-infected patients undergoing antiviral chemotherapy or other kinds of therapeutic procedures to combat AIDS. Sensitive *in vitro* and *in vivo* assays include antigen-specific proliferation,[67] precursor frequency evaluation by limiting dilution,[97] intracytoplasmic cytokine staining,[102] tetramers[103] and delayed T cell hypersensitivity (DTH) to relevant antigenic extracts. However informative these biological parameters may be, no one is singularly a better predictor of therapy-linked immune recovery than clinical effectiveness. This is evaluated as control over opportunistic infections and as a safe withdrawal of chemoprophylaxis.

Since antiretroviral chemotherapy has a number of limitations due to side-effects, selection of multiresistant strains and unknown long-term consequences, we believe that gene therapy, appropriately shaped as an adoptive immunotherapy with genetically treated antigen-specific cells, has the potential for being a powerful new complement in the long-term treatment of HIV infection. If the advent of HAART has overshadowed the initial glamor of GT, it is because GT was meant to protect all HIV susceptible cells in the body. This goal is presently unattainable given the available means for *in vivo* delivery and targeting of the therapeutic genes. So a conceivable role for GT as a systemic treatment may be that of an adjuvant modality, making use of engineered CD4 cells to rescue the immune repertoire specific for opportunistic pathogens and for HIV itself, before HIV-induced antigen-specific T cell depletion.

Therefore, irrespective of the optimal vector and genes to be introduced (available either now or in the future) we proposed a therapeutic strategy aimed at preserving a (quasi) intact lymphocyte repertoire. This protocol should ideally be applied with the aim of aborting HIV infection from the reservoirs, at a time when the patient is still asymptomatic, ideally in the course of primary infection, if future studies prove the effectiveness of early

HAART to preserve immune function.[7] At this time, the patient could also benefit from vaccination and/or exposure to selected cytokines. In case of treatment failure and progressive immunodeficiency, the stored cells could be used to reduce life-threatening episodes of opportunistic infections. In all respects the preserved cells may prove an irreplaceable asset for the patient. A flow chart of this strategy of combined cell-gene therapy is presented in Figure 3, with the caption illustrating more details of the potential interventions that may temporally be adopted.

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