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Hematopoietic Stem Cell-Based Therapy for HIV Disease: A Role for Regulatory T Cells

Josef Bodor[1,2*], **Petr Kobylka**[1] **and Gero Huetter**[3]

[1]*Institute of Hematology and Blood Transfusion, Department of Cell Therapy and Cord Blood Bank CR, U Nemocnice 1, 128 20 Prague, Czech Republic*

[2]*Institute of Immunology, Johannes Gutenberg University, Langenbeck str. 1, D-55131 Mainz, Germany*

[3]*Cellex, GmbH, Fiedlerstrasse 36, 01307 Dresden, Germany*

[*]**Corresponding author:** Josef Bodor, Institute of Immunology, Johannes Gutenberg University, Langenbeck str. 1, D-55131 Mainz, Germany, Tel: +420-722-950-013, Fax: 420-241-062-785; E-mail: bodor@unimainz.de

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Abstract

The goal of this review is to develop insight and understanding of the effect of deleting the chemokine receptor CCR5 in T cells, and its interplay with immune regulation of Human Immunodeficiency Virus type–1 (HIV-1), to enable a novel technology platform to cure HIV disease. A critical point is the use Hematopoietic Stem Cell (HSC) transplantation of the cells resistant to HIV such as CCR5Δ32 cells, which harbor deletion in the CCR5 promoter. Such mutations, which spontaneously occur in 4-15% of the European or US populations confer resistance to CCR5-tropic HIV-1 in homozygous individuals and could cure HIV-1 disease based on the outcome of bone marrow engraftment in HIV$^+$ leukemic patients using a CCR5Δ32 homozygous donor (e.g. 'Berlin Patient'). However, potential shift of HIV tropism to CXCR-4 tropic strains of HIV-1 is limiting after HSC transplantation with CCR5Δ32/Δ32 donor since it could lead to recurrence of viremia (e.g. 'Essen patient'). In addition, patients receiving allogeneic bone marrow transplantation often suffer from Graft-Versus-Host Disease (GvHD), and for that reason HIV infection is not considered an indication, unless a hematologic malignancy warrants transplantation. To advance this field, it is, however, vital i) to search for novel determinants to HIV susceptibility using genome-wide analyses and ii) exploit mechanisms, which play a crucial role in amelioration of GvHD such as repression of conventional CD4$^+$ T cells (Tcons) by naturally occurring regulatory CD4$^+$CD25$^+$ T cells (nT$_{regs}$). Transfer of cyclic AMP (cAMP) from nT$_{regs}$ to Tcons underpins function of potent transcriptional repressor termed inducible cAMP early repressor (ICER) leading to suppression of interleukin-2 (IL-2) synthesis in Tcons. Further understanding of the mechanisms of immunological self-tolerance will also provide insights into how strong immune responses such as graft rejection could be restrained and engraftment of HIV resistant cells in HIV$^+$ leukemic patients could be augmented.

Keywords: Chemokine C-C Motif Receptor 5 (CCR5); Human Immunodeficiency Virus type–1 (HIV-1); Hematopoietic Stem Cell (HSC) Transplantation; Graft-Versus-Host Disease (GvHD); Naturally occurring regulatory CD4$^+$CD25$^+$ T cells (nT$_{regs}$); Conventional CD4$^+$ T cells (Tcons); Cyclic AMP (cAMP); Inducible cAMP Early Repressor (ICER)

Introduction

Infection with the Human Immunodeficiency Virus (HIV) requires entry into target cells by binding of the viral envelope to the CD4 receptor and to either the Chemokine (C-C motif) Receptor 5 (CCR5) or the Chemokine (C-X-C motif) Receptor 4 (CXCR4) [1]. Homozygous carriers of the Δ32 mutation (CCR5Δ32/Δ32) prevent cellular entry of CCR5-tropic (R5- tropic) HIV type 1 (HIV-1) because the mutation prevents functional expression of the CCR5 chemokine receptor used by HIV-1 to enter CD4$^+$ T cells [2]. The first case of the Hematopoietic Stem Cell (HSC) transplantation of the CCR5Δ32/Δ32 cells resistant to HIV has been reported in 2009 [3,4]. This HSC transplantation led not only to the successful cure of leukemia but also to undetectable levels of HIV-1 for more than five years thus mimicking the artificial development of a natural controller phenotype ('Berlin patient'-Timothy Ray Brown) [5,6]. However, concerns about a potential shift of HIV tropism towards CXCR4- tropic (X4-tropic) HIV-1 after HSC transplantation with CCR5Δ32/Δ32 donor remain since expression of CXCR4 receptor and cellular entry of X4-tropic strains of HIV-1 in CCR5Δ32/Δ32 cells is unaffected [2,3,7]. Indeed mere HSC transplantation from donors with non-mutated CCR5 pretreated with antiretroviral therapy (ART) was reported to yield viral rebound in another two cases of HIV-1-infected patients undergoing allogeneic stem-cell transplantation in Brigham and Women Hospital ('Boston patients') [8]. Shift of HIV tropism to X-4 tropic strains of HIV-1 after HSC transplantation with CCR5Δ32/Δ32 mutation lead reportedly to viremia in the case of a 27-year-old patient with HIV-1 infection and anaplastic large cell lymphoma ('Essen patient') [9]. This case highlights the fact that viral escape mechanisms might jeopardize CCR5-knockout strategies to control HIV infection. One possibility how to approach this caveat is based on in depth comparison of 'Berlin' and 'Essen' patients in order to pinpoint determinant(s) responsible for susceptibility towards HIV-1 infection and/or shift of HIV tropism using massively parallel gene expression analysis and New Generation Sequencing (NGS). Despite of these shortcomings CCR5Δ32 mutation still represents attractive approach to enable novel technology platform to master eradication of HIV-1 in HIV$^+$ leukemic patients emphasized by the fact that the Δ32 mutation plays an important role in natural HIV resistance since heterozygous carriers have reduced susceptibility to infection and delayed onset of AIDS, while homozygous carriers are resistant to HIV infection [10]. If successful these determinant(s) in combination with CCR5Δ32 mutation represent attractive approach to master eradication of HIV-1

in HIV$^+$ leukemic patients. Additionally, this approach requires the control over "allo-effect" in order to ameliorate Graft-Versus-Host Disease (GvHD) crucial for successful HSC based therapy. Thus 4 screening for the Δ32 mutation at the CCR5 locus, which is relatively frequent in Europe and US, could enable a novel technology platform aiming for the cure rather than the control of HIV infection. The CCR5Δ32 mutation is a good example of an advantageous allele with a well-characterized geographic distribution [11]. Originally it was presumed that bubonic plague could act as the selective agent, but the subsequent analysis implied a disease like smallpox as a more plausible candidate [12]. Lucotte and Mercier suggested that the geographic distribution indicate a Viking origin [13]. They proposed that the allele present in Scandinavia before 1,000 to 1,200 years ago was then carried by Vikings westward to Iceland, eastward to Russia, and southward to Central and Southern Europe.

In Depth Comparison of 'Berlin and Essen Patients'

The prospect of reaching a 'functional cure' for HIV-1 infection has been raised by recent reports on 'Berlin patient' [5,6]. This case is reminiscent of an earlier case described in 1999 involving an HIV-1-infected patient (also referred as the 'Berlin patient') who controlled viral replication (a natural controller phenotype) [14]. Genotypic analysis revealed that this patient carried the highly protective HLA class I allele HLA-B*57 enriched among patients in whom HIV is spontaneously controlled in the absence of ART. However, shift of HIV tropism to X-4 tropic strains of HIV-1 after HSC transplantation with CCR5Δ32/Δ32 mutation is a major concern (reported in the 'Essen patient') which could be represented as a cautionary tale of drawing broad conclusions from a single patient [9]. Although Timothy Ray Brown does not carry HLA-B*57 allele, there have been reported major differences between 'Berlin' and 'Essen' patients receiving CCR5Δ32 homozygous allogeneic stem cell transplantation [3-7,9] (Table 1). First, discrepancies to the HIV-1 type B envelope (env) V3 consensus sequence (marked in bold and underlined in Table 1) could be also responsible for differential outcome based on distinct virulence of both HIV-1 strains. Furthermore, in the case of 'Berlin' patient ART was discontinued on day of transplantation while in 'Essen' patient ART was discontinued one week before transplantation and more aggressive conditioning regimen was used followed by a very late engraftment (usually between 10-14 days as opposed to 39 days in 'Essen' patient). The CXCR4-predicted minority viruses (X-4 tropic HIV-1) present prior to transplantation were unable to rebound after transplantation in 'Berlin' patient presumably due to their dependence on CCR5 for replication [7].

	Berlin Patient	**Essen Patient**
Age, gender	40y, male	27y, male
Malignancy	acute myeloid leukemia	anaplastic large T-cell lymphoma
Time between infection and ART	7 years	3 years
Time between infection and Tx	12 years	5 years
Tx regimen	intermediate intensity	myeloablative+ 12 Gy TBI
Immunosuppression	ATG, CSA, MTX, MMF	ATG, CSA, MTX
GvHD	max. grade 1 (skin)	max. grade1-2 (skin)
Engraftment	day +11	day +39
ART discont	on day of Tx	7 days before Tx
Viral load at Tx	below detection	below detection
V3 sequence >3 months prior Tx*	CIRPNNNTRK**G**IHIGPGRAFYTTGEIIGDIRQAHC	CTRPNNNTRKGIPLGPGKVFYAT-EIIRDIRKAYC
X4 prediction** 3months prior Tx Immediate prior Tx	capable not determined	intermediate capable
Disease control	partial, relapse after 12 months	no, relapse after 2 months

Table 1: Differences between 'Berlin and Essen patients' receiving CCRΔ32 homozygous allogeneic stem-cell transplantation. The viral tropism of HIV-1 was determined by genotyping the V3 amino acid sequence and applying Geno3Pheno bioinformatic software to predict viral coreceptor use (*discrepancy to the HIV type B V3 consensus sequence CTRPNNNTRKSIHIGPGRFYTTGEIIGDIRQAHC) are marked in bold and underlined; **DNA or RNA according to Geno3Pheno) [3-7,9]. There have been reported important differences in both cases: ART was discontinued one week before transplantation in 'Essen' patient. Furthermore, in the case of 'Essen' patient a more aggressive conditioning regimen was used and the patient had a very late engraftment (usually between 10-14 days as opposed to 39 days in 'Essen' patient).

The genotypic analyses of HIV-1 in 'Essen' patient showed a shift from a dominantly R-tropic HIV-1 strains before stem cell transplantation toward and X-4 tropic HIV-1 strains after transplantation [9]. While discrepancies in env V3 consensus sequence indisputably play a major part in HIV-1 virulence, 5 contributions of genomic variation underlying complex traits such as HIV-1 persistence and/or HIV-1 tropism are being evaluated. Genome-Wide Association Studies (GWASs) have been heralded as a major advance in biomedical discovery, having identified more than 2,000 robust associations with complex diseases since 2005 [15]. More importantly,

the continued values of GWAS comes from efficiency of the method for interrogating low-frequency variants (those with Minor Allele Frequencies (MAFs) of 0-5-5%) that are initially identified through sequencing, using dense genotyping platforms that capture a large proportion of genomic variation underlying complex traits such as resistance or susceptibility to HIV infection (see also Illumina products for NGS). However, such approach is complicated with hurdles since each genome is expected to contain approximately 150,000 novel single-nucleotide variants, including 250 to 300 disruptive variants in human disease genes, and approximately 20 completely inactivated genes [16,17]. Compared with the reference sequence generated by the Human Genome Project, any single individual's genome has about four million sequence variations [17]. Although most of these variants are harmless, some cause disease, and predispose to differential response to HIV infection. Combining all genes from the GWAS together with genes reported in the literature to affect HIV yields 2,410 protein-coding genes, or fully 9.5% of all human genes yielded a more extensively corroborated set of host factors assisting HIV replication [18]. Moreover, genome-wide analysis of primary $CD4^+$ and $CD8^+$ T cell transcriptomes shows evidence for a network of enriched pathways associated with HIV disease [19]. Importantly, a single genetic variant such as CCR5Δ32 mutation may have a very different impact depending on the other genetic variants that exist in the genome (such as HLAB* 57 allele) and/or environmental factors. Therefore GWAS analysis of 'Berlin and Essen patients' may offer appropriate modifier genes, which could predispose for differential response to HIV infection. It is imperative to further validate and characterize these modifier genes of $CD4^+$ and $CD8^+$ T cell transcriptomes to gain additional insights for mechanisms directing either susceptibility towards HIV-1 infection or HIV tropism or both.

HSC-based therapy for HIV disease: A gene therapy approach

GWASs in combination with gene editing revitalized a gene therapy approach and together with documented cure of a HIV-1-infected patient after allogeneic transplantation from a CCR5-null donor ('Berlin patient') [3,4] has renewed optimism that a potential alternative to conventional ART is emerging [20,21] (Figure 1). While allogeneic grafts could lead to complete eradication of viral reservoirs [3,4,6], this remains to be observed following autologous HSC transplantation. Development of curative autologous transplantation 6 strategies such as gene editing of CCR5 and CXCR4 receptors and HSC transplantation of modified cells and/or adoptive transfer of autologous T cells [22,23] would significantly increase the number of treatable patients, eliminating the need for matched donors and reducing the risks of adverse events. Recent studies suggest that gene therapy may provide a mechanism for developing curative therapies based on results from early-stage clinical trials [6] in concordance with recent findings in animal models of gene modified HSC transplantation [20]. Expression of cellular/artificial restriction factors or disruption of CCR5 has been shown to limit viral replication and provide protection of genetically modified cells. One way how to achieve the goal of HSC-based gene therapy for HIV disease stems from efficient and stable introduction of novel gene functions responsible for differential outcome of HIV viremia into HSCs and their subsequent delivery in progeny T cells and/or myeloid cells. Therefore, approaches aimed at modifying HSCs to treat HIV disease based either on targeted disruption of cellular genes involved in HIV entry, such as the CCR5 co-receptor (susceptibility factor) or introduction of gene(s) that interfere with HIV replication, such as fusion inhibitors or host restriction factors (resistance factors) are being implemented [20,22,23]. There are at least two general approaches to achieve this goal: the use of integrating vector systems that permit the introduction of anti-HIV genes into the genome of HSCs and non-integrating vector systems that introduce gene-modifying enzymes to affect gene disruptions or homologous recombination. These approaches are enabled by a number of vector systems available that allow for efficient and stable gene delivery to HSCs. The introduction of multiple hematopoietic cytokines and the Retro Nectin fragment (a recombinant human fibronectin fragment) has successfully facilitated substantial improvements in the genetic manipulation of HSCs [20,24]. Recent protocols have focused on the use of safety modified, HIV-derived lentiviral vectors [20,25], an approach that allows the generation of high-titer vectors and efficient gene transfer to HSCs. Unfortunately these vectors have been associated with a high risk of leukemia in prior transplant protocols. However, improved vector systems have been developed and evaluated, and the currently used lentiviral vectors have limited risk for malignant transformation. The Self-Inactivating (SIN) lentivirus is capable of integration but have a nonfunctional Long Terminal Repeat (LTR) in the integrated provirus and rely on a weaker internal promoter element for expression of transgene [26]. The removal of the strong LTR promoter reduces the potential for insertional activation of nearby genes [27,28].

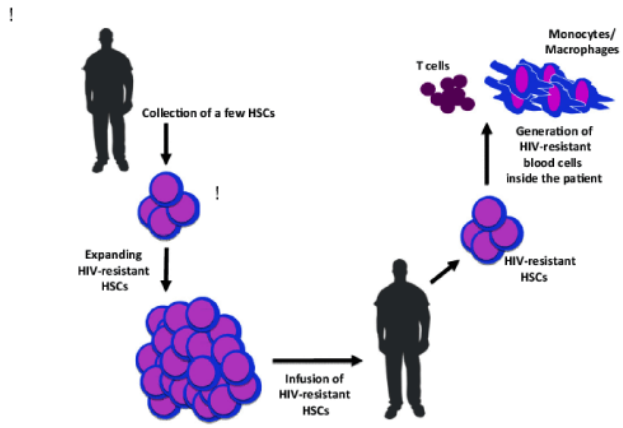

Figure1: Hematopoietic stem cell-based therapy for HIV disease (adopted from Kiem et al. [20]. Long-lived, self-renewing, multilineage hematopoietic stem cells (HSCs) selected such that their progeny resist HIV-1 infection (such as HSCs from donor harboring CCR5Δ32 mutation). The host could thereafter be repopulated with a hematopoietic system (including $CD4^+$ T cells and myeloid targets for HIV) that is resistant to the replication and spread of R5-tropic strain of HIV-1.

An alternative gene transfer approach is to utilize viral vectors that have been modified such that they are unable to integrate into the host genome [29]. The recent emergence of DNA editing proteins, including zinc finger nucleases [30], TAL effector 7 nucleases [31] and homing endonucleases [32], has created a possibility that any genetic locus can be specifically and permanently inactivated. This approach can be applied to CCR5 in any cellular type; including patients own HSCs [31] or peripheral blood lymphocyte (PBL) $CD4^+$ T cells [22].

However, using this approach offers only limited efficacy e.g. zinc finger nucleases used to disrupt CCR5 in human HSCs has shown in a humanized mouse model of HIV infection only 17% of disrupted CCR5 alleles, usually resulting in HSCs with heterozygous disruptions [31]. Therefore, significant obstacles remain with regards to the depletion of established viral reservoirs in an autologous transplantation setting devoid of the "allo-effect". Nevertheless, innovative combination of HSC-based therapies for HIV disease may aid the reduction of viral reservoirs in HIV-1-infected patients and promote the artificial development of a natural controller phenotype.

Prostaglandin-modulated HSC-transplantation

It is generally accepted that 'true' self-renewing human HSCs could be found within the $CD34^+$ population and that engraftment of a suitably conditioned host with a sufficient number of such cells will result in long-term multi-lineage hematopoiesis [20]. Numerous efforts have been made to expand HSCs *in vitro* so that they will be more readily accessible for use *in vivo*. Probably the most successful expansion reagent identified to date has been the purine derivative Stem Regenin 1 (SR1), which promotes the *ex vivo* expansion of $CD34^+$ cells obtained in culture and increases more than 10-fold the number of cells able to engraft in humanized mice [33]. Umbilical Cord Blood (UCB) cells are a valuable source of HSCs for use in allogeneic transplantation [20,34,35]. Key advantages are easy availability and less stringent requirements for HLA matching [20]. However, UCB cells contain an inherently limited HSC count associated with delayed time of engraftment, high graft failure rates, and early mortality. Prostaglandin E2 (PGE2) and its derivative 16, 16 dimethyl Prostaglandin E2 (dmPGE2) was recently identified to be a critical regulator of HSC homeostasis [36]. Recent data have shown that brief *ex vivo* modulation with dmPGE2 could improve patient outcomes by increasing the 'effective dose' of HSCs with preferential long-term engraftment of the dmPGE2-treated HSCs in allogeneic transplantation. Moreover, it was demonstrated that Tcons could be developed *in vitro* into $CD4^+CD25^+Foxp3^+$ inducible regulatory T cells (iT_{regs}) with an equivalent suppressive potential as naturally occurring regulatory T cells (nT_{regs}) by continuous polyclonal activation with anti-CD3/CD28 mAbs or CD28 super agonistic monoclonal antibody (CD28SA) [37]. During the differentiation process, the iT_{regs} express Cyclooxygenase-2 (COX-2) and produce PGE2 [38]. Interestingly, neither 8 resting nor activated nT_{regs} express COX-2. The PGE2 production from iT_{regs} can be fully suppressed by the COX inhibitor indomethacin [39]. These data indicate that PGE2 plays an important role not only in differentiation of HSCs, thus releasing stringency required for HLA-matching donors with potential recipients, but also in dominant suppressive effects of iT_{regs} expressing COX-2 with acquired ability to produce copious amounts of PGE2 responsible for delivery of suppressive function of T_{regs} through elevated levels of cAMP [40,41].

Downregulation of CCR5 and inhibitory pathway of PGE_2

An important precedent of receptor-mediated cAMP formation in iT_{regs} is PGE2 synthesis [39,42]. It has been demonstrated that iT_{reg} cells express COX-2 and produce PGE2 upon differentiation, signaling through any of its four receptors-EP1, EP2, EP3, EP4-often with opposing effects. EP2 and EP4 appear to be the most abundant in naïve cells isolated from peripheral blood and are up-regulated in response to activation. Recent studies have provided significant insights-in particular, a pathway has been described in Tcons where signaling through EP2 or EP4, with its concomitant increase in cAMP levels, leads to Protein Kinase A (PKA) activation and, through an EBP50-Ezrin-PAG scaffold process phosphorylation of the C-terminal Src Kinase (Csk). Phosphorylated Csk in turn inhibits Lck-mediated phosphorylation of the T Cell Receptor (TCR) complex, thus inhibiting TCR signaling and T cell proliferation and function (Figure 2).

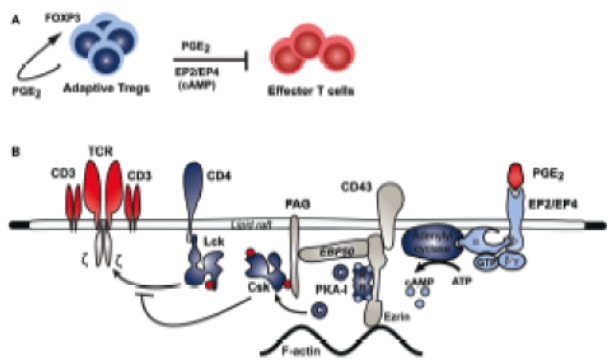

Figure 2: Inhibitory pathway of prostaglandin E2 (PGE2) (adopted from Lone and Tasken [39]). (A) PGE2 mediates T_{reg} inhibition of effector Tcell (Tcon) function through a cAMP and PKA-mediated pathway. (A) In response to continuous antigen exposure, for instance in cancer and HIV, adaptive T_{reg} cells express cyclooxygenase-2 (COX-2) and PGE2, stimulates FOXP3 expression in these cells. The T_{reg} cell-derived PGE2 can signal through EP2 and EP4 receptors on Tcons to inhibit the function of these cells through pathway shown in (B). 21 Binding of PGE2 to its receptors on Tcons stimulates adenylyl cyclase activity, which increases intracellular cAMP levels and thus activates PKA. Aided by an Ezrin-EBP50-PAG scaffold, PKA phosphorylates Csk, which in turn phosphorylates Lck to inhibit its activity. Lck normally acts to promote TCR signaling; thus Lck inhibition through this PGE2-initiated pathway inhibits TCR signaling in Tcons.

Importantly, PGE2-mediated transcriptional attenuation of CCR5 chemokine receptor expression tightly correlates with expression of potent transcriptional regulator-Inducible cAMP Early Repressor (ICER) in peripheral blood human monocytes (Figure 3). These preliminary studies suggest that in addition to ICER mediated down-regulation of IL-2 synthesis in Tcons [43,44], ICER also attenuates expression of CCR5 receptor (Figure 3). Since PGE2 has capacity to down-regulate expression of CCR5 receptor gene this could lead to dual use of dmPGE2-improved engraftment of HIV resistant cells while diminishing residual CCR5 expression in cells from heterozygous donors (CCR5wt/Δ32) thus mimicking CCR5Δ32/Δ32 homozygous (CCR5 null) phenotype. Provided that dmPGE2 has lasting effects in HSCs this could lead to significant increase of the number of treatable patients (frequency of heterozygous CCR5wt/Δ32 donors in North of Europe and US is at least 10 fold higher than in case of homozygous CCR5Δ32/Δ32 donors) [45]. Therefore, potential dmPGE2–mediated down-regulation of CCR5 expression driven from single CCR5 allele in HSCs from donors heterozygous for Δ32 mutation could 9 significantly increase probability of appropriate donor selection for bone marrow transplant in HIV^+ infected leukemic patients.

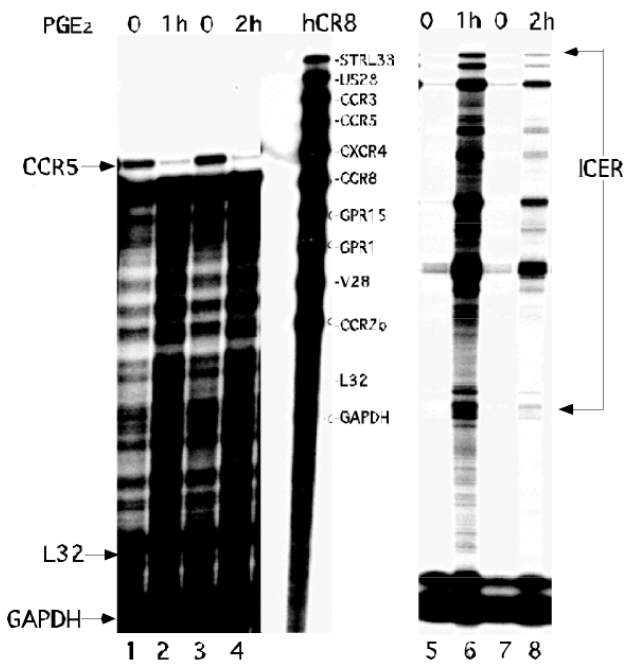

Figure 3: PGE2-mediated transcriptional attenuation of CCR5 chemokine receptor expression correlates with ICER induction. Human monocytes treated with PGE2 (1 and 2 hrs; lanes 2, 4, 6, and 8) were scored for human chemokine receptor expression (left panel) in parallel with ICER expression (right panel), using RNase protection assay (Ambion). For evaluation of human chemokine receptor expression, a Riboquant hCR8 probe set was used. Levels of chemokine receptor were evaluated after PGE2 treatment (500 ng/ml final for 1h and 2h at 370C in regular media). Levels of CCR5 were inversely related to the levels of ICER mRNA after PGE2 treatment. Templates of the housekeeping genes for the analysis of hL32 and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping genes were included to allow assessment of total RNA levels. In particular, downregulation of CCR5 expression could be critical in heterozygous CCR5wt/Δ32 donor in order to mimick CCR5 null cells from CCR5Δ32/Δ32 homozygous donor [45].

HSC-based therapy for HIV disease: A role for Gp120 in amelioration of GvHD

Patients receiving allogeneic bone marrow transplantation often suffer from GvHD, and for that reason HIV infection is not considered an indication, unless a hematologic malignancy warrants transplantation. Earlier reports on anti-CD4-mediated tolerance and T_{reg} activation took advantage of the HIV gp120 protein having been a high-affinity ligand for CD4 and reported that gp120-mediated activation of nT_{regs} through CD4 was sufficient to turn on the suppressive activity of nT_{regs} [46,47] (Figure 4). CD4-mediated activation depends on Lck and low levels of cAMP production and can be blocked by Src family kinase and adenylyl cyclase inhibitors [47]. Functional analysis of the effects of gp120-mediated activation of nT_{reg} *in vivo* in a GvHD model demonstrated that the nT_{reg} activation by gp120 through adenylyl cyclase could abolish the rejection (reviewed in [48]). The data on gp120 are important in the context nT_{reg} cell-mediated suppression *in vivo* as a starting point for potential new therapies to ameliorate GvHD following HSC-transplantation of the cells resistant to HIV-1. To advance this field, it is, however, vital to implement novel insights of the protection from GvHD (control of "allo-effect") e.g. by elevated levels of cAMP through binding of HIV-1 envelope protein gp120 to human T_{regs} (Figure 4) [46,47].

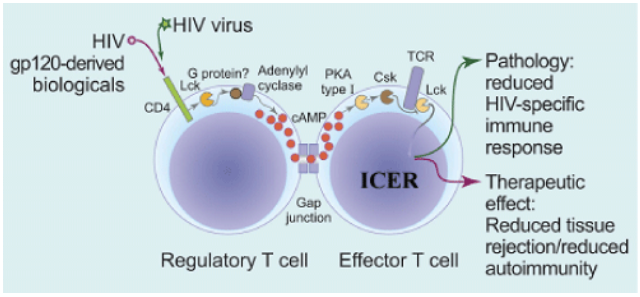

Figure 4: A schematic representation of nT_{reg} cell immunosuppression by cAMP following gp120 ligation of CD4 (adopted from Tasken, K. 2009 [47]). Upon triggering of CD4 on T_{regs} by gp120 protein or possibly gp120-derived agonists, Lck becomes active and turns on cAMP production by adenylyl cyclase possibly through interaction with a G protein [68]. cAMP is transferred from T_{regs} to Tcons through cell-to-cell contacts called gap junctions that allow diffusion of small molecules down the concentration gradient and into Tcons [49]. Once inside effector cells, cAMP inhibits immune function through PKA-Csk inhibitory pathway that turns off T-cell activation proximally under the T-cell Receptor (TCR) in parallel with induction of potent inhibitor of cAMP-mediated transcription ICER (inducible cAMP early repressor) leading to transcriptional attenuation of IL-2 and numerous other NFAT-driven cytokines and chemokines [44,54,71,72]. This could ameliorate Graft Versus Host Disease (GvHD) and lead to reduced tissue rejection and/or autoimmunity [67,73].

nT_{regs} represent a unique T-cell lineage released from thymus endowed with the ability to effectively suppress immune responses. Therefore, approaches to modulate nT_{reg} cell-function *in vivo* could provide ways to enhance or reduce immune responses and lead to novel therapies. It is known that nT_{regs} need to be activated to exert their suppressive function on bystander Tcons. However, it has remained elusive how activation of T_{regs} may occur effectively, as their suppression is not restricted and their antigen specificity may be different from the cells they suppress [48]. In terms of link between Lck and the adenylyl cyclase leading to increased cAMP levels in nT_{regs} this connection could explain how CD4 ligation and subsequent Lck activation increased intracellular cAMP concentration [39]. It is assumed that elevated levels of cAMP inside nT_{regs} may activate and directly suppress Tcons in a contact dependent manner by nT_{reg} cells forming gap junctions with Tcons [49]. Indeed, recently published data from the Taskén group further show that gap junctions opening may be also controlled by cAMP and PKA phosphorylation of connexin 43 (Cx43) gap junctions as Cx43 interacts with ezrin, an A kinase anchoring protein (AKAP), that targets PKA to Cx43 [50] (Figure 5).

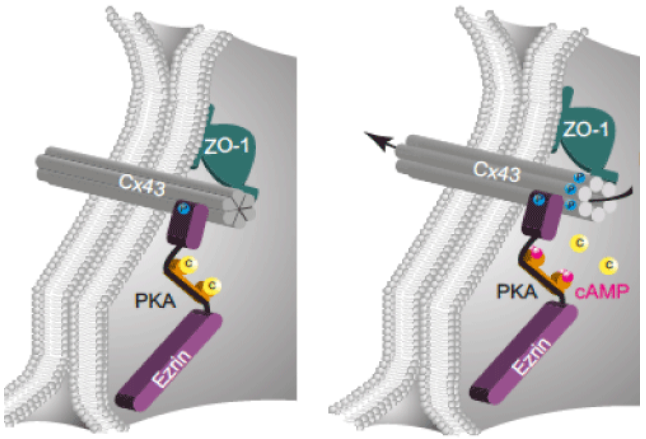

Figure 5: Schematic depiction of the cAMP-PKA regulation of opening and closing of Cx43 gap junctions. Based on data from the Tasken group (Pidoux et al., 2014 [50]), various 22 cellular types including T cells may have a Cx43/ZO-1/Ezrin/PKA supramolecular complex where ezrin targeted PKA by phosphorylation of Cx43 controls it's opening and closing.

As both CD28 and CTLA-4 molecules are implicated in the function of 10 nT_{reg} cells [51-53] the ability of their natural ligands B7-1 (CD80) and B7-2 (CD86) to influence the nT_{reg} suppressive capacity via induction of ICER was investigated [44,54,55]. Collectively, these data indicate that B7 expressed on Dendritic Cells (DCs) and Tcons is directly involved in ICER/CREM (cAMP responsive element modulator) expression during nT_{reg} cell-mediated suppression [55] (Figure 6).

It is conceivable that B7 can trigger elevated levels of cAMP responsible for ICER induction in synergy with cAMP transferred by nT_{regs} via gap junctions [44]. As nT_{reg} gap junctions are formed by Cx43 [50], we anticipate that in synergy with intercellular gap junction formation, cAMP influx leads to the immediate early induction of ICER in TCR-activated Foxp3neg Tcons leading to transcriptional attenuation of IL-2 expression instrumental for contact-dependent nT_{reg}-Tcon regulation (Figure 7A).

cAMP and ICER: A model of nT_{reg} cell-mediated suppression

Since the second messenger cAMP induces Inducible cAMP Early Repressor (ICER) it was hypothesized that ICER as a potent readily inducible transcriptional repressor plays an important role in nT_{reg} cell-mediated suppression (as predicted by Rudensky [40]). ICER is generated by use of an alternative downstream promoter in the gene encoding CREM [43]. Inter alia, during nT_{reg}-mediated suppression we have shown that ICER preferentially inhibits the production of IL-2, an essential growth factor for auto-aggressive Tcons [44].

Moreover, we have shown that the transcription factors ICER and nuclear factor of activated T cell c1 (NFATc1) are decisively involved in the suppression of Tcons by nT_{reg} cells [55,56]. Deficiency in these transcription factors led to a resistance of $CD4^+$ T cells against nT_{reg} cell-mediated suppression. Based on these data, we have proposed a spatiotemporal model of nT_{reg} cell-mediated suppression of Tcons through elevated levels of intracellular cAMP using either direct cell-to-cell communications to transfer cAMP through gap junctions or receptormediated hypoxia-adenosinergic signaling (reviewed in Bodor et al. 2012 [55] (Figure 7A-D)). Both of these mechanisms lead to elevated intracellular levels of cAMP in target Tcons, subsequent ICER expression, its nuclear localization, and transcriptional attenuation of IL-2 synthesis [54,55]. Importantly, dysregulation of hypoxia-adenosinergic signaling during HIV-1 infection decreases frequency of $CD73^+CD8^+$ T cells in HIV-infected patients, which correlates with immune activation and T cell exhaustion [57].

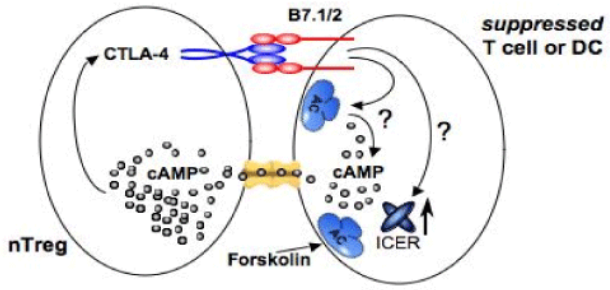

Figure 6: Cyclic AMP underpins suppression by nT_{reg} cells. Upon TCR activation (not shown), CTLA-4 is deployed to the surface of nT_{reg} cells, and a high-affinity CTLA-4/B7 interaction in synergy with intercellular gap junction formation (in yellow) leads to cAMP formation [51,52,53,74] and subsequent cAMP influx followed by the immediate, early induction of ICER in TCR-activated Foxp3neg Tcons [44]. Analogous effect could be achieved by direct activation of Adenylyl Cyclase (AC) by forskolin or inhibition of Phosphodiesterases (PDEs) responsible for degradation of cAMP e.g. by Rolipram [55]. In response to cAMP-ICER is induced (after 2-4 h of delay, necessary for ICER synthesis) in the Foxp3neg Tcons [44] and later ICER protein is enforced to the nucleus in response to cAMP where it attenuates IL-2 expression, induced by TCR activation [55]. TCR-activated Tcons promote CTLA-4 and B7 expression in cAMP-dependent fashion [74,75] during delay in ICER expression (ICER is absent and/or cytosolic) [55]. This could lead to 'processive' ICERmediated transcriptional attenuation of IL-2 expression by CTLA-4/B7 interaction in 'infectious' manner in the next neighboring activated Foxp3neg Tcons. When ICER is in the nucleus (whether this is a result of direct, intracellular Foxp3 expression in nT_{regs}, and/or cAMP influx in suppressed Tcons, or both), autonomous CTLA-4 signaling inhibits ERK and thus protects ICER from ERK-mediated phosphorylation, subsequent ubiquitination, and nuclear de-localization [55]. In this model, nT_{reg} cells modulate activity of autoreactive Tcons and/or Dendritic Cells (DCs) through high-affinity CTLA-4/B7 and Class II-TCR interactions (not shown).

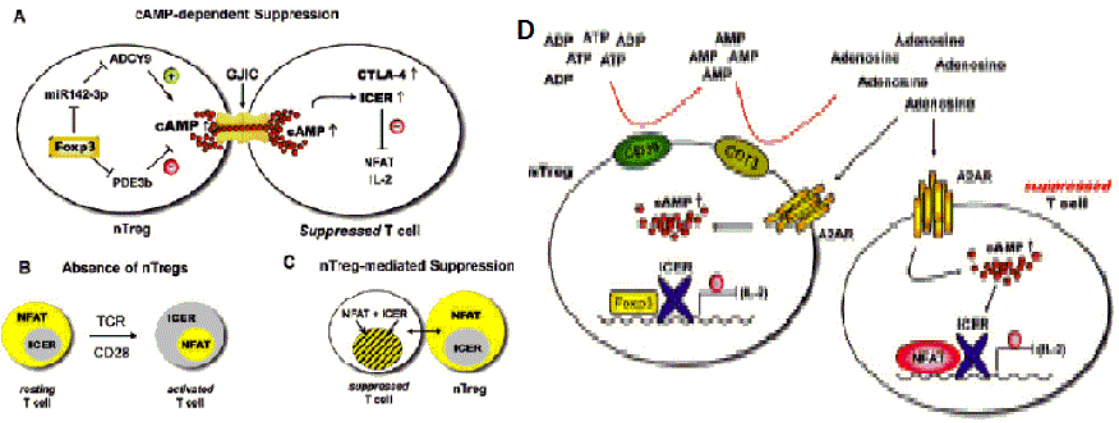

Figure 7: nT_{reg} cells direct ICER into the nucleus of activated $CD4^+$ Tcons via cAMP [55]. (A) 'Supraphysiologically' high intracellular cAMP levels are generated in nT_{reg} cells, at least in part, by Foxp3-mediated down-regulation of the Pde3b gene. Furthermore, Foxp3 also down-regulates miR-142-3p targeting adenylyl cyclase (ADCY9) mRNA resulting in up-regulated cAMP production [76,77]. cAMP is then transferred from nT_{reg} cells to the activated conventional $CD4^+$ T cells via gap junction intercellular communications (GJICs) [49]. There cAMP has at least two effects: first it induces ICER expression and second it enables the nuclear localization of ICER leading to transcriptional attenuation of IL-2 synthesis by suppression of NFATc1/α gene expression and/or formation of inhibitory NFAT/ICER protein complexes responsible for attenuated 23 expression of IL-2 and numerous other NFAT-driven cytokines and chemokines [55]. In addition, cAMP may up-regulate surface expression of CTLA-4 in suppressed conventional $CD4^+$ T cells [74], thus conferring a B7 inhibitory signal to target cell populations [52,53]. (B) In the absence of nT_{reg} cells, for example, after ablation of nT_{reg} in DEREG mice, TCR triggering and CD28-costimulation via CD28 superagonist (CD28SA) mAb results in cytosolic localization of ICER, which disables its function as a transcriptional repressor leading to unopposed NFAT-driven transcription [55]. When ICER is ousted to the cytosol, NFAT is translocated into nucleus and drives vigorous IL-2 expression in Tcons upon CD28 co-stimulation (activated T cell) [55]. (C) Gap junction intercellular communications (GJICs) transferring cAMP from nT_{reg} cells to Tcons lead to the maintenance of ICER in the nucleus of both cell populations during nT_{reg}-cell-mediated suppression [49]. In the presence of a CD28 signal (either CD3/CD28 *in vitro* or CD28SA *in vivo*), ICER and NFAT co-localize in the nucleus of activated Tcons, nuclear co-localization of ICER and NFAT leads to the inhibition of NFATc1/α gene induction and/or formation of inhibitory NFAT/ICER protein complexes, thus inhibiting NFAT-driven transcription of IL-2 and numerous other cytokines and chemokines [44]. (D) Hypoxia-adenosinergic signaling: An additional model for the cAMP-enabled and nT_{reg}-cell-mediated suppressive function of ICER, CD39, and CD73 ectoenzymes on nT_{reg} cells. These cells generate extracellular immunosuppressive adenosine from ATP, which adds to the suppressive effects of inflamed-tissue hypoxic adenosinergic signaling on conventional $CD4^+$ T cells [78,79] acting via the A2A receptor (A2AR) expressed on $CD4^+$ T cells (both in nT_{reg} cells and Tcons). A2AR signaling enhances the levels of intracellular cAMP and, presumably, in synergy with the model described in Figure 6 enforces nuclear localization of ICER leading to transcriptional attenuation of IL-2 production in suppressed Tcons [54,55].

HIV infection deregulates the balance between nT_{regs} and IL-2 Producing Tcons

HIV infection alters balance between nT_{regs} and IL-2 producing Tcons, both of which are $CD4^+$ T cells, by decreasing the expression of the IL-2 receptor α (IL-2Rα) [58]. Indexation 11 of nT_{regs} to the number of activated T cells represents a homeostatic mechanism ensuring that T cell expansion remains under control. However, immune activation observed in HIV-1-infected patients suggests dysfunction of this mechanism. Balance of nT_{regs} versus IL-2-producing Tcons is disturbed in viremic HIV-1 infected patients. In addition to the downregulation of IL-2Rα (CD25) observed in viremic patients $Foxp3^+$ cells with lower expression of CD25 have an impaired suppressive function and are less capable of maintaining Foxp3 expression [59-61]. On the other side immune activation in HIV-1 infected patients is probably a multifactorial phenomenon affected by numerous viral and immunological factors. Namely, the inability of Tcons to detect IL-2 because of defects of IL-2Rα expression or IL-2 signaling is likely to result in uncontrolled Tcon activation and autoimmune disease. Therefore, the HIV-1 mediated IL-2Rα (CD25) downregulation in nT_{regs} and the functional impairment described in HIV-1 infected nT_{regs} could be crucial in the immune activation of viremic HIV-1 infected subjects. Additionally, ART was unable to restore such nT_{reg} cell-associated disturbances because dysregulated balance between nT_{regs} and IL-2 producing Tcons was also found in aviremic HIV-1 infected patients treated for at least two years with ART [62]. Clearly, activated $CD4^+CCR5^+$ T cells predict increased acquisition of SIV infection in rhesus macaques [63].

Kinetics and activation requirements of nT_{regs} in GvHD

nT_{regs} maintain tolerance by dominant suppression of self-reactive Tcons in peripheral tissues. Nevertheless, the activation requirements and mode of action of human nT_{reg} cells display significant variability in suppressive activity. nT_{regs} display significant variability in the suppressive activity as 54% of healthy blood donors examined *ex vivo* had fully suppressive (activated) nT_{reg} cells, whereas in the remaining donors, anti-CD3/CD2/CD28 stimulation was required for nT_{reg} cell-suppressive activity [64]. Furthermore, anti-CD3/CD2/CD28 stimulation followed by fixation in paraformaldehyde left nT_{regs} fully

suppressive in all donors. The fixation-resistant suppressive activity of nT_{regs} was obliterated by trypsin treatment, indicating that a cell surface protein(s) could be directly involved. Fractionation of activated versus resting nT_{regs} identified that CD147 marks the switch between resting ($CD45RA^+$) and activated ($CD45RO^+$) subsets within the $Foxp3^+$ T cell population [65]. Interestingly, treatment of steroid-refractory acute GvHD with anti-CD147 mAb (ABX-CBL) showed significant improvement most likely by differential effect on $CD147^+$ T cells favoring activated nT_{regs} [66]. To assess activation requirements of contactdependent immune suppression by human nT_{reg} cells different protocols e.g. with anti12 CD3/CD28 [55], CD28SA [55,67,68], or anti-CD3/CD2/CD28 were employed [64] in order to ameliorate GvHD underlying ability of these treatments to do both-expansion and activation of nT_{reg} cells.

Functional delineation of human $CD4^+$ T cells expressing the Foxp3

$CD4^+CD25^+Foxp3^+$ T_{regs} develop in the thymus and have been termed 'natural' or 'thymic' T_{reg} cells (nT_{regs}) in contrast to T_{reg} cells that develop in peripheral lymphoid tissues, which are often $Foxp3^-$ and have been termed 'adaptive' or 'induced' T_{reg} cells (iT_{regs}) [69]. $CD4^+Foxp3^+$ cells might be also generated in peripheral lymphoid tissues from naïve $CD4^+Foxp3^-$ progenitors. The cytokine Tumor Growth Factor β (TGF-β) and the mode of antigen presentation represent two major factors involved in the induction of Foxp3 expression in the periphery. The heterogeneity of $CD4^+Foxp3^+$ T cells shows the need for additional markers in order to distinguish between functional nT_{reg} and iT_{reg} cells and $Foxp3^+$ naïve-like non-T_{reg} cells. $CD4^+Foxp3^+$ T cells in humans can be divided into three subgroups; $CD45RA^+Foxp3^{lo}$ naïve T_{reg} cells, CD45RA-$Foxp3^{hi}$ effector T_{reg} cells, and $CD45RA^-Foxp3^{lo}$ T cells, where the last population is cytokine-producing, activated Tcons that do not confer suppressive function [64]. Activated T_{regs} can also be identified by $CD25^+$ or $Foxp3^+$ in combination with $CD147^+$ [65]. One of the consequences of having T_{reg} cellspecific DNA demethylation in FOXP3 locus is enhanced and ensured expression of T_{reg} cell signature molecules by increasing accessibility of enhancers by transcription factors. For instance, FOXP3 CNS2 contains a transcriptional enhancer, which is demethylated during acquisition of suppressive function in iT_{regs} since mice without this region are unable to form these T_{reg} cells [69]. In line with this cyclic AMP responsive element binding (CREB) and Ets1, transcription factors essential for iT_{reg} cell function, bind to CNS2 of FOXP3 depending on methylation status [70]. Since ICER is dominant negative regulator of CREBmediated transcription [55] it is conceivable that cAMP and ICER may have influence on stability of iT_{reg} phenotype at least in some of these populations.

Humanized Mice–a Xenogeneic GvHD Model Applicable for Human nT_{reg}CCR5Δ32 Cell Analysis *in vivo*

To investigate the potential nT_{reg}CCR5Δ32-modulating capacity of gp120 *in vivo* a well-defined xenogeneic GvHD model based on the transfer of human HSCs treated with optimized dose of dmPGE2 or specific subsets of PBLs into immunodeficient mice was described [33]. Intraperitoneal injection of human HSCs or PBLs into newborn NOD-Scid mice results in 13 development of a lethal GvHD leading to death after 30 to 90 days, depending on the number of transferred HSCs or PBLs. GvHD is characterized by decelerated growth, reduced body weight, reduced mobility, and ruffled fur with a total mortality greater than 95% within two months. In this model, GvHD onset is not affected by the limited number of T_{reg} precursors transferred within HSCs. However, co-transfer of resting human nT_{regs} in ratios between 4:1 and 10:1 (PBMCs:nT_{regs}) prevented all signs of GvHD. HIV-1 envelope glycoprotein gp120 showed potent nT_{reg} cell-activating capacity *in vitro* and the xenogeneic GvHD model proved to be useful for analyzing the suppressive function of gp120 activated human T_{reg} cells *in vivo*. Notably, a single administration of 5μg gp120 completely prevented all phenotypic signs of GvHD without transfer of additional T_{regs} [68].

Conclusions

A major innovation is the use HSC transplantation of the cells resistant to HIV-1 such as CCR5Δ32 cells, which do not express CCR5 due to a deletion in the promoter. The mutation confers resistance to R5-tropic HIV-1 in homozygous individuals and could cure HIV-1 disease based on the outcome of bone marrow engraftment in HIV-1 patients with leukemia using a CCR5Δ32 homozygous donor. However, patients receiving bone marrow allo transplantation often suffer from GvHD, and for that reason HIV-1 infection is not considered an indication, unless leukemia warrants transplantation. To advance this field, it is, however, vital with (i) mapping of donors in bone marrow registries to identify CCR5Δ32 donors for world-wide matching to HIV^+ leukemic recipients; (ii) to advance strategies to understand immune dysfunction and immune regulation of HIV-1 and ability to offer suppression of GvHD via expansion and activation of suppressive T_{reg} cell function; and (iii) to explore function of CCR5Δ32 T cells and the capability to manipulate CCR5 and other modifying HIV-1-susceptibility genes in stem cells moving towards future auto-transplantation of CCR5 negative hematopoietic stem cells.

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