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

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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 CXCR4-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 (nTregs). Transfer of cyclic AMP (cAMP) from nTregs 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 (nTregs); 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-) 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 A32 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].

**Table 1: Differences between ‘Berlin and Essen patients’ receiving CCR5Δ32 homozygous allogeneic stem-cell transplantation.**

|                       | 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. grade 1-2 (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* | CIRPNNTKGIIHGPGFRTGTEIIIGDIRQAHC | CTRPNNTKGIPLPGKVFYAT-EIIRDIRKAYC |
| X4 prediction** 3months 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 CCR5Δ32 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 CTRPNNTKGIPLPGKVFYAT-EIIRDIRKAYC). 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 analysis 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 NG5). 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 insertion activation of nearby genes [27,28].

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].
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 (iTregs) with an equivalent suppressive potential as naturally occurring regulatory T cells (nTregs) by continuous polyclonal activation with anti-CD3/CD28 mAbs or CD28 super agonistic monoclonal antibody (CD28SA) [37]. During the differentiation process, the iTregs express Cyclooxygenase-2 (COX-2) and produce PGE2 [38]. Interestingly, neither 8′-resting nor activated nTregs express COX-2. The PGE2 production from iTregs 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, but also in Treg expression in 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.

Figure 2: Inhibitory pathway of prostaglandin E2 (PGE2) (adopted from Lone and Tasken [39]). (A) PGE2 mediates Treg 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 Treg cells express cyclooxygenase-2 (COX-2) and PGE2, stimulates FOXP3 expression in these cells. The Treg 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 CCR5 Δ32/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.

Downregulation of CCR5 and inhibitory pathway of PGE2:

An important precedent of receptor-mediated cAMP formation in iTregs is PGE2 synthesis [39,42]. It has been demonstrated that iTreg 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 EBPSO-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).

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.
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
reg through adenylyl cyclase could abolish the rejection (reviewed in [48]). The data on gp120 are important in the context of 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
reg (Figure 4) [46,47].

Regulatory T cell Effector T cell

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
reg 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
reg 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 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
reg 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
reg need to be activated to exert their suppressive function on bystander Tcons. However, it has remained elusive how activation of T
reg 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
reg, 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
reg 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).
As both CD28 and CTLA-4 molecules are implicated in the function of 10 nTreg cells [51-53] the ability of their natural ligands B7-1 (CD80) and B7-2 (CD86) to influence the nTreg 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 nTreg 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 nTreg via gap junctions [44]. As nTreg 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 nTreg-Tcon regulation (Figure 7A).

cAMP and ICER: A model of nTreg 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 nTreg 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 nTreg-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 nTreg cells [55,56]. Deficiency in these transcription factors led to a resistance of CD4+ T cells against nTreg cell-mediated suppression. Based on these data, we have proposed a spatiotemporal model of nTreg 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 receptor-mediated 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 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 its opening and closing.

Figure 6: Cyclic AMP underpins suppression by nTreg cells. Upon TCR activation (not shown), CTLA-4 is deployed to the surface of nTreg 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 Adenyl 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’ ICER-mediated 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 nTreg, 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, nTreg cells modulate activity of autoreactive Tcons and/or Dendritic Cells (DCs) through high-affinity CTLA-4/B7 and Class II-TCR interactions (not shown).
HIV infection deregulates the balance between nTreg and IL-2 Producing Tcons

HIV infection alters balance between nTreg 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 nTreg cells to the activated conventional CD4+ T cells via gap junction intercellular communications (GJICs) transferring cAMP from nTreg cells to Tcons lead to the maintenance of ICER in the nucleus of both cell populations during nTreg-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 nTreg-cell-mediated suppressive function of ICER, CD39, and CD73 ectoenzymes on nTreg 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 nTreg 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].

Figure 7: nTreg cells direct ICER into the nucleus of activated CD4+ Tcons via cAMP [55]. (A) ‘Supraphysiologically’ high intracellular cAMP levels are generated in nTreg cells, at least in part, by Foxp3-mediated down-regulation of the Pde3b gene. Furthermore, Foxp3 also down-regulates miR-142-3p targeting adenyl cyclase (ADCY9) mRNA resulting in up-regulated cAMP production [76,77]. cAMP is then transferred from nTreg 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 nTreg cells, for example, after ablation of nTreg 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 nTreg cells to Tcons lead to the maintenance of ICER in the nucleus of both cell populations during nTreg-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 nTreg-cell-mediated suppressive function of ICER, CD39, and CD73 ectoenzymes on nTreg 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 nTreg 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].

Kinetics and activation requirements of nTreg in GvHD

nTreg maintain tolerance by dominant suppression of self-reactive Tcons in peripheral tissues. Nevertheless, the activation requirements and mode of action of human nTreg cells display significant variability in suppressive activity. nTreg display significant variability in the suppressive activity as 54% of healthy blood donors examined ex vivo had fully suppressive (activated) nTreg cells, whereas in the remaining donors, anti-CD3/CD2/CD28 stimulation was required for nTreg cell-suppressive activity [64]. Furthermore, anti-CD3/CD2/CD28 stimulation followed by fixation in paraformaldehyde left nTreg, fully
suppressive in all donors. The fixation-resistant suppressive activity of nTregs was ablated by trypsin treatment, indicating that a cell surface protein(s) could be directly involved. Fractionation of activated versus resting nTregs 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 nTregs [66]. To assess activation requirements of contact-dependent immune suppression by human nTregs 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 nTregs cells.

**Functional delineation of human CD4+ T cells expressing the Foxp3**

CD4+CD25+Foxp3+ Treg develop in the thymus and have been termed ‘natural’ or ‘thymic’ Treg cells (nTreg) in contrast to Treg cells that develop in peripheral lymphoid tissues, which are often Foxp3- and have been termed ‘adaptive’ or ‘induced’ Treg cells (iTreg) [69]. CD4+Foxp3+ cells might also be 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 nTreg and iTreg cells and Foxp3+ naïve-like non-Treg cells. CD4+Foxp3- T cells in humans can be divided into three subgroups: CD45RA-Foxp3hi naïve Treg cells, CD45RA-Foxp3hi effector Treg cells, and CD45RA-Foxp3lo T cells, where the last population is cytokine-producing, activated Tcells that do not confer suppressive function [64]. Activated Treg can also be identified by CD25+ or Foxp3 in combination with CD47hi [65]. One of the consequences of having Treg cells specific DNA demethylation in FOXP3 locus is enhanced and ensured expression of Treg 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 iTreg since mice without this region are unable to form these Treg cells [69]. In line with this cyclic AMP responsive element binding (CREB) and Ets1, transcription factors essential for iTreg cell function, bind to CNS2 of FOXP3 depending on methylation status [70]. Since ICER is a dominant negative regulator of CREB-mediated transcription [55] it is conceivable that cAMP and ICER may have influence on stability of iTreg phenotype at least in some of these populations.

**Humanized Mice—a Xenogeneic GVHD Model Applicable for Human nTregCCR5Δ32 Cell Analysis in vivo**

To investigate the potential of nTregCCR5Δ32-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]. Intraportal 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 nTreg precursors transferred within HSCs. However, co-transfer of resting human nTreg in ratios between 4:1 and 10:1 (PBMC:nTreg) prevented all signs of GVHD. HIV-1 envelope glycoprotein gp120 showed potent nTreg cell-activating capacity in vitro and the xenogeneic GVHD model proved to be useful for analyzing the suppressive function of gp120 activated human Treg cells in vivo. Notably, a single administration of 5μg gp120 completely prevented all phenotypic signs of GVHD without transfer of additional Treg [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 homologous 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 Treg 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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