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Tackling HIV: Genetic vs. Immune CCR5 targeting

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Abstract

Recent advances in gene targeting have proposed new approaches to treat HIV infection, focused on CCR5, which is a key molecule in virus entry as well as in infection maintenance. *Ex vivo* cell targeting could make T-cells resistant to infection while reducing cell reservoirs where HIV can escape total eradication. Immunization could provide prompt receptor down regulation and preventive immunity also in limited resources settings. Innovative, gene-based methods and immune-based interventions aimed at silencing CCR5 expression *in vivo* will be compared.

Keywords: HIV; CCR5; Gene Therapy; Vaccine; Humoral Immunity

Introduction

In the Nineties, observations about the role of RANTES and other chemokines in HIV infection were reported [1]; almost at the same time, CCR5-Delta32, a rare mutation in the corresponding chemokine receptor, was found to confer resistance [2-6]. Both findings pointed out the key role of CCR5, the major HIV coreceptor, in establishment and in maintenance of HIV infection.

Since that evidence, CCR5 has become an important potential preventive and therapeutic target for blocking HIV-1 entry *in vivo*; its interest was reinforced by the common observation that homozygous Delta32 carriers show normal inflammatory and immune reactions. CCR5 was associated with inflammation in several conditions, such as age-related degenerative diseases, rheumatoid arthritis and cancer; the absence of CCR5 expression was not definitely associated with any medical dysfunction, hence not excluding the feasibility of anti-CCR5 interventions [7,8]. Consequently, a growing number of strategies aimed at preventing CCR5 function in HIV entry and spread have been designed and tested.

Anti-CCR5 strategies include small molecule drugs, such as maraviroc, *ex vivo* gene targeting introducing Delta32-like mutations in CD4+T lymphocytes and/or in hematopoietic stem cells, administration of chemokine analogues able to block the coreceptor inside cells and prevent its surface signalling, the generation of antibodies aimed at downregulating CCR5 receptors from target cells.

All of these antiviral strategies showed their efficacy and safety in preclinical assays; maraviroc is in clinical therapy since 2007; gene targeting has been already tested in some patients undergoing Hematopoietic Stem Cells (HSC) transplantation; intra- and extracellular immunization have been successfully tested in animal models.

This review will not consider development of drug inhibitors and chemokine analogues; it will focus genetic and immune-based techniques aimed at reducing or preventing CCR5 expression on target cells, i.e. CCR5 gene targeting and anti-CCR5 immunization strategies.

Gene Targeting

Since 1996, clinical observations showed that individuals carrying an homozygous mutations impairing CCR5 expression were highly resistant to HIV infection [2,3,6]. As summarized in Table 1, the status of heterozygous Delta32 carrier does not confer full protection from HIV infection but has been associated with slower progression; in fact, heterozygous Delta32 mutation was frequently observed in Long Term Non Progressor subjects (LTNP) [9-11]. Delta32 mutation, which causes the premature truncation of CCR5 molecule and prevents its surface expression, was not found to cause any immune dysfunction in homozygous subjects; however, it has been associated with increased susceptibility to West Nile virus or to tick-borne encephalitis [12,13].

Conversely, CXCR4 molecule, the second HIV coreceptor, is involved in hematopoiesis and neurogenesis, therefore its function is not dispensable and its mutations similar to Delta32 cannot be observed *in vivo* [14].Other genetic polymorphisms have been associated with HIV protection, as those involving SDF-1alpha (a CXCR4 ligand), RANTES (a CCR5 ligand) or CCR2 (a chemokine receptor sharing high homology to CCR5) [15,16].

However, human CD4+T lymphocytes where CCR5 and CXCR4 were inactivated by intrakine expression, did show normal proliferation and response to antigens, mitogens, cytokines and other chemokines [17]. These findings seem confirmed by a recent study, showed that conditional CXCR4 knock out in T cells was not lethal in mice, allowing the study of CXCR4 involvement in arthritis [18].

Transplantation with CCR5 Delta32/Delta32 hematopoietic cells was first performed in an HIV-infected patient with acute myeloid leukemia; once recovered, the patient remained free from viremia for 20 months without receiving antiretroviral therapy (ART) [19]. Subsequently, CCR5- CD4+ donor cells were found in submucosal gastrointestinal (GI) and in glial brain biopsies some months after transplantation, showing that these cells have slowly colonized host tissues, and suggesting that their expansion could have reduced viral reservoirs [20]. Immune analysis of circulating lymphocytes showed that host memory CD4+ T lymphocytes have been replaced by resistant donor cells, therefore subtracting an important pool of virus targets. Most importantly, CCR5 ablation has not induced virus switch either in CXCR4 dependent (so called X4 viruses) or in dual tropic (so called R5-X4 viruses). As a confirm, the patient interrupted ART without

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Anti-CCR5 gene targeting	Ref	Evidence	Benefits	Limits
Natural Delta32 homozygosis	[2-6]	Spontaneous mutation occurring in 4-18% of European and Askenazi Jews people. Not found in Asian and Pacific indigens.	Lack of CCR5 surface expression. No signs of immune alterations. Resistance to R5 HIV infection.	No resistance to X4 or dual R5/X4 strains. High sensitivity to West Nile virus and tick-borne encephalitis.
Transplantation of CCR5-/- cells (HST or T lymphocytes)	[19,20,29]	Clinical infusion of modified cells in patients undergoing cytoreductive treatment.	Resistance to HIV infection. Long-term cell survival GI mucosal colonization. Brain colonization Reduction of HIV reservoirs ? Heritable DNA modification. Definitive cell cure .	Off-target genotoxicity (e.g. CCR2). Oncogenesis. CXCR4 is required for HSC maturation in bone marrow. Multiple treatment cycles ? Selection of resistant strains. Mucosal efficacy?
CCR5 and/or CXCR4 KO by Zn-finger Nucleases in T cells	[29]	Ex vivo transient expression of viral vectors carrying ZFNs	Resistance to HIV infection. Mucosal protection? Reduction of HIV reservoirs ? Definitive cell cure ?	Off-target toxicity Insertional mutagenesis? Lentivirus-induced activation or mobilization of endogenous LTR? Unknown long-term safety issues.
CCR5 KO by TALENs or CRISPRs in T cells		Preclinical study in mice receiving ex vivo modified T cells	Lower off-target activity than ZFNs.	
Double CCR5+CXCR4 KO by ZFNs in T cells		*Ex vivo* trial	Total resistance to HIV infection.	
Ribozymes		*In vitro* *Ex vivo* trial	Preventing CCR5 expression.	Lentivirus-induced activation or mobilization of endogenous LTR?
Intrabodies-mediated CCR5 intracellular retention		*Ex vivo* assay	Preventing CCR5 expression. Resistance to R5-HIV infection.	Excess of intracellular protein entrapment

Table 1: Anti-CCR5 gene/expression targeting.

incurring viral rebound and antiviral antibodies vanished over time, suggesting that HIV was no longer expressed [20]. After six years of HIV remission, the "Berlin patient" is considered the first (and unique) case of successful HIV cure since now, even if minimal HIV expression could be still present, at levels beyond limits of detection achieved by present technology [21]. Other HIV-positive patients underwent HSC transplantation to treat leukemia or lymphoma, but HIV rebound was observed after transplant and ART and immunosuppressive therapy were required to control viral load and Graft-versus-Host (GVH) disease; most of these patients died after transplant [22]. The mechanisms involved in HIV eradication in "Berlin patient" only are not yet fully understood. This patient underwent severe particular transplant conditions different from those ones applied to the other patients who died, suggesting that total body irradiation or the engraftment with Delta32 cells from a CCR5 donor might have been critical differential aspects in the case of the Berlin patient [22].

Transplantation with Delta32 homozygous HSC associated with ART is considered a promising way to restore immune system with cells resistant to HIV infection and to reduce HIV reservoir cells [23]. However, this approach presents several limitations, such as the shortage of Delta32 homozygous donors [24], the requirement of severe cytoreductive treatments before and of immunosuppressive and antiviral therapies after the procedure and the consequent economic burden, all of whom make this practice uniquely applicable to HIV patients with haematologic tumors in Western countries [22,25].

Other strategies focused CCR5 Delta32 homozygosis by means of gene disruption, antisense RNAs, intracellular expression of chemokines or antibodies (Table 1).

Engraftment of T cells is fater, and CD4+ cells proliferate rapidly *ex vivo* and *in vivo*; HSC grow slower and require more extensive cytoreductive conditioning to achieve an initial advantage over host T-cells, but can differentiate to all cell lineages. Due to their fast growth once engrafted, HSCs are more prone to degenerate in tumors than T-cells, due to the possible off-target, mutagenetic or trans-activating effects [26]. On the other hand, CCR5 gene knock out (KO) could have a protective role towards donor cells activation and dissemination, since T-cell migration towards inflammatory loci depends on CCR5 receptors [31]; especially CCR5 density on cell surface was observed to increase T-cell migration in Graft-versus-Host disease (GVHD) [28].

Gene Knock out

Transient expression of specific endonucleases under the control of adeno- or lentiviral vectors was aimed at introducing CCR5 mutations and at preventing its transcription or translation in CD4+ lymphocytes [22,25]. Different semisynthetic nucleases have been build, which put together a DNA binding domain, such as Zinc Fingers (ZF), and an endonuclease function (N), usually provided by *Fok I* restriction enzyme (Table 1).

Zinc Finger Nucleases (ZFNs) take advantage of the most versatile and most used DNA binding proteins, have been used in many cell types and also tested in *ex vivo* human trials [29]. Other DNA binding proteins, such as the Transcription Activator-Like Effectors (TALEs), large proteins of vegetal origin, have already been assayed *in vitro*, showing a comparable efficiency to ZFNs; no infusion assays, even in humanized mice, have still tested the safety of this approach [30]. Another promising method to be exploited in gene editing is the CRISPR/Cas9 (clustered regularly interspaced palindromic repeats sequences) system, which usually serves to bacteria to inactivate plasmid and phage DNA and to elude host innate immunity by inducing multiple nicks under thecontrol of a guide RNA [25]. CRISPR/Cas9 has been successfully used to target human cells and disrupt CCR5 gene, but off-targeting remains a major limit to be overcome [31,32].

In order to obtain a double strand DNA break and to increase target specificity, a ZF and a nuclease domain work on each DNA strand, and the whole ZFN protein is a dimer. DNA recognition ensured by a ZF domain usually spans 9-18 bp [25]. DNA breaks undergo cell repair

systems, which are often prone to introduce mutations and deletions and therefore lead to reduced or blocked gene expression in a high proportion of treated cells. Even the induction of a single CCR5-Delta32 hit can be helpful in gene editing procedures, since increases the proportion of CCR5-homozygous cell population generated by ZFN expression; in fact, therapeutic success depends on the proportion of cells carrying biallelic CCR5 disruption [29]. On the other hand, the strong DNA affinity or the prolonged expressions of ZFNs within target cells have been associated with off-target effects, i.e. mutagenesis or disruption of undesired or unspecific cellular genes. Due to its homology to CCR5, the CCR2 gene, coding for the chemokine receptor binding the Monocyte Chemo attracting Protein-1 (MCP-1), is one of the most frequent off-targets of ZFN [30,33].

Finally, the complete CCR5 inactivation should prevent virus switch to X4 or dual-tropic viruses in the early phase of HIV infection, but if the CCR5 inactivation happens later during HIV infection, a double CCR5-CXCR4 targeting should be performed. Double CCR5-CXCR4 gene disruption has been performed in human cell lines coinfected with two adenoviral vectors carrying the two specific ZFNs. Coinfection with an R5 and a X4 HIV strains enriched the *in vitro* population of resistant T cells from 9% to 99%, confirming their resistance. Similar results were observed in CD4+ T primary cells, which were infused in humanized mice and challenged with infectious R5 and X4 tropic viruses. Up to 10% of resistant human cells were found in mice spleens; proportion of cells carrying CCR5 and CXCR4 gene inactivation reached 69% and 73%, respectively; these encouraging results need to be further confirmed before applying to human therapy [34].

Antisense RNA

To shut down CCR5 expression, several RNA-based strategies have been assayed for more than ten years (Table 1). Different approaches were shown effective to reduce CCR5 expression, working through RNA silencing (siRNA), antisense RNAs targeting different viral and cellular genes or ribozymes with catalytic activity [35-40].

Differently from gene editing strategies, RNA silencing or antisense shut-down require the continuous expression of therapeutic RNA; suitable viral vectors should work *ex vivo* or possibly *in vivo*, therefore bypassing requirements of cell culture, autologous cells cytoreduction and reinfusionof treated cells.

Adenoviruses and pseudotyped lentivirus vectors have been successfully used to transduce siRNA-coding sequences within cells; while the former vectors remain as episomes and can be lost after some cell cycles, the latter ones can make genes stably integrated in chromosomes, but with a higher risk of mutagenesis. Conditional replication of lentivirus vectors could hypothetically result beneficial, because could contribute to spread therapeutic genes to cells already carrying HIV proviruses, but the risk of insertional mutagenesis and reactivation of endogenous retroviruses cannot be excluded [41]. Similarly to what observed with gene editing, off-targeting activity and over-expression of antisense RNA may result toxic [38]; in addition, undesired activation of innate immunity, driven by double stranded RNA (dsRNA) via Toll-like receptors, could be observed [46,43].

Silencing RNA (siRNA) is an innate cellular mechanism to regulate gene expression that takes advantage of short antisense RNA (about 20 bp) complementary to a specific mRNA and commits it to degradation. ShRNA are short hairpin RNA, precursor of siRNA, which are processed by *Dicer* endonuclease to become guide RNA and address the silencing complex (RISC) to the targetmRNA [44]. Partial or complete CCR5 shut-down in T cells has been achieved with various viral vectors, ranging from SV40 to lentiviruses, under the control of promoters with different strength [36,38,39,45]. Rhesus CD34+ cells, i.e. precursor of T-cells, monocytes and macrophage lineages, transduced with a lentivirus vector carrying an H1-promoter controlled shRNA, showed a 3-10-fold reduction in CCR5 surface expression and partial resistance to SIVmac challenge; once infused in macaques, reconstituted T lymphocytes population without signs of toxicity [37]. Another study assessed the stable expression of a shRNA in macrophages derived from fetal liver CD34+ cells; CCR5 downregulation was over 90% in differentiate macrophages, conferring viral protection without apparent signs of toxicity [46].

The transduction of an antisense RNA complementary to CCR5 sequence reduced receptor surface expression by 98% and blocked R5 HIV strain infectivity more than 50% [47].

Ribozymes are catalytic RNA molecules able to cut target mRNA in one or more fragments (Table1); different CCR5 ribozymes have been designed and successfully tested in human cells [48].Stable expression of a multimeric hammerhead in human T cells decreased receptor expression and nearly abolished infectivity of a R5 HIV strain, without affecting X4-mediated infection [49]. Another ribozyme, transduced in CD34+ cells by a retroviral vector, did not affect cell differentiation in T cells lineages and macrophages cultured in a mouse model of thymic differentiation [35]. Anti CCR5 ribozymes were also included in multitarget vectors; similarly to ART combinations, lentiviral vectors carrying different blocking agents should prevent the development of resistance to genetic therapies.

A triple combination of HIV *tat* and *rev* decoys and an anti-CCR5 ribozyme was tested in CD34+ cells; expression of therapeutic genes in differentiated monocytes reduced HIV R5 infectivity following an additive mode, the triple combination being the most effective among the combinations assayed [50].

Intracellular Chemokines and Antibodies

The increased expression of RANTES, MIP-1α and MIP-1β chemokines, and the consequent internalization of CCR5 receptor, have been considered natural protective factors in HIV infection [4,5]. Therefore, chemokine analogues appeared promising drug candidates to confer mucosal protection from HIV entry, with lower risks of inducing drug resistance [51].

A tricky way to achieve inhibition of CCR5 expression has been accomplished through expression of intracellular CCR5 ligands, such as modified chemokines and antibodies (Table 1).

Genes coding for CCR5 and CXCR4 ligands, i.e. RANTES and SDF-1α, were modified to be targeted within endoplasmic reticulum (ER). Both molecules worked as molecular decoys and effectively prevented both receptors from surface exposure; once transduced in human T-cell lines and in Peripheral Blood Mononuclear Cells (PBMCs), singularly or in pair, conferred the expected resistance to R5 and/or X4 HIV strains upon challenges [13,52,53].

Similar results were confirmed by a recent study, where intracellular RANTES genes, controlled by EF1-alpha promoter, were efficiently transduced in human cells by a lentiviral vector after CD3 and CD28 antibody stimulation [54]. Intrakine expression reduced, but not completely removed, CCR5 molecules from cell surface; expression levels of other surface receptors, such as CCR1 and CCR3, which usually bind RANTES, was found reduced by effect of the intrakine. Interestingly, real-time PCR analysis revealed a low copy number of proviral DNA in transduced cell cultures; differently from control cells,

where viral DNA increased over time, treated lymphocytes maintained a constant amount of viral DNA over three weeks [54].

Moreover, different genetic association between Long Term Non Progressor status and copy number variation of CCL3L1 gene encoding for MIP-1α have been published in the last few years [55-56] and in particular some isoforms of MIP-1α has shown to be very potent agonists of CCR5 [57-58].

The use of intracellular antibodies rather than chemokines could prevent undesired interactions withredundant CCR receptors (Table 1).ST6, a Fab fragment from a mAb recognizing a unique sequence in CCR5 N-terminus, was engineered to become a single chain antibody (scFv) endowed with an ER retention peptide. Intracellular expression of scFv completely blocked surface labelling of CCR5 molecule in cytofluorimetric assays both in human and in rhesus cells, while CXCR4 expression was unaffected. Modified cells became resistant to R5 HIV challenge and to R5-mediated cell-cell fusion, showing the effective removal of surface CCR5 [59]. A subsequent study showed that modified primary T cells carrying CCR5 intrabody were protected from HIV transmission, once interacting with activated, antigen-presenting, dendritic cells pulsed with R5 HIV. Transduced CD34+ human cells, infused in NOD/SCID (nonobese diabetic/severe combined immunodeficiency) mice, differentiated in CD4+ and CD8+ cell lineages, showing stable intrabody expression and retaining HIV resistance [60]. Finally, anti-CCR5, single chain antibodies were also used to specifically target viral pseudotyped lentiviral vectors to CCR5-expressing cells [61].

Similarly to ART, combined gene targeting interventions should be required to achieve complete virus suppression [48]; however, the induction of virus resistance and the survival of long-lasting cell sanctuaries cannot be fully excluded[27,30].

Immunization studies

All attempts to block CCR5 molecule through host immune responses have to cope with two major factors: the highly flexible, poor immunogenic structure of the antigen and the need to elicit host auto-immunity and to break immune tolerance towards a self antigen.

Moreover, anti-CCR5, as all antibodies, could exert antiviral activity through many different mechanisms, including binding competition, steric hindrance, receptor internalization, block of virus transcytosis across epithelial cell layers, complement fixation or Antibody-Dependent T-cell-Mediated Cytotoxicity (ADCC) [62,63]. No present studies have either defined what is the most effective way in which anti-CCR5 antibodies exert anti-HIV activity or what is the CCR5 domain to be preferred to induce the most effective antibodies.

CCR5 molecule is a G-protein coupled receptor (GPCR), with a typical structure made of seven transmembrane domains; N-terminus and three extracellular loops (ECL1, 2 and 3), are the immunogenic regions and account for about a fourth of its whole sequence (90 out of 352 aminoacids). The two longer domains, the N-terminus and the second extracellular loop (ECL2), are involved in HIV binding (chemokines only bind ECL2 domain), and host the immunodominant epitopes recognized by the majority of monoclonal antibodies [64-66]. Chemokines binding to CCR5 determines receptor internalization as well as T-cell and macrophage chemotaxis; chemokine analogue drugs suitable to antiviral therapy should not induce pro-inflammatory, adverse effects [55].

Monoclonal antibodies to N-terminus and ECL2 compete with HIV for binding (ECL2, but not N-terminus, antibodies prevent chemokine binding); some of them may induce receptor internalization. Monoclonal antibodies have been evaluated in several studies [67]; in humanized form, some have been also tested in clinical trials of passive immunization [68-71]. Anti-CCR5 recombinant antibodies have also been isolated by phage libraries, an approach aimed at increasing the chances to obtain highly active, specific antibodies to the expected target [72,73]. Single chain antibodies to CCR5, selected from a phage library displaying cyclic constrained peptides, were found to block specific receptor, but not CXCR4 [74].

The ECL1 domain does not bind HIV; its engagement by natural anti-CCR5 antibodies induces a long-lasting receptor internalization, mediated by clathrin-coated pits [75].

Natural anti-CCR5 antibodies were found in various groups of individuals belonging to different ethnic groups, such as the Delta32 homozygous carriers, exposed to CCR5 through sexual intercourse with CCR5+ partners; haemophilic patients, repeatedly exposed to alloantigens found in blood transfusions; HIV-exposed but uninfected sexual partners of HIV-positive patients (ESN or EU or MEU); HIV-positive patients and especially LTNP subjects, who control the disease for years in absence of ART [75-84]. Strikingly, natural antibodies to CCR5, either IgG and IgA, have been detected in serum as well as in other biological fluids, such as saliva, milk, semen and cervicovaginal secretions, where they are likely to exert direct antiviral activity by inducing receptor internalization or by inhibiting mucosal transcytosis of virus particles (i.e. their transfer across cell membranes of mucosal epithelia) (Table 2). Differently from natural antibodies to ECL1, monoclonal antibodies (mAbs), such as 2D7 (ECL2), do not block HIV transcytosis [78,80-84,86].

Anti-CCR5 antibody	Ref	Study	Immunogen/ Vector	Adjuvant//Route/ Schedule	Biological features	Limits
Natural ECL1 Abs	[75,80,83,84,86]	ESN and LTNP sera.	Natural Ags?	Low dose Ags presented by mucosal route?	Inhibition of MIP-1beta chemotaxis. Binding to native CCR5 on PBMC. CCR5 internalization. Block of HIV transcytosis . Block of R5-HIV isolates from A, B, C, E clades	Natural, uncommon.
Natural Nt/ECL2 Abs	[76-78,85]	Healthy donors Delta32+ ESN Delta32+ CCR5- HIV+ patients	Natural Ags?	Allo Ags exposure?	Competition for chemokine binding. Binding to native CCR5 on transfected or PBMC. Block of R5-HIV laboratory and primary isolates.	Natural response observed in healthy HIVexposed or in chronic HIV pz. Not all Abs internalize CCR5
Anti-CCR5 humanized mAbs	[68-71]	Passive immunization clinical trial	PRO140 HGS001	IV infusion in HIV-positive patients	Well-tollerated, no toxicity.	IM infusion. Immune responses to therapeutic Abs.

Table 2: Anti-CCR5 immune responses in humans (naturally occurring or upon passive immunization).

The maintenance of a proper antigen presentation made difficult to reproduce natural anti-CCR5 responses upon immunization; due to their flexibility, N-terminus and CCR5 loops require a conformed status to retain proper immunogenicity [87]. Table 3 summarizes significant preclinical immunizations performed in rodents. Not surprisingly, first immunization experiments failed in achieving the expected responses, probably due to the epitope presentation in form of conjugated, flexible peptides. Specific, albeit scarce, antibodies obtained from peptide immunizations were nevertheless able to bind CCR5 molecule and to block *in vitro* infection of R5 HIV strains [88,89].Further experiments assayed immunogens endowed with a determined three-dimension conformation: immunization with a cyclic peptide from ECL2 (R168-T177) induced specific antisera in macaques; antibodies bound human and macaque CCR5+ cells and inhibited infection of A and C clades primary R5 HIV and SHIV isolates *in vitro* [90].

As shown in Table 3, other immunizations took advantage of the Flock House capsid protein (FHV), a conformation-constrained vector, to elicit antibodies to ECL1 (Y89-W102). Systemic and mucosal murine immunization elicited IgG and IgA antibodies in serum and in vaginal fluids. Such antibodies recognized and downregulated CCR5 from human and murine cells, inhibited MIP-1β induced chemotaxis and blocked clade B R5 virus infection *in vitro*. Similarly to ESN individuals, CD4+ PBMCs from serum and vaginal washes of immunized mice showed lower amounts of endogenous CCR5 receptors [87].

Aminoacid substitutions introduced in ECL1 peptides displayed in the same carrier were found to increase antibody affinity compared to the wild-type peptide (ECL1, A95-A96 vs. wild type D95-F96); according with NMR analysis, substitutions increase peptide stabilization, enhancing its propensity to assume an helical conformation, therefore confirming the elevated flexibility of native ECL1 domain [91].

In order to increase immunogenicity, vaccine strategies also addressed the construction of high density peptide arrays displayed on Virus Like Particle (VLPs) (Table 3), inspired by the fact that viral envelopes crowded with protein spikes are highly immunogenic and could induce neutralizing responses more easily [92]. As a confirm, such VLPs, based on MS2 bacteriophage backbone, were found to enhance immunogenicity of gp120-V3 and ECL2 peptides [93]. Other bacteriophage-based VLPs, carrying CCR5 peptides, achieved strong systemic and local responses, once administered through airways in a preclinical test in rats [94].

A key aspect in CCR5-targeting immunization is the ability to induce mucosal responses, because HIV entry takes place in mucosal districts almost in all cases [95]. Mucosal districts offer both humoral and physical barriers to infection, due to antiviral factors, such as RANTES and defensins, to IgG and IgA, and to morphological features of epithelia [96,97]. Strikingly, X4 viruses were found to be restricted in transcytosis and in mucosal infection as well [98-100].

Natural HIV-blocking IgA in mucosal secretions, described in ESN, was already supposed to confer natural resistance to HIV [80,82,101,102]. Anti-viral IgA were found to prevent infection in animal challenges and in human trials by exerting a number of antibody-mediated activities, in addition to neutralization of CD4+ cell infection. Most surprisingly, mucosal antiviral activities took place even in the absence of detectable systemic neutralizing responses [103-105].

Immunization by mucosal route (intranasal DNA prima followed by peptide booster) elicited specific IgG and IgA in sera and in mucosal secretions (intestinal, vaginal and lung) to gp120-V3 loop, gp41-ELDKWAS epitope and CCR5-ECL2 (R168–S185) peptides [101]. In the same study, long-term IgG and IgA blocking antibodies were still observed 12 months after boosting, suggesting that intranasal DNA priming followed by one peptide/L3 adjuvant booster immunization could induce long-lasting immunogenicity to conformational epitopes [101]. As described in Table3, immunization with a conformation-constrained ECL1 peptide by IM or IN route elicited IgG andespecially IgA antibodies in serum and in vaginal fluids; besides other anti-CCR5 effects, such antibodies caused a marked downregulation of the receptor

Anti-CCR5 antibody	Ref	Study	Immunogen/Vector	Adjuvant/Route/ Schedule	Biological features	Limits
Nt and ECL2 Abs	[101]	Preclinical, mice	ECL2 (aa 168–182) peptide emulsified in mono-oleate/fatty acid (L3) adjuvant	IN DNA priming and booster IM immunization	Serum and mucosal IgG and IgA (intestinal, vaginal and lung). Long-lasting IgG and IgA (12 months from boosting). IN DNA prime+peptide booster induced HIVblocking antibodies and B memory cells.	Human/simian CCR5 sequences. Not conformed peptide.
ECL1 Abs	[87]	Preclinical, mice	ECL1 conformed in FHV capsid protein	Freund's adjuvant by IM and IN administration	IgG and IgA. Long lasting CCR5 downregulation on PBMC and mucosal fluids. Transcytosis inhibition.	Human ECL1 sequence.
Nt Abs	[89]	Preclinical, rabbits	Nt (M1-S7) conjugated with KHL.	Fusion with a Tspecific peptide from *Tetanus* toxoid	Binding to N-term and full CCR5. HIV block in macrophages in vitro.	Not conformed, human Nt sequences. Low proportion of CCR5-specific antibodies
Nt-and ECL2-CCR5	[94]	Preclinical, rats	Nt, cyclic ECL2 and Nt+cyclic ECL2	Phage Qbeta-based VLPs by IM or aerosol route	Strong IgG and IgA in serum Aerosol boost induced mucosal IgA	Macaque CCR5 sequences.
CCR5 Abs	[106]	Preclinical, mice	Homologous, Nt, ECL1, ECL2 sequences conformed in FHV capsid protein.	Alum best adjuvant than Freund's, RIBI, Montanide. IM priming + mucosal IN boosting best schedule among 13 protocols compared.	Murine CCR5 sequences. Full tolerance break. Serum and mucosal IgG and IgA. Long lasting CCR5 downregulation in PBMC and mucosal fluids. Mucosal IgA priming. Transcytosis inhibition. No signs of autoimmunity.	Low IgA recovery from mucosal fluids.

Table 3: Preclinical anti-CCR5 immunization studies in rodents.

from CD4+ PBMCs, especially observed in cells isolated from vaginal fluids of mice vaccinated by mucosal route [87].

A recent preclinical study performed in mice have systematically addressed all aspects of anti-CCR5 immunization, including the use of adjuvants, in order to define the optimal schedule to elicit strong and long-lasting systemic and especially mucosal responses [106]. Three different immunogens, i.e. N-terminus, ECL1 and ECL2, expressed in the context of FHV capsid protein, were administered in 13 different protocols of immunization, comparing different combination of systemic and mucosal routes and four adjuvants (Table 3). Systemic IM priming and IN mucosal boosting with Aluminium adjuvant resulted the best combination and elicited high-titer specific antibodies. Not surprisingly, analysis of Peyer's patches revealed consistent priming in a high proportion of cells, showing an activated B phenotype and high-levels of IgA, representing up one-third of HIV-blocking antibodies.

ECL1 and ECL2 gave rise to stronger responses than N-terminus, achieving almost total CCR5 downregulation, sustained write block of HIV infection and nearly complete block of transcytosis. Most importantly, histopathological analysis found mild to moderate signs of chronic inflammation in some tissues, consistent with the activity of Aluminium adjuvant, but no signs of autoimmunity were observed [106].

In vivo immunization&challenge studies

Results from immunization and challenge studies led to evaluate *in vivo* efficacy and safety, since CCR5 immune targeting raised concerns about the feasibility of a breakage in immune tolerance and the consequent risk of autoimmunity [107].

As summarized in Table 4, elicited anti-CCR5 antibodies were found to block HIV and SHIV infection *in vitro,* therefore showing that immune tolerance to a self antigen was broken; no immunized animal escaped infection, but reduced viral load and control of viremia in respect tounvaccinated control animals support the role of CCR5 auto-antibodies in controlling the infection [108-110].

Striking results come from a recent *in vivo* study, which confirms protective role of anti-CCR5 antibodies *in vivo* and suggests that this vaccination could really confer long-lasting *in vivo* protection (Table 4).A bacteriophage Qss VLP-based vaccine, presenting N-terminus and cyclic ECL2 peptides, was assayed in macaques before vaginal challenge with SIVmac251 [107]. Four vaccine doses were administered by intramuscular (IM) only or by intravaginal (IV) +IM routes.

After high-dose challenge, vaccinated animals showed 30-fold lower peaks and viremia than controls; in three out of twelve treated animals (25%), one from IM and two from IM+IV groups, SIV nucleic acids become undetectable since six weeks p.i., not only in serum but also in lymph nodes and colon biopsies. Unfortunately, vaginal secretions could not be examined and so IgA levels were not evaluated. *In vivo* depletion of CD8+ T cells after one year p.i. failed to induce viral rebound, suggesting that protection did not depend on cytotoxic cells but mostly relied on humoral immunity; when anti-CCR5 antibodies titer was vanished, i.e. more than one year after immunization, the three animals could become infected upon a new vaginal or intravenous high-dose challenge [111]. High-dose challenge was intended to clearly transmit infection in all controls and treated animals; however, high-dose challenge is far from usual conditions of HIV transmission and could bring to underestimate the actual level of protection [103].

Conclusions

Responses from fresh studies confirm the important role of CCR5 molecule in HIV infection and maintenance, and get to consider its targeting is an attainable therapeutic goal. HIV story tells that many approaches and different therapeutic tools have been helpful to transform the acute infection in a chronic condition, with the aim at full virus eradication. On one hand, CCR5 and possibly CXCR4 gene ablation seem to promise the definitive eradication of HIV infection, albeit at heavy and costly conditions; similar efforts can be uniquely afforded by few chronic patients who cannot be longer treated with drugs or who need anticancer, life-saving interventions.

On the other hand, CCR5 immune targeting could offer effective and long-lasting preventive immunity, with low healthcare and individual burden. Both genetic and immune strategies offer specific rationale to fight HIV and find application in precise contexts; both of these could bring further development and offer new insight towards between the and control write HIV, although a CCR5 vaccine-based approach to induce CCR5 antibodies in groups at risk of contracting

Anti-CCR5 Vaccine	Ref	Study	Immunogen/Vector	Adjuvant//Route/ Schedule	Biological features	Limits
CCR5 auto-Abs	[108]	Preclinical, macaques SHIV challenge	High density Nt peptides conjugated to VLPs from BPV-L1 protein. Homologous, non conformed macCCR5 sequences were used.	9 IM inocula with TiterMax Gold adjuvant	Binding to native macCCR5 in vitro SHIV block. Reduced viral loads and time to clearance upon IV infection with a weakly pathogenic SHIV. SHIV clearance correlated with anti-CCR5 antibody titer and avidity.	Abs titers were found to decline over time but responded to subsequent boosts. No vaccinated macaque escaped challenge, albeit most of them controlled it.
CCR5 Abs	[110]	Preclinical, macaques SHIV challenge	Cyclic ECL2 (Arg168-Tyr177+CyscDDR5) conjugated to MAP poly-lysin resin	Immunizations in Complete (IP, 0, 1wk) and Incomplete (SC, 6 wk) Freund's	Binding to human and macaque PBMCs. In vitro block of laboratory and primary isolates. Reduction of viral load upon challenge.	Infection was partly controlled but not prevented.
CCR5 Abs	[111]	Preclinical, macaques Vaginal SIV challenges	Nt and cyclic ECL2 peptides in bacteriophage Qss VLPs Homologous macCCR5 sequences were used. .	4 IM priming (Freund's) + 3 vaginal boosting	Viremia peak 30-fold lower Undetectable SIV in 3/12 animals since 6 wk p.i. for more than a year in serum, lymph nodes and colon biopsies. Viral control due to humoral but no to cell-mediated immunity.	Vaginal secretions could not be examined. IgA were not evaluated. High dose challenges could have even masked the real extent of protection

Table 4: Preclinical, anti-CCR5 immunization studies with virus challenges.

HIV infection could be a more feasible and safe therapeutic goal than gene therapy, considering the HIV epidemiology and the difficulty of implementing CCR5 gene therapy in individuals residing in developing countries, of HIV infection.

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