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Review

C-C chemokine receptor type five (CCR5): An emerging target for the control of HIV infection☆

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ABSTRACT

When HIV was initially discovered as the causative agent of AIDS, many expected to find a vaccine within a few years. This has however proven to be elusive; it has been approximately 30 years since HIV was first discovered, and a suitable vaccine is still not in effect. In 2009, a paper published by Hutter et al. reported on a bone marrow transplant performed on an HIV positive individual using stem cells that were derived from a donor who was homozygous for a mutation in the CCR5 gene known as CCR5 delta-32 (Δ32) (Hütter et al., 2009). The HIV positive individual became HIV negative and remained free of viral detection after transplantation despite having halted anti-retroviral (ARV) treatment. This review will focus on CCR5 as a key component in HIV immunity and will discuss the role of CCR5 in the control of HIV infection.

Contents

1. Introduction to CCR5

The first chemokine was discovered in 1977 (Walz et al., 1977) and since then a large super family of chemokines has been identified. Chemokines are small proteins with several functions including immune surveillance and immune cell recruitment. The effects of these chemokines are mediated by their G-protein-coupled receptors (GPCR's). Chemokines are classified as C, CC, CXC and CX3C depending on the structure and number of cysteine residues. Moreover, the receptors are designated with an addition of 'R' to indicate receptor (Murphy et al., 2000).

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Chemokine receptors consist of seven transmembrane domains and amino and carboxyl termini (Horuk, 1994). The C-C receptors often share a significant degree of homology, with 'C-C chemokine receptor type five' (CCR5) and 'C-C chemokine receptor type two' (CCR2) sharing 75% homology (Combadiere et al., 1996). Despite the close structure of these receptors, they bind to different ligands and mediate a variety of effects.

The chemokine receptor CCR5 is expressed on various cell populations including macrophages, dendritic cells and memory T cells in the immune system; endothelium, epithelium, vascular smooth muscle and fibroblasts; and microglia, neurons, and astrocytes in the central nervous system (Rottman et al., 1997). Natural ligands for the receptor include 'macrophage inflammatory protein one alpha' (MIP1-α) (Nibbs et al., 1999), 'macrophage inflammatory protein one beta' (MIP1-β), 'regulated upon activation, normal T-cell expressed, and secreted' (RANTES) and 'monocyte chemotactic protein two' (MCP-2) (Combadiere et al., 1996; Raport et al., 1996; Samson et al., 1996a; Gong et al., 1998). The receptor plays a role in the inflammatory response by directing cells to sites of inflammation. Other chemokine receptors work together with CCR5 to stimulate T-cell functions (Contento et al., 2008). The receptors enhance T-cell co-stimulation and cytokine release from $CD4^+$ T-cells. Furthermore, ligands for CCR5 augment the activation of T-cell responses and enhance the production of antigen specific T-cells (Taub et al., 1996). During inflammation, the level of CCR5 expression is up-regulated in $CD8^+$ cells, which allows the cells to move towards sites of $CD4^+$ T-cell and dendritic interactions (Castellino et al., 2006). This increases the chance of $CD8^+$cells encountering antigen specific cells. Thus, CCR5 enhances the adaptive immune response.

In 1996, it was discovered that CCR5 is necessary as a co-receptor for entry of the macrophage tropic HIV strains (Dragic et al., 1996; Deng et al., 1996). Dragic et al. demonstrated that the ligands for CCR5 inhibit viral entry and envelope mediated fusion (Dragic et al., 1996). The virus uses CCR5 especially during initial infection, whereas the alternative co-receptor 'C-X-C chemokine receptor type four' (CXCR4) is used much later in HIV infection when the infected individual is progressing towards AIDS.

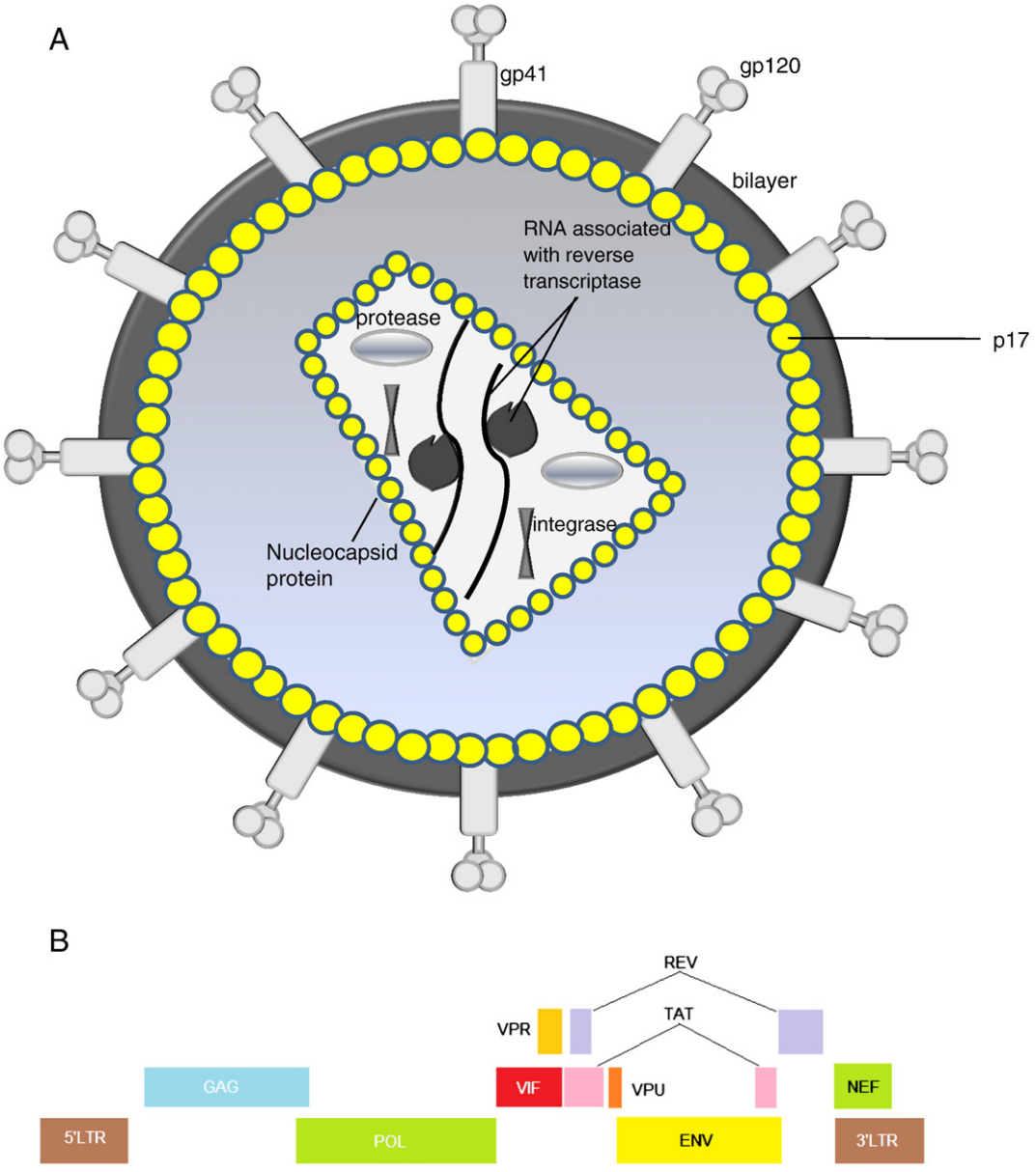

Fig. 1. A. Structure of a mature HIV virion. B. Structure of the HIV genome.

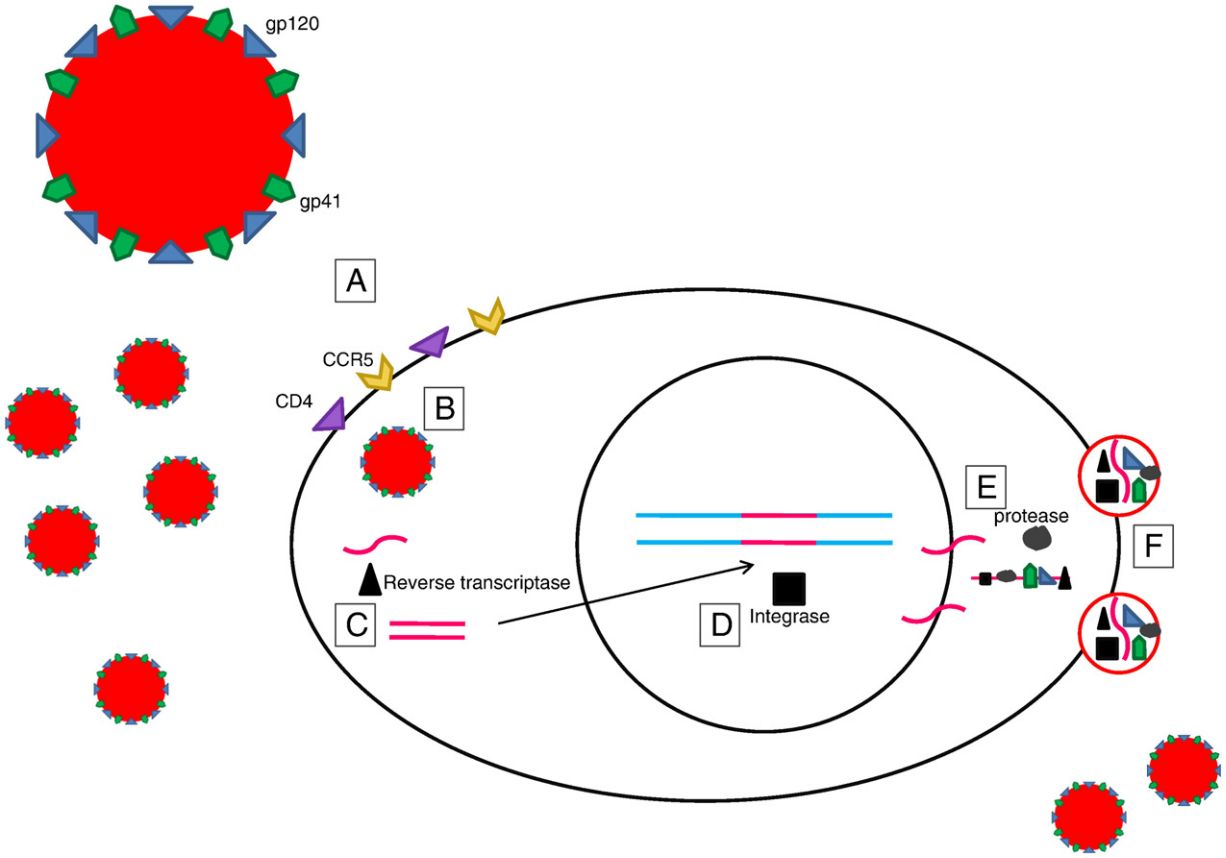

Fig. 2. Diagram illustrating HIV replication. (A) The virus glycoprotein receptors bind to host cell CD4 and a co-receptor CCR5. (B) Fusion of the virus with the host cell membrane results in viral uncoating and the release of the viral nucleocapsid into the cytoplasm. (C) The enzyme reverse transcriptase converts the single stranded RNA into double stranded DNA. (D) The viral DNA is transported to the host nucleus where it is integrated into the host's DNA. (E) Viral DNA is transcribed and translated using host cell machinery and then cleaved by viral protease into functional viral proteins. (F) Viral RNA and proteins assemble at the cell surface and bud off the cellular membrane.

2. HIV replication

Replication of HIV requires entry into a host cell where it uses the host cell machinery to propagate. The virus consists of a lipid bilayer that contains two glycoprotein receptors, gp120 and gp41 (designated gp160), which are used to gain entry into the host cell (Greene, 1997; Phillips, 1996) (Fig. 1A). The viral core contains the p17 matrix protein, p24 capsid protein and the p6 and p7 nucleocapsid proteins. The matrix protein is found beneath the lipid bilayer where it maintains the structural integrity of the virion (Wu et al., 2004). The capsid protein forms a protective case around the genomic material. The nucleocapsid protein, p7, is involved in forming and stabilizing the RNA as well as in nucleocapsid assembly (Goel et al., 2002). The p6 nucleocapsid protein is involved in viral assembly and budding of the virus from the host cell (Sandefur et al., 2000). The core also consists of enzymes required for viral replication such as reverse transcriptase, integrase and protease (Geleziunas and Greene, 1999). The viral genome is made up of nine genes: *gag* (group specific antigen), *pol* (polymerase), *env* (envelope), *tat* (trans-activation of transcription), *rev* (regulator of virion), *nef* (negative factor), *vpr* (viral protein r), *vif* (viral infectivity factor), and *vpu* (viral protein u) (Fig. 1B). The *gag* gene codes for proteins that make up the core, while the *env*- and *pol* genes code for gp160 and enzymes, respectively. The remaining (*tat*, *rev*, *nef*, *vpr*, *vif*, *vpu*) genes are involved in accessory functions that aid viral production and spread (Hirsch and Curran, 1990; Montagnier and Clavel, 1994).

Viral replication begins with attachment of the virus's gp120 surface protein to the host cell CD4 receptor (McDougal et al., 1986) (Fig. 2). This triggers a conformational change in the virus's structure, which reveals the gp41 surface protein (Rizzuto et al., 1998). The latter will bind to a chemokine co-receptor on the host cell being either CCR5 (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996; Doranz et al., 1996) or CXCR4 (Feng et al., 1996). Viruses that bind to CCR5 and CXCR4 are designated R5 and X4, respectively, while viruses that bind to both CCR5 and CXCR4 are designated R5X4. Co-receptor binding brings the virus into close contact with the host cell membrane as gp41 forms pores in the membrane (Srinivas et al., 1992; Miller et al., 1993). This anchors gp120, and facilitates fusion and entry (Helseth et al., 1991).

Cellular factors as well as p17, Vif and Nef from the virus are involved in viral uncoating which unveils the RNA (Hirsch and Curran, 1990; Harrich and Hooker, 2002). The RNA is transcribed into double stranded complementary DNA (cDNA) by the enzyme reverse transcriptase (Varmas, 1988). The doubled stranded DNA is then incorporated into a pre-integration complex (Farnet and Haseltine, 1991), which is transported across the nucleopore into the nucleus by the Vpr protein (Popov et al., 1998). The integrase enzyme inserts the DNA into the host chromosome (Varmas, 1988). After integration, transcription is stimulated by host cellular RNA polymerase to produce Nef, Rev and Tat. The latter is involved in promoting the transcription of further HIV products by binding to promoter regions of the gene and additional host factors. The Rev product binds to transporter regions of the gene transcripts and facilitates the movement of these messenger RNA (mRNA) products to the cytoplasm for translation into protein (Pollard and Malim, 1998). The Env protein product, gp160, undergoes posttranslational modification in the endoplasmic reticulum and then inserts into the cell membrane. The Gag and Pol polyprotein products assemble with the envelope proteins where budding occurs. The process of budding triggers the catalytic activity of the protease enzyme. The enzyme cleaves the Gag and Pol polyproteins into its constituent

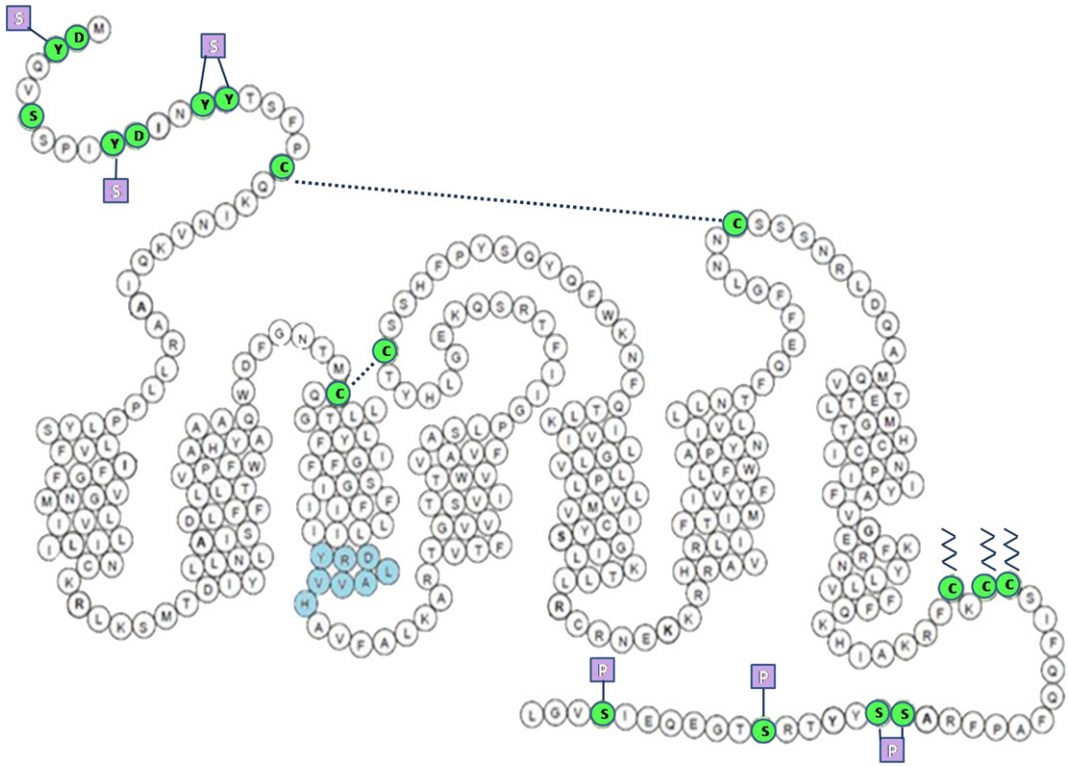

Fig. 3. CCR5 protein structure. Protein structure of CCR5 indicating regions of importance with the dotted line showing disulfide linkage, the boxed S and P indicating sulfate and phosphate moieties and the three zig zag lines showing palmitoylation of C moieties. The DRYLAVVH sequence is highlighted in blue. Image adapted from Blanpain and Parmentier, 2000.

structural and functional proteins, which form the virion [reviewed in (Sierra et al., 2005)].

3. CCR5 protein structure

The CCR5 protein consists of 352 amino acids with a molecular weight of 40.6 kDa (Samson et al., 1996a). The protein contains an amino terminal (N-terminal), seven trans-membrane domains (TMD) made up of hydrophobic residues, three extracellular loops (ECL), three intracellular loops (ICL) and a cytoplasmic or carboxyl tail (C-terminal tail). The protein is composed of conserved residues, specific motifs of charged or hydrophobic regions and post-translational modifications. These regions are important for chemokine ligand binding, functional response of the receptor, and HIV co-receptor activity (Fig. 3).

The N-terminal is rich in tyrosine and acidic amino acids that play an important role in both R5 HIV interaction and chemokine binding (Dragic et al., 1998; Rabut et al., 1998; Farzan et al., 1999). Blanpain et al. used deletion and mutagenesis techniques in this region and discovered the role of these amino acids in the N-terminal (Blanpain et al., 1999a). Mutants lacking amino acids 2–13 responded weakly to elevated concentrations of ligands. In addition, co-receptor function was reduced by 80%. Using alanine-scanning mutagenesis, five amino acid positions (2, 3, 10, 11 and 18) were deemed to be important for ligand and HIV envelope binding affinity.

Similar to other chemokine receptors, cysteine residues are found in all three ECLs and the N-terminal (Blanpain et al., 1999b). The second ICL has a conserved sequence motif, DRYLAVVA, and a short third ICL containing charged amino acids (Opperman, 2004). A motif designated TxP, with x referring to any amino acid, was found in the second TMD creating a structural constraint important for receptor function (Govaerts et al., 2001). The proline and threonine residues in this region provide flexibility to the receptor backbone. Mutation in this area reduces ligand binding and severely affects the functional response of the receptor.

Most GPCRs have a conserved disulfide bond between the first and second ECLs (Fraser, 1989; Dohlman et al., 1990; Savarese et al., 1992; Perlman et al., 1995). Chemokine receptors contain an additional disulfide bond between the N-terminal and third ECL (Baggiolini et al., 1997). These disulfide bonds are important for ligand binding and stable receptor conformation (Perlman et al., 1995). Naturally occurring mutations were found at these positions in long-term non-progressors (Carrington et al., 1999) which led to the study conducted by Blanpain et al. (1999b) on these cysteine residues. Substitution of alanine at any individual cysteine reduced cell surface expression of CCR5 between 40 and 70%. The cysteine mutants did not respond to CCR5 agonists, but allowed R5 HIV infection albeit at decreased levels.

As mentioned previously, the tyrosine and acidic residues in the N-terminal are important in HIV infection and chemokine binding. The tyrosines are post translationally modified by sulfation, which increases the net negative charge of the N-terminal region, facilitating interaction with ligands and HIV (Farzan et al., 1999). Viral gp120 has been shown to bind sulfated moieties on the cell surface (Mondor et al., 1998; Roderiquez et al., 1995) and sulfated compounds can inhibit *in vitro* HIV infection by binding to gp120 (Yang et al., 1996). Farzan et al. showed that prevention of tyrosine sulfation reduced ligand and gp120 binding affinity but did not affect CCR5 expression (Farzan et al., 1999). The serine residue at position six in the N-terminal also contains an O-linked glycosylation modification that affects CCR5 chemokine binding (Bannert et al., 2001).

The C-terminal region of CCR5 consists of various modifications and residues, which affect expression and function of CCR5. Using green fluorescent fusion proteins and immunofluorescence, Blanpain et al. demonstrated that CCR5 is palmitoylated at the C-terminal. Cysteine residues (positions 321, 323 and 324) are acylated and serve as an anchor between the C-terminal and the cell membrane (Blanpain et

al., 2001). This facilitates receptor transport to the cell surface, affects interaction between the receptor and signaling pathways and is involved in receptor-mediated endocytosis (Blanpain et al., 2001; Percherancier et al., 2001; Kraft et al., 2001). Eliminating palmitoylation reduced surface expression by intracellular trapping of the receptor in organelles and subsequent degradation. This reduces the amount of receptor utilized by HIV for entry. Escape mutants that reach the cell surface have impaired signaling but still maintain intact co-receptor function (Blanpain et al., 2001). The C-terminal region is also enriched in serines and threonines that provide phosphorylation sites for G-protein coupled receptor kinases (Oppermann et al., 1999).

Thus, amino acid modifications of CCR5 have important consequences for HIV infection and ligand binding affinity.

4. CCR5 expression and HIV

The density of CD4 receptors on the cell surface is an important factor for HIV infection, although peripheral blood $CD4^+$ cells have been reported to have a relatively consistent density on the cell membrane (Kabat et al., 1994; Poncelet et al., 1991). Nonetheless, Moore et al. found that CCR5 expression displays large inter-individual variability (Moore, 1997). This was shown to affect HIV infectability *in vitro* in cell lines (Platt et al., 1998), macrophages (Tuttle et al., 1998) and lymphocytes (Wu et al., 1997).

In individuals infected with HIV, the percentage of $CD4^+CCR5^+$ T-cells is higher (13.2%) than when compared to uninfected individuals (6.2%) (Ostrowski et al., 1998). The highest percentage of expression was found in an individual with acute HIV syndrome, recorded at around 30–40%. The variation in CCR5 percentages in HIV infected individuals did not correlate with genotype as three individuals with heterozygosity for Δ32, a CCR5 mutation known to reduce HIV infection (reviewed in Section 7), had different levels of expression (2.7%, 13.1% and 17%). In contrast, the activation state of the $CD4^+$ cells as measured by HLA-DR positively correlated with CCR5 expression.

In 1999, a study conducted by de Roda Husman et al. assessed CCR5 expression in terms of CCR5 genotype and HIV infection and progression (de Roda Husman et al., 1999). Individuals with wild-type CCR5 receptors had higher levels of $CD4^+CCR5^+$ T-cells than those with heterozygous Δ32 genotypes, and this was observed in both HIV infected (wild-type — 28%; Δ32 heterozygote — 21%) and uninfected individuals (wild-type — 15%; Δ32 heterozygote — 10%). Furthermore, infected individuals in end stage HIV progression had higher percentages than individuals that had not progressed. The study postulated that the $CD4^+CCR5^+$ T-cell percentage is directly correlated with HIV disease progression due to the constant immune activation associated with HIV. The presence of the CCR5 receptor on memory effector T-cells (Mo et al., 1998) or mature activated T-cells supports the latter finding.

Reynes et al. postulated that CCR5 expression affects virus production and viral load (Reynes et al., 2000). The study found a strong correlation between CCR5 density and viral load, but a weak correlation between $CD4^+CCR5^+$ T-cell percentage and viral load. In addition, cell activation did not affect CCR5 density and there was no correlation between cell activation state and viral load. Infection with HIV did not up-regulate CCR5 density, as there was little difference between density in infected and uninfected individuals. Furthermore, treatment of infected individuals with anti-retroviral drugs (ARVs) reduced viral load but not CCR5 density. As such, the strong correlation between viral load and CCR5 density was independent of activation state or up-regulation of CCR5 by HIV. Platt et al. elucidated a threshold of 10,000 CCR5 molecules per $CD4^+$ cell as a requirement for HIV infection (Platt et al., 1998). Levels below this threshold value showed a marked reduction in cell infectability. Most individuals with a low viral load have CCR5 densities below this value (Reynes et al., 2000). Thus, the *in vivo* importance of CCR5 density in determining viral load was established.

5. Regulation of CCR5 expression

CCR5 expression is regulated at three levels: (1) genetic factors (reviewed in Section 8); (2) factors involved in activation, signaling and trafficking of the receptor including desensitization (the receptor stops responding to stimulus), internalization (receptors are removed from the cell surface after stimulus and move to intracellular organelles) and recycling (receptors return to the cell surface) and; (3) environmental or other triggers.

Chemokine receptor CCR5 is part of the GPCR family, which upon binding to the relevant ligand results in the release of the αi and βγ G-protein subunits. This in turn mediates an effector response (Aramori et al., 1997; Zhao et al., 1998). These responses include the release of adenylyl cyclase and phospholipase Cβ, which release intracellular calcium and form inositol triphosphate. These products activate phosphorylation of the receptor *via* protein kinase C and G-protein coupled receptor kinases (GRK) (Oppermann et al., 1999). Phosphorylation *via* these kinases occurs at the serine and C-terminal residues of CCR5. Binding of β-arrestin 1 and 2, which are regulatory proteins, occurs at the activated serines (Kraft et al., 2001) and the conserved DRY motif in the ICL (Gosling et al., 1997). The β-arrestin proteins desensitize the receptor to further stimulation but also participate in receptor-mediated endocytosis (Huttenrauch et al., 2002).

The level of CCR5 expression is determined by the rates of endocytosis and recycling. The β-arrestin protein facilitates the former process by participating in the binding process between the phosphorylated receptor and clathrin coated pits (Goodman et al., 1996; Laporte et al., 1999). An alternative pathway of endocytosis can occur by cholesterol rich caveolae dependent pathways (Mueller et al., 2001). Once endocytosis is complete, the phosphorylated receptor collects in perinucleur endosomes. These endosomes then return the receptor to the cell surface in a dephosphorylated form (Signoret et al., 1998; Pollok-Kopp et al., 2003). According to Signoret et al. the receptor is not subject to degradation pathways, as it does not accumulate in late endosomes (Signoret et al., 1998). Infection and entry of HIV into cells does not require CCR5 internalization or signaling (Gosling et al., 1997), but the chemokine-induced endocytosis decreases available receptor for HIV use (Amara et al., 1997; Mack et al., 1998). This is the mechanism of chemokine mediated anti-HIV activity.

Environmental factors, affecting CCR5 expression, include infectious agents such as *Mycobacterium tuberculosis* (MTb) and HIV. Santucci et al. showed that MTb infection up-regulates CCR5 expression thereby facilitating HIV cell infectability (Santucci et al., 2004). Endotoxins such as lipopolysaccharide (LPS) can also increase CCR5 expression (Juffermans et al., 2001). The latter study injected LPS into HIV negative males to induce endotoxemia; this resulted in a 2–4 fold increase of CCR5 on $CD4^+$ T-cells. Studies *in vitro* yielded similar results with LPS, MTb and *Staphylococcus aureus*, demonstrating that bacterial infection can aid HIV viral entry by up-regulating CCR5. Shalekoff et al. analyzed the effect of TB and HIV infection in leukocyte populations *in vivo* (Shalekoff et al., 2001). The study found that CCR5 expression was significantly increased in all leukocyte subsets during TB, HIV and dual infection. However, the level of CCR5 expression on $CD4^+$ cells was not increased.

Conversely, Ostrowski et al. showed that HIV affects the level of expression of CCR5, as there is a direct correlation with HIV disease progression (Ostrowski et al., 1998). Individuals with end stage HIV were shown to have the highest percentages of CCR5 expressing $CD4^+$ cells.

Clerici et al. found that immune activation in Africa is environmentally induced and is not due to genetic determinants (Clerici et al., 2000). This results in an up-regulation of CCR5 mRNA and concomitantly CCR5 cell surface expression, increasing R5 HIV infection.

Other factors affecting expression include drugs such as statins that have been shown to reduce the level of CCR5 mRNA and increase

CCR5 ligand expression (Nabatov et al., 2007). These drugs decreased both R5 and X4 HIV infection *in vitro*. Perney et al. studied the effect of chronic alcohol consumption on CCR5, since the receptor is involved in inflammatory reactions (Perney et al., 2004). The study showed that in comparison to control subjects, alcoholic individuals had lower CCR5 densities on $CD4^+$ T-cells.

Cytokine levels are also known to affect CCR5. Cytokines affect CCR5 expression with pro-inflammatory cytokines such as interleukin 2 (IL-2) (Wu et al., 1997; Bleul et al., 1997), interleukin 12 (IL-12), tumor necrosis factor α and INF-γ increasing CCR5 expression on peripheral blood mononuclear cells (Hariharan et al., 1999; Patterson et al., 1999). The anti-inflammatory cytokine, interleukin 10 (IL-10), also increased CCR5 density on monocytes by prolonging the half-life of CCR5 mRNA (Sozzani et al., 1998).

6. CCR5 gene structure

On March 19 1996, Samson et al. published the first report indicating the molecular cloning, expression and ligand binding properties of CCR5 (Samson et al., 1996a). Four months later, two separate reports confirming these findings were published (Combadiere et al., 1996; Raport et al., 1996). Samson et al. cloned and expressed the fifth chemokine receptor, designated Chem13 (Samson et al., 1996a). Using a murine clone they examined a human genomic DNA library, isolated, and purified a positive clone, which was physically linked with the already discovered CCR2 gene. The ORFs of these two genes were separated by 17.5 kb. The similarity between CCR2B and CCR5 was found to be 71% identical in amino acid residues and 55%, 49% and 48% identical in residues to CCR1, CCR3 and CCR4, respectively (Raport et al., 1996). Combadiere et al. (Combadiere et al., 1996) isolated a human CCR5 clone, identical to the clone identified in the Samson paper (Samson et al., 1996a), which differed at position 90 of the protein with leucine substituting for alanine. This finding illustrated that these two sequences are different alleles of the CCR5 gene with a novel variant having been discovered. Additionally, Combadiere et al. demonstrated that the signal transduction pathway is petussis toxin sensitive, confirming its nature as a GPCR (Combadiere et al., 1996).

The CCR5 gene was localized to chromosome 3p21 (Liu et al., 1996) and was found within a cluster of genes encoding for other chemokine receptors which included CCR1, CCR2, CCR3, XCR1 and CCBP2 (Samson et al., 1996a; Maho et al., 1999). The CCR5 gene is composed of three exons, two introns and two promoters (Fig. 4) (Mummidi et al., 1997). The upstream promoter, designated Pu or PR2, consists of a 1.9 kb region preceding exon 1 that is 57 bp in length. Exon 1, the start of the coding region, is followed by the first intron that is 501 bp in length. Exon 2 is intronless and can be found as exon 2a (235 bp) and exon 2b (54 bp). The second promoter, designated Pd or PR1, encompasses the intron 1 and exon 2 regions (Mummidi et al., 2007). A 1.9 kb intron is located between exon 2 and exon 3. Exon 3 is intronless and contains the entire ORF of the CCR5 gene, 11 bp of the 5′ UTR and the complete 3′ UTR (Mummidi et al., 1997).

The CCR5 gene has several ATG transcription sites, prior to the start codon of exon 3, leading to the generation of different CCR5 transcripts, which vary in their 5′ UTR regions (Mummidi et al., 1997). These transcription start sites are usually negated by stop codons preceding exon 3, with the longest resulting cistrons being 9 and 15 amino acids long. The formation of multiple transcripts upstream of exon 3 is a potential regulator of CCR5, through reduction of protein expression. The CCR5 protein containing transcripts are referred to as CCR5A and CCR5B. These two transcripts code for the same CCR5 protein with CCR5B lacking the 235 bp exon 2b fragment.

The two promoters for CCR5, Pu and Pd, contain sequences with high proportions of pyrimidines, which is unusual for promoters, and lack the canonical TATA and CCAAT sequences (Mummidi et al., 1997). However, the Pd promoter consists of a non-consensus TATA box. Furthermore, the upstream or Pu promoter was found to be weaker than the downstream or Pd promoter with the latter having up to five fold greater activity (Liu et al., 1998). Liu et al. characterized the transcriptional activity of the Pd promoter, which contained two TATA motifs as well as a myriad of transcription factor binding sites (Liu et al., 1998). Negative regulatory factors were found in intron 2 and the region upstream of the Pd promoter. The use of two promoters result in different CCR5 transcripts with transcription occurring at multiple start sites found in either exon 1 or exon 2 (Mummidi et al., 1997, 2007).

Transcription of the CCR5 gene driven by the Pu promoter contains exon 1, and results in CCR5A or B (Mummidi et al., 2007). Alternatively, transcription driven by the Pd promoter results in truncated isoforms, which are not individually named. Contrary to previously established data that suggested the Pu promoter was less transcriptionally active and therefore not an important determinant in CCR5 gene expression, Mummidi et al. showed that this was an error (Mummidi et al., 2007). Using RT-PCR based methods, the group identified Pd as the promoter used in unstimulated primary T-cells, whereas Pu was initially used in stimulated T-cells. The erroneous data was a result of transformed T-cells being used in experimentation, thereby affecting the level of CCR5 protein expression *via* the Pu promoter.

The promoter regions include various sites for transcription factor binding, which according to Bream et al. can produce various CCR5 promoter alleles that affect CCR5 expression (Bream et al., 1999). Mummidi et al. found binding sites for activator protein one, octamer one (Oct-1) and GATA transcription factors on the CCR5 gene (Mummidi et al., 1997). In addition, Moriuchi et al. found 11 designated areas deemed to be protected from DNase digestion (Moriuchi et al., 1997). The areas consisted of sequences resembling transcription factor binding sites. Later the group discovered that one of the DNase protected areas contained a third GATA binding site (Moriuchi et al., 1999). Furthermore, the group found that one of the binding sites for GATA, designated GATA-1, up-regulated CCR5 promoter activity and that mutation of the binding site significantly reduced transcriptional activity. In the Pd promoter, Liu et al. found a myriad of transcription factor binding sites, with nuclear factor-kappa-beta being an effective stimulator of the CCR5 promoter (Liu et al., 1998).

The transcription factor Oct-1 was found to negatively regulate the Pu promoter. Octamer two (Oct-2), however, was found to actively stimulate the promoter (Mummidi et al., 2007). The Oct transcription factors have been shown to up-regulate CCR5 protein expression and increase fusion with R5 HIV (Moriuchi and Moriuchi, 2001). Rosati et al. identified multiple binding sites for 'CCAAT/enhancer-binding protein beta' (CEBPβ) within the CCR5 gene, mainly in the intron and promoter (Rosati et al., 2001). Moreover, they found that an increase

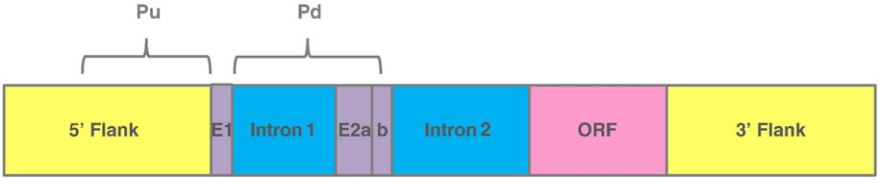

Fig. 4. Structure of the CCR5 gene.

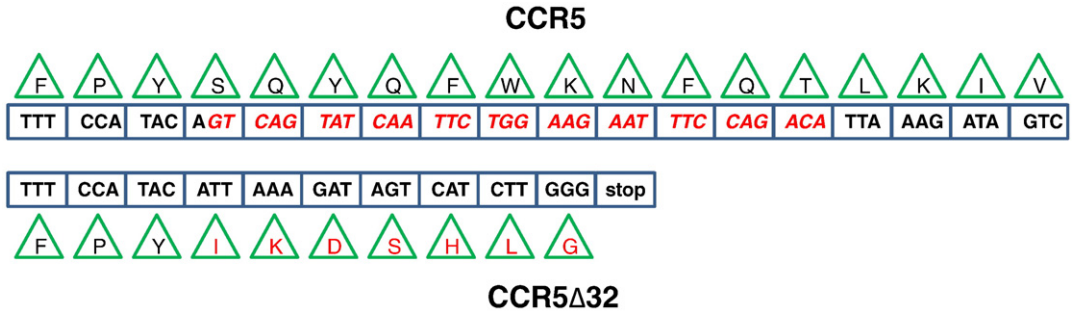

Fig. 5. Diagram of the differences between wild-type CCR5 and Δ32. Illustration of the region involving the Δ32 mutation with the upper section showing the translation of the wild type CCR5 protein while the lower section demonstrates the translation of the mutant protein. The red highlighted region in the wild type sequence refers to the region deleted in Δ32. The red highlighted region in the mutant protein sequence refers to the novel amino acids inserted followed by the stop codon.

in this transcription factor increased the activity of the promoter. In lymphoid and myeloid cell lineages, CEBPβ binds to sites in the intron. Meanwhile, promoter-binding sites were found in specific cell types in the myeloid lineage. The importance of CEBPβ was indicated by the high levels of the factor in HIV positive individual blood cells. Furthermore, these individuals had higher levels of CCR5 positive lymphocytes. Rosati et al. (2001) further supported the study conducted by Liu et al. (1998), in which a negatively regulating sequence was found in the intronic region, emphasizing the importance of intronic regions in CCR5 expression.

The regulation of CCR5 and its effect on protein expression at the level of the cell membrane is complex. The introns as well as sequences in the 5′ and 3′ UTR have been found to affect CCR5 gene regulation (Mummidi et al., 1997). As such, mutations in these regions should be considered important in regulation.

7. The CCR5Δ32 mutation

The CCR5Δ32 mutation was initially discovered in 1996 (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996b) as a genetic mutation that confers protection to cells from infection by HIV. Genetic analysis of the open reading frame (ORF) of the gene by Liu et al. revealed a deletion of 32 basepairs consisting of nucleotides 794 to 825 (Liu et al., 1996). The deletion involves a frameshift mutation with the inclusion of seven novel amino acids following amino acid 174 and a stop codon at amino acid 182 (Fig. 5). The mutant allele contains 215 amino acids in comparison to the full-length 352 amino acid wild type CCR5. Samson et al. found that the region affected was the second extracellular loop (Samson et al., 1996b). The subsequent protein lacked the last three transmembrane domains as well as regions important in G-protein interaction and signal transduction. Both groups discovered that $CD4^+$ cells with the mutated CCR5 prevented HIV envelope fusion.

The Δ32 mutant allele is confined mostly to individuals of European descent, at gene frequencies of approximately 10%, and has a north to south latitude decline in frequency (Martinson et al., 1997). Martinson et al. analyzed the distribution of the Δ32 mutation in more than 3000 individuals from various countries and found a 2–5% gene frequency in Europe, Middle East and some parts of the Indian subcontinent (Martinson et al., 1997). Isolated incidences of Δ32 found in other regions were attributed to European gene flows into these areas. The highest frequency of the mutation was discovered in the Ashkenazi Jewish population at frequencies of 20.93%. In 2005, Novembre et al. confirmed these results when they assessed the Δ32 frequency in various population groups worldwide (Novembre et al., 2005). The mutant allele is absent in Black populations excluding the African American group which may have acquired the mutation through admixture (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996b).

The origin of the Δ32 mutant allele was dated back to between 275 and 1875 years ago, as a unique mutation that increased over the years due to a selective pressure (Stephens et al., 1998). Stephans et al. used haplotype analysis on the chromosomes of 192 Caucasian individuals and estimated the origin using a coalescence theory (Stephens et al., 1998). They hypothesized that the Black plague was the strong selective pressure that caused the mutant allele boom.

Historical data however suggests that the Black plague is not the selective force. The distribution of Δ32 in a north to south gradient does not correspond to casualties of the plague. In fact, the distribution follows a south to north gradient (Cohen and Weaver, 2006). Moreover, the Black plague showed the greatest casualties and effects in areas with the lowest allele frequencies of Δ32, such as the Mediterranean region and China. In 2004, Mecsas et al. infected CCR5 deficient mice with the bacterial pathogen known to cause the Black plague (Mecsas et al., 2004). The experimental data demonstrated no differences in bacteria growth or survival of the deficient mice in comparison to CCR5 containing mice. The finding of ancient DNA in skeletal remains dating back 2900 years presents further evidence. The Δ32 allele frequency found in these remains was similar to that found in individuals ridden by the plague in the same region (Hummel et al., 2005).

Smallpox was another pandemic deemed culpable for the Δ32 mutant allele increase (Galvani and Slatkin, 2003). The pandemic had severe casualties that exceeded those of the plague. Smallpox is a virus similar to HIV, as poxviruses are known to infect lymphocytes using chemokine receptors (Novembre et al., 2005). Conversely, historical evidence refutes this theory as smallpox started outside Europe and did not affect anyone country more significantly than another (Cohen and Weaver, 2006). The discovery of ancient DNA with similar allele frequencies of Δ32 indicated that historic pandemics such as the plague and smallpox did not result in the allele increase.

Faulds and Horuk suggested that Δ32 arose without a selective event (Faulds and Horuk, 1997). Tandem repeats found in the coding region of the CCR5 gene could cause unequal homologous recombination, which results in the Δ32 allele. However, the origins of the Δ32 mutation remain an enigma.

The hype surrounding the Δ32 mutation stems from its ability to protect homozygous individuals from HIV. However, in 1997, studies showed that some individuals with homozygous Δ32 were infected with HIV (Biti et al., 1997; O'Brien et al., 1997; Theodorou et al., 1997). Analysis of the HIV strains in these individuals revealed the presence of X4 utilizing HIV, accompanied by very rapid $CD4^+$ T-cell decline (Michael et al., 1998). This indicates that the mutation does not protect Δ32 homozygous individuals from viral strains which utilize alternative receptors.

The protective effect of the Δ32 mutation towards HIV is a consequence of an inhibition of CCR5 protein expression on the cell surface. This prevents HIV from utilizing the receptor for viral entry. In addition, the Δ32 protein, localized to the endoplasmic reticulum, exerts a *trans*-dominant negative effect on the wild type CCR5 protein, inhibiting its transport to the cell surface (Benkirane et al., 1997;

Table 1
List of previously identified mutations in the protein-coding region of the CCR5 gene.

Variant	Nucleic acid substitution	Protein region
I12T[a,b]	A25C	N-terminal
C20S[b]	T58A	N-terminal
A29S[a,b]	G85T	N-terminal
I42F[b]	A124T	TMD 1
L55Q[b,c]	T164A	TMD 1
R60S[b]	G180T	ICL 1
A73V[b]	C218T	TMD 2
C101X[d]	T303A	ECL 1
G106R[b]	G316A	TMD3
C178R[e]	T532C	ECL 2
S185R[b]	A553C	ECL 2
L215S[a,c]	A187T	TMD 5
I254T[a,b]	T758C	TMD 6
R223Q[b,c]	G668A	ICL 3
228delK[b]	680del3	ICL 3
C269F[b]	G806T	ECL 3
G301V[b]	G902T	TMD 7
FS299[c]	893delC	TMD 7
A335V[b,c]	C1004T	C-terminal
Y339F[a,b,c]	A1016T	C-terminal

Footnote: All variants (except 228delK and FS299) are named with the first letter indicating wild-type amino acid. The number between the wild-type amino acid and the last letter indicates the position on the CCR5 protein, and the last letter indicates the mutant amino acid. 228delK is a deletion of a three-nucleotide codon, which codes for lysine at position 228 while FS299 is a frameshift caused by single basepair deletion. "a" represents variants causing conservative amino acid changes. "b" represents variants discovered in (Carrington et al., 1997), "c" represents variants discovered by (Ansari-Lari et al., 1997), "d" represents variants discovered by (Quillent et al., 1998) and "e" represents variants discovered by (Magierowska et al., 1999).

Chelli and Alizon, 2001). Further *in vitro* investigation by Agrawal et al. showed that the mutant protein reduces the surface expression of wild type CCR5 and CXCR4 by dimerization (Agrawal et al., 2004). This conferred a broad-spectrum inhibition to R5, X4 and R5X4 HIV infection. Homozygous Δ32 individuals with mutant protein expression in $CD4^+$ cells were shown *in vivo* to have down-regulated CXCR4 surface protein expression and decreased susceptibility of the cells to X4 infection. Thus, the Δ32 protein product is of great importance in providing resistance to HIV infection. This was further supported by evidence suggesting that Δ32 homozygous individuals with HIV infection do not stably express the Δ32 protein, and are devoid of the molecular mechanism of protection (Agrawal et al., 2004, 2007). Stable expression of the mutant protein can be affected by polymorphisms in the promoter region of the gene (Jin et al., 2008). Individuals homozygous for Δ32 with the 59537-A/A variant had reduced expression of mutated protein in comparison to homozygous individuals with the wild type 59537-G/G promoter variant.

8. CCR5 mutations

Mutations in the coding and promoter regions of the CCR5 gene have been well documented in relation to HIV infection and progression. Coding region mutations affect the CCR5 protein structure, which can affect production, transport, chemokine binding, signaling and expression of the CCR5 receptor. Mutations in the promoter region can affect the DNA transcription factor binding or regulatory sites leading to aberrations in CCR5 mRNA production.

8.1. Coding region mutations

Six of the variants in Table 1 (I12T, C20S, I42F, L55Q, A73V, and C101X) have been previously identified in individuals with Δ32 (Carrington et al., 1997; Quillent et al., 1998).

The C101X (Quillent et al., 1998) and FS299 (Ansari-Lari et al., 1997) variants result in premature termination of translation. The C101X variant discovered in an HIV negative homosexual male individual, frequently exposed to HIV, results in substitution of cysteine at position 101 of the CCR5 protein with a stop codon and has a high allele frequency in central Africa (Blanpain et al., 2000). Consequently, this premature termination of translation forms a protein that contains only two transmembrane domains. Studies performed *in vitro* indicate that this protein is not expressed on the cell surface, does not function as a HIV co-receptor and is misfolded. The consequence of these factors is that the protein is retained intracellularly (Blanpain et al., 2000). The FS299 variant, found at 3–4% allele frequency in Asia (Blanpain et al., 2000), is a frameshift mutation causing absence of the latter portion of the seventh TMD and complete absence of the C-terminal. Protein expression on the cell surface is reduced because of intracellular retention. Furthermore, it does not bind or respond to chemokines but still has the ability to bind to HIV, albeit with reduced efficiency (Blanpain et al., 2000).

The I12T, C20S and A29S variants are all located in the N-terminal. According to Carrington et al., the variants markedly reduce cell surface expression and ligand binding with the former two not functioning as HIV co-receptors (Carrington et al., 1999). Conversely, Blanpain et al. found the I12T variant mediated HIV entry (Blanpain et al., 2000). The C20S variant prevents disulfide bond formation between the N-terminal and ECL 3. Considering the importance of this bond in chemokine binding (Blanpain et al., 1999b), the variant is unable to function or respond to chemokines *in vitro* (Blanpain et al., 2000). The variant also reduces cell surface expression but does not prevent HIV co-receptor function. Blanpain et al. also refute the findings of the A29S variant, as expression on the cell surface was found to be within the normal wild-type range (Blanpain et al., 2000). However, the variant does not respond to MIP-1α and MIP-1β but does respond to MCP-2 and can function as an HIV co-receptor (Blanpain et al., 2000).

The I42F, L55Q, and A73V variants are found in the first and second TMDs, and according to Carrington et al., these variants support HIV infection and have a ~4–8 fold higher affinity for ligands (Carrington et al., 1999). Howard et al. demonstrated that both I42F and A73V have reduced surface expression in comparison to wild-type receptors, with the former showing increased binding to RANTES and the latter having decreased HIV co-receptor activity (Howard et al., 1999). The L55Q receptor is a variant affecting a highly conserved residue, which is important in mediating receptor activation but not chemokine binding affinity (Blanpain et al., 2000).

The C178R variant, initially discovered in the Vietnamese population (Magierowska et al., 1999), affects a highly conserved cysteine involved in disulfide bonding between ECL-1 and ECL-2, which is important for CCR5 structure and in HIV binding (Wu et al., 1997). Studies show the variant causes a dramatic reduction in cell surface expression with intracellular retention of the misfolded receptor (Blanpain et al., 2000). The mutant receptor does not bind or respond to chemokines but can still bind HIV.

The R223Q variant is also known to affect a conserved residue. Carrington et al. observed a decrease in variant co-receptor function while still maintaining the ability to bind to gp120 (Carrington et al., 1999). Analysis by Capoulade-Metay et al. found no change in CCR5 expression or chemokine binding while Zhao et al. found no effect on HIV infection *in vitro* or the CCR5 mRNA level (Capoulade-Metay et al., 2004; Zhao et al., 2005).

The G106R variant changes the residue hydrophobicity in the third TMD, resulting in reduced surface expression (Capoulade-Metay et al., 2004) without affecting levels of mRNA production (Zhao et al., 2005). The variant also decreases binding to chemokines and HIV.

The S185R, I254T and C269F variants are found in the South East Asian population group (Capoulade-Metay et al., 2004). The former two variants may alter residue charge and hydrophobicity respectively. The receptor function and HIV co-receptor activity, however, remain similar to the wild-type receptor. In contrast, the C269F variant disrupts the conserved cysteine involved in disulphide linkage of ECL-3 to the N-terminal. This results in reduced cell surface expression, decreased binding to MIP-1β and RANTES and weak HIV co-receptor binding.

8.1.1. Mutations discovered in South Africa

South Africa has one of the highest levels of HIV infection in the world with approximately 10.6% of the South African population being infected with HIV (Statistics South Africa). The Δ32 mutation is found heterozygously in the Caucasian population at an allelic frequency of 9.4% but is virtually absent from the Black African population. The C101X variant was not detected in the South African Black population but was found at an allelic frequency of 0.7% in Caucasians (Williamson et al., 2000).

In 2001, Peterson et al. identified seven novel variants (D2V, P35, Y89, L107F, P162, R225X and R225Q) exclusive to the Black African and Coloured population groups as well as six previously discovered variants (L55Q, S75, R223Q, A335V and Y339F) (Petersen et al., 2001). Further investigation by Hayes et al. showed that the novel P35 variant was not significantly different in slow *versus* fast progressors (Hayes et al., 2002). The A335V variant, found homozygously in a Black African female and heterozygously in a Coloured male and female, may have significantly contributed to the long-term non-progression seen in these individuals.

In 2010, mutant receptor constructs from four novel variants (L107F, R225Q, D2V, and R225X) identified in the Peterson paper were analyzed *in vitro* for their effects on expression, chemokine binding and response, and HIV co-receptor properties (Folefoc et al., 2010). The D2V variant found in the N-terminal showed decreased CCR5 expression, reduced chemokine binding and response, and lowered HIV infection. The R225X mutation, on the other hand, resulted in premature termination of translation in the third cytoplasmic loop, showed no surface expression, chemokine binding and response, and could not support HIV infection. The latter mutation was found heterozygously and could partially protect against HIV in these individuals.

Studies in South Africa have focused mostly on determining Δ32 frequency and mutations in the ORF. However, in 2010 a 9.2 kb region encompassing the entire CCR5 gene was analyzed for SNPs, indels and haplotypes in 35 Black and 35 Caucasian individuals in South Africa (Picton et al., 2010). The study revealed 68 SNPS, four indels, the Δ32 mutation as well as seven complex haplotypes, while illustrating that the diverse variation in the CCR5 gene in the South African population may explain differences in response to HIV.

8.2. Promoter region mutations

A plethora of studies surrounding mutations and effects has been completed on the ORF of the CCR5 gene. Although these mutations significantly influence CCR5 and HIV infection, the mutations within the intronic, promoter and untranslated exons should also be included.

Martin et al. performed denaturing high pressure liquid chromatography and single strand confirmation polymorphism analysis on HIV positive patients (Martin et al., 1998). They identified 10 SNPs that in different combinations created four common haplotypes (CCR5 P1–4) and six infrequent haplotypes (CCR5 P5–10). These haplotypes affect the progression of HIV at varying rates.

A CCR2 variant, known as CCR2–64I and located in the first TMD of the CCR2 protein, slows the progression of HIV irrespective of Δ32 status (Smith et al., 1997; Kostrikis et al., 1998). Due to the close proximity of CCR2 and CCR5, linkage disequilibrium between variants in the two genes may cause the slow progression as the CCR2 receptor is rarely used for HIV infection (Deng et al., 1996). A cytosine/thymine (C/T) polymorphism at position 59653 (according to U95626) in intron 2 of the CCR5 gene was shown to be in 100% linkage disequilibrium with the CCR2–64I polymorphism (Kostrikis et al., 1998); however, the protection provided *via* linkage of the variants could not be established (Martin et al., 1998).

An A/G polymorphism at position 59029 (GenBank Accession: U95626) in the promoter region of CCR5 has been shown to affect HIV progression (McDermott et al., 1998). Individuals with an A/A genotype progress rapidly towards AIDS in comparison to individuals with the G/G genotype which progress 3.8 years more slowly. The latter genotype was associated with 45% lower promoter activity (McDermott et al., 1998). The A/A allele is found in the CCR5 P1 haplotype and has potentially greater efficient promoter activity, even though it is not found in any transcription factor binding regions. Shieh et al. found that individuals homozygous for the A/A genotype had higher levels of $CD4^+$ cells expressing CCR5 (Shieh et al., 2000). This polymorphism was shown to determine CCR5 expression and HIV infection *in vitro* (Salkowitz et al., 2003).

A study performed by de Souza et al. on the effect of four promoter polymorphisms on HIV perinatal transmission in Brazilian children, revealed two genotypes affecting HIV transmission (de Souza et al., 2006). The 59353 T/T genotype was associated with an increase in HIV transmission, while the 59402 A/A genotype has a protective effect. Strong linkage disequilibrium between the 59029 A/A and 59353 T/T genotypes has been identified. Individuals lacking these genotypes progress more slowly towards AIDS (Clegg et al., 2000). Promoter variants can also affect transcription factor binding. This was illustrated by Bream et al. who showed that certain variants bind more efficiently than other variants do (Bream et al., 1999).

9. Role of CCR5 deficiency in disease

Individuals homozygous for the Δ32 mutation, which abolishes CCR5 receptor expression, are healthy and at no apparent disadvantage (112, 114). Other than its protective effect in HIV infection, the mutations role in providing a protective or negative impact on other diseases remains controversial.

In rheumatoid arthritis, $CCR5^+$ monocytes are found in the synovial fluid (Kohem et al., 2007) as the receptor plays a role in the inflammatory process. A meta-analysis showed that the Δ32 mutation provides protection in the latter disease (Prahalad, 2006), as the severity of the disease is reduced (Scheibel et al., 2008). In renal allograft outcome, CCR5 deficient mice had a significant decrease in chronic renal transplantation rejection (Dehmel et al., 2010). This favorable outcome is due to a decrease in inflammatory reactions in the acute phase while in the chronic phase the alternative macrophage pathway is activated. During the classic macrophage pathway a strong pro-inflammatory response ensues, but in the alternative pathway a wound healing or immune-suppressive response is characterized (Martinez et al., 2009).

The Δ32 allele has also been associated with protection in coronary artery disease and myocardial infarction (Szalai et al., 2001; Gonzalez et al., 2001; Pai et al., 2006). The mutant allele was associated with lower levels of triglycerides and higher HDL plasma levels, both beneficial in reducing the risk of cardiovascular disease (Hyde et al., 2010). The use of the CCR5 inhibitor, maraviroc, was also associated with better lipid profiles (DeJesus et al., 2008). In 2006, Henckaerts et al. postulated that a deficiency of CCR5 could be protective in primary sclerosing cholangitis (PSC) (Henckaerts et al., 2006). Conversely, the Δ32 allele frequency was significantly higher in PSC patients compared to controls and furthermore the former individuals had more severe liver disease (Eri et al., 2004).

Studies in mice show that CCR5 deficiency increases the severity of brain injury in stroke (Sorce et al., 2010). Deficiency in CCR5 is also associated with impaired osteoclast differentiation and osteoblast maturation leading to defective bone repair in mice (Hoshinoa et al., 2009). Glass et al. showed that in mice, CCR5 is essential for survival against West Nile virus (WNV) infection (Glass et al., 2005). A further study in humans homozygous for Δ32 illustrated a significant correlation between the mutation and fatality in WNV infection (Glass et al., 2006). The mutation has also been implicated in severe tickborne encephalitis symptoms (Kindberg et al., 2008) and adverse reactions to yellow fever virus vaccine (Pulendran et al., 2008).

High levels of CCR5 are significantly associated with non-metastatic colorectal cancer, whereas weak or deficient CCR5 expression is

significantly associated with an advanced form of the disease (Zimmerman et al., 2010). In bone marrow transplants, CCR5 presence on $CD4^+$ T-regulatory cells is important in prolonging graft survival (Wysocki et al., 2005). This was corroborated by a study, which showed that CCR5 genotypes with higher levels of expression have a significant survival outcome (McDermott et al., 2010). However, contrary to these findings, Bogunia-Kubik et al. demonstrated that Δ32 individuals have a reduced likelihood of developing graft *vs.* host disease (Bogunia-Kubik et al., 2006).

10. CCR5 as a target in HIV therapy

10.1. Anti-retroviral drugs and vaccines

As mentioned previously R5 viruses are predominant during initial HIV infection whereas X4 or R5X4 viruses usually appear later in infection when the infected individual is progressing towards AIDS. Considering the role of the CCR5 receptor in HIV infection, Maraviroc (Pfizer), a new class of ARVs which are small molecule CCR5 receptor antagonists was approved in 2007. Maraviroc was indicated for use in infected individuals with R5 tropic strains who were treatment experienced (Selzentry, 2007). Other similar CCR5 antagonists included Vicriviroc (Schering-Plough) and Aplaviroc (GlaxoSmithKine). Small molecule antagonists bind to CCR5 hydrophobic residues in the TMDs and prevent the interaction of HIV with the receptor (Dragic et al., 2000; Maeda et al., 2006, 2008).

The receptor antagonists are atypical in comparison to traditionally used ARVs as the drug binds to host cellular factors instead of HIV directly. However, HIV resistant variants have been discovered. Resistance occurs either as a result of CXCR4 receptor usage (Westby et al., 2006) or the ability to use drug bound CCR5 for entry (Tilton et al., 2010). Safarian et al. proposed the use of a combination of CCR5 directed HIV therapies to synergistically inhibit HIV and slow down the emergence of resistant strains (Safarian et al., 2006).The group illustrated that anti-CCR5 monoclonal antibodies together with small molecule inhibitors were more effective in preventing HIV entry as a result of the different mechanisms of action.

In 2007, a phase IIb HIV vaccine trial was conducted in various countries including South Africa. The study aimed at increasing a cellular immune response towards HIV in uninfected individuals. Results from the study showed that the vaccine did not protect against HIV infection, neither did it inhibit the progress of HIV once individuals were infected. Alarmingly, the vaccine may have increased the susceptibility of participants to HIV infection although the mechanism remains unknown (Sekaly, 2008).

Production of an HIV vaccine is fraught with many challenges including the inability to use live or dead virus due to the risk of infecting participants. However, there is no suitable model to investigate the effects of the vaccine; humanized mouse models may alleviate this problem. In addition, there are many HIV variants due to the rapid mutation rate, which complicates the production of a broadly effective vaccine (Gallo, 2010). The viral properties of HIV also contribute to the hurdles faced. Being a retrovirus allows HIV to integrate into the host cell genome where it can remain latent for many years. The virus also damages the immune system and changes the dynamics of the immune environment. This can affect the vaccine's response and diminish its sustainability (Esparza et al., 2010). A suitable vaccine will therefore, need to be one that prevents initial HIV infection (Esparza et al., 2010).

10.2. Alternative therapeutics

Considering the complex nature of HIV vaccine development and the issues surrounding ARV toxicity and viral resistance, many researchers are looking at alternative mechanisms of therapy. Since the discovery of the Δ32 protective effect in HIV, investigators are targeting the CCR5 receptor at the DNA, transcription and protein levels.

Since the protective effect of Δ32 lies in its ability to induce a *trans* dominant negative effect on wild-type CCR5 and CXCR4 protein expression (Benkirane et al., 1997; Chelli and Alizon, 2001), Luis Abad et al. used truncated CCR5 molecules to induce a similar effect in human T-cells (Luis Abad et al., 2003). Upon expression *in vitro* the CCR5 truncated molecules significantly inhibited CCR5 function and provided protection against R5 viral strains.

Hutter et al. used an HLA matched allogeneic stem cell donor to treat and confer HIV resistance to the patient in their study (Hütter et al., 2009). The likelihood of finding an HLA matched Δ32 homozygous individual to treat each HIV patient is low. Hence the proposal to use autologous cells that have been genetically engineered to resist HIV by using gene therapy is a promising avenue (Baltimore, 1988).

RNA interference (RNAi) using small interfering RNAs (siRNAs) or short hairpin RNA (shRNA) is a mechanism used to target the transcripts of HIV proteins or host cellular components and leads to their subsequent degradation and a reduction in protein expression (Novina et al., 2002; Li et al., 2003; Qin et al., 2003; Li et al., 2005; An et al., 2007; Anderson et al., 2009; Liang et al., 2010).

The study conducted by Anderson et al. used a lentiviral vector to transduce $CCR5^+$ human cells with a shRNA directed at CCR5 (Anderson et al., 2009). The results of the study illustrated the successful and specific CCR5 targeting with a 93% reduction in CCR5 expression and a conferred resistance to HIV. In another study, Liang et al. developed a novel shRNA to suppress CCR5 in hematopoietic stem cells (HSCs) (Liang et al., 2010). Results showed that the CCR5 knockdown cells had a 90% reduction in CCR5 mRNA and were resistant to HIV infection by R5 strains (Liang et al., 2010). Bifunctional siRNAs were designed by Ehsani et al. to target the CCR5 co-receptor and HIV RNA (Ehsani et al., 2010). This ensured that HIV infection and replication was prevented by using this dual mechanism.

Another mechanism being investigated is zinc finger nucleases (ZFNs) which bind to specific predetermined DNA sequences and result in a permanent double stranded break resulting from transient expression of the zinc finger. Holt et al. demonstrated the success of ZFN mediated therapy on human HSCs transplanted into NOD/SCID/$IL2r\gamma^{null}$ mice. Mice that received ZFN induced cells had lower HIV levels and showed rapid proliferation of CCR5 null cells as opposed to untreated mice which had rapid $CD4^+$ T cell decline (Holt et al., 2010). Other studies have also shown the ability of ZFN-mediated CCR5 inactivation to confer HIV resistance (Perez et al., 2008).

Gene therapy strategies utilize retroviral or lentiviral vector systems to transfer the therapeutic product. Both the efficiency and success of such vectors make them popular choices. However, certain risks such as viral mediated oncogenesis can occur, particularly with the former (Dropulic, 2005). The Sleeping Beauty Transposon system offers a non-viral mechanism of gene transfer with investigations showing stable delivery of siRNAs, efficient knockdown of CCR5 and CXCR4 receptors from the cell surface and successful resistance to HIV viral strains (Tamhane and Akkina, 2008).

The disadvantages of expressing siRNAs range from off target mRNA degradation (Jackson et al., 2006) to cytotoxicity (Grimm et al., 2006). However, clinical trials have thus far demonstrated positive results. Four patients diagnosed with lymphoma underwent HSC transplantation with genetically modified $CD34^+$ cells (DiGiusto et al., 2010). The cells were transduced with a lentiviral vector containing three RNA based anti HIV therapeutics one of which was a CCR5 ribozyme. No changes were observed in hematopoietic potential when compared to unmodified cells and there were no related toxicities. The transduced cells efficiently engrafted in all four individuals and showed persistent expression of the therapeutics 24 months after the transplant. In a review by Mitsuyasu et al., the authors described two clinical trials using genetically modified HSCs which illustrated the safety and feasibility of such a procedure (Mitsuyasu et al., 2011).

11. Conclusion

Receptor antagonist drugs are problematic due to drug toxicity and the emergence of escape mutants. The use of gene therapy on HSCs may provide a long-term protection against HIV infection and progression. Transducing HSCs ensures continued self renewal and differentiation potential of HIV resistant cells while ultimately replacing and reconstituting the individual's HIV depleted immune system as has been seen with the patient in the Hutter study (Hütter et al., 2009). The ultimate goal of this therapy is to eliminate the virus completely. The protocol involved in HSC transplantation includes apheresis of peripheral blood cells or bone marrow aspiration, isolation of the target cells and transduction with the vector before subsequent re-administration into the patient. An eventual protocol should aim to minimize the procedure by designing a therapeutic vector that only requires direct injection *in vivo* where the appropriate cells are then specifically selected for transduction.

The notion of eliminating a single receptor as the key to unraveling the enigma of the HIV cure is still an exciting prospect. However, the complexities associated with such a task need to be considered. The idea of CCR5 dispensability is based on the premise that Δ32 homozygous individuals are seemingly healthy with no apparent abnormalities. However these individuals acquired the mutation through a line of ancestry dating back thousands of years. This has allowed these individuals to evolve and possibly adapt to the lack of CCR5 usage by shifting immune and other functions to alternative receptors or structures. Artificially inducing a null CCR5 phenotype may have consequences that we are as yet unaware of. Although the Hutter paper provides a breakthrough strategy towards the long awaited cure for HIV (Hütter et al., 2009), the long-term effect of the treatment on the affected individual will only be revealed in the next few years.

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