R5X4 EVOLVE DISTINCT GP41 ACTIVATION PATHWAYS TO SENSE ALTERNATE CORECEPTOR BINDING

#183

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Background

HIV Envelope glycoproteins gp120 (surface subunit) and gp41 (transmembrane subunit) exist on the surface of virions. gp120 binding to its primary receptor CD4 creates a binding site for one of two obligatory chemokine receptors (CKR), CCR5 or CXCR4.

CKR binding triggers the refolding of gp41, enabling the insertion of its hydrophobic fusion peptide (FP) into the target cell membrane. The outcome of which are conformational changes that force pore formation between the virus and target cells allowing for transfer of viral core **(Fig 1A)**

gp120's interaction with CCR5 and/or CXCR4 classifies Env into R5 or X4 phenotypes, respectively.

Transmitted/founder viruses that establish infection in a new host use CCR5 as a CKR while X4 viruses emerge and predominate in late stage disease in a subset of infected individuals. R5X4 (dualtropic) isolates are able to use both CKRs and emerge prior to the acceleration of T cell decline and the onset of symptomatic disease.

R5X4 are believed to represent an evolutionary intermediate between R5 and X4 isolates **(Fig 1B)**

The molecular events that lead to the activation of the fusion function of gp41 in both monotropic and dualtropic viruses are still unclear.

This study aimed to define how signals induced by engagement of R5X4 Envs are transmitted to gp41 as an activation signal through mutagenic screen of the gp120-gp41 association interface **(Fig 1B)**.

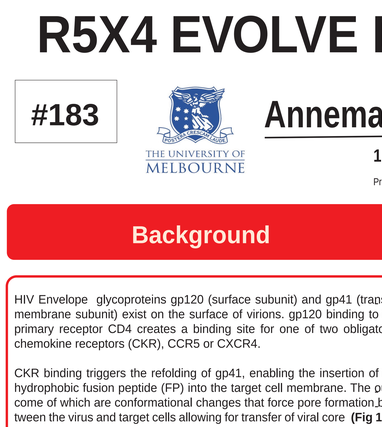


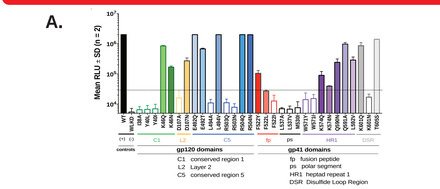
Fig 1: HIV Envelope glycoproteins as mediators of fusion and entry.
(A) Receptor induced fusion cascade. (B) Evolution of CKR use (C) Strategy for identifying amino acids involved in conformational signalling in R5X4 viruses and proposed impact of mutagenesis of Env on CKR and cellular tropism

Methodology

Interfacial residue mutants were generated by mutagenesis in the primary R5X4 dBR07 and coreceptor use of mutants was analysed in U87.CD4.CCR5/CXCR4 stable cell assay systems and subsequently in primary cells.

Key interfacial residue mutants were further tested in a Vpr-Blam fusion assay (Cavrois *et al.* 2002) to isolate the stage at entry impacted by the mutations. Key findings are as summarised.

Screen of gp120-gp41 association interface



B. Summary of phenotypes of dBR07 mutants

location	gene/cpe	gp120 expression	Virions*	Infectivity†	Moderate infectivity‡	High infectivity‡	Functional group§
	WT	+					1
C1	E80A	+					1
C1	V83L	+					1
C1	K86E	+					1
C1	K86Q	+					1
Layer 1	D107A	+					2
C5	E492T	+					4
C5	L494V	+					4
C4	L494A	+					3
C5	R504A	+					1
C5	R504Q	+					1
C5	R504K	+					3
fusion peptide	F522Y	+					1
	F522S	+					3
gp41	W571A	+					1
gp41	L537A	+					1
HR1	W571A	+					1
HR1	K574A	+					1
HR1	K574N	+					1
HR1	Q591A	+					1
HR1	Q591K	+					1
HR1	L592A	+					1
HR1	L592N	+					1
DSR	N625K	+					1
DSR	N625A	+					1

*, gp120 absent; +/-, trace level of gp120; + level of gp120 > trace < WT; ++, WT-levels of gp120

†non-infectious, <30,000 RLU; Low infectivity, >30,000 <100,000; Moderate infectivity> 100,000 <300,000; High infectivity, > 300,000 RLU

‡Functional group 1: gp120 incorporation and infectivity blocked by all substitutions of a particular amino acid
Functional group 2: gp120 incorporation blocked, but low infectivity retained by all mutants
Functional group 3: gp120 incorporation and infectivity blocked by least conservative substitution(s); gp120 incorporation and infectivity retained by conservative substitution mutant(s)
Functional group 4: high levels of infectivity retained by all mutants; usually an incorporation defect associated with the least conservative substitution of a particular residue

Fig 2: Screening of gp120-gp41 near neighbour contacts in the primary R5X4, dBR07.
(A) gp120-gp41 mutants were screened in a TZMbl infectivity assay to identify functional mutants and (B) categorised according to infectivity phenotypes and Env incoporation in virions as determined by western blot (data not shown).(C) Location of functional mutants within the gp120-gp41 association interface (dotted lines) in the context of an Env trimer (PDB ID: 5FYK).

- *In Silico* analysis identified 19 key residues in the gp120-gp41 interface to which conserved substitutions were introduced and subsequent mutants screened for functionality in TZMbl assays (**Fig 2A**) and grouped into 4 categories summarised in **Fig 2B**.
- 15/30 mutants from functional groups 3 and 4 were found to be able to faciliate infectivity (**Fig 2B & 2C**) and underwent further analysis in different coreceptor assays.

Screening of interfacial mutants in U87.CD4.CCR5 and U87.CD4.CXCR4 cells

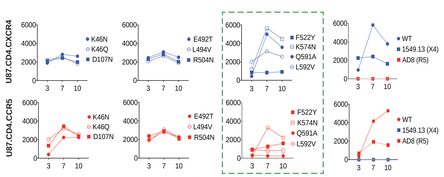


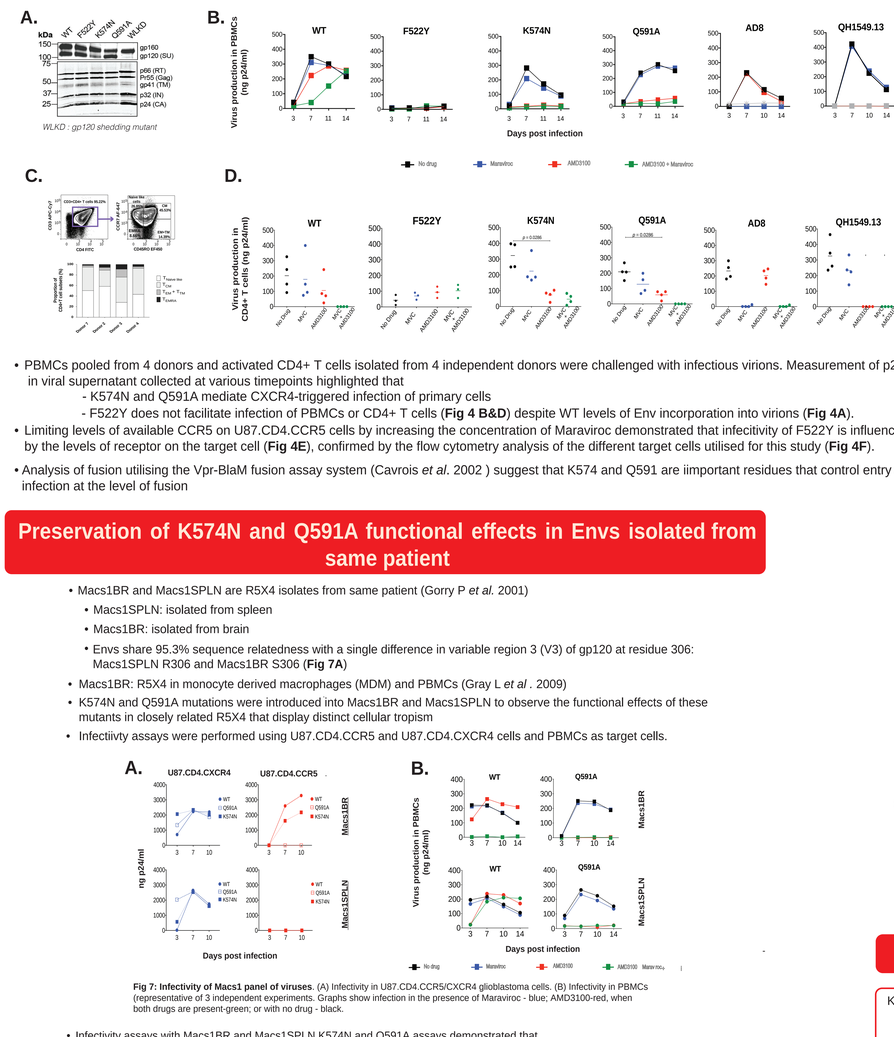
Fig 3: Infectivity of dBR07 functional mutants in U87.CD4.CCR5 and U87.CD4.CXCR4 glioblastoma cells

- 3/15 mutants found to display alternate CKR use in U87.CD4.CCR5 and U87.CD4.CXCR4 cell lines (**Fig 3**).
  - **F522Y** in the fusion peptide maintained CCR5-mediated infection but not CXCR4
  - **K574N** and **Q591** in the HR1 maintained CXCR4 mediated infection but not CCR5

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The gp41 HR1 residues and Q591 are required for fusion mediated CCR5-triggered infection of target cells while infection via fp residue F522 is influenced by receptor numbers



- PBMCs pooled from 4 donors and activated CD4+ T cells isolated from 4 independent donors were challenged with infectious virions. Measurement of p24 in viral supernatant collected at various timepoints highlighted that
  - K574N and Q591A mediate CXCR4-triggered infection of primary cells
  - F522Y does not facilitate infection of PBMCs or CD4+ T cells (**Fig 4 B&D**) despite WT levels of Env incorporation into virions (**Fig 4A**).
- Limiting levels of available CCR5 on U87.CD4.CCR5 cells by increasing the concentration of Maraviroc demonstrated that infecitivity of F522Y is influenced by the levels of receptor on the target cell (**Fig 4E**), confirmed by the flow cytometry analysis of the different target cells utilised for this study (**Fig 4F**).
- Analysis of fusion utilising the Vpr-BlaM fusion assay system (Cavrois *et al.* 2002) suggest that K574 and Q591 are iimportant residues that control entry and infection at the level of fusion

Preservation of K574N and Q591A functional effects in Envs isolated from same patient

- Macs1BR and Macs1SPLN are R5X4 isolates from same patient (Gorry P *et al.* 2001)
  - Macs1SPLN: isolated from spleen
  - Macs1BR: isolated from brain
  - Envs share 95.3% sequence relatedness with a single difference in variable region 3 (V3) of gp120 at residue 306: Macs1SPLN R306 and Macs1BR S306 (**Fig 7A**)
- Macs1BR: R5X4 in monocyte derived macrophages (MDM) and PBMCs (Gray L *et al* . 2009)
- K574N and Q591A mutations were introduced into Macs1BR and Macs1SPLN to observe the functional effects of these mutants in closely related R5X4 that display distinct cellular tropism
- Infectiivty assays were performed using U87.CD4.CCR5 and U87.CD4.CXCR4 cells and PBMCs as target cells.

Fig 7: Infectivity of Macs1 panel of viruses. (A) Infectivity in U87.CD4.CCR5/CXCR4 glioblastoma cells. (B) Infectivity in PBMCs (representative of 3 independent experiments. Graphs show infection in the presence of Maraviroc - blue; AMD3100-red, when both drugs are present-green; or with no drug - black.

- Infectivity assays with Macs1BR and Macs1SPLN K574N and Q591A assays demonstrated that
  - Q591A improves or confers CXCR4 preference in both viruses. abolishing CCR5 use
  - K574N confers CXCR4 preference in both viruses; reduces CCR5 use in Macs1BR
- Macs1BR demonstrating less sensitivity to K574N mutation suggests that it may represent early evolutionary intermediate owing to less selection pressure in CNS
- Macs1SPLN showing CXCR4 preference even in the absence of mutations suggest that it may represent a late evolutionary strain adapted to CXCR4 use.
- Ability of Macs1SPLN WT to infect even in the presence of both drugs suggest potential utilize drug bound receptor

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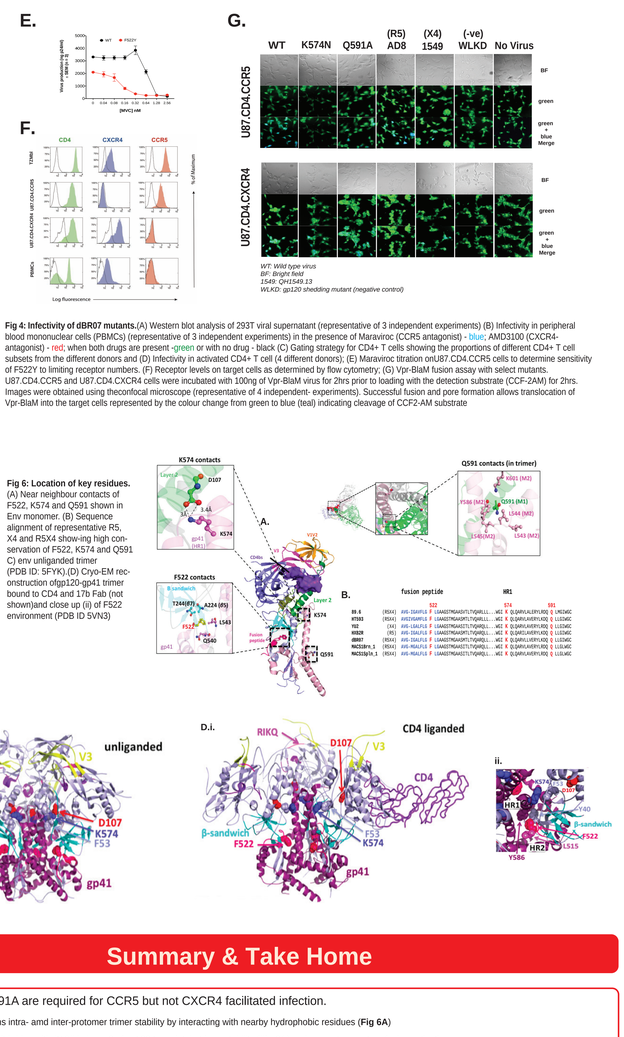


Fig 4: Infectivity of dBR07 mutants.(A) Western blot analysis of 293T viral supernatant (representative of 3 independent experiments) (B) Infectivity in peripheral blood mononuclear cells (PBMCs) (representative of 3 independent experiments) in the presence of Maraviroc (CCR5 antagonist) - blue; AMD3100 (CXCR4-antagonist) - red; when both drugs are present -green or with no drug - black (C) Gating strategy for CD4+ T cells showing the proportions of different CD4+ T cell subsets from the different donors and (D) Infectivity in activated CD4+ T cell (4 different donors); (E) Maraviroc titration onU87.CD4.CCR5 cells to determine sensitivity of F522Y to limiting receptor numbers. (F) Receptor levels on target cells as determined by flow cytometry; (G) Vpr-BlaM fusion assay with select mutants. U87.CD4.CCR5 and U87.CD4.CXCR4 cells were incubated with 100ng of Vpr-BlaM virus for 2hrs prior to loading with the detection substrate (CCF-2AM) for 2hrs. Images were obtained using theconfocal microscope (representative of 4 independent- experiments). Successful fusion and pore formation allows translocation of Vpr-BlaM into the target cells represented by the colour change from green to blue (teal) indicating cleavage of CCF2-AM substrate

Fig 6: Location of key residues.
(A) Near neighbour contacts of F522, K574 and Q591 shown in Env monomer. (B) Sequence alignment of representative R5, X4 and R5X4 show-ing high conservation of F522, K574 and Q591 C) env unliganded trimer (PDB ID: 5FYK).(D) Cryo-EM reconstruction ofgp120-gp41 trimer bound to CD4 and 17b Fab (not shown)and close up (ii) of F522 environment (PDB ID 5VN3)

Summary & Take Home

K574N and Q591A are required for CCR5 but not CXCR4 facilitated infection.

Q591 maintains intra- amd inter-protomer trimer stability by interacting with nearby hydrophobic residues (**Fig 6A**)

K574 forms salt bridge with D107 in layer 2 of gp120 to maintain trimer stability (**Fig 6A**) and prevent premature fusion activation

Receptor binding dissolves K574-D107 salt-bridge forcing new interaction with gp120's F53 (Ozorowski et al 2017) (Fig 6D) proximal to the 7 stranded beta-sandwich priming FP to its fusion conformation. N574 likely disrupts these conformational changes when CCR5 is bound stopping signal propogation to gp41

F522 in the gp41 fusion peptide is buried within hydrophobic pocket formed by complex salt-bridging networks in and adjacent to the 7-stranded beta sandwich (**Fig 6**)

Introduction of hydrohyl group (ie F522Y) results in changes in Env that abolish CXCR4 use and imposes sensitivity of Env to limiting CCR5 numbers

Thus gp41 residues, in addition to gp120 CKR determinants, are important for sensing alternate CKR engagement and may also be key in determining CKR and potentially cell tropism

HIV Env glycoprotein evolve distinct CKR signaling pathways and likely adapt to declining CCR5 numbers available on cells by acquiring the ability to signal /use CXCR4 if CCR5 levels are inadequate