Cross-Clade Inhibition of HIV on Primary Cells by CXCR4 or CCR5 Fused to the C34 Peptide from gp41 HR2

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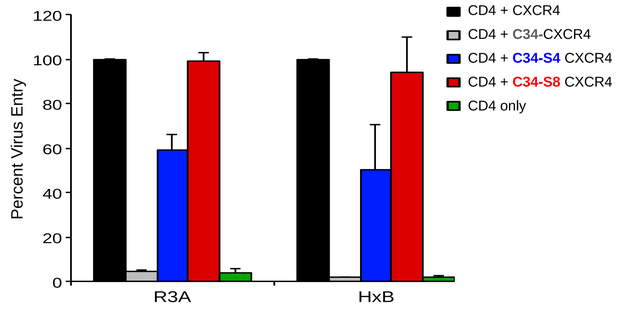
Background: HIV-1 entry into CD4⁺ T cells requires binding to CD4 and either the CCR5 (R5) or CXCR4 (X4) co-receptor. Thus, strategies that disable productive co-receptor (CoR) engagement should provide potent protection from HIV infection. Previously we described a 34 amino acid peptide from the C-terminal heptad repeat-2 domain of gp41 (C34) which, when fused to the amino terminus (NT) of either R5 or X4, inhibits HIV-1 infection in transformed cells *in vitro*. Moreover, our initial studies suggested that C34-R5 or C34-X4 fusions provided trans-dominant resistance to infection irrespective of viral tropism (i.e. either C34-R5 or C34-X4 could inhibit entry of R5, X4 or dual-tropic isolates).

Methods: Using lentiviral vectors, C34-R5 or C34-X4 fusion constructs were transduced into anti-CD3/CD28-stimulated primary human CD4⁺ T-cells. Controls included GFP alone and C34-conjugated to CD4. Susceptibility to infection by R5, X4, or dual tropic HIV-1 from different clades was assessed by flow cytometry and reverse transcriptase activity (RT). Functionality of the transduced T-cells was determined by evaluating their expansion and intracellular cytokine levels after re-stimulation with PMA/ionomycin or anti-CD3/CD28 beads.

Results: In CD4⁺ T-cells from multiple donors, when C34-R5 or C34-X4 were expressed, there was almost complete inhibition (>98%) of HIV-1 infection by intracellular p24 levels and RT. GFP-only and C34-CD4 expressing cells were infected at levels similar to untransduced T-cells. C34/CoR expression was >90% on Day 0 and stable during the 14 days of culture (>85%). Trans-dominant inhibition by C34-R5 or C34-X4 occurred for X4, R5 and dual-tropic primary isolates from clades B and A/E. Remarkably, when C34-CoR transduced and untransduced cells were titrated (1:4, respectively) and challenged with diverse HIV isolates, a condition that provides a more sustained exposure to HIV, selective enrichment of C34-CoR expressing cells occurred from the expected starting levels of ~25% up to 60% C34-CoR+ cells during viral replication. Lastly, PMA/ionomycin and anti-CD3/CD28 stimulation of C34-R5 and C34-X4 expressing T-cells grew normally and produced levels of dwith the potential to control HIV infection in humans.

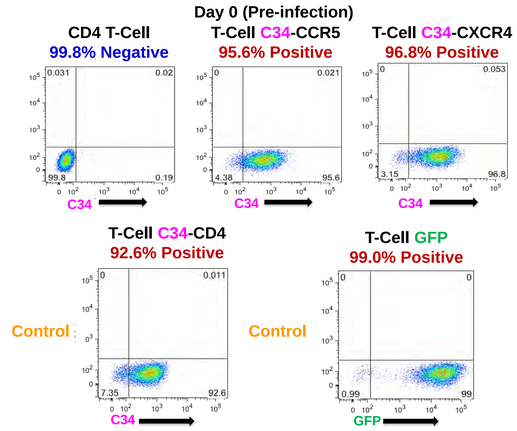
C34-CoReceptor Inhibition: Specificity

C34	WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL
C34- S4	WME**F**DRE**L**NNYTSLIHSLIEESQN**E**QEK**D**EQELL
C34- S8	WME**F**DRE**L**NN**F**TSL**L**HS**I**IEE**A**QN**E**QEK**D**EQELL



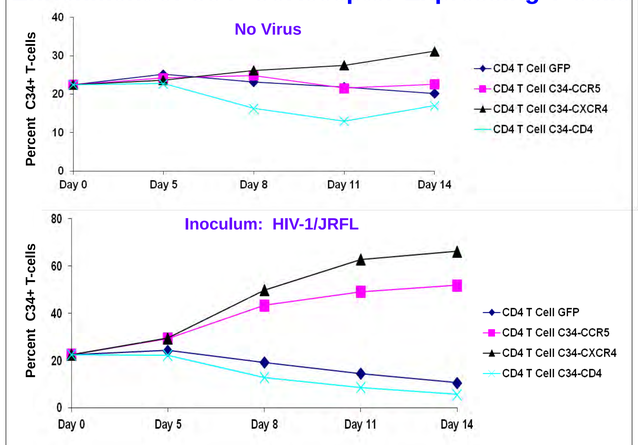
Cf2-Luc reporter cells were transfected with CD4 ± CXCR4 unconjugated or conjugated to C34 or C34 containing mutations of 4 (S4) or 8 (S8) residues that are critical for inhibition of gp41 6-helix bundle formation. Mutations at these positions abrogated C34-mediated inhibition of viral entry (S8 > S4).

Effects of C34-CoReceptors in Primary CD4⁺ T-cells



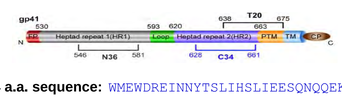
Primary CD4⁺ T-cells were stimulated (CD3/CD28 Dynabeads +IL-2) and then transduced with a lentiviral vector to express C34-CCR5, C34-CXCR4, C34-CD4 or GFP. Expression of C34-conjugated receptors was assessed with an anti-C34 antibody. As shown, C34-R5, -X4 and -CD4 were stably expressed up to 14 days.

Enrichment of C34⁺ CoReceptor-Expressing T-Cells

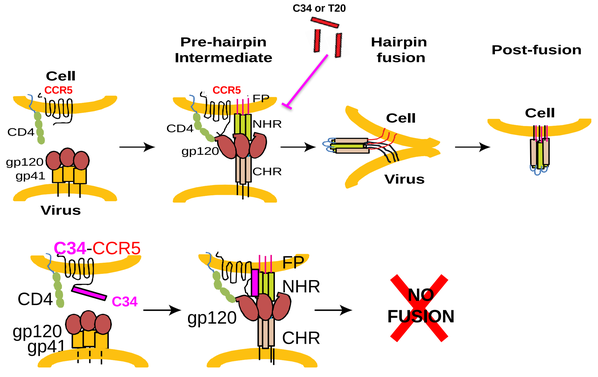


Graphs show selective enrichment of C34-CCR5⁺- and C34-CXCR4⁺-expressing CD4⁺ T-cells (but not of control C34-CD4⁺ and GFP-expressing cells) after HIV-1/JRFL inoculation.

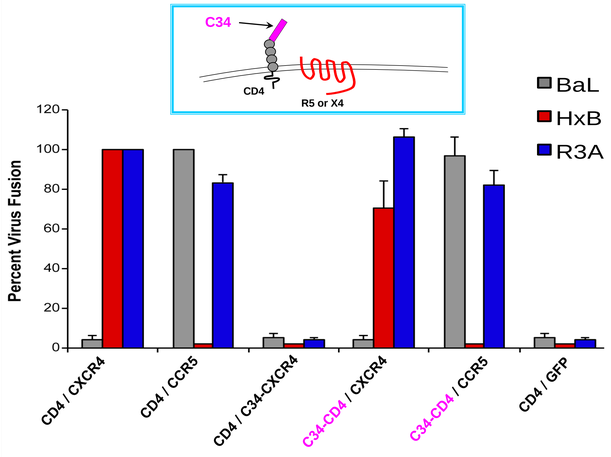
Blocking Fusion Using C34-peptide Fused to the CCR5 or CXCR4 N-terminus



C34 a.a. sequence: WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL

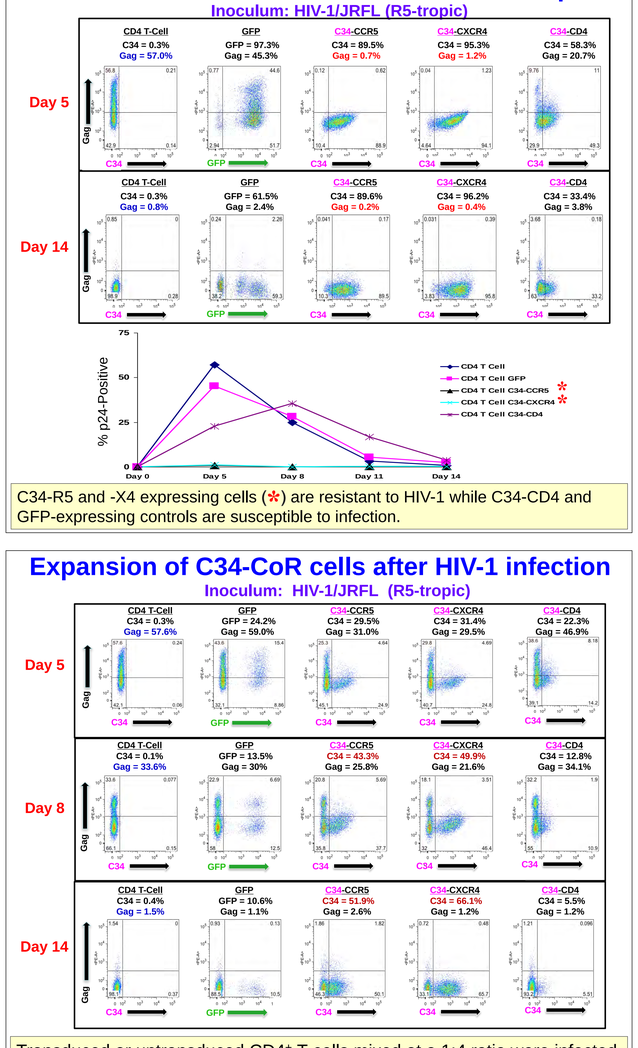


C34-CoReceptor Inhibition: Positioning



On Cf2-Luc reporter cells, C34-conjugated to the amino terminus of CD4 failed to inhibit entry of R5- (BaL), X4- (HxB), or dual-tropic (R3A) HIV-1.

HIV-infection of CD4⁺ T-cells ± C34-CoReceptors



C34-R5 and -X4 expressing cells (✲) are resistant to HIV-1 while C34-CD4 and GFP-expressing controls are susceptible to infection.

Inhibition of diverse HIV-1 isolates (14 DPI)

HIV-1 Viral Isolate	Inhibition C34-CCR5	Inhibition C34-CXCR4
JRFL (R5-topic / Clade B)	>98%	>99%
BaL (R5 Tropic / Clade B)	>98%	>98%
US1 (R5-topic / Clade B)	>99%	>99%
CMU01 (X4-topic / Clade A-E)	>99%	>98%
MN (X4-topic / Clade B)	>99%	>99%
R3A (R5/X4-topic / Clade B)	>98%	>99%
SF2 (R5/X4-topic / Clade B)	>99%	>99%

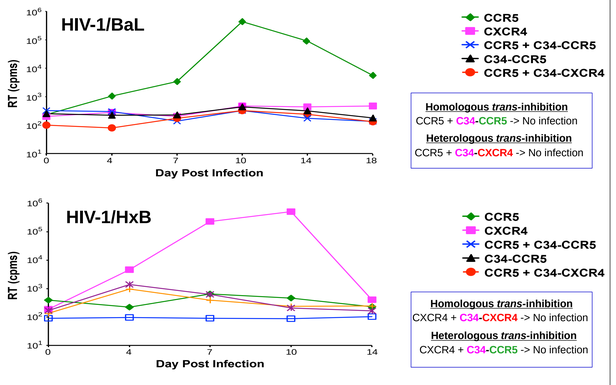
Selective Enrichment of C34⁺ T-Cells (14 DPI)

HIV-1 Viral Isolate	Enrichment C34-CCR5 Start	Enrichment C34-CCR5 Final	Enrichment C34-CXCR4 Start	Enrichment C34-CXCR4 Final
JRFL (R5-topic / Clade B)	24%	51%	25%	66%
BaL (R5 Tropic / Clade B)	21%	45%	27%	53%
US1 (R5-topic / Clade B)	24%	47%	27%	60%
CMU01 (X4-topic / Clade A-E)	24%	52%	25%	59%
MN (X4-topic / Clade B)	21%	50%	27%	78%
R3A (R5/X4-topic / Clade B)	21%	55%	27%	76%
SF2 (R5/X4-topic / Clade B)	24%	53%	25%	69%

Inhibition of Diverse HIV-1 isolates by C34-conjugagted CoReceptors.
Top Panel: C34 CoReceptor-transduced CD4⁺ T-cells were inoculated with HIV-1 isolates and p24 Gag staining assessed. Potent inhibition occurred of all isolates tested.
Bottom Panel: When mixed at a ratio of 1:4 transduced vs. non-transduced cells, enrichment of C34-CCR5 and C34-CXCR4 T-cells was observed following inoculation with all HIV-1 isolates tested and with cells from multiple donors.

C34-CCR5 and C34-CXCR4 Do Not Permit Entry of Either R5 or X4 Tropic HIV-1

Infection of SupT1 T-Cells Stably Expressing the Indicated Co-Receptors



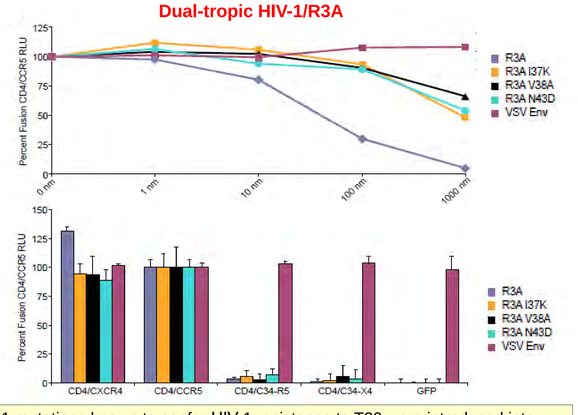
Homologous *trans*-inhibition
CCR5 + C34-CCR5 -> No infection
Heterologous *trans*-inhibition
CCR5 + C34-CXCR4 -> No infection

Homologous *trans*-inhibition
CXCR4 + C34-CXCR4 -> No infection
Heterologous *trans*-inhibition
CXCR4 + C34-CCR5 -> No infection

C34-conjugated coreceptors (CCR5 and CXCR4) when stably expressed on a T-cell line exhibit potent *trans*-dominant inhibition of R5- and X4-tropic viruses in a homologous and heterologous manner.

T20-Resistant HIV is Inhibitable by C34-CoReceptor

Dual-tropic HIV-1/R3A



Gp41 mutations known to confer HIV-1 resistance to T20 were introduced into dual-tropic HIV-1/R3A, and their effects on entry of Env-pseudotype viruses assessed on QT6 cells expressing the indicated coreceptors. **Top Panel** shows T20-resistance conferred by these mutations. **Bottom Panel** shows that T20-resistant Envs remained highly susceptible to C34-conjugated CCR5 and CXCR4.

Expansion of C34-CoR cells after HIV-1 infection

Inoculum: HIV-1/JRFL (R5-tropic)

Transduced or untransduced CD4⁺ T-cells mixed at a 1:4 ratio were infected with HIV-1/JRFL. C34-CCR5⁺ and C34-CXCR4⁺ cells expanded during the course of infection, while C34-CD4⁺- and GFP⁺-transduced cells were not.

Summary & Conclusions

- The C34 peptide from HIV-1 gp41 HR2 is a potent inhibitor of HIV-1 entry when conjugated to the CXCR4 or CCR5 amino termini.
- This inhibition was highly specific and dependent on positioning of the C34 peptide and its amino acid content. (No inhibition was seen when C34 was conjugated to the CD4 amino terminus or when residues required to inhibit formation of the gp41 6-helix bundle were mutated).
- C34-CXCR4 and C34-CCR5 could inhibit R5- and X4-tropic HIV-1 isolates in a *trans*-dominant manner (i.e. C34-R5 or C34-X4 could inhibit both R5 and X4 tropic isolates), suggesting that these molecules are targeted to points of viral entry where the C34 peptide can directly access the site of 6 helix bundle formation irrespective of viral tropism.
- *Trans*-dominant inhibition of R5 and X4 HIV-1 is also seen when C34-CCR5 or C34-CXCR4 are transduced into primary CD4 T-cells. Cells expressing C34-conjugated coreceptors show a significant selective growth advantage during HIV-1 infection.

Future Directions

- Assess the ability of C34-conjugated CCR5 and CXCR4 to exert *trans*-dominant inhibition of R5- and X4-tropic HIV-1 in the NSG humanized mouse model.
- Extend this approach to explore gene-editing methodology in primary CD4⁺ T-cells with a goal of enabling *trans*-dominant HIV inhibition to be exerted from endogenous coreceptors.
- Assess the immunogenicity of the C34 peptide when coupled to an exogenous or endogenous coreceptor.

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