RNA-Associated Early-Stage Antiviral Factor Is a Major Component of Lv2 Restriction

RNA-associated Early-stage Anti-viral Factor (REAF) is a major component of LV2 restriction

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Running Head: REAF is Lv2

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Abstract

HIV and SIV replication in human cells is restricted at early post-entry steps by host inhibitory factors. We previously described and characterised an early phase restriction of HIV-1 and 2 replication in human cell lines, primary macrophages and PBMCs. The restriction was termed Lentiviral restriction 2 (Lv2). The viral determinants of Lv2 susceptibility mapped to the HIV-2 Env and CA. We subsequently reported a whole genome siRNA screen for factors involved in to HIV which identified RNA-associated Early-stage Anti-viral Factor (REAF). Using HIV-2 chimeras of susceptible and non-susceptible viruses we show here that REAF is a major component of the previously described Lv2. Further studies of the viral CA demonstrate that the CA mutation I73V (previously called I207V), a potent determinant for HIV-2, is a weak determinant of susceptibility for HIV-1. More potent CA determinants for HIV-1 REAF restriction were identified at P38A, N74D, G89V and G94D. These results firmly establish that in HIV-1 CA is a strong determinant of susceptibility to LV2/REAF. Similar to HIV-2 the HIV-1 Env can rescue sensitive CAs from restriction. We conclude that REAF is a major component of the previously described Lv2 restriction.

Importance

Measures taken by the host cell to combat infection drive the evolution of pathogens to counteract or side step them. The study of such virus-host conflicts can point to possible weaknesses in the arsenal of viruses and may lead to the rational design of anti-viral agents. Here we describe our discovery that the host restriction factor REAF fulfils the same criteria previously used to describe Lentiviral restriction (Lv2). We show that, like HIV-1 CA, the CA of HIV-1 is a strong determinant of LV2/REAF susceptibility. We illustrate how HIV counteracts LV2/REAF by using an envelope with alternative routes of entry into cells.

Introduction

Infection of cells by human and simian immunodeficiency virus (HIV and SIV) is initiated by binding of the viral envelope (Env) to CD4. Conformational changes in the viral Env expose a site that can interact with a chemokine receptor, either CXCR4 or CCR5, expressed at the cell surface of $CD4^+$ T cells and primary macrophages (1, 2). Viruses in general can enter cells through different routes, either directly at the plasma membrane (PM) or through one of a number of endocytic pathways (3). Influenza is a prototypical virus that enters cells through an endocytic route and requires the acid environment of the late endosome to trigger its fusion and entry into cells. Since the mechanism of HIV fusion is pH independent (4) it has been widely assumed that HIV fuses at the PM (5-7). pH independent endocytic entry has recently been

observed (8-15) and is thus a possible mechanism of HIV entry; this however remains a topic of considerable controversy (16, 17). Regardless of the route, once HIV fuses at the plasma membrane the conical core is released into the cytoplasm. The viral genomic RNA is reverse transcribed by the virally encoded RNA/DNA dependent reverse transcriptase (RT), resulting in virally encoded RNA:DNA, single and double stranded (ss and ds)DNA intermediates. The RNase H activity of RT degrades the RNA from these hybrids resulting in ssDNA from which the second DNA strand is synthesised (18, 19). Once reverse transcription is complete the double stranded proviral DNA is processed for integration into the host cell genome.

HIV must overcome many cellular barriers to its replication as it journey to the nucleus to integrate into the host genome (20, 21). Interferon-induced transmembrane proteins (IFITMs) can inhibit virus-cell membrane fusion (22) and the process of reverse transcription itself is also vulnerable. Immediately after initiation, members of the Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like (APOBEC) family of restriction factors induce deoxycytidine to deoxyuridine mutations in the nascent DNA (23). Further disabling reverse transcription SAMHD1 depletes the dNTP substrates required (24). RNA-associated Early-stage Anti-viral Factor (REAF) was described to inhibit HIV and SIV replication during reverse transcription (25). REAF is intrinsically expressed and provides an initial line of defence against HIV and SIV infection. It associates with reverse transcripts; either ssDNA or RNA:DNA hybrids, however the precise mechanism of its action is not yet understood. A more recently described restriction factor MX2/MXB inhibits replication at a later stage, suppressing nuclear import and proviral formation (26-28). Integration is inhibited by the TRIM28 (KAP1)/SETDB1 complex (29). Once

the provirus is integrated the late phase of the replication cycle begins with the production of viral proteins (30). A plasma membrane located restriction factor tetherin/BST2/CD317, prevents viruses from leaving the cell at the late budding stage of the life cycle (31).

The first Lentiviral restriction factor 1 (Lv1) effective against HIV-1 was identified as rhesus TRIM5α (32, 33). Lv1/TRIM5α is species specific and active against HIV-1 in non-human primate cells. TRIM5α forms a lattice around the capsid (CA) resulting in premature disassembly of the conical core (34). It is not known if Lv2 is species specific. It inhibits HIV-1 and 2 during reverse transcription and susceptibility is determined by the viral CA (35). Lv2 differs from Lv1 in that the Env is an additional determinant of restriction. Approximately half of HIV-1 and HIV-2 viruses are susceptible to Lv2 (35). Lv3 is a post entry restriction to infection of simian MAGI cells by HIV-1 and similar to Lv2 is dependent on fusion events at the cell membrane (36). Lv4 restricts nuclear entry of SIV isolates in human cells (37). A recently described restriction to HIV-1 induced by TLR 7/8 agonist in human monocytes is termed Lv5 (38). So far the identities of Lv3, 4 and 5 are unknown. Here we describe the identification of REAF as a potent component of Lv2.

To identify components of Lv2 we designed a whole genome siRNA screen (20). HeLa-CD4 cells were transfected with an siRNA library targeting 19,121 human genes and then challenged with an HIV-$1^{89.6}$ (MCR) pseudovirus (20). One factor identified was RPRD2 (here called RNA-associated Early-stage Anti-viral Factor; REAF) which we now show fulfils the characteristics used to define Lv2 (35,38).

Materials and Methods

Cells. Buffy coats from seronegative donors were obtained from the National Blood Service (Brentwood, UK). Donors were anonymous and patient consent was not required. Peripheral blood mononuclear cells (PBMC) were prepared by density-gradient centrifugation (Lymphoprep, Axis-Shield). Monocyte-derived macrophages (MDM) were isolated from PBMC using $CD14^+$ MACS Microbeads (Miltenyi Biotec) and left to differentiate for 5 days in RPMI 1640/10% foetal calf serum (FCS) and 15ng/ml granulocyte macrophage colony stimulating factor (GM-CSF; Peprotech). HEK 293T, HeLa-CD4, U87-CD4-CXCR4, HeLa EKV and HeLa EKVΔCPSF6-358 cells and their optimal culture conditions have been described previously (39-42).

Preparation of REAF knockdown cells. The pSUPER RNAi system (pSUPER.retro.puro; Oligoengine) was used for expression of shRNA in mammalian cells (43, 44). For REAF knockdown pSUPER.retro.puro(shREAF) was generated by digestion with BglII and HindIII, annealing of the specific primers and ligation. The shRNA target sequences are shown in upper case within the primers listed below:

shREAF-BglII: 5' gatccccCACGTAAGCCCTCAGATGAttcaagagaTCATCTGAGGGCTTACGTGttttta 3'

shREAF-HindIII: 5' agcttaaaaaCACGTAAGCCCTCAGATGAtctcttgaaTCATCTGAGGGCTTACGTGggg 3'

The vector was either transfected directly into HeLa-CD4 cells for transient knockdown or used to generate stable knockdown cell lines. Briefly, retroviruses were produced by co-transfecting

pSUPER.retro.puro(shREAF) with an HIV-1 *gag-pol* expression vector (p8.91) (45) and pMDG VSV-G Env into HEK 293T cells. Supernatant containing virus was harvested after 48 hours and was used to transduce HeLa-CD4 cells under puromycin selection. REAF silencing in transient and stably knocked down cells was confirmed by Western blot.

Plasmids and virus production. The infectious molecular clone for HIV-$1^{89.6}$ was obtained from the Centre for AIDS Research (NIBSC, UK). Infectious full-length and chimeric HIV clones were prepared by polyethylenimine (PEI; Polysciences) or Lipofectamine 2000 (Invitrogen) transfection of HEK 293T cells. The virus named in parentheses for each pseudotype denotes the Env used.

Production of CA mutant viruses. HIV-1 CA mutants were generated by site directed mutagenesis (SDM) of the HIV-$1^{NL4.3}$-derived viral clone pBR-NL43-IRES-eGFP (46) with further modification to introduce stop codons in the first and third codons of the Env coding sequence. HIV-1 pseudovirus particles were produced by PEI transfection of HEK 293T cells using a 1:1 molar ratio of viral plasmid to MCR/MCN/VSV-G/NL4.3 Env expression plasmid.

Western blot. SDS-PAGE separated proteins were detected with the primary rabbit polyclonal antibody against REAF (Eurogentec) or GAPDH (loading control; Abcam) followed by horseradish peroxidase-conjugated donkey α-rabbit antibody (GE Healthcare). Protein was visualised using a chemiluminescence kit (ECL; GE Healthcare).

siRNA transfection and infection with replication competent virus. HeLa-CD4 cells were seeded at 2.5×10^4 cells/well in 24-well plates. siRNA transfection (30nM) was performed using HiPerfect (QIAGEN) according to the manufacturer's instructions using the following sequences:

siREAF: 5' CACGTAAGCCCTCAGATGATA 3'

siCB: 5' ACAGCAAATTCCATCGTGT 3'

72 hours after siRNA transfection, cells were challenged with virus for up to 5 hr. Infection was assessed up to 48 hours by intracellular p24 staining.

In situ **immunostaining for p24 antigen.** Infected cells were fixed with cold (-20°C) methanol:acetone (1:1), washed with PBS then immunostained for p24 using mouse anti-HIV-1 p24 monoclonal antibodies EVA365 and 366 (NIBSC, UK) (1:50) as previously described (47). Infected cell foci stained blue (regarded as foci of infection (FFU/ml)) and were quantitated by light microscopy.

Statistical analysis. The results presented are derived from a minimum of three independent experiments performed in duplicate at minimum. Differences between two treatments were tested for statistical significance using unpaired two-tailed *t*-Tests. * denotes $p < 0.05$, *n.s.* not significant.

Results

The HIV-2 molecular determinants of Lv2 restriction were previously mapped using two HIV-2 molecular clones of viruses derived from the same patient, HIV-2^{MCR} and HIV-2^{MCN}, which are differentially sensitive to Lv2. A gene swapping approach between the viruses identified the *gag* and *env* genes as critical determinants of Lv2 restriction (39). These chimeric viruses (shown schematically in Fig. 1A) were tested to determine if they had the same pattern of susceptibility to REAF.

HeLa-CD4 cells were knocked down for REAF using specific (siREAF) or non-targeting control siRNA (cyclophilin B, siCB) (Fig. 1B). Fig. 1C shows viral rescue in HeLa-CD4 cells following treatment with siREAF and compared with cells treated with siCB. Repeat experiments consistently show, as expected for a virus highly sensitive to Lv2 (39), that the HIV-2^{MCR} virus is potently rescued in comparison to HIV-2^{MCN} (50 fold vs 10 fold; $p = 0.004$). When the *env* and *gag* from the restricted HIV-2^{MCR} was inserted in place of the relatively insensitive HIV-2^{MCN} *env* and *gag* (HIV-$2^{MCN\,mcr\ env+gag}$), greater sensitivity of this virus to REAF was observed (66 fold). In the reciprocal experiment, where the *env* and *gag* from HIV-2^{MCN} replaced the HIV-2^{MCR} genes (HIV-$2^{MCR\,mcn\ env+gag}$*),* the resulting chimera was only rescued 3 fold ($p < 0.001$). These results for susceptibility to REAF are consistent with the Lv2 phenotype previously described (39). A single point mutation in HIV-2^{MCR} CA at position 73 is known to be a critical determinant of Lv2 restriction (previously labelled position 207). Fig. 1C shows that HIV-$2^{MCR\ CA\ I73V}$ is rescued only 12 fold from REAF restriction compared to 50 fold for wild type HIV-2^{MCR} ($p = 0.003$).

To confirm these results and for further experiments we generated HeLa-CD4 cell lines permanently expressing shRNA specific for REAF mRNA. Western blot (WB) analysis shows that the HeLa-CD4-shREAF cells expressed much less REAF protein than the parental HeLa-CD4 cells

200 (Fig. 1D). The phenotype of knockdown of REAF in this cell line was confirmed using HIV-2^{MCR}
201 and HIV-$2^{MCR\ CA\ I73V}$. The HIV-2^{MCR} virus was restricted 326 fold compared to 33 fold for the HIV-
202 $2^{MCR\ CA\ I73V}$ (Fig. 1E; $p = 0.016$).
203 We previously reported that Lv2 was active in HeLa-CD4, human primary PBMC and MDM but
204 not in U87-CD4-CXCR4 cells (39). REAF mRNA (data not shown) and protein is present in MDM
205 and to much lower levels in PBMC (Fig. 1F) while WB analysis shows it to be barely detectable in
206 U87-CD4-CXCR4 (Fig. 1G).
207
208 As previously reported for Lv2 (39) and further demonstrated here, the HIV-2 determinants for
209 REAF are Env and CA (specifically amino acid 73). We sought to identify the determinants of
210 REAF restriction for HIV-1. Fig. 2A shows that, compared to HIV-$1^{89.6}$, HIV-$1^{NL4.3}$ is more resistant
211 to REAF restriction (3 fold vs 21 fold; $p < 0.001$). We used HIV-$1^{NL4.3}$ to further establish if the
212 equivalent HIV-2 CA mutation 73 plays a role in Lv2/REAF restriction in HIV-1. Using SDM we
213 generated HIV-$1^{NL4.3\ CA\ I73V}$. Both wild type and mutant CA were pseudotyped with HIV-2^{MCR} Env
214 and/or HIV-$1^{NL4.3}$ Env and tested for their susceptibility to REAF using the HeLa-CD4-shREAF cell
215 line. Fig. 2B shows that wild type HIV-$1^{NL4.3}$ CA is only weakly susceptible to REAF when
216 pseudotyped with HIV-$1^{NL4.3}$ Env. However when the CA is mutated (HIV-$1^{NL4.3\ CA\ I73V}$ (NL4.3)) the
217 restriction is more potent but still relatively weak compared to HIV-2 (15 fold, compare to Fig.
218 1E for HIV-2). The CA I73V was further restricted when pseudotyped with an HIV-2^{MCR} Env (21
219 fold, $p = 0.03$), but not with HIV-2^{MCN} Env (16 fold, $p = n.s.$). Thus CA amino acid 73 and Env are
220 determinants of Lv2/REAF restriction for both HIV-1 and 2.
221

222 The CA amino acid at position 73 lies in the binding domain of the cleavage and polyadenylation
223 specific factor 6 (CPSF6) protein (48). This is of particular interest as the adjacent CA mutation
224 (N74D) has been shown to affect the sensitivity of HIV-1 to depletion of RanBP2, Nup153 and
225 TNPO3 nuclear pore proteins (49, 50).
226 N74D is an HIV-1 escape mutant that was generated by passage of HIV-$1^{NL4.3}$ in cells expressing
227 an artificially mutated CPSF6-358 that perturbs HIV-1 nuclear entry (50). CPSF6 is a pre-mRNA
228 processing protein that shuttles between the nucleus and the cytoplasm (51). The mutant form
229 CPSF6-358 lacks a C-terminal nuclear-targeting arginine/serine (RS)-rich domain and so is
230 confined to the cytoplasm and restricts HIV-1 before nuclear entry (50, 52, 53).
231 We tested whether the Lv2/REAF HIV-2 CA determinant I73V was similar to N74D with respect
232 to resistance to CPSF6-358. Another CA mutation, P38A, which was mutated outside the CPSF6
233 CA binding region was included as a negative control (54). HeLa-CD4 target cells permanently
234 expressing the mutant CPSF6-358 (HeLa EKVΔCPSF6-358) or vector alone (HeLa EKV) (42, 48)
235 were challenged with pseudotypes carrying a mutant or wild type CA. Fig. 2C shows that, as
236 expected, infection of the pseudotypes with wild type and P38A CA are inhibited (62 and 73
237 fold respectively). However both CA mutants, HIV-$1^{NL4.3\ CA\ I73V}$ (VSV-G) and HIV-$1^{NL4.3\ CA\ N74D}$ (VSV-
238 G), were resistant to CPSF6-358 (3.3 and 1.6 fold; both $p < 0.001$).
239
240 Given this similarity in resistance to CPSF6-358 we also sought to determine whether, similar to
241 I73V, N74D is more susceptible than wild type virus to REAF restriction. HeLa-CD4 and HeLa-
242 CD4-shREAF cells were challenged by pseudotypes with either wild type or mutated CA. Fig. 3A
243 shows that HIV-$1^{NL4.3}$ (MCR) is rescued 13 fold. Surprisingly HIV-$1^{NL4.3\ CA\ N74D}$ (MCR) is even more

restricted and is rescued 53 fold ($p < 0.001$), suggesting CA N74D is a more potent determinant of REAF restriction in HIV-1

As well as being less sensitive to CPSF6-358, the CA N74D mutation has a more stable conical core which results in delayed disassembly and reverse transcription (55). It is thought that optimal disassembly of the conical core is required for successful infection as mutations interfering with core stability often result in a disturbance of reverse transcription kinetics (54, 56-58). We previously reported that REAF was transiently down modulated shortly after infection (25). We hypothesised that unstable capsids will prematurely disassemble and expose reverse transcripts to REAF. The corollary of this is that capsids that disassemble too late will miss the window of time where REAF is absent. To test this hypothesis that capsid stability was a determinant of REAF/Lv2 restriction we investigated the REAF susceptibility of CA mutations with varying CA stability. P38A, in contrast to N74D, is highly unstable (54, 59) while G94D is unaffected (55). The G89V mutation in the cyclophilin binding loop was chosen because it has previously been shown to affect sensitivity to host restriction factors (42, 54, 57, 58). P38A is also distinct from N74D in that it is sensitive to CPSF6-358 (Fig. 2C). Fig. 3C, D and E show the infectivity of all three capsid mutants. HIV-$1^{NL4.3\ CA\ P38A}$ (MCR), HIV-$1^{NL4.3\ CA\ G89V}$ (MCR) and HIV-$1^{NL4.3\ CA\ G94D}$ were severely compromised on HeLa-CD4 cells (titre of <500 FFU/ml; compared to wild type CA, Fig. 3B). However their replication was rescued in the absence of REAF similar to HIV-$1^{NL4.3\ CA\ N74D}$ (MCR) (Fig. 3F; all $p < 0.001$).

We showed above that in addition to CA, HIV-1 Env also confers sensitivity to REAF (Fig. 2B). We previously determined that HIV-2^{MCN} Env has the ability to overcome Lv2 (35, 39). If REAF is Lv2

the HIV-2^{MCN} Env would overcome REAF restriction. HeLa-CD4-shREAF cells were challenged with HIV-$1^{NL4.3}$ with a wild type CA pseudotyped with HIV-2^{MCN} Env. As expected the wild type HIV-$1^{NL4.3}$ CA was only slightly restricted and the HIV-2^{MCN} Env could reduce this to a small but significant degree (5.6 to 3.1 fold; $p = 0.007$) (Fig. 4A). In contrast the HIV-$1^{NL4.3}$ strains carrying REAF sensitive CA mutations N74D, P38A, G89V and G94V were potently rescued with the HIV-2^{MCN} Env (Fig. 4B-E, all p-values < 0.001) further confirming that REAF and Lv2 are similarly rescued by viral Env.

Discussion

Here we show that REAF is a major component of the previously described restriction Lv2 (35, 60, 61) . HIV chimeric viruses and mutants that delineate susceptible and resistant clones demonstrate that Lv2 and REAF are indistinguishable. Both Lv2 and REAF restriction activity are molecularly determined by the viral Env and CA. The CA amino acid at position 73 in HIV-2 was a crucial determinant of Lv2 and confirmed here for REAF (39). The equivalent amino acid at position 73 also affects HIV-1 susceptibility to REAF. Although the effects of I73V are statistically significant they are much weaker compared to HIV-2. Indeed, the same amino acid substitution rendered HIV-2^{MCN} *less* susceptible but HIV-$1^{NL4.3}$ *more* susceptible to REAF. This led us to the hypothesis that the overall structure or stability of the CA rather than precise molecular interactions is critical. It has been proposed that disassembly too early prior to localisation at the nuclear pore would result in exposure to restriction factors and premature termination of reverse transcription (19, 62-65). Indeed it has been proposed that HIV-1 disassembly involves a regulated collapse of the conical core which protects viral reverse transcription complexes (66).

Also it has been shown that disassembly occurs within an hour of fusion and is facilitated by reverse transcription (67-69). We have observed that cellular REAF is reduced within one hour of viral challenge, but importantly, levels are rapidly replenished an hour later (25). REAF associates with viral nucleic acid and restricts replication during reverse transcription (25). We therefore suggested that the temporary reduction in REAF protein level allows viruses to reverse transcribe in the absence of REAF associated activity. This model is in keeping with the notion that CA stability is a determinant of REAF associated restriction. We further tested the hypothesis that CA stability is a determinant of REAF susceptibility using already well characterised mutants with different CA stabilities. Compared to I73V (20 fold) more potent effects (more than 50 fold) were observed with mutants P38A (unstable), N74D (hyperstable) and G94D (unaffected) (55). Since all these CA mutations result in greater susceptibility to REAF associated activity despite having divergent CA stabilities we cannot conclude that CA stability is a major determinant of susceptibility to REAF. Regardless we show that the CA is a strong determinant of REAF restriction in HIV-1. Given the multifunctional role of CA in HIV-1 replication (70), understanding the role of CA in susceptibility to REAF associated activity may shed light on more specific interactions of CA with host cell factors required for efficient infectivity of HIV.

It is highly controversial whether or not infectious HIV conical cores enter the cytoplasm after fusion at the plasma membrane or through an endocytic route (8-17, 35, 60). Our previous observation of Lv2 suggests that, although either route is possible, infection is more successful when the virus fuses at the plasma membrane and avoids an endocytic entry pathway (35, 60).

Our results here confirm that, like Lv2, the choice of entry route as determined by the viral Env is a determinant of susceptibility to REAF associated activity. HIV-$1^{NL4.3}$ can be rendered sensitive to REAF if pseudotyped with HIV-2^{MCR} while HIV-2^{MCN} Env does not. These previously characterised HIV-2 Envs fuse either at the plasma membrane (MCN) or via an endocytic route (MCR) [35,38]. Furthermore all CA mutants which were rendered highly sensitive to REAF associated activity were protected when pseudotyped with an MCN Env.

We propose that, similar to Lv2, REAF may be more active against viruses attempting to access the cytoplasm via an endosome. Therefore, fusion at the plasma membrane is a more efficient replication pathway. However we cannot eliminate the possibility that some viruses will bypass REAF associated activity regardless of entry route, for example if they have a conical core without the sensitivity conferring mutations described here. Future studies that address the role of Env and CA in determining REAF associated restriction will shed light on these early host cell interactions in the early life cycle of HIV.

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Author contributions

Designed experiments; ÁMcK, KMM, RDS; Performed experiments; ÁMcK, KM, EOS, CEJ, JDD, CP and RDS; Analysed data; ÁMcK, KMM, RDS. Wrote the manuscript; ÁMcK and KMM.

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Figure legends

Figure 1

REAF is an important component of Lv2. A) A schematic representation of the HIV-2 molecular clones, chimeric viruses and site directed mutagenesis (*) used to map the determinants of REAF restriction. B) Western blot of HeLa-CD4 cell lysate following REAF siRNA knockdown compared with non-targeting control (siCB). GAPDH is added as a loading control. C) Titration of constructs on HeLa-CD4 cells transiently transfected with siREAF showing fold change compared to cells transfected with siCB control (compared with HIV-2^{MCR}: HIV-2^{MCN} $p = 0.004$, HIV-$2^{MCNmcr\ env+gag}$ $p = n.s.$, HIV-$2^{MCRmcn\ env+gag}$ $p < 0.001$, HIV-$2^{MCR\ CA\ I73V}$ $p = 0.003$). D) WB of knockdown of REAF in HeLa-CD4-shREAF cells compared to HeLa-CD4 cells. GAPDH is added as a loading control. E) Fold change for HeLa-CD4-shREAF cells infected with HIV-2^{MCR} and HIV-$2^{MCR\ CA\ I73V}$ confirms the Lv2 phenotype in the stable knockdown cells ($p = 0.016$). F) WB of REAF levels in MDM and PBMC compared to HeLa-CD4 cells. GAPDH is added as a loading control. G) WB of REAF levels in U87-CD4-CXCR4 cells compared to HeLa-CD4 cells. GAPDH is added as a loading control.

Figure 2

The HIV-1 CA determinants of REAF associated restriction are the same as those for HIV-2 and are in the CPSF6 binding pocket. A) A comparison of the susceptibility of HIV-$1^{NL4.3}$ and HIV-$1^{89.6}$ following transient knockdown of REAF by siRNA ($p < 0.001$). B) Mutation of the HIV-$1^{NL4.3}$

capsid (HIV-$1^{NL4.3\ CA\ I73V}$ (MCR) or HIV-$1^{NL4.3\ CA\ I73V}$ (NL4.3)) renders it susceptible to restriction in HeLa-CD4-shREAF cells (p = 0.03). C) Fold inhibition of HIV-$1^{NL4.3}$ (VSV-G) with CA mutants I73V and N74D (p < 0.001) in the presence of mutant CPSF6-358 compared to vector alone.

Figure 3

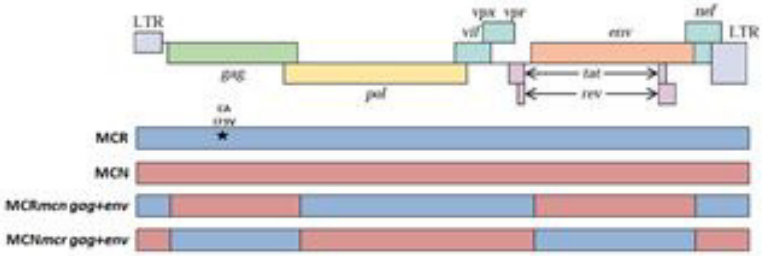
Mutant capsids are sensitive to REAF restriction. A) Infection of HeLa-CD4-shREAF cells with HIV-$1^{NL4.3\ CA\ N74D}$ (MCR) renders it susceptible to REAF compared to viral pseudotype with wild type CA (HIV-$1^{NL4.3}$ (MCR)) (p < 0.001). B-E) Titres of HIV-$1^{NL4.3}$ (MCR), HIV-$1^{NL4.3\ CA\ P38A}$ (MCR), HIV-$1^{NL4.3\ CA\ G89V}$ (MCR) and HIV-$1^{NL4.3\ CA\ G94D}$ (MCR) following challenge of HeLa-CD4 and HeLa-CD4-shREAF cells. F) Fold change for HIV-$1^{NL4.3\ CA\ P38A}$ (MCR), HIV-$1^{NL4.3\ CA\ G89V}$ (MCR) and HIV-$1^{NL4.3\ CA\ G94D}$ (MCR) on HeLa-CD4-shREAF cells compared to wild type CA (HIV-$1^{NL4.3}$ (MCR)) show they are also susceptible to REAF (all p <0.001).

Figure 4

Viral envelope determines susceptibility to REAF associated restriction. A) Infection of HeLa-CD4-shREAF cells with HIV-$1^{NL4.3}$ (MCN) decreases sensitivity to REAF associated restriction compared to HIV-$1^{NL4.3}$ (MCR) (p = 0.007). Comparison of HIV-2^{MCR} and HIV-2^{MCN} Env on pseudotypes carrying B) N74D (p < 0.001), C) P38A (p < 0.001), D) G89V (p < 0.001) and E) G94D (p = 0.001) CA show HIV-2^{MCN} Env makes these viruses less sensitive to REAF.

1

A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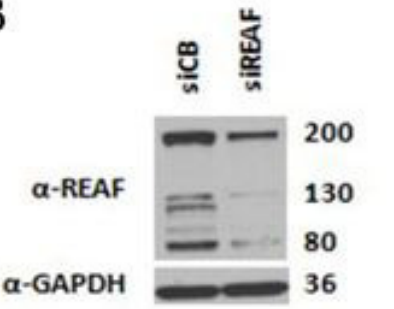
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α-GAPDH
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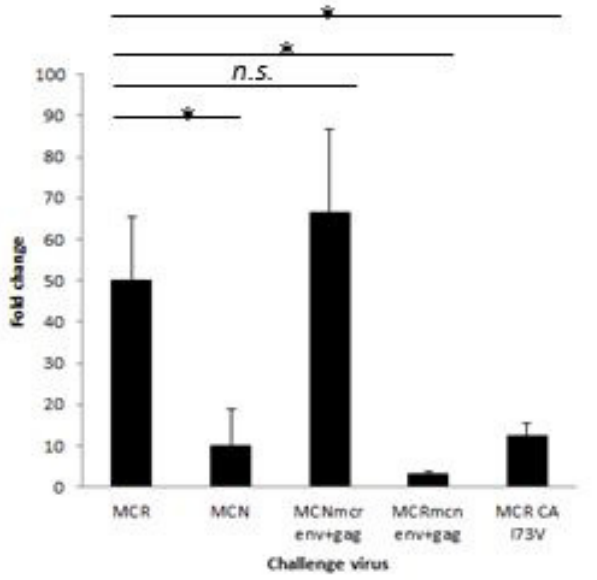
130
80
36

C


n.s.
Fold change
100
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80
70
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30
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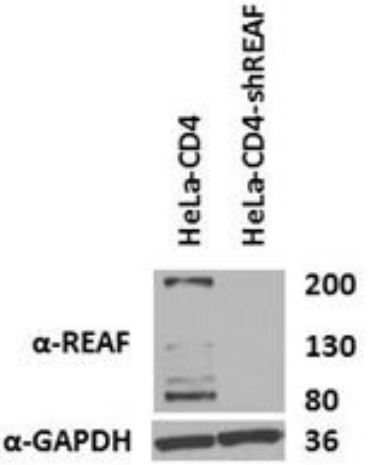
MCR
MCN
MCNmcr env+gag
MCRmcn env+gag
MCR CA I73V
Challenge virus

D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α-REAF
α-GAPDH
200
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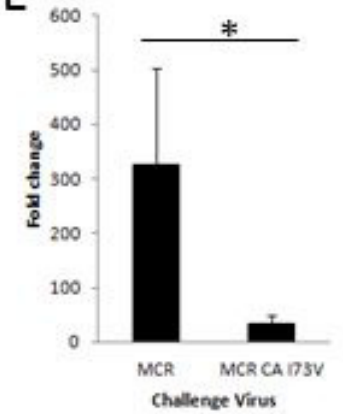
36

E


Fold change
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500
400
300
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100
0
MCR
MCR CA I73V
Challenge Virus

F


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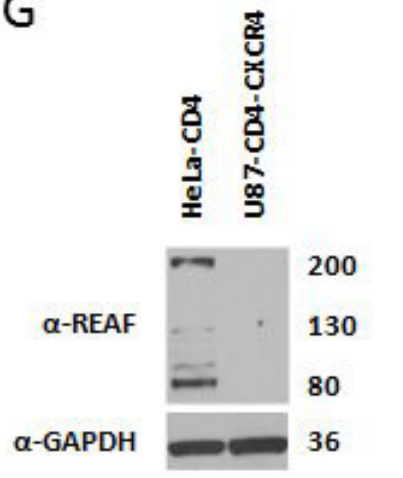
PBMC
α-REAF
α-GAPDH
200
130
80
36

G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80
36

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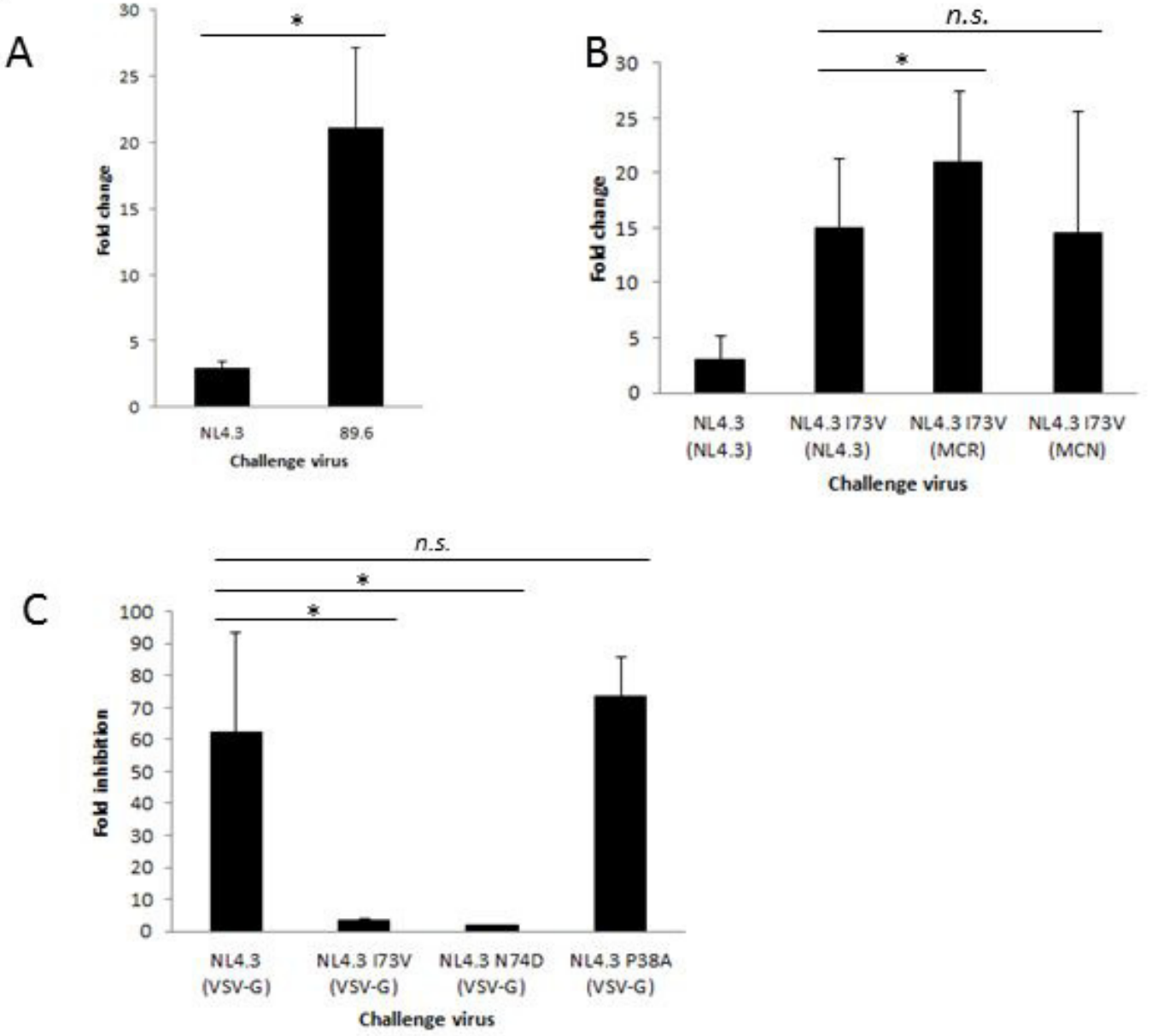
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Fold change
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89.6
Challenge virus
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n.s.
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30
25
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0
Fold change
NL4.3 (NL4.3)
NL4.3 I73V (NL4.3)
NL4.3 I73V (MCR)
NL4.3 I73V (MCN)
Challenge virus
C
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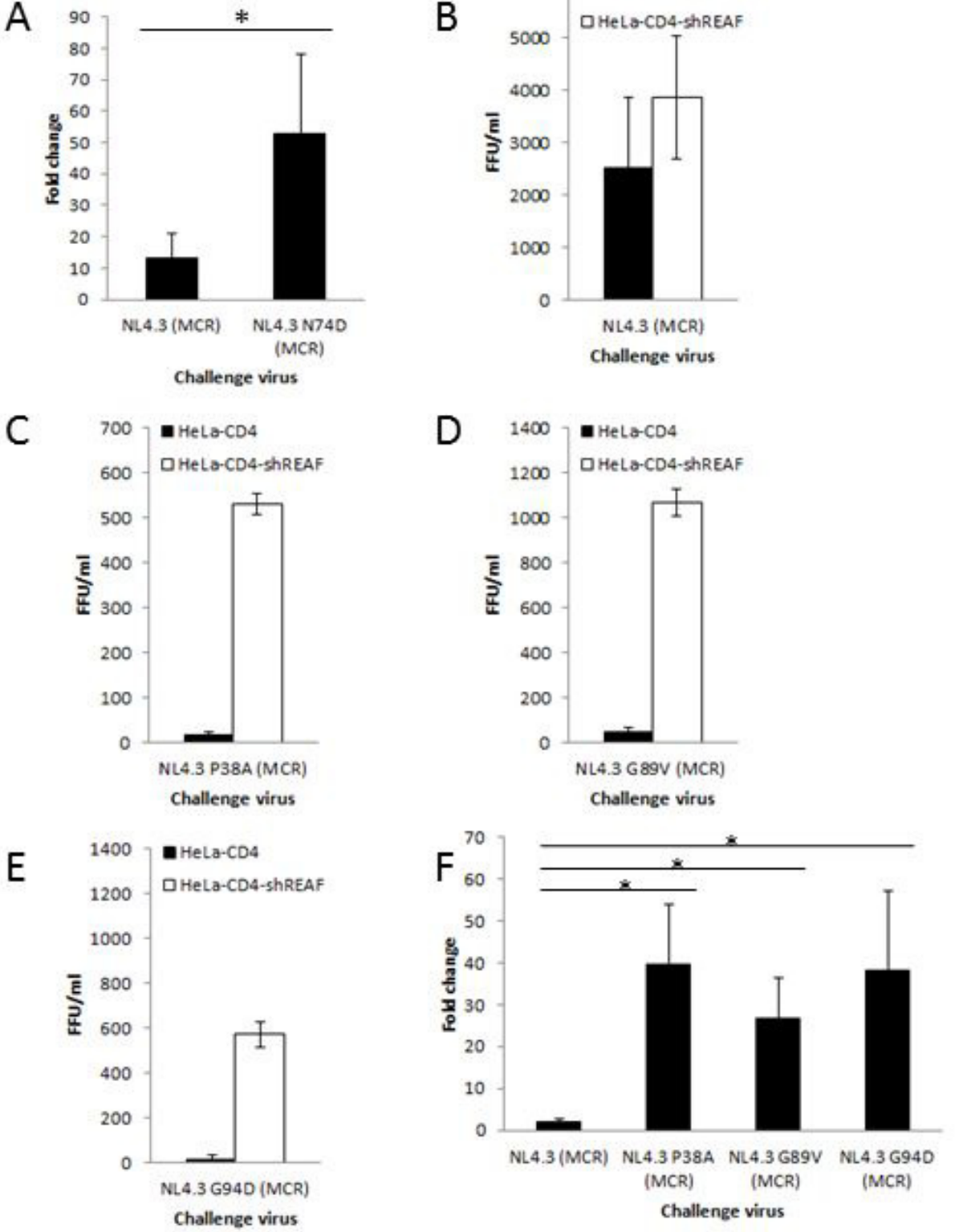
Fold inhibition
NL4.3 (VSV-G)
NL4.3 I73V (VSV-G)
NL4.3 N74D (VSV-G)
NL4.3 P38A (VSV-G)
Challenge virus

3


A
*
Fold change
NL4.3 (MCR)
NL4.3 N74D (MCR)
Challenge virus
B
HeLa-CD4
HeLa-CD4-shREAF
FFU/ml
NL4.3 (MCR)
Challenge virus
C
HeLa-CD4
HeLa-CD4-shREAF
FFU/ml
NL4.3 P38A (MCR)
Challenge virus
D
HeLa-CD4
HeLa-CD4-shREAF
FFU/ml
NL4.3 G89V (MCR)
Challenge virus
E
HeLa-CD4
HeLa-CD4-shREAF
FFU/ml
NL4.3 G94D (MCR)
Challenge virus
F
Fold change
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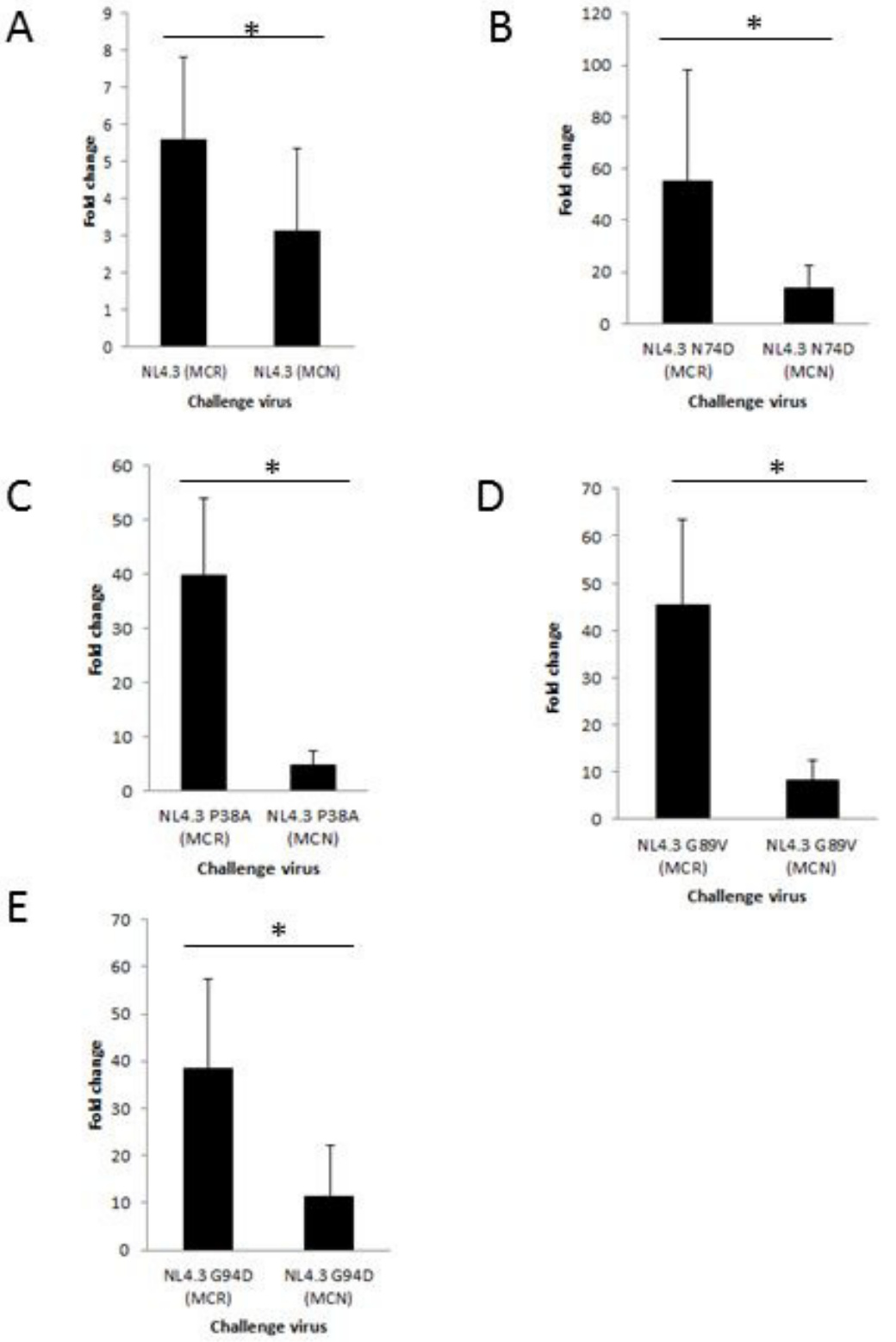
NL4.3 G94D (MCR)
Challenge virus

4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NL4.3 N74D (MCR)
NL4.3 N74D (MCN)
NL4.3 P38A (MCR)
NL4.3 P38A (MCN)
NL4.3 G89V (MCR)
NL4.3 G89V (MCN)
NL4.3 G94D (MCR)
NL4.3 G94D (MCN)
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