The HLA-C*04:01/KIR2DS4 gene combination and HLA alleles with high population frequency drive rate of HIV disease progression

Alex OLVERA[1,$,#], Susana PÉREZ-ÁLVAREZ[1,2,$,*], Javier IBARRONDO[3], Carmela GANOZA[4], Javier R. LAMA[4,5], Aldo LUCCHETTI[4], Steven CATE[6], William HILDEBRAND[6], Nicole BERNARD[7], Lupe GOMEZ[8], Jorge SANCHEZ[4,5], Christian BRANDER[1,2,9,10]

[1]IrsiCaixa AIDS Research Institute - HIVACAT, Hospital Germans Trias i Pujol, Badalona, Barcelona, Spain, [2]Universitat Autònoma de Barcelona, Barcelona, Spain [3]Department of Medicine, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, California, United States of America [4]Asociación Civil IMPACTA Salud y Educación, Lima, Peru, [5] Department of Global Health, University of Washington, Seattle, Washington, United States of America, [6]University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, United States of America, [7]Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada, [8]Departament d'Estadística i Investigació Operativa, Universitat Politècnica de Catalunya, Barcelona, Spain, [9]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain, [10]Universitat de Vic, Spain.

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[#] Address correspondence to Alex Olvera,

IrsiCaixa AIDS Research Institute,

Hospital Germans Trias i Pujol,

Crta del Canyet sn, 08916, Badalona (Barcelona), Spain.

Phone: +34 93 465 63 74 Email: aolvera@irsicaixa.es

*Present address: Biokit Research & Development, Lliçà d'Amunt, Barcelona, Spain.

$A.O. and S.P.A. contributed equally to this work.

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Abstract

OBJECTIVE: To identify HLA class I and KIR genotypes associated with different risks for HIV acquisition and HIV disease progression.

DESIGN: Cross-sectional study of a cohort of 468 high-risk individuals (246 HIV+ and 222 HIV−) from an outpatient clinic in Lima (Perú).

METHODS: The cohort was high-resolution HLA- and KIR-typed and analysed for potential differences in single allele frequencies and allele combinations between HIV+ and HIV− individuals and for associations with HIV viral load and CD4 counts in infected individuals.

RESULTS: HLA class I alleles associated with lack of viral control had a significantly higher population frequency than relatively protective alleles (p=0.0093), in line with a rare allele advantage. HLA-A*02:01 and HLA-C*04:01 were both associated with high viral loads (p=0.0313 and 0.0001 respectively) and low CD4 counts (p=0.0008 and 0.0087 respectively). Importantly, the association between HLA-C*04:01 and poor viral control was not due to its linkage disequilibrium with other HLA alleles. Rather, the co-expression of its putative KIR ligand KIR2DS4f was critically linked to elevated viral loads.

CONCLUSIONS: These results highlight the impact of population allele frequency on viral control and identify a novel association between HLA-C*04:01 in combination with KIR2DS4f and uncontrolled HIV infection. Our data further support the importance of the interplay of markers of the adaptive and innate immune system in viral control.

Keywords: HIV infection, viral load, CD4 counts, HLA class I, KIR

Introduction

In the absence of anti-retroviral treatment most HIV infected subject progress to AIDS, although the rate of disease progression varies widely between HIV (rapid) "progressors" and groups such as "elite controllers" and "long term non-progressors". In addition, relative protection from infection has been described in a number of highly-exposed, seronegative (HESN) individuals [1-3]. The mechanisms behind HIV disease progression and increased/reduced susceptibility to HIV infection remain unclear although a series of host genetic and viral factors have been associated with the different outcomes. While genes involved in innate defense mechanisms and co-receptor usage, like DC-SIGN, Cyclophilin A, TRIM5α, APOBEC3G and CCR5-Δ32 have been shown to contribute to relative resistance to HIV infection [2], polymorphisms in the human leukocyte antigen (HLA) and Killer-cell immunoglobulin-like receptor (KIR) genes have been strongly associated with better or worse viral control and rate of HIV disease progression in infected subjects [2, 4, 5].

Since the early years of the HIV pandemic, particular HLA were noted to influence infection susceptibility and HIV disease progression rates [6, 7]. In particular, HLA class I alleles A*01:01, A*74:01, C*06:02 and C*07:01 were associated with relative protection from infection, whereas A*23:01, B*07:02 and B*42:01 were associated with elevated seroconversion rates [8, 9]. Although cohort size can limit robust associations between HLA alleles and protection from HIV infection [2], individual HLA alleles, usually encoded at the highly variable HLA-B locus, have been repeatedly associated with accelerated (HLA-B*35-PX, -B*53, -B*58:02) or with slower (HLA-B*13, -B*27, -B*51, -B*57, -B*58:01, and -B*81:01) HIV disease progression. Of note, HLA-B*35 and HLA-

C*04 are consistently associated with rapid disease progression in Caucasians, but not in African Americans [10, 11], this deleterious effect has mainly been attributed to the HLA-B*35-PX alleles (HLA-B*35:02, -B*35:03 and -B*35:04) to which the HLA-C*04 allele is in strong linkage disequilibrium (LD) [11]. Furthermore, infrequent HLA-B alleles have been correlated with more favourable disease outcome, probably due to extensive HIV adaptation to the population's most frequent HLA alleles [12, 13]. Indeed, studies in HLA-B*15:03 expressing individuals showed opposite levels of viral control and selective viral adaptation depending on allele frequency in the population [14]. Similarly, in the Japanese population, HLA-B*51:01 has lost its relative protective effects over the years, explained by gradual adaptation of the virus to the local population [15-17].

Aside from effects attributed to individual HLA alleles, HLA haplotypes and combination of HLA alleles with other genes have been reported to exert additive effects on HIV control [18-23]. These reports also include genes in loci other than the commonly described HLA-B locus, highlighting the importance of HLA-C alleles and their expression levels on HIV-1 control [24-26]. This has been thought to be of special relevance for HIV-1 infection because of the described effect of the viral protein Nef on the surface expression of HLA-A and –B, but not -C proteins [27], and the crucial role of HLA-C molecules in natural killer cell (NK) function through their interaction with the NK KIR receptors. Among the latter, the functional KIR2DS4 receptor (KIR2DS4f) has been associated with elevated viral loads and increased transmission rates of HIV-1 [28, 29]. In addition, highly exposed-uninfected female sex workers seem to more frequently possess inhibitory KIR2DL2 and KIR2DL3 genes in the absence of their cognate HLA-C1 ligand or to be homozygous for KIR3DL1 in the absence of HLA-Bw4, while HIV-seropositive female sex workers carry homozygous

inhibitory KIR2DL3 with its corresponding HLA-C1 ligand [30]. On the other hand, KIR3DS1 has a protective effect in regards to HIV-acquisition, similarly to what is seen in individuals with high-expression homozygous KIR3DL1 genotypes and HLA-B*57 co-carriage [31-33]. In HIV-infected subjects, the combination of KIR3DL1 and HLA-B*57 or certain HLA-Bw4 alleles as well as the combination of KIR3DS1 with HLA-B Bw4-80I have been associated with slower progression to AIDS [34, 35].

Despite a wealth of data and association studies, the mechanisms by which protective HLA and KIR alleles and their combinations mediate beneficial effects remain unclear. In addition, many host immunogenetic studies have been focused on Caucasian or African populations but little is known about the impact of HLA and KIR gene polymorphisms on HIV control in other populations. This is the case for Peruvian cohorts that have been part of large HIV vaccine and infection prevention studies [36, 37], but for which only limited host genetics studies are available [38-40]. In the present work we studied the associations between HLA alleles and KIR genes and resistance to HIV infection or virus control and disease progression in a cohort of HIV seropositive and HESN individuals from Lima.

Material and methods

Ethics Statement

Protocols were approved by the IMPACTA Human Research Committee in Lima, Peru. All subjects provided written informed consent before enrolling into the study.

Study Cohort

For the present study a cohort of 468 individuals engaging in high-risk sexual behaviour was recruited and tested at the IMPACTA HIV outpatient clinics in Lima, Perú. Study subjects were mainly (>80%) derived from previously described cohorts of men who have sex with men (MSM) and were all recruited at two clinical sites in Lima [41]. When prompted to self-assign ethnic origin, all recruited individuals considered themselves to be of a mixed Amerindian ethnicity. Of the 468 subjects, 222 were seronegative for HIV-1 (HIV−) and 246 were infected with HIV-1 clade B (HIV+), 11 of these were under cART treatment. Seropositive individuals were estimated to be infected for at least 6 months and had reached stable viral load set point [42]. For 94 HIV-infected individuals additional viral load determinations at least 6 months apart were determined and showed overall stable viral loads, consistent with chronic stages of HIV infection. Median viral load in the cohort was 37,113 HIV copies/ml (range 50-750,000 copies/ml) and a median CD4 count of 384 cell/µl (range 170-1151 cell/µl) as described in the past [41].

HLA and KIR typing

All individuals were 4-digit typed for HLA class I alleles using in-house PCR and DNA sequence-based typing methodologies in the CLIA/ASHI accredited HLA typing laboratory at the University of Oklahoma Health Sciences Center. Sequencing analysis and HLA allele assignment was performed with Assign-SBT v3.5.1 from Conexio Genomics. Ambiguous types were resolved to 4-digits with PEL-FREEZ UNITRAY SSP, Life Technologies as described [41]. KIR genotyping using Olerup SSP® KIR Genotyping according to manufacturer's instruction was completed in a subset of 243 subjects from whom additional samples were available, including 73 HIV− and 170 HIV+ subjects,

Statistical analysis

Comparison of HLA and KIR allele frequencies in the Peruvian population was based on information from the Allele Frequency Net Database [43] (www.allelefrequencies.net). Linkage disequilibrium (LD) was calculated for HLA alleles and KIR genes using Fisher's exact test with correction for multiple comparisons. The significance of differences in the frequencies of individual HLA alleles, KIR genes, 2-locus HLA haplotypes (2HLA) and HLA alleles-KIR gene combinations (HLA-KIR) between HIV+ and HIV− groups was assessed using Fisher's exact test with correction for multiple comparisons. Viral loads and CD4 counts were compared between groups of untreated HIV+ (N=235) individuals carrying or not individual HLA alleles, KIR genes, 2HLA and HLA-KIR by Mann-Whitney test. ART-treated individuals (n=11) were excluded from analyses assessing associations of HLA alleles and KIR genes with HIV viral load and CD4 counts. Multiple comparison correction was performed using the False Discovery Rate (FDR) and *q*-value calculation. Contributions of particular HLA allele and KIR gene combinations to the observed differences in viral loads or CD4 counts were assessed using One-Way ANOVA and two-by-two comparisons using one-sided t-test. The association of the HLA allele population frequency with viral load was evaluated by calculating the individual's total cumulative frequency as the sum of the 6 HLA allele frequencies [19] and the cumulative frequency for HLA-A, -B and –C locus calculated as the sum of the population frequency of the 2 alleles in each locus. Cumulative frequencies were compared among groups of individuals with low (1st quartile), intermediate (2nd and 3rd quartile) and high (4th quartile) viral load using a One-Way ANOVA as well as two-by-two comparisons using one-sided t-test. In all cases the statistical significance threshold was set at $p<0.05$. Statistical analyses were performed using R Statistical Software (http//www.r-project.org/) and GraphPad software.

Results

HLA allele and KIR gene frequency and association with risk of HIV infection

Forty-nine HLA-A, 92 HLA-B and 33 HLA-C alleles were identified in this high-risk cohort enrolled and followed at two IMPACTA clinics in Lima, Peru. HLA allele frequencies for alleles with >1% cohort frequency are shown in Figure 1 (complete list supplementary Table S1). The most common alleles (>10% cohort frequency) for the different loci included HLA-A*02:01 (46.8%), -A*24:02 (19.4%), -A*02:11 (18.6%), -A*31:01 (10.3%); HLA-B*35:01 (12.0%), -B*51:01 (10.5%), HLA-C*04:01 (37.6%), -C*07:02 (29.3%), -C*01:02 (21.6%) and -C*03:04 (18.8%). Twenty-four HLA alleles (6 HLA-A, 10 HLA-B and 8 HLA-C) had not been previously described in Central and South American populations according to the Allele Frequency Net Database (Table S1). In addition to HLA, we also determined the KIR gene cohort frequencies (Figure 1). All framework and KIR genes determining haplotype A, except KIR2DS4, showed population frequencies of >90%, with the KIR3DP1 pseudogene being present in 100% of the tested individuals (Table S1).

The comparison of HLA alleles, KIR genes, 2HLA, and HLA-KIR frequencies between HIV+ and HIV− subjects revealed that HLA-B*40:02 was more frequent in HIV− than in HIV+ subjects (p=0.029) while HLA-B*35:43 showed the strongest association with HIV acquisition (p=0.012). Weaker trends were observed for additional HLA alleles, 2HLA and HLA-KIR combinations (Figure S1), but none of these associations remained significant when the analysis was based on a 20% FDR (q>0.2).

198

199 ***HLA and KIR association with viral load and CD4+ T cell counts***

200 We used high-resolution DNA sequence based typing (SBT) to identify class I HLA and
201 KIR alleles that corresponded to differences in HIV plasma viral load and CD4 counts,
202 focusing on HIV-1 plasma viral load as a strong independent predictor of disease
203 progression [42, 44, 45]. Overall, 5 individual HLA class I alleles were associated with
204 increased and 5 more with reduced viral loads, 5 HLA alleles were associated with lower
205 CD4 counts, and 6 alleles were associated with higher CD4 counts (Figure 2). Of these
206 alleles, A*02:01 and C*04:01 showed significant differences in both increased plasma viral
207 loads and reduced CD4 counts relative to individuals not expressing these alleles (Figure
208 2). Statistical significance was especially strong for associations between HLA-C*04:01
209 and viral load (p= 0.0001, q= 0.0096) and CD4 counts (p= 0.0087, q= 0.1679), and for
210 HLA-A*02:01 with CD4 counts (p= 0.0008, q= 0.0476). HLA–B*18:01 showed an
211 association with lower CD4 counts while subjects with HLA–B*39:14 and –B*39:13
212 showed higher CD4 counts (Figure 2), all p-values <0.05 and FDR q-values <0.2. Since
213 earlier reports have shown that combinations of HLA class I alleles can additively influence
214 viral control [19], we assessed whether similar effects could be observed in our Peruvian
215 cohort. Haplotype associations largely coincided with the single allele results in this
216 population (Figure S2); and the most frequent A*02:01-C*04:01 haplotype was associated
217 with the highest viral loads (p=0.0008 and q=0.1459), and lowest CD4 counts (p=0.0012
218 and q=0.1233). The analysis of HLA class I homozygosis did show a weak trend towards
219 higher viral loads in subjects homozygous for one or more of the three HLA class I loci
220 compared to completely heterozygous individuals (median viral load 50,816 and 34,674
221 HIV copies/ml respectively; one-tailed t-test p=0.1519, data not shown). However, this

222 analysis was limited by the relatively low number of subjects with homozygous locus (n=
223 41)
224
225 ***Rare-HLA allele Advantage***
226 HLA alleles showing significant differences in viral load or CD4 counts also showed
227 differences in their overall cohort frequency, with alleles and haplotypes associated with
228 higher viral loads or lower CD4 counts being more frequent than alleles showing lower
229 viral loads or higher CD4 counts (p= 0.0093 and p= 0.0044, Figure 2 and S2, respectively).
230 To further explore the relationship between HLA allele cohort frequencies and virus
231 control, the cumulative frequencies of the 6 HLA class I alleles was determined for each
232 individual and compared to viral load (Figure 3A). Indeed, individuals in the lowest viral
233 load quartile (range of viral load 50-12,975 HIV copies/ml plasma) showed the lowest
234 cumulative HLA frequencies while higher frequencies led increasingly to higher viral
235 loads. These effects appeared mainly driven by HLA-A and HLA-C alleles, of which
236 HLA-C showed a significant association between viral loads and cumulative frequencies in
237 a loci-specific analysis (Figure 3B). These data further substantiate that high-frequency
238 HLA alleles are associated with less effective T cell control of HIV and that rare alleles can
239 mediate a rare-allele advantage [13].
240
241 ***HLA-A*02:01 and -C*04:01 linkage disequilibrium and association with viral load.***
242 To test whether HLA alleles with the strongest association with viral control (i.e. HLA-
243 A*02:01 and HLA-C*04:01) mediated their effects independently, we carried out LD
244 analyses. Our data show that HLA-A*02:01 and HLA-C*04:01 were in LD (p=0.0030,
245 q=0.0221) with each other and that these alleles mediated an additive negative effect, with

246 individuals carrying both alleles having the highest viral loads and lowest CD4 counts
247 (Figure 4A). No other allele associated with uncontrolled infection was found in LD with
248 A*02:01 while HLA-C*04:01 was found to be in highly significant LD with three HLA-
249 B*35 subtype alleles, B*35:01, B*35:05 and B*35:09 (p= 2.40E-23, p= 4.68E-20 p=
250 2.93E-09 respectively), that were associated with lower CD4 counts (B*35:01) and high
251 viral loads (B*35:05 and B*35:09). Importantly, the associations between HLA-C*04:01
252 and high viral loads remained statistically significant even when individuals who carried
253 HLA-B*35:01, -B*35:05 or -B*35:09 alleles were excluded from the comparison (Figure
254 4B). Earlier studies in Caucasian cohorts have suggested that the HLA-C*04:01 effect on
255 disease progression was due to its LD with particular HLA-B*35-PX alleles [11]. However,
256 the HLA-B*35-PX alleles in the Peruvian cohort were not associated with disease
257 progression on their own. Although the cohort contained insufficient HLA-C*04:01
258 negative individuals expressing HLA-B*35:01, -B*35:05 or -B*35:09 alone to assess their
259 individual effects on viral load, the significantly elevated viral load in individuals
260 expressing HLA-C*04:01 in the absence of these HLA-B*35 alleles (Figure 4B), indicates
261 that the HLA-B*35-C*04 disadvantage may be largely mediated by HLA-C*04:01 in the
262 present Peruvian cohort.
263
264 ***KIR genes and HLA-C*04:01 combined association with HIV viral load.***
265 When integrating KIR polymorphisms into the analyses, the data showed a statistically
266 significantly elevated viral load for individuals not expressing KIR2DL1 or the pseudogene
267 KIR2DP1 (Table S2); however, these associations were severely limited by the small
268 number (N=4 and 3, respectively) of subjects not expressing these common KIR genotypes.
269 On the other hand, KIR2DS4f expression was associated with higher viral load with

borderline statistical significance (p=0.0437, q=0.2040) while presence of KIR2DS1 was related to higher CD4 counts (p=0.0291, q=0.1472), respectively (supplementary Table S2). Of note, there were three KIR genes known to act as ligands for HLA-C*04:01 that were associated with significant differences in median viral loads and CD4 count differences: KIR2DL1, KIR2DS1 and KIR2DS4f. Other KIR genes known to bind HLA-C*04:01 including KIR2DL2 and KIR2DL3 [46] did not show any association with viral control or CD4 counts. The KIR pseudogene KIR2DP1 was also linked to higher viral loads, but since it does not encode a functional protein this association is probably due to its strong linkage with KIR2DL1.

Given that the strongest associations with lack of HIV control were observed for KIR having the potential to use HLA-C*04:01 as a ligand, we explored their contribution to viral loads stratifying by HLA-C*04:01 expression. For both KIR2DL1 and KIR2DS1, possible associations with higher viral loads were likely mediated by HLA-C*04:01 alone, as both KIR genes in the absence of HLA-C*04:01 showed viral loads comparable to the rest of the cohort (Figure 5A). Although the different KIR2DS4 polymorphism were not strongly associated with differences in viral loads (Figure 5B), the homozygous co-expression of the functional KIR2DS4 (KIR2DS4f) gene together with HLA-C*04:01 was associated with the highest viral loads (Figure 5C). These data suggest that KIR2DS4f, but not KIR2DS4d, further enhanced HLA-C*04:01's deleterious association with HIV control.

Discussion

Although Peruvian cohorts are well represented in past and current HIV-1 vaccine trials [36, 47], little is known about the genetic heterogeneity in this population. To fill this gap in our knowledge and to identify which specific HLA and KIR genes, either individually or in combination, are associated with viral control we performed high-resolution HLA and KIR typing on a cohort from Lima, Peru. The present data essentially double the available host genetic data for this country and identify a number of HLA and KIR genes that are associated with reduced or elevated viral loads and CD4 counts [38-40]. This includes HLA alleles that have not previously been identified in Central and South American cohorts, and provides the first KIR frequency information in the Peruvian population.

A number of HLA class I and II alleles have been associated with superior or inferior control of HIV infection, measured as viral load, CD4 count, or time to progression to AIDS [4, 48, 49]. However, not all alleles show the same effects on HIV control in all populations assessed, such as the HLA-B*15:03 and B*51 alleles [14, 16], and some alleles may exert their effects at different stages of HIV infection [50]. In addition, not all studies have employed high resolution molecular typing to resolve HLA subtype differences [51, 52]. When compared to existing literature [41], relatively few individuals in the Lima cohort expressed known protective HLA class I alleles such as HLA-B*57 and B*58:01. This, along with the fact that the most common alleles (HLA-A*02:01 and HLA-C*04:01) were strongly associated with increased viral load and reduced CD4 counts, is in line with a rare-allele advantage as proposed by Trachtenberg et al. [12, 13]. This may be linked to the level of viral adaptation to the most common HLA alleles in the population [12, 14, 17, 53] and the increased chance for transmission of adapted virus and more accelerated disease progression between partly HLA matched individuals [54, 55]. Of note, associations

between allele frequencies and viral loads were observed for HLA-A and, particularly, HLA-C but not HLA-B alleles. In light of past reports attributing the bulk of the anti-viral T cell immunity to HLA-B restricted T cells, these data support the notion that at least a portion of the anti-viral T cell response does not exert effective immune control of HIV and further underlines the potentially beneficial effect of non-HLA-B restricted T cell responses on viral replication [19, 51].

The high frequency of HLA-A*02:01 and HLA-C*04:01 allowed us to assess how these alleles contribute individually or combined to HIV control. While the combined expression of HLA-A*02:01 and HLA-C*04:01 was associated with the highest viral load, our data indicate that this association was largely driven by the expression of HLA-C*04:01. HLA-C*04:01 has been previously associated with differences in time to AIDS progression in African and Caucasian cohorts depending on its LD with beneficial (HLA-B*81:01) or deleterious (HLA-B*35-PX) class I alleles [10, 11, 51]. Of note, the B*35 alleles HLA-B*35:01, -B*35:05 and –B*35:09 showed a slightly additive effect on viral load beyond that of HLA-C*04:01, but the lack of individuals bearing these alleles without co-expressing HLA-C*04:01 prevented us from drawing further conclusions. In addition, B*35:01 is a -PY allele and HLA-B*35:05 and –B*35:09 have not been classified as either PX or PY allele [56, 57] and are present at frequencies in the Peruvian cohort that are too low to establish statistically robust associations with clinical parameters of viral control.

Interestingly, all KIR genes that were associated with differences in HIV control, KIR2DS1, KIR2DL1 and KIR2DS4, are putative ligands for HLA-C*04:01 [46]. Other KIR genes whose products use HLA-C*04:01 as ligands (KIR2LD2 and KIR2LD3) or

other HLA alleles known to bind KIR2DS4 (HLA-A*11:02, HLA-C*05:01 and -C*16:01) were not associated with differences in viral control in this cohort [46, 58]. Among KIR2DS1, KIR2DL1 and KIR2DS4, the strongest association with high viral load was mediated by KIR2DS4f in individuals expressing HLA-C*04:01. KIR2DS4f has been shown to be a weak and highly restricted ligand of HLA-C*04:01 [46], capable of triggering NK clone activation; albeit this may need the presence of additional ligands [59, 60]. In contrast to the functional gene version, the truncated KIR2DS4d variant lacks the intracellular and transmembrane domains required for cell surface expression and effective signal transduction [46, 61, 62]. Individuals in the Lima cohort bearing this KIR2DS4d variant in combination with HLA-C*04:01 did not show the detrimental associations with viral load that was seen for the combination of HLA-C*04:01 with the functional KIR2DS4f gene. Expression of KIR2DS4f alleles has been linked to higher viral load and heterosexual transmission in a cohort from Zambia, although this association was independent of the presence of HLA-C*04:01 [29]. In addition, KIR2DS4f has been found to be more frequent in mothers that transmitted HIV-1 intrapartum to their KIR2DS4f negative children, compared to non-transmitting mothers [28]. Related to this, recent data show that the inhibitory KIR2DL2 can potentiate the detrimental effect of HLA-B*54 on human T lymphotropic virus type 1 viral load [63]. This is in line with the Peruvian data where the activating KIR2DS4f protein appears to be enhancing the negative effects of HLA-C*04:01.

In summary, the present study identifies the common HLA alleles HLA-A*02:01 and, especially HLA-C*04:01 as being related to lack of viral control in a MSM cohort in Lima,

Peru. The data demonstrate that the deleterious effect of HLA-C*04:01 on viral control is independent of other HLA alleles, but dependent on the co-expression of an activating KIR gene (KIR2DS4f). These results implicate HLA-KIR interactions in the in vivo control of chronic HIV infection and may also help explain reported viral adaptations to KIR genotypes [64].

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Conflicts of interest

There are no conflicts of interest.

Author's contributions

Alex Olvera performed ANOVA and t-test analysis of the data, multiple variable analysis was performed with Susana Pérez-Álvarez under supervision of Lupe Gomez. Alex Olvera together with Javier Ibarrondo, Nicole Bernard and Christian Brander drafted the first version of the manuscript. Steve Cate and William Hildebrand performed HLA and KIR typing. Aldo Lucchetti, Javier Lama, Carmela Ganoza and Jorge Sanchez were in charge of cohort recruitment, management and clinical data collection.

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596 **Figure legends.**

597

598 **Figure 1. HLA allele and KIR gene population frequency**. HLA-A, -B and –C allele,
599 KIR gene and 2-locus HLA haplotype (2HLA) population frequencies. High-resolution
600 HLA and KIR typing was conducted on 246 HIV infected (HIV+) and 222 HIV non-
601 infected (HIV-) subjects in a cohort in Lima, Peru, and their population frequencies
602 calculated. Only HLA alleles with frequencies higher than 1% and 2HLA with frequencies
603 higher than 5% are shown. KIR framework genes are highlighted by bold type red letters
604 while genes determining the group A haplotype are highlighted by bold black letters.

605

606 **Figure 2. Viral load and CD4 count association with HLA.** Viral load and CD4 counts
607 were compared between HIV positive (HIV+) subjects carrying or not a particular HLA
608 allele. Only HLA alleles showing significant (p<0.05, Mann-Whitney test) differences in
609 viral load or CD4 counts are shown. For each HLA allele, the distribution of CD4 counts is
610 shown in the left panel, viral loads in the middle panel and their cohort frequencies (%) in
611 the right hand panel. Alleles are ordered by median viral load in subjects expressing each
612 allele. Alleles with statistically significant differences (p<0.05) are highlighted by red box-
613 plot if they were associated with higher viral loads or lower CD4 counts and with green
614 box-plots if they were associated with either lower viral loads or higher CD4 counts. Boxes
615 indicate the median, 25th and 75th quartile and whiskers the upper and lower range limits.
616 Alleles with p-values below 0.01 are indicated by ** and with p-values below 0.001 by
617 ***. HIV+ individuals under treatment (n=11) were excluded from this analysis. The
618 median CD4 counts and viral load of the entire HIV+ cohort are indicated by the vertical

dashed lines in the left and middle panels. Median population frequencies were compared between alleles showing significantly higher or lower median viral loads or CD4 counts.

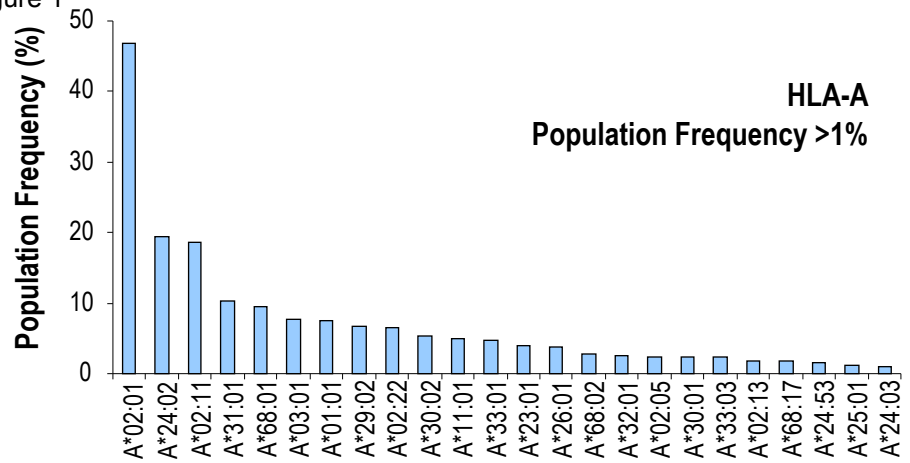
Figure 3. **Comparison of cumulative HLA cohort frequency (%) with median viral loads**. A) The total cumulative cohort frequency of all 6 HLA class I alleles was determined for each individual and compared between viral load quartiles. B) Cumulative cohort frequencies were broken down by HLA-A, -B and –C loci. The range of the viral load quartiles is indicated below the quartile number. One-way ANOVA was performed and median cumulative allele frequencies for individuals with low (first quartile), intermediate (second and third quartile) or high (fourth quartile) viral loads were compared by two-by-two comparisons (t-test). *p*-values > 0.05 are not indicated, between 0.05 and 0.01 are indicated by *, between 0.01 and 0.001 by ** and below 0.001 by ***

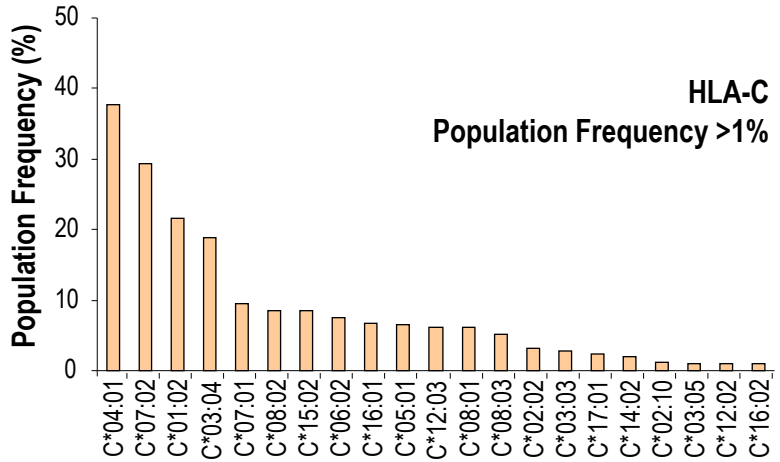
Figure 4. Effect of HLA-C*04:01 linkage disequilibrium with other HLA alleles. A. Viral loads (circles) and CD4 counts (squares) in individuals carrying HLA-A*02:01 with HLA-C*04:01 compared to those in individuals carrying one or none of these alleles. **B.** Viral loads (circles) and CD4 counts (squares) in individuals carrying B*35:01, B*35:05 or B*35:09 with C*04:01 compared to those in individuals carrying one or none of these alleles. Median population values are indicated by a dotted line. One-way ANOVA and two-by-two comparisons using t-tests were performed, *p*-values of <0.05 are indicated by *, <0.01 by ** and <0.001 by ***.

Figure 5. HLA-C*04:01 and its putative KIR ligands combined effect on the viral load. A. Viral loads in individuals carrying type 1 KIR genes 2DL1 and 2DS1 together

643 with HLA-C*04:01 compared with those in individuals carrying one or none of these
644 gene/alleles. **B.** Viral loads in individuals homozygous or heterozygous for either the full
645 length KIR2DS4 variant (2DS4f) or its deleted version (2DS4d). **C**. Viral loads in HLA-
646 C*04:01 positive and negative individuals homozygous or heterozygous for 2DS4f and
647 2DS4d. In all figures the whole cohort median viral load is indicated by a dotted line. One-
648 way ANOVA and two-by-two comparisons using t-tests were performed, *p*-values of <0.05
649 are indicated by *, <0.01 by ** and <0.001 by ***.

Figure 1


HLA-A
Population Frequency >1%
Population Frequency (%)
0
10
20
30
40
50
A*02:01
A*24:02
A*02:11
A*31:01
A*68:01
A*03:01
A*01:01
A*29:02
A*02:22
A*30:02
A*11:01
A*33:01
A*23:01
A*26:01
A*68:02
A*32:01
A*02:05
A*30:01
A*33:03
A*02:13
A*68:17
A*24:53
A*25:01
A*24:03


HLA-C
Population Frequency >1%
Population Frequency (%)
0
10
20
30
40
50
C*04:01
C*07:02
C*01:02
C*03:04
C*07:01
C*08:02
C*15:02
C*06:02
C*16:01
C*05:01
C*12:03
C*08:01
C*08:03
C*02:02
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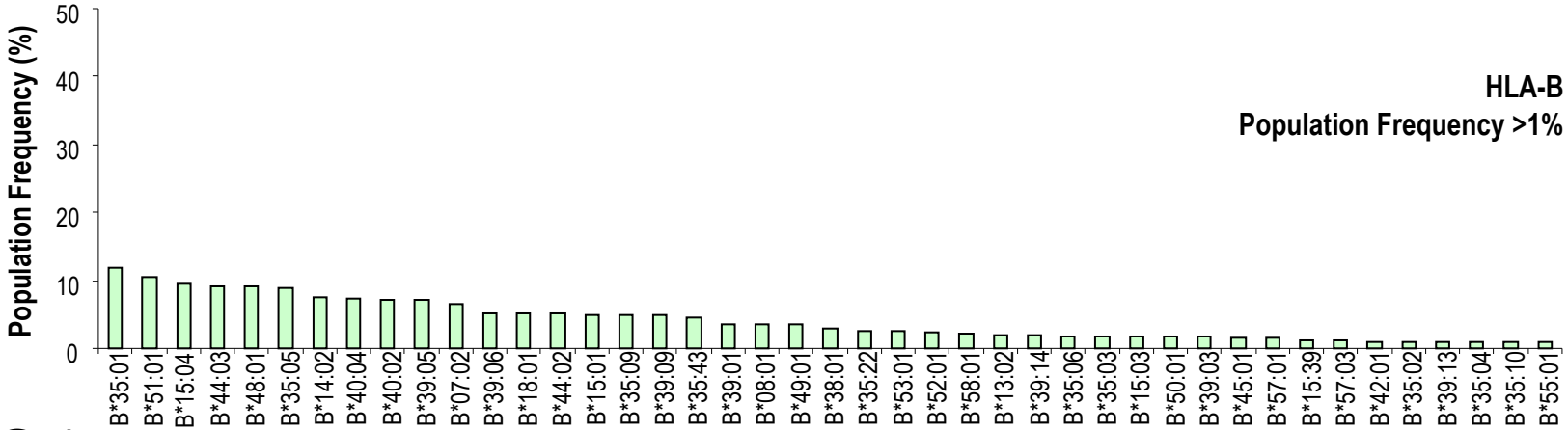
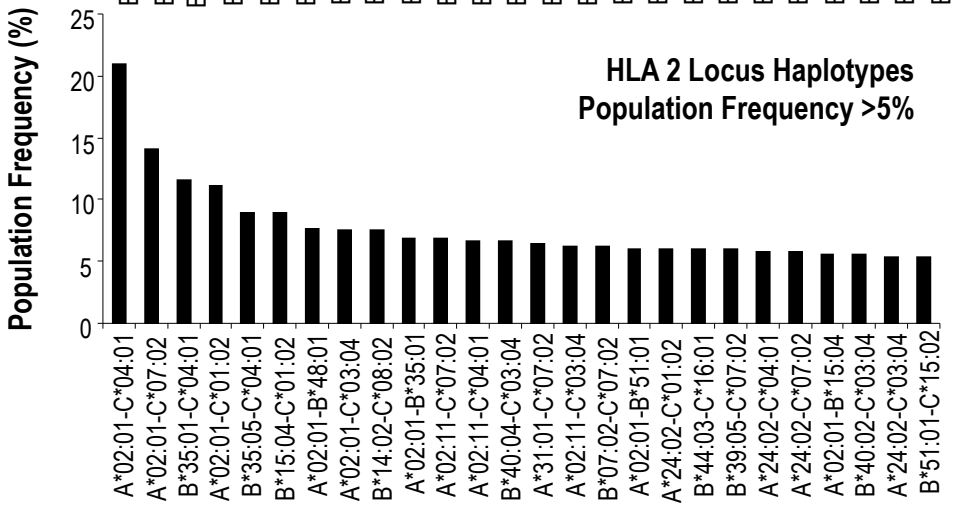
C*17:01
C*14:02
C*02:10
C*03:05
C*12:02
C*16:02


HLA-B
Population Frequency >1%
Population Frequency (%)
0
10
20
30
40
50
B*35:01
B*51:01
B*15:04
B*44:03
B*48:01
B*35:05
B*14:02
B*40:04
B*40:02
B*39:05
B*07:02
B*39:06
B*18:01
B*44:02
B*15:01
B*35:09
B*39:09
B*35:43
B*39:01
B*08:01
B*49:01
B*38:01
B*35:22
B*53:01
B*52:01
B*58:01
B*13:02
B*39:14
B*35:06
B*35:03
B*15:03
B*50:01
B*39:03
B*45:01
B*57:01
B*15:39
B*57:03
B*42:01
B*35:02
B*39:13
B*35:04
B*35:10
B*55:01


HLA 2 Locus Haplotypes
Population Frequency >5%
Population Frequency (%)
0
5
10
15
20
25
A*02:01-C*04:01
A*02:01-C*07:02
B*35:01-C*04:01
A*02:01-C*01:02
B*35:05-C*04:01
B*15:04-C*01:02
A*02:01-B*48:01
A*02:01-C*03:04
B*14:02-C*08:02
A*02:01-B*35:01
A*02:11-C*07:02
A*02:11-C*04:01
B*40:04-C*03:04
A*31:01-C*07:02
A*02:11-C*03:04
B*07:02-C*07:02
A*02:01-B*51:01
A*24:02-C*01:02
B*44:03-C*16:01
B*39:05-C*07:02
A*24:02-C*04:01
A*24:02-C*07:02
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A*24:02-C*03:04
B*51:01-C*15:02

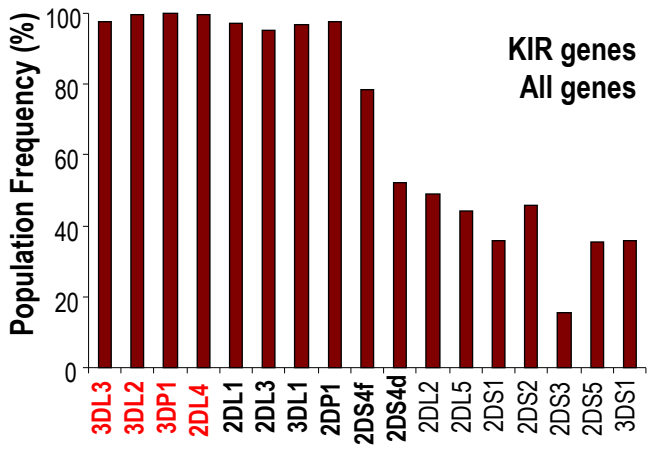

KIR genes
All genes
Population Frequency (%)
0
20
40
60
80
100
3DL3
3DL2
3DP1
2DL4
2DL1
2DL3
3DL1
2DP1
2DS4f
2DS4d
2DL2
2DL5
2DS1
2DS2
2DS3
2DS5
3DS1

Figure 2

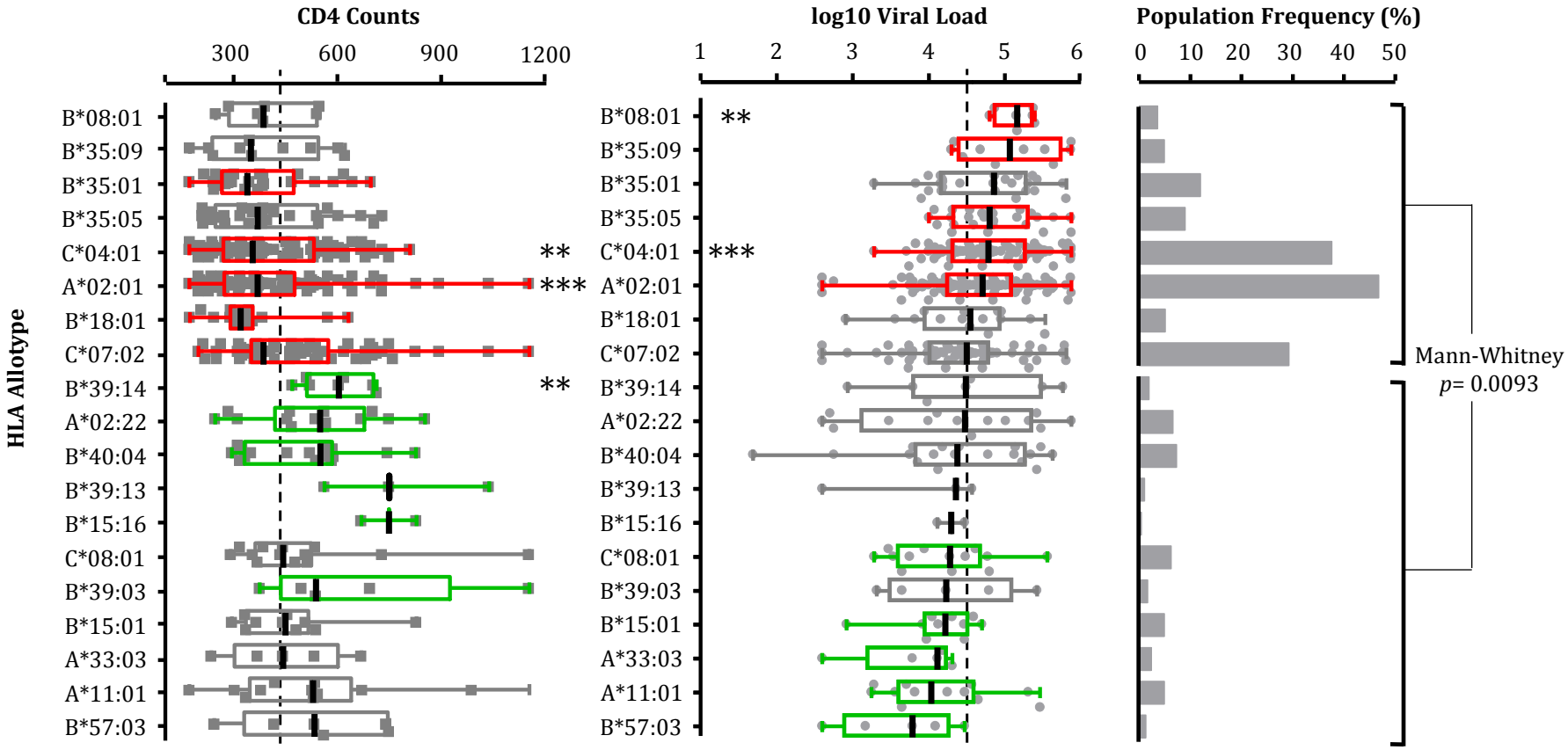

CD4 Counts
300
600
900
1200
log10 Viral Load
1
2
3
4
5
6
Population Frequency (%)
0
10
20
30
40
50
HLA Allotype
B*08:01
B*35:09
B*35:01
B*35:05
C*04:01
A*02:01
B*18:01
C*07:02
B*39:14
A*02:22
B*40:04
B*39:13
B*15:16
C*08:01
B*39:03
B*15:01
A*33:03
A*11:01
B*57:03
**
***
**
**
***
Mann-Whitney
p= 0.0093

Figure 3

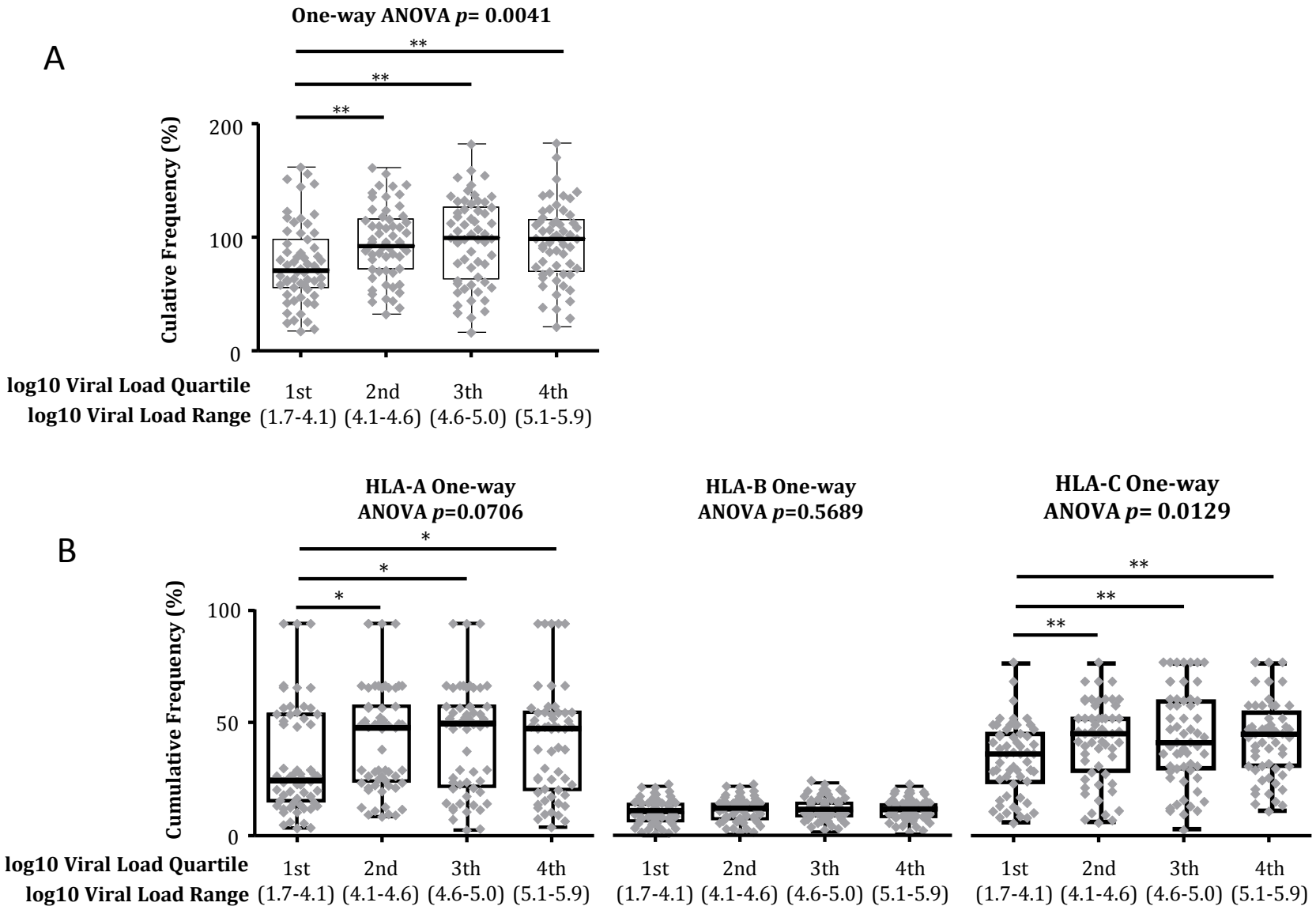

A
One-way ANOVA p= 0.0041
**
**
**
Culative Frequency (%)
200
100
0
log10 Viral Load Quartile
log10 Viral Load Range
1st (1.7-4.1)
2nd (4.1-4.6)
3th (4.6-5.0)
4th (5.1-5.9)
B
HLA-A One-way ANOVA p=0.0706
HLA-B One-way ANOVA p=0.5689
HLA-C One-way ANOVA p= 0.0129
*
*
*
**
**
**
Cumulative Frequency (%)
100
50
0
log10 Viral Load Quartile
log10 Viral Load Range
1st (1.7-4.1)
2nd (4.1-4.6)
3th (4.6-5.0)
4th (5.1-5.9)

Figure 4

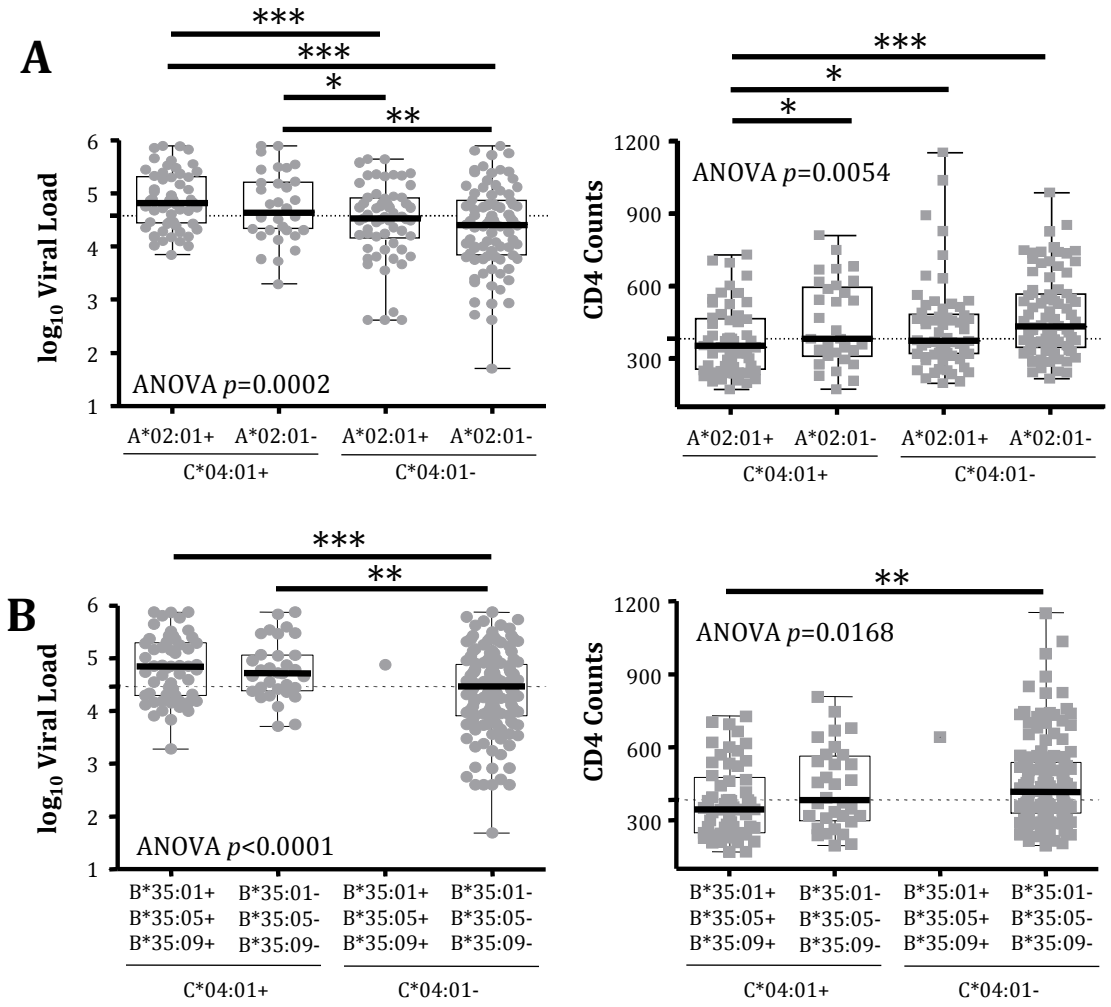
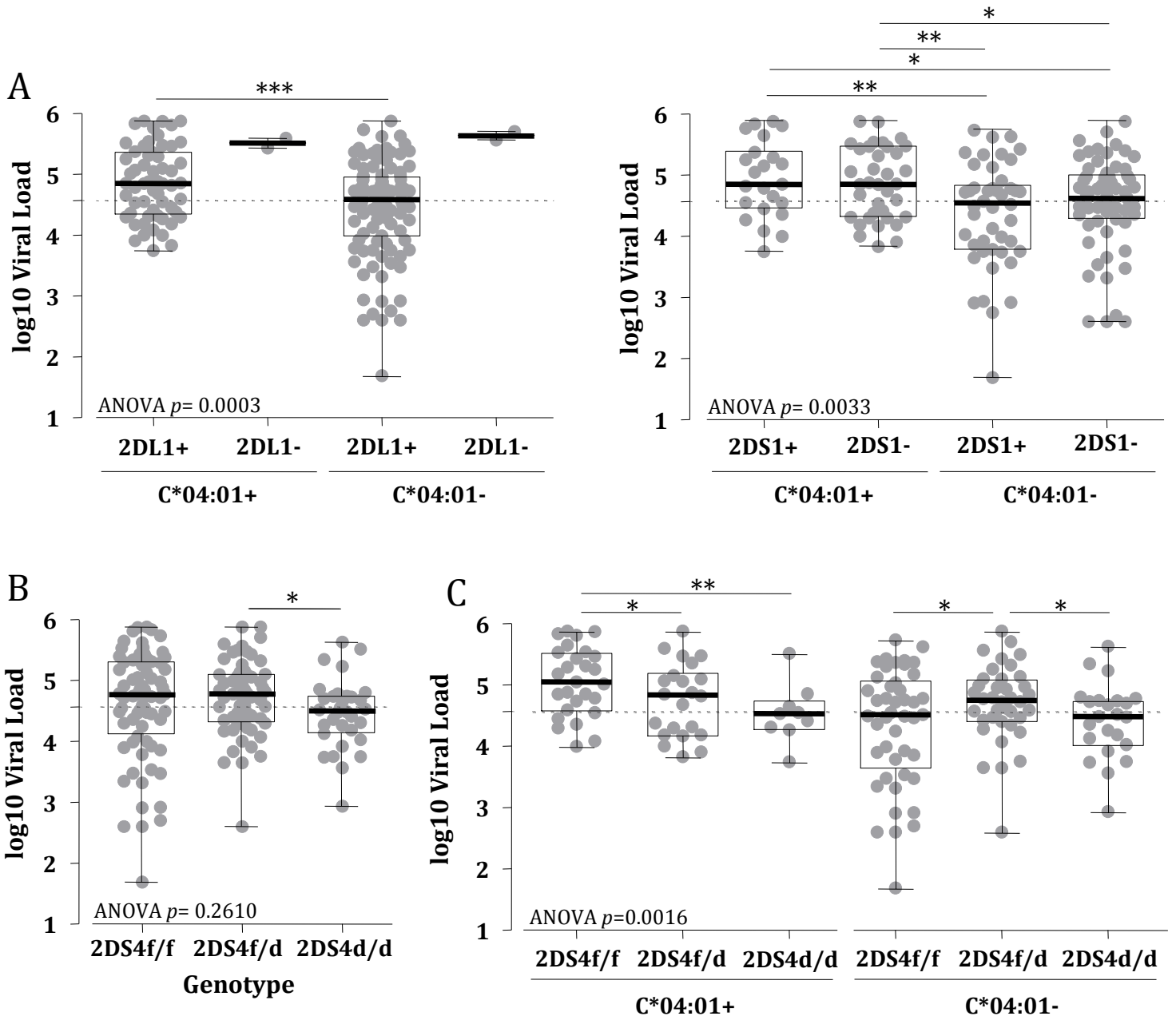

A
***
***
*
**
log10 Viral Load
ANOVA p=0.0002
A*02:01+ A*02:01- A*02:01+ A*02:01-
C*04:01+ C*04:01-
***
*
*
CD4 Counts
ANOVA p=0.0054
A*02:01+ A*02:01- A*02:01+ A*02:01-
C*04:01+ C*04:01-
B
***
**
log10 Viral Load
ANOVA p<0.0001
B*35:01+ B*35:01- B*35:01+ B*35:01-
B*35:05+ B*35:05- B*35:05+ B*35:05-
B*35:09+ B*35:09- B*35:09+ B*35:09-
C*04:01+ C*04:01-
**
CD4 Counts
ANOVA p=0.0168
B*35:01+ B*35:01- B*35:01+ B*35:01-
B*35:05+ B*35:05- B*35:05+ B*35:05-
B*35:09+ B*35:09- B*35:09+ B*35:09-
C*04:01+ C*04:01-

Figure 5


A
log10 Viral Load
ANOVA p= 0.0003
2DL1+
2DL1-
2DL1+
2DL1-
C*04:01+
C*04:01-
ANOVA p= 0.0033
2DS1+
2DS1-
2DS1+
2DS1-
C*04:01+
C*04:01-
B
ANOVA p= 0.2610
2DS4f/f
2DS4f/d
2DS4d/d
Genotype
C
ANOVA p=0.0016
2DS4f/f
2DS4f/d
2DS4d/d
2DS4f/f
2DS4f/d
2DS4d/d
C*04:01+
C*04:01-