Inhibitory Killer Immunoglobulin-like receptors to self HLA-B and HLA-C ligands contribute differentially to Natural Killer cell functional potential in HIV infected Slow Progressors

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1. ABSTRACT

Inhibitory Killer Immunoglobulin-like Receptors (iKIR) interact with their ligands, HLA molecules, to license Natural Killer (NK) cells for functional competence. Previous studies stimulating peripheral blood mononuclear cells (PBMCs) with the HLA-devoid K562 cell line revealed that NK cells from individuals with an iKIR encoded by the KIR3DL1 locus with self HLA-Bw4 as their ligands, had increased frequencies of tri-functional NK cells that expressed the degranulation marker CD107a and secreted Interferon-γ and Tumor Necrosis Factor-α than those from individuals who were homozygous for HLA-Bw6 alleles, which are not ligands for these iKIR. To assess the effect of other iKIR to self-HLA (S-iKIR) on the NK cell response, we compared HIV-infected slow progressors (SP) carrying S-iKIR to HLA-C alleles with or without S-iKIR to HLA-Bw4. We show that S-iKIR to HLA-B and C alleles differ in their contribution to NK cell functional potential in HIV-infected SP upon stimulation with K562 targets.
Natural Killer (NK) cells are important in innate immune defenses to transformed and virus infected cells [20]. NK cell activation is governed by the integration of signals transmitted through a stochastically expressed repertoire of germ-line encoded activating and inhibitory receptors, which trigger cytotoxicity and cytokine/chemokine secretion without prior exposure to antigen. Dominant control of NK cell activation is mediated by inhibitory NK cell receptors (iNKR) that bind to major histocompatibility complex (MHC) class I (HLA) molecules as markers of self on normal cells [9]. The most widely studied HLA-specific iNKR in humans are encoded by the polygenic and polymorphic Killer Immunoglobulin-like Receptor (KIR) gene family that includes up to 17 KIR genes or pseudo-genes [16].

NK cells gain functional competence during development through an HLA-dependent process termed licensing [2;13]. Licensing ensures that tolerance to self is maintained by endowing NK cells that express inhibitory KIR (iKIR) to self-HLA (S-iKIR) with functional competency, whereas NK cells that lack S-iKIRs remain unlicensed and hyporesponsive [2;13]. Experiments using single MHC class I transgenic mice demonstrated a crucial role for these alleles in NK licensing by showing that the responsiveness of mature NK cells varied according to the MHC class I environment [7;11]. Although the molecular mechanisms underlying the interactions between MHC class I and iNKR that confer NK cell responsiveness remain incompletely defined, the immunoreceptor tyrosine-based inhibitory motif (ITIM) of the iNKR is required [13].

Several models have been proposed to explain how MHC class I molecules deliver educational signals to NK cells. The rheostat model of NK licensing postulates
that NK cell education is a quantitative and dynamic process whereby the strength of the signals delivered by iKIR following engagement with MHC class I molecules is proportional to a threshold of activation that is set in the NK cells [5]. Different degrees of inhibitory and activating receptor input quantitatively tune the activation pathways of the NK cell and translate into corresponding levels of responsiveness [6;11;23;24].

Epidemiological studies have linked the carriage of certain KIR3DL1/HLA-B genotypes with slower time to AIDS and lower viral load (VL) [17]. KIR3DL1 is one of the most polymorphic loci among the KIR region genes, encoding both activating (KIR3DS1) and inhibitory (KIR3DL1) receptors [16]. A subset of HLA-B alleles, known as Bw4, act as ligands for KIR3DL1 NK receptors [8]. Bw4 alleles differ from the remaining Bw6 alleles, which do not interact with KIR3DL1, at amino acids 77 to 83 of the HLA-B heavy chain [22]. Compared to Bw6 homozygotes (hmz), the KIR3DL1/HLA-B genotype most strongly associated with slower time to AIDS and VL control is the KIR3DL1*h/*y and HLA-B*57 combined genotype (*h/*y+B*57) [17]. The KIR3DL1*h/*y genotype is defined by having no low expression alleles [17]. Furthermore, individuals who carry the *h/*y+B*57 genotype have higher percentages of NK cells able to express the degranulation marker CD107a and secrete both Interferon-γ (IFN-γ) and Tumor Necrosis Factor-α (TNF-α) in response to stimulation with the HLA-devoid K562 cell line when compared to NK cells from individuals who carry either KIR3DL1*h/*y or HLA-B*57 alone [4]. The fact that NK cells from individuals carrying *h/*y+B*57 demonstrate higher functionality when stimulated with HLA-devoid target cells may explain the slower HIV disease course and superior VL control observed in HIV-infected slow progressors (SP) [12]. However, in a setting where the HLA-devoid
K562 cell line used to stimulate NK cells, the absence of both HLA-C and HLA-B molecules abrogates inhibitory signals mediated through KIR3DL1, KIR2DL1 and/or KIR2DL2/3 receptors. Therefore we questioned whether NK functional heterogeneity observed among KIR3DL1 hoz can be attributed to iNKR other than KIR3DL1.

To address whether carriage of S-iKIR to HLA-B and C differentially contributes to NK cell function, we compared the frequencies of mono-functional, bi-functional and tri-functional NK cells from SP categorized according to the number and type of their S-iKIR. We report here that K562 stimulated NK cells from SP with S-iKIR to HLA-Bw4 exhibit a higher frequency of poly-functional NK cells compared to those from subjects without this S-iKIR. NK cells from subjects with S-iKIR to HLA-C alone have a missing self response skewed towards a narrower functional profile characterized by secretion of IFN-γ alone, expression of CD107a alone or secretion of both IFN-γ and TNF-α.
3. MATERIAL AND METHODS

3.1 Study population

The study population included 46 HIV-infected SP. Thirty-four were recruited from the Canadian Cohort of HIV Infected Slow Progressors and 11 were from a cohort followed at the National Institutes of Allergy and Infectious Diseases [19]. Informed consent was obtained from all study participants and research adhered to the ethical guidelines of the authors’ institutions. We defined SP as HIV-infected subjects who maintained absolute CD4 counts above 400 cells/mm³ for more than 7 years, Elite Controllers (EC) followed for at least 1 year with VL <50 copies/ml of plasma and Viral Controllers (VC) followed for at least 1 year with VL <3000 copies/ml of plasma. All were treatment naïve. Age, CD4 counts, log₁₀ VL and duration of infection for SP with 1, 2 or 3 S-iKIR are shown in Table 1. No between-group differences in age, CD4 count, log₁₀ VL or duration of infection was noted (not shown).

3.2 MHC and KIR genotyping

Genomic DNA was extracted from PBMC or EBV-transformed cells using a QIAamp DNA blood kit (QIAGEN, Inc., Mississauga, Ontario, Canada). All subjects were HLA typed as previously described (4). KIR3DS/L1 genotyping was performed by PCR with 2 sets of sequence-specific primers (PCR-SSP) as previously described [4]. Only KIR3DL1 homozygotes were included in this study to minimize the effect carriage of KIR3DS1 may have on NK function. The presence of alleles encoded by the KIR2DL1 and KIR2DL2/3 loci was determined by PCR amplification using 2 sets of primers specific for KIR2DL1, KIR2DL2 and KIR2DL3 allele sets [15]. For the purposes of this study the
following iKIR-HLA receptor ligand pairs were considered: 1) KIR2DL1 with HLA-C2
2) KIR2DL2/3 with HLA-C1 alleles and 3) KIR3DL1 with HLA-Bw4 alleles. The HLA-
C1 allele subset has an asparagine at position 80 and includes HLA-Cw1, Cw3, Cw7,
Cw8, Cw12, Cw14 and Cw1601. The HLA-C2 allele subset has a lysine at position 80
and includes HLA-Cw2, Cw4, Cw5, Cw6, Cw15, Cw1602, Cw17 and Cw18.

3.3 Cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient
centrifugation (Ficoll-Paque Pharmacia Upsala, Sweden) from whole blood obtained by
venipuncture into tubes containing EDTA anti-coagulant or by leukaphoresis as
previously described [12]. Cells were cryopreserved in 10% DMSO (Sigma-Aldrich, St.
Louis, MO) with 90% fetal bovine serum (FBS, Wisent, St. Bruno, Quebec, Canada).

3.4 Assessment of NK cell functional potential

3.4.1 NK cell activation

The functional potential of 46 SP stimulated with HLA-devoid K562 cells was
assessed as previously described [4]. Briefly, cryopreserved PBMC were thawed and
resuspended at 10^6 cells/ml in RPMI 1640 that contained 10% FBS, 2mM L-glutamine,
50 IU penicillin and 50µg/ml streptomycin (cRPMI) (all from Wisent). Brefeldin A
(5µg/ml, Sigma-Aldrich), Monesin (6µg/ml, Golgi-Stop; BD Biosciences, Mississauga,
Ontario, Canada) and anti-CD107a–FITC mAb (BD Biosciences) were added to the cells.

PBMC (10^6 per condition) were stimulated with HLA-devoid K562 cells
(American Type Culture Collection Manassas, VA) at a PBMC: target ratio was 5:1 for 6
hours at 37°C in a humidified 5% CO₂ incubator. Included in all experiments were conditions where PBMC were stimulated with medium alone as a negative control and with 1.25µg/ml phorbol 12-myristate 13-acetate (PMA); 0.25µg/ml ionomycin, (Sigma Aldrich) as a positive control. Only data from cells that responded to the PMA; ionomycin positive control stimulus, were included in analyses.

3.4.2 NK cell staining for phenotype and function

After stimulation, cells were stained for viability using the Aqua LIVE/DEAD® fixable dead cell stain kit (Invitrogen, Burlington, Ontario, Canada). Live PBMC were stained for cell surface markers with anti-CD56-APC, anti-CD16-Pacific Blue (BD Biosciences), and anti-CD3-ECD (Beckman Coulter, Mississauga, Ontario, Canada) for 30 min. After washing with phosphate buffered saline (PBS) containing 1% FBS (Wisent) and 0.1% sodium azide (Sigma Aldrich), cells were fixed and permeabilized using the Fix and Perm Kit (Invitrogen) and stained for intracellular cytokines using anti-IFN-γ-Alexa 700 and anti-TNF-α-PE-Cy7 mAbs (BD Biosciences). Cells were washed and fixed with 1% paraformaldehyde (Fisher Scientific, Ottawa Ontario, Canada) and stored in the dark at 4°C until acquisition.

3.5 Flow cytometry analysis

Between 400,000 and 500,000 events were acquired on an LSRII flow cytometer (BD Biosciences). Data analysis was performed using FlowJo software version 8.7.1 (Tree Star, San Carlos, CA). The functional profiles of NK cells stimulated with K562 were determined using the gating strategy described previously (12). NK cells were
defined as CD3–CD56+/−CD16+/−. Boolean gating was used to identify seven NK cell functional profiles, i.e. tri-functional NK cells (CD107a+ IFN-γ+ TNF-α+), bi-functional NK cells (any combination of two of these functions) and mono-functional NK cells (any single one of these functions). The negative control (medium alone) value for each of these functional subsets was subtracted from the corresponding K562 stimulated functional subset response to correct for background.

3.6 Statistical analysis

GraphPad Instat 3.05 and GraphPad Prism 4.01 (GraphPad Software, San Diego CA) were used for statistical analyses and graphical presentations. A Mann Whitney U test or a Kruskal-Wallis test with Dunn’s post-test comparisons were used to test the significance of between-group differences in NK functional subsets based on S-iKIR combinations and in log10VL for comparisons of 2 and more than 2 groups, respectively. P-values <0.05 were considered significant.
4. RESULTS

4.1 Differential contribution of carriage of S-iKIR to HLA-B and HLA-C to NK cell responses to missing self.

*KIR2DL1* is carried by over 99% of Caucasians and by 45 of the 46 individuals in our study population [10] (Table 1). *KIR2DL2* and *KIR2DL3* are alleles at the *KIR2DL2/3* locus, which is carried by all KIR region haplotypes and by all the individuals in our study population [10]. All study subjects carried 1 or 2 *KIR2D-HLA-C* S-iKIR combinations depending on whether they were *HLA-C1* or *HLA-C2* homozygous versus *HLA-C1/C2* heterozygous. Therefore, all *HLA-Bw6* hmz carry either 1 or 2 S-iKIR while *HLA-Bw4* positive subjects carry 2 or 3 S-iKIR. If these iNKR are co-expressed with their HLA ligands, developing NK cells have the potential to be licensed through this interaction for subsequent functional competence [13]. In a setting where K562 cells are used to stimulate NK cells, the absence of both HLA-C and HLA-B on this cell line abrogates inhibitory signals mediated through KIR3DL1, KIR2DL1 and KIR2DL2/3 receptors.

Figure 1 shows results for the frequency of K562 stimulated tri-functional NK cells from 46 *KIR3DL1* hmz SP categorized into groups according to the number and type of S-iKIR they carried. Group 1 included 11 Bw6 hmz who carried only one S-iKIR to either HLA-C1 or C2. Group 2 included 4 Bw6 hmz who carried two S-iKIR, i.e. 2 *KIR2D* genes in combination with both an HLA-C1 and C2 ligand. Group 3 included 17 *HLA-Bw4* positive SP with 2 S-iKIR, i.e. a *KIR3DL1-HLA-Bw4* and 1 *KIR2D-HLA-C* combination. Group 4 included 14 SP with 3 S-iKIR, i.e. a *KIR3DL1-Bw4* and 2 *KIR2D-*
HLA-C combinations. No significant between-group differences in the percent of K562 stimulated tri-functional NK cells were observed between Groups 1 and 2 or between Groups 3 and 4 (p>0.05, Dunn’s post-test comparisons) indicating that the carriage of 1 versus 2 KIR2D-HLA-C genetic combinations does not influence frequencies of tri-functional NK cells. On the other hand, when NK cells from SP with the same number of S-iKIR (Groups 2 and 3) were compared, those from Group 3 subjects (who carried a KIR3DL1/HLA-Bw4 combination) had significantly higher frequencies of tri-functional NK cells compared to those from Group 2 subjects (who carried 2 KIR2D-HLA-C combinations) (p<0.05, Dunn’s post-test comparisons). The frequency of tri-functional NK cells was significantly higher in SP from Groups 3 and 4 combined (who carried a KIR3DL1-HLA-Bw4 combination) versus those from Groups 1 and 2 combined (who do not carry KIR3DL1-HLA-Bw4) (p<0.05, Mann-Whitney test). Together these results suggest that the carriage of a KIR3DL1-HLA-Bw4 genotype contributes to the level of NK cell tri-functional potential significantly more than carriage of KIR2D-HLA-C genotypes.

Next, we examined whether this pattern of between-group differences in NK tri-functional potential was also observed for other NK functional subsets. The frequencies of the 3 bi-functional NK cells are shown in Figures 2 A-C. Significant between-group differences were detected for the frequency of NK cells that were CD107a^+IFN-γ^+ and IFN-γ^+TNF-α^+ (p<0.05, Kruskal-Wallis test). No significant differences were seen between NK cells from Groups 1 and 2 or between Groups 3 and 4 for any of the 3 K562-stimulated NK bi-functional subsets.
No between-group differences were observed for the frequency of NK cells with mono-functional CD107a+, IFN-γ+ and TNFα+ responses to K562 stimulation (p>0.05, Kruskal-Wallis test) (Figure 3). If the results are analyzed in a different manner by pooling together subjects in Groups 1 and 2 (with no S-iKIR to HLA-Bw4) and those in Groups 3 and 4 (with an S-iKIR to HLA-Bw4) and comparing these 2 larger groups, NK cells from subjects without a KIR3DL1-HLA-Bw4 combination had significantly lower frequencies of NK cells in all functional subsets tested except for those characterized by CD107a+TNF-α+ and TNF-α+ functional patterns (p<0.05 for all comparisons, Mann-Whitney test) (Figure 2 and 3).

Previous studies have reported a dis-regulation in NK cell function and subset distribution with increasing levels of viremia in HIV-infected subjects [1]. Our study population included some SP with detectable VL. We therefore compared between-group VL levels to address whether differences in NK cell function could be accounted for by differences in VL and found none (p>0.05; Kruskal-Wallis test, Figure 4). As well, no significant differences were detected in the proportion of EC with VL below the level of detection by standard assays between groups (p>0.05; chi-square test) (data not shown). In summary these results suggested that between-group differences in NK functional subset frequency were not driven by differences in log_{10} VL.
5. DISCUSSION

In this report we stimulated NK cells from HIV-infected SP with the HLA-devoid K562 cell line and compared the frequency of NK functional subsets characterized by all possible permutations of IFN-γ and TNF-α secretion and CD107a expression. We showed that the frequency of tri-functional, bi-functional IFN-γ⁺CD107a⁺ and IFN-γ⁺TNF-α⁺, and mono-functional IFN-γ⁺ and CD107a⁺ NK functional subsets differed depending on whether the NK cells originated from individuals who did or did not carry genes coding S-iKIR to HLA-Bw4. Carriage of S-iKIR to either HLA-C1 or HLA-C2 supertypes or both did not modify the frequency of NK cell responses to missing self. We also confirmed that VL alone did not account for variations in the frequencies of poly-functional NK cell subsets from SP who carried S-iKIR for HLA-B versus HLA-C.

According to the rheostat model of NK licensing, the strength of the inhibitory input received by NK cells during the education process determines a threshold of activation that is set in each NK cell [5;6]. Both the type of MHC class I molecules and the number of expressed self-specific iNKR capable of interacting with the MHC class I molecules govern the strength of inhibitory input. The stronger the inhibitory input, the more likely the NK cell will pass the threshold of activation required to respond to stimuli with an increased number of effector functions and exhibit a poly-functional profile (5, 6). Therefore, NK cells vary in responsiveness to stimulation in accordance with variations in iNKR/HLA interactions [5;6].

NK cells from Bw6 hmz individuals carrying 1 or 2 S-iKIR recognizing HLA-C and no S-iKIR for HLA-Bw4 were indistinguishable in terms of the frequency of any of the 7 possible functional subsets measured. NK cells from individuals carrying 1 or 2 S-
iKIR recognizing HLA-C with an S-iKIR for HLA-Bw4 were also indistinguishable in terms of the frequency of any of the 7 possible functional subsets measured. This observation suggests that S-iKIR to HLA-C have a weaker impact on NK education compared to that of S-iKIR to HLA-Bw4. One explanation for this may be related to the lower expression of HLA-C versus HLA-B on the surface of many cell types [3;18] which may translate into a lower impact on NK cell education.

Kim et al. also report differences in the strength of the NK cell response depending on the type of self-HLA-B and HLA-C ligands expressed indicating that certain KIR and HLA alleles strongly impact the potency of the NK cell response [14]. However, contrary to our findings the authors report that the quantity of S-iKIR also influences the potency of the NK cell response [24]. We found that carrying S-iKIR to HLA-C supertypes did not significantly contribute to the NK cell functional potential whereas S-iKIR to HLA-Bw4 did. One reason for this discrepancy may arise from differences in the functional output of NK cells measured. Whereas our study assessed differences in the simultaneous production of cytokine secretion and CD107a expression, Kim et al. compared IFN-γ production and NK cytotoxicity as separate measures of the NK cell response potency [14]. Furthermore, to the best of our knowledge ours is the first study to address whether S-iKIR to HLA-Bw4 and HLA-C1/C2 alone or in combination influence NK cell poly-functionality.

NK cells from individuals carrying S-iKIR to HLA-C1/C2 without S-iKIR to HLA-Bw4 do respond to missing self but tend to do so by secreting IFN-γ alone, expressing CD107a alone or by secreting both IFN-γ and TNF-α. This finding is also in line with predictions of the rheostat model of NK cell education, which would predict
that lower-impact educational inputs translate into the induction of fewer functions in stimulated NK cells [6].

NK cells with tri-functional potential produce more IFN-γ, TNF-α and express more CD107a on a per cell basis than the same function in mono-functional NK cells [12]. Therefore, NK cells from individuals carrying S-iKIR to HLA-C supertypes without S-iKIR to HLA-Bw4 would be expected to elicit lower levels of IFN-γ, TNF-α and CD107a than NK cells from subjects who are also positive for S-iKIR to HLA-Bw4. Whether higher levels of inhibitory input during NK cell education and enhanced NK cell poly-functionality translates into a more potent missing self response to virally infected cells warrants further investigation.

A potential limitation of this study is that the impact of other iNKRs to self HLA was not considered. We reasoned that the influence on NK education of NKG2A:CD94, which recognizes the leader sequence of several HLA alleles complexed to HLA-E would be comparable from one person to another, as has been reported (24). The iNKR LILRB1 also has broad range HLA recognition [21]. Stimulated NK cells bearing this receptor as their only iNKR are hyporesponsive suggesting the educational impact of signaling through this receptor may be minimal [23]. The iKIR KIR3DL2, which binds HLA-A*03/*11 in the presence of a restricted set of peptides may also contribute minimally to NK education (24). Single iNKR positive NK cells bearing KIR3DL2 were anergic to missing self stimuli [23]. In summary, these iNKRs would be expected to contribute minimally to between-subject variability in NK functional potential.
Another limitation of this study is that we did not consider the influence of activating NK receptors on functional potential. Although the ligands for many of the activating NK receptors are unknown, their role in tuning NK functional potential is starting to be addressed [16]. It is possible that some of the variability within our groups arises from the influence of activating receptors on NK education.

In this study we used HLA-devoid K562 cells as a stimulus. These cells interrupt multiple inhibitory signals mediated by iNKR recognizing HLA alleles as ligands. This stimulus precludes isolating the influence of individual S-iKIR. A more comprehensive examination of the link between the strength of the inhibitory input received by NK cells during their education and NK cell functionality will be possible by using antibody panels that detect functional NK subsets based on iNKR expression. Data generated using such an approach can provide information on which NK subsets are responding, their relative contribution to the total response and the functional potential of each NK cell subset.

Whereas the iKIR receptors KIR2DL1, KIR2DL2/3 and KIR3DL1 may all contribute to the level of NK functional competence in an individual that co-carry the appropriate ligands for these iKIR, we show that the contribution of S-iKIR to HLA-C supertypes to the NK missing self response is more restricted than that of the S-iKIR to HLA-Bw4 in terms of magnitude and the breadth of functions measured. These results are consistent with the interpretation that differences in the strength of inhibitory inputs received by NK cells during the licensing process contribute to variations in the frequencies and functional potential of NK cells among SP with diverse HLA alleles [5].
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7. REFERENCES


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8. LEGENDS

Figure 1

Frequencies of tri-functional NK cell responses to missing self are higher when from HIV infected slow progressors (SP) who do versus do not carry inhibitory Killer Immunoglobulin-like Receptors (iKIR) to self HLA-Bw4 alleles.

Scatter plots show the frequency tri-functional NK cells following K562 stimulation for SP categorized according to the type and number of iKIR to self HLA (S-iKIR). Group 1 includes subjects with only one S-iKIR to either an HLA-C1 or C2 allele, Group 2 includes subjects with only two S-iKIR to both an HLA-C1 and C2 allele, Group 3 includes subjects with S-iKIR to HLA-Bw4 and either an HLA-C1 or C2 allele, Group 4 includes subjects with an S-iKIR to HLA-Bw4 and both an HLA-C1 and C2 allele. Each data point represents results from a single individual. The line through each scatter plot is the mean for the group. P-values of <0.05 and <0.005 are indicated by a “*” and “**”, respectively over the line linking the 2 groups compared.

Figure 2

Frequencies of bi-functional CD107a⁺IFN-γ⁺ and dual cytokine secreting TNF-α⁺IFN-γ⁺ NK cells responses to missing self are higher when from subjects who do versus do not carry S-iKIR to HLA-Bw4.

Scatter plots show the frequency of bi-functional CD107a⁺IFN-γ⁺ (Panel A), IFN-γ⁺ TNF-α⁺ (Panel B) and CD107a⁺TNF-α⁺ (Panel C) NK cells following K562 stimulation for SP categorized into four groups according to the type and number of their S-iKIR. Each data point represents results from a single individual. The line through each scatter plot is the
mean for the group. Comparisons between pooled subjects in Groups 1 and 2 with no S-iKIR to HLA-Bw4 and Groups 3 and 4 with S-iKIR to HLA-Bw4 are shown with bracketed lines. P-values of <0.05 were considered significant and indicated with an * over the line linking the groups compared.

Figure 3

**Frequencies of mono-functional CD107a⁺ and IFN-γ⁺ NK cells responses to missing self are higher when from subjects who do versus do not carry S-iKIR to HLA-Bw4.**

Scatter plots show the frequency of mono-functional IFN-γ⁺ (Panel A), CD107a⁺ (Panel B) and TNF-α⁺ (Panel C) NK cells following K562 stimulation for SP categorized into four groups according to the type and number of their S-iKIR. Each data point represents results from a single individual. The line through each scatter plot is the mean for the group. Comparisons between pooled subjects in Groups 1 and 2 with no S-iKIR to HLA-Bw4 and Groups 3 and 4 with S-iKIR to HLA-Bw4 are shown with bracketed lines. P-values of <0.05 were considered significant and indicated with an * over the line linking the groups compared.

Figure 4

**SP categorized according to the type and number of S-iKIR do not differ in their Log₁₀ Viral Load (VL)**

Scatter plots showing the Log₁₀VL of SP categorized into four groups according to the type and number of S-iKIR. Each data point represents the Log₁₀VL measurement for a single subject.