Full title: Resistance to HIV-1 infection among African female sex workers is associated with inhibitory KIR in absence of their HLA ligands[1]

Running title: KIR and HLA in resistance to HIV-1 infection

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Key words: AIDS, Natural Killer Cells, MHC,

Word count of the abstract: 148

Total calculated character count: 31191 (i.e. < 4 printed pages in the JI)

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Abstract

NK cells are regulated in part by killer immunoglobulin-like receptors (KIR) which interact with HLA molecules on potential target cells. *KIR* and *HLA* loci are highly polymorphic and certain *KIR/HLA* combinations were found to protect against HIV disease progression. We show here that KIR/HLA interactions also influence resistance to HIV transmission. HIV-exposed but seronegative female sex workers in Abidjan, Côte d'Ivoire frequently possessed inhibitory *KIR* genes in absence of their cognate *HLA* genes: *KIR2DL2/KIR2DL3*-heterozygosity in absence of *HLA-C1* and *KIR3DL1*-homozygosity in absence of *HLA-Bw4*. HIV-seropositive female sex workers were characterized by corresponding inhibitory *KIR/HLA* pairings: *KIR2DL3*-homozygosity together with *HLA-C1* and a trend towards *KIR3DL1*/*HLA-Bw4*-homozygosity. Absence of ligands for inhibitory KIR could lower the threshold for NK cell activation. In addition, exposed seronegatives more frequently possessed AB KIR genotypes which contain more activating *KIR*. The data support an important role for NK cells and KIR/HLA interactions in antiviral immunity.

Introduction

NK cells play an important role in the innate immune system by providing the first line of defense against viral infections and tumors (1). NK cell activity is partially controlled by distinct inhibitory and activating killer immunoglobulin-like receptors (KIR)[4] which recognize specific ligands on potential target cells (2). NK effector functions occur only when inhibitory signals are overcome by activating signals. KIR are encoded by 15 polymorphic genes located on chromosome 19 and contain two or three external immunoglobulin-like domains (2D, 3D) with either long (2DL, 3DL) or short (2DS, 3DS) cytoplasmic tails, corresponding to their function as inhibitory or activating receptors, respectively (3). Several inhibitory KIR have well-defined HLA class I ligands. KIR2DL2 and KIR2DL3 bind to HLA-Cw group 1 (HLA-C1) which have asparagine at position 80, while KIR2DL1 binds the mutually exclusive HLA-Cw group 2 (HLA-C2) with a lysine at this position (4). KIR3DL1 binds HLA-B molecules with the serologically defined epitope Bw4, specified by five variable amino acids spanning positions 77-83 (ref. 5). The alternative serotype, Bw6, is not known to bind any KIR. Despite high sequence similarity with inhibitory receptors, activating 2DS and 3DS KIR show either weak or undetectable binding to HLA class I (ref. 6-8).

The extreme levels of population diversity and rapid evolution of both *KIR* and *HLA* genes suggest that they are under pathogen-mediated selection (9). *KIR* and *HLA* loci map to separate chromosomes resulting in variation in the number and kind of KIR/HLA ligand pairs, potentially influencing disease outcome at the individual level. Indeed, specific *KIR*/*HLA* genotypes favoring NK cell activation were found to

protect against disease progression after HIV-1 or HCV infection (10, 11) and were associated with increased susceptibility to autoimmune disorders like psoriatic arthritis (12) and type I diabetes (13).

Rare individuals remain HIV-seronegative despite frequent unprotected exposure to the virus and several mechanisms of resistance have been proposed (HIV-exposed seronegative or ESN, reviewed in ref. 14). *HLA* polymorphism has been shown to play a role in protection against HIV infection (15, 16), but its mode of action remains incompletely resolved. In this study, we analyzed the genes encoding KIR and their HLA ligands in ESN female sex workers (FSWs), HIV-1-seropositive (SP) FSWs, and HIV-seronegative female blood donors (FBDs) from Abidjan, Côte d'Ivoire.

Materials and methods

Study subjects

Twenty one ESN and 20 SP FSWs were enrolled at a confidential FSW clinic in Abidjan, Côte d'Ivoire between June 1998 and May 2000. The women were followed as part of a clinical trial testing the efficacy of a nonoxynol-9 microbicide gel (17). Twenty five HIV-seronegative FBDs were enrolled at the blood transfusion center in Abidjan, Côte d'Ivoire. The study was approved by ethical committees of the Ministry of Health, Côte d'Ivoire and the Institute of Tropical Medicine, Antwerp, Belgium, and by the Institutional Review Board of the Centers for Disease Control and Prevention, Atlanta, GA. All subjects gave written informed consent prior to enrolment.

Laboratory methods

Whole blood was drawn in EDTA tubes (Becton Dickinson). Plasma was separated from whole blood by centrifugation and tested for HIV by ELISA and Western blot, and confirmed by HIV RT-PCR. PBMC were separated from whole blood by gradient centrifugation and stored in liquid nitrogen.

KIR and HLA class I genotyping

Genomic DNA was extracted from PBMC using a QIAamp DNA blood mini kit (Qiagen). KIR typing was performed with the PCR-sequence specific primer technique like previously reported (18). In the assessment of KIR genotypes, group A haplotypes were defined by the presence of *KIR2DL1*, *KIR2DL3*, *KIR3DL1* and *KIR2DS4*. Group B haplotypes were defined by lack of *KIR3DL1* and *KIR2DS4* in the

presence of inhibitory *KIR2DL2* and *KIR2DL5* and one or more of the activating *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5*, and *KIR3DS1* (19). *HLA-B* and -*C* typing at the allele group level was performed by PCR-sequence specific oligonucleotides methodology (Tepnel Lifecodes).

Statistical methods

Differences in continuous variables were analyzed with non-parametric Mann-Whitney U tests. Fisher's exact tests were used to calculate statistical significances and exact 95% confidence intervals of odds ratios (OR) of allele frequency differences. Occurrence of trend was analyzed with the exact non-parametric Cochran-Armitage test. Level of statistical significance for all analyses was set at $p < 0.05$. Statistical analyses were performed with SAS version 9.1 (The SAS Institute).

Results and discussion

Twenty one ESN FSWs, 20 SP FSWs, and 25 FBDs were included in the study (Table 1). First, we analyzed the frequencies of individual *KIR* genes and *KIR* genotypes. ESN FSWs showed significantly higher frequencies of inhibitory *KIR2DL2* and *KIR2DL5* than SP FSWs (Fig. 1A). ESN FSWs also showed higher frequencies of activating *KIR2DS2* and *KIR2DS3*, but the differences were not statistically significant (Fig. 1B). All study subjects displayed *KIR3DL1* and *KIR2DS4* genes, therefore only AA and AB genotypes were present. ESN FSWs more frequently displayed AB genotypes, while SP FSWs more frequently displayed AA genotypes (Fig. 1C). In general, FBDs showed frequencies of individual *KIR* genes and *KIR* genotypes in between those from ESN and SP FSWs (Fig. 1A-C).

Next, we analyzed the presence of inhibitory *KIR* in association with their known HLA ligand genes (Tables 2 and 3). First, we analyzed gene frequencies of the inhibitory receptors KIR2DL2 and KIR2DL3, which segregate as alleles of the same gene locus, together with their HLA-C1 ligand (Table 2). There were significantly more *KIR2DL2/KIR2DL3* heterozygotes among ESN FSWs, while SP FSWs showed a markedly higher proportion of *KIR2DL3/KIR2DL3* homozygotes. There was a trend towards a higher frequency of *HLA-C1/C2* heterozygotes among SP FSWs, potentially resulting in more inhibitory NK signals via KIR2DL receptors. More specifically, we found that the increased frequency of homozygous *KIR2DL3* among SP FSWs was significant only if both *HLA-C1* and *C2* were present. ESN FSWs retained an increased frequency of heterozygous *KIR2DL2/KIR2DL3* only in the absence of their HLA-C1 ligand gene (i.e. *C2/C2*-homozygous). The trends for the

KIR/HLA comparisons between ESN FSWs and FBDs were similar to those between ESN and SP FSWs, although they did not always reach statistical significance probably because of the low sample size.

Next, we analyzed frequencies of inhibitory *KIR3DL1* and activating *KIR3DS1*, also alleles of the same gene locus, together with their HLA-Bw4 ligand (Table 3). *KIR3DL1* and *KIR3DS1* frequencies were similar among ESN and SP FSWs. SP FSWs tended to be more frequently homozygous for *HLA-Bw4*, on its own and in combination with *KIR3DL1*, potentially resulting in more NK inhibition via KIR3DL1. A significantly higher proportion of ESN FSWs who were homozygous for *KIR3DL1* lacked *HLA-Bw4* (i.e. *Bw6*-homozygous), resulting in absence of NK inhibition via KIR3DL1. Here, the trends for the KIR/HLA comparisons between ESN FSWs and FBDs did not confirm those between ESN and SP FSWs: similar proportions of ESN FSWs and FBDs were *HLA-Bw4*- and *KIR3DL1/HLA-Bw6*-homozygous. None of the subjects included in the study were homozygous for *KIR3DS1* (Table 3). Slow progression towards AIDS was previously associated with *KIR3DS1* in the presence of *HLA-Bw4* alleles with isoleucine at position 80 (*Bw4-80Ile*) (10). In our study, frequencies of *Bw4-80Ile* were similar among ESN, SP FSWs, and FBDs. Compared with ESN FSWs, SP FSWs displayed a higher proportion of the alternative *HLA-Bw4* allele with a threonine at position 80 (p = 0.028). No statistically significant interactions were observed between *KIR3DS1* and *HLA-B* alleles, possibly as the result of the low frequency of *KIR3DS1* in this population (12% compared with 42% among healthy Caucasians, ref. 18). No correlations were found between the observed *KIR2DL/HLA-C* and *KIR3DL1/HLA-B* interactions (data not shown).

At the time of sample collection, HIV-1 seroprevalence was 32% among FSWs (20), and 14% among their clients (21). HIV-1 seroincidence among FSWs participating in the nonoxynol-9 trial was 4% (17). Using self-reported data, ESN FSWs in Abidjan were estimated to have on average 52 unprotected exposures to HIV-1 per year (22). None of the FSWs tested in Abidjan showed the *CCR5* 32-bp deletion (23), which is in agreement with other populations in Africa (24). Together, this suggests that ESN FSWs in Abidjan are at high risk for acquiring HIV-1 infection. ESN FSWs enrolled in this study reported a median duration of commercial sex work of 3.5 years, ranging from as short as 2 months to as long as 20 years (Table 1). Putative HIV-1 resistance factors may be selected for in ESN subjects with a longer history of high-risk behavior. To test this, we correlated the observed KIR/HLA associations with the duration of commercial sex work of the ESN FSWs. For the *KIR* and *KIR/HLA* combinations that reached statistical significance in Tables 2 and 3, we observed a statistically significant trend over SP and ESN FSWs who had done more and less than 3 years of commercial sex work (Fig. 2).

In this study, the ESN status of FSWs was associated with the occurrence of certain inhibitory *KIR* genes in the absence of their cognate *HLA* genes: *KIR2DL2/KIR2DL3* heterozygosity in the absence of *HLA-C1*, and independent from this, *KIR3DL1*-homozygosity in the absence of *HLA-Bw4*. Together, 38% of ESN FSWs showed at least one such *KIR/HLA* mismatch compared to 0% of SP FSWs ($p = 0.006$). Absence of HLA ligands for inhibitory KIR may lower the threshold for NK cell activation via activating KIR, resulting in NK cytotoxic activity and early elimination of HIV-infected cells. In agreement with this, a higher frequency of ESN FSWs possessed *AB* KIR genotypes, which contain a higher number of activating *KIR*. In that respect, our

data corroborate a recent study showing increased NK cell-mediated cytotoxicity and cytokine and β-chemokine secretion among ESN intravascular drug users (25). In contrast with this, SP FSWs were characterized by corresponding inhibitory KIR/HLA ligand pairs which can directly inhibit NK cell effector functions: *KIR2DL3*-homozygosity in the presence of heterozygous *HLA-C1/C2*, and independent from this, a trend towards *KIR3DL1/HLA-Bw4*-homozygosity. Together, 61% of SP FSWs showed at least one such inhibitory KIR/HLA pair compared to 19% of ESN FSWs ($p = 0.009$). In addition, SP FSWs more frequently showed *AA* KIR genotypes which contain lower numbers of activating *KIR*.

Although SP FSWs showed a wide range of CD4 counts (median, 437 cells/µl; range, 144-1712 cells/µl; Table 1), it is unclear to what extent rapid and slow progressor patients were equally represented; this cannot be known in a cross-sectional study. Thus, it cannot be ruled out that KIR/HLA combinations related to differential disease progression in the SP group have influenced our results. However, although less pronounced, the KIR and HLA frequency differences among ESN and SP FSWs were generally confirmed by comparisons between ESN FSWs and FBDs. This supports the conclusion that the observed associations are related to HIV susceptibility rather than differential HIV disease progression.

Despite large gene frequency differences, the small sample size resulted in KIR/HLA interactions with relatively weak statistical significance. Because of the exploratory nature of the study, the testing of distinct hypotheses rather than a comprehensive screening for genes and associations, and restriction of *HLA-B* and *-C* allele analysis to 4 distinct KIR binding groups, correction for multiple testing was not applied.

Exact testing for small sample sizes was performed for all analyses. Nevertheless, confirmation of our findings in larger populations of healthy versus HIV-infected subjects, and in functional studies, is needed.

Our data are the first to show that KIR/HLA interactions may influence susceptibility to virus transmission. Moreover, the *KIR/HLA* gene combinations favoring NK activation over inhibition by lack of HLA ligands for inhibitory KIR may be more straightforward than those previously described for protection against viral disease progression. Indeed, delayed onset of AIDS was associated with the activating KIR3DS1 receptor in combination with its only presumptive HLA-B Bw4-80Ile ligand (10). Resolution of hepatitis C virus infection was found to be associated with corresponding *KIR2DL3/HLA-C1* gene combinations, only putatively resulting in the weakest inhibitory signals (11).

The non-classical HLA class I molecules HLA-E and -G are ligands for the inhibitory NK cell receptors CD94/NKG2A and KIR2DL4, respectively. A recent study found genetic variants of HLA-E and HLA-G with a potentially lower affinity for their inhibitory NK cell receptors to be associated with a decreased risk of HIV transmission (26). These findings further support a role for NK cells in protection against virus transmission and suggest that parallel inhibitory NK receptor/HLA ligand mechanisms may be at play.

Acknowledgments

We thank the community of female sex workers in Abidjan for their cooperation, Joris Menten and Odette Tossou for help with data analysis.

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Footnotes

[1] This work was supported by the Belgian Fund for Scientific Research (FWO-Vlaanderen), grant G.0660.06, and the Centers for Disease Control and Prevention, Division of HIV/AIDS prevention, Atlanta, GA, USA. S.V. was supported by a grant from the Scientific Fund W. Gepts AZ-VUB.

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[4] Abbreviations used in this paper: ESN, HIV-exposed seronegative; FBD, female blood donor; FSW, female sex worker; SP, HIV-1-seropositive; KIR, killer immunoglobulin-like receptor; OR, odds ratio.

Figure legends

Figure 1. Frequencies of individual *KIR* genes and *KIR* genotypes among ESN FSWs, SP FSWs and FBDs. **A**, inhibitory *KIR* genes. **B**, activating *KIR* genes. **C**, *KIR* genotypes. Gene and genotype frequencies were compared between ESN and FBDs and between ESN and SP FSWs with non-parametric Fisher's exact tests, *p*-values < 0.1 are shown. SP, HIV-1-seropositive female sex workers, n = 20, white bars; FBD, HIV-seronegative female blood donors, n = 25, hatched bars; ESN, HIV-exposed seronegative female sex workers, n = 21, black bars.

Figure 2. Test for trend of *KIR* and *HLA* frequencies with duration of commercial sex work of ESN FSWs. **A**, *KIR* alleles. **B**, *KIR/HLA* allele combinations. *KIR* alleles and *KIR/HLA* allele combinations that reached statistical significance in Tables 2 and 3 were tested. Tests for trend were performed with exact non-parametric Cochran-Armitage tests. SP, HIV-1-seropositive female sex workers, n = 20 (except for *KIR3DL1/3DL1 + HLA Bw6/Bw6*, n = 16), white bars; ESN < 3 y, HIV-exposed seronegative female sex workers with less than 3 years of commercial sex work, n = 9, hatched bars; ESN ≥ 3 y, HIV-exposed seronegative female sex workers with more than 3 years of commercial sex work, n = 12, black bars.

Table 1. Characteristics of female sex workers and female blood donors included in the study.

	ESN (n = 21)	FBD (n = 25)	SP (n = 20)	FBD vs. ESN	SP vs. ESN
				P	*p*
Age (years)	30 (26-35)	22 (21-27)	33 (25-38)	0.008	0.592
Duration of commercial sex work (months)	43 (25-76)	NA	33 (16-91)	NA	0.554
$CD4^+$ T cells (cells/µl)	1221 (987-1444)	NA	437 (296-747)	NA	< 0.001

Data are median values (interquartile range). ESN, HIV-exposed seronegative female sex workers; FBD, HIV-seronegative female blood donors; SP, HIV-1-seropositive female sex workers; NA, not available. *p*-values calculated with non-parametric Mann-Whitney U tests.

Table 2. Frequencies of inhibitory *KIR2DL2* and *KIR2DL3* alleles and their HLA-C1 ligand gene among female sex workers and female blood donors.

	ESN (n = 21)	FBD (n = 25)	SP (n = 20)	ESN vs. FBD			ESN vs. SP		
				OR	95% CI	*p*	OR	95% CI	*p*
***KIR* alleles**									
2DL2/2DL2	24	20	15	1.25	0.24-6.48	1.000	1.77	0.28-13.1	0.697
2DL2/2DL3	62	44	25	2.07	0.55-7.98	0.253	4.88	1.08-23.5	**0.028**
2DL3/2DL3	14	36	60	0.30	0.05-1.50	0.176	0.11	0.02-0.60	**0.004**
***HLA-C* alleles**									
C1/C1	29	28	15	1.03	0.23-4.49	1.000	2.27	0.39-16.2	0.454
C1/C2	43	56	70	0.59	0.16-2.20	0.554	0.32	0.07-1.38	0.118
C2/C2	29	16	15	2.10	0.41-11.8	0.475	2.27	0.39-16.2	0.454
***KIR-HLA* combinations**									
2DL2/2DL3 + C1/C1	19	12	10	1.73	0.25-13.2	0.686	2.12	0.26-25.8	0.663
2DL2/2DL3 + C1/C2	24	32	15	0.66	0.14-2.92	0.744	1.77	0.28-13.1	0.697
2DL2/2DL3 + C2/C2	19	0	0	∞	1.16-∞	**0.037**	∞	0.92-∞	0.107
2DL3/2DL3 + C1/C1	0	8	5	0.00	0.00-4.11	0.493	0.00	0.00-18.1	0.488
2DL3/2DL3 + C1/C2	10	20	45	0.42	0.04-3.02	0.428	0.13	0.01-0.82	**0.015**
2DL3/2DL3 + C2/C2	5	8	10	0.58	0.01-12.0	1.000	0.45	0.01-9.51	0.606

Data are percentages. ESN, HIV-exposed seronegative female sex workers; FBD, HIV-seronegative female blood donors; SP, HIV-1-seropositive female sex workers. Statistical significance and exact 95% confidence intervals (CI) of odds ratios (OR) were calculated with Fisher's exact tests. *p*-values < 0.05 are in bold.

Table 3. Frequencies of *KIR3DL1/KIR3DS1* alleles and their HLA-Bw4 ligand gene among female sex workers and female blood donors.

	ESN (n = 21)	FBD (n = 25)	SP (n = 20)	ESN vs. FBD			ESN vs. SP		
				OR	95% CI	*p*	OR	95% CI	*p*
***KIR* alleles**									
3DL1/3DL1	95	84	85	3.81	0.33-197	0.357	3.53	0.25-194	0.343
3DL1/3DS1	5	16	15	0.26	0.01-3.03	0.357	0.28	0.01-4.02	0.343
3DS1/3DS1	0	0	0	NA	NA	NA	NA	NA	NA
***HLA-B* alleles**[a]									
Bw4/Bw4	10	13	38	0.70	0.05-6.90	1.000	0.18	0.02-1.28	0.055
Bw4/Bw6	62	52	56	1.49	0.38-5.88	0.557	1.26	0.28-5.75	0.749
Bw6/Bw6	29	35	6	0.75	0.17-3.21	0.752	6.00	0.59-294	0.113
***KIR-HLA* combinations**[a]									
3DL1/3DL1 + Bw4/Bw4	10	4	31	2.32	0.11-143	0.599	0.23	0.02-1.78	0.202
3DL1/3DL1 + Bw4/Bw6	57	48	50	1.45	0.38-5.63	0.563	1.33	0.30-5.96	0.746
3DL1/3DL1 + Bw6/Bw6	29	35	0	0.75	0.17-3.21	0.752	∞	1.37-∞	**0.027**
3DL1/3DS1 + Bw4/Bw4	0	9	6	0.00	0.00-3.77	0.489	0.00	0.00-14.5	0.432
3DL1/3DS1+ Bw4/Bw6	5	4	6	1.10	0.01-90.2	1.000	0.75	0.01-62.8	1.000
3DL1/3DS1 + Bw6/Bw6	0	0	6	NA	NA	NA	0.00	0.00-14.5	0.432

Data are percentages. ESN, HIV-exposed seronegative female sex workers; FBD, HIV-seronegative female blood donors; SP, HIV-1-seropositive female sex workers; NA, not available. Statistical significance and exact 95% confidence intervals (CI) of odds ratios (OR) were calculated with Fisher's exact tests. *p*-values < 0.05 are in bold.

[a]Data available for 16 SP FSWs and 23 FBDs due to insufficient amounts of DNA.

Figure 1

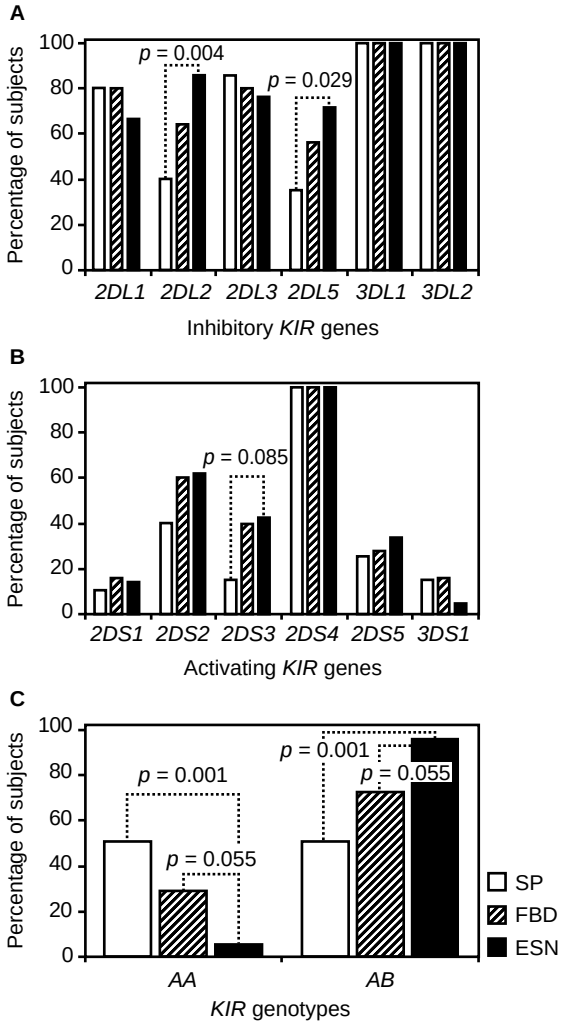
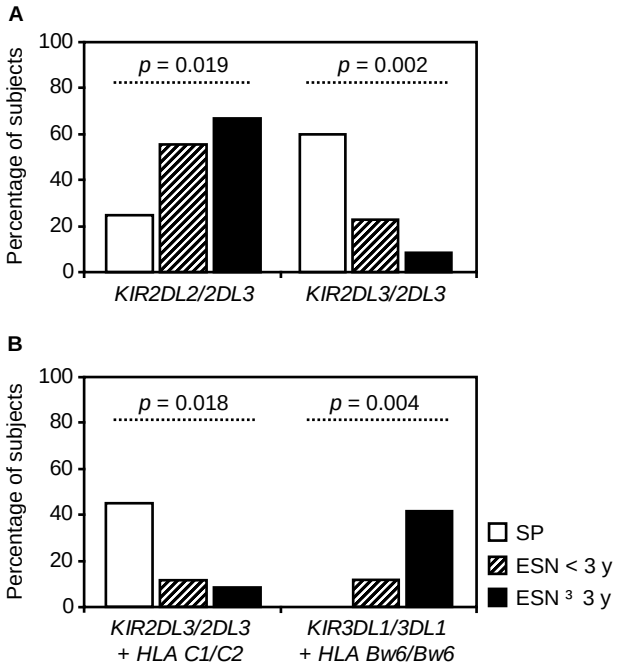

A
Percentage of subjects
100
80
60
40
20
0
p = 0.004
p = 0.029
2DL1
2DL2
2DL3
2DL5
3DL1
3DL2
Inhibitory KIR genes
B
Percentage of subjects
100
80
60
40
20
0
p = 0.085
2DS1
2DS2
2DS3
2DS4
2DS5
3DS1
Activating KIR genes
C
Percentage of subjects
100
80
60
40
20
0
p = 0.001
p = 0.055
p = 0.001
p = 0.055
SP
FBD
ESN
AA
AB
KIR genotypes

Figure 2


A
100
80
60
40
20
0
Percentage of subjects
p = 0.019
p = 0.002
KIR2DL2/2DL3
KIR2DL3/2DL3
B
100
80
60
40
20
0
Percentage of subjects
p = 0.018
p = 0.004
KIR2DL3/2DL3
+ HLA C1/C2
KIR3DL1/3DL1
+ HLA Bw6/Bw6
SP
ESN < 3 y
ESN ³ 3 y