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Class I HLA haplotypes form two schools that educate NK cells in different ways

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Summary

Natural killer (NK) cells are lymphocytes having vital functions in innate and adaptive immunity, as well as placental reproduction. Controlling education and functional activity of human NK cells are various receptors that recognize HLA class I on the surface of tissue cells. Epitopes of polymorphic HLA-A,-B and –C are recognized by equally diverse killer cell immunoglobulin-like receptors (KIR). In addition, a peptide cleaved from the leader sequence of HLA-A,-B or –C must bind to HLA-E for it to become a ligand for the conserved CD94:NKG2A receptor. Methionine/threonine dimorphism at position -21 of the leader sequence divides HLA-B allotypes into a majority having -21T that do not supply HLA-E binding peptides and a minority having -21M, that do. Genetic analysis of human populations worldwide shows how haplotypes with -21M

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SUPPLEMENTARY MATERIALS

Figure S1 – Worldwide analysis of *HLA B–C* haplotype frequencies
Figure S2 – Primate MHC class I leader sequence derived peptides
Figure S3 – Evolution and variation of position -21 in the leader sequence of Catarrhine primate MHC-B
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Figure S11 – Effects of NKG2C expression on frequency of NKG2A$^+$ NK cells
Table S1 – Antibody panel used for mass cytometry

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HLA-B rarely encode the KIR ligands: Bw4$^+$HLA-B and C2$^+$HLA-C KIR. Thus there are two fundamental forms of HLA haplotype: one preferentially supplying CD94:NKG2A ligands, the other preferentially supplying KIR ligands. -21 HLA-B dimorphism divides the human population into three groups: M/M, M/T and T/T. Mass cytometry and assays of immune function, shows how M/M and M/T individuals have CD94:NKG2A$^+$ NK cells which are better educated, phenotypically more diverse and functionally more potent than those in T/T individuals. Fundamental new insights are given to genetic control of NK cell immunity and the evolution that has limited the number of NK cell receptor ligands encoded by an HLA haplotype. These finding suggest new ways to dissect the numerous clinical associations with HLA class I.

Introduction

The education of Natural Killer (NK) cells and their response to infection, cancer and allogeneic tissue are guided by interactions between NK cell receptors and MHC class I ligands. These engagements enable NK cells to distinguish diseased cells, which have perturbed expression of MHC class I, from normal healthy cells. Such surveillance by NK cells is achieved with a bipartite system, which combines conserved receptors that recognize non-polymorphic MHC class I with diverse receptors that recognize polymorphic MHC class I (1).

In humans, polymorphic determinants of HLA-A, -B and –C are recognized by diverse and rapidly evolving killer cell immunoglobulin-like receptors (KIR). These bind to the upper face of the HLA class I molecule, making contact with the amino-terminal part of the α_1 helix, the carboxy-terminal part of the α_1 helix, and the bound peptide (2). Key polymorphisms in the α_1 helix determine the three major epitopes recognized by KIR. The C1 epitope of HLA-C is defined by asparagine at position 80, whereas lysine at the same position defines the C2 epitope. In this way, every HLA-C allotype carries either C1 or C2 and is a KIR ligand. By contrast, a minority of HLA-A and –B allotypes are KIR ligands. This function is conferred by a sequence motif at residues 77–83, which defines the Bw4 epitope carried by subsets of HLA-A and –B allotypes. Interactions of the C1, C2 and Bw4 epitopes with their cognate KIR are diversified by sequence variation in the KIR, the bound peptide and other residues of HLA class I that do not contact KIR directly. According to their HLA class I type, individual humans can have one, two or all three of these epitopes recognized by KIR (3).

In comparison to the highly diversified interactions of KIR with HLA-A, -B and -C, the recognition of HLA-E by the CD94:NKG2A receptor is conserved (2). HLA-E, CD94 and NKG2A have little polymorphism and the binding site of HLA-E is specific for peptides corresponding to residues - 22 to -14 of the leader sequence of HLA-A, -B and -C (4, 5). Because HLA-E must bind such a peptide, in order to fold properly and reach the cell surface, the amount of HLA-E detected by CD94:NKG2A correlates with how much HLA-A, -B and –C is being made by the cell. This property ensures that CD94:NKG2A$^+$ NK cells are sensitive to the overall expression of HLA class I and to its perturbation in cells compromised by stress or disease.

In the nonamer peptides that bind to HLA-E, the anchor residue at position 2 corresponds to residue -21 of the classical HLA class I leader sequence. Methionine -21, the residue present in all HLA-A and -C allotypes and a minority of HLA-B allotypes, provides a good anchor residue that facilitates the folding and cell-surface expression of HLA-E (6). In contrast, threonine -21, the residue present in the majority of HLA-B allotypes, does not bind effectively to HLA-E. The dimorphism at position -21 of the HLA-B leader sequence, has been correlated with susceptibility to HIV infection (7) and with capacity for NK cell mediated killing of HIV-infected cells (8).

In reviewing the effects of NKG2A and HLA-E on HIV infection, Yunis et al noted that there is a strong tendency for Bw4^{+}HLA-B to have -21T (9). One implication of this bias is that disease correlations made with Bw4 (10), could be due either to direct interaction of the Bw4 epitope with cognate KIR or to the indirect effect that a reduced supply of HLA-E binding peptide has on CD94:NKG2A recognition of HLA-E. A second implication is that Bw4^{+}HLA-B allotypes, which function as KIR ligands, should contribute less to formation of CD94:NKG2A ligands than the Bw4^{-}HLA-B allotypes that do not function as KIR ligands. To explore these implications, we investigated if such genetic and functional segregation is a more general principle in the human system of NK cell receptors and MHC class I ligands.

Results

C2^{+}HLA-C and -21M HLA-B segregate on different HLA haplotypes in Eurasian populations

Comparison of 16,384 *HLA* haplotypes, representing 51 anthropologically well-defined human populations worldwide (fig. S1) shows that *HLA* haplotypes encoding C2^{+}HLA-C almost always encode -21T HLA-B. Thus 92.6% of the 5,439 haplotypes encoding C2^{+}HLA-C also encode - 21T HLA-B, whereas only 7.4% of haplotypes combine C2^{+}HLA-C with -21M HLA-B. In contrast, the 10,945 haplotypes encoding C1^{+}HLA-C exhibit a more even distribution of the -21T (68.6%) and -21M (31.4%) forms of HLA-B leader peptide (Fig. 1A). These results show that C2, a stronger KIR ligand than C1, is usually paired with forms of HLA-B unable to contribute peptides that bind HLA-E and form CD94:NKG2A ligands. This bias points to persisting selection against haplotypes that contribute both a C2 ligand for KIR and an HLA-E ligand for CD94:NKG2A.

Dimorphism at position -21 of HLA-B modulates the supply of CD94:NKG2A ligands, whereas dimorphism at position 80 of HLA-C determines the presence and abundance of C1 and C2 ligands. Together the two dimorphisms define four different haplotypes. The haplotype frequencies were used to calculate D', a measure of linkage disequilibrium (LD), for each population. This analysis revealed a striking difference between African and non-African populations. The seven African populations exhibit no LD between -21 HLA-B and 80 HLA-C, whereas 27 of the 44 non-African populations exhibit complete LD (D' = 1) and eight others have D’ ≥0.9 (Fig. 1B). All populations of European origin have high D’, as do 16 of the 23 Asian populations. In Eurasian populations there is an effective exclusion of -21M HLA-B from haplotypes encoding the C2 epitope, whereas Africans have no such exclusion.

The seven Asian populations with D'<0.9 (Fig. 1B) have particularly high frequencies of -21T HLA-B and C1$^+$HLA-C (N80), an imbalance that reduces the robustness of the D' calculation. Although these populations have low frequencies of C2$^+$HLA-C haplotypes, in absolute number they have many of these haplotypes, and a majority of them (90.2%) encode -21T HLA-B. We can therefore conclude with confidence, that haplotypes combining -21M HLA-B and C2$^+$ HLA-C are rare in all Eurasian populations.

Worldwide, we found only five different *HLA* haplotypes that combine -21M HLA-B with C2$^+$HLA-C (fig. S1). These three African and two Asian haplotypes form three groups. Group I comprises two common African haplotypes that have African-specific *HLA-B* and -*C* alleles: *B*42:01-C*17:01* and *B*81:01-C*18:01* (Fig. 1C). Distinguishing HLA-C*17, -C*18 and -C*07 from other HLA-C allotypes is alanine at position -17 in the leader peptide. For HLA-C*07:01, this residue prevents production of a peptide that binds and stabilizes HLA-E (11, 12). Group II is represented by the third African haplotype, which combines African-specific *HLA-B*81:01* with globally widespread *HLA-C*04:01* (Fig. 1C). Peptides derived from the HLA-B*81:01 leader sequence are predicted to promote surface expression of HLA-E.

Group III comprises the two Asian haplotypes. Both are rare and combine *HLA-C*15:05* with either *HLA-B*73:01* or *HLA-B*07:05* (Fig. 1C). Modern humans acquired the *B*73:01-C*15:05* haplotype, by introgression in Asia from archaic humans (13). HLA-B*73:01 is an unusual HLA-B allotype that carries the C1 epitope (14). Consequently, the *B*73:01-C*15:05* haplotype supplies both the C1 (*B*73:01*) and C2 (*C*15:05*) epitopes. *HLA* haplotypes that combine C2$^+$HLA-C with -21M HLA-B are rare in Eurasia and could all have derived from a single event of archaic introgression. Haplotypes combining C2$^+$HLA-C and -21M HLA-B are more common in Africa, but mainly involve African-specific *HLA-C* allotypes having leader sequences that poorly promote HLA-E expression.

In cells and individuals, *HLA* haplotypes do not function alone but in diploid combinations: genotypes. Of 8,192 individuals in the 51 populations, 6.1% are -21M HLA-B homozygotes (M/M), 31.6% are -21M/-21T heterozygotes (M/T), and 62.3% are -21T HLA-B homozygotes (T/T) (Fig. 1D). Thus a large majority of human individuals (93.9%) has -21T HLA-B, whereas a minority (37.7%) has -21M HLA-B. Although differing in their relative frequencies, -21M and -21T HLA-B are both present in all human populations. The frequency of the minor allele, -21M HLA-B, is highest in Europe, lowest in Australia and at intermediate levels in Asia, Africa, America and Polynesia. In populations with higher frequencies of *-21M HLA-B*, there is complete segregation of *-21M HLA-B* and C2$^+$*HLA-C* onto different subsets of *HLA* haplotypes.

Methionine -21 MHC-B is the ancestral form from which threonine -21 MHC-B was derived

That all HLA-A and -C and some HLA-B have -21M, suggests -21M was the ancestral form of the leader sequence, from which -21T derived. To test this hypothesis, we examined MHC class I leader sequences of apes and Old World monkeys, the species most related to humans (15). Closest are the great apes, who have equivalents of HLA-A, -B and –C (generically called MHC-A, -B and –C). More distantly related, Old World monkeys have MHC-A and MHC–B, but not MHC-C (fig. S2A). In macaque monkeys, -21M is present in 239 of 241

MHC-A and 365 of 399 MHC-B, whereas -21T occurs only in 4 MHC-B. Of 34 MHC-B lacking -21M, 24 have -21V and 6 have -21L. For orangutan, the Asian great ape, the dataset is small but all 5 MHC-A, 6 MHC-B and 5 MHC-C allotypes have -21M. Such dominance of -21M in orangutan and Old World monkey MHC class I, argues for -21M MHC-B being the ancestral form (fig. S2B).

Chimpanzee, human's closest living relative, has HLA-A, -B and –C orthologs, named Patr-A, -B and –C (fig. S2A). Of 28 Patr-A, 27 have -21M and one has -21L, a dominance of -21M like that at HLA-A. Of 41 Patr-B allotypes, 38 have -21T, 3 have -21L and none has -21M. Thus the trend for -21T to replace -21M at HLA-B has gone to near completion at Patr-B. Whereas -21M is fixed at HLA-C, replacement of -21M by -21T is seen in 5 of 22 Patr-C allotypes. All these patterns of substitution are consistent with -21T having derived from -21M in a common humanchimpanzee ancestor.

Only one point mutation can convert -21M to -21T. This is replacement of 'T' in methionine codon ATG with 'C' to give threonine codon ACG. In addition to methionine, only five other residues – threonine, arginine, isoleucine, leucine and valine – are present at position -21 in primate MHC-A, -B and –C (fig. S2). Each of these residues is specified by a codon that can be formed by point mutation of the methionine codon. In contrast, no other codon present at position -21 (threonine, arginine, isoleucine, leucine, or valine) can give rise to all the other codons by point mutation (fig. S3). These various lines of evidence make it almost certain that methionine was the ancestral residue at position -21 of primate MHC-A, -B and –C.

All Bw4$^+$HLA-A have -21 methionine and almost all Bw4$^+$HLA-B have -21 threonine

HLA-B was first identified as a dimorphism of Bw4 and Bw6 antigens. Their epitopes are determined by sequence motifs at positions 77–83 in the α_1 domain. Bw4 is the ligand for KIR3DL1 (16), whereas Bw6 is not recognized by human KIR. Of 33 HLA-B allotype groups, 12 have the Bw4 motif and 21 the Bw6 motif (fig. S4A). The correlation between Bw4$^+$HLA-B and -21T HLA-B is striking, as noted previously (9). Eleven of the twelve Bw4$^+$HLA-B allotype groups have -21T. Bw4 and -21M are combined only in HLA-B*38:01, an allotype that could be of archaic origin (13). Considering all combinations of Bw4$^+$ and Bw6$^+$ with -21T and -21M present in 3,019 HLA-B allotypes, gives an odds ratio (OR) of 0.08 (99% CI = 0.06, 0.11; $p = 1 \times 10^{-15}$) for Bw4$^+$ HLA-B having -21M. Thus the odds of a Bw4$^+$HLA-B allotype having -21M are very small. Contrasting with Bw4$^+$HLA-B, the segregation of -21M and -21T among Bw6$^+$HLA-B allotypes is relatively even. Nine of the Bw6$^+$ HLA-B groups have -21M and twelve have -21T. Four HLA-A allotype groups carry the Bw4 epitope (HLA-A*23, -A*24, -A*25 and -A*32) (fig. S4B). These are recognized by KIR3DL1 (17), with the possible exception of HLA-A25 (18). Phylogenetic analysis points to a gene conversion between *Bw4$^+$HLA-B* and *HLA-A* having transferred the Bw4 motif to an HLA-A allotype (19). Subsequent conversions among *HLA-A* alleles transferred the Bw4 epitope to three other HLA-A allotypes. Unlike Bw4$^+$HLA-B, all Bw4$^+$HLA-A have -21M, consistent with the original gene conversion having involved a short segment of exon 2 sequence that included the Bw4 motif, but not -21T of exon 1. Although *Bw4$^+$HLA-A* alleles are present on many different *HLA-A* haplotypes, it is

striking and significant ($p = 1 \times 10^{-15}$, Fisher's exact test) that haplotypes encoding both $Bw4^+$ HLA-A and $Bw4^+$ HLA-B are rare in the 51 populations we studied (fig. S5).

Position -21 dimorphism of HLA-B marks two functionally distinctive groups of HLA haplotypes

Our genetic and population analyses show that *HLA* haplotypes having -21M HLA-B rarely encode $Bw4^+$HLA-B or $C2^+$HLA-C. This subset of *HLA* haplotypes encode HLA-B that promote expression of HLA-E, the CD94:NKG2A ligand, as well as $C1^+$HLA-C, the ligand for KIR2DL2/3. On the other hand, the subset of *HLA* haplotypes that have -21T HLA-B, can provide the Bw4, C2 and C1 ligands for KIR3DL1, KIR2DL1/S1 and KIR2DL2/3, respectively, but cannot supply peptides derived from the HLA-B leader sequence that promote the formation of HLA-E ligands for CD94:NKG2A. Thus dimorphism at position -21 in the HLA-B leader peptide is seen to divide *HLA* haplotypes into two functional groups; one biased to the supply of CD94:NKG2A ligands, the other biased towards the supply of KIR ligands. The goal of subsequent experiments was to test this hypothesis with analyses of immunological function.

-21 HLA-B genotype correlates with surface levels of HLA-E and CD94:NKG2A

To investigate the effects of -21 HLA-B dimorphism on surface expression of HLA-E and its functional interactions with CD94:NKG2A, we compared three cohorts of healthy, CMV-negative, blood donors. These cohorts comprise 20 homozygotes for -21M HLA-B (M/M), 20 homozygotes for -21T HLA-B (T/T) and 20 heterozygotes for -21M and -21T (M/T) (fig. S6). The surface abundance of HLA-E on PBMC increases with the copy number of -21M HLA-B (Fig. 2A). The difference between M/T and T/T donors is highly significant (p=0.007; n=40; Mann-Whitney test), as is the difference between M/M and T/T donors (p=0.00002; n=40; Mann-Whitney test). The second copy of -21M HLA-B causes a relatively minor increase in HLA-E expression, showing that the important difference is between donors who have -21M HLA-B and donors who lack -21M HLA-B. Thus the level of HLA-E expression set by peptides derived from HLA-A and -C is particularly sensitive to the presence and absence of -21M HLA-B. Also affecting surface abundance is the dimorphism at position 107 of HLA-E, which distinguishes lower expressing HLA-E*01:01 (arginine 107) from higher expressing HLA-E*01:03 (glycine 107) (20, 21). HLA-E typing the 60 donors (fig. S6) showed HLA-E*01:03 homozygotes have highest expression, HLA-E*01:01 homozygotes have lowest expression and heterozygotes have intermediate expression. HLA-E*01:01 expression is 92% that of HLA-E*01:03 (fig. S7). Individuals of all three HLA-E genotypes are included in the cohorts of M/M, M/T and T/T donors (Fig. 2A) and individuals of all three -21 HLA-B genotypes are included in the groups of donors defined by HLA-E genotype. Thus HLA-E genotype is not a cause of the expression differences between the M/M, M/T and T/T donors. In addition, examining the Thousand Genomes database of whole exome sequences for 2,352 individuals found no evidence for LD between alleles of HLA-E and -21 HLA-B.

A second functional effect of increasing -21M copy number is to decrease the frequency of NK cells that express CD94:NKG2A (Fig. 2B and S8). Again the big difference is between donors who lack -21M (T/T) and donors who have -21M (M/M+M/T) (R^2=0.44;

$p=1.5\times10^{-10}$), with M/M donors exhibiting a small decrease additional CD94:NKG2A$^+$ NK cell number from that seen for M/T donors. A third effect of higher -21M copy number is to reduce the amount of CD94:NKG2A expressed the NK cell surface ($R^2 = 0.5$, $p=5\times10^{-10}$) (Fig. 2C).

These results show that for T/T donors, the supply of peptides coming from the leader peptides of HLA-A and -C limits the surface expression of HLA-E. This limitation is largely overcome by presence of one -21M HLA-B allele, as in M/T donors. There is, however, a small additional increase of surface HLA-E on cells of M/M donors. who express two -21M HLA-B alleles. Again, we see how the system is acutely sensitive to the difference between M/T and T/T genotypes. With population frequencies of 31.6% and 62.3%, respectively, the M/T and T/T donors comprise a large majority (93.9%) of the human species. That increased frequency of CD94:NKG2A$^+$NK cells, with decreased surface expression of CD94:NKG2A, correlate with - 21M copy number, suggest these effects are caused by increased supply of HLA-E binding peptides.

-21 HLA-B genotype correlates with cell surface abundance of HLA-C

The amount of HLA-C on cell surfaces varies with -*21 HLA-B* (Fig. 3A). M/M donors have low levels, correlating with their relatively restricted HLA-C diversity. As expected from the observed LD (Fig. 1), no M/M donor has C2$^+$HLA-C. Of the 20 M/M donors, twelve are homozygous for *HLA-C*07*, and seven are heterozygous (fig. S6). This weakly expressed allele is subject to miR-148a-mediated downregulation (22). Other *HLA-C* alleles expressed by M/M donors, *HLA-C*08* and *HLA-C*12*, are not affected by miR-148a and exhibit higher expression. Only one M/M donor is homozygous for higher-expressing *HLA-C* alleles. The cluster of 20 data points (Fig. 3B) comprises three phenotypic subclusters correspond to three genotypes: twelve *HLA-C*07* homozygotes with extremely low expression, seven *HLA-C*07* heterozygotes with very low expression and one *HLA-C*08* homozygote with low expression. The levels of *HLA-C* expression we observe are consistent with known differences in the properties of *HLA-C* alleles (fig. S6). Having *HLA-C*07* are characteristic European haplotypes, in which the linked -21M *HLA-B* allele is either *HLA-B*07* or *HLA-B*08*. Both haplotypes have characteristics suggestive of Neanderthal origin (13).

Within the T/T donor cohort, HLA-C expression varies widely, forming clusters of data points at high, low and intermediate values (Fig. 3A). M/T donors, have HLA-C levels that span the low to intermediate range of the T/T donors. The difference between M/M and T/T donors is striking ($p=0.0008$; $n=40$; Mann-Whitney test), and that between M/M and M/T donors is significant ($p=0.002$; $n=40$; Mann-Whitney test). The data points obtained for M/T donors form two clusters, corresponding to the intermediate and low clusters of T/T donors (Fig. 3A). The three clusters of HLA-C expression correlate with polymorphism at position 80 and its phenotypic expression as C1 and C2 epitopes. The low level cluster is enriched with C1/C1 homozygotes, and includes all M/M donors. The high level cluster is enriched with C2/C2 homozygotes: all T/T donors. The intermediate level cluster is enriched with C1/C2 heterozygotes (Fig. 3C).

A dimorphism that markedly affects *HLA-C* expression is in the miR-148a binding site of the 3'-untranslated region (22). Transcription is lower for *HLA-C* alleles with intact (I) miR-148a binding sites, but higher for alleles that acquired a nucleotide deletion (D) in the binding site (Fig. 3D). In *HLA-C*, there is LD between codon 80 encoding C2 and the deleted form of the miR-148a binding site. They are also in LD with -21T of *HLA-B*. This extended LD explains the similar patterns observed in Fig. 3 A, B, C and D. This shows how *HLA-B* and *-C* dimorphisms act in concert to modulate *HLA-C* expression.

Dimorphism at position -21 of HLA-B divides the human population into three subpopulations (M/M, M/T and T/T) that have distinctive patterns of HLA-C expression (Fig. 3E, F and G). Restricted to $C1^+$HLA-C, M/M donors have low HLA-C expression (<100 msi) and genotypes dominated by *HLA-C*07* (Fig. 3E). For the twelve HLA-C*07 homozygotes, and 23 heterozygotes, the mean surface expression is 40 and 60 msi, respectively. In contrast, the 25 donors who lack *HLA-C*07* (these include M/M, M/T and T/T individuals) have a mean expression of 140 msi. Accompanying *HLA-C*07* in the 23 heterozygotes are 11 other *HLA-C* alleles (*HLA-01:02*, *02:02*, *03:04*, *04:01*, *05:01*, *06:02*, *08:02*, *12:03*, *14:02*, *15:02* and *16:01*). These data (Fig. 3E, F and G) demonstrate that HLA-C*07 has the lowest expression and also suggest that in heterozygotes *HLA-C*07* can suppress expression of the other *HLA-C* allele.

M/T donors (Fig. 3F) exhibit more variety in *HLA-C* genotype and wider range of surface expression (<200 msi) than M/M donors (Fig. 3E). M/T donors with higher HLA-C expression (100–200 msi) than M/M donors typically have *HLA-C* alleles that are unaffected by miR-148a (Fig. 3F). Nonetheless, the dominant effect of *HLA-C*07* is still observed for M/T donors. This is not so for T/T donors, with their greater variety of *HLA-C* genotypes and expanded range (<300 msi) of surface expression (Fig. 3G). The cluster of high-expressing individuals comprises four $C2^+$HLA-C homozygotes, a genotype specific to T/T donors and two $C1^+$HLA-C homozygotes (fig. S6).

P2M HLA-B increases phenotypic diversity in the population of CD94:NKG2A$^+$ NK cells

Because -21M HLA-B acts to decrease the size of the CD94:$NKG2A^+$NK cell population (Fig. 2), we further investigated this subset of NK cells. For PBMC of 60 donors, the expression of 35 cell-surface markers (Table S1) was analyzed by mass cytometry. These data were subjected to hierarchical clustering analysis using SPADE (Fig. 4) (23, 24). Within the donor panel, the diversity of CD94:$NKG2A^+$ NK cells is considerable: individuals have 337–989 different phenotypes. In general, the CD94:$NKG2A^+$ NK cells of M/M donors have greater phenotypic than those of T/T donors ($p=2\times10^{-6}$; n=40; Mann-Whitney test) (Fig. 4A). M/T donors have highest phenotypic diversity, but their differences with M/M donors did not reach statistical significance. This analysis shows how -21M HLA-B has two important effects: reducing the size of the CD94:$NKG2A^+$ NK cell population and increases its phenotypic diversity.

-21M HLA-B copy number also influences the expression of other inhibitory receptors by CD94:$NKG2A^+$ NK cells. The frequencies of CD94:$NKG2A^+$ NK cells expressing one or more of KIR2DL1 (Fig. 4B), KIR3DL1 (Fig. 4C), KIR3DL2 (Fig. 4D) and LILRB1 (Fig. 4E) are highest in M/M donors, lowest in T/T donors and intermediate in M/T donors.

Because M/M donors lack Bw4 and C2 ligands, these data (Fig. 4) show that CD94:NKG2A$^+$ NK cells most frequently co-express KIR2DL1 and KIR3DL1 in the absence of their ligands. In T/T donors that have C2, or Bw4, the frequencies of CD94:NKG2A$^+$ NK cells expressing the C2-specific KIR2DL1, Bw4-specific KIR3DL1, are much reduced. During development and education, the acquisition of a self-reactive KIR by CD94:NKG2A$^+$ NK cells usually leads to downregulation and loss of surface CD94:NKG2A (25, 26).

SPADE analysis shows that -21M copy number correlates with increasing numbers of CD57-expressing CD94:NKG2A$^+$ NK cells (Fig. 5A). Qualitative differences were also observed. M/M and M/T donors have comparable CD57 expression by their subsets of CD94:NKG2A$^+$ NK cells expressing KIR2DL1, KIR3DL1, KIR3DL2 and LILRB1. The pattern is different for T/T donors, where CD57 is expressed by fewer subsets of CD94:NKG2A$^+$ NK cells. These subsets express KIR3DL2 and LILRB1, but little KIR2DL1 or KIR3DL1. Consistent with CD57 being characteristic of educated NK cells (27, 28), components of activating pathways - CD16, CD122, NKG2D and 2B4 - are expressed at significantly higher frequencies by CD57$^+$CD94:NKG2A$^+$ NK cells than CD57$^-$CD94:NKG2A$^+$ NK cells (Fig. 5B–G).

-21M HLA-B correlates with functionally stronger CD94:NKG2A$^+$ NK cells

Phenotypic analysis points to the small, diverse populations of CD94:NKG2A$^+$ NK cells of M/M and M/T donors being better educated and functionally more potent than the large, but less diverse populations present in T/T donors. This hypothesis was tested using three assays of NK-cell function. The first assay compared the capacity of CD3$^-$CD56^{dim} NK cells from M/M, M/T and T/T donors to mediate antibody-dependent cellular cytotoxicity (ADCC) (Fig. 6A). NK cells were challenged with Raji cells coated with anti-CD20 and assessed for cytotoxic activity, as measured by surface expression of CD107a. At antibody concentrations of 1.25–2.5μg/ml, NK cells from M/M donors made stronger, more sensitive ADCC responses than NK cells from T/T donors, with NK cells from M/T donors exhibiting intermediate responses (Fig. 6A).

The second assay assessed the IFN-γ response of NK cells activated by a fixed concentration of IL-12 (10 IU/ml) combined with different concentrations of IL-15 (Fig. 6B). At low IL-15 concentrations (0.001–1ng/ml) M/M donor NK cells produced more IFN-γ than NK cells from T/T donors ($p = 0.002$; n=40; Mann-Whitney test), with NK cells from M/T donors giving intermediate responses. At higher IL-15 concentrations, the trend was similar, but not statistically significant.

The third assay compared the missing-self response of NK cells from M/M, M/T and T/T donors (Fig. 6C and 6D). PBMC, cultured overnight with IL-12 and IL-15, were then challenged with MHC class I deficient K562 target cells. In assays of degranulation (Fig. 6C) and IFN-γ production (Fig. 6D), NK cells from M/M and M/T donors responded more effectively than NK cells from T/T donors. Correlating with these differences is an abundance of CD122 on the CD94:NKG2A$^+$ NK cell surface (Fig. 5C). In all three functional assays, the sensitivity and potency of the NK cell response correlates with presence of -21M HLA-B.

-21M HLA-B correlates with better educated CD94:NKG2A$^+$ NK cells

To examine how -21 HLA-B dimorphism influences NK cell education, ADDC assays were performed and the NK cells responding with IFN-γ or cytokicity (cell surface CD107a) were analyzed using mass cytometry and Boolean gating (Figure S9). CD57 expression was used to distinguish educated NK cells that are differentiated from those that are undifferentiated. Comparison of M/M, M/T and T/T donors identified significant differences in the size of several NK cell subsets, effects that differed between the cytokine producing NK cells and the cytotoxic NK cells (Figure 7A).

The subset of KIR$^-$CD94:NKG2A$^+$ NK cells are the NK cells that can only be educated by the inhibitory HLA-E receptor, CD94:NKG2A. The subset of KIR$^-$CD94:NKG2A$^+$ cells that produces IFN-γ in the ADCC assay is of similar size in M/M (33.3% of total NK cells), M/T (31.5%) and T/T (34.4%) donors (Fig. 7B). However, the extent to which these educated cells are differentiated (CD57$^+$) varies with the -21M copy number. In T/T donors 53.8% of the KIR$^-$CD94:NKG2A$^+$ cells are differentiated, whereas in M/M and M/T donors this rises to 82.8% in M/M donors and 81.3% in M/T donors ($p=0.00001$, n=40; Mann-Whitney test). The dominant effect of -21M is striking and shows that the additional HLA-E binding peptides contributed by one copy of -21M HLA-B are responsible for increasing the differentiated subpopulation of the educated KIR$^-$CD94: NKG2A$^+$ cells from just over half to more than 80%.

The KIR$^+$CD94:NKG2A$^-$ subset of NK cells are the NK cells that can only be educated by KIR. They are a small subset in M/M donors (14.3% of total NK cells), of intermediate size in M/T donors (28.2%) ($p=2\times10^{-8}$, M/T vs. T/T; n=40; Mann-Whitney test) and a larger subset in T/T donors (37.7%) ($p=2\times10^{-10}$, M/M vs. T/T; ; n=40; Mann-Whitney test). The proportions of these educated cells that become differentiated are 71.3% in M/M 74.8% in M/T and 81.1% in T/T donors. Here the major effect of -21 HLA-B dimorphism is to change the size of the KIR$^+$CD94:NKG2A$^-$ NK cell subset, and its effect on the extent of education and differentiation is minor. Unlike the dominant effect that -21M HLA-B exerts on CD94:NKG2A-mediated education, the effect of -21M HLA-B on KIR-educated NK cells is one of gene dosage. One copy of -21T HLA-B doubles the number of KIR$^+$CD94:NKG2A$^-$ NK cells and two copies further increases their number to 2.6 fold that seen in M/M donors.

The number of KIR$^-$CD94:NKG2A$^+$ NK cells that make a cytotoxic response (CD107a) in the ADCC assay is similar for the M/M (30.5%), M/T (26.2%) and T/T (26.5%) donors. Of these, the CD57$^+$ NK cells comprise 39.3% in M/M, 38.1% in M/T and 52% in T/T donors. This trend towards increased education of KIR$^-$CD94:NKG2A$^+$ NK cells with increased -21T HLA-B is the opposite of the effects observed for the IFN-γ producing KIR$^-$CD94:NKG2A$^+$ NK cells.

The proportion of NK cells in the KIR$^+$CD94:NKG2A$^-$ subset is 25.3% in M/M, 37.4% in M/T and 40% in T/T donors. Of these the CD57$^+$ cells comprise 66% in M/M, 59.9% in M/T and 52.7% in T/T donors. This trend towards increased education with -21T copy number is also the opposite to that observed for IFN-γ producing KIR$^+$CD94:NKG2A$^-$ NK cells. Overall, the observed effects of -21HLA-B dimorphism on cytotoxic NK cells are qualitatively different and quantitatively weaker than observed for cytokine-producing NK

cells. These differences are reflected in the comparisons of Figure 7A. For CD107A expression the significant correlations are seen only for NK cells expressing both KIR and CD94:NKG2A; cells for which the educating receptor is unknown. In contrast, for the IFN-γ producing NK cells, significant correlations were made for NK cells expressing only CD94:NKG2A or only KIR, and for which the educating receptor is defined.

-21 HLA-B dimorphism shapes the inhibitory NK cell receptor repertoire

For each donor we determined the frequency of $CD56^{dim}$ NK cells that express KIR2DL1, the inhibitory C2-specific receptor. For the three donor groups we see similarly wide frequency ranges and similar mean frequencies (Fig. 8A). For T/T donors, presence of the educating C2 ligand give a mean frequency of 23% $KIR2DL1^{+}$NK cells, compared to 17.4% for T/T donors who lack C2. For M/T donors an educating effect was barely detectable, 22.8% $KIR2DL1^{+}$NK cells of in $C2^{+}$ donors compared to 21.4% in $C2^{-}$ donors. Applying the same analysis to KIR2DL2/3, the inhibitory C1-specific receptor, gave similarly wide frequency ranges for $KIR2DL2/3^{+}$NK cells and comparable mean frequencies for the three donor groups (Fig. 8B). Because of the high C1 frequency, an educating effect could only be assessed for T/T donors. In the presence of C1, the mean frequency of $KIR2DL2/3^{+}$NK cells was 18.9%, compared to 13.3% in its absence. Thus presence of an educating C1 or C2 ligand increases the proportion of NK cells expressing the cognate KIR (29).

Within the $CD56^{dim}$ NK cell population, the combinatorial patterns of expression for six inhibitory HLA class I receptors (KIR2DL1, KIR2DL2/3, KIR3DL1, KIR3DL2, CD94:NKG2A and LILRB1) were determined by mass cytometry and Boolean gating analysis (Fig. 8C and S10). Of 64 possible phenotypes, 38 were present in all donors. 34 of these phenotypes accounts for >1% of the $CD56^{dim}$ NK cells. Having highest frequency (mean of 7.3%, 60 donors) is the null phenotype having no inhibitory receptors. The 33 other common phenotypes have between one and three inhibitory receptors. Thus phenotype comprising 4 or more inhibitory receptors are rare.

Comparison of the inhibitory receptor repertoires for the M/M and T/T donors identified four phenotypes that are significantly more abundant in M/M ($p = 0.0001$; n=40; Mann-Whitney test). These phenotypes, which are at low frequency in M/M donors and absent from T/T donors, combine either four or five inhibitory receptors (Fig. 8C). NK cells with these phenotypes are also present in M/T donors. Six phenotypes are significantly more abundant in T/T donors ($p = 0.00005$; n=40; Mann-Whitney test). These six phenotypes are well represented in M/M and T/T donors, but have higher frequencies, comparable to the null phenotype frequency, in T/T donors. Of the six phenotypes, two comprise one receptor: CD94:NKG2A alone and KIR3DL1 alone. Common to the other four phenotypes is CD94:NKG2A, which is paired in turn with each of the four inhibitory KIR. The increased abundance of NK cells having these six phenotypes is largely responsible for T/T donors having larger $CD94{:}NKG2A^{+}$NK cell populations than M/M donors. The striking differences between the NK cell subpopulations that are elevated in M/M and T/T donors reflect the mature, well educated NK cells of the former and the immature, less educated NK cells of the latter.

Discussion

Two structurally unrelated and functionally complementary forms of inhibitory HLA class I receptor shape the education and function of human NK cells (30, 31). The older and more conserved receptor, CD94:NKG2A, recognizes the complex of HLA-E and a nonamer peptide cleaved from the leader sequence of HLA-A, -B or –C (4, 5). A corresponding CD94:NKG2A receptor is present in mice, which recognizes the complex of Qa1 and an a nonamer peptide cleaved from the leader sequences of polymorphic H-2 polypeptides (32). More recently evolved than CD94:NKG2A are the highly diversified KIR, which recognize conformational determinants of HLA-A, -B and -C (the Bw4, C1 and C2 epitopes) and are present only in higher primates (33). Thus the KIR system of receptors and MHC class I ligands emerged and evolved within the context of an established system of CD94:NKG2A receptors that had been in place for more than 90 million years. In this setting, the epitopes recognized by KIR evolved in a stepwise fashion over a period of 40 million years, first the Bw4 epitope, then C1 and most recently C2 (33). By combining worldwide genetic analysis of human populations, with phylogenetic comparison and functional analysis, our study demonstrates how stepwise addition of each KIR ligand was accompanied by changes and adaptation in the way that NK cell education and response are mediated by CD94:NKG2A recognition of HLA-E.

The nonamer peptides that bind HLA-E have methionine at position 2, which corresponds to position -21 in the MHC class I leader sequence. Prior to emergence of the *KIR* gene family, and during its early expansion, methionine -21 was fixed in the leader sequences of polymorphic MHC class I. That is still true for HLA-A and -C, but not HLA-B. In ~80% of HLA-B allotypes, methionine -21 was replaced by threonine, which does not promote surface expression and recognition of HLA-E by CD94:NKG2A. Because of this functional dimorphism, HLA-B is predicted to make variable contributions to the pool of HLA-E binding peptides, whereas HLA-A and –C are not. We show here that surface expression of HLA-E is reduced by 16% in homozygous -21T HLA-B individuals compared to homozygous -21M HLA-B individuals. Although modest in magnitude, we find this effect has considerable impact on the education and function of human NK cells.

A striking bias is that eleven of twelve groups of Bw4^{+}HLA-B allotypes have -21T threonine, whereas the groups of Bw4^{-}HLA-B allotypes more evenly divide into ones having -21M and -21T. This linkage disequilibrium, between functional dimorphisms of the leader peptide and the α_1 domain of HLA-B, is consistent with there being selection against HLA-B allotypes that contribute both to CD94:NKG2A and KIR ligands.

Like Bw4^{+}HLA-B, C2^{+}HLA-C is rarely present on haplotypes having -21M HLA-B. Although such haplotypes have C1^{+}HLA-C and potential for C1-mediated NK cell education, it is reduced because their HLA-C alleles are subject to miR-148a-mediated transcriptional down-regulation. Thus there is linkage disequilibrium between -21M/T HLA-B dimorphism and dimorphism in the HLA-C 3'-untranslated region, which determines which HLA-C alleles are affected by miR-148a. The net effect is that -21M HLA-B haplotypes encode HLA-C that has lower cell-surface expression than HLA-C encoded by -21T HLA-B haplotypes.

Our population genetic and phylogenetic analyses identified two qualitatively different groups of *HLA class I* haplotype. Although defined by the simple methionine/threonine dimorphism at position -21 in the leader peptide, this dimorphism has had a profound impact on the evolution of the *HLA* complex and of human NK cell diversity. The subset of *HLA* haplotypes defined by -21M HLA-B increases cell-surface expression of HLA-E and its capacity to mediate NK cell education and effector function through interaction with CD94:NKG2A. In contrast, the group of *HLA* haplotypes defined by -21T decrease expression and functional potential of HLA-E, but increase the frequency and surface expression of the Bw4, C1 and C2 ligands and their potential to mediate NK cell education and effector function through engagement of KIR.

The dimorphism at position -21 of HLA-B defines three groups of individuals, M/M homozygotes, M/T heterozygotes and T/T homozygotes, that comprise about 6%, 32% and 62% of the human population, respectively. Each group exhibits functional differences, but the effect of -21M is so dominant that M/M and M/T individuals are similar, whereas T/T is quite different from both of them. We will therefore focus on comparison of M/M and T/T homozygotes.

M/M donors lack the Bw4 and C2 epitopes and in general have a more restricted set of HLA types than T/T donors. Their cells express low levels of $C1^{+}$HLA-C and high levels of HLA-E. Education through HLA-E produces a phenotypically diverse population of CD94:$NKG2A^{+}$ NK cells that express markers of activation, differentiation and education, such as CD16 and CD57. These NK cells are effective in mediating ADCC, in secreting cytokines and in killing HLA class I deficient cells with a ‘missing-self’ response; they are also responsive to cytokines, such as IL-15.

With their wide range of HLA types, T/T donors express C1, C2 and Bw4 in a variety of combinations. Their cells have higher levels of HLA-C and lower levels of HLA-E than the cells of M/M donors. Although T/T donors have larger populations of circulating CD94:$NKG2A^{+}$ NK cells than M/M donors, the T/T NK cells exhibit less phenotypic diversity than M/M NK cells. They also have higher surface expression of CD94:NKG2A and exhibit a generally immature and uneducated phenotype. T/T donors have an overall reduced ADCC and missing-self response at lower CD20 antibody concentrations, but their activated NK cells are predominantly expressing differentiated and educated $CD57^{+}KIR^{+}$ phenotypes. The common phenotypes for NK cells of T/T donors have only one or two of the six inhibitory HLA class I receptors (CD94:NKG2A, KIR2DL1, KIR2DL2/3, KIR3DL1, KIR3DL2 and LILRB1) consistent with the involvement of KIR, and possibly LIR, in NK cell education. By contrast, common phenotypes for the NK cells of M/M individuals can have four to five of the six inhibitory HLA class I receptors including multiple KIR. This pattern indicates that the KIR have not been subject to functional selection by their cognate ligand, and is consistent with CD94:NKG2A recognition of HLA-E being the principal mechanism for NK cell education in M/M individuals.

Immature $CD56^{dim}$CD94:$NKG2A^{+}$ NK cells, of the type characterizing T/T donors, can respond to autologous HIV-infected target cells with degranulation and secretion of the cytokines: IFN-γ and MIP-1β (34). By binding to CCR5, MIP-1β prevents HIV from

engaging its coreceptor, which inhibits both the progress and spread of infection (35). These observations suggest that CD94:NKG2A$^+$ NK cells of T/T donors could be more effective in responding to some types of infection, like HIV, than the more educated CD94:NKG2A$^+$ NK cells of M/M donors. Consistent with this thesis, are results from a study of heterosexual couples in which one partner is HIV infected and the other is not, despite exposure to the virus. Uninfected partners of T/T genotype were more resistant to infection, than uninfected partners of either M/T or M/M genotype. Moreover, the NKG2A$^+$ NK cells from uninfected T/T partners were more effective at killing autologous HIV-infected target cells *in vitro*, than the NKG2A$^+$ NK cells of uninfected M/T or M/M partners (7, 8). Here the effect of -21M is dominant and -21T is subordinate, the hierarchy observed throughout our analysis.

The characteristic CD56^{dim}CD94:NKG2A$^+$ NK cells of T/T donors are also implicated in controlling HIV viremia and providing protection from AIDS. These beneficial effects were first correlated with homozygosity for Bw4$^+$HLA-B (10). Subsequent appreciation that Bw4 correlates with -21T HLA-B led to a reanalysis of the clinical data which showed that the beneficial effects correlate well with -21T HLA-B homozygosity (9). Thus the correlation with Bw4 homozygosity can also be interpreted in terms of a mechanism involving HLA-E recognition by the CD56^{dim}CD94:NKG2A$^+$ cells of T/T donors, NK cells known to be effective against HIV (7, 8). Thus the protection associated with Bw4$^+$ HLA-B homozygosity could be mediated by KIR3DL1$^+$ cells or CD94:NKG2A$^+$ cells, or by their combination. Similarly, because C2$^+$HLA-C is also in linkage disequilibrium with -21T, disease associations with C2$^+$HLA-C could be mediated by direct interaction of the C2 epitope with the KIR2DL1 or KIR2LS1 NK cell receptors, by the effect of -21T HLA-B on HLA-E and CD94:NKG2A$^+$ NK cells, or by some combination of the three ligand-receptor interactions.

There are limitations to our study. The donor panel was not large in size and particular HLA class I alleles and haplotypes of European origin are highly represented. We did not type the KIR genes of the donor panel. There are known differences in cell-surface expression levels and in the strength of signaling associated with specific KIR alleles. Neither did we perform any studies to correlate the -21M/T HLA-B dimorphism with disease.

Here we have shown how two groups of *HLA B-C* haplotypes provide different schools of human NK cell education. The ancestral haplotype encodes -21M HLA-B and C1 and promotes NK cell education by CD94:NKG2A, HLA class I. In contrast, the younger haplotypes encode -21T HLA-B, in various combinations with Bw4, C1 and C2 and promote NK cell education mediated by KIR. The balance between the two groups of *B-C* haplotypes provides heterozygotes with a greater NK cell diversity than either homozygote.

Materials and Methods

Study Design

Regulating human NK cells are two distinctive HLA class I receptors: variable KIR that recognize polymorphic HLA-A, -B and –C and invariant CD94:NKG2A that recognizes the complex of HLA-E and a peptide cleaved from an HLA-A, -B and –C leader sequence. Dimorphism at position -21 of the HLA-B leader sequence was known to give rise to

functional HLA-E binding peptides with methionine (M) and non-functional peptides with threonine (T). The objective of this study was to define the genetics of this dimorphism in the context of the world's human population and its functional impact on NK cell mediated immunity. Population genetic analysis (using publicly available and well-curated HLA data) revealed linkage disequlibrium between the -21 HLA-B dimorphism and other HLA-B and -C dimorphisms that affect KIR recognition. Immunological studies, ex vivo phenotyping with mass cytometry and in vitro functional assays, were performed on PBMC from a 60 donor panel giving equal representation of the three key genotypes: M/M, M/T and T/T. All donors were CMV negative. The sample size was determined to be large enough to give representative sampling of donors with different HLA genotypes, but of a size that was feasible for the functional assays. Randomization and blinding were not used in this observational study. Not all ex vivo experiments were run in duplicate and functional assays were reported based on mean averages. With this approach we were able to demonstrate how the different HLA haplotype groups educate human NK cells: one by CD94:NKG2A, the other by KIR.

Donors and samples

Peripheral blood mononuclear cells (PBMC) were obtained from 60 healthy individuals and cryopreserved. These samples were the source of the NK cells analyzed. PBMC were isolated from leukocyte reduction system (LRS) chambers through Ficoll density-gradient centrifugation. LRS chambers were purchased from the Stanford Blood Center. Age and ethnicities for all donors are shown in Fig. S6.

HLA Genotyping and CMV serology

Serological CMV typing and *HLA-A and -B* genotyping of the sixty healthy individuals were determined at the Stanford Blood Center. *HLA-C* genotypes were determined by polymerase chain reaction-based sequence-specific oligonucleotide probe with a Luminex 100 instrument (Luminex Corp.). HLA-E genotyping was generated by polymerase chain reaction using primers: HLA-E*01:01F (CGAGCTGGGGCCCGACA), HLA-E*01:03F (CGAGCTGGGGCCCGACG) and HLA-E*01R (TTCCAGGTAGGCTCTCTGG) HLA-A, -B, -C and -E genotypes and NK cell receptor ligand assignments are shown in Fig. S6.

Functional assays

Three assays of NK cell function were performed. The first test measured the ADCC response of NK cells after challenge and culture with Raji cells coated with anti-CD20Ab. In the second test, PBMC were cultured overnight in the presence of IL-12, IL-15 or IL-12 and IL-15. In the third test, PBMC previously cultured in the presence of IL-12 and/or IL-15 were further challenged and cultured with class I-deficient K562 cells. PBMC (5×10^5) were mixed with K562 or Raji cells at a ratio of 10:1 in V-bottom 96-well plates, centrifuged at 1000 rpm for 3 min, and incubated at 37C for 5 h. Brefeldin A and monensin (both from BD Biosciences) were added to cultures after 1 h. For K562 and cytokine stimulation, PBMC cultured in complete medium and nothing else were used as the negative control. For ADCC assays, Raji cells (2×10^6/ml) were pre-coated with either Rituximab (Genentech) at 10 μg/ml or with murine IgG at 10 μg/ml for 30 min. Raji cells were washed in RPMI 1640 containing 10% heat-inactivated FBS and then mixed with PBMC. ADCC assays were also

repeated at a single concentration of anti-human CD20 antibody [2.5mg/ml], and cells were stained for mass cytometric analysis. We gated on all IFN-γ^+ and CD107a$^+$ NK cells and then performed Boolean gating analysis using NKG2A, KIR2DL1, KIR2DL2/L3/S2, KIR3DL1, KIR3DL2 and LILRB1 (Figure 7 and S9). We grouped NK cells into subsets based on presence or absence of CD57 followed by any KIR and LILRB1 (collectively termed KIR$^+$) or NKG2A.

Staining, data acquisition, and analysis

PBMC samples were thawed and washed with RPMI-1640 (Corning Cellgro) containing 10% fetal bovine serum (heat-inactivated), 2mM L-Glutamine and antibiotics (penicillin [100 units/ml] and streptomycin [100mg/ml]) (Gibco BRL/Life technologies) and incubated at 37°C with 5% CO_2 for 4 hours. Four to eight million PBMC were stained for mass cytometry analyses, which were performed as described (24) using the 39 antibodies listed in Table S1 as well as cisplatin (used as a cell viability reagent). NK cells were identified with a serial gating strategy as described (36) (Figure S7). The data were acquired with a CyTOF 2® instrument (Fluidigm, Inc.) and analyzed using FlowJo software v9.4.8 (Treestar, Inc) and Cytobank (Cytobank, Inc). Spanning-tree progression analysis of density normalized events (SPADE) analyses were performed on 20,000 CD94:NKG2A$^+$ NK cells from each donor using Cytobank and were restricted to a limit of 1,000 phenotypes. For functional assays, NK cells were gated on using anti-human antibodies (BD Biosciences): CD3 FITC (UCHT1), CD56 PE-Cy7 (NCAM 16.2). NK cell function was measured by using antibodies (BD Biosciences): CD107a PE (H4A3) and IFN-γ APC (4S.B3). Samples were acquired on a BD Accuri flow cytometer (BD Biosciences).

Effects of NKG2C expression and/or CMV serostatus on expression of CD94:NKG2A

Historical data comparing the frequency of NKG2A$^+$ and NKG2C$^+$ NK cells from 204 healthy donors (37) was used to corroborate our observation of the decrease in frequency and signal intensity of CD94:NKG2A staining associating with increased number of P2M leader peptides (Figure S11). A cut-off value of 3% CD56^{dim} NK cells expressing CD94:NKG2C was used as selection criteria for the sixty individuals in this current study.

Antibody Conjugation

Antibodies were purchased from the companies specified in Table S1 and labeled using Maxpar-X8 labeling reagent kits (Fluidigm) according to the manufacturer's instructions and as previously reported (36).

Immunogenetic and Statistical Analyses

Statistical analyses were performed using Excel (Microsoft Corp), Prism v5 (GraphPad Software, Inc.) and the Open Source statistical package R (http://www.r-project.org); ISBN 3-900051-07-0). Sequence data used in analyses of leader sequence peptides was publicly available from the immunopolymorphism database (www.ebi.ac.uk/ipd/). HLA-A,-B and -C genotype dataused as input for haplotype analyses was publicly available from the National Center for Biotechnology Information (NCBI) database, dbSNP (http://www.ncbi.nlm.nih.gov/SNP) as well as from Allele*Frequencies

(www.allelefrequencies.net). *HLA B-C and A-B* haplotype frequency and linkage disequilibrium (LD) analyses were performed using the Genetic Analysis Package (GAP) in R. Briefly, haplotypes were first inferred from genotypes using the genecounting function, *gc.em*. The haplotype frequencies were then used as input for generating LD and chi-square statistics using the function, *LD22*. P values for LD measurement (D') were calculated using chi-square test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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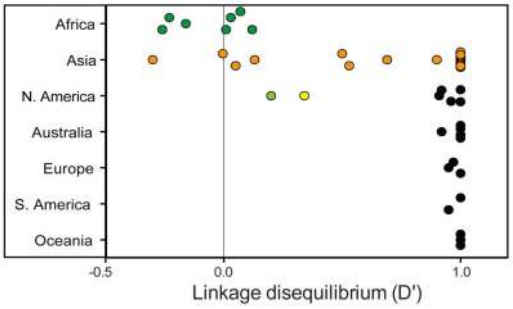
A

Population group	Proportion of total *HLA B–C* haplotypes			
	HLA-C2		HLA-C1	
	HLA-B -21M	HLA-B -21T	HLA-B -21M	HLA-B -21T
African	0.12	0.39	0.13	0.36
European	0.01	0.39	0.27	0.33
Asian	0.01	0.18	0.19	0.62
Australian	0.0	0.56	0.05	0.39
Oceanian	0.0	0.25	0.22	0.53
North American	0.0	0.37	0.22	0.41
South American	0.0	0.35	0.31	0.34

B



C

Five HLA-B -21M — C2⁺HLA-C haplotypes			
Haplotype group	Haplotype	Population group	Group characteristics
I	B*42:01 — C*17:01	Africans	African-specific HLA-B and HLA-C alleles
	B*81:01 — C*18:01		
II	B*81:01 — C*04:01		African-specific HLA-B allele and widespread HLA-C allele
III	B*73:01 — C*15:05	Asians	All or part of the haplotype arising by introgression from archaic humans
	B*07:05 — C*15:05		

D

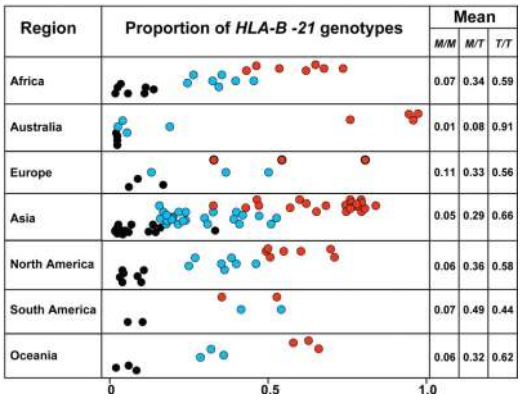


Figure 1. Alleles encoding HLA-B -21M and C2^{+}HLA-C segregate on different haplotypes outside of Africa

A. The C1/C2 dimorphism at position 80 of HLA-C and the -21 M/T dimorphism of HLA-B define four *HLA B-C* haplotypes. Shown are the proportions of each haplotype in major human population groups: 51 populations and 16,384 *HLA* haplotypes were analyzed.

B. plots the linkage disequilibrium (D') between the *HLA-B* and *HLA-C* dimorphisms: Africans (dark green circles), Asians (orange circles), admixed US populations of African ancestry (light green circles) and Asian ancestry (yellow circles), all other populations (black circles). The haplotype frequencies used to calculate D' are given in Figure S1.

C. Described are characteristics of the five haplotypes that encode -21M HLA-B and $C2^{+}$HLA-C.

D. Frequencies of the three -*21 HLA-B* genotypes in major population groups: -*21T/T* homozygotes (red circles), -*21M/T* heterozygotes (blue circles) and-*21M/M* homozygotes (black circles). (8,192 genotypes were analyzed)

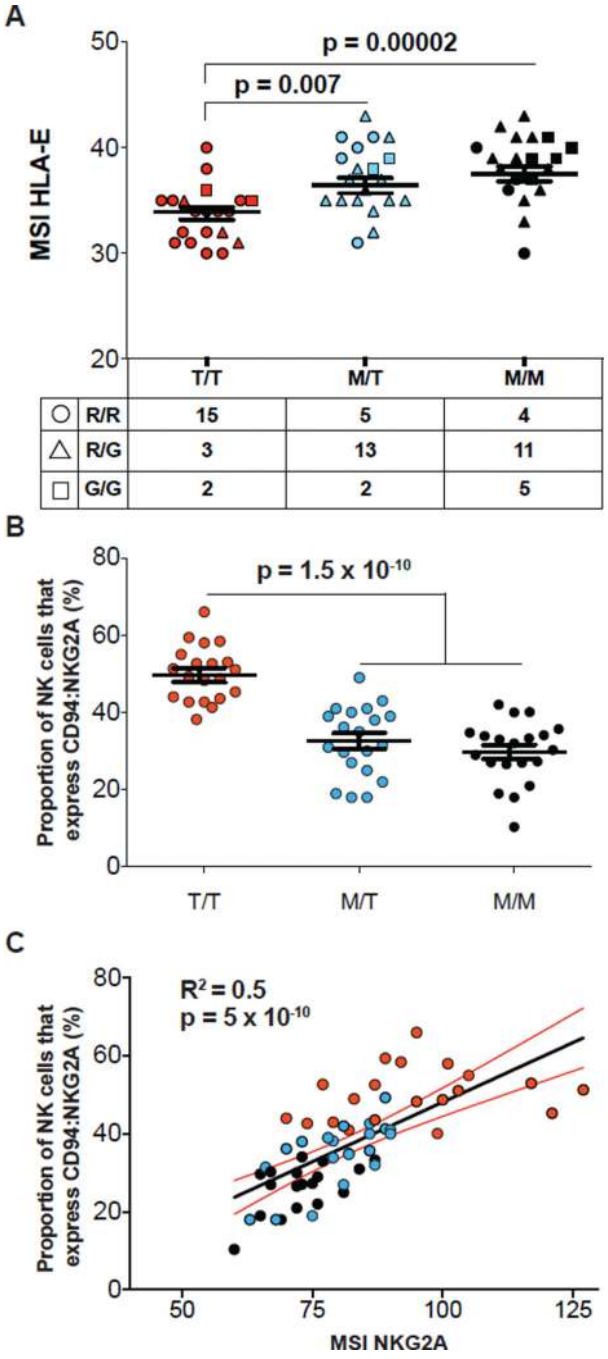


Figure 2. -21 HLA-B dimorphism modulates expression of HLA-E and CD94:NKG2A

Mass cytometry analysis of PBMC from 20 donor cohorts defined by -21 *HLA-B* genotype: homozygous -21T (T/T) red circles, heterozygous -21M/T (M/T) blue circles and homozygous -21M (M/M) black circles.

A. Compares the cell-surface HLA-E expression given by the median signal intensity (msi). Donors are also defined by 107 HLA-E genotype: homozygous 107 R (R/R) circles, heterozygous 107 R/G (M/T) triangles and homozygous 107 G (G/G) squares.

B. Compares the frequency of CD94:NKG2A^{+}NK cells.

C. Compares the proportion (%) of NK cells expressing CD94:NKG2A with their level of surface expression (msi). The thick black line shows the goodness of fit (R^2) determined by Spearman correlation analysis. The thin red lines denote the 95% confidence interval (range 0.44–0.77). The correlation coefficient (R^2) is shown with a two-tailed p value. All donors were CMV negative.

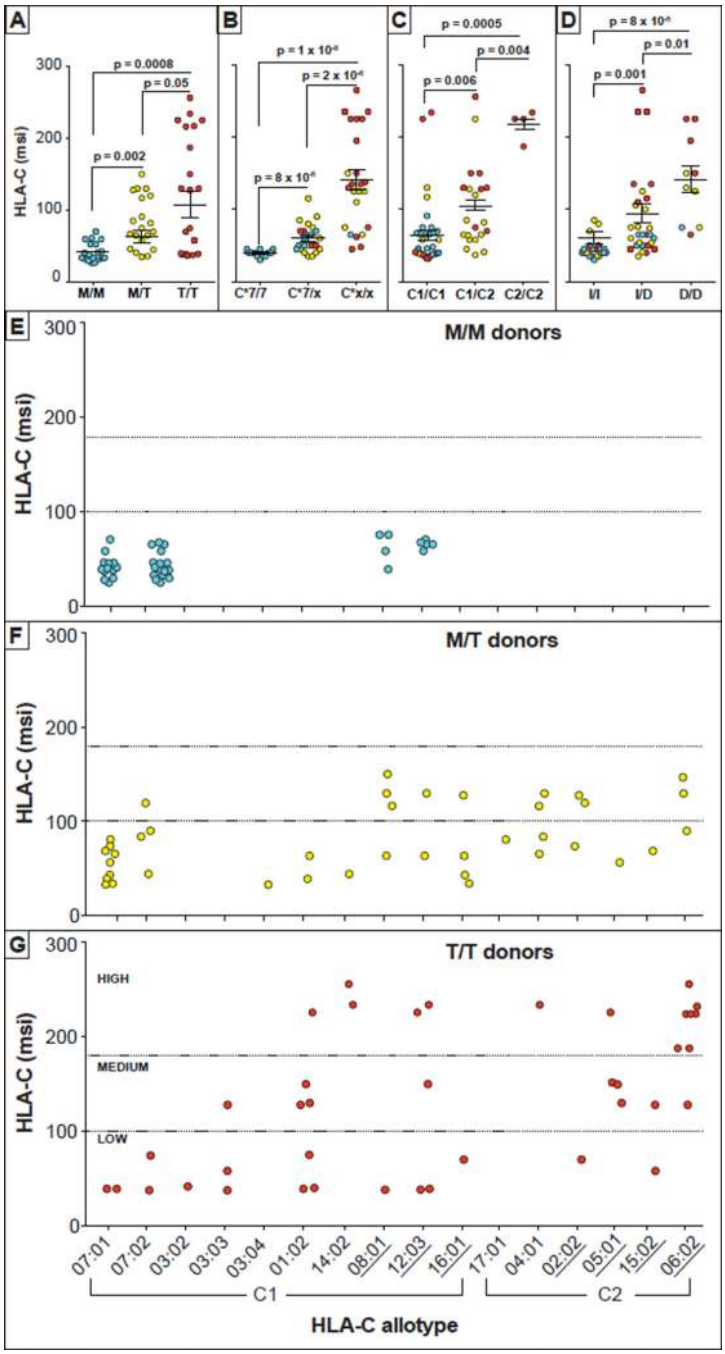


Figure 3. -21 HLA-B dimorphism correlates with differences in HLA-C expression

Surface expression of HLA-C by donor PBMC was determined by flow cytometry and quantified by median signal intensity (msi).

A. Donors are grouped by -21M HLA-B genotype.

B. Donors are grouped by presence and absence (x) of HLA-C*07.

C. Donors are grouped according to the C1 and C2 epitopes of HLA-C.

D. Donors grouped by genotype of the miRNA-148a binding site in the 3'-untranslated region of *HLA-C*: 'I' is the intact functional allele, 'D' is the non-functional allele that

contains a deletion. Statistical significance was determined by two-tailed unpaired Mann Whitney tests and given as mean p values ± standard error.

E, F and G compares the surface expression of HLA-C, with HLA-C genotype for M/M donors, M/T donors and T/T donors, respectively. Every donor is represented by two red circles one for each *HLA-C* allele, and placed above the allele's name. *HLA-C* homozygotes have two circles with identical msi in the same column.

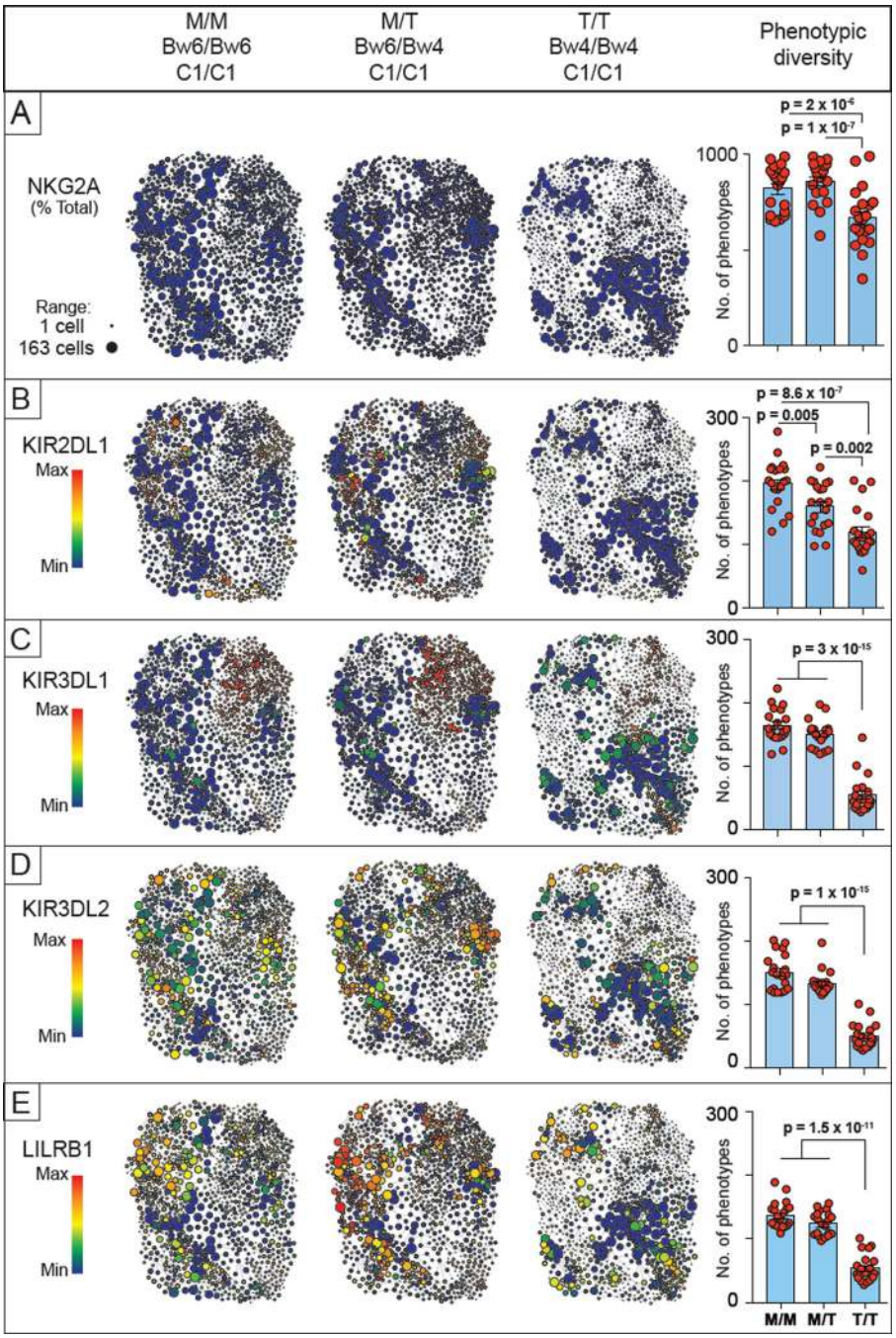


Figure 4. -21 HLA-B dimorphism modulates the co-expression of inhibitory receptors by educated CD94:NKG2A$^+$ NK cells

A. NK cell expression of CD4:NKG2A.

B. Coexpression of KIR2DL1 and CD94:NKG2A.

C. Coexpression of KIR3DL1 and CD94:NKG2A.

D. Coexpression of KIR3DL2 and CD94:NKG2A.

E. Coexpression of LILRB1 and CD94:NKG2A.

In each panel there are three diagrams representing Spanning-tree Progression Analysis of Density normalized Events (SPADE) for CD94:NKG2A$^+$NK cells. Each NK cell

subpopulation is represented by a circle, its area corresponding to the subpopulation's size. Shown are data for representative M/M (*left*), M/T (*left center*) and T/T donors (*right center*). On the right are histograms showing the phenotype diversity in the three donor cohorts. Phenotypic data was obtained by mass cytometric analysis using antibodies specific for 35 cell-surface markers.

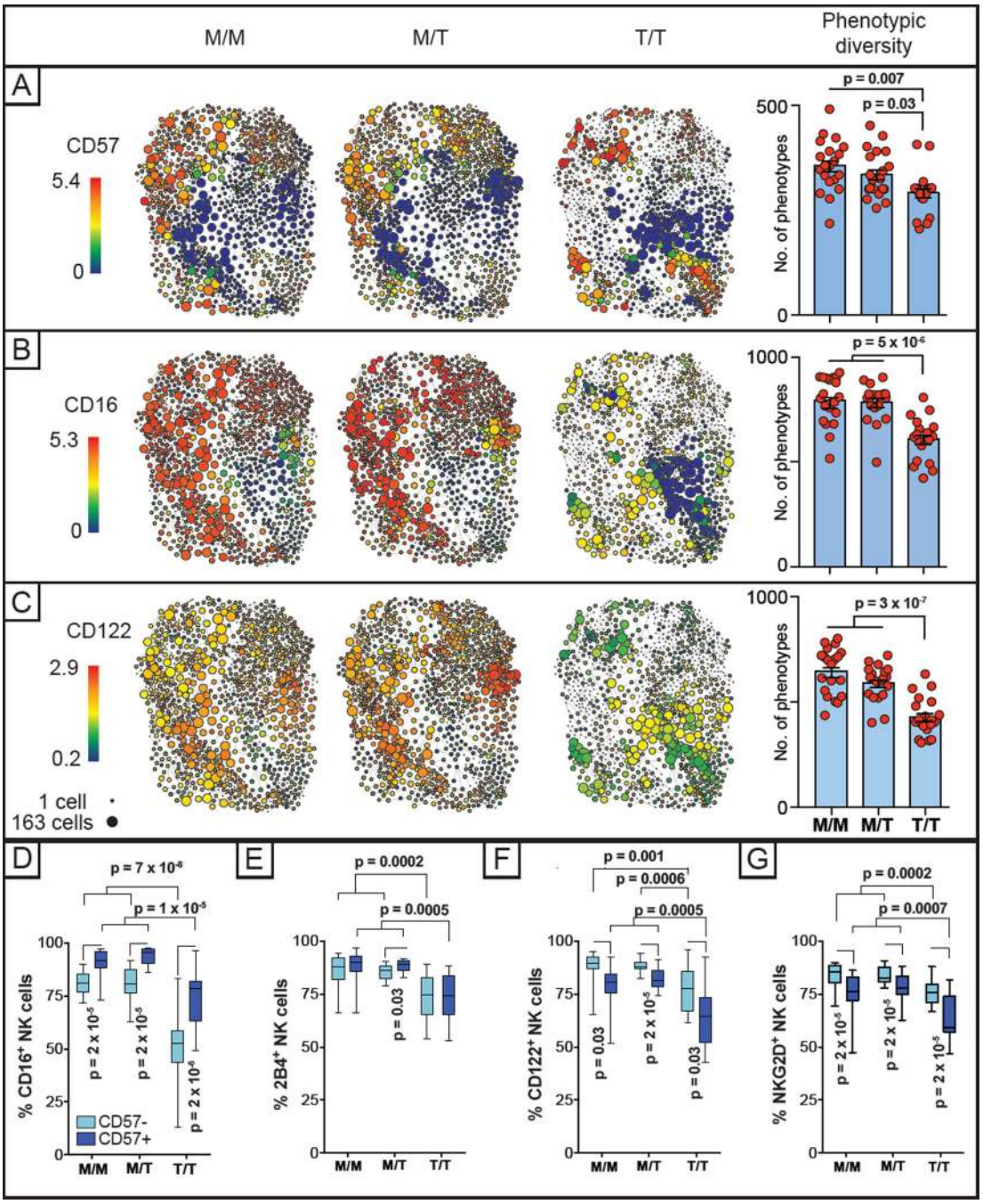


Figure 5. -21M HLA-B drives expansion of educated CD94:NKG2A$^+$ NK cells expressing enhanced levels of activating receptors

SPADE for three representative M/M (*left*), M/T (c*enter left*) and T/T (*center right*) donors combined with histograms giving the phenotypic diversity of CD94:NKG2A$^+$ NK cells in the cohorts of 20 M/M, M/T and T/T donors (right).

A. CD57-expressing CD94:NKG2A$^+$ NK cells.

B. CD16-expressing CD94:NKG2A$^+$ NK cells.

C. CD122 CD57-expressing CD94:NKG2A$^+$ NK cells.

D. Frequencies of CD57$^-$ and CD57$^+$ CD94:NKG2A$^+$ NK cells.

E. Frequencies of $CD16^-$ and $CD16^+$ CD94:$NKG2A^+$ NK cells.
F. Frequencies of $2B4^-$ and $2B4^+$ CD94:$NKG2A^+$ NK cells.
G. Frequencies of $NKG2D^-$ and $NKG2D^+$ CD94:$NKG2A^+$ NK cells.
Statistical significance (p values) derives derived from two-tailed unpaired Mann Whitney (A–C) and paired Wilcoxon (D–G) tests (mean ± SE).

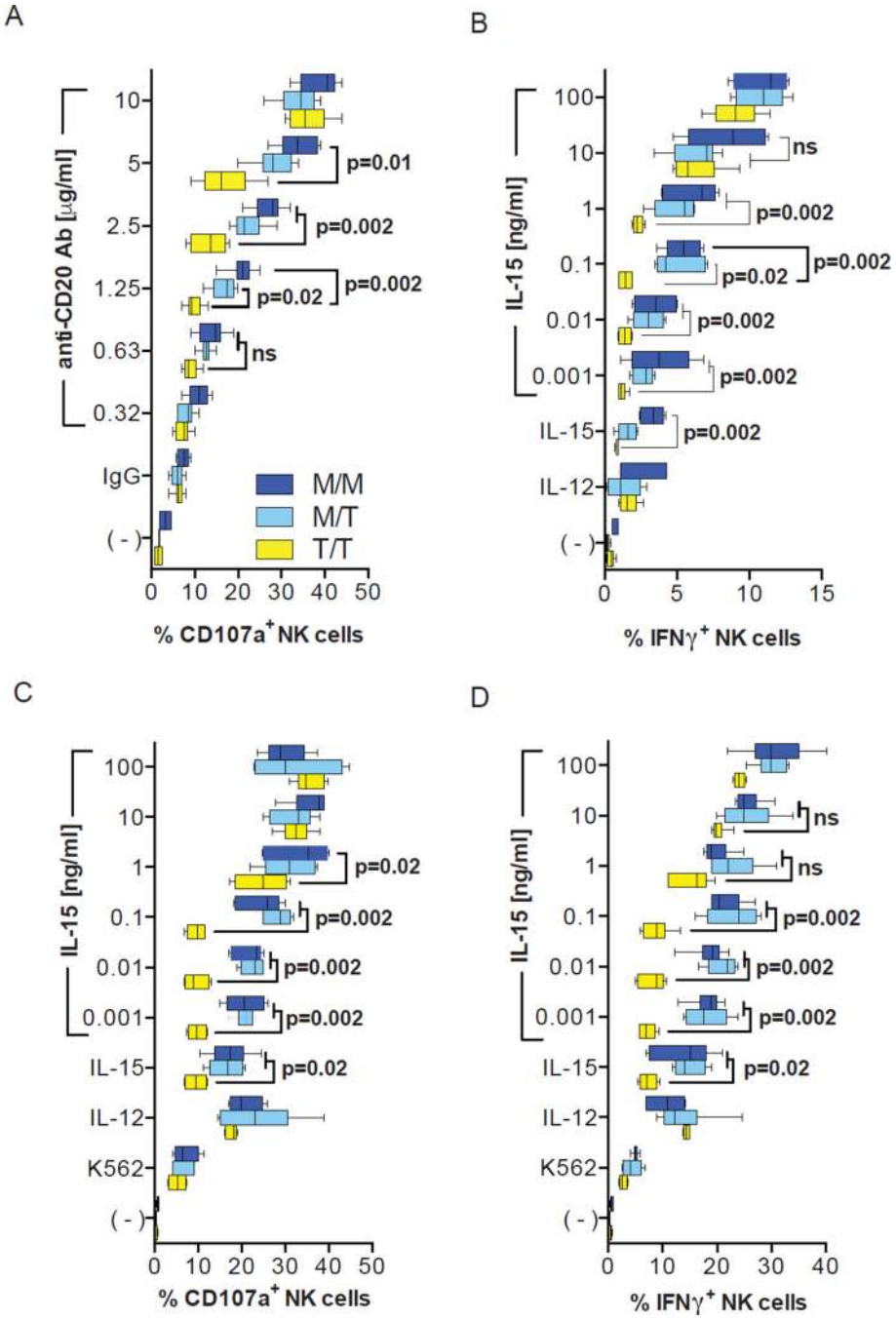
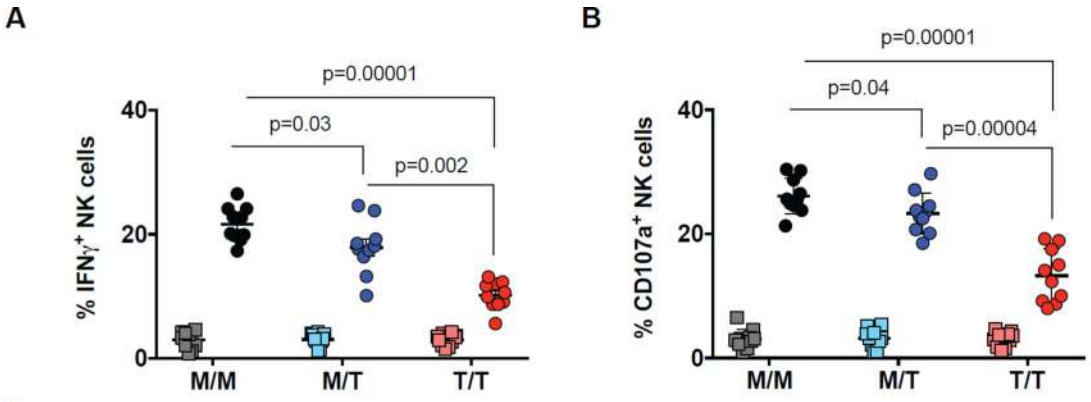


Figure 6. -21 HLA-B dimorphism modulates NK cell effector function

A. PBMC were cultured for 6 hours in an ADCC assay with Raji cells (E:T = 10:1) coated with with anti-CD20 Ab or murine IgG (IgG) (range: 0.32–10μg/ml) and the $CD3^{-}CD56^{dim}$ NK cells assayed for surface expression of CD107a.

B. PBMC were cultured for 18 hours with cytokines and the frequency of IFN-γ^{+} $CD3^{-}CD56^{dim}$ NK cells determined. Cytokines were IL-12, IL-15, or IL-12 and IL-15 (range: 0.001–100ng/ml).

C and D. PBMC were cultured with Raji cells for 18 hours and then with K562 cells for 6 hours (E:T = 10:1). $CD3^{-}CD56^{dim}$ NK cells were assayed for CD107a (C) or IFN-γ (D). PBMC from all 60 donors were included in these analyses. M/M donors (*dark blue boxes*) M/T donors (*light blue*) and T/T donors (*yellow*). Statistical significance (p values) derives from two-tailed unpaired Mann Whitney tests (mean ± SE).



C

NK cell phenotype	Percentage of NK cells:		M/M vs. T/T: Mann Whitney test (p value)	
	IFN-γ+	CD107a+	IFN-γ+	CD107a+
CD57- KIR- CD94:NKG2A+				
M/M donors	5.7 ± 0.8	18.2 ± 2.1	**0.00005**	0.1
T/T donors	15.9 ± 1.7	12.7 ± 1.4		
CD57+ KIR- CD94:NKG2A+				
M/M donors	27.6 ± 2.1	12.0 ± 1.7	**0.0008**	0.57
T/T donors	16.5 ± 1.9	13.8 ± 2.1		
CD57- KIR+ CD94:NKG2A+				
M/M donors	10.3 ± 0.9	11.8 ± 1.3	0.27	0.42
T/T donors	13.2 ± 1.8	10.0 ± 1.1		
CD57+ KIR+ CD94:NKG2A+				
M/M donors	40.0 ± 2.2	30.5 ± 2.9	**0.00001**	**0.01**
T/T donors	14.5 ± 2.4	21.0 ± 1.8		
CD57- KIR+ CD94:NKG2A-				
M/M donors	4.1 ± 0.7	8.8 ± 1.7	0.25	**0.0004**
T/T donors	7.1 ± 1.6	19.2 ± 1.6		
CD57+ KIR+ CD94:NKG2A-				
M/M donors	10.2 ± 0.9	16.7 ± 2.4	**0.00001**	0.13
T/T donors	30.6 ± 0.8	21.4 ± 1.9		
CD57- KIR- CD94:NKG2A-				
M/M donors	2.1 ± 0.2	2.0 ± 0.3	0.24	0.73
T/T donors	1.8 ± 0.3	1.9 ± 0.4		

Figure 7. Substantial influences of -21 HLA-B dimorphism on NK cell education

A and B. In the ADCC assay, PBMC from 30 donors (10 M/M, 10 M/T and 10 T/T donors) were cultured for 6 hours with Raji cells (E:T = 10:1) coated with murine IgG (IgG) (squares) or with anti-CD20 Ab (circles) (2.5μg/ml). $CD3^{-}CD56^{dim}$ NK cells were assayed for IFN-γ (A) or CD107a (B).

C. Using mass cytometry analysis and Boolean gating, the populations of IFN-$\gamma^{+}CD3^{-}CD56^{dim}$ NK cells and $CD107a^{+}CD3^{-}CD56^{dim}$ NK cells were each divided into eight subpopulations. Listed in the table, under the percentage of IFN-γ^{+} and $CD107a^{+}$ NK

cells, is the relative size of each subpopulation ± SE. Differences in the frequencies of these subpopulations in M/M and T/T donors were assessed with Mann-Whitney tests. Statistically significant differences are denoted with bold text. The gating strategy used to define the seven NK cell subsets is shown in fig. S9.

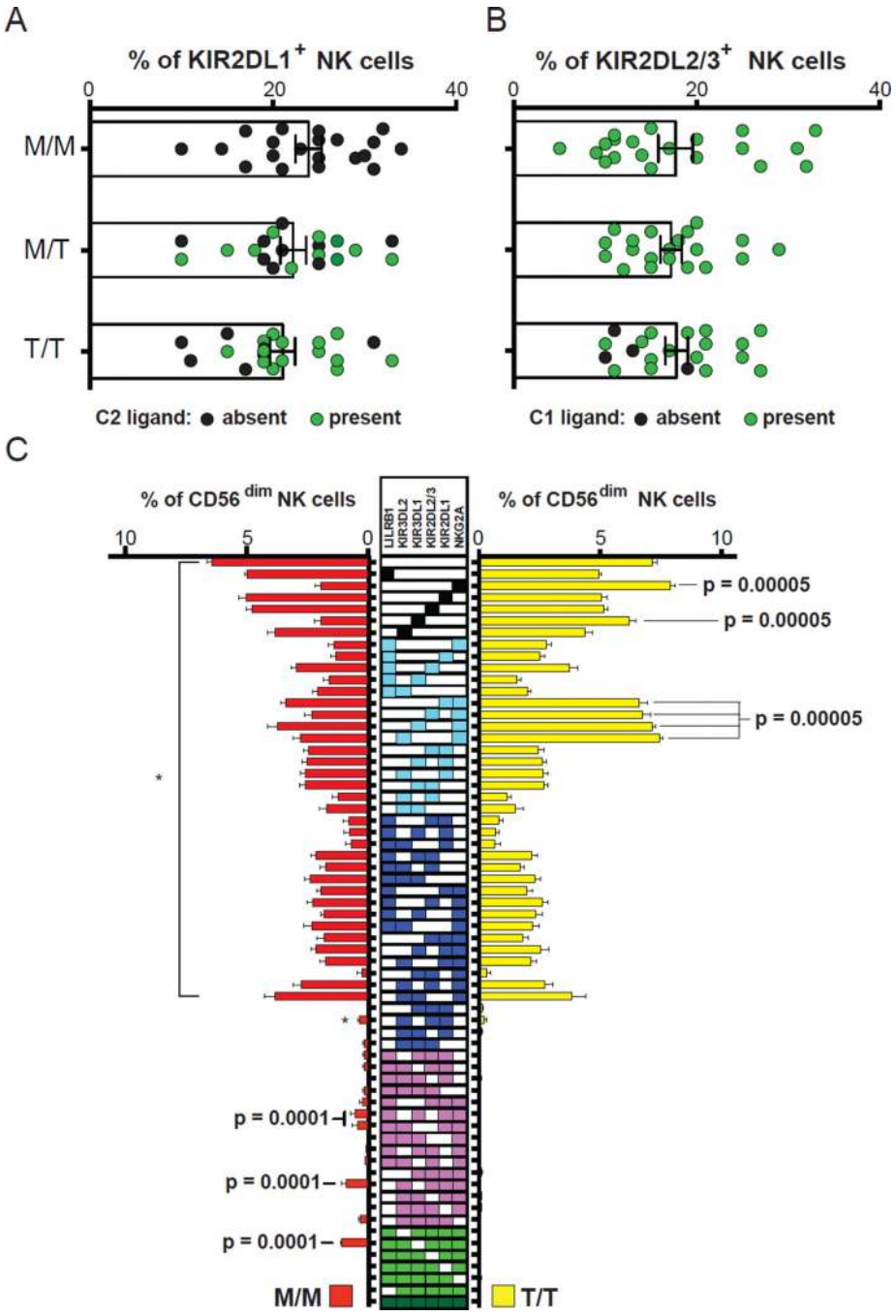


Figure 8. Substantial influence of -21 HLA-B dimorphism on the NK cell repertoire of expressed inhibitory HLA class I receptors

A. The frequencies of CD56^{dim} NK cells expressing C2-specifc KIR2DL1 in M/M, M/T and T/T donors. The color of a data point indicates the presence (green) or absence (black) of the C2 epitope in a donor.

B. The frequencies of CD56^{dim} NK cells expressing C1-specifc KIR2DL2DL3 in M/M, M/T and T/T donors. The color of a data point indicates the presence (green) or absence (black) of the C1 epitope in a donor.

C. The frequency in M/M (red bars) and T/T (yellow bars) of NK cells expressing the 64 phenotypic combinations of six inhibitory receptors that recognize HLA class I: LILRB1, KIR3DL2, KIR3DL1, KIR2DL2/3, KIR2DL1 and CD94:NKG2A. The asterisks indicate the 39 phenotypes that are present in all 60 donors. Data represent means (± SE) from 20 T/T and 20 M/M donors. *p* values are derived from unpaired Mann-Whitney tests to test for differences between M/M and T/T donors.