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Chimpanzees Use More Varied Receptors and Ligands Than Humans for Inhibitory Killer Cell Ig-Like Receptor Recognition of the MHC-C1 and MHC-C2 Epitopes

Achim K. Moesta, Laurent Abi-Rached, Paul J. Norman, and Peter Parham

Humans and chimpanzees have orthologous MHC class I, but few orthologous killer cell Ig-like receptors (KIR). Most divergent are lineage III KIR, which in humans include the inhibitory KIR2DL1 and 2DL2/3 specific for HLA-C. Six lineage III chimpanzee KIR were identified as candidate inhibitory MHC-C receptors and studied using cytolytic assays, to assess the capacity of a defined KIR to function with a defined MHC class I allotype, and direct binding assays with KIR-Fc fusion proteins. Pt-KIR2DL6 and 2DL8 were demonstrated to be inhibitory C2 receptors with a specificity and specificity-determining residue (lysine 44) like KIR2DL3. Analogously, Pt-KIR2DL7 is like KIR2DL1, an inhibitory C2 receptor having methionine 44. Pt-KIR3DL4 and 3DL5 are unusual lineage III KIR with D0 domains, which are also inhibitory C2 receptors with methionine 44. Removal of D0 from KIR3DL, or its addition to KIR2DL2, had no effect on KIR function. Pt-KIR2DL9, a fourth inhibitory C2 receptor, has glutamate 44, a previously uncharacterized specificity-determining residue that is absent from human KIR. Reconstruction of the ancestral hominoid KIR sequence shows it encoded lysine 44, indicating that KIR have methionine 44 and glutamate 44 subsequently evolved by independent point substitutions. Thus, MHC-C2-specific KIR have evolved independently on at least two occasions. None of the six chimpanzee KIR studied resembles KIR2DL2, which interacts strongly with C1 and cross-reacts with C2. Whereas human HLA-B allotypes that have functional C1 epitopes are either rare (HLA-B*73) or geographically localized (HLA-B*46), some 25% of Patr-B allotypes have the C1 epitope and are functional KIR ligands.

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atural killer cells are implicated in several functions: innate immunity to viral infection (1), recruitment of adaptive immunity (2, 3), immune surveillance for cancer (4), implantation during pregnancy (5, 6), and graft-vs-leukemia effects following hematopoietic stem cell transplantation (7, 8). Evidence for the diverse and varying selective forces that have acted on NK cells is the variety of their cell surface receptors, in particular the families of inhibitory receptors that recognize polymorphic determinants of MHC class I molecules. Two structurally unrelated types of receptor have independently adapted and expanded to fulfill this role: the lectin-like Ly49 receptors and the killer cell Ig-like receptors (KIR). Mammalian species differ widely in their use of these receptors. In primates and cattle, diverse KIR families have evolved (9), whereas Ly49 remains a single copy gene that in humans became nonfunctional. Conversely, rodents (10, 11) and horses (12) have diverse Ly49 receptors, but little or no expansion of KIR.

All KIR derive from one of two ancient forms, KIR3DL and KIR3DX, which diverged from a common ancestor ~135 million years ago, long before the diversification of modern placental mammals (9). In cattle, KIR diversity derives from KIR3DX, whereas KIR3DL remained a single-copy gene. Conversely, primate KIR diversity derives from KIR3DL, whereas KIR3DX remained a single-copy gene that in humans is nonfunctional (9). The diversity of KIR in cattle and primates, which initially seemed similar, is now seen to represent independent expansions of different progenitor genes. Thus, from the available data, expansion of the KIR3DL genes appears restricted to primates. Even among primates there is extensive species-specific variation (13–15), such that the most informative comparisons have come from studying the chimpanzee (16), the human species’ closest living relative.

Four of the 15 human KIR genes encode inhibitory receptors specific for polymorphic determinants of HLA-A, B, and C (17–20). Phylogenetically, these receptors are of two distinct lineages. In lineage II is KIR3DL1 that recognizes Bw4 epitopes of HLA-A and B (21), and KIR3DL2 that recognizes HLA-A3 and A11 (19, 22); lineage III encompasses KIR2DL1 and KIR2DL2/3, which are specific for the mutually exclusive C2 and C1 epitopes of HLA-C defined by residue 80 of the α1 helix (C1, asparagine; C2, lysine) (23, 24). Whereas all individuals can combine at least one HLA-C epitope with its cognate KIR, it is not the case for HLA-A- and B-specific KIR, because their target epitopes are carried by only a minority of the allotypes (25, 26). Consequently, the functional effects of the inhibitory KIR are dominated by the HLA-C-specific receptors (27).

Chimpanzees have fixed Patr-A, B, and C genes that are orthologous to HLA-A, B, and C (28). Although no individual alleles are shared, Patr-C allotypes carry the C1 and C2 epitopes like HLA-C; some Patr-B allotypes carry the Bw4 epitope like HLA-B; and some Patr-A allotypes resemble HLA-A3 and A11. Although
the genetic complexity of the chimpanzee KIR system is comparable to the human one, only three KIR genes are shared (KIR2DL4, 2DL5, and 2DS4), and they do not include ones specifying inhibitory MHC-A, B, or C receptors (16). Functional analysis identified clones of chimpanzee NK cells with patterns of alloreactive cytolysis indicating expression of inhibitory C1 or C2 receptors (16). Analysis of cDNA from these cells correlated C1 specificity with Pt-KIR2DL6 and C2 specificity with Pt-KIR3DL4. Although both belong to lineage III, neither KIR closely resembled its functional human counterpart, nor did their reactivity with anti-KIR Abs. Therefore, we performed this study to directly define the functional specificities of Pt-KIR2DL6 and 3DL4, and also those of other putative inhibitory MHC-C receptors that have emerged from genomic studies.

Materials and Methods

Chimpanzee KIR

Chimpanzee KIR, Pt-KIR3DL4, Pt-KIR3DL5, and Pt-KIR2DL6 were described in a previous report from our laboratory (16). Additional receptors were described during the characterization of three KIR haplotypes isolated from two chimpanzees (29) (L. Abi-Rached, manuscript in preparation) and isolated from cDNA of those individuals (Clint and Donald). KIR typing a panel of 22 unrelated individuals indicates that Pt-KIR2DL6, Pt-KIR2DL8, Pt-KIR3DL4, and Pt-KIR3DL5 are very common (phenotypic frequency >80%); Pt-KIR2DL9 has intermediate frequency (phenotypic frequency ~40%); and Pt-KIR2DL7 is less common (phenotypic frequency ~10%) (L. Abi-Rached, manuscript in preparation).

Cell lines and Abs

NKL, a leukemia-derived cell line, was maintained as described (30). KIR were individually expressed in NKL using lentiviral delivery, as described (31). Full-length coding regions of chimpanzee KIR were amplified by PCR from cDNA clones and cloned into the pBMN-I-GFP retroviral vector. Domain-addition and domain-deletion mutants were generated using two-step rPCR and subsequently cloned into pBMN-I-GFP. Retrovirus was generated in the packaging cell line 293T cells using standard protocols.

FIGURE 1. Chimpanzees have inhibitory KIR that distinguish MHC-C1 and MHC-C2 allotypes similarly to human KIR2DL1 and KIR2DL3. Shown are the results of cytotoxicity assays in which the target cells were 221 cells expressing C1 (left panels) or C2 (right panels), and the effector cells were NKL cells expressing either chimpanzee (solid lines; Pt-KIR2DL6, ○; Pt-KIR3DL4, ◊) or human KIR (dashed lines; KIR2DL1, □; KIR2DL3, Δ).

FIGURE 2. The number of lineage III KIR in chimpanzees is biased toward inhibitory receptors and in humans toward activating receptors. Shown is a schematic depiction of the structure and domain organization for the receptors encoded by chimpanzee and human lineage III KIR genes (St = extracellular stem, Tm = transmembrane domain, Cy = cytoplasmic domain). Only KIR encoded by orthologous genes (i.e., KIR2DS4 and Pt-KIR2DS4) are on the same line. Expressed D0 domains are shaded black; short-tailed cytoplasmic domains are white. Residues at the specificity-determining position 44 of D1 are shown in standard single-letter amino acid code, as is the lysine residue encoded at position 233 in the transmembrane domain of activating receptors. Indicated by diamonds (♦) are the ITIMs present in the cytoplasmic domain of inhibitory KIR. The reactivity of anti-KIR mAbs (EB6, DX27, and NKVFS1) with Pt-KIR was determined either using bead-bound soluble Fc-KIR fusion proteins and/or transduced NKL cells. For human KIR, the Ab reactivities were either determined using the same methods (KIR2DL1, KIR2DL2, KIR2DL3) or are as reported previously (57, 58).
Supernatants were used to infect growing NKL cells, and stable, KIR-expressing cells were sorted for equivalent GFP expression using a FACSVantage cell sorter (BD Biosciences). Transfectants were stained with PE conjugates of EB6 (Beckman-Coulter), DX27 (BD Biosciences), and NKVSF1 (SeroTec), and then analyzed by flow cytometry to confirm cell surface expression of KIR receptors, where possible. Pt-KIR2DL6 and Pt-KIR2DL8 were recognized by EB6, but no receptors interacted with DX27. With the exception of Pt-KIR2DL7 and Pt-KIR3DL5, all chimpanzee and human lineage III KIR receptors tested were recognized by NKVSF1. Fluorescence was analyzed on a FACScan flow cytometer (BD Biosciences).

Individual Patr-B and Patr-C cDNAs in the pBIneo vector were transfected into the MHC-A-, B-, and C-deficient cell line 221, as previously described (21). A residue of the MHC class I leader peptide was mutated to prevent cd94/NKG2A-mediated inhibition (31, 32). Cells were sorted for equivalent levels of MHC class I cell surface expression using the class I-specific Ab W6/32.

Cytotoxicity assay

Killing of transfected and untransfected 221 target cells by NKL cells was assayed, as described (31). Briefly, effector cells were mixed with C11+-loaded target cells at ratios ranging from 20:1 to 5:1 and incubated for 4 h at 37°C. Supernatants were harvested and Cr51 release was quantified using loaded target cells at ratios ranging from 20:1 to 5:1 and incubated for 4 h. Untransfected 221 target cells and untransduced NKL cells were included as internal controls in all cytotoxicity experiments. For specific lysis, results from one representative experiment are depicted.

KIR-Fc fusion proteins

Sequences coding for the extracellular Ig-like domains and stem region of KIR were genetically fused to the Fc portion of human IgG by rPCR and cloned into the pACgp67 vector (BD Biosciences). Recombinant baculovirus was generated in Sf9 insect cells by cotransfection with linearized baculovirus (BD Biosciences), and soluble fusion protein was produced in Hi5 insect cells using the Baculogold (BD Biosciences) baculovirus expression system, as described (31).

Ab capture of fusion proteins

KIR-Fc fusion proteins were incubated with anti-human IgG-coated beads (Bangs Laboratories) at a concentration of 100 μg/ml for 20 min at 4°C. Beads were washed in PBS (1% FCS) and then incubated with PE conjugates of EB6, DX27, NKVSF1, and anti-human Fc-PE (One Lambda). Fluorescence was analyzed on a FACScan flow cytometer (BD Biosciences). Abs recognizing KIR3DL1 (Z27; Beckman Coulter) and KIR3DL2 (DX31; gift from L. Lanier, University of San Francisco, San Francisco, CA) were also tested, but did not react with any of the receptors. Ab reactivities of recombinant proteins were consistent with those seen for transduced NKL.

Single-Ag bead analysis of KIR-Fc specificity

Binding of KIR-Fc fusions to a broad panel of HLA-A, B, and C allotypes was performed, as described (31), using LABScreen single-Ag bead sets (One Lambda). The following bead sets were used for the analysis: set 1 (Lot 008), set 2 (Lot 008), and set 3 (Lot 008). Binding of W6/32 Ab was used as the positive control to assess bead quality and account for bead-to-bead variation in HLA class I bound. Bead-bound fusion proteins were stained with anti-human Fc-PE and visualized using a Luminex100 reader (Luminex).

Phylogenetic analysis and ancestral sequence reconstruction

To reconstruct the evolution of the lineage III KIR D1 sequences, a genomic segment containing the exon encoding the D1 domain as well as 300 bp of intron on each side of the exon was used. The same approach was used for the D2 domain (two recombinant sequences, Pt-KIR2DL7 and KIR3DL3, were excluded). Nucleotide sequences were aligned with MAFFT (33), and the resulting alignments were corrected manually. Phylogenetic analyses were performed with three methods, as follows: maximum likelihood (ML), neighbor joining (NJ), and parsimony. NJ analyses were performed with MEGA4 (34) using the Tamura-Nei method with 500 replicates. PAUP*4.0b10 (35) and the tree bisection-reconnection branch swapping algorithm were used for parsimony analyses with 500 replicates and a heuristic search. ML analyses were performed with RAxML7 (36) under the GTR + CAT model with 500 replicates (rapid bootstrapping). The ML tree topologies were subsequently used for ancestral sequence reconstructions. Ancestral sequence reconstruction for the D1 and D2 domains was performed with CODEML (37) using the marginal reconstruction approach and the M0 model.

Results

Chimpanzees have inhibitory NK cell receptors specific for the MHC-C1 and MHC-C2 epitope

Previous study showed that some clones of chimpanzee NK cells exhibit alloreactions corresponding to the C1 and C2 specificities originally defined in the human system, and distinguished by the asparagine/lysine polymorphism at position 80 (16). Correlation of these alloreactive specificities with the variegated cell surface expression of KIR suggested that Pt-KIR2DL6 is an inhibitory receptor specific for C1 and that Pt-KIR3DL4 is an inhibitory receptor specific for C2. To test these predictions, we made transductants of the NKL cell line that expressed either Pt-KIR2DL6 or Pt-KIR3DL4 as the only cell surface KIR and examined their capacity to kill transfected 221 cells expressing single MHC class I allotypes (Fig. 1).

Unmanipulated NKL cells effectively killed the MHC class I-deficient 221 cells. In contrast, NKL cells expressing Pt-KIR2DL6 could not kill 221 cells expressing chimpanzee or human MHC-C allotypes bearing the C1 epitope (Fig. 1, left panels), although they...
killed 221 cells expressing C2-bearing allotypes (Fig. 1, right panels). This pattern of killing paralleled that observed for NKL cells expressing the human C1 receptor, KIR2DL3. Conversely, NKL cells expressing Pt-KIR3DL4 could not kill 221 cells expressing chimpanzee or human MHC-C allotypes that bear the C2 epitope defined by lysine 80 (Fig. 1). This pattern of killing was like that observed for NKL cells expressing KIR2DL1, the human C2 receptor. These results demonstrate that Pt-KIR2DL6 is an inhibitory C1 receptor and Pt-KIR3DL4 an inhibitory C2 receptor; specificities consistent with the residues found at the specificity-determining position 44 in the D1 domain of the KIR, Pt-KIR2DL6 and Pt-KIR2DL3, have lysine 44, whereas Pt-KIR3DL4 and KIR2DL1 have methionine 44. The inhibitory KIR-MHC interactions were removed by addition of the blocking anti-HLA class I Ab DX17 (data not shown). No inhibition was seen with any of the four KIR-transduced cell lines against a panel of target cell lines expressing chimpanzee or human MHC-A (Patr-A*1501, Patr-A*0601, HLA-A*0201, HLA-A*2403) or MHC-B (Patr-B*1801, Patr-B*2001, HLA-B*5701, HLA-B*0801), demonstrating the specificity of the inhibition (data not shown).

FIGURE 4. Distinct events have given rise to the multiple specificity-determining residue of primate lineage III KIR. A, Phylogenetic analysis was performed on genomic segments that encode the D1 (left panel) and D2 (right panel) domains from both lineage II and III KIR using NJ, parsimony, and ML approaches. The NJ trees were used for display and rooted at the midpoint. Support is indicated for nodes in which bootstrap support was >50 with two of the three methods, or signified by ● in which support was >80 with all three methods. Ancestral sequences were reconstructed, and the residue for position 44 is indicated at several nodes (see arrows). At position 44 of D1, the presence of lysine is indicated by K, glutamate by E, methionine by M, and threonine by T. B, Summary of substitutions from the ancestral sequence. The average number of differences from the ancestral sequence is given according to species and signaling function. For activating KIR, averages are shown for all activating receptors and without KIR2DS4/Pt-KIR2DS4 shown in parentheses. C, Comparison of substitutions in the amino acid sequences of the D1 and D2 domains of human and chimpanzee inhibitory lineage III KIR with the sequence of the predicted ancestral hominoid lineage III KIR. Identity with the ancestral sequence is indicated by dots. All residues in the ancestral sequence were predicted with p=0.99, except underlined residues, for which p=0.95, and residues in italics, which were statistically unresolved and for which the most likely residue is given. Positions that have been subject to positive selection are in grey shaded boxes (L. Abi-Rached, manuscript in preparation); arrowheads denote positions that contact MHC class I.
Chimpanzees have more inhibitory MHC-C receptors than humans

A characteristic of the human lineage III KIR is that the two genes encoding inhibitory HLA-C receptors (KIR2DL1 and KIR2DL2/3) are outnumbered by the five genes encoding activating receptors. For the chimpanzee, there is also a bias, but it is in the opposite direction: genomic and cDNA analyses have uncovered six chimpanzee lineage III Pt-KIR with potential to be inhibitory MHC-C receptors and only three activating receptors (Fig. 2). Of the six inhibitory receptors, Pt-KIR2DL6 and 2DL8 have lysine 44; Pt-KIR3DL4, 3DL5, and 2DL7 have methionine; and Pt-KIR2DL9 has glutamate, a residue not present in human inhibitory KIR, but previously identified in orangutan lineage III KIR (13).

We assessed the capacity of the four additional chimpanzee lineage III KIR to function as inhibitory MHC-C receptors, using the approach that was successfully applied to Pt-KIR2DL6 and 3DL4 (Fig. 1). As predicted from their position 44 specificity-determining residues, the two allotypes of Pt-KIR2DL8 were proven to be inhibitory C1 receptors (Fig. 3, A and B), and both Pt-KIR2DL7 and 3DL5 were shown to be inhibitory C2 receptors (Fig. 3, C and D). The glutamate 44-containing receptor Pt-KIR2DL9 was also unexpectedly discovered to be specific for C2 (Fig. 3, E and F). These results show that the chimpanzee has greater number and diversity of functional inhibitory MHC-C receptors than the human species. This is also reflected at the level of genotype: chimpanzee haplotypes can contain multiple genes encoding C1- and C2-specific receptors, and on average chimpanzee KIR genotypes contain four genes for inhibitory HLA-C receptors (L. Abi-Rached, manuscript in preparation).

KIR specific for C2 evolved from C1-specific KIR on at least two occasions

Presence of methionine, lysine, and glutamate at position 44 in hominoid inhibitory KIR begs the question: which of these came first? To address this issue, an analysis to predict the sequence of the D1 and D2 domains of the ancestral lineage III KIR was performed (Fig. 4). This was done using phylogenetic trees of lineage II and III KIR sequences (Fig. 4A) and reconstruction of the ancestral lineage III sequence (Fig. 4, B and C). Our analysis predicts with high confidence (p > 0.99) that lysine was the ancestral residue at position 44 for all hominoid lineage III KIR. This assignment indicates that hominoid KIR with C1 specificity evolved first, and that evolution of the C2 specificity occurred subsequently. The analysis also shows that chimpanzee lineage III KIR with methionine and glutamate at position 44 were independently derived by single-point substitutions from KIR with lysine 44. Thus, these two types of C2-specific receptor independently evolved from C1-specific receptors. Evolution of glutamate 44-containing KIR in the orangutan was also shown to be due to an independent event of point mutation in a lysine 44-containing KIR. The functional consequences of this change, however, have yet to be determined. Finally, a more recent human-specific mutation in a lysine 44-containing KIR gave rise to the threonine 44 now present in the KIR2DS3- and 2DS5-activating receptors. For these two KIR, no binding to MHC class I has been detected (38).

As a group, the human inhibitory lineage III KIR show fewer amino acid substitutions from the common ancestor than chimpanzee KIR (16 vs 20 substitutions on average; Fig. 4B), with KIR2DL3 being the least divergent receptor. At several positions that have been subject to positive selection (residues 50, 131, and 190), humans retain the ancestral residue, whereas chimpanzee receptors have diversified this position (Fig. 4C). Conversely, human activating KIR show a larger number of differences, with KIR2DS3 and KIR2DS5 showing the most changes of any lineage III receptors (26 and 24 differences, respectively; data not shown).

Selection analysis showed that polymorphisms at nine positions in D1 and six in D2 are the result of natural selection in the course of hominoid evolution (Fig. 4C). Three of these positions (residues 44, 70, and 71) are contact residues for bound MHC class I, including residue 44. This finding, combined with the result that mutation of lysine to methionine and glutamate independently gave rise to C2-specific KIR, suggests that episodes of selection for C2-specific inhibitory KIR have occurred on more than one occasion.
FIGURE 6. Common chimpanzee MHC-B allotypes carry C1 epitopes that function as ligands for C1-specific KIR. A, Summarizes the results of binding assays between Fc-KIR fusion proteins and beads coated with different HLA class I allotypes. Fusion proteins were made from Pt-KIR2DL6, Pt-KIR2DL7, Pt-KIR2DL8 (allele Pt-KIR2DL8t7 shown), and Pt-KIR2DL9. Mean values are given for nine HLA-C1, seven HLA-C2, and for the combination of 29 HLA-A and 48 HLA-B allotypes. HLA-B*4601 and B*7301 are shown individually (and were not included in the group of HLA-A and HLA-B) because they carry the C1 epitope. B, Shows an alignment of the amino acid sequences for positions 65–83 of MHC-B and MHC-C molecules. Positions 76 and 80, for which a valine 76, asparagine 80 motif is essential for the C1 epitope, are highlighted by grey-shaded boxes. C, Shows the results of cytotoxicity assays of NKL transductants expressing single KIR (Pt-KIR2DL7, 2DL6, 2DL8, and human KIR2DL3) as effector cells and 221 cells transfected with single MHC class I allotypes (Patr-C*0401, C*0501, B*1601, B*0801, B*2001, and B*1801). Bars are shaded according to the sequence motif at positions 76 and 80, as shown on the right (C1, □; C2, ■; E76/I80, □; no MHC, ■). Results are shown for an E:T ratio of 20:1. D, Comparison of the allele frequencies of the C1 and C2 epitopes for the MHC-C locus for chimpanzee and human populations. Human population data were compiled from the International Histocompatibility Working Group database, and the number of individual populations within each world region is indicated. The mean frequency and range (in parentheses) of all allotypes with the C1 and C2 epitope are shown. Note the more even frequency of C1 and C2 in the chimpanzee than human.
The D0 domain of lineage III KIR does not influence ligand binding or specificity

The common ancestor of the lineage III KIR had three extracellular Ig-like domains (14). All human lineage III KIR have only two Ig-like domains (D1 and D2), although the genes retain non-expressed pseudoexons 3, a relic of an ancestral exon 3 that encoded a D0 domain (39). For chimpanzees, the situation is different. Among the chimpanzee lineage III KIR, several express the D0 domain, whereas others lack it (Fig. 2). However, rather than representing an ancient form of lineage III, the D0 domain of these receptors appears to be the result of recombination with another lineage (14). To assess the role of the D0 domain, we examined the function of mutant KIR in which D0 had been either removed from KIR3DL or added onto KIR2DL (Fig. 5).

In these experiments, the removal or addition of the D0 domain did not perturb the synthesis and cell surface expression of the KIR. When the D0 domain was removed from the C2 receptors, Pt-KIR3DL4 and Pt-KIR3DL5, we found no detectable effect on the strength and specificity of their interaction with C2 (Fig. 5A). Similarly, addition of the D0 domain from Pt-KIR3DL4 to either chimpanzee Pt-KIR2DL6 or human KIR2DL3 also had no effect on C1 recognition. Neither was the strength and specificity of KIR2DL1 for C2 affected by addition of the D0 domain from Pt-KIR3DL4 (Fig. 5B). These results clearly demonstrate that the presence or absence of the D0 domain does not significantly affect the interactions of the lineage III KIR with their cognate MHC-C ligands, at least in the assays we used. This behavior provides a strong contrast to that of KIR3DL1, a lineage II KIR specific for Bw4 epitopes of HLA-A and HLA-B. The D0 domain of KIR3DL1 enhances the binding of ligand to the D1 and D2 domains and is essential for the correct folding and cell surface expression of the receptor (40, 41). That all human and some chimpanzee lineage III genes have acquired mutations that eliminate expression of exon 3 may reflect a limited functional contribution of the D0 domain to MHC-C recognition.

MHC-B and MHC-C are both common sources of C1 ligands in the chimpanzee

Bivalent KIR-Fc fusion proteins were made from Pt-KIR2DL6, 2DL7, 2DL8, and 2DL9 and tested for binding to a diverse panel of beads coated with single HLA-A, B, and C allotypes (Fig. 6A). The results were consistent with the C1 and C2 specificities determined by cytotoxicity assays (Fig. 3). Pt-KIR2DL7 and Pt-KIR2DL9 bound only to beads coated with C2-bearing HLA-C allotypes. This pattern of reactivity was indistinguishable from that obtained with human KIR2DL1. Conversely, Pt-KIR2DL6 and Pt-KIR2DL8 gave binding patterns like that of KIR2DL3: positive reactions being observed with HLA-C allotypes having the C1 epitope and two HLA-B allotypes, B*4601 and B*7301, that also have C1. Distinguishing B*4601 and B*7301 from other HLA-B allotypes is the presence of valine at position 76, which, in combination with the asparagine present at position 80 in all HLA-B allotypes, gives B*4601 and B*7301 the C1 epitope.

Whereas valine 76 is a rarity in human HLA-B allotypes, it is present in a substantial proportion (~25%) of chimpanzee Patr-B allotypes (Fig. 6B). Approximately 50% of chimpanzees carry one or two MHC-B allotype with valine 76, whereas HLA-B allotypes with the C1 epitope are found only at very low frequencies in specific populations (with the exception of HLA-B*4601 in Southeast Asian populations). To determine whether these chimpanzee allotypes can function as ligands for C1-specific KIR, we tested their capacity to protect transfected 221 cells from lysis by NKL cells transduced with various KIR2DL (Fig. 6C). In these assays, the valine 76-containing Patr-B*0801 and B*1601 protected 221 cells from lysis by NKL cells expressing C1-specific Pt-KIR2D and KIR2DL3, but not NKL cells expressing C2-specific Pt-KIR2D. The inhibition was less than that achieved by the C1-bearing Patr-C*0501, but was clearly above that observed for the C2-bearing Patr-C*0401. These results indicate that significant subsets of Patr-B and Patr-C allotypes provide ligands for the two C1-specific inhibitory KIR of the chimpanzee. The allele frequencies of 0.48 for C1 and 0.52 for C2 at Patr-C are well balanced (Fig. 6D) and give phenotypic frequencies of 68 and 73%, respectively (28) (L. Abi-Rached, manuscript in preparation). Conversely, C1 is the dominant form in many human populations, particularly ones that have undergone bottlenecks, such as Amurians and East Asians, in which allele frequencies of C1 outweigh C2 by 2:1 (Fig. 6D). However, when both the Patr-C and Patr-B allotypes that have C1 are taken into account, ~80% of chimpanzees have the C1 epitope.

Discussion

Of the seven human and nine chimpanzee lineage III KIR genes, only KIR2DS4 and Pt-KIR2DS4 are orthologous. All other lineage III KIR are the products of species-specific evolution that occurred after the separation of human and chimpanzee ancestors. In humans, the genes encoding activating KIR outnumber by 2.5 to 1 those encoding inhibitory KIR, the latter consisting of KIR2DL1 and KIR2DL2.3. Whereas KIR2DL1 is exquisitely specific for C2 epitopes, the KIR2DL2 and KIR2DL3 allotypes of the KIR2DL2/3 locus are less specific; they principally react with C1, but exhibit cross-reactivities with some C2-bearing allotypes (31, 42). These cross-reactions are more apparent for KIR2DL2, a stronger receptor than KIR2DL3. By contrast to the human situation, in chimpanzees the activating KIR are outnumbered 2-fold by the inhibitory KIR, for which the functions and specificities have been the focus of this investigation. Thus, the ratio of inhibitory to activating KIR in these two closely related species differs by a factor of 5 (Fig. 2).

On the basis of genomic and cDNA analysis, we identified six chimpanzee lineage III KIR genes that encode inhibitory receptors with potential specificity for MHC class I. Despite the considerable sequence divergence in the human and chimpanzee KIR, all six chimpanzee KIR were demonstrated to have functional specificities for MHC-C epitopes that were essentially the same as the established C1 and C2 specificities of human KIR. Like human KIR2DL3, chimpanzee Pt-KIR2DL6 and 2DL8 are C1 specific and have lysine at position 44, the specificity-determining position. Four chimpanzee KIR have similar C2 specificity to human KIR2DL1, but three of them differ from KIR2DL1 in distinctive structural features. Pt-KIR3DL4 and 3DL5 provide the first examples of MHC-C specific KIR that have three extracellular domains, and Pt-KIR2DL9 exhibits C2 specificity, although its specificity-determining residue is glutamate, rather than the methionine found in other C2 receptors. Only Pt-KIR2DL7 has the same domain structure and specificity-determining residue as KIR2DL1. In our experiments, these differences had no detectable effects on the capacity to bind MHC-C and inhibit the function of effector NKL cell function, nor did mutagenesis to add or remove the D0 domain. One interpretation of the latter result is that the D0 domain is of marginal benefit, which could explain why the exon encoding this domain has been silenced in all human and a majority of chimpanzee lineage III KIR. Alternatively, D0 may have functions that are not well replicated by the NKL cell line, or pertain to the role of inhibitory MHC-C receptors in NK cell development (43).

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Pt-KIR2DL6 and 2DL8 both resembled KIR2DL3 in their functional specificity for C1, and thus, a potentially significant difference between the species is that no chimpanzee equivalent of KIR2DL2 was identified. Human KIR haplotypes are divided into two broad groups, A and B, according to gene content (26). KIR2DS4, which has a chimpanzee ortholog, is the only activating lineage III KIR of the A haplotype. In contrast, the human-specific activating lineage III KIR are only present on B haplotypes, and the same is true for KIR2DL2. Thus, the formation of KIR2DL2, which most likely involved recombination between KIR2DL1 and KIR2DL3, as well as the emergence of A and B haplotypes, could also be products of human-specific evolution, as is consistent with the absence of A- and B-like differences between chimpanzee KIR haplotypes (L. Abi-Rached, manuscript in preparation).

Reconstructing the sequence of the common ancestor of the hominoid lineage III KIR showed that it had lysine 44 as its specificity-determining residue. This result strongly indicates that the first MHC-C receptor was C1 specific. Supporting this conclusion are studies of the orangutan, a species in which the MHC-C locus is not fixed (44), as it is in humans and chimpanzees. That all known orangutan MHC-C are C1 suggests this epitope is ancestral, and C2 evolved subsequently. Ancestral reconstruction also showed that C2-specific KIR with methionine and glutamate at position 44 independently evolved from C1-specific KIR by point mutation. Today, all modern human populations have both C1 and C2, indicating the presence and persistence of selection pressures that retain them.

One possible explanation for the independent evolution of C2 receptors with methionine and glutamate at position 44 is that they are functionally different and complementary. Our results show that their specificity and avidity for C2-bearing MHC allotypes are similar, suggesting that if such difference exists, it does not involve ligand binding, but another aspect of their function not investigated in this study. An alternative possibility is that in some circumstances (as appears for the chimpanzee) there is advantage to having two genes encoding C2 receptors, because this would increase the proportion of NK cells expressing C2 receptors (45). Such selection would not necessarily discriminate between receptors having glutamate or methionine at position 44, but pick the first two variants to emerge, which by chance happened to be one with glutamate 44 and one with methionine 44. In other circumstances, either population bottleneck or selection could cause the loss of one of the C2 receptors, explaining the absence of inhibitory C2 receptors having glutamate 44 in humans.

The MHC-B and MHC-C loci emerged from a common ancestor during hominoid evolution. In humans, the C1 and C2 epitopes are almost exclusively carried by HLA-C allotypes. Notable, and informative, exceptions are HLA-B*4601 and B*7301, which both carry the C1 epitope and interact with human and chimpanzee C1-specific KIR. These two allotypes have very different histories. HLA-B*4601 was relatively recently formed in Southeast Asia by gene conversion (46). Subsequently, it has reached allele frequencies of nearly 20% in some Southeast Asian populations. In contrast, HLA-B*7301 is a rare, but geographically widespread allotype (47) that appears to be the last survivor of an old and divergent lineage of MHC-B alleles, distinguished from the dominant lineage by having valine at position 76, an essential feature of the C1 epitope. Contrasting with the strong bias for HLA-B allotypes not to have valine 76, the Patr-B locus has a diverse and substantial representation of valine 76-containing allotypes that we demonstrate are ligands for C1-specific KIR. In the panel of chimpanzees that we studied, ~50% of individuals express a Patr-B allotype that carries the C1 epitope.

Thus, in the chimpanzee, Patr-B and Patr-C are both substantial contributors of C1 ligands. During human evolution, this function of MHC-B was lost because the frequency of B allotypes with valine 76 declined. In this context, the formation and selection of B*4601 can be seen as a reacquisition through gene conversion of a valine 76-containing B allotype. Thus, in humans, the epistatic interaction between lineage III KIR and HLA-B that was lost due to selection or drift has been restored in particular populations. Notably, B*4601 contributes an additional C1 ligand in populations that already have high frequencies of C1 within HLA-C (Fig. 6D).

We have shown in this study that humans and chimpanzees have a similar system of C1 and C2 ligands that interact with cognate lineage III inhibitory KIR. This system existed in the common ancestor and has been preserved throughout ~8 million years of independent evolution. During this time, significant differences also occurred in the human and chimpanzee lineages. The overall trend during human evolution has been to concentrate the C1 and C2 epitopes at the HLA-C locus, which can be seen as becoming increasingly specialized in the regulation of NK cells via their KIR. In the chimpanzee, the specialization appears to have been less extreme, because a diversity of both Patr-B and Patr-C allotypes can serve as ligands for inhibitory lineage III KIR. In humans, the inhibitory C1 and C2 receptors are encoded by single-copy genes, which are essentially fixed. Related activating receptors evolved and now define the A and B groups of KIR haplotypes, which are associated with C1 receptors of different strength and cross-reactivity with C2. An equivalent bifurcation of KIR haplotypes is not apparent in the chimpanzee, in which there is a multiplicity of inhibitory C1- and C2-specific KIR and fewer activating lineage III KIR.

Environmental and behavioral differences have most likely shaped the distinctive MHC and KIR systems that we describe in humans and chimpanzees. Humans differ from the extant apes in their capacity to populate new and varying habitats by dispersal and adaptive radiation. Human-specific traits such as increased postbreeding lifespan and shorter interbirth intervals (48) enable rapid population growth following periods of restricted population size due to migration, disease, or resource depletion (49). Human reproductive strategy most likely evolved under constraints, which became increasingly lifted following the advent of recent (postneolithic) modern human behavioral patterns (49). In the case of extreme population bottleneck, as most likely occurred during the evolution of premodern humans when genetic diversity diminished (50), subsequent population expansion favors rapid evolution of crucial epistatic interactions and selection for new variants (51).

The extensive species differences in inhibitory KIR and MHC frequencies could also be a reflection of species-specific expansion within the chimpanzee, as suggested by their unusually high number of positively selected genes (52). Although chimpanzee populations have most likely remained stable in terms of geographic distribution and total population numbers (53), behavioral adaptations may have contributed specific selection on immune loci. For example, hunting and consumption of other primate species expose chimpanzees to broad array of trans-species pathogenic infections and superinfections (54, 55). This in turn can render chimpanzees a resource for infectious diseases that emerge in humans, such as HIV type 1 (56).
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Disclosures
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