



Allelic Polymorphism Determines Surface Expression or Intracellular Retention of the Human NK Cell Receptor KIR2DL5A (CD158f)

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KIR2DL5 (CD158f) is the most recently identified inhibitory member of human killer-cell Ig-like receptors (KIRs), which enable NK cells to sense self-HLA. Unlike KIR2DL1–3, recognizing HLA-C allotypes through Ig-like domains of the D1–D2 type, KIR2DL5 shares a D0–D2 configuration with KIR2DL4, and its ligands have not been identified. KIR2DL5 is encoded by two paralogous genes displaying copy number variation and allelic polymorphism—*KIR2DL5A* and *KIR2DL5B*. UP-R1 mAb, raised against the common allele *KIR2DL5A*001*, enables specific KIR2DL5 detection. However, not every *KIR2DL5+* individual has NK cells staining with UP-R1, discrepancy explained in part by epigenetically silent *KIR2DL5B* alleles with a distinctive substitution in a promoter RUNX-binding site. Furthermore, we show here that the transcribed allele *KIR2DL5A*005*, second most common of its locus, fails to confer NK cells UP-R1 reactivity, phenotype explained by inefficient transport of its product to the cell surface. Two amino acid substitutions distinguish the *KIR2DL5A*005* and **001* coding regions. Western blot, flow cytometry, and confocal microscopy analyses of cells transfected with tagged constructs demonstrate that a serine substitution for glycine-174, conserved in most KIR, is mainly responsible for KIR2DL5A*005 intracellular retention, and it also affects mAb recognition. In contrast, substitution of aspartate for asparagine 152 has only a minor effect on surface expression, despite destroying an otherwise conserved N-glycosylation site. Our results help to explain the variable expression profile of *KIR2DL5+* subjects and indicate that functional polymorphisms in both its promoter and its coding regions are critical for understanding the KIR2DL5 role in immunity and its importance for human health.

Keywords: natural killer cells, repertoire development, KIR, NK-cell receptors, allelic polymorphism

INTRODUCTION

Several inhibitory members of the human killer-cell Ig-like receptors (KIRs) family recognize allotypes of classical HLA class I molecules, and they enable NK cells to monitor, through the missing-self mechanism, selective subversion of antigen presentation in infected and tumor cells (1, 2). Besides, other KIRs have less well-defined functions in immunity (3). Among these is KIR2DL5 (CD158f),

Abbreviations: HEK, human embryonic kidney; KIR, killer-cell Ig-like receptor.

which is identified in surveys of the human genome years after the other KIR (4, 5). Like the divergent KIR 2DL4, 2DL5 has Ig-like domains homologous to the D0 and D2 of three-domain KIR and is conserved in several hominoid species. But unlike KIR 2DL4, 2DL5 shares with HLA-C-specific KIR 2DL1–3 (i.e., those with D1–D2 domains) a clearly inhibitory capacity and clonal expression on NK cells and T-lymphocytes (6). The ligand recognized by KIR2DL5 remains, however, elusive.

KIR2DL5 has a complex genetics due to copy number variation and allelic polymorphism (7). It is encoded in the human genome by two closely related genes—*KIR2DL5A* in the telomeric segment of KIR haplotypes and *KIR2DL5B* in the centromeric interval (8, 9). Each of these loci is present or absent in different common haplotypes. Moreover, *KIR2DL5A* has undergone further duplication in some rare haplotypes (10–12). *KIR2DL5A* and *B* are represented by 15 and 28 alleles, respectively, in the Immuno Polymorphism Database release 2.6.0 (5 and 13 alleles, without taking into account non-coding polymorphisms) (13). *KIR2DL5A* is most commonly represented by allele *2DL5A*001*, followed in frequency by *2DL5A*005*, whereas the centromeric locus is usually represented by allele *2DL5B*002* and also by *2DL5B*006* in Black populations [Ref. (11, 14–17) and our own unpublished data].

Allelic polymorphism affects the coding and the non-coding regions of *KIR2DL5* (7, 13). Of particular functional relevance is one polymorphic G > A substitution at nucleotide 97 before the initiation codon that destroys a RUNX-binding site conserved in the proximal promoter of most *KIR*. Such change correlates exactly with complete epigenetic silencing of the common alleles of *KIR2DL5B* and the *KIR3DP1* pseudogene, while all known *KIR2DL5A* and a few *KIR2DL5B* and *KIR3DP1* alleles have intact RUNX-binding sites and are clonally transcribed (8, 14, 18–20).

Also of interest are two linked dimorphisms in the exon coding for the membrane-proximal D2 Ig-like domain (codons 152 and 174), since they sort all alleles of both the centromeric and the telomeric *KIR2DL5* loci into two mutually exclusive groups (Table 1). Asparagine and glycine are seen in the common *2DL5A*001* allele, in *2DL5B*006*, and in other less-common alleles of both loci, besides being conserved in virtually all KIR, while *2DL5A*005* on the telomeric side, the dominant centromeric allele *2DL5B*002* and others encode aspartate and serine at those positions (7). No *KIR2DL5* allele with a mixed motif has been described.

TABLE 1 | Common dimorphisms of the *KIR2DL5* coding and promoter regions.

Locus	Promoter nucleotide -97	Representative alleles	
		Asn152-Gly174	Asp152-Ser174
<i>KIR2DL5A</i>	G	*001	*005
<i>KIR2DL5B</i>	A/G ^a	*006	*002

Guanidine at nucleotide -97 correlates with clonal transcription; adenine with complete silencing; Asn152-Gly174 and Asp152-Ser174 with cell surface and intracellular expression, respectively.

^aEach of *KIR2DL5B*006* and **002* comprises multiple alleles coding for identical polypeptides, but differing from each other by changes in nucleotide -97 (besides other non-coding or synonymous substitutions) (13).

Generation of a specific monoclonal antibody, UP-R1, enabled us to substantiate *KIR2DL5* protein expression on NK cells and to characterize its biochemical and functional features. However, characterization was limited to NK cells expressing the dominant allele *KIR2DL5A*001*, against which the mAb was raised (6). Some discrepancies between genotyping and flow cytometry studies were subsequently observed, and they suggested that the aforementioned polymorphisms in the *KIR2DL5* primary structure might somehow affect protein expression or recognition by UP-R1, still the only available mAb specific for *KIR2DL5*. In this study, we build on those observations and demonstrate that phenotypic, functional, and structural diversity of *KIR2DL5* depends, not only on copy number variation and variable transcription but also on allelic polymorphism targeting *KIR2DL5* to either NK-cell surface expression or intracellular retention.

MATERIALS AND METHODS

KIR2DL5-FLAG Constructs, Cell Lines Culture, and Transfection

A *KIR2DL5A*001* cDNA clone with a FLAG epitope (DYKDDDDK) inserted between the leader peptide and the D0 Ig-like domain (6) was used to generate, by site-directed mutagenesis, three additional constructs bearing each of the missense mutations that distinguish the *KIR2DL5A*001* and **005* primary structures—constructs N152D (152 AAT → GAT, asparagine to aspartate), G174S (GGC → AGC, glycine to serine), and *KIR2DL5A*005* (both changes). Plasmids were purified using the EndoFree Plasmid Maxi and Midi Kits (Qiagen, Valencia, CA, USA) and sequenced with the universal primer SP6 and the internal primer R5e61 (5'-gttttgagcttggtcag-3'). Only error-free clones were used for transfection. Transfection experiments were replicated using up to five different plasmid batches per construct to control for random variations in expression attributable to DNA quality.

Human embryonic kidney (HEK)-293T cells were cultured in DMEM (Corning Inc., Corning, NY, USA) supplemented with 2 mM L-glutamine, 1% penicillin–streptomycin, and 10% FBS (Gibco Life Technologies, Carlsbad, CA, USA), and transiently co-transfected by the calcium phosphate method with 5 µg of each *KIR2DL5* construct and 0.1 µg of pEGFP-N1 vector (Clontech Laboratories, Palo Alto, CA, USA). Jurkat cells were cultured in RPMI-1640 Glutamax (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS and 1% penicillin–streptomycin, and transiently transfected with 2 µg of each *KIR2DL5* construct along with 0.1 µg of pEGFP-N1, using Solution V and the X-01 program of a Nucleofector I apparatus (Amaxa Biosystems, Cologne, Germany).

Western Blot

Forty-eight hours after transfection, 2×10^5 HEK-293T cells were lysed in 1% Non-idet P-40 Substitute (Sigma-Aldrich, St. Louis, MO, USA), 20 mM Tris-HCl, pH 8, 137 mM NaCl, 2 mM Na₂EDTA lysis buffer containing 1% protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Proteins were reduced and denatured in 5× Laemmli buffer, run in 10%

SDS-PAGE, and blotted to nitrocellulose membranes (iBlot Gel Transfer Stacks Nitrocellulose, Mini, Novex Life Technologies, Grand Island, NY, USA), which were treated for 1 h with blocking buffer (Li-Cor Bioscience, Lincoln, NE, USA). KIR2DL5-FLAG protein bands were detected with 1:1,000 dilution of anti-FLAG mAb M2 (Sigma-Aldrich, St. Louis, MO, USA), and 1:5,000 secondary antibody directed against mouse IgG (IRDye800, Li-Cor Bioscience). Beta-actin was detected, as a positive control, with 1:2,000 rabbit anti-human β -actin antibody (Abcam, Cambridge, UK) and the secondary reagent IRDye700 (Li-Cor Bioscience). Signals were detected with the Odyssey Infrared Imaging System (Li-Cor Bioscience). For N-deglycosylation, 400 μ g of total cell lysates were denatured for 10 min at 100°C and treated with 2.5 U of Peptide-N-Glycosidase F (PNGase F, New England Biolabs, Ipswich, MA, USA) for 5 h at 37°C before Western blotting.

Flow Cytometry

PBMCs or IL-2-expanded NK cells from healthy donors of known *KIR* genotype were incubated at 4°C with the following antibodies: APC anti-CD56 (eBioscience, Inc., San Diego, CA, USA), PE/Cy7 anti-CD3 (Biolegend, San Diego, CA, USA), and either PE anti-KIR2DL5 (UP-R1, Biolegend) or PE anti-KIR2DL2/L3/S2 (DX27, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Isotype-matched negative controls were PE IgG1 (clone MOPC-21, Sigma-Aldrich) and PE IgG2a (clone S43.10, Miltenyi). Flow cytometry analysis was performed in MACSQuant Analyzer (Miltenyi) using MACSQuantify software. Percentages of KIR2DL5-positive cells were determined after gating on the CD3-CD56⁺ NK-cell population. The genotype of each donor was assessed using previously published polymerase chain reaction primers (14) targeting specific polymorphisms in the *KIR2DL5* coding and promoter regions; the latter primers also enabled to rule out potential confounding presence of transcribed *KIR2DL5B* alleles. This study was carried out in accordance with the recommendations of the Ethical Committee of Clinical Research of *Instituto de Investigación Sanitaria Puerta de Hierro*, which approved the protocol (270910-258). All participating volunteers provided written informed consent in accordance with the Declaration of Helsinki.

Primary mAbs used for flow cytometry of *KIR2DL5*-transfectants were anti-FLAG mAb M2 (Sigma-Aldrich), *KIR2DL5*-specific mAb UP-R1 (6), and mouse IgG1 isotype-matched negative control MOPC-21 (Sigma-Aldrich). PE-conjugated goat anti-mouse IgG + IgM, F(ab')₂ fragments (Jackson ImmunoResearch, West Grove, PA, USA) were used as secondary Ab. Jurkat and HEK-293T cells were stained 24 and 48 h post-transfection, respectively, and transient surface expression of *KIR2DL5*-FLAG was determined in Epics XL (Coulter Electronics, Hialeah, FL, USA) or MACSQuant (Miltenyi) flow cytometers, using Expo32 and MACSQuantify software, respectively. EGFP-positive cells ranged ~8–12% (Jurkat) and 30–40% (HEK-293T). Mean fluorescence intensities (MFIs) of different *KIR2DL5* constructs, calculated after gating on EGFP⁺ cells, were compared using the paired Student's *t*-test. To assess the effect of *KIR2DL5* polymorphisms on recognition by mAb UP-R1, we calculated in each experiment the ratio of the MFIs obtained with *KIR2DL5A**001 and each transfectant using M2 (anti-FLAG) and

UP-R1, and ratios obtained for each mAb were compared using the non-parametric Wilcoxon signed-rank test.

Confocal Microscopy

Forty-eight hours after transfection, HEK-293T cells were stained with anti-FLAG M2 mAb, fixed in PBS with 4% paraformaldehyde, and incubated with Alexa546-conjugated anti-mouse IgG (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) to detect *KIR2DL5*-FLAG molecules on the surface. Then, cells were treated with 0.3% Triton X-100, and re-incubated with anti-FLAG M2 mAb, followed by Alexa448-labeled anti-mouse IgG (Invitrogen), to detect intracellular *KIR2DL5* molecules. After this staining strategy, based on Ref. (21), cells were visualized on poly-L-lysine-coated glass-bottom dishes on a confocal laser-scanning microscope (TCS SP5, Leica Microsystems CMS GmbH, Mannheim, Germany) using Argon (488 nm) and Helium-Neon (543 nm) lasers and a 20 \times /0.5 lens. Images were acquired using the LAS AF SP5 software (Leica).

KIR2DL5A Structure Model

We based on the *KIR2DL1* X-ray structure (22) (PDB accession code: 1NKR) to predict the structures of the D2 Ig-like domains of *KIR2DL5A**001 and *005, using the SWISS-MODEL automated protein structure homology-modeling system (23), accessible via the ExPASy web server.¹ Quality of each model was assessed with the QMEAN Z-score (24), which describes the likelihood that a model quality is comparable to high-resolution experimental structures.² Structures were visualized and edited using the PyMOL Molecular Graphics System (version 1.7.4, Schrödinger, LLC).

RESULTS

Allelic Polymorphism Affects *KIR2DL5A* Detection on the NK-Cell Surface by Flow Cytometry

Flow cytometry assays of PBMCs from donors with a *KIR2DL5A**001 allele identify discrete subpopulations of NK cells reacting with the only available anti-*KIR2DL5* mAb, UP-R1 (average of positive NK cells in 11 donors \pm SD: 5.18 \pm 2.15%; range: 1.71–7.89%), consistently with the clonal expression pattern reported for the receptor (4, 6). In contrast, much fewer UP-R1-positive NK cells are seen in donors carrying allele *KIR2DL5A**005 (0.50 \pm 0.24%; range: 0.23–0.93%; *n* = 9; *p* = 0.0001), their proportions being not significantly different from the background seen in donors lacking a transcribed *KIR2DL5* gene (0.33 \pm 0.20%; range: 0.15–0.78%; *n* = 11). In fact, the flow cytometry plots of donors pertaining to the latter two categories are hardly distinguishable on an individual basis (Figure 1), indicating that NK cells of *KIR2DL5A**005 subjects are, for some reason, unreactive with UP-R1 in these assays.

*KIR2DL5A**001-positive NK cells expanded *in vitro* express higher levels of the receptor on the surface than resting cells,

¹<http://swissmodel.expasy.org/>.

²<https://swissmodel.expasy.org/qmean/cgi/index.cgi?page=help>.

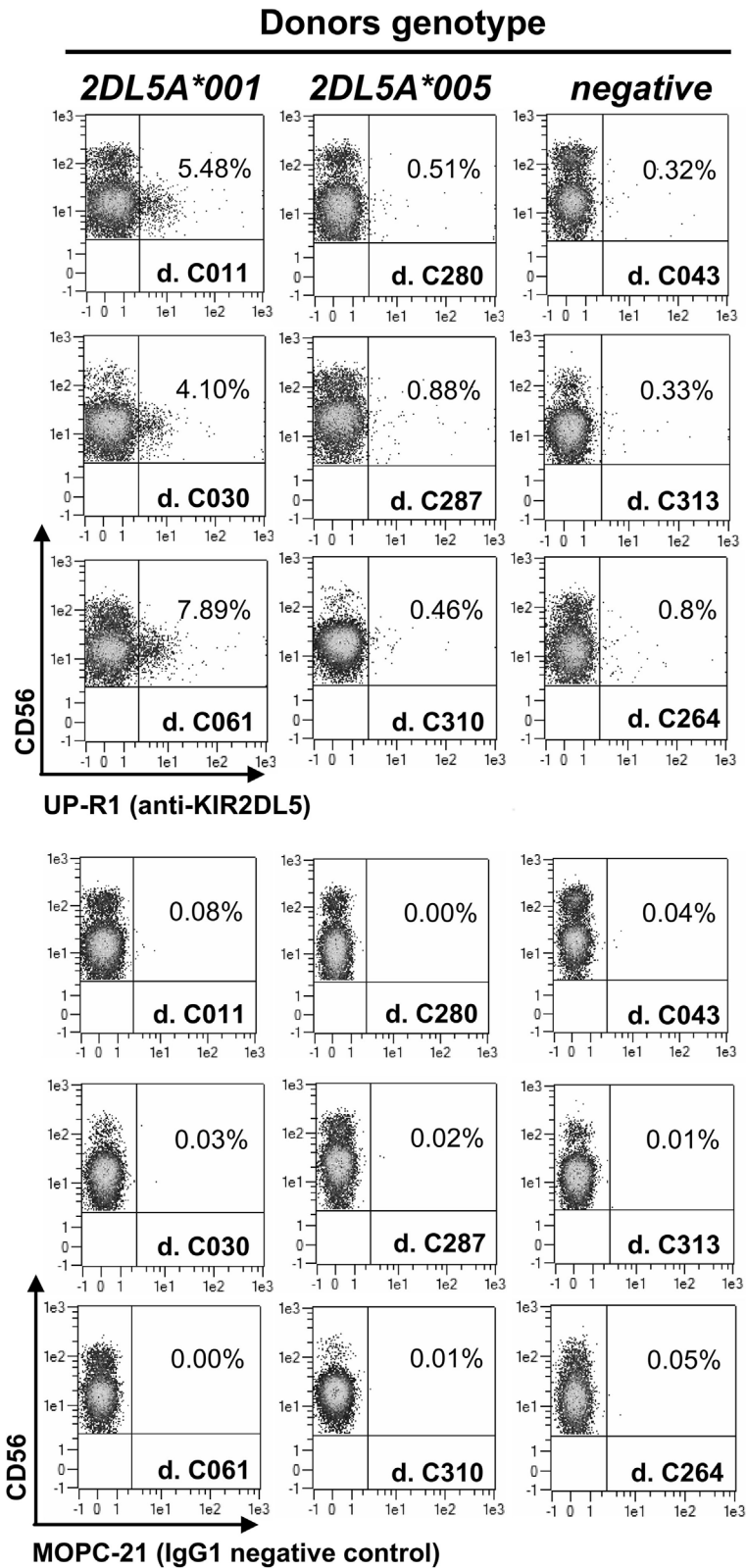


FIGURE 1 | Flow cytometry profiles of NK cells from donors with different *KIR2DL5A* genotypes. Unmanipulated NK cells from peripheral blood of three donors expressing *KIR2DL5A*001* (left, positive control), three carrying *KIR2DL5A*005* (middle), and three lacking a transcribed *KIR2DL5A* or *KIR2DL5B* gene (right, negative control).

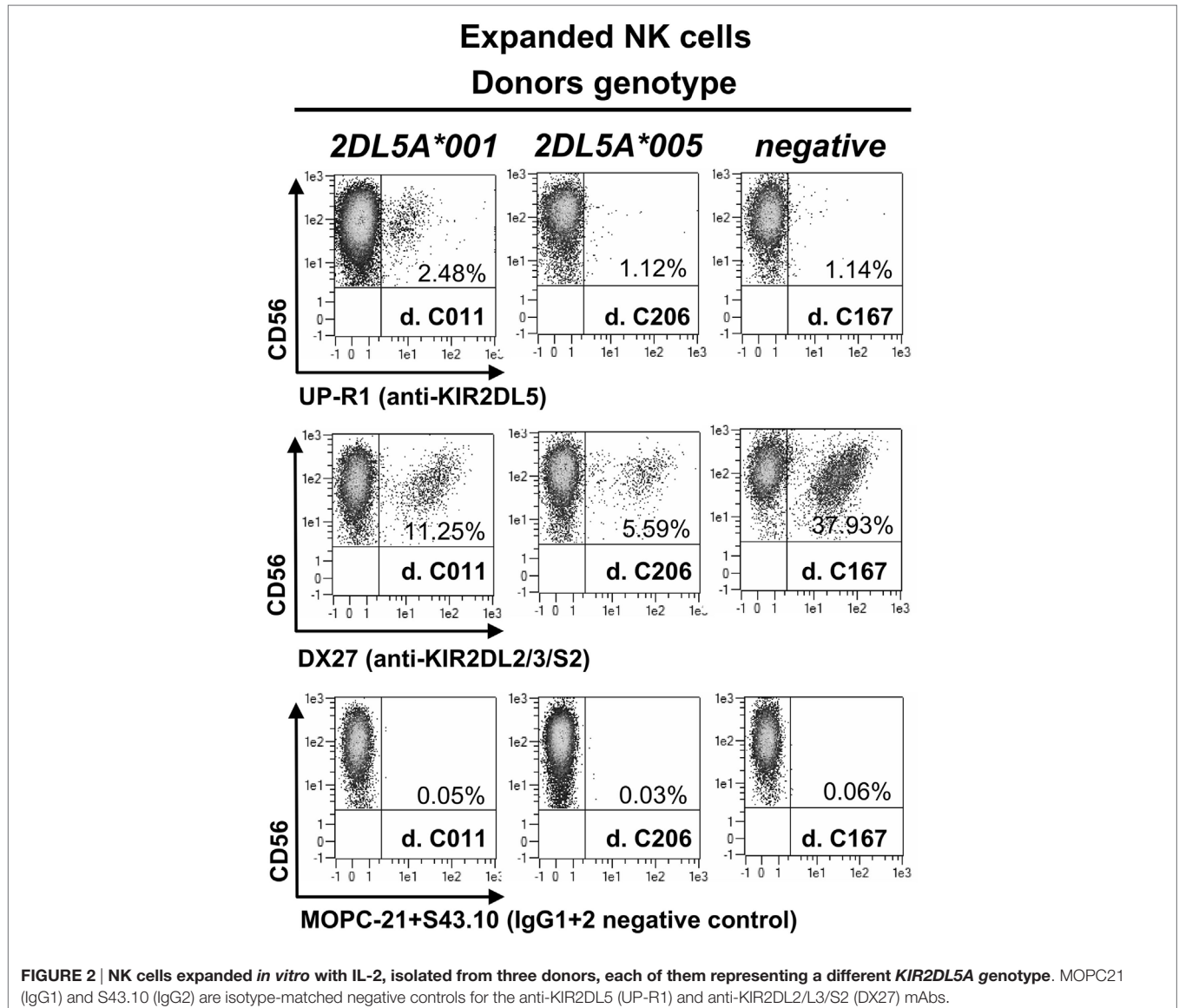
as it happens with other KIR (**Figure 2**). In contrast, NK cells expanded from individuals carrying *KIR2DL5A*005* appear to upregulate no product detectable with UP-R1, remaining indistinguishable from those of a *KIR2DL5A*-negative donor (**Figure 2**). This argues against the possibility of a *KIR2DL5A*005* product being expressed at low levels on the surface of freshly isolated NK cells. Given that *KIR2DL5A*005* mRNA is as readily detected as that of the common allele *KIR2DL5A*001* (14), these results suggest that the putative *KIR2DL5A*005* product is not transported to the cell surface or fails to react with mAb UP-R1.

Predictive Analysis of Posttranslational Modifications, Subcellular Location, and Folding Motifs in *KIR2DL5A* Alleles

Because of the crucial role of protein posttranslational modifications on several cellular processes, including maturation and

protein location, we analyzed whether potential *KIR2DL5A* posttranslational processing could be predictably affected by its allelic polymorphism. The two substitutions that distinguish the primary structures of *KIR2DL5A*001* and **005* are located in the D2 Ig-like domain and involve amino acids with side chains susceptible to glycosylation—asparagine/aspartate-152 and glycine/serine-174. Therefore, we performed a predictive search of possible O- and N-glycosylation motifs in the sequences of their D2 domains. None of the predicted O-glycosylation sites involves *KIR2DL5* polymorphic positions (not shown). In contrast, substitution of asparagine-152 by aspartate in *KIR2DL5A*005* destroys an N-glycosylation site (**Figure 3**) highly conserved in all KIR (25), whose loss might affect *KIR2DL5A*005* surface expression.

We also explored possible presence in the *KIR2DL5A*005* primary structure of sequence motifs that could affect its subcellular location or folding. However, we found no signals for endoplasmic reticulum (ER) retention, such as di-basic (i.e., arginine/lysine)



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D2 domain 98 . . . . . ↓ . . . . . ↓ . . . . . ↓ . . . . . 195
KIR2DL5A*001 GLFGKPSLSAQPGPTVRTGENNVTLSCSRSSFDMYHLSREGRAHEPRLPAVPSVNGTFQADFPPLGPATHGGTYTCFGSLHDSPYEWSDPSDPLLVSVT
KIR2DL5A*005 -----d-----s-----

KIR2DL1*001 --ye-----la-----y-----e--r--g-k-----r---f-----ks-----
KIR2DL1*014 --ye-----la-----y-----e--r--g-k-----r--s-f-----ks-----
KIR2DL4*001 --ye---t-r-----q---i-----e--l-----i-----e--r---f-g-----a---p---

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FIGURE 3 | Comparison of the primary structures of the *KIR2DL5A001 and *005 D2 Ig-like domains, along with those of *KIR2DL1* alleles *001 and *014, and *KIR2DL4**001.** Arrows mark *KIR2DL5A* polymorphic positions 152 and 174, and Phe-129, predictably confronted to Gly/Ser-174 in the receptor architecture. Other residues mentioned in the text are underlined. Amino acid numbering is based on the mature *KIR2DL5A* polypeptide (the *KIR2DL1* N-terminal Ig-like domain, not represented, is five amino acids longer than those of Killer-cell Ig-like receptors 2DL4 and 2DL5).

motifs, or variants of an H/KDEL sequence seen in ER-resident proteins (26). Furthermore, *KIR2DL5A**005, like allele *001, has intact WSXPS motifs in each of its Ig-like domains, mutations of which have been reported to hamper correct folding and surface expression of the *KIR3DL1**004 allele (27), as well as cytokine receptors (28).

Allele *KIR2DL5A**005 Is Weakly Expressed on the Surface of Transfected Cells and Is Poorly Recognized by *KIR2DL5*-Specific mAb UP-R1

To reliably track the expression of *KIR2DL5A**001 and *005, we tagged cDNA constructs of these alleles with a FLAG epitope which should enable us to detect their products independently of UP-R1 recognition. Such constructs were transfected into HEK-293T cells, and expression was verified by western blot with an anti-FLAG mAb. As shown in **Figure 4A**, specific bands of 50–60 kDa were seen in both *KIR2DL5A**001 and *005-transfected cells, in comparison with mock-transfected HEK-293T. Differences in the relative mobility of *KIR2DL5A**001 and *005 were observed, and they are attributable to their differential N-glycosylation, since they disappear after treatment of the cell lysates with PNGase F (**Figure 4B**). Also, due to variable transfection efficacy, the relative amounts of each synthesized protein varied between experiments (e.g., experiments 1 and 2 of **Figure 4A**). To compensate for such variations, transfections were replicated six times using different plasmid batches, EGFP was co-transfected in all experiments, and *KIR2DL5* expression was evaluated on EGFP⁺ cells.

Flow cytometry analysis of *KIR2DL5A*-transfected HEK-293T cells with anti-FLAG mAb revealed discrete subpopulations of cells showing specific surface expression of both *KIR2DL5A**001 and *005 (**Figures 5A,B**). Density of the former molecule was, however, consistently and significantly higher than that of the minor allele *KIR2DL5A**005 (average MFI of six experiments \pm SD: 38 ± 11 vs. 9 ± 8 ; $p = 0.0004$). Similarly, flow cytometry with the *KIR2DL5*-specific mAb UP-R1 showed that *KIR2DL5A**001-transfected cells are stained more intensely than those expressing *KIR2DL5A**005 (MFI 47 ± 16 vs. 4 ± 6 ; $p = 0.0001$, **Figures 5C,D**). These results roughly replicate the observed natural behavior of these alleles, except for the fact that *KIR2DL5A**005 is only detectable on transfected cells. Furthermore, comparison of the staining profiles obtained with anti-FLAG M2 and anti-*KIR2DL5*

UP-R1 mAbs showed that, despite following similar trends, differences in apparent expression levels of *KIR2DL5A**001 and *005 were somewhat more pronounced when cells were stained with UP-R1 mAb (**Figure 5**). For instance, the MFI values of *KIR2DL5A**001 cells were 5.1- to 75.0-fold higher than those of *005 ones when measured with UP-R1, but only 2.0- to 21.6-fold higher when assessed with the anti-FLAG mAb, the difference being statistically significant ($p < 0.05$) and consistently seen in every experiment. This behavior suggests that *KIR2DL5A**005 detection by UP-R1 is limited, not only by lower expression of the molecule but also by poorer recognition by the antibody.

Differential Contribution of the G174S and N152D Polymorphisms to the *KIR2DL5A**005 Phenotype

To assess the relative contribution of the *KIR2DL5A**005 polymorphisms to reduced surface expression and UP-R1 mAb recognition of this allele, we introduced separately the G174S and N152D mutations into the *KIR2DL5A**001 coding region, and analyzed expression on transfected HEK-293T cells as above, using anti-FLAG and anti-*KIR2DL5* mAbs.

Examination of the mutants flow cytometry profiles with the anti-FLAG antibody (**Figures 5A,B**) showed that substitution of aspartate for asparagine-152 induced only a modest effect on *KIR2DL5A* expression levels (MFI: 26 ± 11 vs. 38 ± 11 in *KIR2DL5A**001, $p = 0.0695$). In contrast, the serine for glycine-174 mutant displayed a significant reduction in surface expression in comparison with the wild-type allele (MFI: 18 ± 10 vs. 38 ± 11 , $p = 0.0013$). Western blot analyses of the two mutants revealed comparable levels of *KIR2DL5* protein synthesis (not shown).

Analysis of expression with the *KIR2DL5*-specific mAb UP-R1 replicated the results obtained with the anti-FLAG antibody, except for the facts that both mutants differed significantly from *KIR2DL5A**001 (**Figure 5D**); and that, as it happened in the comparison of *KIR2DL5A**001 and *005, the decrease in recognition of the G174S mutant was more pronounced with UP-R1 than with the anti-FLAG antibody (*KIR2DL5A**001/G174S MFI ratios: UP-R1, 2.7–29.5; anti-FLAG, 1.4–4.6, $p < 0.05$). In fact, G174S mutants showed an average MFI similar to that of the low-expressed allele *KIR2DL5A**005 (8 ± 11 vs. 4 ± 6), supporting that serine-174 hampers recognition by the *KIR2DL5*-specific antibody besides reducing expression levels.

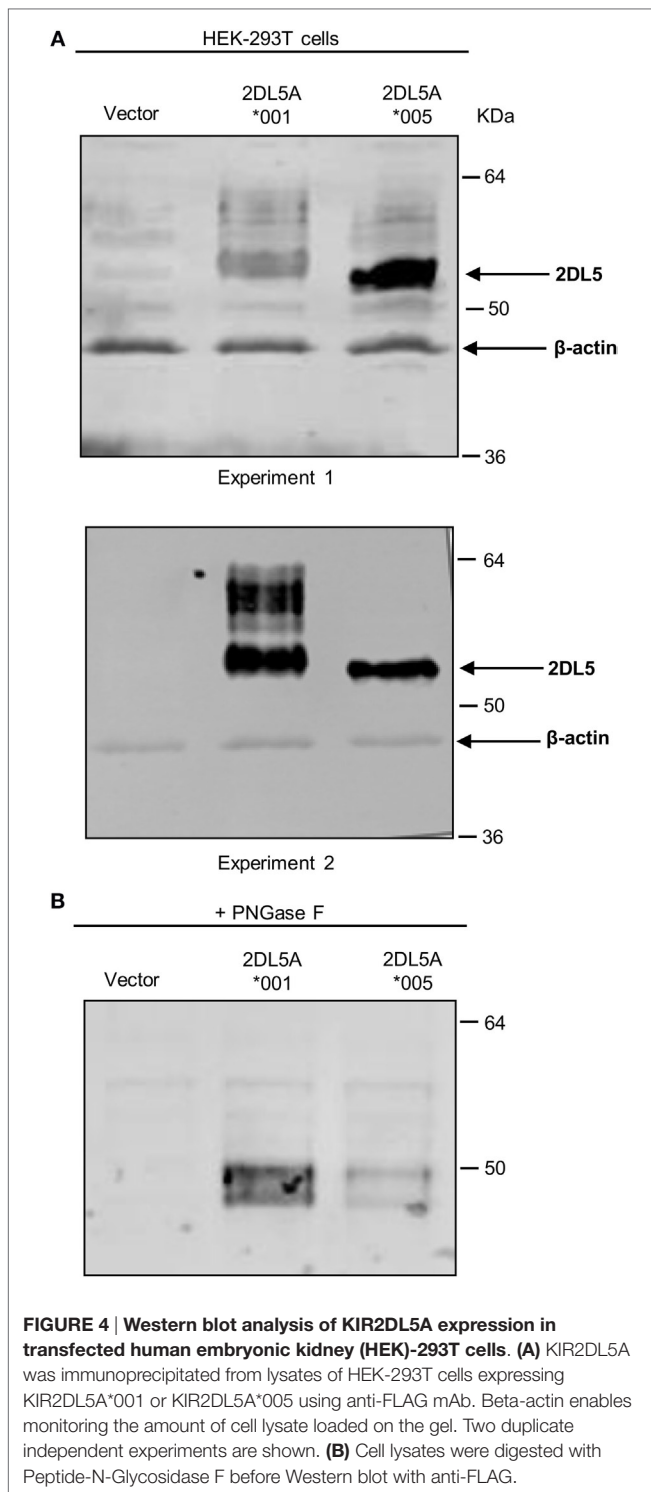


FIGURE 4 | Western blot analysis of KIR2DL5A expression in transfected human embryonic kidney (HEK)-293T cells. (A) KIR2DL5A was immunoprecipitated from lysates of HEK-293T cells expressing KIR2DL5A*001 or KIR2DL5A*005 using anti-FLAG mAb. Beta-actin enables monitoring the amount of cell lysate loaded on the gel. Two duplicate independent experiments are shown. **(B)** Cell lysates were digested with Peptide-N-Glycosidase F before Western blot with anti-FLAG.

To further validate our findings, we replicated the flow cytometry analysis of each *KIR2DL5* construct after nucleofection into Jurkat, a T-cell line previously used in studies of KIR expression (27, 29, 30). In line with the less effective transfection of Jurkat, expression levels of all *KIR2DL5* constructs emulated more closely those seen in NK cells of *KIR2DL5A*001*- and **005*-positive

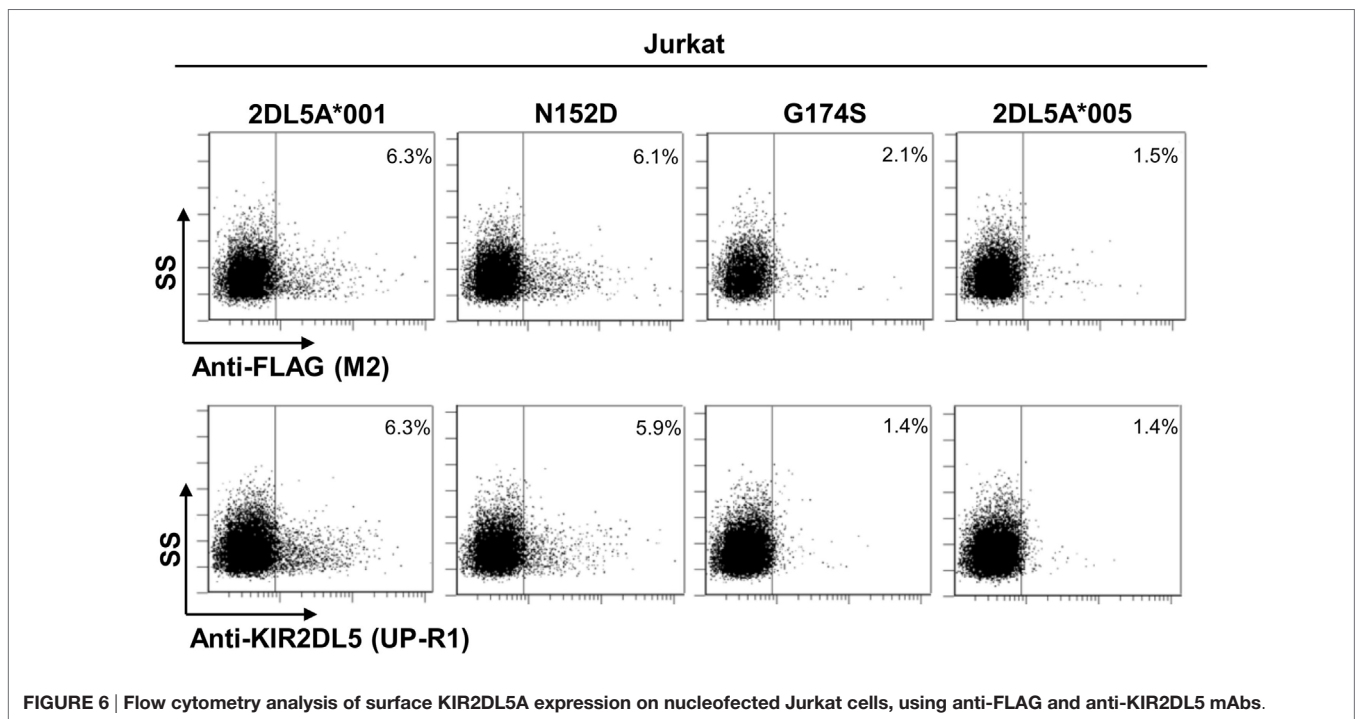
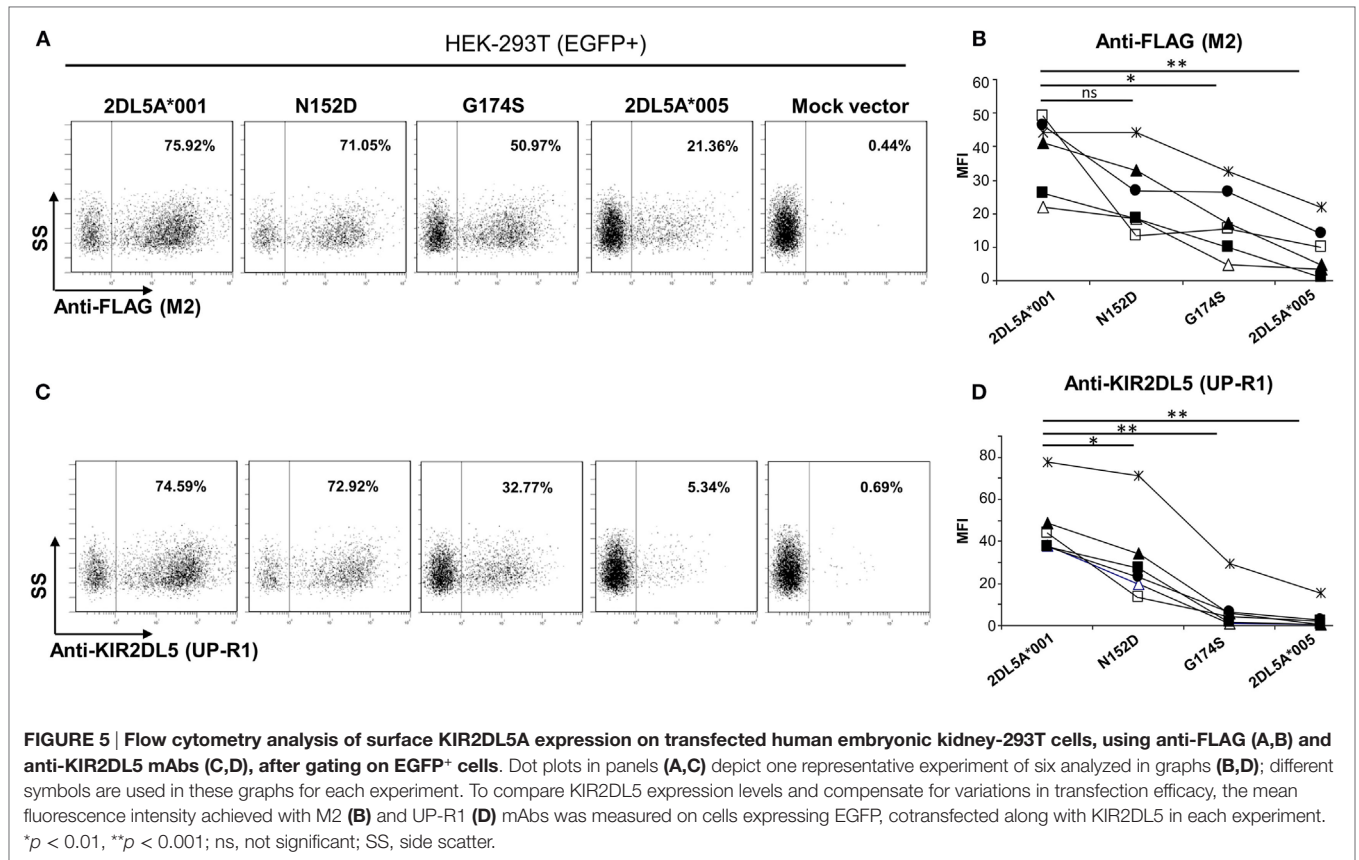
subjects, being 2- to 10-fold lower than those obtained with HEK-293T cells. Despite this difference, the relative expression of each construct in comparison with *KIR2DL5A*001*, determined with either UP-R1 or anti-FLAG mAbs, roughly reproduced that previously obtained in HEK-293T cells; i.e., percentages of cells with appreciable receptor density on the surface were conspicuously lower for *KIR2DL5A*005* and the G174S mutant than for *KIR2DL5A*001*, whereas the N152D mutation had only a minor effect on *KIR2DL5* expression and recognition by UP-R1 (Figure 6).

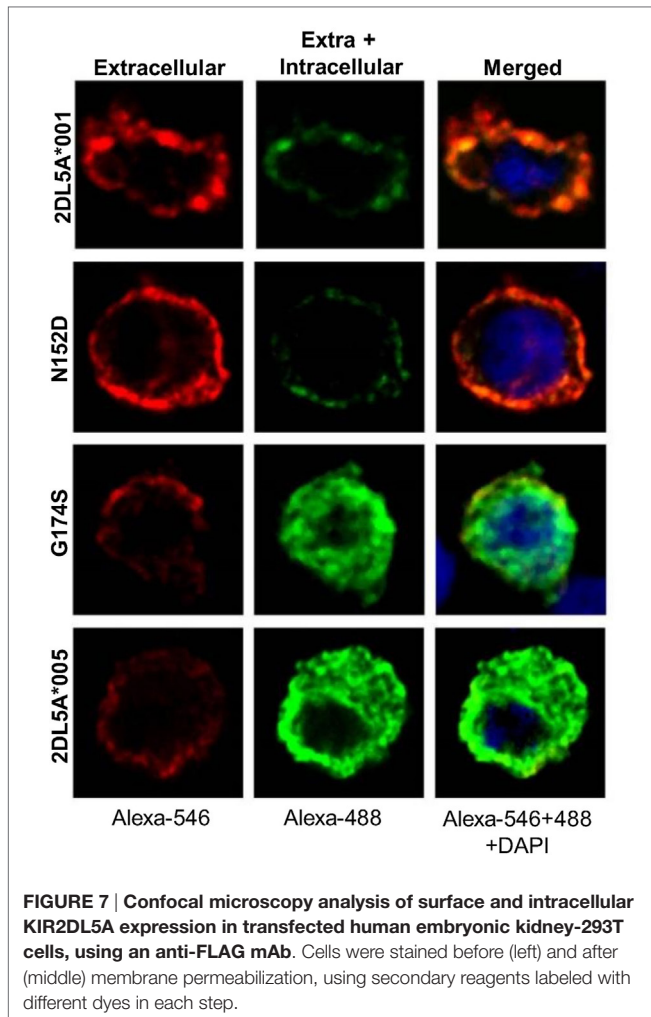
Allele *KIR2DL5A*005* Is Retained Intracellularly Due to a Serine for Glycine Substitution in the D2 Ig-Like Domain

Our previous results show that *KIR2DL5A*005* translation is not followed by efficacious expression on the cell surface. To assess whether the *KIR2DL5A*005* product is retained intracellularly, we performed confocal microscopy experiments of HEK-293T cells transfected with FLAG-tagged constructs of alleles *KIR2DL5A*001* and **005*, and with the above described intermediate mutants N152D and G174S.

As shown in Figure 7, remarkable differences were seen in the cellular distribution of the four transfected constructs, as assessed with an anti-FLAG mAb. In cells expressing *KIR2DL5A*001* or the N152D mutant, fluorescence was localized on the plasma membrane, without appreciable intracellular staining. In contrast, *KIR2DL5A*005*, as well as mutant G174S, were predominantly detected inside transfected cells, despite a small amount of molecules colocalizing with the plasma membrane, in agreement with the flow cytometry results. Unfortunately, *KIR2DL5*-specific mAb UP-R1 behaved poorly after cell fixation–permeabilization, which precluded subsequent studies of intracellular staining with this mAb to track *KIR2DL5A*005* localization in NK cells expressing naturally this molecule.

To gain insight on the mechanism by which a serine for glycine-174 change in the D2 Ig-like domain could hinder *KIR2DL5A* surface expression, we analyzed this substitution in a predicted three-dimensional model of the receptor. To that end, and since the crystallographic structure of *KIR2DL5* has not yet been solved, we modeled its D2 domain on the structure of *KIR2DL1* (31), which, among all KIR, shares with *KIR2DL5* the highest amino acid sequence identity in that region (86.73% with no gaps). QMEAN6 *Z*-scores, estimating the absolute quality of the *KIR2DL5*001* and **005* models, were 0.25 and -0.43 SDs, respectively, from the *KIR2DL1* experimental structure. *KIR2DL5* residue 152 (Asn/Asp) is exposed on the protein surface (segment connecting β -strands D and E), while amino acid 174 (Gly/Ser) is partially hidden inside the receptor architecture (strand F), confronted with phenylalanine-129 (loop 5, near strand C). Replacement of glycine-174 in the *KIR2DL5A*001* model by serine of *KIR2DL5A*005* induces displacement of phenylalanine-129 (Figure 8), as a consequence of the bigger size of the serine hydroxymethyl group in comparison with the hydrogen side chain of glycine-174. Of note, a similar displacement of *KIR2DL1* tyrosine-134 (paralogous of *KIR2DL5* Phe-129, Figure 3), resulting in intracellular retention, has been attributed to an identical

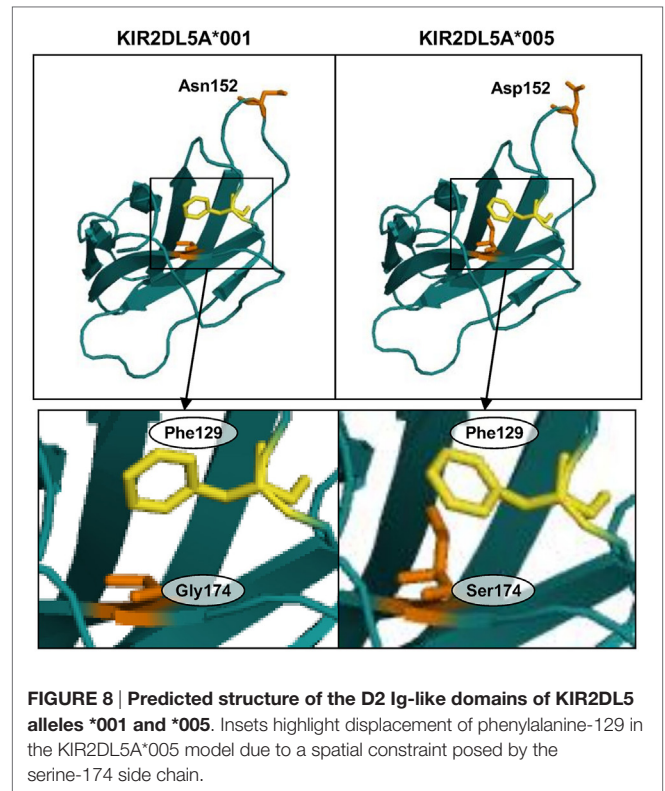




Gly-to-Ser substitution in allele KIR2DL1*014 residue 179 (32), paralogous of KIR2DL5 amino acid 174. KIR2DL5 substitutions G174S and N152D appear to affect no other bonds or interactions between surrounding amino acids, and we found no additional clues to the KIR2DL5A*005 behavior by modeling on KIR2DL4 (PDB accession code 3WYR, results not shown), receptor that, in contrast with KIR2DL1, shares with KIR2DL5 the D0–D2 Ig-like domain organization, but slightly lower percentage of identity in the primary structure.

DISCUSSION

The KIR2DL5 function and importance in human health are poorly understood. Studies on this KIR are complicated by its entangled genetics and expression profiles. Most conspicuous in this regard is frequent discrepancy between detection of the receptor by flow cytometry and simple forms of *KIR* genotyping that inform only of presence or absence of a *KIR2DL5* gene. Such contradicting results (i.e., no protein detectable by flow cytometry in a *KIR2DL5*⁺ individual) are partly explained by most *KIR2DL5B* alleles being epigenetically silenced, which can be



predicted reliably by genotyping the -97 A/G dimorphism of the *KIR2DL5* promoter—only alleles with G (part of a RUNX-binding site) are transcribed (14). In this study, we have addressed another source of apparent discrepancy between KIR2DL5 phenotyping and basic genotyping—lack of a detectable product despite presence of a *KIR2DL5* allele with an intact RUNX site. We have shown here that this situation is regularly seen in *KIR2DL5A**005, part of the common telomeric *KIR* motif *3DS1-2DL5A**005-*2DS3**002-*2DS1-3DL2* (7, 10, 11, 17). According to our results, such phenotype can be anticipated also for *KIR2DL5B**0020106 and *00202, centromeric alleles governed by predictably functional promoters and encoding mature polypeptides identical to *KIR2DL5A**005 (16).

Western blot, flow cytometry, and confocal microscopy studies of cells transfected with tagged *KIR2DL5A* constructs show existence of a *KIR2DL5A**005-gene product that, in comparison with the common allele *KIR2DL5A**001, scarcely reaches the plasma membrane and is mostly retained intracellularly. Comparison of the flow cytometry profiles of mAbs directed against the tag and the receptor also reveal that the only available KIR2DL5-specific mAb, UP-R1, binds to 2DL5A*005 with lower efficacy than to 2DL5A*001, suggesting that its epitope is somehow altered in the former allele. In consequence, we cannot exclude completely that *KIR2DL5A**005 traces reach the NK-cell surface at levels undetectable by the mAb, as shown for another KIR retained intracellularly, 3DL1*004 (30). Against this possibility is that NK cells expanded from *KIR2DL5A**005 subjects remain negative for UP-R1. We have also not ruled out that intracellular KIR2DL5 might exert some function. In this regard, 2DL4, the only other

human KIR with D0–D2 domains, has been reported to signal from its preferential intracellular location (33).

The two amino acid changes in the membrane-proximal D2 Ig-like domain that distinguish KIR2DL5A*005 from allele *001 (and most other KIR) appear to have independent and different contributions to its phenotype, according to the behavior of cells transfected with the individual mutant sequences. Replacement of asparagine-152 by aspartate appears to have only a minor effect on expression and UP-R1 mAb recognition in spite of destroying an N-glycosylation site well conserved in KIR, while substitution of serine for glycine-174 is enough for inducing intracellular retention, reducing drastically surface expression and impairing mAb recognition.

The reason for serine-174 hampering KIR2DL5 expression is apparently subtle. Three-dimensional modeling only shows that the serine side chain forces, through steric hindrance, displacement of the phenylalanine-129 aromatic residue from its place in KIR2DL5A*001. It is possible that such displacement results in some disorganization of the D2 Ig-like domain architecture, hampering proper folding and favoring intracellular retention of the receptor, rather than trafficking to the cell surface. In support of this possibility is that a similar phenomenon has been reported for KIR2DL1, in which an identical polymorphism also associates with lack of surface expression (32). Such disorganization could also explain that a hardly exposed amino acid affects UP-R1 mAb recognition.

KIR2DL5 is, in many aspects, an intriguing molecule. Contrasting with most human KIR, it is seen in several primate species, feature shared by KIR2DL4. But, unlike the latter KIR, 2DL5 is not conserved in all humans; on the contrary, despite its gene being duplicated (trait specific of our species), nearly a half of human genomes lack both *KIR2DL5A* and *KIR2DL5B* (7). Furthermore, the two loci are represented by alleles known or expected to be expressed on the NK-cell surface (e.g., *2DL5A*001*, *2DL5B*00602*); and by ones that are not, either due to silenced transcription (e.g., *2DL5B*00601*), to amino acid substitutions that induce intracellular retention (e.g., *2DL5A*005*), or both (the common *2DL5B*002* alleles). The fact that surface and intracellularly expressed and also non-expressed *KIR2DL5* alleles are all maintained at significant frequencies in different human populations suggests that balancing selection favors persistence of functionally divergent KIR2DL5 allotypes that might help humans to survive to different selective pressures. Elucidation of such pressures and comprehension of other aspects of KIR2DL5 function await identification of the molecules recognized by the receptor.

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In summary, we have shown that a common polymorphism in the *KIR2DL5* coding sequence targets the encoded receptor for intracellular expression, which, together with variations in its promoter, explains all apparent discrepancies between KIR2DL5 geno- and phenotyping. A major implication of our results is that analyses of NK-cell repertoire and genetic association studies aimed at exploring or understanding relationship of KIR2DL5 with different health conditions will only be meaningful if its allelic polymorphism and different expression profiles are taken into account.

AUTHOR CONTRIBUTIONS

EC designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. EE designed and performed experiments, and analyzed and interpreted data. CV designed the study, directed research, and wrote the manuscript.

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Conflict of Interest Statement: The Immunogenetics and Histocompatibility group of *Instituto de Investigación Sanitaria Puerta de Hierro*, directed by CV, shares with Dr. Miguel López-Botet (*Universitat Pompeu Fabra*, Barcelona, Spain) rights to monoclonal antibody UP-R1 and has received license fees and royalties from companies selling the antibody. The authors declare that the research was conducted in the absence of any other commercial or financial relationships that could be construed as a potential conflict of interest.

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