ORIGINAL ARTICLE

Natural LILRB1 D1-D2 variants show frequency differences in populations and bind to HLA class I with various avidities

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Abstract

Leukocyte immunoglobulin-like receptor B1 (LILRB1) is widely expressed on various immune cells and the engagement of LILRB1 to HLA class I and pathogen-derived proteins can modulate the immune response. In the current study, 108 LILRB1 alleles were identified by screening the LILRB1 locus from the 1000 Genomes Phase 3 database. Forty-six alleles that occurred in three or more individuals encode 28 LILRB1 allotypes, and the inferred LILRB1 allotypes were then grouped into 9 LILRB1 D1-D2 variants for further analysis. We found that variants 1, 2, and 3 represent the three most frequent LILRB1 D1-D2 variants and the nine variants show frequency differences in populations. The binding assay demonstrated that variant 1 bound to HLA class I with the highest avidity, and all tested LILRB1 D1-D2 variants bound to HLA-C with lower avidity than to HLA-A and -B. Locus-specific polymorphisms at positions 183, 189, and 268 in HLA class I and dimorphisms in HLA-A (positions 207 and 253) and in HLA-B (position 194) affect their binding to LILRB1. Notably, the electrostatic interaction plays a critical role in the binding of LILRB1 to HLA class I as revealed by electrostatic analysis and by comparison of different binding avidities caused by polymorphisms at positions 72 and 103 of LILRB1. In this paper, we present a comprehensive study of the population genetics and binding abilities of LILRB1. The data will help us better understand the LILRB1-related diversity of the immune system and lay a foundation for functional studies.

Introduction

Leukocyte immunoglobulin-like receptor B1 (LILRB1), also known as LIR-1, ILT-2, MIR-7, and CD85j, is an inhibitory receptor that was first identified by Cosman et al. in 1997 (Cosman et al. 1997). LILRB1 is encoded within the leukocyte receptor complex, which harbors the LILR and killer cell immunoglobulin-like receptor (KIR) families, and occupies about 1 Mb of human chromosome 19q13.4 (Wende et al. 1999; Canavez et al. 2001). LILRB1 is widely expressed on various immune cells that include macrophages, monocytes, dendritic cells, B cells, and subsets of NK cells and T cells (Borges et al. 1997; Burshtyn and Morcos 2016). It is also expressed on decidual immune cells (McIntire et al. 2008). The expression pattern of LILRB1

suggests that it plays crucial roles in both immune responses and in supporting fetal tolerance in pregnancy.

LILRB1 is a type I transmembrane glycoprotein with four extracellular immunoglobulin-like (Ig-like) domains and four immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within the cytoplasmic region for signal transduction (Cosman et al. 1997). The two membrane distal Ig domains (D1-D2) are responsible for interacting with HLA class I, with the D1 domain engaging with the α3 region of the HLA class I heavy chain, and the D2 domain interacting with beta 2 microglobulin (β2m) (Willcox et al. 2003). No major binding sites have been identified within the D3 and D4 domains of LILRB1 (Nam et al. 2013). Since the α3 region of HLA class I antigens and β2m are relatively conserved, LILRB1 binds to a broad range of HLA class I. The LILRB1-HLA class I binding pattern is different from that of KIRs and T cell receptors (TCR), which recognize the polymorphic α1 and α2 domains of subsets of HLA class I (Djaoud and Parham 2020). Jones et al. found that LILRB1 bound to a broad range of HLA class I by testing the binding of one LILRB1 variant (unspecified, but most likely variant 1) to a panel of 97 HLA class I antigens

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in a single antigen bead assay (Jones et al. 2011). The effect of amino acid changes in the D1-D2 domains of LILRB1 on binding to select HLA class I has been investigated. The four most-studied amino acid changes in this region are L68P, A93T, I142T, and S155I, with the numbers corresponding to the full-length protein sequence. Yu et al. found that LILRB1 bound to HLA-B*58, with the LILRB1 LAIS variant binding to a lesser extent than the PTTI variant (Yu et al. 2018), and Kuroki et al. demonstrated that the LILRB1 LAIS and PTTI variants bind to HLA-A*11, HLA-C*04, HLA-B*35, and HLA-G1 to a similar degree (Kuroki et al. 2005). These studies focused on the two common LILRB1 variants and a small number of HLA ligands. As the differences in binding avidities between LILRB1 and HLA class I may substantially be related to the diversity of immune response among individuals, we determined the range of natural variation in LILRB1 and tested binding against a comprehensive panel of HLA allotypes.

It has also been shown that LILRB1 is related to pathogen infection and several diseases. LILRB1 binds to the HLA class I mimic UL18 encoded by human cytomegalovirus (Yang and Bjorkman 2008) with a binding affinity > 1000-fold higher than that for HLA class I (Chapman et al. 1999). LILRB1 also binds to a subset of repetitive interspersed families of polypeptides (RIFINs) of the *P. falciparum* parasite, which can inhibit LILRB1-expressing B cells and NK cells and help to evade the host immune system (Saito et al. 2017). LILRB1 is also associated with several autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Monsiváis-Urenda et al. 2007; Kuroki et al. 2005). LILRB1 plays dual roles in cancer biology, acting both as an immune checkpoint molecule and as a tumor-sustaining factor. These observations of the roles of LILRB1 in disease have highlighted the importance of analyzing the LILRB1 diversity across populations to lay a foundation for functional studies to determine disease mechanism. However, the natural LILRB1 alleles and their frequencies in each human population largely remain unknown.

In the present study, we analyzed the LILRB1 locus from the 1000 Genomes Phase 3 database. LILRB1 alleles and allotypes were inferred, and their frequencies in populations were calculated. Eight natural LILRB1 D1-D2 variants were produced and their binding avidities for a panel of 97 HLA class I antigens by single antigen bead assay were tested. The data will help us better understand the LILRB1-related diversity of the immune system and lay a foundation for functional studies.

Materials and methods

Selection of individuals from the 1000 Genomes database

In order to assess global genetic variation in *LILRB1* locus, we selected individuals from the 1000 Genomes Project database (http://www.internationalgenome.org) that had read depth sufficient for robust SNP determination (Clarke et al. 2017; Auton et al. 2015). We correlated read depth to mapped.bam file size by selecting 15 individuals with file sizes ranging from 15 to 40 Gb and determining the read depth for each coding exon (E3–E16) of LILRB1 using MOSDEPTH (Pedersen and Quinlan 2018). Sufficient read depth for each coding exon of LILRB1 was obtained when individuals had mapped.bam file sizes of 30 Gb and larger. Using this cutoff, 429 individuals (out of 2504 assessed) from 26 sub-populations were selected from the 1000 Genomes Project Phase 3 database.

Inference of LILRB1 allele and allotype for selected individuals

We obtained the SNPs for the complete LILRB1 region on human chromosome 19 (55,085,346–55,148,979) using Data Slicer (http://grch37.ensembl.org/Homo_sapiens/Tools/DataSlicer). The focus of this analysis was variation in the coding region so we excluded SNPs in the introns, 5′-UTR, and 3′-UTR. In the coding region, 149 SNPs were found, 66 of which only occur once and were excluded in the present study. The remaining 83 SNPs were analyzed by PHASE2.1 (Stephens et al. 2001) using the default parameters to infer the alleles. The inferred alleles were then translated into corresponding allotypes for further analysis. This analysis provided all common naturally occurring variants and their population frequencies.

Production, purification, and integrity analysis of LILRB1-D1D2-Fc proteins

The sequence encoding the D1-D2 domains of LILRB1 variant 1 and the hinge-CH2-CH3 region of human IgG1 was synthesized by GenScript, with *XbaI* and *NotI* restriction sites at the 5′-end and 3′-end respectively. The synthesized sequence was first cloned into the NotI/XbaI sites of the pcDNA3.1(+) vector and the sequence confirmed by Sanger sequencing (MCLAB, CA, USA). The sequencing primers are listed in Table 1. Site-directed mutagenesis was conducted using the plasmid pcDNA3.1-LILRB1-D1D2-Fc to generate the sequences encoding the remaining eight LILRB1-D1D2-Fc proteins. The primers used for site-directed mutagenesis are listed in Table 1. Sequences of all variants were confirmed by Sanger sequencing. The pcDNA3.1-LILRB1-D1D2-Fc constructs were digested with *XbaI* and *NotI* (New England Biolabs, UK) and the target sequences were cloned into *XbaI* and *NotI* digested pAcGP67A vector to be used for protein production. The sequences in the pAcGP67A-LILRB1-D1D2-Fc constructs

Table 1 Primers used in the present study

	Primer	Sequence (5′–3′)
Sequencing	pcDNA3.1-T7	TAATACGACTCACTATAGG
	pcDNA3.1-BGH	TAGAAGGCACAGTCGAGG
	pAcGP67A-F	AAATGATAACCATCTCGC
	pAcGP67A-R	GTCCAAGTTTCCCTG
	Internal-F	AACCAGGCTCTGTGATCACC
Mutagenesis	68-F	GAGAAAAGAAAACAGCAC*CC*TGGATTACACGGATCCC
	68-R	GGGATCCGTGTAATCCAGGGTGCTGTTTTCTTTTCTC
	72-F	CAGCACTCTGGATTACAC*A*GATCCCACAGGAGCTTGTG
	72-R	CACAAGCTCCTGTGGGATCTGTGTAATCCAGAGTGCTG
	93-F	CCATCACCTGGGAACAT*A*CAGGGCGGTATCGCTG
	93-R	CAGCGATACCGCCCTGTATGTTCCCAGGTGATGG
	103-F	CGCTGTTACTATGGTAGC*C*CACACTGCAGGCCGCTCAG
	103-R	CTGAGCGGCCTGCAGTGTGGCTACCATAGTAACAGCG
	142-F	GAACTCAGGAGGGAATGTAA*CC*CTCCAGTGTGACTCACAG
	142-R	CTGTGAGTCACACTGGAGGGTTACATTCCCTCCTGAGTTC
	155-F	GGTGGCATTTGATGGCTTCA*T*TCTGTGTAAGGAAGGAGAAG
	155-R	CTTCTCCTTCCTTACACAGAATGAAGCCATCAAATGCCACC
	107,110F	AGCGACACTGCAGGC*T*GCTCAGAG*CC*CAGTGACCCCCTGGA
	107,110R	TCCAGGGGGTCACTGGGCTCTGAGCAGCCTGCAGTGTCGCT

The letters in italics indicate the mutated nucleotide bases. The digits indicate the positions of mutated residues

were again confirmed by Sanger sequencing. Despite repeated attempts at mutagenesis, we were unable to successfully produce the variant 8 clone and continued the analysis with the remaining eight variants.

Protein production and purification was conducted as described (Hilton et al. 2015). Briefly, the pAcGP67A-LILRB1-D1D2-Fc constructs were co-transferred into *Sf9* insect cells with linearized baculovirus (Expression System, Cat. #91–002, USA) using Cellfectin (Invitrogen, CA, USA). SDS-PAGE was performed to measure the protein yield after each round of viral amplification. For large-scale protein production, 1 L of *Sf9* cells at a cell density of 2 M/mL was infected with 1 mL of high viral titer P2 supernatant for 96 h. Cell supernatant was collected by centrifugation (2500 g, 10 min, 4 °C) and filtered through a 0.2-μm filter (Nalgene, Cat. #450–0020). After incubation with protein A sepharose beads overnight at 4 °C, the beads were washed with 500 mL of PBS. The LILRB1-D1D2-Fc proteins were eluted from the beads with 0.1 M glycine (pH 2.7), with the eluted protein being immediately neutralized with 0.2 M Tris-base (pH 9). The proteins were adjusted to 1 mg/mL and stored at −20 °C for further use.

The integrity of the purified proteins was assessed by flow cytometry using a Cytek Aurora spectral cytometer. Briefly, 20 μL of anti-human IgG-coated beads was added to 500 μL of LILRB1-D1D2-Fc fusion protein at a concentration of 100 μg/mL. After incubation at 4 °C for 30 min, the

beads were collected by centrifugation (250 g for 3 min) and washed 3 times with FACS buffer (FCB). Following the last wash, the beads were resuspended in 50 μL of FCB with 2 μL of APC conjugated anti-human LILRB1 antibody (Thermo Fisher, Cat. #17–5129-42). After incubation at 4 °C for 30 min, the beads were collected by centrifugation (250 g for 3 min) and washed 3 times with FCB. The beads were then resuspended in 150 μL of PBS and analyzed by flow cytometry.

HLA binding assay

LILRB1-D1D2-Fc proteins were tested for binding to 97 classical HLA class I antigens (31 HLA-A, 50 HLA-B, and 16 HLA-C) using the single antigen bead assay developed by One Lambda (Thermo Fisher Scientific, Cat. #LS1A04) as described (Hilton et al. 2017). Briefly, LILRB1-D1D2-Fc proteins were diluted to 100 μg/mL with PBS and incubated with the LABScreen microbeads (60 min at 4 °C, gently shaking). As a positive control for antigen density on individual beads, beads were incubated with W6/32, which is a mouse mAb that binds a common epitope to all HLA-A, -B, and -C (W6/32 (Barnstable et al. 1978)). Beads incubated with same volume of PBS were the negative control. After 4 washes, LILRB1-D1D2-Fc binding beads and negative control beads were resuspended in 100 μL PBS with 1% PE-conjugated goat anti-human IgG-Fc antibody and W6/32 binding beads were resuspended in 100 μL PBS

with 1% PE-conjugated goat anti-mouse IgG-Fc antibody. They were then incubated for 60 min at 4 °C, gently shaking. After 4 washes, the beads were resuspended in 100 μL PBS and amount of bound antibody was quantitated using a BioPlex 200. The binding of LILRB1-D1D2-Fc to HLA class I was normalized to W6/32 binding, calculated using the following formula: (LILRB1-D1D2-Fc binding − negative control binding) / (W6/32 binding − negative control binding).

Statistical analysis

The data were analyzed statistically using the SPSS statistics package 25 (SPSS Inc. Chicago, USA). One-way analysis of variance (ANOVA) and LSD post hoc test were used to analyze the binding data, with $P \leq 0.05$ between groups considered significant.

Results

The overall goals of our study were to determine frequency differences for LILRB1 polymorphisms in worldwide populations, and to assess the effect of these polymorphism on LILRB1 binding to ligands. We analyzed individuals from the 1000 Genomes Project database to determine the number and frequency of SNPs in the coding region of LILRB1. The results of this study were used to determine the common LILRB1 D1-D2 allotypes which were used in binding experiments.

Selection of individuals for SNP analysis

The 1000 Genomes Project was designed to provide a comprehensive description of human genetic variation by sequencing multiple individuals from worldwide populations. Over three phases of the 1000 Genomes Project, the genomes of 2504 individuals from 26 sub-populations were sequenced and reconstructed. The average read depth for whole-genome sequencing of the 2504 individuals is 7.4X (Auton et al. 2015). While Auton et al. have already qualified the imputation of accuracy for the whole dataset, we still want to know the sequencing quality of our targeted *LILRB1* region. We expected that read depth in the coding region of *LILRB1* would correlate with the file size of available mapped reads. To determine a cutoff for file size that would be sufficient for robust SNP calling, fifteen individuals with mapped.bam files ranging from 15 to 40 Gb were selected from the 1000 Genomes Phase 3 database:

HG01912, HG03432, HG02502, HG02580, HG02477, HG01956, HG02558, HG03950, HG02508, HG03085, HG02974, NA19984, HG02420, NA19900, and HG02284 were analyzed. The read depth for each coding exon (E3–E16) of LILRB1 was determined using MOSDEPTH. We found that an average read depth of about 8X, sufficient for robust SNP calling, for the complete coding region of LILRB1 was obtained when individuals had mapped.bam file sizes of 30 Gb and larger (Sup. Fig. 1A). A representative read depth analysis is shown in Sup. Fig. 1A. Using this file size cutoff, 429 individuals were selected from 26 sub-populations (Fig. 1).

Determining common LILRB1 D1-D2 allotypes

Analysis of the coding region of LILRB1 in the 429 selected individuals identified 149 SNPs. Of these, 66 SNPs occurred only once and were excluded from further analysis. The remaining 83 SNPs were found to be 62.7% nonsynonymous and 37.3% synonymous (Sup. Fig. 1B). Analysis of the 83 SNPs by PHASE revealed 108 alleles, of which 46 alleles occur once, 16 alleles occur twice, and 46 alleles occur three or more times (Sup. Figs. 1C and 2). These 46 LILRB1 alleles that occur three or more times encode 28 LILRB1 allotypes and are listed in Fig. 2. As our goal was to assess the effect of LILRB1 polymorphism on binding to HLA class I, we then grouped the 28 LILRB1 allotypes into nine LILRB1 D1-D2 variants based on the amino acid sequences of the D1-D2 region (Fig. 3A, B) for assessment by binding assay, as crystal structure analysis of LILRB1-HLA class I showed these domains are responsible for ligand interactions (Willcox et al. 2003).

LILRB1 D1-D2 variants show frequency differences in populations

We also calculated the frequency of these nine natural LILRB1 D1-D2 variants in populations (Fig. 3C). Variants 1, 2, and 3 represent the three most frequent LILRB1 variants in populations worldwide. Variant 1 (previously referred to as the LAIS variant) is the most frequent variant in European and South Asian populations, with frequencies of 63.6% and 62.6%, respectively. Variant 2 (previously referred to as the PTTI variant) is at low frequency in African populations (3.1%), whereas it is the most frequent variant in East Asian populations (46.2%). Variant 3 is enriched in African populations with a frequency of 35.3%, while it is less frequent in South Asian populations with a frequency of only 4.4%. Some rare variants (4–9) were also found, and demonstrated variable fre-
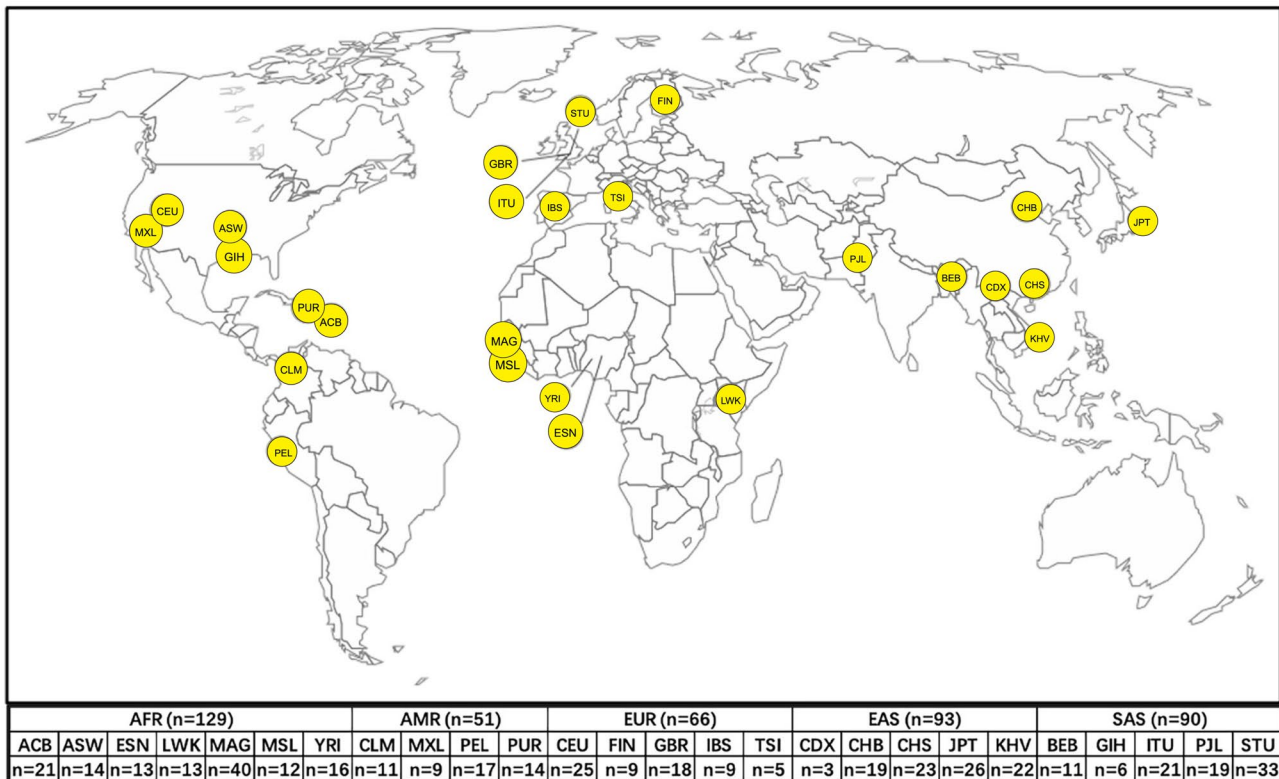
AFR (n=129)							AMR (n=51)				EUR (n=66)					EAS (n=93)					SAS (n=90)				
ACB	ASW	ESN	LWK	MAG	MSL	YRI	CLM	MXL	PEL	PUR	CEU	FIN	GBR	IBS	TSI	CDX	CHB	CHS	JPT	KHV	BEB	GIH	ITU	PJL	STU
n=21	n=14	n=13	n=13	n=40	n=12	n=16	n=11	n=9	n=17	n=14	n=25	n=9	n=18	n=9	n=5	n=3	n=19	n=23	n=26	n=22	n=11	n=6	n=21	n=19	n=33

Fig. 1 Individual selection from the 1000 Genomes Phase 3 database. The location of each sub-population is shown in the map and the abbreviation of its name is indicated by capital letters in the yellow circle. The number of individuals in each sub-population is shown below the map. Regional and population identifiers are the same as used for the 1000 Genomes Project database (http://www.internationalgenome.org)

quencies in global populations. These LILRB1 variants with distinct frequencies in populations may possess different binding affinities for HLA class I and pathogen-derived proteins.

LILRB1 D1-D2 variants bind to HLA class I with different avidities

All LILRB1 D1-D2 variants except variant 8 were successfully produced and purified from *Sf9* cells (Fig. 4A). The integrity of the purified proteins was assessed by flow cytometry using APC conjugated anti-human LILRB1 antibody. The data demonstrated that all produced proteins bind to anti-human LILRB1 antibody efficiently, indicating that the D1-D2 portion is functional (Fig. 4B). The binding of LILRB1 D1-D2 variants to HLA class I was tested by single antigen bead assay (Fig. 4C). Variant 1 binds to HLA class I with the highest avidity. Variant 5 binds to HLA class I with higher avidity than other variants except variant 1. Variants 2, 3, 6, and 9 have similar binding avidities for HLA class I, with the binding avidity of variant 2 for HLA class I slightly lower than that of variant 9. Variants 4 and 7 bind to HLA class I with the lowest binding avidity, and there is no significant difference between their binding avidities. The detailed binding data of LILRB1 D1-D2 variants for HLA class I are shown in Sup. Fig. 3. Although their binding avidities differ to some extent, they have a similar binding specificity for HLA class I.

LILRB1 D1-D2 variants bind to HLA-A, -B, and -C with different avidities

We analyzed the binding to HLA-A, -B, and -C separately to assess binding differences for the LILRB1 D1-D2 variants (Fig. 5). Our data demonstrated that all tested LILRB1 D1-D2 variants bound to HLA-C with significantly lower avidity compared to binding to HLA-A or -B. Variants 3, 4, 5, 6, 7, and 9 bound to HLA-A with slightly higher avidity than to HLA-B. However, variants 1 and 2 binding to HLA-A and HLA-B had no significant difference.

Polymorphisms in the α3 domain of HLA class I affect the binding to LILRB1

Since the α3 domain of HLA class I is responsible for interacting with the D1 domain of LILRB1 as shown by the crystal structure (Willcox et al. 2003), we then exam-

cDNA coord.	12	203	215	277	307	319	329	425	464	659	705	847	926	954	1048	1115	1203	1212	1298	1342	1375	1408	1690	1748	1919	1933	Alleles
Codon	4	68	72	93	103	107	110	142	155	220	235	283	309	318	350	372	401	404	433	448	459	470	564	583	640	645	
Domain	SP	D1-D2									D3-D4								STEM			TM	CYT				
Allotype 1	I	L	R	A	D	R	S	I	S	L	I	T	A	I	G	T	Q	K	P	G	L	I	E	G	V	A	A1, 3, 5, 27, 34, 36, 37
Allotype 2	-	P	-	T	-	-	-	T	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A2, 16, 20, 25
Allotype 3	-	P	-	-	-	-	-	T	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A4, 6
Allotype 4	-	P	-	-	-	-	-	T	I	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	A7, 28, 29, 42
Allotype 5	-	-	-	-	-	-	-	-	I	-	-	A	-	-	-	M	L	N	-	R	-	V	-	-	-	-	A8
Allotype 6	-	P	-	-	-	-	-	T	I	-	M	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	A9
Allotype 7	-	P	-	-	H	-	-	T	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A10
Allotype 8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	V	V	-	E	-	-	A11
Allotype 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	V	-	-	-	-	A12
Allotype 10	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	A13
Allotype 11	-	P	-	-	-	-	-	T	I	-	-	-	-	-	R	-	-	-	-	R	V	V	-	-	-	-	A14, 19
Allotype 12	-	P	-	-	-	-	-	T	I	P	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	A15
Allotype 13	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A17
Allotype 14	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A18
Allotype 15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	A21
Allotype 16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	E	-	A22
Allotype 17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-	A23
Allotype 18	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	A24
Allotype 19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	T	A26
Allotype 20	-	P	-	-	-	-	-	T	I	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	A30
Allotype 21	M	P	-	-	-	C	P	T	I	-	-	-	-	M	R	-	-	-	-	R	V	V	-	-	-	-	A31, 32, 46
Allotype 22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	V	V	-	-	-	-	A33
Allotype 23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	A35
Allotype 24	-	P	-	-	-	-	-	T	I	-	-	-	-	-	-	-	L	N	-	R	-	-	-	-	-	-	A38, 41
Allotype 25	-	P	-	-	-	-	-	T	-	-	-	-	-	-	R	-	-	-	-	R	V	V	-	-	-	-	A39, 40
Allotype 26	-	P	-	-	H	-	-	T	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	A43
Allotype 27	-	P	-	T	-	-	-	T	I	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	A44
Allotype 28	M	P	-	-	-	C	P	T	I	-	-	-	-	M	R	-	-	-	-	R	V	V	-	E	-	-	A45

Fig. 2 Human LILRB1 is moderately polymorphic. The 46 LILRB1 alleles found in three or more individuals encode 28 LILRB1 allotypes. The amino acid changes are summarized by different domains, including the signal peptide (SP), D1-D2 domains, D3-D4 domains, STEM region, transmembrane region (TM), and the cytoplasmic tail (CYT)

ined whether the amino acid changes in the α3 domain of HLA class I affect binding to natural LILRB1 D1-D2 variants. In total, there are 21 polymorphisms in the α3 domain for the 97 tested HLA class I antigens (Fig. 6A–C). Four of the polymorphisms were not dimorphic within at least one of the three HLA class I types. These polymorphisms distinguished the HLA-A, -B, and -C from each other and may be responsible for the avidity differences seen between them. All HLA-A and HLA-B have 183D, and they bind to all LILRB1 D1-D2 variants with significantly higher avidity than HLA-C with 183E (Fig. 7A). All HLA-A have 189M, and all HLA-B and HLA-C have 189V, and the binding avidity of HLA-A with 189M is significantly higher than that of HLA-B and HLA-C with 189V (Fig. 7B). All HLA-A and HLA-C have 239G, and HLA-B (except HLA-B*73:01) have 239R, and there is no significant difference in the binding avidity for LILRB1 D1-D2 variants between the

two groups (Sup. Fig. 4A). HLA-A (except HLA-A*80:01) and HLA-B (except HLA-B*73:01) have 268K, and all HLA-C have 268E, and the binding avidity of HLA-C with 268E for LILRB1 D1-D2 variants is significantly lower than of HLA-A and HLA-B with 268K (Fig. 7C).

For the remaining 17 polymorphisms, 11 of them occur in less than two HLA class I allotypes and were not used for statistical comparison. Finally, the remaining six polymorphisms are dimorphic within at least one of the three HLA class I types. Our data demonstrated that 194I in HLA-B is associated with higher binding to all tested LILRB1 D1-D2 variants (Fig. 7D). 207G in HLA-A is associated with higher binding to LILRB1 variants 2, 3, 4, 6, 7, and 9 (Fig. 7E), and 253E in HLA-A is associated with higher binding to LILRB1 variants 3, 4, 6, 7, and 9 (Fig. 7F). However, dimorphisms at positions 184 (184P vs 184A), 193 (193A vs 193P), 194 (194V vs 194I), and 246 (246A vs 246S) in
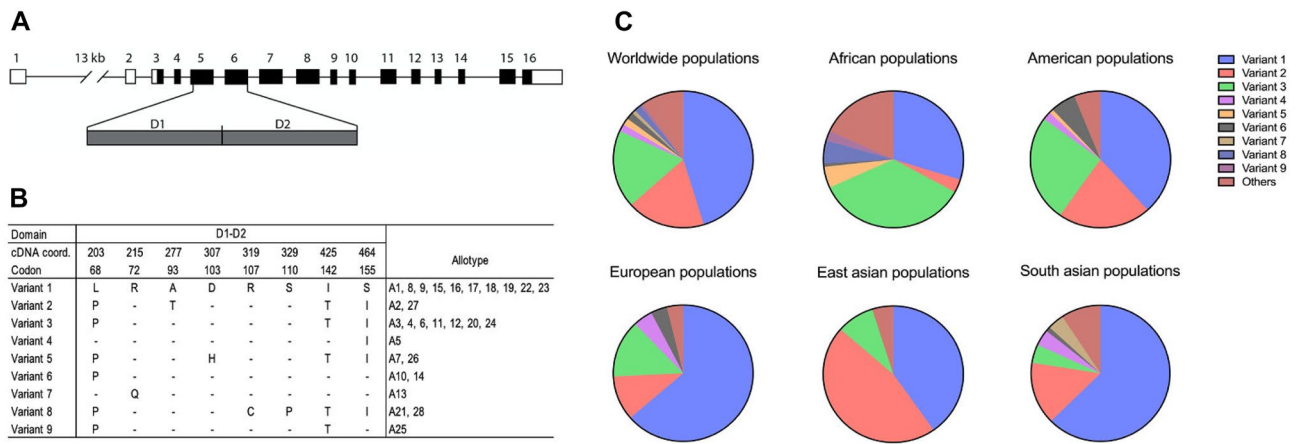
Fig. 3 LILRB1 D1-D2 variants and their frequencies in populations. **A** Exon 5 and exon 6 encode the D1 and D2 domains respectively. **B** Nine natural LILRB1 D1-D2 variants revealed in the present study and corresponding allotypes. **C** Pie charts of global population LILRB1 variant incidence. Variants 1, 2, and 3 represent the three most frequent LILRB1 D1-D2 variants and they show frequency differences in populations

HLA-A, and in HLA-C at position 219 (219W vs 219R) do not affect the binding to the tested LILRB1 D1-D2 variants (Sup. Fig. 4B–F).

Positively charged residues at D1 domain of LILRB1 enhance the binding to HLA class I

The structure of LILRB1 in complex with HLA-A*02 (PDB acc. no.: 1P7Q) was retrieved and visualized in ChimeraX (Pettersen et al. 2021). Electrostatic analysis showed that the contact sites between the LILRB1 D1-D2 domains and α3 domain of HLA-A*02 and β2m are oppositely charged (Fig. 8A–D). The contact sites of the D1 domain of LILRB1 are positively charged, binding to the negatively charged contact sites of the α3 domain of HLA class I. However, the contact sites of the D2 domain of LILRB1 are negatively charged, binding to the positively charged contact sites of the β2m domain. It is worth noting that there are

two pairs of LILRB1 D1-D2 variants that differ at only one position and at that position the amino acids have different charges (Fig. 3B). Variant 1 and variant 7 have arginine (R) and glutamine (Q) at position 72 respectively in the D1 domain, with arginine being positively charged and glutamine being neutral at physiological pH. Similarly, variant 3 and variant 5 have aspartate (D) and histidine (H) at position 103 respectively in the D1 domain, with aspartate being negatively charged and histidine being positively charged at physiological pH. The binding data demonstrated that variant 1 with 72R binds to HLA class I with significantly higher avidity compare to variant 7 with 72Q (Fig. 8E). Variant 3 with 103D binds to HLA class I with significantly lower avidity than variant 5 with 103H (Fig. 8F). These data suggest that electrostatic interaction plays a critical role in the binding of LILRB1 to HLA class I.

It is unfortunate that we were unable to produce variant 8. It has two substitutions at positions 107 and 110 differen-
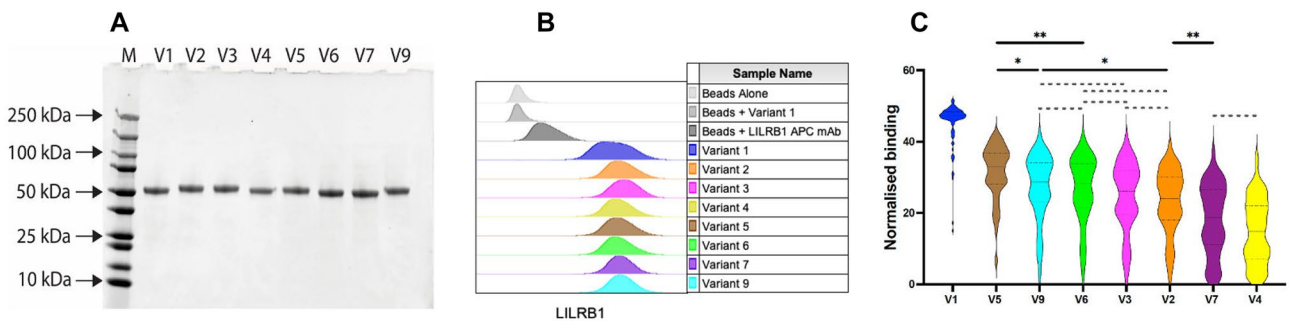

Fig. 4 LILRB1 D1-D2 variants bind to HLA class I with different avidities. **A** SDS-PAGE analysis of the eight successfully produced and purified LILRB1-D1D2-Fc proteins. **B** Representative integrity analysis of the D1-D2 portion of the produced proteins by flow cytometry using anti-human LILRB1 antibody. **C** Violin plots of the binding data of each of LILRB1 D1-D2 variants to 97 combined HLA class I tested by single antigen bead assay. The dashed line indicates there is no significant difference between variants, the single star indicates $p \leq 0.05$, and the double stars indicate $p \leq 0.01$. For all other comparisons, $p \leq 0.0001$
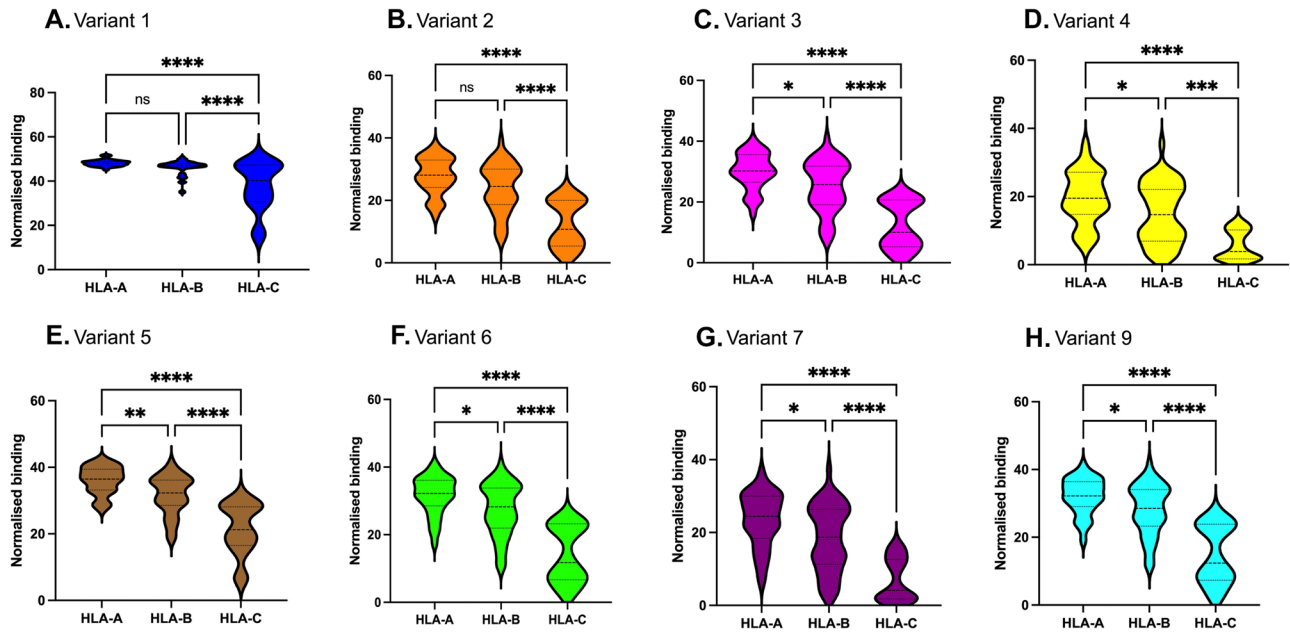
Fig. 5 The binding of variants 1 (**A**), 2 (**B**), 3 (**C**), 4 (**D**), 5 (**E**), 6 (**F**), 7 (**G**), and 9 (**H**) to HLA-A, -B, and -C. The ns indicates no significant difference, single star indicates $p \leq 0.05$, double stars indicate $p \leq 0.01$, three stars indicate $p \leq 0.001$, and four stars indicate $p \leq 0.0001$
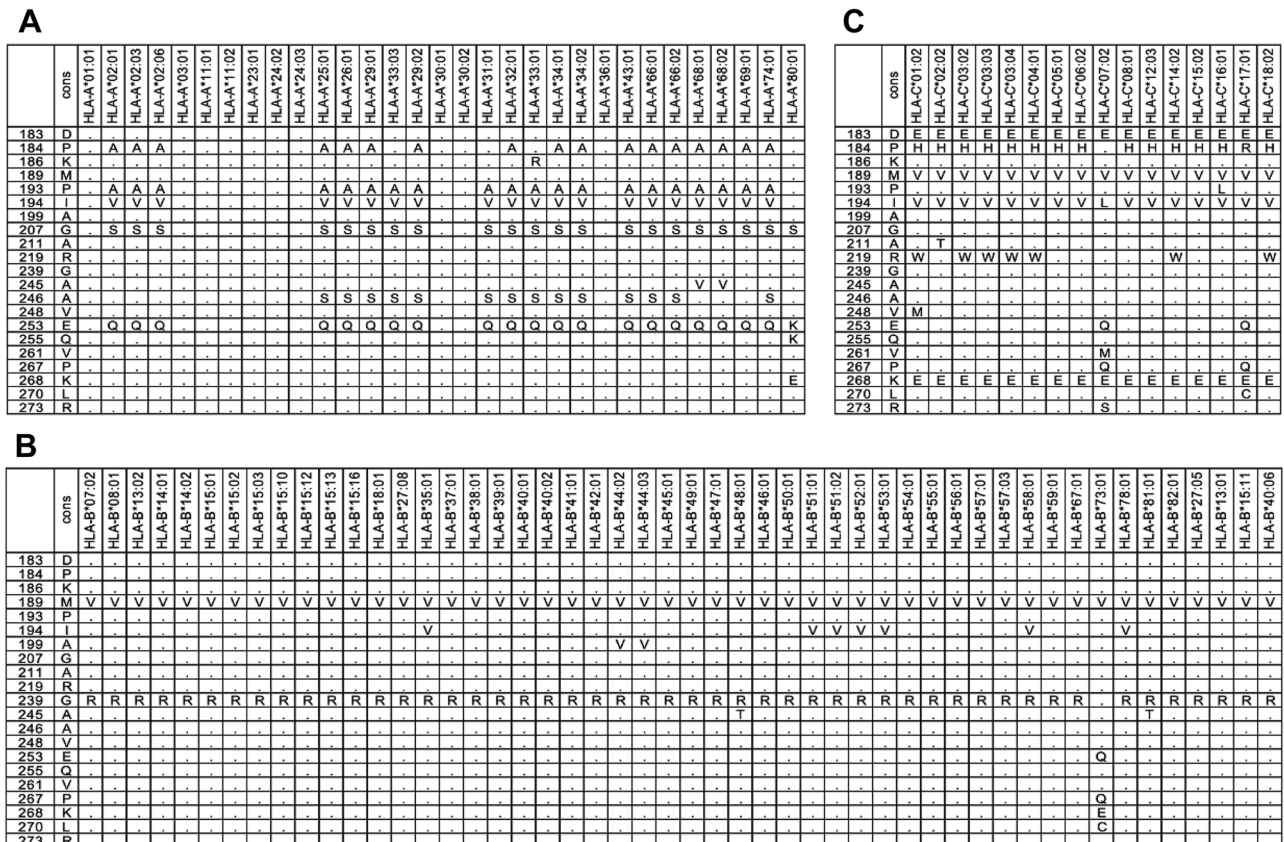

Fig. 6 Polymorphisms in the α3 domain of 31 tested HLA-A (**A**), 50 tested HLA-B (**B**), and 16 tested HLA-C (**C**). Amino acids matching the consensus sequence are indicated by a dot
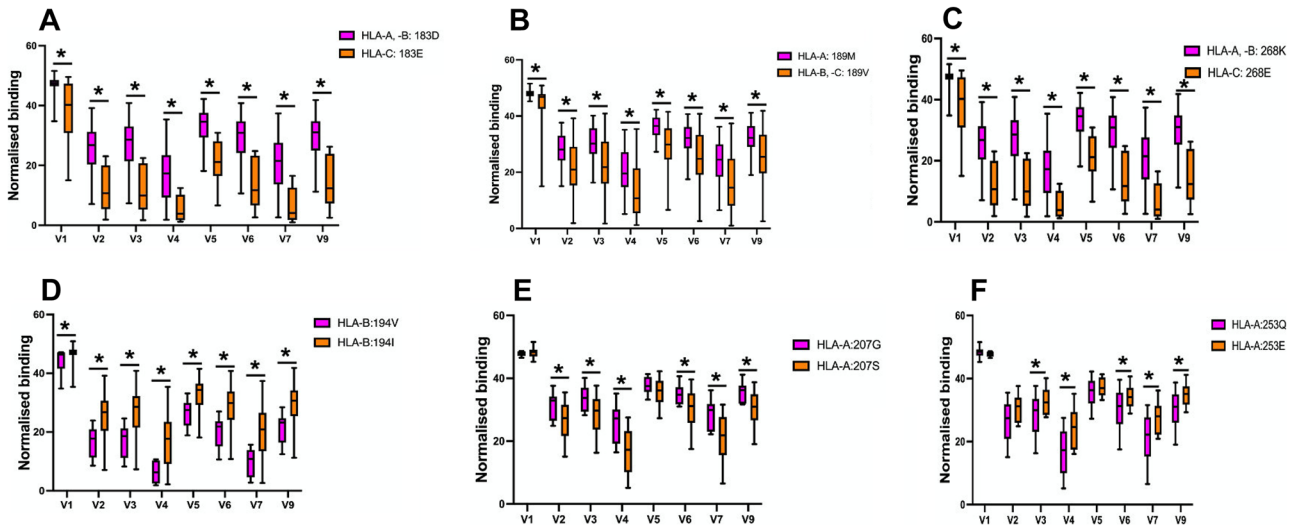
Fig. 7 Polymorphisms in the α3 domain of HLA class I affect the binding to LILRB1. Locus-specific polymorphisms at position 183 (**A**), 189 (**B**), and 268 (**C**) affect the binding to LILRB1. Dimor- phisms at position 194 (**D**) in HLA-B and at positions 207 (**E**) and 253 (**F**) in HLA-A affect the binding to LILRB1. The star indicates $p \leq 0.05$
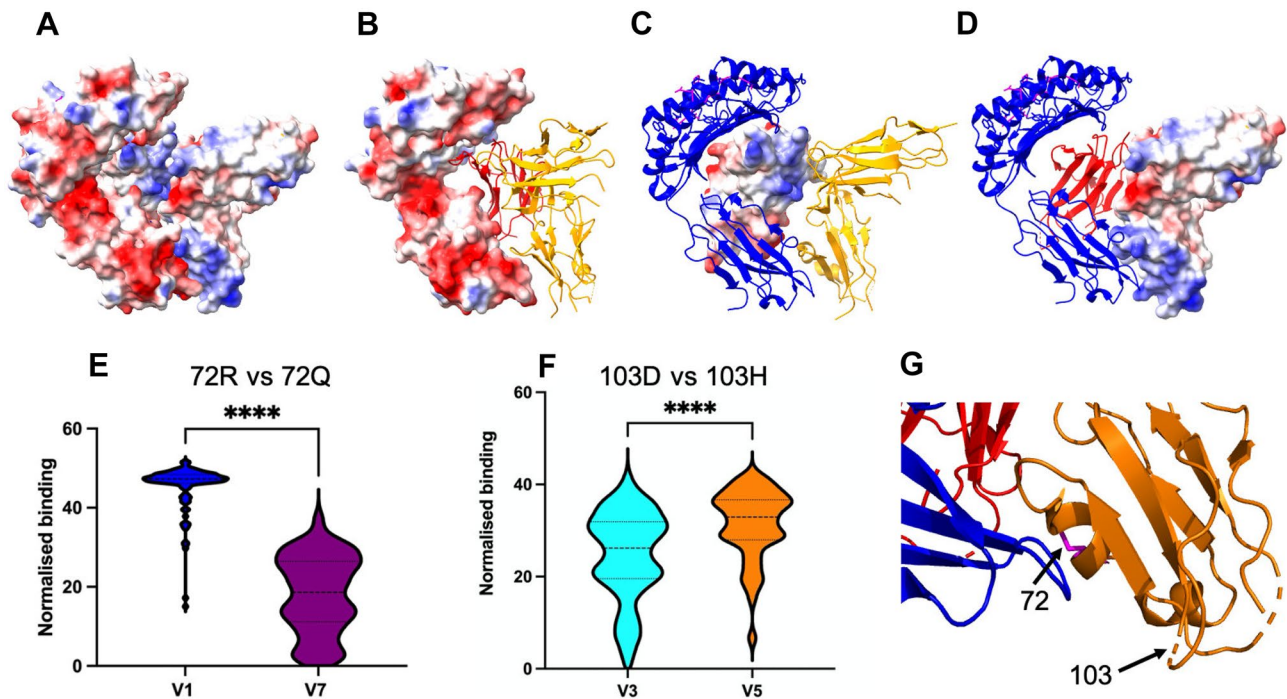
Fig. 8 Positively charged residues at D1 domain of LILRB1 enhanced the binding to HLA class I. Electrostatic analysis of the LILRB1-HLA-A*02 structure complex (**A**), and the HLA class I (**B**), β2m (**C**), and D1-D2 of LILRB1 (**D**) are highlighted. Blue shad- ing indicates positive charge and red indicates negative charge. In the ribbon diagrams, HLA class I is colored dark blue, β2m is red, and LILRB1 is orange. The effects of differently charged residues at positions 72 (**E**) and 103 (**F**) on the binding avidity of LILRB1 for HLA class I were determined by binding assay. Mean and SD are shown. Four stars indicate $p \leq 0.0001$. **G** Ribbon diagram of LILRB1 and HLA-A2 complex, with positions 72 and 103 indicated by black arrows. Residues 101–106 were missing in the structure, indicated by the dashed line. The ribbon diagram shows a zoomed view of the LILRB1 D1 domain binding to HLA-A2 with residues 72 and 103 of LILRB1 indicated by arrows. Dark blue indicates the HLA-A2, red indicates the β2m, and orange indicates the LILRB1 D1 domain

tiating it from the more common variant 3. Variant 8 has a cysteine at position 107 compared to the positively charged arginine present in all other variants. We would predict this to reduce the binding affinity of variant 8. In addition, at position 110, variant 8 has a proline instead of the serine that is present in all other variants. Introduction of the proline could cause an alteration in the structure that may affect binding. Future comparison of the binding avidity of variant 8 to other variants will give us more information on the interaction mechanism between LILRB1 and HLA class I.

Discussion

This study investigated the genetic diversity of LILRB1 in worldwide populations and assessed the binding of eight natural LILRB1 D1-D2 variants to a panel of 97 classical HLA class I antigens. We showed that LILRB1 is moderately polymorphic and the frequency of LILRB1 D1-D2 variants varies in populations. Variant 1 binds to HLA class I with the highest avidity, and variants 2, 3, 5, 6, and 9 bind to HLA class I with moderate avidity, whereas variants 4 and 7 bind to HLA class I with the lowest avidity. All tested LILRB1 D1-D2 variants bind to HLA-C with lower avidity compared with binding to HLA-A and HLA-B. Additionally, polymorphisms in the α3 domain of HLA class I influence the binding to LILRB1, partially accounting for the variation in binding avidity of LILRB1 for HLA class I. Lastly, we found that the electrostatic interaction plays a critical role in the binding of LILRB1 to HLA class I, which is evidenced by electrostatic analysis and by comparison of different binding avidities caused by polymorphisms at positions 72 and 103 of LILRB1.

We comprehensively investigated the genetic diversity of human LILRB1. Although there are a few studies genotyping the LILRB1 locus, they are limited to a small number of cohorts or specific to a certain population. Kuroki et al. screened polymorphisms in the LILRB1 locus of 18 unrelated Japanese healthy individuals and revealed 17 SNPs, including 4 nonsynonymous SNPs (L68P, A93T, I142T, and S155I) in the D1-D2 domains (Kuroki et al. 2005). Davidson et al. genotyped a group of 11 healthy donors and found 10 LILRB1 alleles and 85 SNPs, which is greater than that reported in Japanese populations (Davidson et al. 2010). More recently, Yu et al. deduced the natural LILRB1 variants by analyzing the polymorphisms L68P, A93T, I142T, and S155I in the 1000 Genomes Project and calculated their proportions in worldwide populations (Yu et al. 2018). The limitations in that study are as follows: (1) detailed LILRB1 polymorphisms in the entire coding region and the inferred alleles were not reported; (2) only 4 nonsynonymous SNPs in the D1-D2 domain were included to infer the D1-D2 variants; (3) the haplotype frequency was only calculated for

the worldwide populations; and (4) only LAIS and PTTI variants were produced and functionally examined in the further analysis. We found 149 SNPs in the coding region of LILRB1, and 108 alleles were inferred by analyzing 83 SNPs that occurred twice or more. Nine LILRB1 D1-D2 variants were further revealed by analyzing all polymorphisms in the D1-D2 domain. We also found that the frequency of LILRB1 D1-D2 variants shows population difference and variants 1–3 represent the three most frequent LILRB1 D1-D2 variants in worldwide populations. Additionally, polymorphisms in the introns and UTRs were also examined in several studies (Kuroki et al. 2005; Davidson et al. 2010; Wiśniewski et al. 2015; Yu et al. 2020). However, we did not include the polymorphisms in the non-coding region in our study. Our findings will enable the investigation of LILRB1 variant association to diseases within global populations and lay a foundation for functional studies.

Several studies have demonstrated that LILRB1 binds to a broad range of HLA class I (Willcox et al. 2003; Jones et al. 2011; Burshtyn and Morcos 2016). However, whether LILRB1 binds to HLA class I with various avidities and how natural LILRB1 variants differently bind to HLA class I largely remain unknown. Previously, the binding of LILRB1 to HLA-A*01:01, HLA-A*03:01, HLA-B*07:02, HLA-B*08:01, HLA-B*15:01, HLA-B*27:02, HLA-B*27:05, HLA-B*35, HLA-B*51, and HLA-B*58 has been determined by flow cytometry analysis (Fanger et al. 1998; Cosman et al. 1997; Baía et al. 2016; Yu et al. 2018), measuring the binding of LILRB1 protein to HLA class I transfectants. The binding of HLA-A*11, HLA-C*04, HLA-C*06:02, and HLA-C*07:02 has been determined by surface plasmon resonance (Kuroki et al. 2005; Chapman et al. 1999; Shiroishi et al. 2003). The binding of HLA-A*02:01, HLA-G1, and HLA-F was revealed by structural analysis (Willcox et al. 2003; Wang et al. 2020; Dulberger et al. 2017). Nevertheless, only one LILRB1 variant was tested in their studies and the number of tested HLA class I antigens is limited. Our results demonstrated that LILRB1 D1-D2 variant 1 bound to HLA class I with the highest avidity, and variants 2, 3, 5, 6, and 9 bind to HLA class I with moderate avidity, whereas variants 4 and 7 bind to HLA class I with the lowest avidity. This comprehensive comparison of the binding avidity for HLA class I between LILRB1 D1-D2 variants will greatly benefit the future functional studies on LILRB1. Jones et al. tested the interaction of one LILRB1 variant (unspecified, but most likely variant 1) to a panel of 97 HLA class I antigens, and found that LILRB1 bound to HLA class I with low variations in their binding avidity (Jones et al. 2011). Indeed, we found that variant 1 bound to HLA class I with low variation, which is consistent with their results to some extent. However, other tested LILRB1 D1-D2 variants bound to HLA class I with higher binding variations. What's more, we found that all tested LILRB1 D1-D2 variants bound to HLA-C with the lowest avidity, and LILRB1 D1-D2 vari-

ants bound to HLA-A with slightly higher avidity than to binding to HLA-B. We identified that 183E and 268E only occur in the α3 domain of HLA-C, whereas HLA-A and HLA-B have 183D and 268 K instead. These locus-specific polymorphisms may be responsible for the low binding of HLA-C to LILRB1, although they are not in direct contact (Sup. Fig. 5). Notably, three dimorphisms in HLA-A and HLA-B are also associated with binding to LILRB1. 207G and 253E in HLA-A and 194I in HLA-B are associated with higher binding to LILRB1. Taken together, LILRB1 D1-D2 variants bind to HLA class I with various avidities and the polymorphisms in the α3 domain of HLA class I have significantly contributed to the binding variations for LILRB1.

Variations in the binding avidity of individual HLA class I antigens for LILRB1 D1-D2 variants were also observed in the binding assay, which may alter the balance between inhibitory and activating signals within immune cells and subsequently influence the progression of pathogen infection and development of disease. We found that LILRB1 D1-D2 variants displayed higher binding to HLA-A*11:01 and A*11:02. HLA-A*11 is part of a haplotype A11-Cw4-B35-DR1-DQ1 that is associated with rapid progression of HIV (Roger 1998). It is possible that the strong interaction of HLA-A*11:01 and A*11:02 with LILRB1 on immune cells, such as macrophages, CD8 T cells, and NK cells, results in the inhibition of these cells' anti-viral responses. In contrast, several HLA class I antigens bind to LILRB1 D1-D2 variants with weaker avidity, and reduced inhibition of immune cells may enhance the production of cytokines and subsequently influence the disease outcome. HLA-C*04:01 and HLA-C*17:01 have been reported to be associated with severe outcomes in COVID-19 (Weiner et al. 2021; Bonaccorsi et al. 2021). We found that both HLA-C*04:01 and HLA-C*17:01 bind to LILRB1 D1-D2 variants with extremely low avidity (Sup. Fig. 3), which could lead to hyper-responsive APCs, CD8 T cells, and NK cells, a subsequent cytokine storm, and poorer outcomes in COVID-19. In addition, we found that LILRB1 D1-D2 variants bind to HLA-A*26:01, HLA-B*13:01, HLA-B*15:02, HLA-B*15:11, HLA-B*15:13, HLA-B*58:01, HLA-C*06:02, and HLA-C*07:02 with weaker avidity (Sup. Fig. 3). HLA-A*26:01 has been reported to play a predominant role in causing eye inflammations, particularly in Northeast Asian populations (Nakamura et al. 2019; Kaburaki et al. 2010). Several studies have demonstrated that HLA-B*13:01, HLA-B*15:02, HLA-B*15:11, HLA-B*15:13, and HLA-B*58:01 are strongly associated with severe skin conditions in East Asian populations (Satapornpong et al. 2021; Chang et al. 2017; Wong et al. 2022; Dean and Kane 2013). It is worth noting that LILRB1 D1-D2 variant 2 is enriched in Eastern Asian populations with a frequency of 46.2%, and it binds to HLA class I with weaker avidities. Thus, it is very possible that low interaction of these HLA class I antigens with

LILRB1 D1-D2 variants, particularly variant 2, plays critical roles in causing severe skin conditions (or other inflammations) in East Asian populations. Lastly, both HLA-C*06:02 and HLA-C*07:02 are risk alleles associated with psoriasis (Dand et al. 2019; Li et al. 2020); the low binding of them to LILRB1 D1-D2 variants may partially account for it.

We found that the electrostatic interaction plays a critical role in the binding of LILRB1 to HLA class I. Electrostatic analysis revealed that the contact sites of D1 domain of LILRB1 are mainly positively charged, while the contact sites of the α3 domain of HLA class I are negatively charged. What's more, the comparison of LILRB1 D1-D2 variants that vary at position 72 (72R vs 72Q) or position 103 (103D vs 103H) revealed that positively charged residues at these two positions significantly enhance the binding to HLA class I. In addition, several studies have demonstrated that electrostatic interaction can be important to protein structures because they are potent attractions (Sheinerman et al. 2000; Froloff et al. 1997). One good example is that the ionic charge of the loaded peptide significantly influences the stability of HLA class I antigens (Shiroishi et al. 2006; Grifoni et al. 2015). Thus, we concluded here that electrostatic interaction is an important mechanism that used by interactions between LILRB1 and HLA class I, and this interaction can be precisely modified by mutation of charged residues at the contact sites.

Declarations

Conflict of interest The authors declare no competing interests.

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