Human Molecular Genetics

Extensive polymorphisms of LILRB1 (ILT2, LIR1) and their association with HLA-DRB1 shared epitope negative rheumatoid arthritis

Kimiko Kuroki, Naoyuki Tsuchiya, Mitsunori Shiroishi, Linda Rasubala, Yumi Yamashita, Kunio Matsuta, Toru Fukazawa, Makio Kusaoi, Yoshinori Murakami, Masafumi Takiguchi, Takeo Juji, Hiroshi Hashimoto, Daisuke Kohda, Katsumi Maenaka and Katsushi Tokunaga

Hum. Mol. Genet. 14:2469-2480, 2005. First published 13 Jul 2005;
doi:10.1093/hmg/ddi247

Human Molecular Genetics, 2005, Vol. 14, No. 16 **2469–2480**
doi:10.1093/hmg/ddi247
Advance Access published on July 13, 2005

Extensive polymorphisms of *LILRB1* (*ILT2*, *LIR1*) and their association with *HLA-DRB1* shared epitope negative rheumatoid arthritis

Kimiko Kuroki[1,2], Naoyuki Tsuchiya[2,*], Mitsunori Shiroishi[1], Linda Rasubala[1], Yumi Yamashita[3], Kunio Matsuta[4], Toru Fukazawa[5], Makio Kusaoi[5], Yoshinori Murakami[6], Masafumi Takiguchi[7], Takeo Juji[8], Hiroshi Hashimoto[5], Daisuke Kohda[1], Katsumi Maenaka[1] and Katsushi Tokunaga[2]

[1]Division of Structural Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan, [2]Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, [3]Department of Medicine II, Hokkaido University, Sapporo 060-8638, Japan, [4]Matsuta Clinic, Tokyo 155-0032, Japan, [5]Department of Rheumatology and Internal Medicine, Juntendo University, Tokyo 113-8421, Japan, [6]Tumor Suppression and Functional Genomics Project, National Cancer Center Research Institute, Tokyo 104-0045, Japan, [7]Division of Viral Immunology, Center for AIDS Research, Kumamoto University, Kumamoto 860-0811, Japan and [8]Central Blood Institute, Blood Service Headquarters, Japanese Red Cross Society, Tokyo 105-8521, Japan

Received June 21, 2005; Revised and Accepted July 5, 2005

New polymorphisms described in this study have been deposited in GenBank/EMBL/DDBJ accession nos AB204834–AB204850

Leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1/LIR1/ILT2) is an inhibitory receptor broadly expressed on leukocytes and recognizes HLA-class I and human cytomegalovirus UL18. *LILRB1* is encoded within the leukocyte receptor complex on 19q13.4, previously implicated to be a susceptibility region to systemic lupus erythematosus (SLE). In this study, we screened for polymorphisms of *LILRB1* and examined their association with SLE and rheumatoid arthritis (RA). In the 5′ portion of *LILRB1*, three haplotypes containing four non-synonymous substitutions within the ligand-binding domains and two single nucleotide polymorphisms within the promoter region were identified and designated as *PE01–03*. In the 3′ portion, two haplotypes (*CY01, 02*) containing a non-synonymous substitution of the cytoplasmic region were identified. *CY01* and *02* did not co-segregate with *PE01–03*. Significant association with susceptibility to SLE or RA was not observed; however, among the subjects not carrying RA-associated *HLA-DRB1* shared epitope (SE), *LILRB1.PE01/01* diplotype was significantly associated with RA (odds ratio 2.05, $P = 0.019$ and $Pc = 0.038$). Gross difference was not observed in the crystal structures, thermostabilities and binding affinities to HLA-class I ligands among *LILRB1.PE01–03* haplotype products; however, surface expression of LILRB1 was significantly decreased in lymphocytes and monocytes from the carriers of *PE01* haplotype. These findings demonstrated that *LILRB1* is highly polymorphic and is associated with susceptibility to RA in *HLA-DRB1* SE negative subjects, possibly by insufficient inhibitory signaling in leukocytes. In addition, these observations suggested that the polymorphisms of LILR family members may be substantially involved in the diversity of human immune responses.

*To whom correspondence should be addressed. Tel: +81 358413693; Fax: +81 358028619; Email: tsuchiya-tky@umin.ac.jp

INTRODUCTION

Leukocyte immunoglobulin-like receptor [*LILR*, also known as *LIR*, immunoglobulin-like transcript (*ILT*), *CD85* or monocyte/macrophage inhibitory receptor (*MIR*)] family consists of 13 member genes including two pseudogenes (*ILT9* and *ILT10*) (1–4). LILRs can be divided into three classes. Group A (LILRA1, -A2, -A4, ILT8 and LIR9m/ILT11) delivers positive signals by pairing with the Fc receptor common γ chain, which contains an immunoreceptor tyrosine-based activation motif (ITAM). Group B (LILRB1, -B2, -B3, -B4 and -B5) contains two to four immunoreceptor tyrosine-based inhibitory motif (ITIM)-like sequences within the cytoplasmic region and inhibits cell activation by recruiting SHP-1 or SHIP. LILRA3 and LIR9s may be secreted as soluble receptors. The *LILR* gene family is located in the leukocyte receptor complex (LRC), which also contains a number of closely related immunoglobulin-like receptor genes such as killer cell immunoglobulin-like receptors (*KIRs*) and *FCAR* on human chromosome 19q13.4 (5–7). This region has been suggested to be one of the candidate susceptibility regions for systemic lupus erythematosus (SLE) (8,9).

LILRB1 is broadly expressed on hematopoietic cells, including antigen presenting cells (1–3,10), and can bind to a variety of HLA-class I molecules and to cytomegalovirus UL18 through two membrane-distal extracellular domains (D1 and D2) (11–13). Although most KIRs recognize polymorphic epitopes within the α1 and α2 domains of HLA-class I molecules, the binding site for LILRB1 has been mapped to HLA-class I heavy chain α3 domain and β_2-microglobulin (β2m) (13–15). It is consistent with the broad-binding specificity of LILRB1, because the α3 domain is relatively conserved among HLA-class I molecules.

In mice, *Pira* and *Pirb* represent orthologs of activating and inhibitory human *LILR*, respectively (16,17). Mice lacking *Pirb* showed impaired dendritic cell maturation and increased Th2 responses (18). Furthermore, it was recently reported that both PIR-A and PIR-B proteins bind to various mouse MHC class I molecules, and *Pirb* deficient mice showed exacerbated graft versus host disease (GVHD) (19). These observations suggest that LILRs may be critically involved in the regulation of immune system in various aspects.

It has been demonstrated that *KIR* genes are highly polymorphic not only in the nucleotide sequences, but also in the number of loci (20,21). Association with *KIR* polymorphisms has been demonstrated for autoimmune diseases such as rheumatoid arthritis (RA) (22,23), psoriatic arthritis (24), type I diabetes (25) and psoriasis (26,27), as well as for the clinical course of HIV (28) or hepatitis C virus (29) infection.

It is thus probable that *LILR* genes are also polymorphic, and if this is the case, then functional polymorphisms of *LILR* genes may substantially be related to the diversity of immune response among individuals and to the susceptibility to diseases. To date, systematic screening of *LILR* polymorphisms has not been reported, except for *LILRA3* (30) and *LILRB4* (31) genes. In this study, we made an attempt to systematically screen for the polymorphisms of *LILRB1* gene and to examine whether they are associated with the susceptibility to RA and SLE.

RESULTS

Identification of *LILRB1* polymorphisms and haplotypes

Polymorphism screening of the entire length of *LILRB1* revealed 17 single nucleotide polymorphisms (SNPs). Nine SNPs were within the coding region, six within the introns and two within the promoter region (Fig. 1). Among the SNPs in the coding sequence, four encoded non-synonymous substitutions (L68P, A93T, I142T and S155I) were found within the putative ligand-binding domains (D1 and D2) and the other one (E625K) was found between the two ITIM-like motifs important for SHP-1 recruitment (32).

Rather than testing the disease association of every identified SNP individually, we adopted a study design to experimentally define the major haplotypes in the Japanese population based on the segregation patterns in 31 families, to determine the diplotype configuration of each case and control by genotyping only the tag SNPs and to examine the association between the haplotypes and the disease. This design can greatly reduce the number of statistical comparisons involved, if the number of haplotypes is limited when compared with the number of SNPs.

The segregation patterns indicated that 12 SNPs from *−634A>G* in the promoter region to *c.464G>T* (S155I) in exon 5 constitute three major haplotypes in Japanese, which were designated as *LILRB1.PE01, LILRB1.PE02* and *LILRB1.PE03* (PE for promoter and extracellular regions) (Table 1). These three haplotypes could account for all 124 haplotypes of the parents of 31 unrelated families. The haplotype frequencies among 62 unrelated parents were *LILRB1.PE01*: 47.6%, *LILRB1.PE02*: 42.7% and *LILRB1.PE03*: 9.7%.

In contrast, two SNPs in the exon 15 encoding the cytoplasmic region, *c.1866C>T* (L622L) and *c.1873G>A* (E625K), were completely co-segregated in the families and designated as *LILRB1.CY01* (c.1866C–c.1873G) and *LILRB1.CY02* (c.1866T–c.1873A) (CY for cytoplasmic region). These haplotypes did not co-segregate with *LILRB1.PE01–03* haplotypes.

Association of *LILRB1* polymorphisms with rheumatic diseases

We first compared the diplotype frequencies of *LILRB1* between patients with RA or SLE and healthy controls. The diplotype frequencies were compatible with Hardy–Weinberg equilibrium. Significant association was observed neither in RA nor in SLE, although a tendency of increase in *PE01/01* diplotype was observed in RA [$\chi^2 = 3.26$, $P = 0.071$, Pc = 0.64, odds ratio (OR) = 1.40, 95% confidence interval (CI): 0.97–2.03. Pc was derived by multiplying the *P*-value by 9, because association with RA was tested for six *PE* diplotypes and three *CY* diplotypes] (Table 2).

To detect a minor genetic risk more sensitively, we next examined the association of *LILRB1* polymorphisms in individuals with and without the established susceptibility alleles of *HLA-DRB1* separately. As previously described, striking association of *HLA-DRB1* shared epitope (SE) with susceptibility to RA was present in our subjects (33). SE encoding alleles present in the Japanese population are *DRB1*0101,*

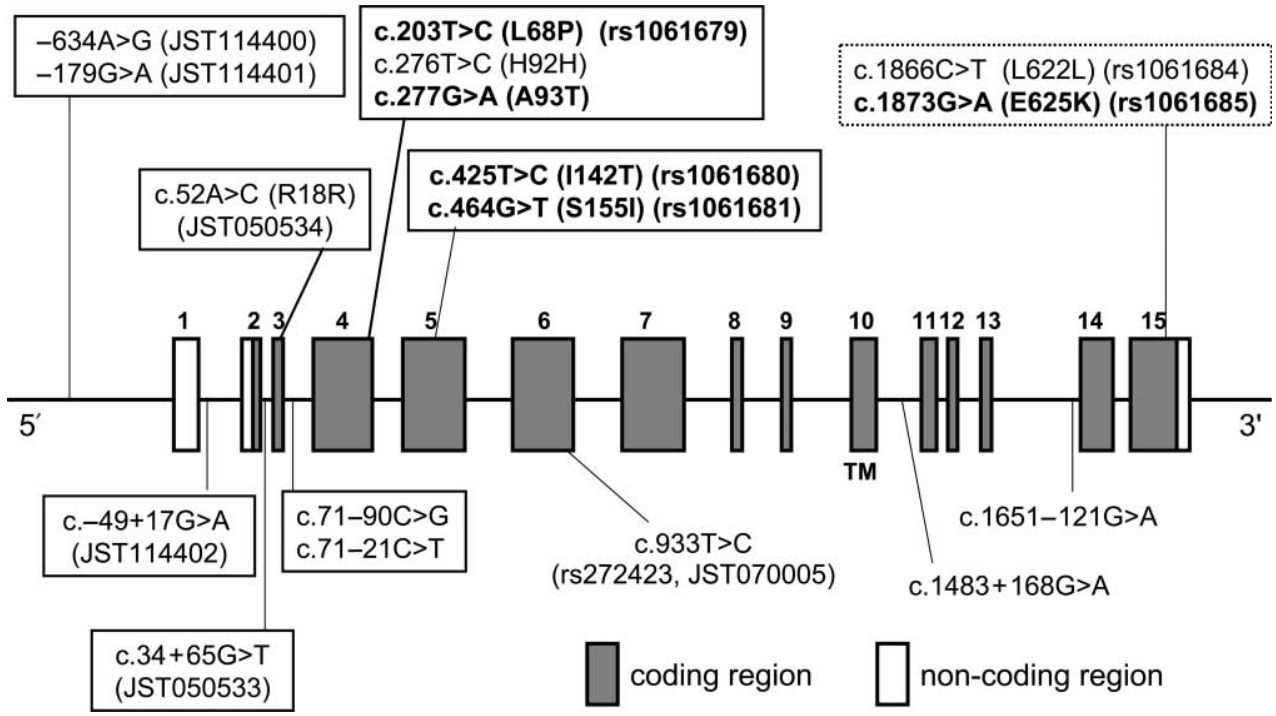

Figure 1. Genetic variations of *LILRB1* detected in this study. Twelve SNPs in the solid boxes and two SNPs in a dotted box indicate PE (promoter and extracellular) and CY (cytoplasmic) haplotypes, respectively. Five non-synonymous substitutions are represented in bold. The designations of the variations are based on the recommendation by Human Genome Variation Society (http://www.hgvs.org/). SNP IDs in parentheses are according to dbSNP (rs, http://www.ncbi.nlm.nih.gov/projects/SNP) or JSNP: a database of common gene variations in the Japanese population (JST, http://snp.ims.u-tokyo.ac.jp). Other SNPs had not previously been registered.

0401, 0404, 0405, 0408, 0410, 1001, 1402 and *1406*. Thus, the diplotype frequency of *LILRB1.PE01/01* that was slightly increased in RA was compared after stratification, according to the presence or the absence of SE. Significant increase was observed in the frequency of diplotype *LILRB1.PE01/01* in the patients without SE when compared with controls without SE [$\chi^2 = 5.51$, $P = 0.019$, Pc = 0.038, OR = 2.05, 95% CI: 1.13–3.72. Pc was derived by multiplying the *P*-value by 2, because two independent comparisons (group A versus B and group C versus D) were made] (Table 3). This difference was not observed in the comparison between SE-positive RA and controls.

In the case of SLE, neither stratification by *HLA-DRB1*1501*, an established susceptibility gene in Japanese (34,35) nor by the clinical characteristics of SLE (age at onset, nephropathy, CNS lupus, serositis, hypo-complementemia, anti-dsDNA and anti-Sm) resulted in a significant association of *LILRB1* haplotypes (data not shown).

As LILRB1 molecule interacts with HLA-class I but not with class II and HLA-DRB1 is in linkage disequilibrium with HLA-class I, it was possible that primary genetic interaction exists between *LILRB1* and HLA-class I alleles rather than DRB1. To examine such a possibility, *LILRB1* haplotype frequency was examined in combination with *HLA-B* alleles. A modest association of *LILRB1.PE01/01* with RA was detected between patients and healthy individuals without *HLA-B54* ($\chi^2 = 3.86$, $P = 0.05$, OR = 1.78, 95% CI: 1.00–3.16), which is in linkage disequilibrium with *HLA-DRB1*0405*, the most frequent allele constituting SE in Japanese. However, the association was weaker in the subjects without *HLA-DRB1* SE, suggesting that *HLA-DRB1*, rather than *HLA-B*, is primarily responsible for the genetic interaction with *LILRB1*. Stratification by other *HLA-B* alleles did not reveal significant association with *LILRB1* with RA.

Structure, stability and HLA-class I binding of LILRB1 haplotype products

Because *LILRB1.PE01* haplotype associated with RA contains four non-synonymous substitutions within the ligand-binding domains (D1D2), among which three (L68, I142 and S155) are unique to this haplotype, we postulated that structural and/or functional changes associated with the amino acid substitutions may be responsible for the susceptibility to RA. To test such a hypothesis, we prepared D1D2 polypeptides encoded by *LILRB1.PE01, 02* and *03* haplotypes, and crystal structures, thermostability and binding affinity to an array of HLA-class I ligands were examined.

Crystal structures of all three haplotype products were determined by molecular replacement and did not reveal substantial difference, except for the polymorphic sites (Fig. 2). The thermostabilities were also not significantly different among three haplotype products, with the melting temperatures of 62.4°C (LILRB1.PE01), 59.0°C (LILRB1.PE02) and 62.8°C (LILRB1.PE03).

Table 1. *LILRB1* haplotypes formed by SNPs within promoter and extracellular regions in Japanese

Position[a]	−634	−179	c. − 49 + 17	c.34 + 65	c.52	c.71 − 90	c.71 − 21	**c.203**	c.276	**c.277**	**c.425**	**c.464**	Haplotype frequency[b]
Nucleotide Amino acid	A>G	G>A	G>A	G>T	A>C R18R	C>G	C>T	**T>C** **L68P**	T>C H92H	**G>A** **A93T**	**T>C** **I142T**	**G>T** **S155I**	(%)
LILRB1.PE01	A	G	G	G	A	C	C	**T** **Leu**	T	**G** **Ala**	**T** **Ile**	**G** **Ser**	59 (47.6)
LILRB1.PE02	G	A	A	T	C	G	T	**C** **Pro**	C	**A** **Thr**	**C** **Thr**	**T** **Ile**	53 (42.7)
LILRB1.PE03	G	A	A	T	C	G	T	**C** **Pro**	T	**G** **Ala**	**C** **Thr**	**T** **Ile**	12 (9.7)

Non synonymous substitutions are shown in bold and each amino acid residue is described below. These haplotypes can be distinguished by the combination of *c.203T>C* and *c.277G>A*.
[a]Numbering starts at the *ATG* translation initiation codon.
[b]Haplotype frequency was calculated from the parents of 31 Japanese families (124 chromosomes).

Table 2. Frequencies of *LILRB1* haplotypes in Japanese patients with RA, SLE and healthy controls

LILRB1.PE	RA (%) $n = 557$	SLE (%) $n = 169$	Controls (%) $n = 396$	LILRB1.CY	RA (%) $n = 557$	SLE (%) $n = 169$	Controls (%) $n = 396$
Diplotype frequency							
PE01/01	94 (16.9)	17 (10.1)	50 (12.6)	CY01/01	464 (83.3)	147 (87.0)	323 (81.6)
PE01/02	228 (40.9)	83 (49.1)	162 (40.9)	CY01/02	85 (15.3)	21 (12.4)	66 (16.7)
PE01/03	35 (6.3)	9 (5.3)	26 (6.6)	CY02/02	8 (1.4)	1 (0.6)	7 (1.8)
PE02/02	147 (26.4)	45 (26.6)	112 (28.3)				
PE02/03	46 (8.3)	14 (8.3)	44 (11.1)				
PE03/03	7 (1.3)	1 (0.6)	2 (0.5)				
Haplotype carrier frequency							
PE01+	357 (64.1)	109 (64.5)	238 (60.1)	CY01+	549 (98.6)	168 (99.4)	389 (98.2)
PE02+	421 (75.6)	142 (84.0)	318 (80.3)	CY02+	93 (16.7)	22 (13.0)	73 (18.4)
PE03+	88 (15.8)	24 (14.2)	72 (18.2)				
Haplotype frequency							
PE01	451 (40.5)	126 (37.3)	288 (36.4)	CY01	1013 (90.9)	315 (93.2)	712 (89.9)
PE02	568 (51.0)	187 (55.3)	430 (54.3)	CY02	101 (9.1)	23 (6.8)	80 (10.1)
PE03	95 (8.5)	25 (7.4)	74 (9.3)				

The binding affinities to a wide range of HLA-class I were examined by means of surface plasmon resonance (SPR), using HLA-A11, -B35, -Cw4 and -G1 recombinant proteins as representative ligands of LILRB1. Substantial differences in the binding affinities with HLA-class I molecules were not observed among LILRB1 haplotype products (Fig. 3; Table 4). Furthermore, affinities of two different monoclonal antibodies (mAbs) to LILRB1 were not substantially different among the haplotype products (Table 4).

Association of surface expression level of LILRB1 with polymorphisms

LILRB1.PE01 haplotype contains two SNPs in the promoter region, which are not present in other haplotypes. We therefore compared the expression levels of LILRB1 on the surface of peripheral blood lymphocytes and monocytes from 11 healthy individuals carrying *LILRB1.PE01* and five non-carriers of *LILRB1.PE01*. Although most of the monocytes were found to express LILRB1, only a proportion of lymphocytes were stained positive, compatible with the LILRB1 expression on most of the B cells but only a subset of T and NK cells (3,36). The average mean fluorescence intensity (MFI) of LILRB1 was significantly weaker in the individuals with *LILRB1.PE01* when compared with those without *LILRB1.PE01*, both in the lymphocytes (mean ± SD, MFI 2.96 ± 0.19 versus 3.22 ± 0.25; $P = 0.03$) and in the monocytes (mean ± SD, MFI 8.36 ± 1.65 versus 10.32 ± 1.39; $P = 0.04$) (Fig. 4A). This difference was not caused by weaker affinity of mAb HP-F1 to LILRB1.PE01 product, because affinity of HP-F1 to LILRB1.PE01D1D2 protein was not weaker in the SPR analysis (Table 4).

To examine whether the reduction of surface expression of LILRB1 is specifically observed in any of the T and B lymphocyte subsets, three-color flow cytometric analyses were conducted using mAbs against CD4, CD8, CD19 and CD27 on eight *LILRB1.PE01* carriers and on five non-carriers. The representative profiles are shown in Figure 4B. LILRB1 expression was observed in most of the CD19+CD27+ (memory B cells) and CD19+CD27− (naïve B cells) with a comparable intensity (data not shown), as well as in a substantial proportion of CD8+ T cells, but only in a tiny fraction of

Table 3. Association of *LILRB1.PE01/01* with RA among HLA-DRB1 shared epitope negative subjects

Group	SE	*LILRB1*[a] (*PE01/01*)	RA (%) $n = 552$	Controls (%) $n = 296$	OR[b]	95% CI[c]
A	+	+	59 (10.7)	18 (6.1)	3.75	2.16–6.50
B	+	−	319 (57.8)	99 (33.4)	3.68	2.69–5.04
C	−	+	34 (6.2)	19 (6.4)	2.05	1.13–3.72
D	−	−	140 (25.4)	160 (54.1)	1.0	

[a]+: *LILRB1.PE01/01* and −: other diplotypes of *LILRB1.PE*.
[b]OR was calculated against individuals in group D.
[c]95% CI, SE positive group (A versus B): $\chi^2 = 0.0034$, $P = 0.95$, $Pc > 1$, OR = 1.02 (0.57–1.81), SE negative group (C versus D): $\chi^2 = 5.51$, $P = 0.019$, $Pc = 0.038$, OR = 2.05 (1.13–3.72).

CD4+ T cells. The average MFI of LILRB1 in the *PE01* carriers was ∼10% lower when compared with the non-carriers in all subsets (CD19+CD27+, CD19+CD27− and CD8+), although the difference did not reach statistical significance probably because of the smaller number of subjects. Nevertheless, these results suggested that the lower expression of LILRB1 associated with the genotype is not restricted to any particular subset of T and B cells.

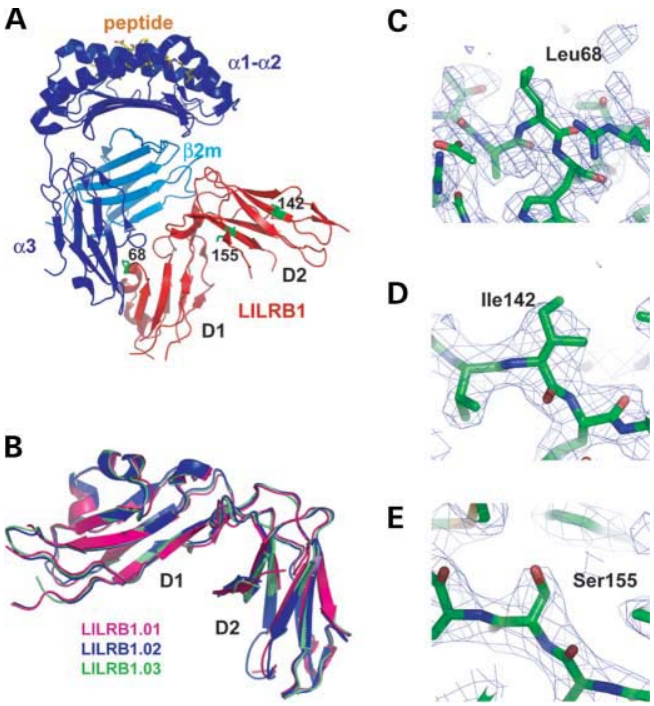

Figure 2. Crystal structures of LILRB1.PE01, PE02 and PE03 products. (**A**) The crystal structure of the LILRB1–HLA-A2 complex is reported by Willcox *et al.* (13). The HLA-A2 heavy chain and β2m are represented in ribbon model (dark blue: heavy chain, light blue: β2m and yellow stick model: the peptide). The three amino acids unique to LILRB1.PE01 are shown in green stick model. (**B**) Structural comparison of MHC binding domains [N-terminal two domains (D1 and D2)] among LILRB1.PE01 (magenta), PE02 (blue) and PE03 (green). (**C**–**E**) The final 2Fo − Fc electron density maps (blue mesh) of the LILRB1.01 structure in the substitution sites [residues 68 (C), 142 (D) and 155 (E)] are contoured at 1σ. The LILRB1.PE01 is shown in green stick model with oxygen (red) and nitrogen (blue) atoms.

DISCUSSION

In the present study, we identified a number of SNPs in the *LILRB1* gene. Although the *KIR* loci have been known to be highly polymorphic (20,21), polymorphism screening has not previously been performed in most *LILR* genes in a systematic manner. This study demonstrated that *LILRB1* gene is also highly polymorphic. We also demonstrated that a haplotype containing four amino acid substitutions within the ligand-binding domains and two promoter SNPs is associated with the susceptibility to RA in *HLA-DRB1* SE negative individuals. Association of *LILRA3* (*ILT6*) deficiency with multiple sclerosis has recently been reported (37). Although disease-associated changes in the expression levels of *LILR* family genes in the tissues have been reported in some diseases including RA (38), this is the first study to demonstrate the role of genomic polymorphisms of *LILR* family gene in the susceptibility to rheumatic diseases.

Because only modest linkage disequilibrium is observed between polymorphisms of *LILRB1* and adjacent genes (unpublished data), the polymorphism of *LILRB1* appears to be primarily associated with RA. The crystal structure of D1D2 and binding affinity with HLA-class I molecules of LILRB1.PE01 protein did not show substantial difference when compared with those of LILRB1.PE02 and PE03 proteins, despite four non-synonymous substitutions in these domains. This is probably because these amino acid positions (L68P, A93T, I142T and S155I) are not in direct contact with HLA-class I α3 and β2m (13). Essentially, similar affinities to two different clones of LILRB1 mAbs also support that there is no gross structural difference among the three haplotype products. Note that LILRB1.PE01 protein loses a potential N-linked glycosylation signal (140N-141V-142T) present in LILRB1.PE02 and LILRB1.PE03 proteins due to the non-synonymous substitution of position 142, the effect of which cannot be addressed in our recombinant protein system. In addition, LILRB1.PE01 protein has higher hydrophobicity than the others. The possibility that these changes might affect protein–protein interactions *in vivo* cannot be excluded. Furthermore, it is theoretically possible that alteration in the affinity with human cytomegalovirus UL18, or other unidentified ligands, is somehow involved in the susceptibility to RA. However, at this point, it is more likely that the decreased expression level of LILRB1, possibly related to the two SNPs in the promoter region and/or post-transcriptional regulation, results in insufficient inhibitory signaling and leads to autoimmunity and inflammation. Such a scenario needs to be tested in future functional analyses.

The precise regulation of the LILRB1 cell surface expression level has been suggested to be important for the control of immune responses. Mice deficient in *Pirb*, an ortholog of human *LILRB1*, exhibit augmented Th2 responses after immunization with T cell-dependent antigens, enhanced IgG and IgE production (18) and accelerated GVHD after transfer of allogeneic splenocytes (19). These phenotypes are associated with augmented activation of dendritic cells and concomitant upregulation of activating PIR-A, which shares its ligands with PIR-B. These observations suggest that PIR-A

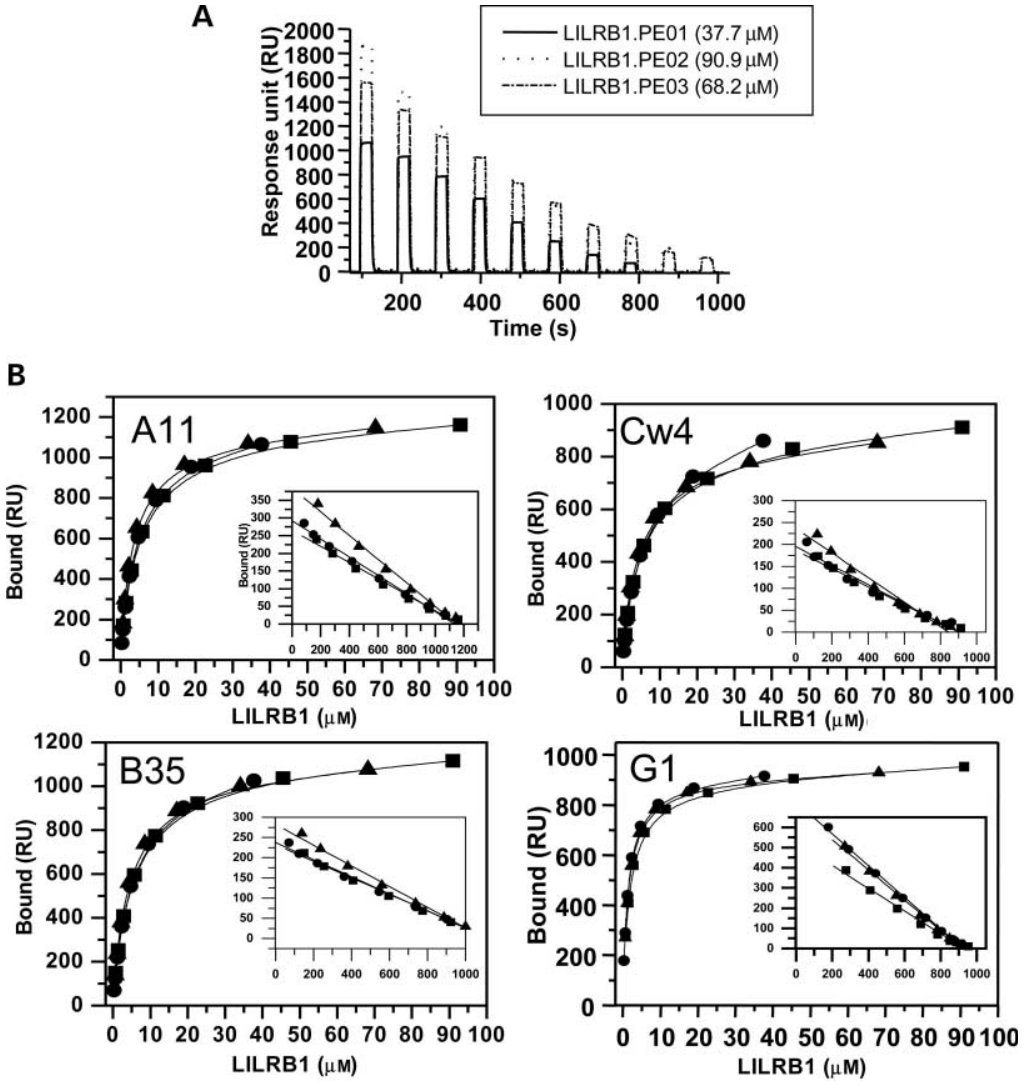

Figure 3. The affinity of LILRB1 D1D2s binding to HLA-class I molecules. (**A**) Representative data of equilibrium binding of LILRB1 D1D2s to HLA-A11. Serially diluted LILRB1 D1D2s (from 37.7 to 0.29 μM for LILRB1.PE01, from 90.9 to 0.18 μM for LILRB1.PE02 and from 68.2 to 0.13 μM for LILRB1.PE03) were injected at a flow rate of 5 μl/min through flow cells with the peptide-HLA-class I complex immobilized to the sensor chip surface. (**B**) Plots of the equilibrium binding responses of LILRB1.PE01 (●), PE02 (■), PE03 (▲) versus concentration. The solid lines represent direct non-linear fits of the 1:1 Langmuir binding isoform to the data. (Insets) Scatchard plots of the same data are shown. The solid lines are linear fits. RU means response units.

and PIR-B play a role in the regulation of the thresholds for the activation of immune system, and PIR-B prevents excessive immune responses in a constitutive manner. The broad recognition of MHC class I molecules by LILRB1 might suggest its role for the maintenance of immunological tolerance. Thus, the lower expression level of LILRB1 might result in the autoimmune response once strong activating signals were transmitted through the activating receptors into the immune effector cells.

In the present study, significant association of *LILRB1* was only detected in *HLA-DRB1* SE negative RA patients. *HLA-DRB1* SE is an established risk factor for RA confirmed in most of the populations and has been estimated to account for a substantial proportion of the genetic background of RA (39). The reason for this differential association remains unclear. One possible explanation is that the contribution of *LILRB1* polymorphisms is much weaker than that of *HLA-DRB1* SE and becomes apparent only when the genetic effect of *HLA-DRB1* SE is excluded. Another possibility is that *LILRB1.PE01* product may interact differentially with some HLA-class I allele products, which are in linkage disequilibrium with *HLA-DRB1.* Although differences in the affinity to HLA-A11, -B35, -Cw4 and -G1 among LILRB1 allele products were not detected, possible change in the affinity to some of other HLA products cannot be excluded. However, as the LILRB1 binding site on HLA-class I (α3 and β2m) is conserved among alleles, such a possibility appears unlikely.

In the clinical settings, genetic risk factors for *HLA-DRB1* SE negative RA have been largely unknown, although association of *NRAMP1* (40) and PD-1 (*PDCD1*) (41) has been

Table 4. Summary of affinity constants between LILRB1 haplotype products and HLA-class I molecules or anti-LILRB1 mAbs

Immobilized	Injected		
	LILRB1.PE01	LILRB1.PE02	LILRB1.PE03
HLA-A11[a]	4.6 ± 0.1 μM	5.3 ± 0.2 μM	3.4 ± 0.1 μM
HLA-B35[b]	5.3 ± 0.03 μM	5.1 ± 0.2 μM	4.0 ± 0.3 μM
HLA-Cw4[b]	5.9 ± 0.1 μM	5.6 ± 0.01 μM	4.2 ± 0.1 μM
HLA-G1[b]	1.4 ± 0.01 μM	1.7 ± 0.1 μM	1.3 ± 0.1 μM
HP-F1 mAb[c]	2.9 ± 0.9 nM	3.5 ± 0.3 nM	3.4 ± 0.3 nM
KY1 mAb[d]	90.5 nM	97.2 nM	98.1 nM

Values indicate *Kd* at 25°C.
[a]Mean ± SD of four independent experiments.
[b]Mean ± SD of two independent experiments.
[c]Mean ± SD of three independent experiments.
[d]Owing to the limited availability of the KY1 mAb, only one experiment was performed.

reported in such a group of patients. Our present observations may eventually provide valuable information for genetic diagnosis or prediction of disease progression in patients with arthritis lacking *HLA-DRB1* SE.

There are some limitations in this study. There was a bias of sex ratio in the samples examined in the present study. However, as the frequencies of *LILRB1* haplotypes were not significantly different between males and females, the results are essentially identical after adjustments. The evidence for association observed in this study is marginal and could have suffered from genetic heterogeneity of the subjects, if present. Although the possibility of population stratification has not been formally excluded for our case–control subjects, the Japanese population has been shown to be relatively homogeneous with respect to genetic background from HLA studies (42), and indeed, in a recently published large-scale case–control association study in Japanese, where 830 patients with RA and 658 unrelated controls were analyzed for population stratification using 2069 tag SNPs distributed in autosomes, no evidence for population stratification was observed (43). With respect to our case–control cohorts, we have conducted association studies on more than 20 genes distributed throughout the genome using partially overlapping subgroups of RA ($n = 101–382$ patients each) and controls ($n = 207–304$ each) randomly chosen from the same cohorts, and no significant difference in the genotype frequencies was observed for most of the polymorphic sites (35). Taken together, it seems unlikely that population stratification had a substantial impact on our findings. Nevertheless, our observations need to be confirmed by independent studies in the future.

Possible roles of *LILR* genes for human inflammatory or infectious diseases are beginning to emerge. LILRB2 and LILRA1 have been reported to recognize HLA-B27 free heavy chain implicated in seronegative spondylarthropaties (11). In lepromatous leprosy lesions where Th2 cytokines are dominantly expressed, LILRs (LILRB5, -B3, -A2, and -A3), especially LILRA2, were shown to be upregulated, suggesting that LILR signaling can regulate Th1/Th2 balance (44). Taken together with our observations indicating extensive polymorphism and disease association, it is suggested that diversity in *LILR* genes may be profoundly involved in the regulation of immune responses and also in the susceptibility to diseases. Studies on the polymorphisms of other *LILR* genes are underway.

MATERIALS AND METHODS

Patients and controls

Polymorphism screening was performed on genomic DNA from 18 unrelated Japanese healthy individuals. To determine major haplotypes of *LILRB1* in Japanese, 31 unrelated families that consist of probands and their parents (93 individuals in total) were analyzed. Case–control association studies were carried out on 557 patients with RA (50 males and 507 females, 44.5 ± 13.1 years), 169 patients with SLE (12 males and 157 females, 40.1 ± 14.3 years) and 396 controls (211 males and 185 females, 34.1 ± 10.4 years). The patients were recruited from Matsuta Clinic, Juntendo University Hospital and the University of Tokyo Hospital, the healthy controls at the University of Tokyo and Japanese Red Cross Central and Kanagawa Blood Centers, all from Tokyo and adjacent areas. The patients were diagnosed according to the classification criteria of American College of Rheumatology (45,46). This study was approved by the research ethics review committees of the participating institutes.

Genomic DNA was purified from the peripheral blood leukocytes from the patients and healthy individuals, using a QIAamp DNA blood mini kit (Qiagen, Hilden, Germany).

Polymorphism screening and haplotype determination

Polymorphism screening and haplotype determination were performed using Japanese family samples by polymerase chain reaction (PCR)-direct sequencing method. The primers were designed according to the genomic DNA sequence of human *LILRB1* (GenBank accession no. AF189277). Because the genomic DNA sequences of *LILR* family genes are highly homologous, nested PCR was employed to achieve specific amplification of *LILRB1*. The entire length of *LILRB1* gene (−820 upstream from the translation start site to +528 downstream of the 3′ end of exon 15) was amplified as two overlapping fragments by means of a long PCR using two primer sets. One primer set was placed within the promoter region (LILRB1-long1F: 5′-CTGCTCATGACATTGATGCT CTG-3′) and exon 4 (LILRB1-long1R: 5′-CGCTACCAT AGTAACAGCGATAC-3′) and the other was placed within exon 4 (LILRB1-long2F: 5′-GTATCGCTGTTACTATGGTA GCG-3′) and at 3′ flanking region of *LILRB1* (LILRB1-long2R: 5′-ATTACAGGCACTGCCACCACAT-3′). Both PCRs were performed in 25 μl reaction mixtures containing 2.5 U of LA Taq DNA polymerase (TAKARA, Otsu, Shiga, Japan), using a T-GRADIENT Thermocycler (Biometra GmbH, Göttingen, Germany). The amplification procedure consisted of initial denaturation at 96°C for 3 min, 35 cycles of denaturation at 96°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 2 min or 5 min 30 s for each fragment, followed by the last extension at 72°C for 7 min.

Each first PCR product was divided into several overlapping segments for second PCR followed by direct sequencing. The

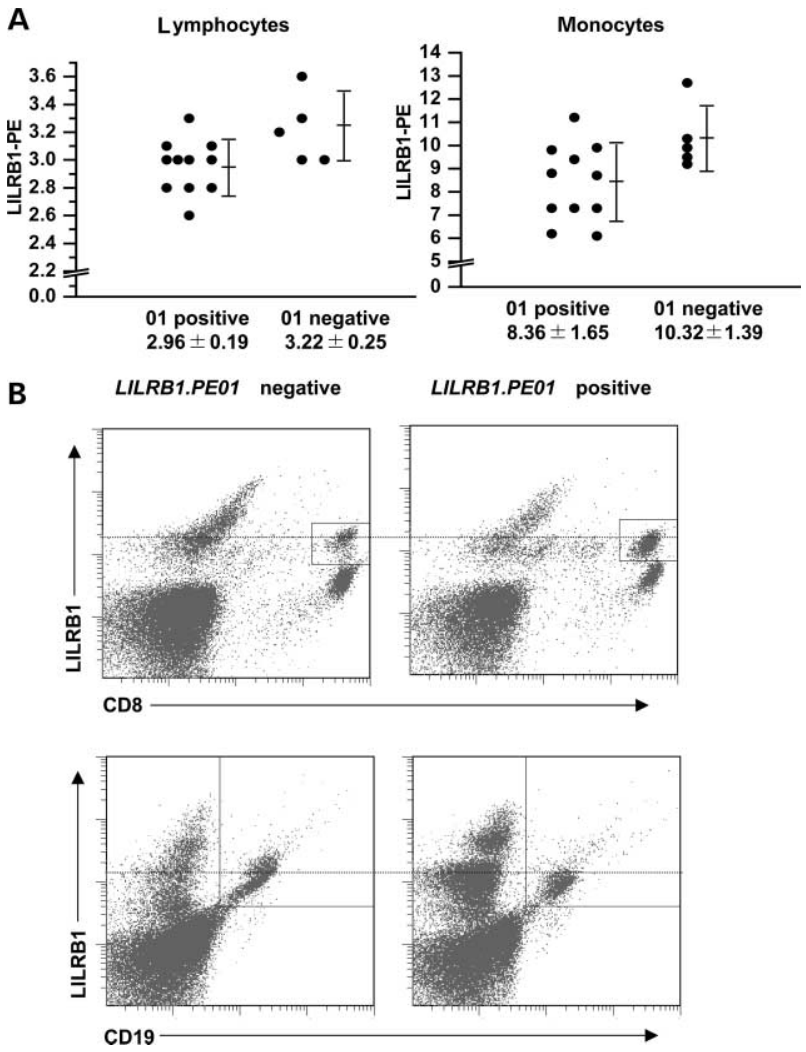

Figure 4. Association of *LILRB1.PE01* haplotype with reduced LILRB1 expression levels on peripheral blood lymphocytes and monocytes. (**A**) Cell surface LILRB1 expression levels on peripheral blood lymphocytes (left panel) and monocytes (right panel) from healthy donors with ($n = 11$) and without ($n = 5$) *LILRB1.PE01* haplotype. Values represent MFI of the cells stained for LILRB1, gated as shown in (A). Individuals carrying *LILRB1.PE01* haplotype exhibited significantly lower MFI both in lymphocytes ($P = 0.03$) and in monocytes ($P = 0.04$). The mean ± SD of each group is indicated below. (**B**) Representative flow cytometric profiles of healthy individuals with and without *LILRB1.PE01* showing the surface expression intensity of LILRB1 in a proportion of CD8+ T cells (upper panel) and B cells (lower panel). The horizontal dashed lines represent MFI of LILRB1 in the CD8+LILRB1+ (upper panel) and CD19+LILRB1+ subsets (lower panel) of the *LILRB1.PE01* negative individual (left panel), provided for reference.

second amplification was performed using the primer sets described in Table 5. Each second PCR was performed in 25 μl reaction mixture containing 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA, USA), using a T-GRADIENT Thermocycler or a GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems). The amplification condition consisted of initial denaturation at 96°C for 10 min, 35 cycles of denaturation at 96°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 40 s, followed by the last extension at 72°C for 4 min. Fluorescence-based automated cycle sequencing of PCR products was performed using ABI 377, 310 or 3100 sequencer (ABI PRISM, Applied Biosystems) with the dye-terminator method, according to the manufacture's instructions (ABI PRISM dRhodamine Terminator Cycle Sequencing-Ready Reaction Kit or BigDye ver3.1 Cycle Sequencing-Ready Reaction Kit).

Haplotypes were determined from the segregation patterns of detected polymorphisms among family members. Although the population-based analysis can only statistically elucidate the haplotype structure, this method can definitely determine the haplotypes from genotyping data of the families.

Genotyping

The three major haplotypes identified within the promoter and extracellular regions (*LILRB1.PE01, 02* and *03*) could be determined from the combination of two SNPs (*c.203T>C* and *c.277G>A*). These 'tag SNPs' were genotyped by direct sequencing of the E fragment (LILRB1-EF–LILRB1-ER).

Table 5. Primers for PCR-direct sequencing used in this study

Fragment	Name	Primer[a]	Position
A	LILRB1-AF	TGACATTGATGCTCTGGG	Promoter
	LILRB1-AR	ACCTCAATCCCTGTTCAACG	Promoter
B	LILRB1-BF	CCAACTCCCCAAATCAAAGC	Promoter
	LILRB1-BR	GGTCCTTACCATGGCAGTC	ex1–int1
C	LILRB1-CF	CAGCATGGACCTGGGTCTT	ex1
	LILRB1-CR	CCTGCTCTGTGGATGGATG	ex2
D	LILRB1-DF	CATCCATCCACAGAGCAGG	ex2
	LILRB1-DR	CCTGGAAGGAAATCAGGAGT	int3–ex4
E	LILRB1-EF	TTGGGTGGGAAATGAGTTAG	int3
	LILRB1-ER	CCATAGTAACAGCGATACCG	ex4
1	LILRB1specificF	GTATCGCTGTTACTATGGTAGCG	ex4
	LILRB1-1R	GAATTTCTCACCTAGGACCAGGA	ex5–int5
2	LILRB1-2F	GGTGCTATGCTTATGACTCG	ex5
	LILRB1-2R	GGTTGTGTGCACCGTAGCAT	ex6
3	LILRB1-3F	TCCCAGGCCAACTTCACCCT	ex6
	LILRB1-R	TGACTGACACAGCAGGGTCAC	ex7
4	LILRB1-4F	AGGACAGTTCTATGACAGAG	int6–ex7
	LILRB1-4R	GAGCTGAGACTTTGAGCTCA	int7
5	LILRB1-5F	TGGAGCTCGTGGTCTCAGG	ex7–int7
	LILRB1-5R	GACTCACCAGATGTGGAGGT	ex8–int8
6	LILRB1-6F	TCTTTTACCCAGGACCGTCT	int7–ex8
	LILRB1-6R	GACTGAACCTAGGACAGAAC	int9
7	LILRB1-7F	GTGGTGAGTGACGGGCTCT	ex9–int9
	LILRB1-7R	TGACCTGCAGCCCTTGTTC	int9
8	LILRB1-8F	GAAGAGTCATGGTTCAGGAC	int9
	LILRB1-8R	CCATTCCCTACTCACTCGAT	ex10–int10
9	LILRB1-9F	CCTCATCCTCCGACATCGA	ex10
	LILRB1-9R	GGAAGAAGGACAGAGCCTC	int10
10	LILRB1-10F	GAACATGGAGGCAGGAGTGT	int10
	LILRB1-10R	TGAGATGATCTCACCCTGAG	int12
11	LILRB1-11F	TGAGAGGAAGAGGTGACCAG	int12
	LILRB1-11R	CCACCGTGACGATGCTGAG	int13
12	LILRB1-12F	TCCTGGTGCCAGATCTAATC	int13
	LILRB1-12R	GTTCCTTACAGCACGTTGCA	int13
13	LILRB1-13F	CCTGAGCTCACCTCGTGG	int13
	LILRB1-13R	GTCAATTTTCCTCACTGTTCC	int13
14	LILRB1-14F	GATTCCATCGGGAAAGGGAT	int13
	LILRB1-14R	TTCCAGACTCCATGGAGTGT	ex15
15	LILRB1-15F	TCTACGCCACTCTGGCCATC	ex15
	LILRB1-15R	AGGCACTGCCACCACATCCA	3′ gene flanking

ex, exon; int, intron.
[a]The primers were designed according to the genomic DNA sequence of human *LILRB1* (GenBank accession no. AF189277).

Determination of the haplotypes encompassing the cytoplasmic region, *LILRB1.CY01* (*c.1866C–c.1873G*) and *LILRB1.CY02* (*c.1866T–c.1873A*), was performed using PCR-single strand conformation polymorphism (SSCP) method. A 289 bp fragment (forward primer: 5′-GACAGGC AGATGGACACTGAGG-3′ and reverse primer: 5′-TTCC AGACTCCATGGAGTGT-3′) containing both polymorphic sites was amplified, and then, 1 μl of solution containing the PCR product mixed with 7 μl of denaturing solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF) was applied to polyacrylamide gel (acrylamide: bisacrylamide = 49:1) after denaturation and immediate cooling on ice. Electrophoresis was carried out in 0.5 × BE [45 mM Tris-borate (pH 8.0), 1 mM EDTA] under constant current of 20 mA/gel, 20°C for 90 min in 10% polyacrylamide gel using a minigel electrophoresis apparatus with a constant temperature control system ($90 \times 80 \times 1$ mm^3, AE-8450, AB-1600 and AE-6370; ATTO, Tokyo, Japan). Single-strand DNA fragments in the gel were visualized by silver staining (Daiichi Chemicals, Tokyo, Japan).

Association was statistically analyzed using StatView-J5.0 (SAS Institute Inc., Cary, NC, USA). χ^2-tests were used to analyze association of the *LILRB1* polymorphisms with susceptibility to RA and SLE. Hardy–Weinberg equilibrium was estimated from typing results using the EH program (47,48). *P*-values were corrected for multiple testing by Bonferroni correction. Specifically, for the association of *LILRB1* diplotype frequencies with RA (Table 2), the *P*-value was multiplied by 9, because independent tests were performed for six *PE* and three *CY* diplotypes. For the analysis of *LILRB1.PE01/01* diplotype with RA stratified by HLA-DRB1 SE (Table 3), the *P*-value was multiplied by 2, because two comparisons (*LILRB1.PE01/01*+ versus − in *HLA-DRB1* SE carriers, *LILRB1.PE01/01* + versus − in *HLA-DRB1* SE non-carriers) were performed.

HLA-DRB1 and *HLA-B* allele typing was performed using PCR-microtiter plate hybridization assay (33,49).

Preparation of recombinant soluble LILRB1 haplotype products and HLA-class I proteins

The recombinant soluble polypeptide representing the ligand-binding site (D1 and D2, amino acid residues 1–197) of LILRB1 was produced in our previous study and was designated as ILT2D1D2 (15). Briefly, D1D2 was expressed by *Escherichia coli* as inclusion bodies, solubilized in denaturant solution containing 6 M GuHCl, refolded by diluting into refolding buffer containing 0.4 M L-arginine and stirred for 48 h at 4°C. Then, the refolding mixture was concentrated with a VIVA-FLOW50 system (Viascience, Hanover, Germany) and Amicon Ultra (Millipore, Billerica, MA, USA), and the D1D2 polypeptide was purified with Superdex 75 26/60 (Amersham Biosciences, Piscataway, NJ, USA).

As the amino acid sequence of the ILT2D1D2 turned out to be the *LILRB1.PE03* haplotype product, expression vectors of LILRB1.PE01D1D2 and LILRB1.PE02D1D2 were prepared from pGEMILT2D1D2 vector using ordinary mutagenesis methods. Then, the LILRB1D1D2 proteins were expressed and purified as described earlier.

Soluble biotinylated HLA-A*1101 (with peptide AIFQSSMTK), HLA-B*3501 (with peptide IPLTEEAEL), HLA-Cw*0401 (with peptide QYDDAVYKL) and HLA-G1 (with peptide RIIPRHLQL) were prepared as described (15).

Crystallization and data collection

The LILRB1.PE01 and LILRB1.PE02 D1D2 proteins were crystallized from the mixture of 2 μl of the protein (~5 mg/ml) and 1 μl of 0.595 M sodium potassium tartrate, 0.085 M Tris–HCl (pH 8.5) and 15% ethylene glycol at 20°C by hanging drop method. Data were collected at 100 K at SPring-8 BL38B2 and BL41XU. The LILRB1.PE01 crystals belong to the space group of $P2_1$ with the cell dimension of $a = 41.0$ Å, $b = 93.2$ Å, $c = 57.8$ Å and $\beta = 107.8°$ [two molecules per asymmetric unit (a.u.)]. The LILRB1.PE02 crystals belong to the space group of $P2_12_12$ with the cell dimension of $a = 36.4$ Å, $b = 103.4$ Å and $c = 53.0$ Å (one molecule per a.u.). Data were processed and scaled with HKL program package (50). The structures were determined by molecular replacement with the initial model of LILRB1.PE03 structure (PDB accession no. 1UFU) (unpublished data). The LILRB1.PE01 and LILRB1.PE02 D1D2 structures were refined using CNS (51) with 20.0–2.75 and 8.0–1.8 Å reflections, respectively. The R_{free} and R_{work} of the final models were 29.5 and 25.0% for LILRB1.PE01 and 23.9 and 20.5% for LILRB1.PE02, respectively. The PDB accession nos for LILRB1.PE01 and LILRB1.PE02 D1D2 structures are 1VDG and 1UGN, respectively.

Surface plasmon resonance

In order to examine the interactions between recombinant LILRB1 D1D2 proteins and MHC class I complexes, SPR experiments were performed using a BIAcore2000 (BIAcore AB, St Albans, UK).

The biotinylated HLA-class I molecules and control biotinylated bovine serum albumin (BSA) were immobilized on the research-grade CM5 chip (BIAcore), onto which streptavidin was covalently coupled. Soluble D1D2 protein of each haplotype, after its buffer was exchanged to HBS-EP [10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20], was injected over the chip at 25°C. The binding response at each concentration was calculated by subtracting the equilibrium response measured in the control flow cell from the response in the HLA-class I flow cell.

Kinetic constants were derived by using the curve-fitting facility of the BIAEVALUATION 3.0 program (BIAcore) to fit rate equations derived from the simple 1:1 Langmuir binding model ($A + B \leftrightarrow AB$). Other curve fittings were performed by ORIGIN 5 (Microcal Software, Northampton, MA, USA). Affinity constants (Kd) were derived by Scatchard analysis or by non-linear curve fitting of the standard Langmuir binding isotherm.

Binding affinity assays between LILRB1D1D2 proteins and anti-LILRB1 (CD85j/ILT2) mAbs, HP-F1 (Beckman Coulter, Fullerton, CA, USA) and KY1 (36) were performed similarly, except that the mAbs and control BSA were immobilized onto the CM5 chip directly.

Circular dichroism

Recombinant LILRB1 D1D2 proteins were diluted to 10 μM in phosphate-buffered saline. In order to compare the thermostability of each LILRB1 D1D2 protein, circular dichroism (CD) spectroscopy was performed with JASCO-J720 spectropolarimeter (Jasco, Tokyo, Japan). The thermostabilities of the LILRB1 D1D2 proteins were analyzed by monitoring the change in the CD value at 217 nm as a function of temperature determined by preliminary experiment. During the alteration of temperature from 30 to 90°C at a constant heating rate (0.25°C/min), the changes of the secondary structure of LILRB1 (mainly β-sheet) are detected as CD values.

Flow cytometric analysis

Surface expression intensity of LILRB1 in peripheral blood leukocytes was examined in 16 healthy controls (11 with *LILRB1.PE01* and five without *LILRB1.PE01*), using PE-conjugated anti-LILIRB1, HP-F1 (Beckman Coulter). Differences in the MFIs of LILRB1-expressing lymphocytes and monocytes were compared between individuals with and without *LILRB1.PE01* haplotype. In order to examine the LILRB1 expression level on the subsets of T and B lymphocytes, three-color analyses were performed using FITC-conjugated anti-CD19, PC5-conjugated anti-CD27 mAbs for B cells and FITC-conjugated anti-CD4, PC5-conjugated anti-CD8 mAbs for T cells (Beckman Coulter). The differences were statistically analyzed using Mann–Whitney's *U*-test and Student's *t*-test.

ACKNOWLEDGEMENTS

The authors are indebted to Dr Toshio Yabe (Tokyo Metropolitan Red Cross Blood Center, Tokyo, Japan) and Dr Jun Ohashi (Department of Human Genetics, The University of Tokyo) for stimulating discussions and to M. Kawamoto, H. Sakai and K. Miura for their assistance in data collection at SPring-8 (Hyogo, Japan). This work was supported by Grant-in-Aid for Scientific Research on Priority Areas (C) 'Medical Genome Science', Grant-in-Aid for Scientific

Research (B) from the Ministry of Education, Science, Sports and Culture of Japan, Health and Labour Sciences Research Grant from the Ministry of Health Labour and Welfare of Japan and by Takeda Science Foundation.

Conflict of Interest statement. None declared.

REFERENCES

1. Cosman, D., Fanger, N., Borges, L., Kubin, M., Chin, W., Peterson, L. and Hsu, M.L. (1997) A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity*, **7**, 273–282.
2. Borges, L., Hsu, M.L., Fanger, N., Kubin, M. and Cosman, D. (1997) A family of human lymphoid and myeloid Ig-like receptors, some of which bind to MHC class I molecules. *J. Immunol.*, **159**, 5192–5196.
3. Colonna, M., Navarro, F., Bellon, T., Llano, M., Garcia, P., Samaridis, J., Angman, L., Cella, M. and Lopez-Botet, M. (1997) A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J. Exp. Med.*, **186**, 1809–1818.
4. Samaridis, J. and Colonna, M. (1997) Cloning of novel immunoglobulin superfamily receptors expressed on human myeloid and lymphoid cells: structural evidence for new stimulatory and inhibitory pathways. *Eur. J. Immunol.*, **27**, 660–665.
5. Wende, H., Colonna, M., Ziegler, A. and Volz, A. (1999) Organization of the leukocyte receptor cluster (LRC) on human chromosome 19q13.4. *Mamm. Genome*, **10**, 154–160.
6. Wende, H., Volz, A. and Ziegler, A. (2000) Extensive gene duplications and a large inversion characterize the human leukocyte receptor cluster. *Immunogenetics*, **51**, 703–713.
7. Wilson, M.J., Torkar, M., Haude, A., Milne, S., Jones, T., Sheer, D., Beck, S. and Trowsdale, J. (2000) Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc. Natl Acad. Sci. USA*, **97**, 4778–4783.
8. Moser, K.L., Neas, B.R., Salmon, J.E., Yu, H., Gray-McGuire, C., Asundi, N., Bruner, G.R., Fox, J., Kelly, J., Henshall, S. *et al.* (1998) Genome scan of human systemic lupus erythematosus: evidence for linkage on chromosome 1q in African-American pedigrees. *Proc. Natl Acad. Sci. USA*, **95**, 14869–14874.
9. Lindqvist, A.K., Steinsson, K., Johanneson, B., Kristjansdottir, H., Arnasson, A., Grondal, G., Jonasson, I., Magnusson, V., Sturfelt, G., Truedsson, L. *et al.* (2000) A susceptibility locus for human systemic lupus erythematosus (hSLE1) on chromosome 2q. *J. Autoimmun.*, **14**, 169–178.
10. Colonna, M., Samaridis, J., Cella, M., Angman, L., Allen, R.L., O'Callaghan, C.A., Dunbar, R., Ogg, G.S., Cerundolo, V. and Rolink, A. (1998) Human myelomonocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules. *J. Immunol.*, **160**, 3096–3100.
11. Allen, R.L., Raine, T., Haude, A., Trowsdale, J. and Wilson, M.J. (2001) Leukocyte receptor complex-encoded immunomodulatory receptors show differing specificity for alternative HLA-B27 structures. *J. Immunol.*, **167**, 5543–5547.
12. Chapman, T.L., Heikema, A.P., West, A.P., Jr and Bjorkman, P.J. (2000) Crystal structure and ligand binding properties of the D1D2 region of the inhibitory receptor LIR-1 (ILT2). *Immunity*, **13**, 727–736.
13. Willcox, B.E., Thomas, L.M. and Bjorkman, P.J. (2003) Crystal structure of HLA-A2 bound to LIR-1, a host and viral major histocompatibility complex receptor. *Nat. Immunol.*, **4**, 913–919.
14. Chapman, T.L., Heikeman, A.P. and Bjorkman, P.J. (1999) The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. *Immunity*, **11**, 603–613.
15. Shiroishi, M., Tsumoto, K., Amano, K., Shirakihara, Y., Colonna, M., Braud, V.M., Allan, D.S., Makadzange, A., Rowland-Jones, S., Willcox, B. *et al.* (2003) Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G. *Proc. Natl Acad. Sci. USA*, **100**, 8856–8861.
16. Kubagawa, H., Burrows, P.D. and Cooper, M.D. (1997) A novel pair of immunoglobulin-like receptors expressed by B cells and myeloid cells. *Proc. Natl Acad. Sci. USA*, **94**, 5261–5266.
17. Yamashita, Y., Ono, M. and Takai, T. (1998) Inhibitory and stimulatory functions of paired Ig-like receptor (PIR) family in RBL-2H3 cells. *J. Immunol.*, **161**, 4042–4047.
18. Ujike, A., Takeda, K., Nakamura, A., Ebihara, S., Akiyama, K. and Takai, T. (2002) Impaired dendritic cell maturation and increased T_H2 responses in PIR-B$^{-/-}$ mice. *Nat. Immunol.*, **3**, 542–548.
19. Nakamura, A., Kobayashi, E. and Takai, T. (2004) Exacerbated graft-versus-host disease in *Pirb*$^{-/-}$ mice. *Nat. Immunol.*, **5**, 623–629.
20. Uhrberg, M., Valiante, N.M., Shum, B.P., Shilling, H.G., Lienert-Weidenbach, K., Corliss, B., Tyan, D., Lanier, L.L. and Parham, P. (1997) Human diversity in killer cell inhibitory receptor genes. *Immunity*, **7**, 753–763.
21. Shilling, H.G., Guethlein, L.A., Cheng, N.W., Gardiner, C.M., Rodriguez, R., Tyan, D. and Parham, P. (2002) Allelic polymorphism synergizes with variable gene content to individualize human KIR genotype. *J. Immunol.*, **168**, 2307–2315.
22. Warrington, K.J., Takemura, S., Goronzy, J.J. and Weyand, C.M. (2001) CD4+,CD28− T cells in rheumatoid arthritis patients combine features of the innate and adaptive immune systems. *Arthritis Rheum.*, **44**, 13–20.
23. Yen, J.H., Moore, B.E., Nakajima, T., Scholl, D., Schaid, D.J., Weyand, C.M. and Goronzy, J.J. (2001) Major histocompatibility complex class I-recognizing receptors are disease risk genes in rheumatoid arthritis. *J. Exp. Med.*, **193**, 1159–1167.
24. Martin, M.P., Nelson, G., Lee, J.H., Pellett, F., Gao, X., Wade, J., Wilson, M.J., Trowsdale, J., Gladman, D. and Carrington, M. (2002) Cutting edge: susceptibility to psoriatic arthritis: influence of activating killer Ig-like receptor genes in the absence of specific HLA-C alleles. *J. Immunol.*, **169**, 2818–2822.
25. van der Slik, A.R., Koeleman, B.P., Verduijn, W., Bruining, G.J., Roep, B.O. and Giphart, M.J. (2003) KIR in type 1 diabetes: disparate distribution of activating and inhibitory natural killer cell receptors in patients versus HLA-matched control subjects. *Diabetes*, **52**, 2639–2642.
26. Suzuki, Y., Hamamoto, Y., Ogasawara, Y., Ishikawa, K., Yoshikawa, Y., Sasazuki, T. and Muto, M. (2004) Genetic polymorphisms of killer cell immunoglobulin-like receptors are associated with susceptibility to psoriasis vulgaris. *J. Invest. Dermatol.*, **122**, 1133–1136.
27. Luszczek, W., Manczak, M., Cislo, M., Nockowski, P., Wisniewski, A., Jasek, M. and Kusnierczyk, P. (2004) Gene for the activating natural killer cell receptor, KIR2DS1, is associated with susceptibility to psoriasis vulgaris. *Hum. Immunol.*, **65**, 758–766.
28. Martin, M.P., Gao, X., Lee, J.H., Nelson, G.W., Detels, R., Goedert, J.J., Buchbinder, S., Hoots, K., Vlahov, D., Trowsdale, J. *et al.* (2002) Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat. Genet.*, **31**, 429–434.
29. Khakoo, S.I., Thio, C.L., Martin, M.P., Brooks, C.R., Gao, X., Astemborski, J., Cheng, J., Goedert, J.J., Vlahov, D., Hilgartner, M. *et al.* (2004) HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science*, **305**, 872–874.
30. Norman, P.J., Carey, B.C., Stephens, H.A.F. and Vaughan, R.W. (2003) DNA sequence variation and molecular genotyping of natural killer leukocyte immunoglobulin-like receptor, LILRA3. *Immunogenetics*, **55**, 165–171.
31. Heinzmann, A., Blattmann, S., Forster, J., Kuehr, J. and Deichmann, K.A. (2000) Common polymorphisms and alternative splicing in the ILT3 gene are not associated with atopy. *Eur. J. Immunogenet.*, **27**, 121–127.
32. Bellon, T., Kitzig, F., Sayos, J. and Lopez-Botet, M. (2002) Mutational analysis of immunoreceptor tyrosine-based inhibition motifs of the Ig-like transcript 2 (CD85j) leukocyte receptor. *J. Immunol.*, **168**, 3351–3359.
33. Shibue, T., Tsuchiya, N., Komata, T., Matsushita, M., Shiota, M., Ohashi, J., Wakui, M., Matsuta, K. and Tokunaga, K. (2000) Tumor necrosis factor alpha 5′-flanking region, tumor necrosis factor receptor II, and HLA-DRB1 polymorphisms in Japanese patients with rheumatoid arthritis. *Arthritis Rheum.*, **43**, 753–757.
34. Hashimoto, H., Nishimura, Y., Dong, R.P., Kimura, A., Sasazuki, T., Yamanaka, K., Tokano, Y., Murashima, A., Kabasawa, K. and Hirose, S. (1994) HLA antigens in Japanese patients with systemic lupus erythematosus. *Scand. J. Rheumatol.*, **23**, 191–196.
35. Tsuchiya, N., Ohashi, J. and Tokunaga, K. (2002) Variations in immune response genes and their associations with multifactorial immune disorders. *Immunol. Rev.*, **190**, 169–181.

36. Yamashita, Y., Amasaki, Y., Yamashita, T., Ichikawa, K., Atsumi, T. and Koike, T. (2002) ILT2/LIR1 inhibitory receptor, a new marker for cytotoxic CD8+ T cells, regulates cytotoxic T cell function. (Abstract). *FASEB J.*, **16**, A316.
37. Koch, S., Goedde, R., Nigmatova, V., Epple, J.T., Müller, N., de Seze, J., Vermersch, P., Momot, T., Schmidt, R.E. and Wille, T. (2005) Association of multiple sclerosis with ILT6 deficiency. *Genes Immun.* Advance online publication, April 7, 2005; doi:10.1038/sj.gene.6364187.
38. Tedla, N., Gibson, K., McNeil, H.P., Cosman, D., Borges, L. and Arm, J.P. (2002) The co-expression of activating and inhibitory leukocyte immunoglobulin-like receptors in rheumatoid synovium. *Am. J. Pathol.*, **160**, 425–431.
39. Seldin, M.F., Amos, C.I., Ward, R. and Gregersen, P.K. (1999) The genetics revolution and the assault on rheumatoid arthritis. *Arthritis Rheum.*, **42**, 1071–1079.
40. Rodiguez, M.R., Gonzalez-Escribano, M.F., Aguilar, F., Valenzuela, A., Garcia, A. and Nunez-Roldan, A. (2002) Association of NRAMP1 promoter gene polymorphism with the susceptibility and radiological severity of rheumatoid arthritis. *Tissue Antigens*, **59**, 311–315.
41. Prokunina, L., Padyukov, L., Bennet, A., de Faire, U., Wiman, B., Prince, J., Alfredsson, L., Klareskog, L. and Alarcon-Riquelme, M. (2004) Association of the PD-1.3A allele of the *PDCD1* gene in patients with rheumatoid arthritis negative for rheumatoid factor and the shared epitope. *Arthritis Rheum.*, **50**, 1770–1773.
42. Tokunaga, K., Imanishi, T., Takahashi, K. and Juji, T. (1996) On the origin and dispersal of East Asian populations as viewed from HLA haplotypes. In Akazawa, T. and Szathmary, E.J. (eds), *Prehistoric Mongoloid Dispersals.* Oxford University Press, Oxford, UK, pp. 187–197.
43. Kochi, Y., Yamada, R., Suzuki, A., Harley, J.B., Shirasawa, S., Sawada, T., Bae, S.-C., Tokuhiro, S., Chang, X., Sekine, A. *et al.* (2005) A functional variant in FCRL3, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities. *Nat. Genet.*, **37**, 478–485.
44. Bleharski, J.R., Li, H., Meinken, C., Graeber, T.G., Ochoa, M.T., Yamamura, M., Burdick, A., Sarno, E.N., Wagner, M., Rollinghoff, M. *et al.* (2003) Use of genetic profiling in leprosy to discriminate clinical forms of the disease. *Science*, **301**, 1527–1530.
45. Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H., Luthra, H.S. *et al.* (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.*, **31**, 315–324.
46. Tan, E.M., Cohen, A.S., Fries, J.F., Masi, A.T., McShane, D.J., Rothfield, N.F., Schaller, J.G., Talal, N. and Winchester, R.J. (1982) The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.*, **25**, 1271–1277.
47. Xie, X. and Ott, J. (1993) Testing linkage disequilibrium between a disease gene and marker loci. (Abstract). *Am. J. Hum. Genet.*, **53** (suppl.), 1107.
48. Terwilliger, J. and Ott, J. (1994) *Handbook for Human Genetic Linkage.* Johns Hopkins University Press, Baltimore and London.
49. Kawai, S., Maekawajiri, S., Tokunaga, K., Kashiwase, K., Miyamoto, M., Akaza, T., Juji, T. and Yamane, A. (1996) Routine low and high resolution typing of HLA-DRB gene using PCR-MPH (microtiter plate hybridization) method. *Eur. J. Immunogenet.*, **23**, 471–486.
50. Otwinowski, A. and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.*, **76**, 307–326.
51. Brunger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S. *et al.* (1998) Crystallography & NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr. D. Biol. Crystallogr.*, **54**, 905–921.