JDI **Journal of Diabetes Investigation** Open access
Official Journal of the Asian Association for the Study of Diabetes

ORIGINAL ARTICLE

HLA-A*33-DR3 and A*33-DR9 haplotypes enhance the risk of type 1 diabetes in Han Chinese

Juanjuan Zhang[1†], Liebin Zhao[1†], Bokai Wang[1], Jie Gao[1], Li Wang[1], Li Li[1], Bin Cui[1], Min Hu[2], Jie Hong[1], Weiqiong Gu[1]*, Weiqing Wang[1], Guang Ning[1,3]

[1]Department of Endocrine and Metabolic diseases, Ruijin Hospital, Shanghai Jiao-Tong University, School of Medicine, Shanghai Key Laboratory for Endocrine Tumors, Shanghai Clinical Center for Endocrine and Metabolic Diseases, Shanghai Institute of Endocrine and Metabolic Diseases and Shanghai E-institute for Endocrinology, [3]Laboratory for Endocrine & Metabolic Diseases, Institute of Health Science, Shanghai JiaoTong University, School of Medicine and Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China, and [2]Center for Transplant and Renal Research, Westmead Millennium Institute, University of Sydney, Sydney, New South Wales, Australia

ABSTRACT

Aims/Introduction: To investigate the typing for human leukocyte antigen (HLA) class I in Chinese patients with type 1 diabetes as a complement screening for HLA class II.

Materials and Methods: A total of 212 type 1 diabetic patients and 200 healthy controls were enrolled. The genetic polymorphisms of HLA class I and II were examined with a high-resolution polymerase chain reaction sequence-based typing method.

Results: The haplotype, A*33:03-B*58:01-C*03:02(A33), was associated with type 1 diabetes ($P = 1.0 \times 10^{-4}$, odds ratio 3.2 [1.738–5.843]). The A33-DR3 and A33-DR9 haplotypes significantly enhanced the risk of type 1 diabetes (A33-DR3, odds ratio 5.1 [2.40–10.78], $P = 4.0 \times 10^{-6}$; A33-DR9, odds ratio 13.0 [1.69–100.32], $P = 0.004$). In type 1 diabetic patients, compared with A33-DR3-negative carriers, A33-DR3-positive carriers had significantly lower percentages of $CD3^+CD4^+$ T cells (42.5 ± 7.72 vs $37.0 \pm 8.35\%$, $P = 0.023$), higher percentages of $CD3^+CD8^+$ T cells (27.4 ± 7.09 vs $32.8 \pm 5.98\%$, $P = 0.005$) and T-cell receptor α/β T cells (70.0 ± 7.00 vs $73.6 \pm 6.25\%$, $P = 0.031$), and lower CD4/CD8 ratios (1.71 ± 0.75 vs 1.16 ± 0.35, $P = 0.003$).

Conclusions: It is the first time that the haplotypes A33-DR3 and A33-DR9 were found with an enhanced predisposition to type 1 diabetes in Han Chinese. A33-DR3 was associated with a reduction in the helper-to-cytotoxic cell ratio and preferential increase of T-cell receptor α/β T cell. The typing for HLA class I and its immunogenetic effects are important for more accurate HLA class II haplotype risk prediction and etiology research in type 1 diabetic patients.

INTRODUCTION

Type 1 diabetes is an autoimmune disease characterized by immune-mediated destruction of pancreatic β-cells, resulting in permanent β-cell loss and insulin deficiency. It is widely recognized that the major component of human leukocyte antigen (HLA) susceptibility to type 1 diabetes involves the DRB1, DQA1 and DQB1 genes[1]. Recent studies have suggested that genes in the HLA region other than DR and DQ also contribute to type 1 diabetes susceptibility[2–4].

The association of HLA class I polymorphisms with the age at onset of type 1 diabetes in Caucasian populations has been previously reported[2,3]. HLA class I molecules, given their role in target-cell recognition by $CD8^+$ cytotoxic T lymphocytes, play a role in the ongoing immune response and, therefore, could affect the rate of pancreatic β-cell destruction. This hypothesis might help explain the observed associations of HLA class I with type 1 diabetes, particularly the association of HLA class I with lymphocyte subpopulation. In addition, it has

recently been observed that innate immune cell cross-talk also occurs in the pancreas of young NOD mice and leads to the initiation of type 1 diabetes, with the interaction of immunoglobulin G (IgG)-producing B-1a cells, neutrophils and interferon-α-producing pDCs[5].

With the requirement of secondary type 1 diabetes prevention trials and the selection of participants with impending diabetes, and the fact that thus far, scarce research is available regarding the association of T lymphocyte subpopulations and innate immunity with high-risk HLA class I genes in patients with type 1 diabetes at the disease onset, we investigated whether typing for HLA-A, HLA-B and HLA-C might complement screening for HLA-DRB1, DQB1 and DQA1 in Chinese patients in Eastern China, and sought to characterize the clinical and immunological features of HLA-typed youth with new-onset type 1 diabetes.

MATERIALS AND METHODS
Study Cohort
A total of 212 patients with new-onset type 1 diabetes included in this dataset were identified and examined at the Department of Endocrine and Metabolic diseases, Ruijin Hospital Affiliated to Shanghai Jiao-Tong University School of Medicine, Shanghai, China, from April 2006 to October 2012. The average age at diagnosis was 20.2 ± 5.5 years, and the average duration of diabetes was 10 ± 6.4 weeks with 87 men and 125 women. A total of 200 healthy volunteers were included as controls, including 117 men and 83 women, with an average age of 23.6 ± 4.2 years, and had no family history of diabetes or overt autoimmune diseases and any chronic diseases. All the cases and controls were Han Chinese and from the East Chinese population belonging to the East Chinese states of Shanghai, Jiangsu and Zhejiang. Individuals with type 1 diabetes or not were diagnosed according to the diagnosis criteria of the World Health Organization and American Diabetes Association[6]. All cases are typical acute-onset, ketosis prone, positive for glutamic acid decarboxylase antibodies. All the patients and 10 of the healthy volunteers were followed up for age at onset, relative with type 1 diabetes (yes vs no), diabetic ketoacidosis history (yes vs no), glucose level, glycated hemoglobin, fasting C-peptide, peak value of C-peptide during a 75-g oral glucose tolerance test and area under C-peptide release curve during an oral glucose tolerance test. To find the relationship between immunophenotypic changes and high-risk haplotypes, the total percentages of neutrophils, natural killer (NK), T and B cells were examined, as well as the coexpression of surface molecules on lymphocytes associated with helper/cytotoxic capacity, activation status and other functions. In addition, the levels of several serum proteins, including glutamic acid decarboxylase antibodies, high-sensitivity C-reactive protein (hs-CRP), IgG and interferon-γ (IFN-γ) were evaluated. All samples were collected with appropriate informed consent, and the protocol was approved by the local ethics committee and carried out in agreement with the Declaration of Helsinki as revised in 2000.

Genotyping Methods
HLA genotyping detection was carried out on 212 patients with type 1 diabetes and 200 healthy controls, with six loci, including A, B, C, DRB1, DQB1 and DQA1, with a polymerase chain reaction sequence-based typing system (Shanghai Tissuebank Biotechnology Co., Ltd., Shanghai, China). Genomic deoxyribonucleic acid was isolated from whole blood using the QiaAMP® DNA mini kits (Qiagen GmbH, Hilden, Germany), and used to amplify HLA class I and class II genes by polymerase chain reaction with primers listed in Table S1. Amplicons were purified using ExoSAP protocols (Exonuclease I and Alkaline Phosphatase kits; Takara, Dalian, China) and sequenced using the ABI Prism BigDye Teminator Cycle Sequencing Reaction Ready kits (Applied Biosystems, Foster City, CA, USA) with the specific sequencing primers (Table S2). Sequences were analyzed using the ABI 3730xl genetic analyzer (Applied Biosystems). The sequences were then genotyped using uTYPE3.0 software and database 3.7 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Class I HLA alleles were sequenced for exons 2, 3 and 4 in both directions, and class II HLA alleles were sequenced for exon 2 and 3 in both directions. To determine the ambiguity, allele-specific sequencing was carried out using a set of group-specific primers (HLA-SBT kit; CATB, Shanghai, China). The deoxyribonucleic acid database of the same sequences of all known alleles were thereby compared to obtain accurate allele coding.

Flow Cytometry
Blood collection was carried out in 212 patients with type 1 diabetes and 10 healthy controls. The measurements of forward and side scatter were combined with CD45 to identify lymphocytes and exclude monocytes. The absolute subpopulations of lymphocyte numbers were calculated based on the total lymphocyte counts and the percentage of subpopulations of lymphocyte cells, as identified by flow cytometry using the EPICS XL (Beckman Coulter, Brea, CA, USA) and Divasoftware (BD Biosciences, Franklin Lakes, NJ, USA). The fraction of lymphocyte cell subsets was determined by multiple-colors fluorescence activated cell sorter analysis using appropriate surface markers: anti-CD3-FITC (Clone UCHT1), anti-CD4-PE-Cy7 (Clone 13B8.2), anti-CD8-APC-H7 (Clone B9.11), anti-CD45-PerCP-Cy5.5 (Clone J.33), anti-CD45RA-FITC (Clone ALB11), anti-C62L-PE (Clone DREG56), anti-CD19-APC (Clone J3.119) anti-CD16-PE (Clone 3GB), anti-CD56-PE (Clone N901), anti-TCR PAN α/β-FITC (TCR 1, Clone IP26A) and anti-TCR PAN γδ-PE (TCR 2, Clone IMMU510; Beckman Coulter).

Laboratory Assessment of Diabetes Status
The serum C-peptide levels were measured by a radioimmunoassay using a commercially available kit (Roche Diagnostics, Penzberg, Germany). The lower limit of detection was 0.1 ng/mL, and undetected values were reported as 0.1 ng/mL. The serum levels of anti-glutamic acid decarboxylase antibodies were also measured by a radioimmunoassay using a commercial

kit (RSR Limited, Cardiff, UK), and the results were considered positive if the values were >7.5 units/mL. Glycated hemoglobin was measured by high-pressure liquid chromatography. The serum levels of hs-CRP were measured using the latex enhanced immunoturbidimetric method. Both IgG and IFN-γ were measured by enzyme-linked immunosorbent assay (Gentaur, Kampenhout, Belgium) and flow cytometry (Beckman, High Wycombe, UK), respectively.

Statistical Analysis

The Hardy–Weinberg test of *HLA* alleles and their linkage disequilibrium test results were analyzed using Arlequin 3.11 software (Institute of Ecology and Evolution, University of Bern, Bern, Switzerland). Statistical analysis was carried out with SPSS 17.0 (SPSS, Armonk, NY, USA). The results are shown as the mean ± standard deviation or as median (range), or otherwise documented as positive cases, constituent ratio or ratio. The difference between classified variables was tested using the χ^2-test or Fisher's exact test if the expected number of participants in any cell was less than five. ANOVA analyses were carried out among the groups with a model that included the baseline values of the age of onset and duration of diabetes as covariates. C-peptide, anti-glutamic acid decarboxylase antibodies and hs-CRP were studied on the logarithmic scale. All hypothesis testing was two-tailed. We made the Bonferroni correction for the *P*-values by the number of alleles with substantial frequencies (≥5%) in six loci including *HLA-A*, *B*, *C*, *DRB1*, *DQB1* and *DQA1* respectively[7]. Statistical significance was defined as $P < 0.05$.

RESULTS

HLA Alleles and Haplotypes

The association of *HLA* alleles and haplotypes with type 1 diabetes is shown in Tables 1, S3 and S4. The frequencies of *A*24:02*, *B*58:01*, *C*03:02* and *A*33:03-B*58:01-C*03:02*, *DRB1*03:01-DQA1*05:01-DQB1*02:01(DR3)*[1], *DRB1*04:XX-DQA1*03:01-DQB1*03:02(04XX: 04:01,04:04,04:05)* (DR4)[1] and *DRB1*09:01-DQA1*03:02-DQB1*03:03(DR9)*[8] haplotypes were significantly higher in the patients with type 1 diabetes than the healthy controls (all *P* < 0.05).

The differences in allele frequencies between controls and cases for these alleles could be exclusively due to the strong linkage disequilibrium between the *HLA* class I and class II loci. To address this issue, we computed the normalized linkage disequilibrium in the type 1 diabetic patients (Table 2). The strongest linkage disequilibrium observed in both samples was between the well-known high-risk haplotypes, *DRB1*03:01-DQB1*02:01* and *DRB1*09:01-DQB1*03:03*, respectively. The other apparently predisposing allele, *A*24:02*, showed no significant linkage disequilibrium to any particular *DR-DQ* haplotype. Of the apparently predisposing alleles, *A*33:03* showed a very strong linkage disequilibrium with *B*58:01*, which explains why *A*33:03* often appeared with *B*58:01* among patients, whereas both these two alleles showed no significant linkage

disequilibrium with the high-risk haplotypes *DR3*, *DR4* and *DR9* (Tables S5–10). Accordingly, primary association data for *HLA* region markers remained significantly associated with type 1 diabetes after computation of linkage disequilibrium.

We then assessed the risk of both the *A33* haplotype and *A*24:02* allele with these stratified *HLA-DR-DQ* haplotypes compared with the results presented earlier, which were not stratified by *HLA-DR-DQ* haplotype. Haplotype *A33* was associated with case chromosomes on *DR3* ($P = 4.0 \times 10^{-6}$, odds ratio 5.09) and is also associated with *DR9* haplotypes ($P = 0.004$, odds ratio 13.00; Table 3), with the exception of *DR4*, where there was no increased risk associated with the *A33* haplotype ($P = 0.499$).

Clinical Characteristics in Patients with High-risk A33-DR3 and A33-DR9 Haplotypes

To explore whether there were cooperative effects between the *A33* and high-risk *DR3* or *DR9* haplotypes, we assessed the correlation between clinical characteristics and *A33* haplotype on *DR3* or *DR9*. The participants were divided into two groups according to different high-risk haplotypes. Haplotypes *A33-DR3* and *A33-DR9* were detected in 41 and 13 out of 212 patients with type 1 diabetes, respectively (19.3% and 6.1%). There were 126 and 92 participants in the *A33-DR3*-negative and *A33-DR9*-negative subgroups, respectively (59.4% and 43.4%). *A33-DR3*-positive carriers were referred to as patients with both haplotype *A33* and haplotype *DR3*, whereas *A33-DR3*-negative carriers were identified as participants without either haplotype *A33* or haplotype *DR3*. The identification of *A33-DR9*-positive and *A33-DR9*-negative subgroups was the same as that of the *A33-DR3* subgroups. Clinical features, such as the age at onset, duration of diabetes, frequency of presence with diabetic ketoacidosis history and relative with type 1 diabetes, levels of fasting C-peptide, peak value of C-peptide during a 75-g oral glucose tolerance test, and area under C-peptide release curve during an oral glucose tolerance test, showed no significant differences between *A33-DR3*-positive carriers and *A33-DR3*-negative carriers (Table S11). For the *A33-DR9*-positive and *A33-DR9*-negative subgroups, there were also no significant differences in any clinical characteristics.

Immunological Features in Type 1 Diabetic Patients Consistent with High-risk A33-DR3 and A33-DR9 Haplotypes

Haplotype *A33-DR3* was associated with the imbalance of T lymphocyte subpopulations at the onset of type 1 diabetes (Figure 1), and an example of the gating strategy and fluorescence activated cell sorter plots is shown in Figure 2. Compared with *A33-DR3*-negative carriers, *A33-DR3*-positive carriers had a significantly lower percentage of $CD3^+CD4^+$ T cells (42.5 ± 7.72 vs 37.0 ± 8.35%, $P = 0.023$), higher percentage of $CD3^+CD8^+$ T cells (27.4 ± 7.09 vs 32.8 ± 5.98%, $P = 0.005$) and lower level of ratio of CD4/CD8 (1.71 ± 0.75 vs 1.16 ± 0.35, $P = 0.003$). In addition, *A33-DR3*-positive carriers had a significantly higher percentage of T-cell receptor (TCR)

Table 1 | Comparison of human leukocyte antigen gene frequencies between type 1 diabetic patients and healthy controls

Gene	Type 1 diabetic patients (n = 212)	Healthy controls (n = 200)	P	Pc	OR	OR (95% CI)
Alleles						
HLA-A						
A*24:02	85 (40.1)	44 (22.0)	7.6×10^{-5}	6.86×10^{-4}	2.373	1.539–3.658
A*33:03	55 (25.9)	36 (18.0)	0.052	0.468	1.596	0.994–2.563
HLA-B						
B*58:01	62 (29.2)	21 (10.5)	2.1×10^{-6}	2.7×10^{-5}	3.523	2.052–6.048
HLA-C						
C*03:02	60 (28.3)	21 (10.5)	5.5×10^{-6}	5.0×10^{-5}	3.365	1.957–5.785
Haplotypes						
A-B-C						
A*33:03-B*58:01-C*03:02	46 (21.7)	16 (8.0)	1.0×10^{-4}	–	3.187	1.738–5.843
DRB1-DQB1-DQA1						
DR3	81 (38.2)	26 (13.0)	5.5×10^{-9}	–	4.138	2.518–6.799
DR4	31 (17.1)	3 (1.5)	3.2×10^{-6}	–	11.247	3.380–37.420
DR9	87 (41.0)	42 (21.0)	1.2×10^{-5}	–	2.618	1.692–4.052

Data are expressed as n (%). χ^2 or Fisher's exact test. DR3 = DRB1*03:01-DQA1*05:01-DQB1*02:01, DR4 = DRB1*04:XX-DQA1*03:01-DQB1*03:02(04:XX: 04:01, 04:04, 04:05), DR9 = DRB1*09:01-DQA1*03:02-DQB1*03:03. –, Not available; CI, confidence interval; HLA, human leukocyte antigen; OR, odds ratio; Pc, corrected P-values.

Table 2 | Normalized linkage disequilibrium between human leukocyte antigen class I and human leukocyte antigen class II alleles (n = 212)

Haplotype		D'	r^2	χ^2	P-value
HLA-A	HLA-B				
33:03	58:01	0.76	0.47	171.06	<0.001
DRB1	DQB1				
03:01	02:01	0.90	0.80	288.70	<0.001
09:01	03:03	0.72	0.52	188.98	<0.001
HLA-B	DQA1				
58:01	05:01	0.80	0.39	140.84	<0.001

Only haplotypes that showed significant linkage disequilibrium are listed. HLA, human leukocyte antigen.

α/β(TCR1$^+$) T cells than the *A33-DR3*-negative carriers (73.6 ± 6.25 vs 70.0 ± 7.00%, P = 0.031). However, the percentages of CD45RA$^+$CD62L$^+$ lymphocytes, neutrophils, natural killer (CD16$^+$CD56$^+$) cells and the IgG-producing B (CD19$^+$) cells were not significantly different between the two subgroups. None of the serum protein levels measured, such as glutamic

acid decarboxylase antibodies, hs-CRP, IgG and IFN-γ, appeared to be associated with *A33-DR3*. No significant differences were found in any immunological features between *A33-DR9*-positive carriers and *A33-DR9*-negative patients, except for CD3$^+$CD4$^+$ T cells and CD4/CD8, respectively (34.8 ± 13.71 vs 44.3 ± 8.48%, P = 0.022; 1.07 ± 0.46 vs 1.76 ± 0.72, P = 0.042), To evaluate whether the observed associations of an altered phenotype in lymphocytes is associated with the A33-DR3 haplotype or with type 1 diabetes, we tested the T lymphocyte subpopulations in 10 healthy participants to evaluate controls by flow cytometry. The data is organized as Figures 1, S1 and S2. Although there were no significant differences in the percentages of CD3$^+$CD4$^+$ T cells and CD3$^+$CD8$^+$ T cells, A33-DR3-positive carriers had the lowest ratio of CD4/CD8. Compared with healthy controls, A33-DR3-positive carriers and A33-DR3-negative carriers had a significantly higher percentage of TCRα/β T cells, respectively (58.7 ± 9.12 vs 73.6 ± 6.25%, P = 2.6×10^{-6}; 58.7 ± 9.12 vs 69.7 ± 7.00%, P = 9.5×10^{-5}). Compared with *A33*-negative carriers, *A33*-positive carriers also had a significantly lower level of ratio of CD4/CD8 (1.75 ± 0.68 vs 1.16 ± 0.35,

Table 3 | Analysis of case and control chromosomes for the risk associated with the haplotype *A*33:03-B*58:01-C*03:02*, stratified by *HLA-DR-DQ* haplotype

Haplotype	Type 1 diabetes patients (n = 212)		Healthy control (n = 200)		OR (95% CI)	P
	With A33*	% with A33	With A33	% with A33		
DR3	41	19.3	9	4.5	5.09 (2.40–10.78)	4.0×10^{-6}
DR4	2	0.9	0	0.0	NA	0.499
DR9	13	6.1	1	0.5	13.00 (1.69–100.32)	0.004

*A33 = A*33:03-B*58:01-C*03:02. χ^2 or Fisher's exact test. CI, confidence interval; NA, not available; OR, odds ratio.
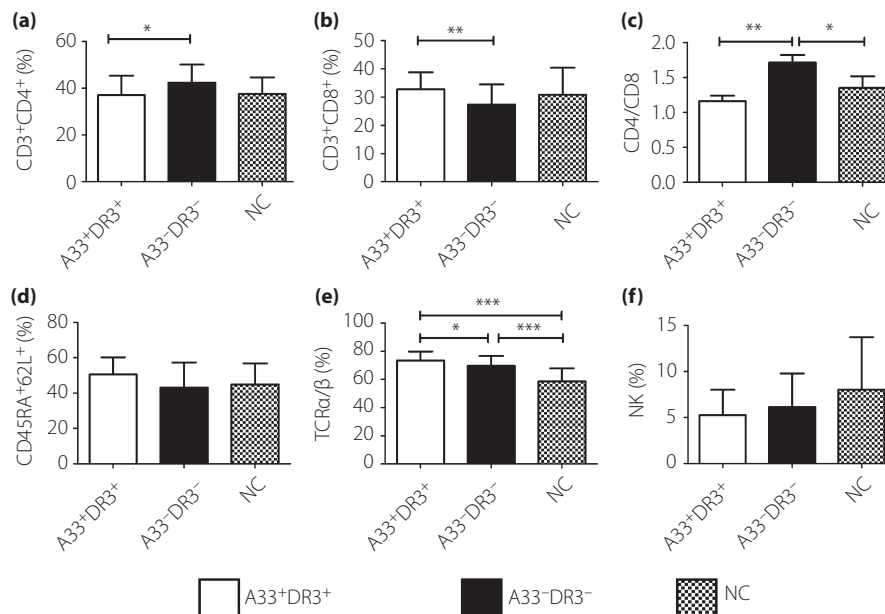
Figure 1 | Association of *A33-DR3* haplotype and T lymphocyte subpopulations in type 1 diabetes patients (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (a) CD3+CD4+ T cells. (b) CD3+CD8+ T cells. (c) Ratio of CD4/CD8. (d) CD45RA+CD62L+ lymphocytes. (e) TCRα/β(TCR1+) T cells. (f) Natural killer (NK; CD16+CD56+) cells. Vertical lines indicate one standard deviation above and below the mean. There were 41, 126 and 10 participants in the A33+DR3+, DR3−A33− subgroups and healthy control (NC) group, respectively. ANOVA after adjustments for age of onset and duration of diabetes.

$P = 2.3 \times 10^{-6}$). No significant differences were found in CD4/CD8 ratio between DR3-positive carriers and DR3-negative patients.

DISCUSSION

Several studies have suggested that extended or ancestral major histocompatibility complex haplotypes provide an even greater risk to develop autoimmunity compared with individual alleles of any single gene[9,10]. Thus, we mainly focused on the susceptibility of haplotypes for *HLA* class I/II in the present study.

In our study, we defined two sets of *HLA-A33*+ haplotypes that provide maximum risk of type 1 diabetes development in Chinese: the *A33-DR3* haplotype and *A33-DR9* haplotype. According to published literature, *A*33:03-B*58:01-C*03:02-DRB1*03:01* is reported as a relatively common haplotype in the Han Chinese population[11–13]. As there were no linkage disequilibrium between the haplotype A33 and DR3, the positive association of *A33-DR3* with type 1 diabetes might be the accumulation of specific *HLA* class I and class II. In the North Indian population, *A2-B8-DR3* contributed the maximum risk (relative risk = 48.7) for type 1 diabetes, followed by *A2-B50-DR3* (relative risk = 9.4) and *A33-B58-DR3* (relative risk = 6.6)[14]. It is interesting to note that the *A33-B58-DR3* haplotype also shows positive disease-association in Mongoloid racial groups[15]. These results support the hypothesis that certain combined haplotypes have subsequently expanded in frequency across populations in different geographical location. The fact that neither *A*33:03* nor *B*58:01* appears type 1

diabetes predisposing when seen in Caucasians (e.g., in the T1DGC data) suggests that the effect seen in Asians might not be due to the alleles themselves, but to something else on the haplotype.

The mechanism of the accumulation of specific *HLA* class I and class II (*DR* and *DQ*) alleles causing higher risk in type 1 diabetes remains unclear. The combination of specific *HLA-A*, -*DR* and -*DQ* alleles might have synergic and complementary effects on various steps of the immune response. Furthermore, the odds ratio of *A33-DR3* and *A33-DR9* was just 5.09 and 13.0, respectively, in new-onset type 1 diabetes, so there remains the possibility that there is synergy between these two haplotypes and non-*HLA* genes[16] or environmental factors, such as viral infection[17], in promoting insulitis in type 1 diabetes. According to the study by Chang *et al.*[18], multivariate analysis showed that HLA-A33 was the gene most significantly susceptible to enterovirus 71. There have been several studies to investigate enterovirus's potential role as an environmental factor in predisposing to type 1 diabetes, though these results remain controversial[19–23].

The significant association of the haplotype *A33-DR3* with the abnormality in the ratio of helper to cytotoxic T cells is consistent with our hypothesis. Given that a previous study found that the persistence of a reduced CD4/CD8 lymphocyte ratio (less than 1.5) might reflect the ongoing process leading to β-cell destruction[24], longitudinal observation of β-cell function is necessary to better elucidate the genetic factors that regulate the natural course of β-cell destruction in type 1 diabetes.
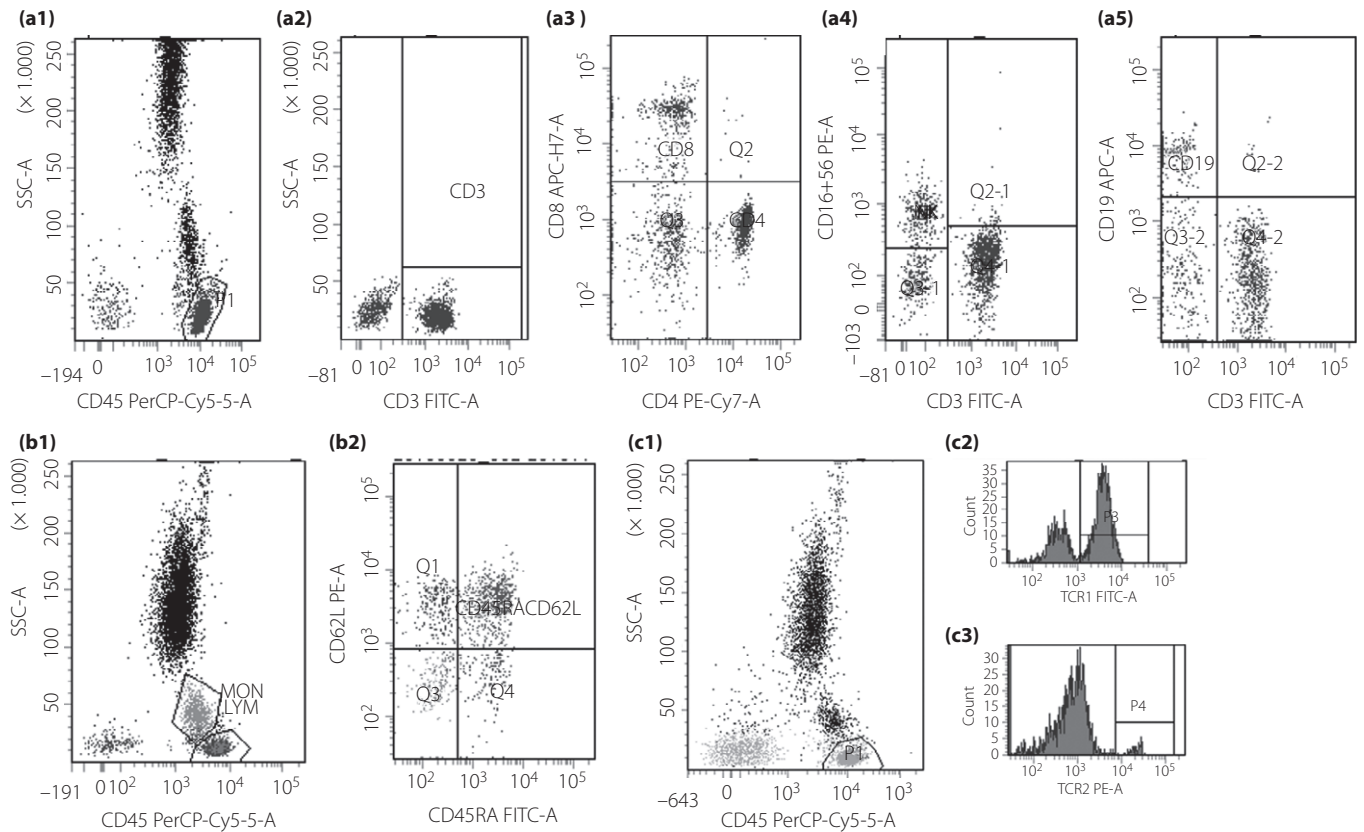
Figure 2 | Flow cytometry results in a sample patient. Gating of lymphocytes were adjusted according to forward scatter (FSC) and side scatter (SSC). (a) Six color: anti-CD45-PerCP-Cy5.5, anti-CD3-FITC, anti-CD4-PE-Cy7, anti-CD8-APC-H7, anti-CD19-APC and anti-CD16+56-PE. Having gated on (a1) CD45$^+$ cells, we counted (a2) CD3$^+$, (a3) CD3$^+$CD4$^+$, (a3) CD3$^+$CD8$^+$, (a4) NK (CD16$^+$CD56$^+$) and (a5) B (CD19$^+$) cells. (b) Three color: anti-CD45-PerCP-Cy5.5, anti-CD45RA-FITC and anti-C62L-PE. Having gated on (b1) CD45$^+$ cells, we counted (b2) CD45RA$^+$CD62L$^+$ lymphocytes. (c) Three color: anti-CD45-PerCP-Cy5.5, anti-TCR1-FITC (TCRαβ) and anti-TCR2-PE (TCRγδ). Having gated on (c1) CD45$^+$ cells, we counted (c2) TCRα/β T cells.

Furthermore, as the decreased CD4/CD8 ratio <1 is one of the features leading to the introduction of an immune-risk phenotype (i.e., immunosenescence), the present results suggest that 'immunosenescence,' or premature aging of the immune system, could contribute to the development of autoimmune diseases[25]. One surprising finding is that the CD4/CD8 ratio was significantly lower in A33(+) carriers than A33(−), but was not significantly changed between DR3(+) and DR3(−). This point is very interesting compared with the absence of the association of the A33 haplotype alone with type 1 diabetes, and might indicate important roles of *HLA* class I in immune modulation in the disease.

TCRα/β chains are heterodimeric membrane proteins expressed on the surface of T cells, and they contribute to direct recognition of antigen peptide presented on the major histocompatibility complex in the target cells[26,27], so it is reasonable to observe higher TCRα/β T cells in the *A33-DR3*-positive carriers.

In contrast, none of the serum protein and cells in innate immunity appeared to be associated with the haplotype *A33-DR3*. According to the study by Diana *et al.*[5], IgG-producing B cells (CD19$^+$), neutrophils and IFN-γ production in circulation are required for the initiation of the diabetogenic T cell response and type 1 diabetes development. This suggests that repertoire selection in type 1 diabetes could preferentially favor self-reactive T cells, because the HLA-A molecule presents the insulin self-peptide, favoring both a smaller number of surviving T cells and a higher percentage of self-reactivity[28].

In regard to clinical parameters, male sex, a younger age at onset, diabetic ketoacidosis history at onset, a father or sibling with type 1 diabetes and longer duration are independent risk factors for early complete β-cell destruction in type 1 diabetes[29,30]. However, in the present study, clinical features showed no significant differences between the patients with or without the above-mentioned *HLA* haplotypes. This might have been due to a narrow range of the age of onset and short duration (less than 6 months) in the present study population as a result of including young patients with new-onset type 1 diabetes, who are mostly in the primary stage of disease. In a Japanese longitudinal study (median 10 years), patients who possessed *HLA-A24*, *HLA-DQA1*03* and *HLA-DR9* together showed complete loss of β-cell function much

earlier than those who did not have this three-allele combination[31].

Although the present study showed the temporal profile of a disruption of particular immunological cell networks in type 1 diabetes and detected two *HLA* haplotypes that enhanced the risk of type 1 diabetes, its retrospective design is a limitation with respect to delineating the association between genetic factors and longitudinal changes of residual β-cell function. In addition, as the frequency of *A33-DR3* and *A33-DR9* was just 19.3% and 6.1%, respectively, in the new-onset type 1 diabetes group, the low frequencies of these haplotypes make it possible that the study was insufficiently powered to find an association with clinical phenotypes. Long-term prospective studies with larger samples on residual β-cell function using the stimulated C-peptide response would be preferable.

This is the first study to report that haplotype *A33-DR3* and *A33-DR9* were found to predispose patients, and to enhance the risk of type 1 diabetes. Furthermore, *A33-DR3* and *A33-DR9* were shown to be associated with a reduction in the helper-to-cytotoxic cell ratio (CD4/CD8 ratio) in the initiation of type 1 diabetes. In particular, a preferential increase of TCRα/βT cell subpopulations was found in the *A33-DR3*-positive carriers compared with *A33-DR3*-negative carriers. In contrast, no relationship was observed between these two haplotypes and the clinical features at onset or the innate immunology. These findings show that typing for *HLA* class I and its immunogenetic effects are important for more accurate *HLA* class II haplotype risk prediction and further revelatory etiology research in type 1 diabetic patients.

DISCLOSURE
The authors declare no conflict of interest.

REFERENCES
1. Noble JA, Erlich HA. Genetics of type 1 diabetes. *Cold Spring Harb Perspect Med* 2012; 2: a007732.
2. Noble JA, Valdes AM, Bugawan TL, *et al.* The HLA class IA locus affects susceptibility to type 1 diabetes. *Hum Immunol* 2002; 63: 657–664.
3. Nejentsev S, Howson JM, Walker NM, *et al.* Localization of type 1 diabetes susceptibility to the MHC class I genes HLA-B and HLA-A. *Nature* 2007; 450: 887–892.
4. Howson JM, Walker NM, Smyth DJ, *et al.* Analysis of 19 genes for association with type I diabetes in the Type I Diabetes Genetics Consortium families. *Genes Immun* 2009; 10(Suppl 1): S74–S84.
5. Diana J, Simoni Y, Furio L, *et al.* Crosstalk between neutrophils, B-1a cells and plasmacytoid dendritic cells initiates autoimmune diabetes. *Nat Med* 2012; 19: 65–73.
6. Expert Committee on the D, Classification of Diabetes M. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 2003; 26 (Suppl 1): S5–S20.
7. Bland JM, Altman DG. Multiple significance tests: the Bonferroni method. *BMJ* 1995; 310: 170.
8. Thomson G, Valdes AM, Noble JA, *et al.* Relative predispositional effects of HLA class II DRB1-DQB1 haplotypes and genotypes on type 1 diabetes: a meta-analysis. *Tissue Antigens* 2007; 70: 110–127.
9. Degli-Esposti M, Leelayuwat C, Dawkins R. Ancestral haplotypes carry haplotypic and haplospecific polymorphisms of BAT1: possible relevance to autoimmune disease. *Int J Immunogenet* 1992; 19: 121–127.
10. Alper CA, Larsen CE, Dubey DP, *et al.* The haplotype structure of the human major histocompatibility complex. *Hum Immunol* 2006; 67: 73–84.
11. Trachtenberg E, Vinson M, Hayes E, *et al.* HLA class I (A, B, C) and class II (DRB1, DQA1, DQB1, DPB1) alleles and haplotypes in the Han from southern China. *Tissue Antigens* 2007; 70: 455–463.
12. Hei AL, Li W, Deng ZH, *et al.* Analysis of high-resolution HLA-A, -B, -Cw, -DRB1, and -DQB1 alleles and haplotypes in 718 Chinese marrow donors based on donor-recipient confirmatory typings. *Int J Immunogenet* 2009; 36: 275–282.
13. Li XF, Zhang X, Chen Y, *et al.* An analysis of HLA-A, -B, and -DRB1 allele and haplotype frequencies of 21,918 residents living in Liaoning, China. *PLoS One* 2014; 9: e93082.
14. Kumar N, Kaur G, Tandon N, *et al.* Genomic evaluation of HLA-DR3+ haplotypes associated with type 1 diabetes. *Ann N Y Acad Sci* 2013; 1283: 91–96.
15. Feng M, Ji Y, Lu Q, *et al.* [Study on HLA haplotypes in Jiangsu-Zhejiang-Shanghai Han population]. *Yi Chuan Xue Bao* 2003; 30: 584.
16. Reijonen H, Concannon P, Kahn C, *et al.* Genetics of type 1 diabetes. *Joslins Diabetes Mellitus* 2005; 14: 355–370.
17. Graves PM, Norris JM, Pallansch MA, *et al.* The role of enteroviral infections in the development of IDDM: limitations of current approaches. *Diabetes* 1997; 46: 161–168.
18. Chang LY, Chang IS, Chen WJ, *et al.* HLA-A33 is associated with susceptibility to enterovirus 71 infection. *Pediatrics* 2008; 122: 1271–1276.
19. Tuvemo T, Dahlquist G, Frisk G, *et al.* The Swedish childhood diabetes study III: IgM against coxsackie B viruses in newly diagnosed type 1 (insulin-dependent) diabetic children–no evidence of increased antibody frequency. *Diabetologia* 1989; 32: 745–747.
20. Graves PM, Rotbart HA, Nix WA, *et al.* Prospective study of enteroviral infections and development of beta-cell autoimmunity. Diabetes autoimmunity study in the young (DAISY). *Diabetes Res Clin Pract* 2003; 59: 51–61.

21. Juhela S, Hyoty H, Roivainen M, *et al.* T-cell responses to enterovirus antigens in children with type 1 diabetes. *Diabetes* 2000; 49: 1308–1313.

22. Sarmiento L, Cabrera-Rode E, Lekuleni L, *et al.* Occurrence of enterovirus RNA in serum of children with newly diagnosed type 1 diabetes and islet cell autoantibody-positive subjects in a population with a low incidence of type 1 diabetes. *Autoimmunity* 2007; 40: 540–545.

23. Moya-Suri V, Schlosser M, Zimmermann K, *et al.* Enterovirus RNA sequences in sera of schoolchildren in the general population and their association with type 1-diabetes-associated autoantibodies. *J Med Microbiol* 2005; 54(Pt 9): 879–883.

24. Al-Sakkaf L, Pozzilli P, Tarn A, *et al.* Persistent reduction of CD4/CD8 lymphocyte ratio amd cell activation before the onset of Type 1 (insulin-dependent) diabetes. *Diabetologia* 1989; 32: 322–325.

25. Prelog M. Aging of the immune system: a risk factor for autoimmunity? *Autoimmun Rev* 2006; 5: 136–139.

26. Bassing CH, Swat W, Alt FW. The mechanism and regulation of chromosomal V(D)J recombination. *Cell* 2002; 109(Suppl): S45–S55.

27. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. *Nature* 1988; 334: 395–402.

28. Kronenberg D, Knight RR, Estorninho M, *et al.* Circulating preproinsulin signal peptide-specific CD8 T cells restricted by the susceptibility molecule HLA-A24 are expanded at onset of type 1 diabetes and kill β-cells. *Diabetes* 2012; 61: 1752–1759.

29. Alford FP, Henriksen JE, Rantzau C, *et al.* Impact of family history of diabetes on the assessment of β-cell function. *Metabolism* 1998; 47: 522–528.

30. Klaff LJ, Taniborlane WV, Cleary PA, *et al.* Effects of age, duration and treatment of insulin-dependent diabetes mellitus on residual beta-cell function: observations during eligibility testing for the Diabetes Control and Complications Trial (DCCT). The DCCT Research Group. *J Clin Endocrinol Metab* 1987; 65: 30–36.

31. Nakanishi K, Inoko H. Combination of HLA-A24,-DQA1* 03, and-DR9 contributes to acute-onset and early complete β-cell destruction in type 1 diabetes longitudinal study of residual β-cell function. *Diabetes* 2006; 55: 1862–1868.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 | Sequences of primers for amplifying *HLA-A, B, C, DRB1, DQB1* and *DQA1*.
Table S2 | Sequences of primers for sequencing *HLA-A, B, C, DRB1, DQB1* and *DQA1*.
Table S3 | Human leukocyte antigen class I allele frequencies in 212 type 1 diabetic patients and 200 people in the control group (positive cases [%]).
Table S4 | Human leukocyte antigen class II allele frequencies in 212 type 1 diabetic patients and 200 people in the control group (positive cases [%]).
Table S5 | Normalized linkage disequilibrium between *A*33:03* and *DRB1* alleles in type 1 diabetes.
Table S6 | Normalized linkage disequilibrium between *B*58:01* and *DRB1* alleles in type 1 diabetes.
Table S7 | Normalized linkage disequilibrium between *A*33:03* and *DQB1* alleles in type 1 diabetes.
Table S8 | Normalized linkage disequilibrium between *B*58:01* and *DQB1* alleles in type 1 diabetes.
Table S9 | Normalized linkage disequilibrium between *A*33:03* and *DQA1* alleles in type 1 diabetes.
Table S10 | Normalized linkage disequilibrium between *B*58:01* and *DQA1* alleles in type 1 diabetes.
Table S11 | Clinical characteristics in patients with high-risk *A33-DR3* and *A33-DR9* haplotypes.
Figure S1 | Association of *A33-DR3* haplotype and T lymphocyte subpopulations (%) in type 1 diabetes patients.
Figure S2 | Association of *A33-DR3* haplotype and T lymphocyte subpopulations ($*10^9$/L) in type 1 diabetes patients.