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The non-conservative *CD177* SNP c.1291G>A is a genetic determinant for HNA-2 atypical/low expression and deficiency

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Abstract

BACKGROUND: Human neutrophil antigen-2 (HNA-2) is exclusively expressed on neutrophils. HNA-2 deficient individuals (HNA-2 null) are susceptible to produce isoantibodies. The nonsense *CD177* coding SNP c.787 A>T has been demonstrated as the primary genetic mechanism for HNA-2 deficiency. We hypothesized that the other genetic variants also contribute to HNA-2 expression and deficiency.

STUDY DESIGN AND METHODS: The deficiency, density, and percentage of HNA-2 antigen on neutrophils from 292 healthy blood donors were determined in flow cytometry. *CD177* genotypes were determined by genomic DNA sequence analyses. The full-length *CD177* cDNAs were amplified and sequenced. Additionally, the whole *CD177* genomic sequence in eight HNA-2 null immunized women and four HNA-2 positive donors were analyzed with next-generation sequencing (NGS). The associations of *CD177* SNP genotypes with HNA-2 expression variation were statistically analyzed.

RESULTS: A functional *CD177* SNP c.1291G>A was identified in the current study. Atypical (trimodal) HNA-2 expression phenotype was consistently observed in donors carrying the heterozygous c.1291G/A genotype. Phenotype-genotype analyses of SNP c.787A>T and SNP c. 1291G>A revealed that all homozygous 787T-1291G (TG/TG) genotype donors were HNA-2 null

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Contributions

J.W. conceived and designed research. Y.L., R.M.S. and J.W. performed experiments and analyzed data. L.L. contributed reagents/analytical tools and revised the manuscript. J.W. wrote the manuscript. B.B., G.B. and U.S. designed research. A.L. performed the experiments. B.B. analyzed data and wrote the manuscript. G.B and U.S. revised the manuscript.

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in healthy blood donors. On the other hand, five out of eight HNA-2 immunized females were homozygous for the 787T-1291G (TG/TG) genotype while the other three HNA-2 immunized females had the 787T-1291G/787A-1291A (TG/AA) genotype and the lowest HNA-2 expressions were observed in healthy subjects with the 787T-1291G/787A-1291A (TG/AA) and 787A-1291A/787A-1291A (AA/AA) genotype.

CONCLUSION: The *CD177* SNP c.1291G>A is a genetic determinant for the atypical and low HNA-2 expressions, which also contribute to HNA-2 deficiency phenotype.

INTRODUCTION

Human neutrophil antigen 2 (HNA-2) is encoded by *CD177* gene,[1–3] which is also known as PRV-1 because *CD177* gene is over-expressed in polycythemia rubra vera patients.[4] HNA-2 typically has a bimodal expression pattern with the presence of both HNA-2 positive and HNA-2–negative populations in most of the human subjects. HNA-2 expression on neutrophils is very heterogeneous with the percentages of HNA-2^+ neutrophils ranging from 0% to 100%. The mean percentage of HNA-2^+ neutrophil subpopulation is between 45% and 65% in human populations. Approximately 3 – 5% Caucasians do not express HNA-2 and are referred as HNA-2 null subjects.[5] HNA-2 null individuals are prone to produce HNA-2 isoantibodies when HNA-2 antigen is introduced through transfusion, pregnancy, or bone marrow transplantation. HNA-2 isoantibodies are involved in a number of disorders such as neonatal alloimmune neutropenia, drug-induced immune neutropenia, and graft failure following marrow transplantation.[6–9] Additionally, HNA-2 isoantibodies are linked to the development of transfusion-related acute lung injury (TRALI) and various pulmonary disorders.[10–13] Consequently, HNA-2 is considered as one of the most important neutrophil antigens in human medicine.[5,14]

Recently, we have unraveled the primary genetic mechanism of HNA-2 deficiency and expression variations, which is caused by the nonsense SNP c.787A>T (rs201821720A>T) within the CD177 coding region.[15] Directly adjacent to CD177 gene, a pseudogene consists of highly homolog sequence to exon 4 through 9 of CD177 is located. The HNA-2^{null} allele (stop codon allele) likely originated from ectopic allelic conversion of the *CD177* pseudogene.[16] We recently found that several HNA-2 null individuals were heterozygous for the SNP c.787A>T.[17] In addition, the genetic determinant of atypical (trimodal) HNA-2 expression is not clear so far. We hypothesize that the genetic factors underlying HNA-2 deficiency are complex and that additional *CD177* SNPs may cause HNA-2 deficiency in humans as well. In this study, we revealed that *CD177* SNP c.1291G>A is a genetic determinant for low HNA-2 expression and HNA-2 deficiency.

MATERIALS AND METHODS

Study subjects

The study was conducted in parallel at two sites (American Red Cross, North Central Blood Services, National Neutrophil Reference Laboratory, St. Paul, Minnesota, USA and Institute for Clinical Immunology and Transfusion Medicine, Giessen, Germany). Healthy blood donors were recruited at the Memorial Blood Center in St. Paul, Minnesota as described

previously.[15] Immunized HNA-2 null mothers with a history of neonatal alloimmune neutropenia were from Giessen, Germany. The age of healthy control donors ranged from 19 to 84 years-old. The human study has been approved by the Institutional Review Board for Human Use at the University of Minnesota and by the Ethical Committee of the Medical Faculty, Justus Liebig University, Giessen, Germany.

Nucleic acid isolation

Human genomic DNA was isolated from EDTA anti-coagulated peripheral blood using the Wizard Genomic DNA Purification kit (Promega, Madison, WI) following the vendor's instruction.

Assessment of HNA-2 expressions on neutrophils

The expression of HNA-2 and the percentage of HNA-2^+ neutrophils in healthy blood donors were determined as described.[15] Briefly, fresh whole blood samples were stained with FITC-conjugated mouse anti-human CD177 (HNA-2) mAb MEM-166 or FITC-conjugated mIgG1 isotype control (ThermoFisher Scientific). Blood samples were subsequently treated with 1× FACS Lysing Solution (BD Biosciences) before being analyzed on a FACS Canto flow cytometer (BD Biosciences). The flow cytometry data were analyzed using FlowJo software (Tree Star Inc., http://www.flowjo.com/). The same criteria were used to identify HNA-2 null individuals.[18]

CD177 sequence analyses and SNP genotype determination

CD177 cDNA and genomic DNA sequences were analyzed as previously described.[15] Total RNA was isolated from peripheral blood leukocytes and used for cDNA synthesis. RT-PCR was carried out to amplify full-length *CD177* cDNAs from 130 healthy donors for coding SNP discovery with nucleotide sequence analyses. The long-template PCR strategy was used to amplify the *CD177* genomic DNA from 292 healthy blood donors. The *CD177*-specific products (8728 base pairs) were sequenced with BigDye v3.1 Sequencing kit on an ABI 3730xl DNA Analyzer using the primer (5'-TCTTTGCCCCACACTAAACA-3') for the SNP c.787A>T genotype determination and the primer (5'-AGGTTG AGTGTGGGTGGTCAGC-3') for the SNP c.1291G>A genotype determination. Linkage disequilibrium patterns of the *CD177* SNP c.787A>T and c.1291G>A were analyzed by Haploview 4.2 (Broad Institute, Cambridge, MA, USA; http://www.broad.mit.edu/mpg/haploview). Haplotype information was also inferred from homozygous human subjects. Genotypes and haplotype frequencies were estimated using the haplo.stats R package version 1.7.9. (https://CRAN.R-project.org/package=haplo.stats).

Library preparation and NGS

DNA samples from eight HNA-2 immunized females and four HNA-2^+ control donors were isolated using DNA isolation kit (Qiagen). The whole *CD177* gene was amplified with long-range polymerase chain reaction kit (Qiagen) in a 25 μL reaction volume using primers flanking exon 1 to 9 as previously described.[17] All the amplicons were purified using the Agencourt AMPure XP PCR Purification systems (Beckman Coulter, Pasadena, CA). The purified amplicons were then quantified using the Qubit dsDNA BR Assay system

(Invitrogen, Carlsbad, CA). The sequencing library was constructed using the Nextera XT sample preparation kit (Illumina, San Diego, CA) and the adapter and barcoding sequences were added simultaneously. The barcoded libraries were subsequently sequenced with v2 kits on a MiSeq sequencer. The sequencing data were aligned to *CD177* gene reference sequence and analyzed with Geneious software.

Statistical analysis

The ANOVA and nonparametric t-test (Mann-Whitney test) were used to determine whether HNA-2 positive cell population sizes, HNA-2 density, and the HNA-2 null phenotype are statistically associated with the *CD177* SNP genotypes.

RESULTS

Association of a non-conservative *CD177* SNP c.1291G>A with the atypical HNA-2 expression

The analyses of HNA-2 expression levels and the HNA-2^+ neutrophil percentages in healthy blood donors revealed that HNA-2 expressions were very heterogeneous among normal healthy blood donors (Fig. 1). Atypical (trimodal) HNA-2 expression (Fig. 1C) showing three populations of neutrophils (HNA-2-negative, HNA-2-low, and HNA-2-high) were observed in 42 out of 292 (14.3%) healthy donors. However, we failed to find an association of atypical HNA-2 expressions with *CD177* SNPs including c.787A>T (rs201821720A>T), rs45553433A>T (His31Leu), rs12980412A>G (Asn204Asp), rs12981771G>T (Arg205Met), and rs61625631G>A (Ala348Thr). By sequencing analyses of cDNAs from 130 healthy donors, we identified a non-conservative *CD177* coding SNP c.1291G>A (rs78718189G>A or Gly431Arg) that changes residue 431 from glycine (Gly) to arginine (Arg) (431G>R, Fig. 2). More importantly, we found that all 42 healthy donors showing the atypical HNA-2 expression had the heterozygous 1291G/A genotype. Our data suggest that the heterozygosity of the SNP 1291G>A is the primary genetic determinant of atypical HNA-2 expressions in healthy donors.

Association of a haplotype of *CD177* SNP c.787A>T and SNP c.1291G>A with low HNA-2 expressions

We subsequently analyzed haplotypes formed by the SNP c.787A>T and SNP c.1291G>A. Haplotype analyses revealed that 787T is in complete linkage disequilibrium with 1291G while 1291A is in complete linkage disequilibrium with 787A. Therefore, two SNPs from three haplotype alleles (Table 1). The 787A-1291G (AG) was the most common SNP haplotype (allele frequency = 0.743). Both 787T-1291G (TG) haplotype (allele frequency = 0.156) and 787A-1291A (AA) haplotype (allele frequency = 0.101) are minor alleles compared to the most common 787A-1291G (AG) allele (Table 1). We examined whether genotypes of two SNPs are associated with the percentages of HNA-2^+ neutrophils and HNA-2 expression levels (represented by MFI or mean fluorescent intensities) on HNA-2^+ neutrophils. As shown in Fig. 3A, all homozygous TG/TG genotype donors were HNA-2 null phenotype. In addition, TG/AG donors had significantly lower percentages of HNA-2^+ neutrophils compared to the AG/AG donors. The percentages of HNA-2^+ neutrophils were not significantly different among AG/AG, AG/AA, AA/AA donors ($P > 0.05$).

In healthy blood donors (non-immunized), all HNA-2 null subjects were homozygous TG/TG genotype, which could not be used for the subsequent analyses of relationship between *CD177* SNP genotypes and HNA-2 expression levels on HNA-2^+ neutrophils. As shown in Fig. 3B, HNA-2 expression levels on HNA-2^+ neutrophils were significantly lower in AA/AA donors than in AG/AG donors ($P = 0.001$). HNA-2 expression levels on HNA-2^+ neutrophil were also significantly lower in TG/AA donors than in TG/AG donors ($P = 0.0007$). In addition, HNA-2^+ neutrophils from AG/AA donors also had significantly lower levels of HNA-2 expression than those from the AG/AG donors ($P < 0.0001$). Taken together, our data demonstrate that presence of *CD177* SNP 787A-1291A (AA) allele leads to significantly lower HNA-2 expressions on HNA-2^+ neutrophils in healthy blood donors (Fig. 3B).

Association of the *CD177* SNP 1291A allele with HNA-2 null phenotype

In a cohort of immunized HNA-2 null females, the whole *CD177* gene was amplified using long-range PCR and subsequently subjected for NGS. The sequences were then analyzed in Geneious software. Five out of eight HNA-2-null individuals were homozygous for 787T-1291G (TG/TG) genotype while the other three were heterozygous 787T-1291G/787A-1291A (TG/AA) genotype. Therefore, a perfect association between heterozygous 787T-1291G/787A-1291A (TG/AA) genotype and the HNA-2 null phenotype was observed in all three immunized HNA-2 null females (Table 1). Our data suggest that the SNP 787A-1291A (AA) allele has a distinctive impact on HNA-2 deficiency in the presence of 787T-1291G allele. None of the HNA-2 positive individuals had the homozygous 787T-1291G genotype, confirming the previous observations.[15,17] Among immunized HNA-2 null females, the 787T-1291G represented the most common haplotype with an allele frequency of 0.81, while the 787A-1291A haplotype had an allele frequency of 0.19 (Table 1). Most importantly, 787A-1291G haplotype was not identified in any immunized HNA-2 null women while all HNA-2 positive donors carry at least one copy of 787A-1291G allele in the German cohort (Table 2). Our data further confirm a predominant role of the SNP c.787A>T in HNA-2 expression.

DISCUSSION

We previously reported that the nonsense SNP c.787A>T (rs201821720) is the primary genetic determinant for HNA-2 deficiency.[15] In the present study, we revealed that neutrophils from heterozygous donors of the *CD177* SNP c.1291G>A always had atypical (trimodal) HNA-2 expressions in healthy blood donors. Additionally, the lowest levels of HNA-2 expression were observed in healthy blood donors with the homozygous 787A-1291A (AA/AA) genotype while 37.5% (3 out of 8) HNA-2 immunized females with HNA-2 null phenotype had the heterozygous 787T-1291G/787A-1291A (TG/AA) genotype. Our data suggest that the 787A-1291A (AA) haplotype allele could lead to low levels and/or the absence of HNA-2 expression in human subjects.

Epigenetic mechanisms control the *CD177* allele-silencing process and one of two *CD177* alleles is silenced during neutrophil differentiation, which may cause monoallelic HNA-2 expression in mature neutrophils.[19] In neutrophils from 787A-1291G/787A-1291A

(AG/AA) heterozygous donors, neutrophils epigenetically silenced for HNA-2 expression on both chromosomes will appear as the HNA-2 negative population. Since the 787A-1291A (AA) haplotype allele results in much lower HNA-2 expression than 787A-1291G (AG) allele (Fig. 1D, 3B), the monoallelic expression of 787A-1291A (AA) allele and 787A-1291G (AG) allele in neutrophils from the same donors will produce two HNA-2^+ populations: a population of low HNA-2 from 787A-1291A (AA) allele and a population of high HNA-2 from 787A-1291G allele. Our data strongly suggest that the SNP c.1291G>A is a primary genetic factor for atypical (trimodal) and low HNA-2 expressions in humans.

HNA-2 is a glycosylphosphatidylinositol (GPI)-linked membrane protein.[20] GPI attachment is achieved by a multi-subunit GPI transamidase in the endoplasmic reticulum lumen after translocation of the pro-peptide across the endoplasmic reticulum membrane.[21] The upstream GPI-attachment site, a short stretch of hydrophilic amino acids, and a carboxyl-terminal hydrophobic domain form the GPI-attachment signal (or GPI-signal) with a quite narrow range of hydrophobicity, which is required for efficient GPI attachment.[22] The non-conservative SNP Gly431Arg (c.1291G>A) is located within the carboxyl-terminal hydrophobic region of HNA-2 GPI-signal modules. The changes of the residue from 431Gly (1291G allele) to 431Arg (1291A allele) would significantly alter the hydrophobicity of HNA-2 GPI-signal as arginine has a hydrophilic side chain while glycine has an uncharged side chain. Therefore, 431Arg allele would produce an HNA-2 pro-peptide with the unfavorable GPI-attachment signal, which would destabilize HNA-2 on the cell membrane and cause low or absence of HNA-2 expressions. Nevertheless, the precise mechanism for the SNP c.1291G>A to affect HNA-2 expressions requires further investigation.

In the presence of nonsense 787T-1291G (TG) allele, the heterozygous 787T-1291G/787A-1291A (TG/AA) subjects had significantly lower levels of HNA-2 expression than the heterozygous 787T-1291G/787A-1291G (TG/AG) subjects in healthy blood donors (Fig. 3B). Furthermore, HNA-2 immunized females with heterozygous 787T-1291G/787A-1291A (TG/AA) genotype were all HNA-2 null, suggesting that the 787A-1291A (AA) allele responsible for the low levels of HNA-2 expression is susceptible to be completely silenced in the presence of nonsense 787T-1291G (TG) allele under some physiological conditions. Nevertheless, the epigenetic mechanism controlling the silence of 787A-1291A allele is required for further investigation.

In summary, the *CD177* SNP c.1291G>A is the primary genetic mechanisms for low levels of HNA-2 expression, which also contributes to HNA-2 deficiency.

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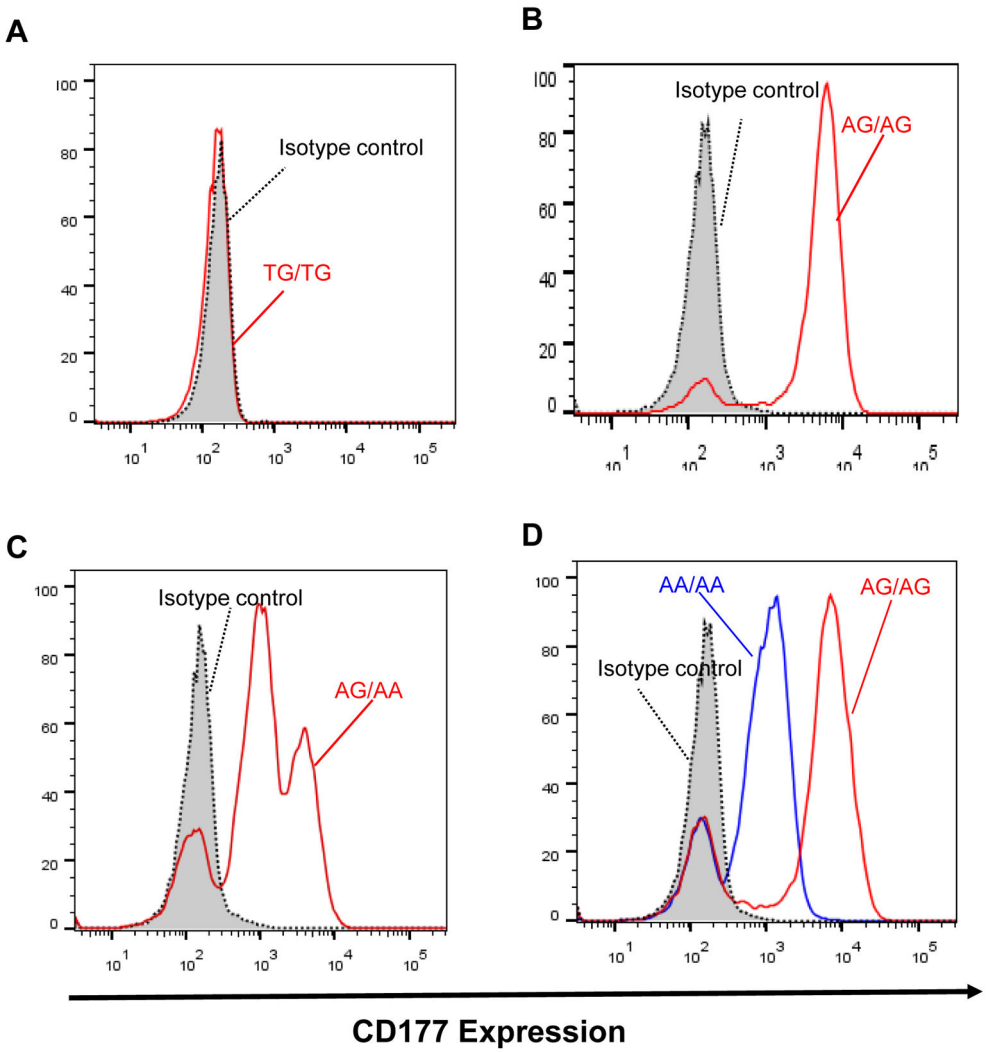

Fig. 1. Relationship between *CD177* SNP c.787A>T-1291G>A genotypes and heterogeneous HNA-2 expression.

A). HNA-2 expression is absent on neutrophils from homozygous 787T-1291G (TG/TG) donors. **B)**. Neutrophils from homozygous 787A-1291G (AG/AG) donors typically express high density of HNA-2. **C)**. Atypical (tri-modal) HNA-2 expression pattern is always displayed on neutrophils from heterozygous 787A-1291G/787A-1291A (AG/AA) donors. **D)**. Neutrophils from homozygous 787A-1291A (AA/AA) donors express a significantly lower level of HNA-2 compared to homozygous 787A-1291G (AG/AG) donors.

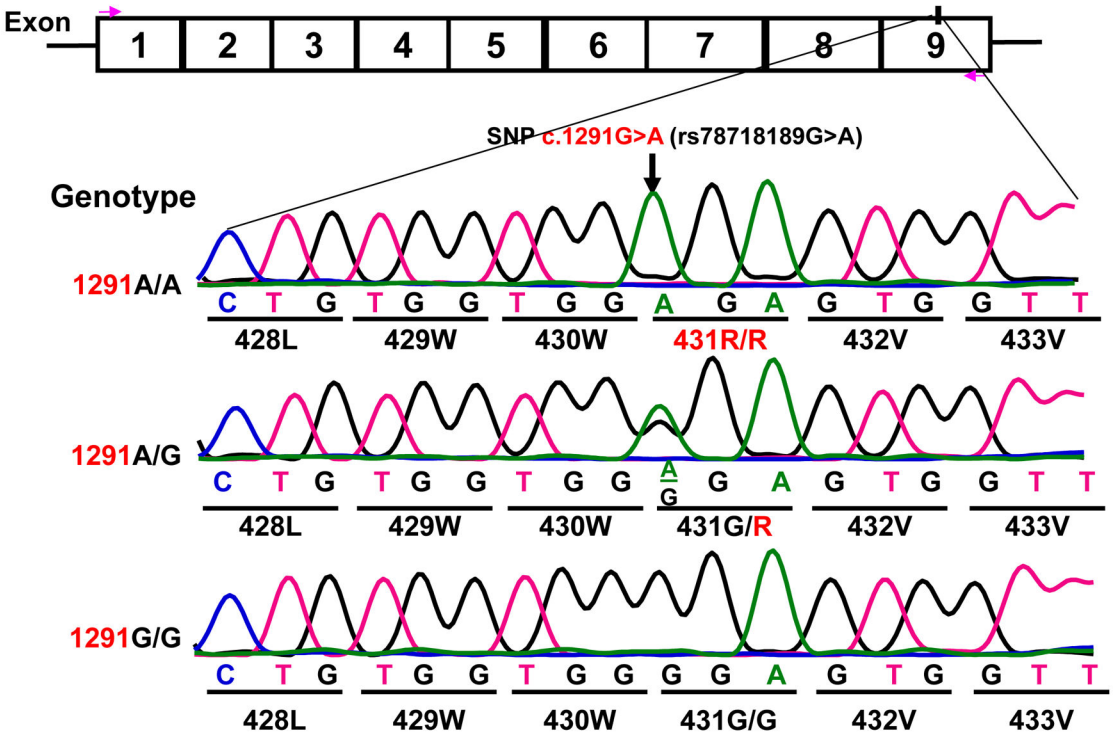

Fig. 2. Non-conservative *CD177* SNP c.1291G>A within the hydrophobic region for GPI-signal of HNA-2 processing.

CD177 SNP c.1291G>A causes the residue 431G to 431R substitution. Chromatograms of three SNP c.1291G>A genotypes (homozygous 1291A/A, heterozygous 1291A/G, and homozygous 1291G/G) were shown.

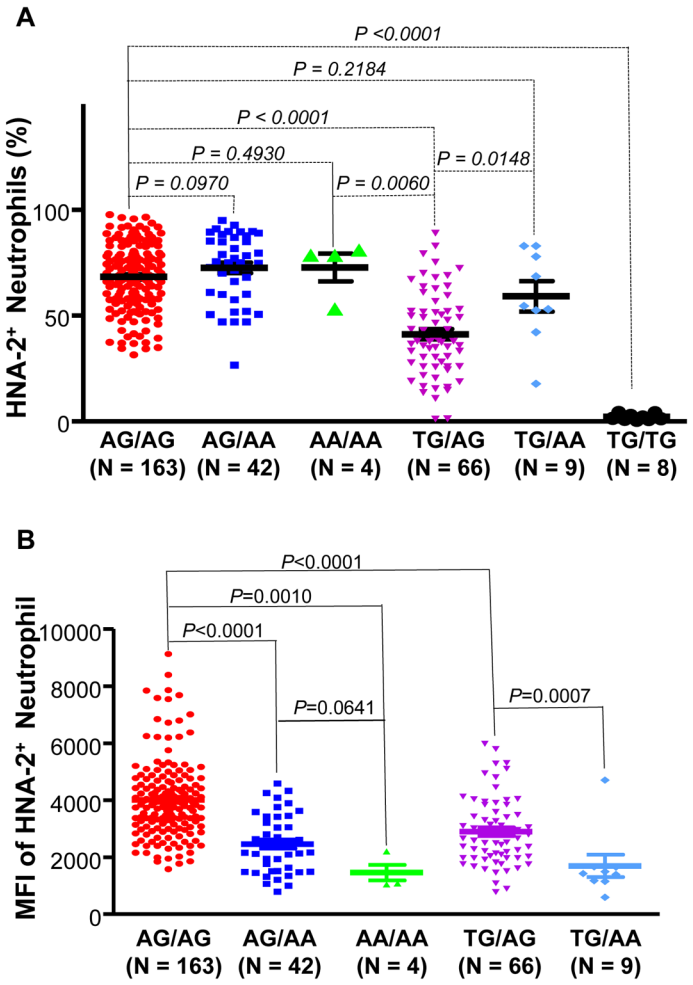

Fig. 3. *CD177* SNP c.787A>T and 1291G>A genotypes are associated with variable HNA-2 expressions in normal healthy blood donors.

A). SNP c.787A>T and c.1291G>A genotypes were associated with the percentage of HNA-2$^+$ neutrophils. All homozygous 787T-1291G/787T-1291 (TG/TG) genotype donors were HNA-2 null. Heterozygous 787T-1291G/787A-1291G (TG/AG) donors had significantly lower percentages of HNA-2$^+$ neutrophils than the homozygous 787A-1291G/787A-1291G (AG/AG) donors ($P < 0.0001$). Heterozygous 787T-1291G/787A-1291A (TG/AA) donors had significantly higher percentages of HNA-2$^+$ neutrophils than the heterozygous 787T-1291G/787A-1291G (TG/AG) donors ($P = 0.0148$). **B**). SNP 787A>T and 1291G>A genotypes were associated with HNA-2 expression levels on HNA-2$^+$ neutrophils. Neutrophils from the homozygous 787A-1291A/787A-1291A (AA/AA) donors expressed significantly lower levels of HNA-2 than those from the homozygous 787A-1291G/787A-1291A (AG/AG) donors ($P = 0.001$). Neutrophils from the heterozygous 787T-1291G/787A-1291A (TG/AA) donors also had much lower levels of HNA-2 expressions than those from heterozygous 787T-1291G/787A-1291G (TG/AG) donors ($P = 0.0007$). Neutrophils from heterozygous 787A-1291G/787A-1291A (AG/AA) donors also

expressed significantly lower levels of HNA-2 than those from the homozygous 787A-1291G/787A-1291G (AG/AG) donors ($P < 0.0001$).

Table 1.

Distributions of *CD177* SNP haplotype alleles and genotypes in study subjects

Haplotype alleles and genotypes	Subjects
US cohort	**N = 292**
Genotypes	**Number (%)**
787A-1291G/787A-1291G (AG/AG)	163 (55.8)
787A-1291G/787A-1291A (AG/AA)	42 (14.3)
787A-1291A/787A-1291A (AA/AA)	4 (1.4)
787A-1291G/787T-1291G (AG/TG)	66 (22.6)
787A-1291A/787T-1291G (AA/TG)	9 (3.1)
787T-1291G/787T-1291G (TG/TG)	8 (2.7)
SNP haplotype alleles	**Number (%)**
787A-1291G (AG)	434 (74.3)
787T-1291G (TG)	91 (15.6)
787A-1291A (AA)	59 (10.1)
German HNA-2 null cohort	**N = 8**
Genotypes	**Number (%)**
787T-1291G/787T-1291G (TG/TG)	5 (62.5)
787A-1291A/787T-1291G (AA/TG)	3 (37.5)
SNP haplotype alleles	**Number (%)**
787A-1291G (AG)	0 (00.0)
787T-1291G (TG)	13 (81.0)
787A-1291A (AA)	3 (19.0)
German HNA-2 positive cohort	**N = 4**
Genotypes	**Number (%)**
787A-1291G/787T-1291G (AG/TG)	2 (50.0)
787A-1291G/787A-1291G (AA/AG)	2 (50.0)
SNP haplotype alleles	**Number (%)**
787A-1291G (AG)	6 (75.0)
787T-1291G (TG)	2 (25.0)
787A-1291A (AA)	0 (00.0)

Table 2.

Percentage of HNA-2^+ neutrophils and HNA-2 expression levels of HNA-2^+ neutrophils from immunized HNA-2 null females and HNA-2 positive control donors.

Phenotype	Genotype	HNA-2+ neutrophils	MFI*
HNA-2 null	787T-1291G/787T-1291G (TG/TG)	0%	-
HNA-2 null	787T-1291G/787T-1291G (TG/TG)	0%	-
HNA-2 null	787T-1291G/787T-1291G (TG/TG)	0%	-
HNA-2 null	787T-1291G/787T-1291G (TG/TG)	0%	-
HNA-2 null	787T-1291G/787T-1291G (TG/TG)	0%	-
HNA-2 null	787A-1291A/787T-1291G (TG/TG)	0%	-
HNA-2 null	787A-1291A/787T-1291G (TG/TG)	0%	-
HNA-2 null	787A-1291A/787T-1291G (TG/TG)	0%	-
Control donors	787A-1291G/787A-1291G (AG/AG)	49%	2100
Control donors	787A-1291G/787A-1291G (AG/AG)	76%	3460
Control donors	787A-1291G/787T-1291G (AG/TG)	77%	3700
Control donors	787A-1291G/787T-1291G (AG/TG)	43%	2000

*MFI: mean fluorescent intensity, representing the expression levels