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Multicenter Study on Differential Human Neutrophil Antigen 2 Expression and Underlying Molecular Mechanisms

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Keywords

Human neutrophil antigens · Neutropenia · Molecular analysis · Monoallelic expression

Abstract

Background: The human neutrophil antigen 2 (HNA-2), which is expressed on CD177, is undetectable in 3–5% of the normal population. Exposure of these HNA-2_{null} individuals to HNA-2-positive cells can cause immunization and production of HNA-2 antibodies, which can induce immune neutropenia and transfusion-related acute lung injury. In HNA-2-positive individuals, neutrophils are divided into a $CD177^{pos.}$ and a $CD177^{neg.}$ subpopulation. The molecular background of HNA-2 deficiency and the bimodal expression pattern, however, are not completely decoded. ***Study Design:*** An international collaboration was conducted on the genetic analysis of HNA-2-phenotyped blood samples, including HNA-2-deficient individuals, mothers, and the respective children with neonatal immune neutropenia and regular blood donors. ***Results:*** From a total of 54 HNA-2_{null} individuals, 43 were homozygous for the *CD177*787A>T* substitution. Six carried the *CD177*c.1291G>A* single nucleotide polymorphism. All HNA-2-positive samples with >40% $CD177^{pos.}$ neutrophils carried the **787A* wild-type allele, whereas a lower rate of $CD177^{pos.}$ neutrophils was preferentially associated with **c.787AT* heterozygosity. Interestingly, only the **c.787A* allele sequence was detected in complementary DNA (cDNA) sequence analysis carried out on all **c.787AT* heterozygous individuals. However, cDNA analysis after sorting of $CD177^{pos.}$ and $CD177^{neg.}$ neutrophil subsets from HNA-2-positive individuals showed identical sequences, which makes regulatory elements within the promoter unlikely to affect *CD177* gene transcription in different CD177 neutrophil subsets. ***Conclusion:*** This comprehensive study clearly demonstrates the impact of single nucleotide polymorphisms on the expression of HNA-2 on the neutrophil surface but challenges the hypothesis of regulatory epigenetic effects being implicated in the bimodal CD177 expression pattern.

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Introduction

The human neutrophil antigen 2 (HNA-2) plays an important role in granulocyte immunobiology. Antibodies to HNA-2 can cause severe neonatal immune neutropenia and transfusion-related acute lung injury (TRALI) [1–3]. HNA-2 is located on CD177, a 56- to 64-kDA glycoprotein that is restricted to neutrophil granulocytes and is anchored to the neutrophil membrane by glycosylphosphatidylinositol (GPI) [4, 5]. The expression can be upregulated by different conditions like bacterial infection, pregnancy, and G-CSF stimulation [6, 7]. At least 3% of Caucasians are completely devoid of HNA-2 (HNA-2_{null}) and only these individuals are prone to develop HNA-2 isoantibodies upon exposure to HNA-2-positive cells [8–11]. Most HNA-2-positive individuals present a peculiar bimodal expression pattern with 2 neutrophil subsets. One subset is negative whereas the second subset expresses HNA-2 at a high or intermediate level [5, 12]. The latter fraction may vary from person to person between <5% and nearly 100%, but the percentage is a lifelong trait [13].

The molecular background regulating the HNA-2_{null} phenotype and the bimodal expression pattern in HNA-2-positive individuals is not completely understood. In 2 individuals previously studied, an incorrect splicing was considered as the molecular reason for the HNA-2_{null} phenotype [14]. Our own unpublished data and observations of others [15], however, demonstrated correctly spliced cDNA in all HNA-2-deficient individuals investigated so far. Numerous *CD177* gene single nucleotide polymorphisms (SNPs) are listed in the NCBI dbSNP database. A low or high HNA-2 expression, as well as the presence of 1 or 2 antigen-positive subsets, had initially been attributed to various sequence variations [16–18]. A homozygous *CD177*c.787A>T* substitution within exon 7 introducing a stop codon was identified as the main cause for the HNA-2_{null} phenotype [13, 15, 19]. The SNP was primarily assigned to either *CD177*c.829A>T* [15] or *CD177*c.843A>T* [19], but in accordance with the Human Genome Variation Society (HGV) the SNP is now assigned to *CD177*c.787A>T* (taking position 1 as the A of the start ATG codon of the CDS) [20]. The mutation was regarded to result from an allelic gene conversion event involving exon 7 of the *CD177P1* pseudogene, which would replace the equivalent exon 7 sequence within the CD177gene [13]. *CD177P1* is located downstream of the *CD177* gene in opposite orientation and comprises exons 4–9 that are highly homologous to the *CD177* gene. In rare cases, additional mutations have also been described in HNA-2_{null} individuals [15, 19]. Copy number variation has been considered as the reason for both HNA-1 deficiency and expression variation. However, the involvement of copy number variation in the regulation of *CD177* gene expression has been excluded [15].

The *CD177*c.787A>T* exchange has also been attributed to the percentage of $CD177^{pos.}$ neutrophils in CD177-positive individuals. Heterozygosity for ectopic *CD177P1* gene conversion correlated with an increased $CD177^{neg.}$ neutrophil fraction where both alleles were transcribed [13]. Recently, a *CD177*c.1291G>A* substitution in combination with the **c.787A>T* SNP has been associated with both the HNA-2_{null} phenotype and the rather rare trimodal expression pattern, depending on the respective haplotype [21]. A monoallelic regulation for CD177 expression on neutrophil subpopulation has been suggested where in HNA-2-positive and HNA-2-negative subpopulations only one allele of *CD177* gene is expressed [22].

In order to verify these controversial findings, we initiated an international multicenter study on the molecular basis of *CD177* gene expression in HNA-2_{null} and HNA-2-positive phenotyped donors as well as in immunized women with HNA-2 isoantibodies, including some of their neonates and the respective fathers. This included analysis of the *CD177* mRNA content in sorted neutrophil subpopulations of HNA-2-positive donors.

Subjects

In this study, a total of 107 samples were included. Samples were provided by each of the contributing centers: German Red Cross Blood Service West, Hagen and Bad Kreuznach (Germany), University Hospital Heidelberg (Germany), Institute for Clinical Immunology and Transfusion Medicine, Justus-Liebig-University Giessen (Germany), Blood and Tissue Bank, Barcelona (Spain), Sanquin, Amsterdam (The Netherlands), University Hospital, Nantes (France), Karolinska University Hospital, Stockholm (Sweden), and Medical University, Vienna (Austria). The study provided advanced diagnostics in some cases of clinically suspected neonatal alloimmune neutropenia (NIN) when samples were submitted to the respective laboratory. This included samples of 9 mothers, 10 neonates, and 5 respective fathers as well as 3 further patients. Additionally, the cohort comprised 80 regular blood donors and volunteers from the contributing laboratories. All donors gave informed consent.

Methods

Serological Assays

Maternal sera of NIN newborns were tested for the presence of granulocyte-specific antibodies in the laboratories of the contributing centers, applying validated and standardized granulocyte immunobiology methods. These included the granulocyte agglutination test (GAT) [23], granulocyte immunofluorescence test

Table 1. Amplification and sequencing primers

Name	Position	Usage	Sequence (5′–3′)	Direction	Ref.
CD177_LR_s	5′ UTR, –39 to –8	gDNA long-range PCR a	ctgaaaaagcagaaagagattaccagccacag	Sense	19
CD177_LR_as	3′ UTR, +115 to +144	gDNA long-range PCR a	gtccaaggccattaggttatgaggtcaga	Anti-sense	19
CD177_LR2_s	5′ UTR, –71 to –43	gDNA long-range PCR b	cttaagggctggtataaaggactt	Sense	
CD177_LR2_as	3′ UTR, +534 to 562	gDNA long-range PCR b	cgctacaatgttcctatggtcataaaatc	Anti-sense	
CD177R_1F	5′ UTR, –24 to –7	cDNA nested PCR 1; Seq. exon 1+2, cDNA+gDNA	gagattaccagccacaga	Sense	8
CD177R_1R	3′UTR, +53 to +29	cDNA nested PCR1	ggaggttgagtgtgggtggtcagca	Anti-sense	8
CD177R_9F	5′UTR, –6 to 15	cDNA nested PCR2	cgggtcATGAGCCCGGTATTA	Sense	8
CD177R_11R	1,289 to 1,311	cDNA nested PCR2; Seq. exon 9, cDNA	GCAGGAAGGGCAAACCACTCCCC	Anti-sense	
CD177R_3F	11 to 30	Seq. exon 2+3, cDNA + gDNA	TATTACTGCTGGCCCTCCTG	Sense	16
CD177R_4F	163 to 183	Seq. exon 3+4, cDNA + gDNA	TGCCAGGACACGTTGATGCTC	Sense	16
CD177R_4R	404 to 385	Seq. exon 3, cDNA	ACTGGGCACCTCAAGGATCC	Anti-sense	16
CD177R_5F	680 to 699	Seq. exon 6+7, cDNA	CCACTGATTGGACCACATCG	Sense	16
CD177R_6F	Intron 5, –86 to –67	Seq. exon 7–9, cDNA	gacctgtgcaatagtgccagc	Sense	16
CD177R_7F	380 to 399	Seq. exon 4–6, cDNA	CAGAAGAGATCTGCCCCAAG	Sense	
CD177_E7F	Intron 6, –134 to –115	Seq. exon 7+8, gDNA	tgacccagcagttgtgatca	Sense	
CD177g_E3F	Intron 2, –18 to exon 3 194	Seq. exon 3, gDNA	ctccctctttcggtccagG	Sense	
CD177g_E4–5R	Intron 5, 82 to 63	Seq. exon 4+5, gDNA	ttggtgtgatggctctggat	Anti-sense	
CD177g_E6F	Intron 5, –86 to –67	Seq. exon 6, gDNA	tgtgatcaccttccctagcc	Sense	
CD177g_E9F	Intron 8, –61 to –42	Seq. exon 9, gDNA	gggtttacaacttggctggg	Sense	

Position numbers within exons refer to CDS (NC_000019.10), whereas UTR and intron positions refer to the nearest exon. Intronic and UTR sequences are printed in lower-case letters.

(GIFT) [24, 25] with either microscopic or flow cytometric evaluation (Flow GIFT) [26], and the monoclonal antibody immobilization of granulocyte antigen (MAIGA) test [25, 27] as well as simultaneous analysis of specific granulocyte antigens (SASGA) assay [28] based on HNA-typed neutrophil cell panels and CD177-specific monoclonal antibodies for the MAIGA test.

HNA-2 phenotypes were only available from blood donors and volunteers because shipping of patient samples after 24 h of blood withdrawal impedes the isolation of intact neutrophils. Individuals with proven HNA-2 isoantibody were assessed as HNA-2 negative without serological phenotyping. Phenotyping of blood donors was performed in the GIFT on either freshly isolated neutrophils or EDTA anticoagulated blood using CD177-specific FITC-labeled mouse monoclonal antibody (moab), clone MEM-166 (Serotec distributed by BioRad, Munich, Germany), and CD16-specific PE/CY7-labeled moab, clone 3G8 (BioLegend, San Diego, CA, USA) to gate the neutrophils followed by flow cytometric evaluation (Beckman Coulter Epics XL; Beckman Coulter, Krefeld, Germany). In Vienna, phenotyping was performed by Flow-GIFT [26] using human allo-anti-HNA-2 and Alexa Fluor 488® goat anti-human IgG Fab Fragment (Jackson ImmunoResearch, New Market, UK). In few further cases fluorescence was assessed microscopically.

Genomic DNA Sequencing

DNA was isolated by the contributing laboratories. The *CD177* gene was separated from the *CD177P1* pseudogene by long-range PCR amplification (Long-Range PCR kit; Qiagen, Hilden, Germany) as described previously [19]. Genomic typing was conducted based on a template-specific long-range amplification of the whole *CD177* coding region encompassing exons 1–9 (8729 bp or alternatively 9323 bp). All amplification and sequencing primers are listed in Table 1. Sequencing reaction in most genomic DNA (gDNA) samples covered only exon 7, which comprises the *CD177*c.787A>T* substitution. Some samples were subjected to sequencing of all *CD177* exons, including exon 9, which contains the *CD177*c.1291G>A* substitution. Sequencing was done using a cycle sequencing kit followed by electrophoretical separation in the ABI Prism 310 DNA Analyzer (Applied Biosystems, ABI, Weiterstadt, Germany).

Sorting of HNA-2-Positive and HNA-2-Negative Subpopulations

Neutrophils were isolated from 2 $CD177^{pos.}$ individuals by dextran sedimentation. Isolated neutrophils were stained with FITC-labeled MEM-166 or mouse IgG. The stained $CD177^{pos.}$ and $CD177^{neg.}$ neutrophil subpopulations were then sorted using FACSAria (Becton Dickinson, Heidelberg, Germany). The neutrophil subpopulation with mean fluorescence intensity values identical to those of mIgG was considered as the $CD177^{neg.}$ subpopulation.

Isolation and Reverse Transcription of RNA from Peripheral Blood Cells

RNA was isolated either from human whole blood or isolated neutrophils. Whole blood was taken in PAXgene storage tubes (Becton Dickinson Diagnostics, Heidelberg, Germany) followed by mRNA isolation using the PAXgene mRNA kit (Qiagen) according to the manufacturer's instructions. Neutrophil granulocytes were isolated from EDTA anticoagulated blood as described elsewhere [29]. The purity of the preparations, determined microscopically from stained smears, was about 95%. RNA was isolated from up to 10^7 isolated neutrophils using the RNeasy Mini Kit (Qiagen). Reverse transcription into cDNA was performed with the Omniscript RT kit (Qiagen) according to the instructions of the manufacturer by use of an oligo-dT-primer (TibMolbiol, Berlin, Germany). A *CD177*-specific fragment was amplified from the first-strand cDNA template in a nested PCR reaction covering the 5′ UTR to the 3′ end of exon 9 as described elsewhere, with slight modifications [8]. In the first PCR reaction, denaturation for 3 min at 95 °C was followed by 25 cycles with 30 s at 95 °C, 30 s at 58 °C, 180 s at 72 °C, and a final elongation of 7 min at 72 °C. The products were checked electrophoretically for the presence of clearly

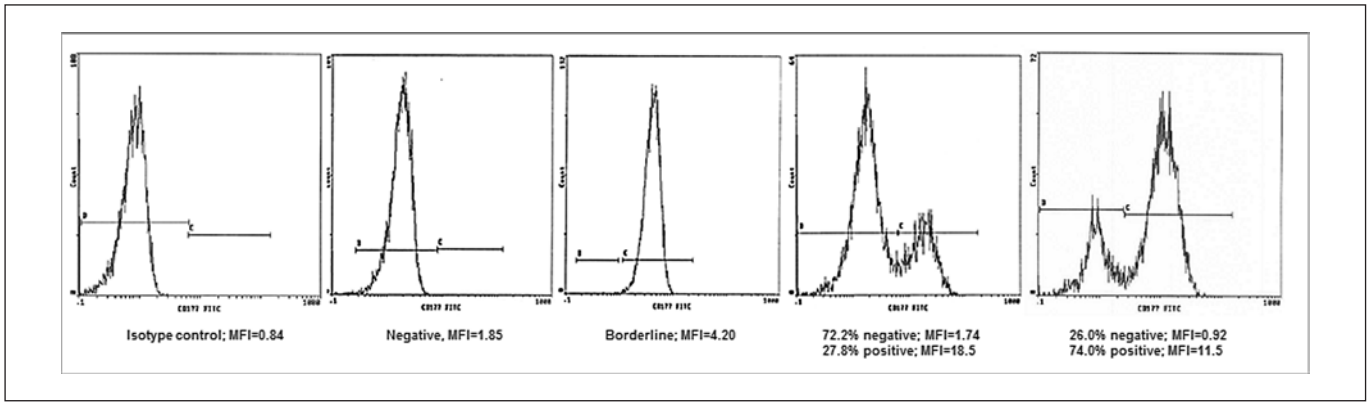

Fig. 1. Neutrophil CD177 expression patterns in HNA-2_{null} and HNA-2-positive individuals demonstrated by flow cytometry.

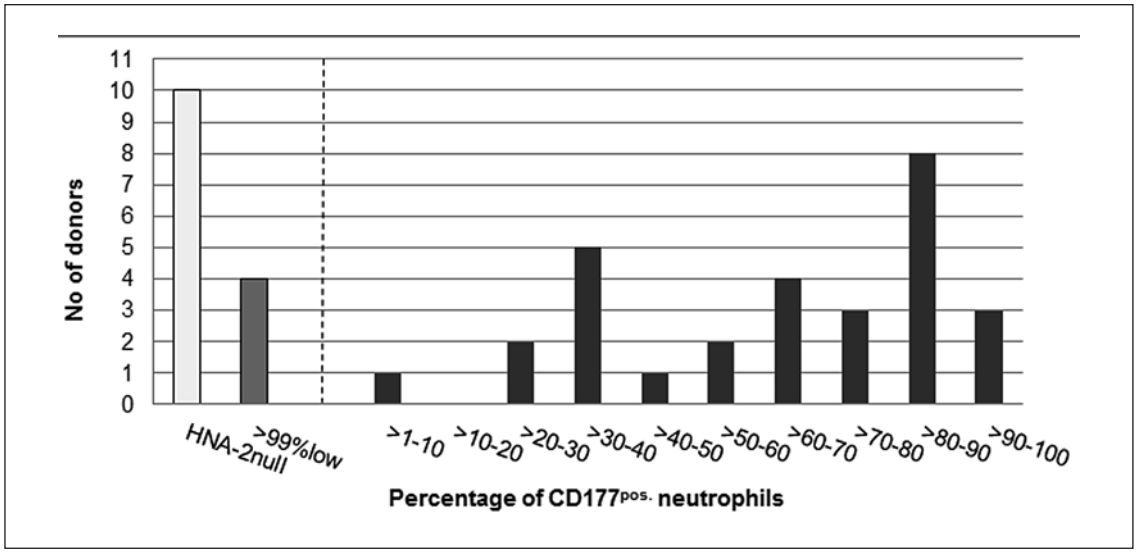

Fig. 2. Range of $CD177^{pos.}$ neutrophil subsets in HNA-2-positive donors as well as the number of negative and borderline phenotypes from 43 donors of the Transfusion Center Bad Kreuznach. The number of individuals with the respective percentage of $CD177^{pos.}$ neutrophils is shown.

visible bands and afterwards 2 µL were subjected to the 2nd PCR reaction, which was performed in analogy to the first one with the exception of 40 cycles und otherwise unchanged conditions. The products were subjected to cycle sequencing applying primers covering the complete coding region (CDS; Table 1).

Results

The study comprised 107 individuals, including 80 regular blood donors from different centers, 9 maternal samples corresponding to NIN cases, 10 of the respective children, and 5 fathers as well as 3 individuals with HNA-2 isoantibodies. Indeed, the plasma of one of the donors included in this study was involved in a case of TRALI. The selection of blood donors in the whole panel was not random because only the transfusion centers Giessen and Bad Kreuznach added samples of blood donors with an HNA-2-positive phenotype whereas the focus of the other centers was put on HNA-2_{null} individuals. Donor samples were assigned to 4 groups, with (1) an HNA-2_{null} phenotype (≤1% $CD177^{pos.}$ neutrophils), (2) HNA-2 low expression (>99% neutrophils with a borderline or questionable expression), (3) ≤40% clearly $CD177^{pos.}$ neutrophils, and (4) >40% clearly $CD177^{pos.}$ neutrophils (Fig. 1, 2). Overall, 29 of the 43 phenotyped blood donors from Bad Kreuznach were clearly $CD177^{pos.}$ with a bimodal expression and a mean positive subset of 63.5%. Four had low expression of HNA-2 (Table 2) and 10 were negative. No trimodal expression pattern with 2 CD-

Table 2. Distribution of the genomic *CD177*787A>T* variation within the phenotyped blood donors, patients, NIN mothers, and their neonates

HNA-2 phenotype/HNA-2 immunization	*CD177* c.787A*	*CD177* c.787A+T*	*CD177* c.787T*	Total
Donors				80
HNA-2 negative	3	7	32[a]	42
Borderline/questionable	3	4	2	9
≤40% CD177$^{pos.}$ neutrophils	3	5	0	8
>40% CD177$^{pos.}$ neutrophils	21	0	0	21
Patients with HNA-2 alloantibody	0	0	3	3
NIN mothers with HNA-2 alloantibody	0	1	8	9
NIN neonates	4	6	0	10
Fathers of the neonates	4	1	0	5

Values indicate the number of subjects. [a] Three of these had HNA-2 antibody, and the blood product of one was involved in a case of TRALI.

177$^{pos.}$ subsets was detected. Samples from blood donors of the other centers were assigned as either negative (n = 32) or questionable (in cases of a borderline fluorescence intensity; n = 5) according to the protocols and defined classifications of the respective laboratories.

Sequencing of gDNA

Genomic sequencing of *CD177* exon 7 and flanking intron sequences in all individuals studied so far has detected 2 haplotypes; wild-type (WT) **c.782G, 786A, 787A, 790G*, and *799A* or the mutated **c.782A, 786C, 787T, 790A*, and *799G*, or heterozygosity for both (Fig. 3). In 90 out of 105 samples, an intron *7+19c>t* substitution, located 178 nucleotides downstream of *CD177*c.787*, was detected. This substitution was associated with the mutant haplotype in exon 7. Genomic sequencing of all 9 *CD177* exons in 16 individuals (11 CD177$^{neg.}$ or questionable and 5 CD177$^{pos.}$) demonstrated a number of different polymorphisms (at positions *CD177*c.92, 114, 551, 610, 614, 751, 1038, 1042, 1097, 1178*, and *1291*) that were not necessarily in the same haplotype as the exon 7 mutations and did not exhibit a uniform pattern (data not shown).

In total, 79.6% of all individuals with an HNA-2$_{null}$ phenotype, including 8 of the 9 NIN mothers but only 2 of 9 donors with borderline or questionable CD177 expression, were homozygous for the *CD177*c.787A>T* substitution (Table 2) whereas all donors with a >40% CD177$^{pos.}$ neutrophil subset were homozygous for the WT allele. A low percentage of ≤40% CD177$^{pos.}$ neutrophils preferentially went along with heterozygosity for the mutation (Table 2).

Fourteen of the total 15 individuals with HNA-2 isoantibodies, including the 9 NIN mothers, 3 immunized donors, and 3 patients with HNA-2 isoantibodies, were homozygous for the *CD177*c.787A>T* (**787TT*) mutation (Table 2). One NIN mother, however, was heterozygous for the **c.787A>T* mutation within exon 7 and negative for the **c.955delG* and c.**1291G>A* mutations within exons 8 and 9, respectively. For each of the 8 homozygous **c.787TT* mothers, pedigrees were available, demonstrating that only 6 of 10 neonates were heterozygous (Table 2; Fig. 4). The 5 available paternal samples that were included to complete the pedigrees carried at least one WT allele. In 1 family with affected twin babies, the pregnancy resulted from an oocyte donation. The immunized gestational mother exclusively carried the **c.787T* allele whereas both neonates were homozygous for the WT alleles that had been inherited by the genetic parents (Fig. 4). Maternal inheritance was unresolved in 2 additional cases.

Further sequencing analysis, spanning all *CD177* gene exons was carried out with DNA samples from 12 donors with CD177$_{null}$ or questionable phenotype and 1 NIN mother without the homozygous *CD177*c.787A>T* substitution. In 3 *CD177*c.787A* homozygous and 1 **c.787AT* heterozygous donor samples, a homozygous *CD177*c.1291G>A* substitution was observed. In 2 other CD177$_{null}$ donors, heterozygosity for both **c.787AT* and **c.1291GA* was detected. The remaining 7 individuals carried the **c.1291G* WT allele in combination with further heterozygous mutations within exon 5 (**c.551G>T*→p.Gly184Val, **c.610A>G*→p.Asn204Asp, and **c.614G>T*→p.Arg205Met), exon 6 (**c.751C>A*→p.Leu251Ile), exon 8 (**c.1042G>A*→p.Ala348Thr), and exon 9 (**c.1178G>C*→p.Arg393Pro).

Sequencing of cDNA

Complementary DNA was available for sequencing from 28 blood donors. Full-length *CD177* cDNA was detected in all samples but consistent genotypes with gDNA sequencing were only obtained in 19 cases (Table 3). Nine

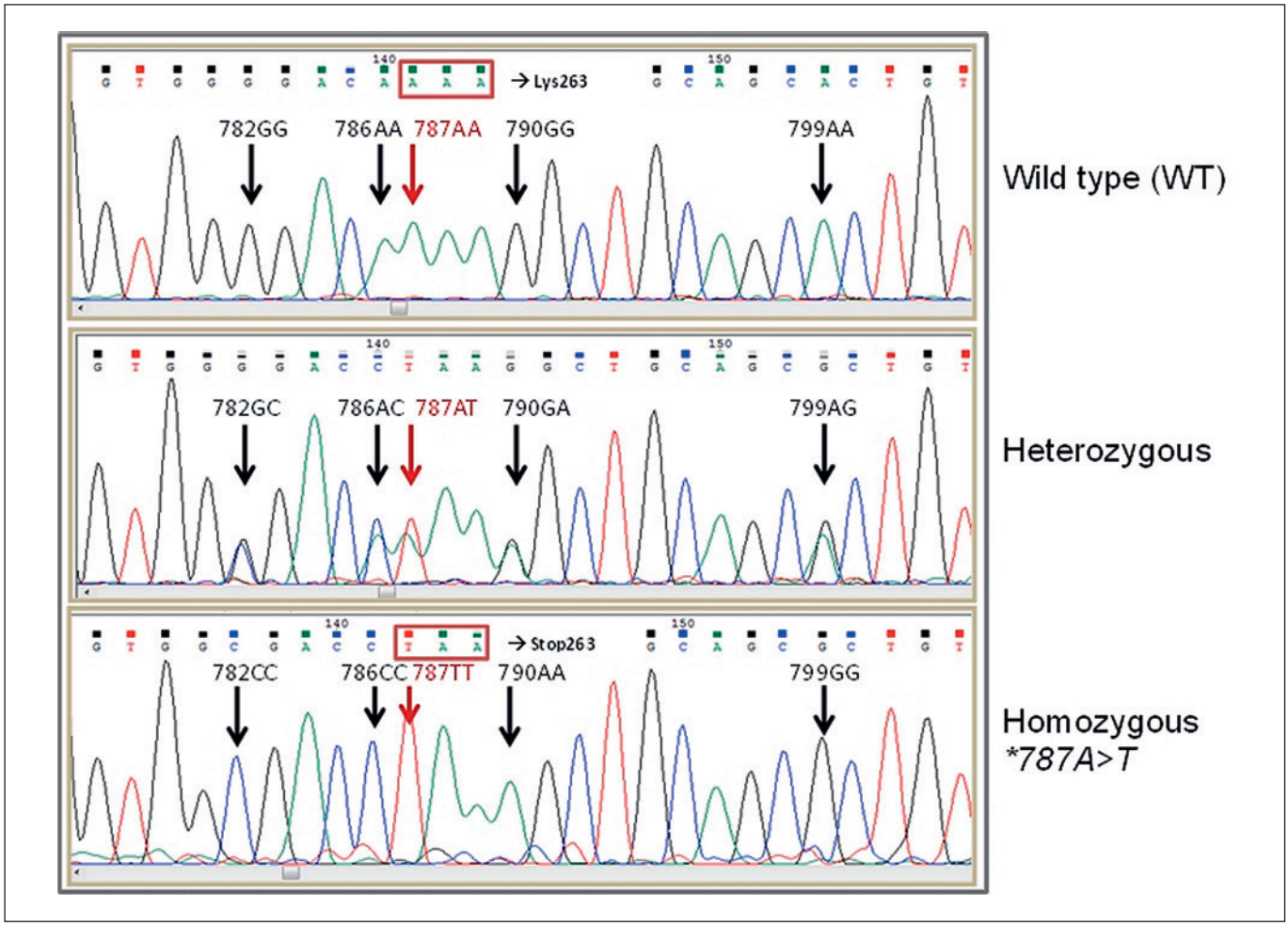

Fig. 3. DNA sequencing of *CD177* exon 7 covering position **c.787* in donors with different genotypes.

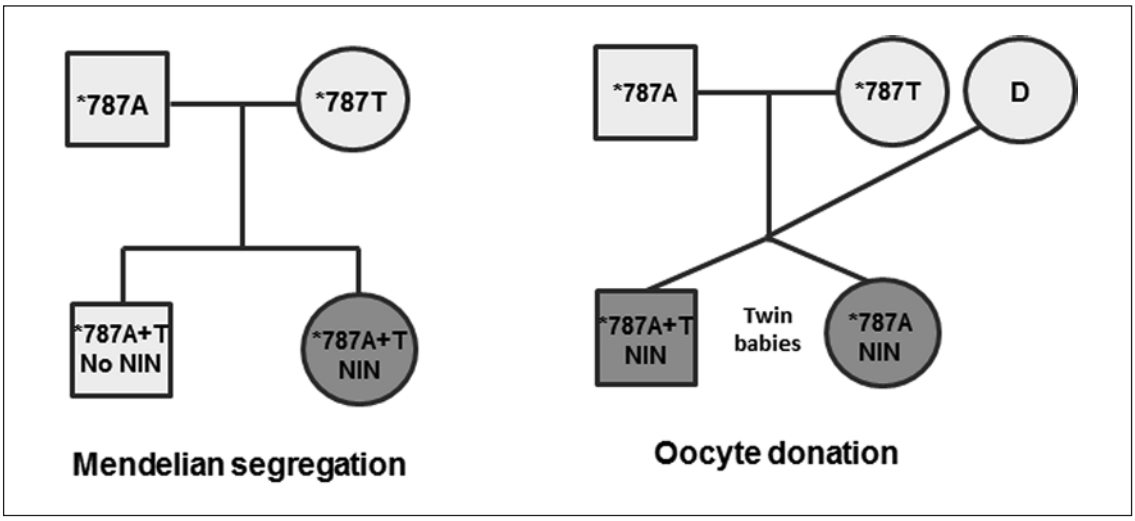

Fig. 4. Pedigrees with differential segregation of the *CD177*c.787* SNP in cases of NIN. In the right pedigree only *CD177* typing of the gestational mother but not of the oocyte donor (D) was available.

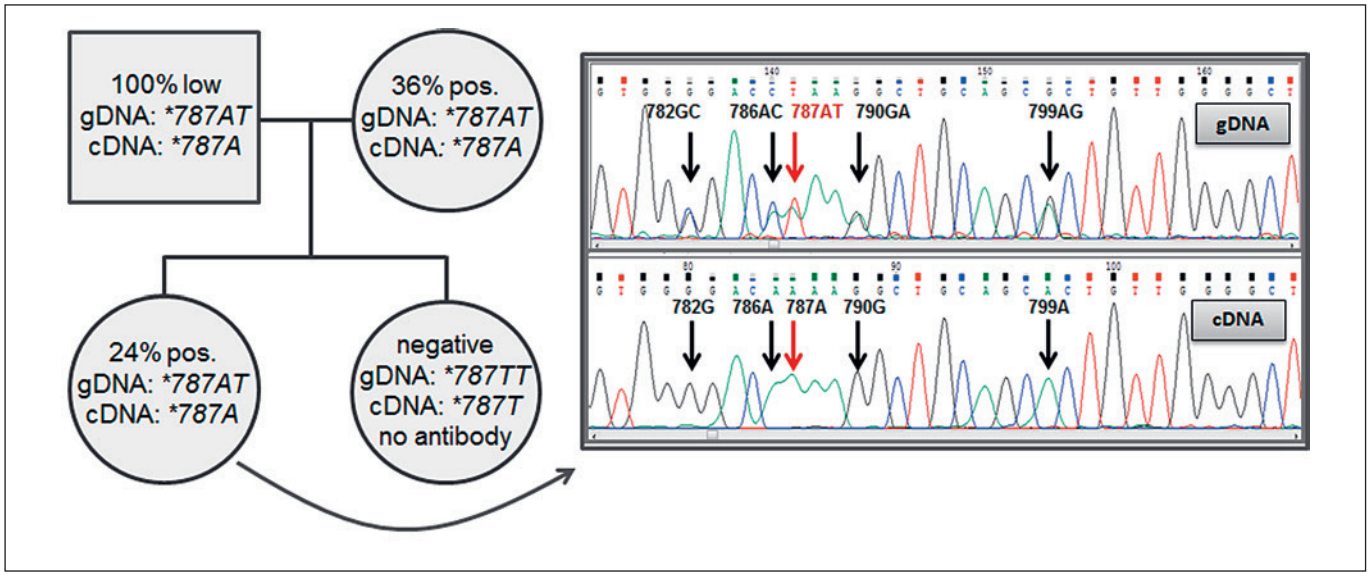

Fig. 5. Pedigree of a donor family with differential cDNA and gDNA allele expression. The antigen-negative daughter was not immunized against HNA-2.

Table 3. Comparison of gDNA and cDNA sequencing results in blood donors in relation to the HNA-2 phenotype

gDNA/cDNA HNA-2 expression	*787TT/*787T	*787AT/*787AT	*787AT/*787A	*787AA/*787A	Total
HNA-2_{null}	9	0	1	0	10
Questionable/borderline	1	0	4	3	8
>40% $CD177^{pos.}$	0	0	1	5	6
≤40% $CD177^{pos.}$	0	0	3	1	4
Total	10	0	9	9	28

Values indicate the number of subjects. In order to conform to a possible MAE only one allele is given for cDNA expression.

samples with heterozygosity for *c.787A and *T* on gDNA only showed the WT *c.787A allele on cDNA level. This finding included 1 donor family with an HNA-2_{null} daughter where both parents and her sibling with low $CD177^{pos.}$ expression exhibited heterozygosity for *c.787A and *T* on gDNA but exclusively *c.787A on cDNA (Fig. 5).

Analysis of cDNA in Sorted $CD177^{pos.}$ and $CD177^{neg.}$ Neutrophil Subpopulations

Recently, monoallelic expression (MAE) regulation for CD177 on neutrophil subsets has been suggested [22]. To clarify whether *CD177* MAE in neutrophil subpopulations is responsible for the discrepancy between gDNA and cDNA analysis, the isolated neutrophils from 2 $CD177^{pos.}$ phenotyped individuals were stained with FITC-labeled MEM166 antibody. The $CD177^{pos.}$ and $CD177^{neg.}$ subpopulations were gated. The neutrophil subpopulation with a peak congruent with that of the negative control was considered as the HNA-$2^{neg.}$ subpopulation (Fig. 6, left panel). *CD177* cDNA content of both neutrophil subpopulations was amplified for *CD177* exons 1–9 and the products of the first PCR reaction, which exhibited a comparably weak band intensity after electrophoretic separation was used as a template for the nested PCR reaction (amplification of exons 1–9) in order to obtain enough template for subsequent cDNA sequencing.

According to MAE, it was expected that methylation mechanisms would suppress CD177 expression in the $CD177^{neg.}$ subpopulation, which would consequently restrict the presence of *CD177* cDNA to $CD177^{pos.}$ positive neutrophil subpopulations. In contrast, our results

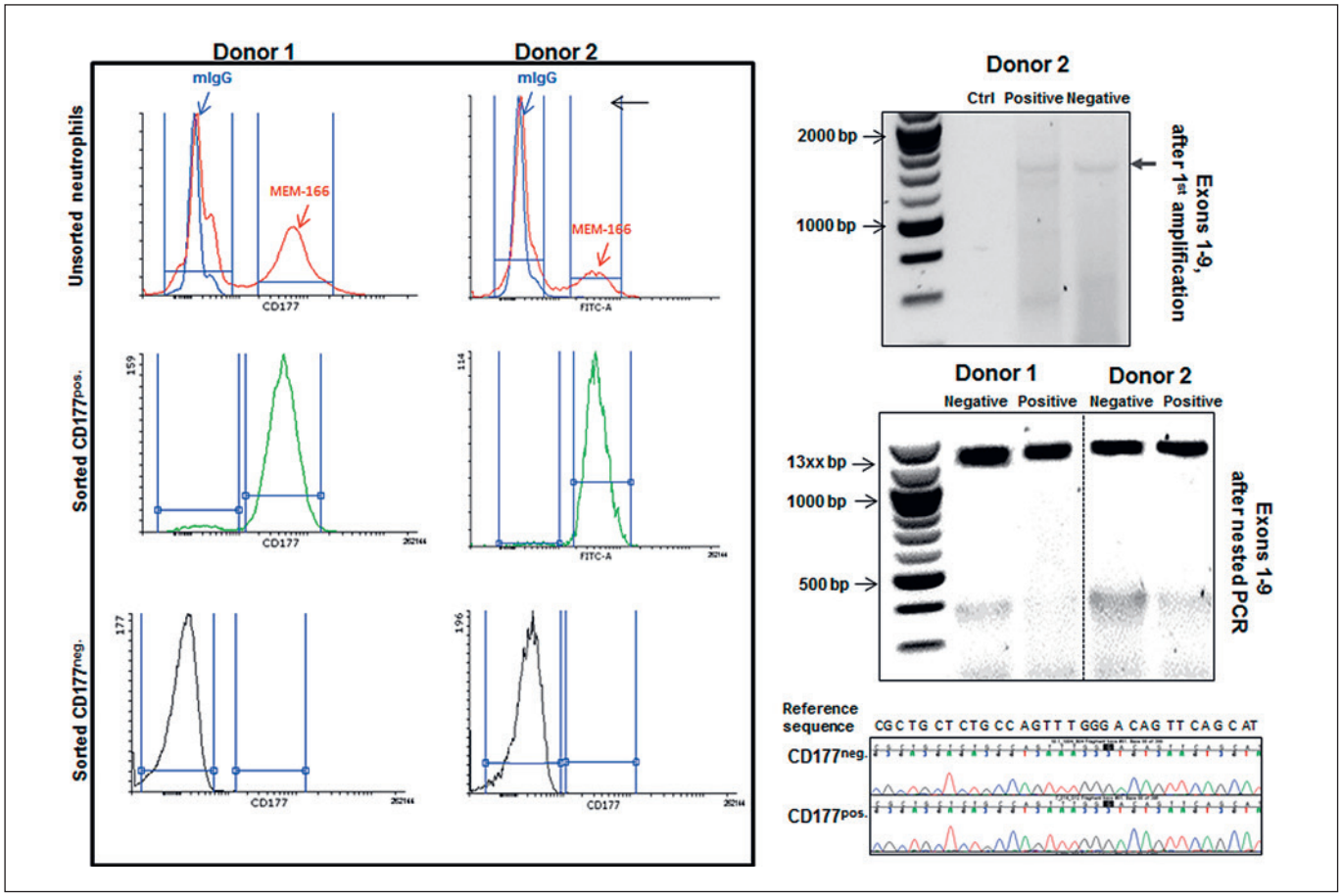

Fig. 6. Analysis of sorted HNA-2-positive and HNA-2-negative neutrophil subpopulations. Neutrophils were labeled with FITC-MEM166 or FITC-mIgG and the subsets were sorted to positive and negative subpopulations according to mean fluorescent intensity (left panel). mRNA content of sorted subpopulations was isolated and converted to cDNA. *CD177* cDNA was amplified by primers flanking exons 1–9. The very weak bands representing the products of the first PCR reaction covering exons 1–9 of the CD-177$^{pos.}$ and the CD177$^{neg.}$ fraction of one donor as well as an aqua control are shown in the upper right panel. After the 2nd round of amplification of the nested PCR, *CD177* cDNA is presented in the CD177$^{neg.}$ and CD177$^{pos.}$ fractions of 2 donors. The sequencing chromatograms exemplarily show sequence homology in the exon 2 amplicons of both CD177$^{neg.}$ and CD177$^{pos.}$ subpopulations of donor 2 with the *CD177* reference sequence (NM_020406.4) for comparison.

showed the presence of full-length *CD177* cDNA in both CD177$^{pos.}$ and CD177$^{neg.}$ subpopulations at comparable amounts (according to band intensities), indicating active *CD177* gene transcription in both neutrophil subpopulations (Fig. 6, right panel).

Sequencing analysis of amplified bands in neutrophil subpopulations demonstrated identical sequences in both negative and positive subpopulations (Fig. 6, right panel).

Discussion

In this study on a relevant number of blood donor and clinical samples, we tried to corroborate known information on the complex genetic basis of HNA-2 phenotypes and to add further data. The *CD177*c.787A>T* substitution that creates a premature stop codon within the *CD177* coding region was identified as the main cause for complete HNA-2 deficiency only within 79.6% of HNA-2$_{null}$ individuals who were homozygous for this mutation [15]. The **c.787* SNP was in complete linkage with the nucleotides at positions *782, 786, 790*, and *799* [13, 15] but not constantly with additional SNPs within other exons or flanking intron sequences, for example, at positions *CD177*c.551, 610, 614, 751, 1,042, and 1,178*. The *CD177*c.787>T* substitution has also recently been found in a TRALI case caused by a male donor that carried HNA-2 isoantibodies [30]. However, this mutation cannot explain the missing HNA-2 expression in all HNA-2$_{null}$ phenotyped blood donors tested so far, as discussed before [15, 19]. However, a definite discrimination between the true HNA-2$_{null}$ phenotype on the one hand and a very small CD177$^{pos.}$ neutrophil subset or a borderline

mean fluorescence intensity on the other hand is sometimes challenging such that samples might be falsely phenotyped as HNA-2_{null} [11, 16]. An unequivocal HNA-2_{null} phenotype is only given in immunized individuals with an HNA-2 isoantibody, either neutropenic patients, mothers of NIN babies, or donors without clinical symptoms, which was the reason to include 15 immunized individuals. But even in this group, 1 of 15 samples carried only one allele with the *CD177***c.787A>T* mutation and did not exhibit an additional *c.1291G>A* or *c.955delG* mutation as observed before in other studies [13, 15]. Compound *CD177***c.787A>T* and *CD177***c.955delG* (originally assigned to position **c.997*) heterozygosity was not detected in any of our samples, which might be due to differential ethnicities of the probands [15, 19].

In 6 of 12 donor samples with negative or borderline CD177 expression, an additional *CD177***c.1291G>A* substitution leading to a p.Gly431Arg exchange might explain the $CD177^{neg.}$ phenotype when the premature stop caused by *CD177***c.787A>T* is missing or only found in a heterozygous manner. The *CD177***c.1291G>A* substitution abrogates the GPI binding site of the CD177 GP so that the molecule is synthesized by the neutrophil but cannot be fixed to the membrane [21]. Three of our HNA-2_{null} samples and one with borderline expression were homozygous for *CD177***c.1291A*, which can explain the HNA-2-deficient phenotype. Provided that within the 2 samples that were heterozygous for *CD177***c.787A* and **c.1291A* both forms are located in trans, this could also abolish CD177 expression. However, 2 further HNA-2_{null} donors and 4 with borderline expression did not carry the *CD177***c.1291G>A* mutation. Unfortunately, no mRNA was available from these individuals to test for a potential MAE. In one of them an additional heterozygous *CD177***c.1178G>C* (p.Arg393Pro) substitution might account for the deficiency or a substantially reduced expression in the case of compound heterozygosity for both mutations. The effect of further single nucleotide polymorphisms on the CD177 expression remains unclear because there was no definite pattern of the SNPs at positions *CD177***c.551, 610, 614, 751*, and *1042*.

Unexpectedly, gDNA of only 6 of 10 NIN neonates was heterozygous for the *CD177***c.787A>T* substitution, while 4 babies only carried the WT allele although their mothers were homozygous for the *CD177***c.787A>T* substitution. In a twin baby pair this could be explained by the fact they resulted from an oocyte donation where the **c.787A* WT twins immunized the gestational mother who was homozygous for the **c.787A>T* mutation. In 2 further cases no information on potential oocyte donations was available, leaving the cases unresolved.

*The CD177***c.787A>T* substitution was not only associated with neutrophil HNA-2 deficiency but also affected the percentage of $CD177^{neg.}$ neutrophils in individuals with a bimodal expression pattern. Consistent with previous findings, heterozygosity for the mutation was preferentially associated with a borderline HNA-2 expression or a small $CD177^{pos.}$ neutrophil subset, whereas all 21 donors with >40% $CD177^{pos.}$ neutrophils carried the WT form, which is consistent with previous findings [15].

Most interesting were the differential typing results of gDNA and cDNA sequencing obtained in 9/28 blood donors whose gDNA was heterozygous for the *CD177***c.787T* haplotype within exon 7 but only exhibited the WT haplotype within cDNA. The data sets of the remaining 19 donors concordantly either showed the *CD177***c.787* WT or the mutated allele. Different approaches try to explain this effect. Monoallelic gene expression is the phenomenon of gene expression when only 1 of the 2 gene copies is actively transcribed. An unstable nature of mRNA from the *CD177***c.787T* allele that is rapidly degraded by the mechanism of nonsense-mediated mRNA decay and thus undetectable by cDNA sequencing has been discussed as the reason of MAE [15], but this is rebutted by the status of the 10 *CD177***c.787T* homozygous donors (gDNA) where the respective cDNA was detected. Second, gene silencing by DNA methylation in the *CD177* promoter region may induce the MAE of the *CD177* gene, which consequently leads to the presence of only one allele in mRNA analysis [22]. In a HeLa cell model this epigenetic mechanism was associated with the $CD177^{neg.}$ fraction, whereas CpG demethylation converted monoallelic into biallelic expression within the respective fraction. During differentiation of CD34+ hematopoietic stem cells into neutrophils 1 of 2 different parental alleles was silenced, thus inducing MAE, which also might explain our own findings [22]. *CD177* mRNA expression has been reported to increase during differentiation of neutrophils, whereas there is no de novo synthesis of protein in mature neutrophils [31]. A possible mechanism for MAE could be a preferential usage of the *CD177***c.787A* allele for transcription during maturation so that only one mRNA version is found in mature neutrophils. Another mechanism that might explain the discrepancy between gDNA and cDNA is the nonspecific amplification of the CD177 pseudogene along with the *CD177* gene. However, this fault was eliminated by the amplification of a *CD177* gene-specific template for the downstream application [19].

To clarify whether the MAE mechanism is regulating CD177 expression on neutrophil subpopulations, the mRNA extracted from sorted $CD177^{pos.}$ and $CD177^{neg.}$ neutrophils was analyzed. Analysis of the *CD177* mRNA content of both sorted $CD177^{pos.}$ and $CD177^{neg.}$ granulocytes showed the presence of identical *CD177* cDNA sequences in both subpopulations. As demonstrated semiquantitatively by agarose gel electrophoresis after both the 1st and the 2nd round of amplification of the nested

PCR reaction, product quantities were comparable in the CD177$^{pos.}$ and CD177$^{neg.}$ neutrophil subpopulations. Although it is always possible to argue that the *CD177* mRNA from the CD177$^{neg.}$ fraction was derived from a minute contamination by CD177$^{pos.}$ neutrophils, we interpret our data as an indication of the presence of an active *CD177* gene in CD177$^{neg.}$ neutrophils, which at least indirectly would exclude gene silencing by methylation of CpG regions as the regulatory mechanism implicated in the bi- or even tri-modal CD177 expression. Thus, other not yet identified upstream regulatory mechanisms are likely responsible for the formation of the CD177$^{neg.}$ neutrophil subset. On the contrary, in another report not only CD177 protein but also *CD177* mRNA expression was restricted to the CD177$^{pos.}$ subset, whereas no CD177 mRNA was detected within the negative subset [22]. Further experimental data are needed to resolve this seeming discrepancy.

Taken together, these results prove the multiple complex mechanisms regulating CD177 expression on the neutrophil surface as described before. The *CD177*c.787A>T*-induced premature stop codon is definitely the main reason for the HNA-2$_{null}$ phenotype and a low proportion of the CD177$^{neg.}$ neutrophil subset. *CD177*c.1291G>A* accounts for the absence of CD177 expression in most of the remaining cases. However, a definite identification of all molecular mechanisms is indispensable for molecular typing of HNA-2, which moreover is aggravated by the fact that an additional long-range PCR reaction is necessary to discriminate a *CD177*-specific template from the *CD177* pseudogene [19]. Recently, a fast screening method based on TaqMan™-PCR was introduced for the identification of HNA-2$_{null}$ individuals homozygous for the **c.787A>T* mutation in both *CD177* and *CD177P1* genes [32]. However, HNA-2$_{null}$ individuals who are able to develop the HNA-2 isoantibody cannot be reliably identified by molecular methods until the molecular basis has been entirely decoded.

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Statement of Ethics

The study was approved by the ethics boards of the contributing institutions. All donors gave informed consent.

Disclosure Statement

The authors disclose no conflicts of interest.

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Author Contributions

B.K.F. initiated and coordinated the study, characterized and analyzed samples, and wrote the manuscript. B.B. added new features, analyzed samples, and actively contributed to the manuscript. A.R., N.N., C.C., P.B., T.J.S., E.H., L.P., P.H., P.R., M.S., H.K., and J.K. all characterized and provided samples, discussed the topic, and carefully revised the manuscript.

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