Therapeutic targets in sickle cell disease
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Non-classical FCGR2C haplotype is associated with protection from red blood cell allo-immunization in sickle cell disease

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Statement of equal authors’ contribution:
JS and SM contributed equally to this work.
FP and TvdB contributed equally to this work.

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Abstract
Red blood cell (RBC) transfusions are of vital importance in patients with sickle cell disease (SCD). However, a major complication of transfusion therapy is allo-immunization. The low-affinity Fc gamma receptors (FcγRs), expressed on immune cells, are important regulators of antibody responses. Genetic variation in FCGR genes has been associated with various auto- and allo-immune diseases. The aim of this study was to evaluate the association between genetic variation of FCGR and RBC allo-immunization in SCD.

In this case-control study, DNA samples from 2 cohorts of transfused SCD patients were combined (France and the Netherlands). Cases had a positive history of allo-immunization, having received ≥1 RBC unit. Controls had a negative history of allo-immunization, having received ≥20 RBC units. Single nucleotide polymorphisms and copy number variation of the FCGR2/3 gene cluster were studied in a FCGR-specific multiplex ligation-dependent probe amplification assay. Frequencies were compared using logistic regression.

Two-hundred-seventy-two patients were included (130 controls, 142 cases). The non-classical open reading frame in the FCGR2C gene (FCGR2C.nc-ORF) was strongly associated with a decreased allo-immunization risk (OR 0.26, 95% CI 0.11-0.64). This association persisted when only including controls with exposure to ≥100 units (OR 0.30, CI 0.11-0.85), and appeared even stronger when excluding cases with Rh or K antibodies only (OR 0.19, CI 0.06-0.59).

In conclusion, SCD patients with the FCGR2C.nc-ORF polymorphism have over a threefold lower risk for RBC allo-immunization compared to patients without this mutation. This protective effect was strongest for exposure to antigens other than the immunogenic Rh or K antigens.
Introduction

Red blood cell (RBC) transfusions are of vital importance in patients with sickle cell disease (SCD). However, an important and frequent complication of transfusion therapy is allo-immunization. The development of allo-antibodies against foreign RBC antigens complicates donor matching procedures and significantly limits the availability of matching blood for future transfusions. In addition, allo-immunized patients are at risk of developing delayed hemolytic transfusion reactions (DHTR), a potentially lethal complication.

Allo-immunization occurs significantly more frequently in SCD patients as compared to the general population with incidences ranging from 18-76% with ABO and RhD matching only. This high allo-immunization rate in SCD patients can partly be explained by the differences in RBC antigens between donors, primarily of European descent, and recipients, primarily of African descent. Extended antigen matching for Rhesus (Rh) phenotype and Kell (K) has significantly reduced the rate of allo-immunization, whereas additional antigen matching for Fy, Jk and MNS appears to be even more effective. Unfortunately, extended matching is not always applied due to limited availability of matching units and the high costs involved.

Interestingly, only a subgroup of patients appears to develop allo-antibodies after transfusion. It is yet unclear why some patients develop allo-antibodies whereas others remain tolerant despite high transfusion exposures. A better understanding of the pathophysiology of RBC allo-immunization may help to identify these high risk patients and could add to the detection of targets for preventive strategies, ultimately promoting a more safe and cost-effective application of transfusion therapy in SCD.

The basic concept of allo-immunization involves the uptake of transfused allogenic RBC antigens by antigen-presenting cells, presentation of antigen by the HLA class II complex to CD4+ T helper cells and subsequent B cell activation with antibody production. However, the occurrence of an actual antibody response is determined by a complex interplay of both genetic predisposition and circumstantial factors at time of transfusion, such as the extent of antigenic incompatibility between donor and host, the immunogenicity of a specific allo-antigen and the level of systemic inflammation. As for genetic predisposition, the HLA class II genotype of a patient is a potential predictor of the allo-immunization status, both in SCD as in general transfused populations. In addition, polymorphisms in immunoregulatory genes have been implicated in RBC allo-immunization (TRIM21, CD81).

This is the first study to assess the association between RBC allo-immunization in
SCD and the genes encoding for low-affinity Fc gamma receptors (FcγRs) for IgG. FcγRs are glycoproteins, expressed mainly by immune effector cells such as antigen-presenting cells, macrophages and monocytes. They allow these cells to bind to the Fc portion of IgG, attached to circulating antigen, facilitating activation of the cell or presentation of antigenic peptides. FcγRIIb and FcγRIII are believed to mediate antigen internalization and presentation to T cells. Evidence supporting this importance of FcγRs in antigen uptake and presentation is provided in studies that show that a single amino acid mutation in the FcγRIIb or FcγRIII cytoplasmic tail prevents antigen internalization and presentation. FcγRIIb appears to play an important role in the regulation of B cells and antibody producing plasma cells. The receptor has been proposed to maintain peripheral tolerance for B cells, a theory that is supported by the fact that FcγRIIb knockout mice lose peripheral B cell tolerance, produce auto-antibodies and develop auto-immune disease. Furthermore, FcγRIIb regulates affinity maturation and memory B cell development. Moreover, FcγRs can shape the antibody repertoire by modulating B cell receptor-mediated cell activation and proliferation. These effects emphasize the crucial role of FcγRs in the formation of allo-antibodies.

The family of FcγRs includes the high affinity FcγRI and the low affinity FcγRII and FcγRIII, comprising of various subclasses (FcγRIIa, FcγRIIb, FcγRIIC, FcγRIIIa and FcγRIIIb). These low affinity FcγRs provide both pro- and anti-inflammatory regulation of immune responses and are encoded by the genes FCGR2A, FCGR2B, FCGR2C, FCGR3A and FCGR3B. Polymorphisms in these genes are common and may affect the function of these proteins, thereby affecting the balance between activating and inhibitory signaling pathways. Various polymorphisms in FCGR genes have been associated with the occurrence of certain auto- and allo-immune diseases, such as idiopathic thrombocytopenic purpura, systemic lupus erythematosus and inhibitor development in hemophilia A.

The aim of this study was to evaluate whether polymorphisms in the FCGR2 and FCGR3 gene are associated with RBC allo-immunization in a cohort of SCD patients. A secondary aim of this study was to assess if variation within these genes confers susceptibility for the occurrence of a DHTR.
Methods

Patients
In this observational, case-control study, DNA was available from two longitudinal cohorts of transfused SCD patients: one from the Netherlands and one from France. All patients of the Dutch cohort were eligible for this study. The French cohort was a random selection of transfused SCD patients included in the SCDTRANSFU cohort, a study population of patients exclusively transfused at the Henri Mondor Hospital in Créteil, France. Patients from both cohorts were included in this study if a) a DNA sample was available, and b) they had a negative history of allo-immunization with a minimum transfusion exposure of at least 20 RBC units (controls), or a positive history of allo-immunization, with a minimum transfusion exposure of at least 1 RBC unit (cases). A minimum of 20 units was applied in negative patients to assure that patients had sufficient transfusion exposure to potentially form any allo-antibodies. The institutional review boards of all participating centers approved the study. This study was conducted in accordance with the Declaration of Helsinki.

Data collection and definitions
In the primary analysis of this study, we assessed the association between polymorphisms in the FCGR2 and FCGR3 genes, and RBC allo-immunization. Cases were defined here as all patients with a clinically significant allo-antibody. Controls were defined as all patients with a negative history of allo-immunization, with a minimum transfusion exposure of at least 20 RBC units. Patients with exclusively one of the following antibodies were excluded from this analysis: auto-antibodies, cold antibodies and naturally occurring antibodies such as anti-A, anti-B, anti-A1, anti-IH, anti-H, anti-A1, anti-I, anti-Lea, anti-Leb and anti-P1. Anti-M was defined as naturally occurring if demonstrated upon first antibody screening with no history of prior transfusion exposure. To test the robustness of our findings a sensitivity analysis was performed to assess the effect of immunization against the highly immunogenic Rh and K antigens (RhD, C, c, E, e and/or K) on the association between FCGR polymorphisms and allo-immunization. We hypothesized that the genetic constitution of a patient is less important for the formation of Rh and K specific allo-antibodies, since these antibodies are so readily formed. To test this hypothesis, we categorized allo-immunized patients (cases) in two subgroups: the first group contained patients with exclusively Rh or K specific antibodies, the second group contained patients with at least one antibody other than Rh or K. Associations between FCGR polymorphisms and allo-immunization were assessed and compared between these two subgroups. Lastly, we performed a separate analysis in the French cohort to assess associations between FCGR polymorphisms and the occurrence of DHTR. DHTR information was solely available for patients from the French cohort. In this analysis, cases were defined as
all patients with a positive history of DHTR. Controls were defined as patients with a negative history of DHTR, with a minimum transfusion exposure of at least 20 RBC units, irrespective of the allo-immunization status. DHTR was defined as described previously.\textsuperscript{42}

**DNA extraction**
Genomic DNA was isolated from whole blood according to manufacturer’s instructions using the Gentra Puregene kit (Qiagen, Hilden, Germany).

**Multiplex ligation-dependent probe amplification (MLPA)**
CNV and SNPs in the low-affinity FCGR genes FCGR2A, FCGR2B, FCGR2C, FCGR3A and FCGR3B were routinely determined with an FCGR-specific MLPA assay (MRC-Holland, Amsterdam, The Netherlands). The MLPA assay was performed according to the manufacturer’s protocol, essentially as previously described.\textsuperscript{43-45} In short, specific MLPA probes recognizing the FCGR2A, FCGR2B, FCGR2C, FCGR3A and FCGR3B genes were designed. To effectively investigate CNV at least 3 probes per gene were designed. Moreover, specific probes for the following polymorphisms and haplotypes were used: FCGR2A (131H/R), FCGR2A (27Q/W), FCGR2B (232I/T), FCGR2C (exon 3 ORF/STOP), FCGR3A (158V/F) and FCGR3B (NA1/NA2/SH). Probes were added for the splice site mutation at the border of exon 7 intron 7 in FCGR2C (rs76277413 c.798 +1 A > G), to distinguish the non-expressed non-classical FCGR2C-ORF variant from the classical FCGR2C-ORF.\textsuperscript{46} The assay also contained non-specific probes for the promoter regions of FCGR2B and FCGR2C for which the polymorphisms make up the promoter haplotypes 2B.1, 2B.2 and 2B.4 as described by Su et al.\textsuperscript{47} allocations of these haplotypes as described in Tsang et al.\textsuperscript{48}

**Statistical analysis**
The allele frequencies of single nucleotide polymorphisms (SNPs) and copy number variation (CNV) were compared between allo-immunized and non-allo-immunized, and DHTR and non-DHTR patients, using single variant logistic regression, adjusted for cohort (French or Dutch) as covariate and assuming an additive genetic effect (in case of variants with more than two genotypes). Comparisons were expressed in odds ratios (OR), 95% confidence intervals (CI) and corresponding P-values. In our primary analysis, we applied a Bonferroni correction to adjust for multiple testing. A P-value less than 0.0038 (0.05/13 variants) was considered statistically significant here. In all subsequent sensitivity analyses, a P-value of less than 0.05 for significance was applied. Expression levels of FcγRs were analyzed using GraphPad Prism 7.02. For comparison of MFI\s the one-way ANOVA-test was used followed by Sidak post-hoc test for correction of multiple comparison. ****P < .0001; ***P < .001; **P < .01; *P < .05

A detailed description and additional methods are available in supplemental Methods.
Results

Study population

Our study population of eligible patients was composed of transfused SCD patients from a cohort from France and a cohort from the Netherlands (figure 1). Baseline characteristics of these 2 cohorts are described in table 1. Patients in the French cohort were slightly older, had a higher cumulative transfusion exposure and had a higher proportion of patients of African ethnicity, as compared to the Dutch cohort. Of the total number of 282 eligible patients, 10 patients were excluded as they exclusively had either auto-antibodies or naturally occurring antibodies. The remaining 272 patients (130 controls and 142 allo-immunized cases) formed our primary study population.

FCGR gene CNV or polymorphism, and susceptibility to allo-immunization

We have analyzed the low-affinity FCGR2/3 genes, including FCGR2A, FCGR2B, FCGR2C, FCGR3A, and FCGR3B, of SCD patients with or without allo-immunization by genotyping copy number variation (CNV) and single nucleotide polymorphisms (SNPs) by multiplex ligation dependent probe amplification (MLPA).

The FCGR2/3 gene locus contains 4 distinct CNV regions (CNR)\(^45\), as shown in figure 2. Table 2 shows the frequencies of the tested CNRs. CNV was found most frequently in CNR1 (FCGR2C/FCGR3B) where 56 patients showed a change in copy number. Copy number changes in the four CNRs were not

| Table 1. Patient characteristics for both cohorts |
|-----------------------------------|-----------------|-----------------|
| N (%) or median (IQR) | France N=169 | The Netherlands N=113 |
| Age at last follow-up in years | 34 (29-45) | 27 (21-38) |
| Female sex | 92 (54) | 66 (58) |
| Hemoglobin genotype | | |
| HbSS / HbS\(^0\) | 163 (96) | 98 (88) |
| HbSC / HbS\(^+\) | 6 (4) | 15 (12) |
| Ethnicity * | | |
| Africa | 144 (88) | 30 (27) |
| Latin-America | 18 (11) | 76 (67) |
| Asia | 0 (0) | 3 (3) |
| Other | 2 (1) | 4 (4) |
| Cumulative transfusion exposure - units | 72 (13-179) | 47 (22-136) |

* Ethnicity was unknown in 5 patients in the French cohort (N=164)
A DNA sample was available of 282 patients from both the French (FR) and the Netherlands (NL) cohorts. Ten patients were excluded as they exclusively had naturally occurring or auto-antibodies (patients excluded FR N=1; NL N=9), leaving a total of 272 patients in our primary study population. We performed 2 sensitivity analyses, I) excluding controls with <100 units transfusion exposure (patients excluded FR N=36, NL N=33); II) dividing cases into patients with exclusively antibodies with Rh or K specificity, and patients with at least one antibody other than Rh or K. A separate analysis was performed to assess the association of FCGR polymorphisms with the occurrence of delayed hemolytic transfusion reactions (DHTR). Data on the history of DHTR status was only available in the FR cohort (N=157; 12 controls were excluded as they had <20 units exposure).
Figure 2. Schematic overview of the FCGR2/3 locus.
Nine SNPs and haplotypes are indicated with orange boxes. Orange bars depict the approximate extent of CNRs in which duplication or deletion can occur. CNR: copy number variable region; SNP: single nucleotide polymorphism; HNA: human neutrophil antigen.
significantly associated with susceptibility to allo-immunization in our study population. In addition, polymorphisms in the FCGR2/3 locus were analyzed for association with allo-immunization (table 2). Single variant logistic regression revealed a highly significant association for FCGR2C.nc-ORF (OR 0.26, CI 0.11-0.64, \( P=0.003 \)). This OR indicates a protective effect of this polymorphism for allo-immunization. Only seven of the 142 (5%) allo-immunized patients had this polymorphism, against 21 of the 130 (16%) non-immunized control patients. This association was consistent in our sensitivity analysis when including only controls with a transfusion exposure of \( \geq 100 \) units (figure 1; OR 0.30, CI 0.11-0.85, \( P=0.023 \)). Other polymorphisms were not associated with allo-immunization.

Interestingly, when excluding cases with exclusively antibodies against the relatively immunogenic Rh and K antigens, the protective association of the FCGR2C.nc-ORF polymorphism with allo-immunization appeared to be even stronger (figure 1 and table 3A; OR 0.19, CI 0.06-0.59, \( P=0.004 \)). In contrast, when only including these cases with exclusively Rh or K specific antibodies in our analysis, the association was not present anymore (OR 0.50, CI 0.14-1.81, \( P=0.292 \)).

<table>
<thead>
<tr>
<th>Copy number region</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNR1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>CNR4</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Allele frequencies</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 2. Copy numbers and allele frequencies of FCGR2/3 genetic variation in 130 control patients (>20 transfused units, no allo-antibodies) and 142 allo-immunized patients

Text in bold indicates significant values. CNR: copy number region, Fc\( \gamma \)R: Fc gamma receptor, OR: odds ratio, CI: confidence interval, n.a.: not applicable.
Despite the clear protective association, 7 patients with the FCGR2C.nc-ORF polymorphism in the total study population still formed allo-antibodies. Table 3B demonstrates that of these seven patients, 3 patients developed only antibodies with Rh specificity within 1-9 units of RBC transfusions. Two patients primarily developed a Rh antibody, and subsequently formed additional allo-antibodies. The remaining 2 patients developed anti-Jsa (KEL 6) or anti-Kpa (KEL 3) antibodies, which both belong the K antibody system.49

Lastly, we also evaluated if the association was consistent in both the French and the Dutch subcohort of patients. In the French subcohort, the association was strongly present (N=165, OR 0.13, CI 0.04-0.47, P=0.002), while the association did not persist in Dutch patients (N=104, OR 0.82, CI 0.21-3.23, P=0.77). Yet, patients in the Dutch subcohort had a higher exposure to units matched only for AB0 and RhD antigens, as the study observation window of this cohort was wider. Therefore, the proportion of cases with exclusively Rh or K antibodies was significantly higher in the Dutch subcohort compared to the French subcohort (respectively 26/51 cases [51%]; and 14/91 cases [15%], P<0.001). Overall, these data indicate that the protec-
Table 3. The influence of highly immunogenic Rhesus or Kell allo-antibodies on the association between the FCGR2C.nc-ORF polymorphism and allo-immunization

A. Controls vs allo-immunized cases, Cases, exclusively Rh or K antibodies*

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
<th>N (control/cases)</th>
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<tr>
<td>FCGR2C.nc-ORF</td>
<td>0.50</td>
<td>0.14-1.81</td>
<td>0.292</td>
<td>130/40</td>
</tr>
</tbody>
</table>

Controls vs allo-immunized cases, Cases, at least 1 antibody other than Rh or K

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
<th>N (control/cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCGR2C.nc-ORF</td>
<td>0.19</td>
<td>0.06-0.59</td>
<td><strong>0.004</strong></td>
<td>130/102</td>
</tr>
</tbody>
</table>

Text in bold indicates significant values. OR: odds ratio, CI: confidence interval.
* RhD, C, c, E, e and/or K antibodies

B. Allo-immunized cases FGCR2C. nc-ORF

<table>
<thead>
<tr>
<th>Allo-immunized cases FGCR2C. nc-ORF</th>
<th>Antibody</th>
<th>Units to first antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient nc-1</td>
<td>Anti-C</td>
<td>1</td>
</tr>
<tr>
<td>Patient nc-2</td>
<td>Anti-Jsa</td>
<td>31</td>
</tr>
<tr>
<td>Patient nc-3</td>
<td>Anti-E</td>
<td>2</td>
</tr>
<tr>
<td>Patient nc-4</td>
<td>Anti-E</td>
<td>9</td>
</tr>
<tr>
<td>Patient nc-5</td>
<td>Primary: Anti-E/M/Jkb, Following: Anti-S, anti-Fya, anti-H</td>
<td>&gt;3</td>
</tr>
<tr>
<td>Patient nc-6</td>
<td>Anti-Kpa</td>
<td>16</td>
</tr>
<tr>
<td>Patient nc-7</td>
<td>Primary: Anti-E, Following: Anti-Knops- auto (RH4-RH6-RH5); FY5-MNS1-MNS3-LE1-LE2-YT2-Htl-ASP</td>
<td>2</td>
</tr>
</tbody>
</table>
tive effect of FCGR2C.nc-ORF appears to be strongest for allo-immunization against antigens other than Rh or K, and is therefore most relevant if extended matching for Rh and K antigens is performed.

**FCGR gene CNV and SNPs and susceptibility to DHTR**

An important clinical complication of allo-immunization is DHTR. Besides their role in the formation of allo-antibodies, FcγRs are especially known for their capacity to induce antibody-mediated phagocytosis or antibody-mediated cellular toxicity (ADCC) associated with hemolysis once an allo-antibody is present. Therefore, we have analyzed the low-affinity FcγR genes of SCD patients to investigate the presence of a potential association with DHTR after allo-immunization. The history of DHTR status was available in 157 patients of the French cohort (figure 1). One-hundred-six of these patients did not have a history of DHTR and served as controls. Fifty-one patients did develop DHTR and were defined as cases. Although a trend of association was found again for FCGR2C.nc-ORF (OR 0.48, CI 0.15-1.51, \( P=0.210 \)), single variant logistic regression analysis did not show any significant associations between FcγR polymorphisms or CNV, and DHTR (table S1).

**Functional consequence of the FCGR2C. nc-ORF polymorphism**

Due to a premature stop codon, the FCGR2C gene is a non-expressed pseudogene in FCGR2C-stop individuals. A SNP in exon 3 can lead to an open reading frame (ORF) which results in expression of the gene (FCGR2C-ORF variant). An additional splice site mutation near exon 7 can generate another stop codon which in turn results in no expression of FcyRIIc. This last variant is the FCGR2C.nc-ORF polymorphism.

To explain the association between this polymorphism in a non-expressed gene and allo-immunization, we assessed FcγR expression profiles of patients with the FCGR2C.nc-ORF polymorphism and compared this to patients with FCGR2C-stop or FCGR2C-ORF, by use of flow cytometry (figure 3). First, we looked at FGCR2C expression. Since the extracellular domain of FcγRIIb and FcγRIIc are identical, we used an antibody that recognizes FcγRIIb/c. We have analyzed NK-cells which do not express FcγRIIb but can express FcγRIIc. In parallel to what can be found in Caucasian donors, no expression of FcγRIIc was found in patients with FCGR2C-stop or FCGR2C.nc-ORF (figure 3A).

Alternatively, the FCGR2C.nc-ORF polymorphism could be linked to a functional unknown polymorphism elsewhere in the
Figure 3. FcγR expression on various cell types in SCD patients.

A. FcγRIIc expression on NK cells of patients expressing FCGR2C.nc-ORF variant (n=6), FCGR2C-stop variant (n=7), FCGR2C-ORF variant (n=2). In red, NK cells of healthy controls with the FCGR2C-ORF variant (n=4). B. FcγRIIb/c expression on neutrophils, monocytes, B cells, T cells and NK cells on patient cells expressing FCGR2C.nc-ORF variant (n=4), FCGR2C-stop variant (n=6), FCGR2C-ORF variant (n=2). C. FcγRIIIa expression on patient cells expressing the FCGR2C.nc-ORF variant (n=6), FCGR2C-stop variant (n=8), FCGR2C-ORF variant (n=2). D. FcγRIIIa expression on neutrophils, monocytes, B cells, T cells and NK cells on patient cells expressing FCGR2C.nc-ORF variant (n=7), FCGR2C-stop variant (n=6), FCGR2C-ORF variant (n=2). Error bars denote the standard error of the mean. Stars represent highly significant differences (**P < .01, ****P < .0001). ns, nonsignificant differences.
Figure 4. FcγRII expression on B cells and monocytes of SCD patients and healthy controls.

A. FcγRIIb/c expression on B cells on cells expressing the FCGR2C.nc-ORF variant (patients, n=6; healthy donors, n=6) or FCGR2C-stop variant (patients, n=4; healthy donors, n=10).

B. FcγRIIa expression on monocytes expressing the FCGR2C.nc-ORF variant (patients, n=4; healthy donors, n=5) or FCGR2C-stop variant (patients, n=6; healthy donors, n=12).

To investigate differential FcγR expression due to a potential unknown polymorphism linked to the FCGR2C.nc-ORF polymorphism, we next assessed FCGR2A, FCGR2/B/C and FCGR3A/B expression on neutrophils, monocytes, B cells and T cells among patients with FCGR2C.nc-ORF, FCGR2C.nc-stop or FCGR2C-ORF. The large spread seen for FcγRIII can be attributed to CNV in tested patients. Interestingly, FcγRIIb/c expression was significantly lower on B cells in FCGR2C.nc-ORF patients compared to FCGR2C.nc-stop patients (figure 3B). When adding healthy (Caucasian) controls with FCGR2C.nc-ORF or FCGR2C.nc-stop, we still find a lower FcγRIIb/c expression on FCGR2C.nc-ORF individuals (figure 4A). In addition, FcγRIIa expression was lower on monocytes in FCGR2C.nc-ORF patients compared to FCGR2C.nc-stop patients (Figure 3C). However, when we add healthy (Caucasian) controls with FCGR2C.nc-ORF or FCGR2C.nc-stop, the difference in FcγRIIa expression is no longer present (figure 4B). Lastly, no significant differences in FcγR expression were found on other cell types (figure 3A-D).
Discussion
This is the first study investigating the association between polymorphisms in the FCGR2/3 gene cluster and allo-immunization against RBC antigens. In a combined cohort of French and Dutch patients with SCD, the FCGR polymorphism FCGR2C.nc-ORF was strongly associated with a lower risk of allo-immunization, conferring a more than threefold lower risk for patients with this polymorphism. The protective effect appeared to be strongest when cases with exclusively Rh or K allo-antibodies were excluded. These findings suggest that FCGR2C.nc-ORF is involved in the pathophysiology of RBC allo-immunization in SCD, especially for immunization against antigens other than Rh and K.

We did not find a significant association between FCGR2/3 polymorphisms and DHTR. It must be noted that DHTR is a multifaceted complication of allo-immunization that is often difficult to diagnose since the symptoms mimic vaso-occlusive crisis. Moreover, the exact mechanism of DHTR is still unclear. DHTR has also been reported in the absence of detectable allo-antibodies. It is hypothesized that FcγR-mediated phagocytosis or ADCC phagocytosis is mediated by complement receptors, or hemolysis may be induced via complement activation and the formation of the membrane attack complex. In addition, bystander hemolysis or continuation of autologous RBCs during vaso-occlusive crisis have been postulated to mediate DHTR. Therefore, DHTR is a complex process that most likely cannot solely be attributed to FcγRs.

The protective effect of FCGR2C.nc-ORF was strongest when we excluded cases with only Rh or K antibodies. Moreover, in patients with the protective FCGR2C.nc-ORF haplotype that paradoxically did form allo-antibodies, the majority primarily formed antibodies of Rh or K specificity, beyond any other RBC antigen-specific antibodies. These data suggest that allo-immunization against the highly immunogenic Rh or K antigens appears to be less dependent on the immunogenetic background of a patient and supports the clinical relevance of extended antigen matching for these antigens in patients with SCD. This would also explain why the protective association did not persist in the Dutch subcohort of patients, as Rh or K antibodies were significantly more frequent here.

The protective marker for allo-immunization that we have identified, lies within the FCGR2C gene. FCGR2C expression depends on a combination of three minor alleles, namely the c.169T>C variant in exon 3 which can lead to an ORF (FCGR2C-ORF); and two splice-site mutations in intron 6 just before exon 7, c.798+1A>G and c.799-1G>C, which can introduce a premature stop codon (FCGR2C.nc-ORF). When expressed, FcγRIIc is an activating receptor on NK cells, B cells, monocytes and neutrophils that can induce innate immune responses such as ADCC. Moreover, FcγRIIc counterbalances the inhibitory FcγRIIb on B cells and is thought to enhance antibody responses to immuni-
zation,\textsuperscript{54} which suggests a pivotal role for this gene in (allo-)immunization. However, FCGR2C\_nc-ORF is a polymorphism in a non-expressed gene. It is unlikely that this polymorphism is of any direct functional effect. Instead, it may act as a marker for a linked functional variation located elsewhere within the relevant region of chromosome 1. FCGR2C polymorphisms in non-coding variants of the gene have previously been associated with HIV-1 vaccine protection.\textsuperscript{55} In a follow-up study by Peng et al., it was suggested that these FCGR2C polymorphisms (present in introns) were associated with expression of Fc\textgamma RIIa and Fc receptor-like A (FCRLA).\textsuperscript{56} FCRLA is a FCGR homologue that can be selectively expressed on B cells and is suggested to be involved in B cell development.\textsuperscript{57}

In our phenotype analysis, we found a reduced expression of Fc\textgamma RIIb on B cells in patients with FCGR2C\_nc-ORF. This may provide a clue for a functional explanation of the observed, protective effect against RBC allo-immunization. It is possible that FCGR2C\_nc-ORF is linked to an unknown polymorphism in the promotor region of FCGR2B, thereby indirectly affecting Fc\textgamma RIIb expression on B cells. However, additional genotype and phenotype analysis of a larger number of FCGR2C\_nc-ORF and FCGR2C stop patients will be required to further explore this hypothesis.

FCGR2C\_nc-ORF and its potential linkage did not seem to induce differential expression of Fc\textgamma RII or Fc\textgamma RIII on neutrophils, monocytes, T cells or NK cells (figure 3). We have not been able to check Fc\textgamma R expression on freshly isolated tissue dendritic cells or macrophages which are the principal antigen presenting cells. Moreover, since the extracellular domain of Fc\textgamma RIIc is identical to Fc\textgamma RIIb\textsuperscript{58}, we cannot discriminate between these two receptors with flow cytometry using blood cells other than NK cells.\textsuperscript{45} Although previously reported in SLE patients to be relevant on B cells, we have not observed any Fc\textgamma RIIc expression in these cells either.\textsuperscript{59} In addition, due to their high degree of homology, our antibodies cannot discriminate between Fc\textgamma RIIIf and Fc\textgamma RIIIfb (the latter being solely expressed on neutrophils). It is therefore possible that our phenotype analysis was not sensitive enough to identify subtle differences in Fc\textgamma R expression. Besides inducing differential expression of Fc\textgamma Rs, a polymorphism can also alter the affinity of the receptor for IgG. This type of functional variation will not be detected with our phenotype analysis but requires extensive genotype analysis.

Previous studies have highlighted the ethnic variation in the FCGR2/3 gene locus.\textsuperscript{54,60-70} While Africa holds the most genetically diverse populations, it is striking that the FCGR2C gene seems to be less polymorphic in our SCD population compared to Caucasians.\textsuperscript{60} FCGR2C is expressed in approximately 18-33\% of Caucasians\textsuperscript{43,60}, while it is rarely found in African individuals.\textsuperscript{60} In
our cohort, 3% of the patients had the FCGR2C-ORF variant. This frequency may have been too low for us to detect associations for this variant with allo-immunization. It would be interesting to assess this association in a larger SCD cohort, since FCGR2C-ORF has been found to be associated with idiopathic thrombocytopenic purpura, an antibody-mediated auto-immune disease.\textsuperscript{43}

This also addresses a limitation to our study. We may have missed weak associations of genetic variations that are less common in our study due to our limited sample size. Therefore, this study does not exclude the possibility that other FCGR polymorphisms can be associated with allo-immunization, although they did not reach significance in the current study. Secondly, our study is specific to allo-immunization in patients with SCD with originally an African background. These results can therefore not directly be extrapolated to other transfused populations, and should be subject of future study.

In conclusion, we have found a protective association between FCGR2C.nc-ORF and RBC allo-immunization in SCD. This association was strongest for immunization against antigens other than the highly immunogenic Rh or K. These findings suggests that the genetic constitution of patients is of less importance in the formation of Rh and K allo-antibodies, emphasizing the importance of extended matching for these RBC antigens. Future studies are needed to understand the functional and immunological mechanism behind the protective effect of FCGR2C.nc-ORF on allo-immunization. This may ultimately add to the development of preventive strategies.
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Authorship contributions
JS and SM analyzed the data and wrote the manuscript. JS, SP, AH, FP and KF coordinated the collection of DNA samples. SM, SN and JG performed the MLPA experiments and analysed the data. SM, JS and MT performed the statistical analysis. SM and CB performed the phenotype analysis. SM, JS, SN, JG, BB, KF, RvB, FNP, TK and TvdB interpreted the data. TvdB, TK, FNP, RvB, and KF supervised the study and edited the manuscript. TvdB, FNP, TK, KF designed the study, and supervised the interpretation and statistical analysis of the data. All other authors enrolled patients in the study and revised and approved the final manuscript.

Conflict-of-interest disclosure
The authors declare no competing financial interests.
References


25. Tatari-Calderone, Z. et al. rs660 polymorphism in Ro52 (SSA1; TRIM21) is a marker for age-dependent tolerance induction and efficiency of allo-immunization in sickle cell disease. Molecular immunology 47, 64-70 (2009).


39. Eckhardt, C. L. et al. The Fc gamma receptor Ila R131H polymorphism is associated with inhibitor development in severe hemophilia A. Journal of throm-


57. Miller, I., Hatzivassiliou, G., Cattoretti, G., Mendesohn, C. & Dalla-Favera, R. IRTAs: a new family of


Supplementary methods

Data collection and definitions
The following data were available for both the Dutch and French study cohorts: date of birth, gender, ethnicity, sickle cell genotype, total transfusion exposure at the end of follow-up (number of RBC units), history of antibody formation. Information on the occurrence of a DHTTR was only available for the French cohort.

In the primary analysis of this study, we assessed the association between polymorphisms in the FCGR2 and FCGR3 genes, and RBC allo-immunization. Cases were defined here as all patients with a clinically significant allo-antibody. Controls were defined as all patients with a negative history of allo-immunization, with a minimum transfusion exposure of at least 20 RBC units. Patients with exclusively one of the following antibodies were excluded from this analysis: auto-antibodies, cold antibodies and naturally occurring antibodies such as anti-A, anti-B, anti-A1, anti-IH, anti-H, anti-A1, anti-I, anti-Lea, anti-Leb and anti-P1. Anti-M was defined as naturally occurring if demonstrated upon first antibody screening with no history of prior transfusion exposure.

To test the robustness of our findings in the primary analysis, we performed the following sensitivity analyses. Firstly, a more strict definition for controls was applied where only non-immunized patients with a transfusion exposure of at least 100 units served as controls. A second sensitivity analysis was performed to assess the effect of immunization against the highly immunogenic Rh and K antigens (RhD, C, c, E, e and/or K) on the association between FCGR polymorphisms and allo-immunization. We hypothesized that the genetic constitution of a patient is less important for the formation of Rh and K specific allo-antibodies, since these antibodies are so readily formed. To test this hypothesis, we categorized allo-immunized patients (cases) in two subgroups: the first group contained patients with exclusively Rh or K specific antibodies, the second group contained patients with at least one antibody other than Rh or K. Associations between FCGR polymorphisms and allo-immunization were assessed and compared between these two subgroups.

Lastly, we performed a separate analysis in the French cohort to assess associations between FCGR polymorphisms and the occurrence of DHTTR. DHTTR information was solely available for patients from the French cohort. In this analysis, cases were defined as all patients with a positive history of DHTTR. Controls were defined as patients with a negative history of DHTTR, with a minimum transfusion exposure of at least 20 RBC units, irrespective of the allo-immunization status. DHTTR was defined as described previously.

Transfusion and laboratory policies
Patients in this study may have been exposed
to both units matched only for AB0 and RhD antigens, units additionally matched for Rh phenotype and K, and units more extensively matched also for at least Fy(a) and, if possible, Jk(b), S and s. In addition, once a patient developed an antibody, matching was extended to the antigen against which the antibody was produced.

In both cohorts patients were routinely screened for allo-antibodies before transfusion, using a 3-cell panel. Screening was repeated at least every 72 hours if further transfusions were required. Gel column agglutination methods were used to assess both RBC antigen phenotype and potential antibodies. In case of a positive allo-antibody screening, a standard panel was used to specify the allo-antibody.

**Flow cytometry**

Peripheral blood mononuclear cells (PB-MCs) were isolated from fresh heparinized whole blood from SCD patients with the FCGR2C.nonclassical-ORF or FCGR2C.stop polymorphism by lysis of red blood cells by an isotonic ammonium-chloride buffer. To define leukocyte populations, the following monoclonal antibodies were used: anti-CD3 (clone SK7, PE-Cy7 labeled, BD pharmin-gen), anti-CD14 (clone M5E2, PE-Cy7 labeled, BD pharmin-gen), anti-CD19 (clone HIB19, APC labeled, BD pharmin-gen) and anti-CD56 (clone B159, APC labeled, BD Pharmingen). Fcγ-receptors expression was measured by use of the following monoclonal antibodies: anti-CD64 (clone 10.1, FITC labeled, BD pharmingen), anti-CD32a,b,c (clone AT10, FITC-labeled, Bio-connect), anti-CD32b,c (clone 2B6, Alexa Fluor 488 labeled, a generous gift from MacroGenics) and anti-CD16 (clone 3G8, FITC labeled, BD pharmin-gen). Non-specific binding and background fluorescence were corrected for by subtracting the median fluorescence intensity (MFI) of relevant isotype controls with the same fluorescent label from the MFI of the FcγR specific antibodies. Cells were analyzed using a FACS CANTO II (BD Biosciences) equipped with FACSDiva Software (BD Biosciences).

**References in supplementary methods**

Table S1. Copy numbers and allele frequencies of FcγR II and III in 106 control patients (no DHTR) and 51 patients with DHTR

<table>
<thead>
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<th>Copy number region</th>
<th>Controls/no allo-immunization, n (%)</th>
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<td>CNR1</td>
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<tr>
<td>CNR3</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CNR4</td>
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Allele frequencies

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<tr>
<td></td>
<td>39 (37%)</td>
<td>92 (87%)</td>
<td>52 (49%)</td>
<td>103 (97%)</td>
<td>96 (86%)</td>
<td>43 (41%)</td>
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<td>14 (13%)</td>
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<td>3 (3%)</td>
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<td>0 (0%)</td>
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DHTR: Delayed Hemolytic Transfusion Reaction, FcγR: Fc gamma receptor, CNR: copy number region, OR: odds ratio, CI: confidence interval, n.a.: not applicable
### Table S1. Continued

<table>
<thead>
<tr>
<th>Copy number region</th>
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<th>OR</th>
<th>95% CI</th>
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<td>CNR3</td>
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<td>.05-19.19</td>
<td>1.000</td>
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<td>n.a.</td>
<td>n.a.</td>
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### Allele frequencies

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<tr>
<th>Allele frequencies</th>
<th>Cases/allo-immunized patients, n (%)</th>
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<th>95% CI</th>
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<td>FCGR2A-131H</td>
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<td>FCGR2C.nc-ORF</td>
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<td>0.15-1.51</td>
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<tr>
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