Regulation of complement activation on red blood cells

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Citation for published version (APA):
Thielen, A. J. F. (2019). Regulation of complement activation on red blood cells.

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Chapter 6

CRISPR/Cas9 generated human CD46, CD55 and CD59 knockout cell lines as tool for complement research


Astrid J.F. Thielen, Iris M. van Baarsen, Marlieke L. Jongsma, Sacha Zeerleder, Robbert M. Spaapen and Diana Wouters
Chapter 6

ABSTRACT

**Background:** To prevent unwanted complement activation and subsequent damage, complement activation must be tightly regulated on healthy host cells. Dysregulation of the complement system contributes to the pathology of diseases like Paroxysmal Nocturnal Hemoglobinuria and atypical Hemolytic Uremic Syndrome. To investigate complement regulator deficiencies, primary patient cells may be used, but access to patient cells may be limited and cells are heterogeneous between different patients. To inhibit regulator function on healthy host cells, blocking antibodies can be used, though it may be difficult to exclude antibody-mediated effects. To circumvent these issues, we created single and combined complement regulator human knockout cells to be able to *in vitro* investigate complement activation and regulation on human cells.

**Methods:** CRISPR/Cas9 was used to knockout (KO) complement regulatory proteins CD46, CD55 and/or CD59 in human HAP1 cells. Single cell derived cell lines were profiled by Sanger sequencing and flow cytometry. To confirm the lack of complement regulatory function, the cells were exposed to complement in normal human serum and subsequently C3 and C4 deposition on the cell surface were detected by using flow cytometry.

**Results:** We created single KO cell lines that completely lacked CD46, CD55 or CD59. We additionally generated double CD46/CD55, CD46/CD59 and CD55/CD59 KOs and triple CD46/CD55/CD59 KOs. Upon classical pathway activation, deletion of CD46 resulted in increased C3 and C4 deposition, while deleting CD55 mainly resulted to increased C3 deposition, confirming their reported function in complement regulation. Upon alternative pathway activation, C3 deposition was only observed on the triple CD46/CD55/CD59 KO cells and not on any of the other cell lines, suggesting that human cells are resistant to spontaneous complement activation and suggesting a role for CD59 in C3 regulation.

**Conclusions:** The generation of complement regulator KO cell lines provides a relevant tool for future *in vitro* investigations of complement activation and regulation on human cells. Furthermore, these cell lines may also be helpful to evaluate therapeutic complement inhibitors and may shed light on novel roles of complement regulatory proteins as we here observed for CD59.
INTRODUCTION

The complement system is part of the innate immune system and consists of circulating and membrane-associated proteins that are involved in the clearance of pathogens, dying cells and immune complexes. Because of its potent pro-inflammatory and potentially destructive effects, complement activation must be tightly regulated on healthy host cells. Dysregulation of the complement system, due to inefficient regulation and/or overstimulation, can result in damage to host cells and contributes to the pathology of many inflammatory diseases\(^1-3\). A hallmark disease of defective complement regulation is Paroxysmal Nocturnal Hemoglobinuria (PNH), which is an acquired condition in which hematopoietic stem cells lack the surface complement regulators CD55 and CD59 due to a mutation in the PIG-A gene that is responsible for GPI-anchorage of these proteins. This complement regulator deficiency renders the cells very sensitive to complement activation, resulting in complement mediated destruction of affected hematopoietic cells.

Surface complement regulators such as CD55 and CD59 are important in the regulation of the complement system, which can be activated via one of three different pathways: the classical (CP), the lectin (LP) and the alternative pathway (AP). In the end, all three pathways trigger the facilitation of phagocytosis by opsonization, formation of the membrane attack complex (MAC) causing osmolytic lysis of target cells and the formation of anaphylatoxins that contribute to inflammation by attracting leukocytes to the site of infection\(^4,5\). On human cell surfaces complement regulatory proteins CD46, CD55 and CD59 can be found that regulate complement activation in different ways (Fig. 1). CD55 promotes the rapid dissociation of C3 convertases of all three pathways via decay accelerating activity. CD46 acts as a cofactor for factor I that enzymatically inactivates C3b and C4b into iC3b and iC4b, respectively.

![Figure 1: Mechanism of complement regulation by surface complement regulators CD46, CD55 and CD59. CD55 promotes dissociation of all C3 convertases via decay accelerating activity. CD46 has cofactor activity for factor I to enzymatically inactivate C3b and C4b into iC3b and iC4b, respectively. CD59 prevents C9 from polymerization and thereby inhibits formation of the MAC.](image-url)
Finally, CD59 prevents C9 from polymerization and thereby inhibits formation of the MAC\textsuperscript{1,6,7}.

To investigate complement regulator deficiencies, primary patient cells may be used or blocking antibodies to inhibit the function of the regulators \textit{in vitro}. However, access to patient cells may be limited, as diseases associated with complement regulator deficiency are rare and cells can be heterogeneous between different patients. Blocking antibodies may induce unwanted antibody-mediated effects and moreover, it may be very costly to use commercially available monoclonal antibodies. Furthermore, mouse models with deficiency of complement regulators are available, but it is known that with respect to their complement system mice are not comparable to humans. To be able to study complement regulation on human cell surfaces in more detail, and to circumvent above mentioned issues, we created single and combined complement regulator knockout (KO) cells using CRISPR/Cas\textsuperscript{9,9}. We specifically targeted CD46, CD55 and CD59 in a human cell line (HAP1 cells) and validated the functional consequence of lacking complement regulators on the cell surface by incubating the cells with normal human serum and measuring C3 and C4 deposition by flow cytometry as readout for complement activation. We conclude that these complement regulator KO cells are a relevant tool for future \textit{in vitro} complement research and may also be used to evaluate novel therapeutic complement inhibitors. Therefore, complement regulator deficient cell lines are useful for \textit{in vitro} investigations of complement activation and regulation on human cells.

**MATERIALS AND METHODS**

**Serum samples**

Normal human serum (NHS) from eight healthy male volunteers with blood group type A, was pooled (NHS pool) after informed consent, and aliquots were stored at -80°C. As a negative control for complement activation, NHS was heat inactivated (HI-NHS) for 30 minutes at 56°C.

**Antibodies**

In house generated monoclonal antibody (mAb) anti-CD46-3 and anti-CD55-1, and anti-CD59 (clone VJ1/12.2; Abcam, Cambridge, United Kingdom) were used to detect cell surface expression by FACS. In-house generated mAbs anti-C3-19 and anti-C4-10\textsuperscript{10,11} were used to measure complement deposition and polyclonal antibodies anti-IgG-FITC and anti-IgM-FITC (Sanquin Reagents, Amsterdam, The Netherlands) were used to measure antibody binding. In-house blocking mAb anti-C1q-85\textsuperscript{12} was used to inhibit the CP and blocking mAb anti-FH-2 was used to inhibit
FH. Blocking mAb anti-C5 (Eculizumab; Alexion Pharmaceutical, Cheshire, CT) was obtained from remnants of used Soliris® injection bottles and was used to inhibit terminal complement activation.

Cell culture
HAP1 cells\textsuperscript{13,14} (Haplogen Genomics, Wien, Austria) were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM; Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (v/v) (FCS; Sigma-Aldrich, Steinheim, Germany), 100 U/mL penicillin (Invitrogen, Paisley, United Kingdom) and 100µg/ml streptavidin (IMDM++; Invitrogen) at 37°C and 5% CO2 and used to generate human cell lines deficient for CD46, CD55 and/or CD59.

Phoenix cells were cultured in Dulbecco’s Modified Eagle Medium (IMDM; Lonza, Basel, Switzerland) supplemented with 10% FCS (v/v) at 37°C and 5% CO2 and used to generated virus for the viral transduction.

Cells were washed with phosphate buffered saline (PBS; Fresenius Kabi, Zeist, The Netherlands) and detached using 0.1% trypsin-EDTA (Gibco, Carlsbad, CA). Subsequently, trypsin was inactivated by FCS present in the culture medium.

CRISPR/Cas9 approach to target CD46, CD55 and/or CD59 in HAP1 cells
A pX330 vector (Addgene, Cambridge, MA) containing CRISPR guide RNA (gRNA) CD46, CD55 or CD59 and X-tremeGENE HP DNA Transfection Reagent (Sigma-Aldrich) were used (ratio 1:3) to transfect HAP1 cells. Single cell derived clonal cell lines were generated and to confirm the KO phenotype of the cells, firstly conventional Sanger sequencing was performed and subsequently flow cytometry was performed. Next, phenotypical confirmed human cells deficient for CD55 were transfected with pX330 vector containing CRISPR gRNA CD46 and the phenotypical confirmed human cells deficient for CD59 were transfected with pX330 vector containing CRISPR gRNA CD46 or CD55 to create human cells deficient for CD46/CD55, CD46/CD59 and CD55/CD59 (double KO cell lines). Lastly, phenotypical confirmed human cells deficient for CD46/CD59 were transfected with pX330 vector containing CRISPR gRNA CD55 to create human cells deficient for CD46/CD55/CD59 (triple KO cell line) and also this phenotype was confirmed using conventional Sanger sequencing and subsequently flow cytometry.

CRISPR gRNA sequences are shown in Table 1 and 1.0µg/mL DyLight 488-conjugated anti-CD46-3, 1.0µg/mL DyLight 647-conjugated anti-CD55-1 and 1:12.5 CF405M-conjugated anti-CD59 were used to detect surface complement regulators. LSR Canto II flow cytometer (BD Biosciences, Breda, The Netherlands) was used for measuring and data analysis was performed using FlowJo software v1.0 (Treestar, Ashland, OR).
Viral transduction

Phoenix cells were transfected with pMXs puro vector containing CD46, CD55, CD59 or nothing (empty vector control) using 2.5M CaCl2 and a 50mM Hepes buffer supplemented with 280mM NaCl and 1.5mM Na2HPO4, 12mM D(+)-Glucose and 10mM KCl. After 48 hours, virus was collected and stored at -80°C. Subsequently, human cells deficient for CD46/CD55/CD59 were transduced with virus containing CD46, CD55, CD59 or nothing by using IMDM supplemented with 10% FCS and 80µg/ml protamine sulphate (Sigma-Aldrich). Thereafter a complement deposition assay (see section below) was performed and both surface complement regulator expression and complement deposition were analyzed using flow cytometry.

Table 1. Sequences of CRISPR gRNAs used for transfection of HAP1 cells to generate human cells deficient for CD46, CD55 and/or CD59. Bold: consensus sequence needed for cloning into pX330 vector. gRNA: guide RNA.

<table>
<thead>
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<th>gRNA sequence (5’-3’)</th>
<th>forward</th>
<th>reverse</th>
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<tr>
<td>CD46</td>
<td>CACCGAAGGGACACTCGCGGCGGC</td>
<td>AAACGCCGCAGGTGTCCTTTTC</td>
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<tr>
<td>CD55</td>
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<tr>
<td>CD59</td>
<td>CACCAGAAGGTCTCTGCTTTC</td>
<td>AAACACAGGACAGACCTCTTG</td>
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Complement deposition assay

The collected HAP1 cells were washed three times with PBS. Complement activation via all three complement pathways (CP, LP and AP) was allowed by incubating 1*10^6 HAP1 cells for 1 hour at 37°C with 25% NHS-pool (v/v), diluted in veronal buffer (VB; 3mM barbital, 1.8mM sodium barbital, 145mM NaCl, pH 7.4) supplemented with 0.05% gelatin (w/v), 10mM CaCl2 and 2mM MgCl2 (VBG++) in the presence of at least equimolar blocking mAb anti-C5. To allow only AP complement activation, VB with 0.05% gelatin (VBG) supplemented with 20mM EGTA and 10mM MgCl2 (VBGMgEGTA) was used. After incubation, HAP1 cells were washed with PBS supplemented with 0.5% (v/v) bovine serum albumin (BSA) before staining with fluorescently labeled antibodies.

C3 and C4 deposition was detected by using 1.0µg/ml DyLight 488-conjugated or DyLight 647-conjugated anti-C3-19 and 1.0µg/ml DyLight 647-conjugated anti-C4-10 and antibody binding was detected by using 1:100 antibodies anti-IgG-FITC and anti-IgM-FITC. HAP1 cells were incubated with the mentioned antibodies in the dark at room temperature for 30 minutes. In addition, 1µM 4′,6-diamidino-2-phenylindole (DAPI; Thermo Scientific, Rockland, IL) was added to discriminate between living and dead cells.
RESULTS

**Human cell lines were genome-edited to lack surface complement regulators CD46, CD55 and/or CD59**

Surface complement regulators CD46, CD55 and CD59 were targeted in HAP1 cells by CRISPR/Cas9 using gRNAs targeting the first exon of each gene. We next generated single cell derived clonal cell lines to confirm the KO phenotype of the cells by conventional Sanger sequencing. All KO cell lines we generated were confirmed to contain a frameshifting base pair mutation in their first exon, indicating that the respective genes were not encoding functional proteins anymore (Fig. 2A). Next, we analyzed the cells by FACS and found that, in contrast to wild type (WT) HAP1 cells, targeted cells completely lacked CD46, CD55 or CD59 surface expression (Fig. 2B). Only the genome-edited regulators were affected, leaving the other surface complement regulators intact, indicating that we generated very specifically single KO cell lines and that the expression of each of these regulators is independent of the others. In addition, we created double KO cell lines for surface complement regulators CD46/CD55, CD46/CD59 and CD55/CD59, by targeting a second surface complement regulator in established single KO cells (Fig. 2C+D). Finally, a triple KO cell line was generated by targeting CD55 in the double KO cell line for CD46/CD59. These cells were completely lacking CD46, CD55 and CD59 on the cell surface as shown by FACS (Fig. 2E+F). Thus, by unbiased selection of genome-edited cell lines based on their genomic sequence, we acquired a collection of cell lines each expressing a unique combination of complement regulators.

**CP-mediated complement deposition is increased on human cells deficient for surface complement regulators**

In order to test to what extent complement is activated on WT HAP1 cells, these cells were incubated with 25% NHS diluted in a buffer containing calcium and magnesium allowing complement activation via all three pathways. Both C3 and C4 deposition were observed on WT HAP1 cells, while incubation with HI-NHS was completely negative. In the presence of a blocking anti-C1q mAb, all complement deposition was inhibited. No spontaneous AP activation seems to have occurred, as we observed no residual C3 deposition after blocking the CP (Fig. 3A). Since we also observed binding of both IgG and IgM from NHS to the WT HAP1 cells (Fig. 3B), we concluded that these antibodies activated the CP leading to C4 and C3 deposition.
Chapter 6

A CD46 KO

B CD46 KO

C CD46/CD55 KO

CD55 KO

D CD46/CD55/CD59 KO

CD59 KO

E CD46/CD55/CD59 KO

CD55/CD59 KO

F CD46/CD55/CD59 KO

CD55 KO (CD55KO on CD46/CD59KO background)

C46/CD95 KO (CD55KO on CD46KO background)
Next, we investigated the functional consequence of deleting one or more surface complement regulators during CP initiated complement activation on human HAP1 cells. To prevent MAC-mediated lysis of the cells, all incubations were in the presence of blocking anti-C5 mAb. As expected, knocking out CD55 resulted in increased C3 deposition on the cell surface as compared to WT cells, while no effect on C4 deposition was observed. By knocking out CD46, both C3 and C4 deposition were strongly increased on the cell surface. Remarkably, the observed effect on C3 deposition was much stronger on CD46 KO cells than on CD55 KO cells. CD59 deficiency had no effect on C3 or C4 deposition (Fig. 3C). Double KO cells for CD46/CD55 and CD46/CD59 and the triple KO cells for CD46/CD55/CD59 showed the same phenotype as CD46 single KO cells, that is increased C3 and C4 deposition. Unexpectedly, no effect on complement deposition was observed on the human cells deficient for CD55/CD59, while a single CD55 deficiency resulted in increased C3 deposition (Fig. 3D+E). Blocking C1q fully inhibited C3 and/or C4 deposition on all cell lines (data not shown), indicating that complement activation was initiated via the CP. Our results show that the makeup of cell surface complement regulators determines the extent of CP initiated complement deposition. In these experiments CD46 was the most dominant regulator for both C3 and C4 deposition upon CP activation.

Spontaneous AP-mediated complement deposition only observed on human cells lacking all surface complement regulators

Next, we investigated the effect of knocking out surface complement regulators on spontaneous AP activation, by incubating the cells with 25% NHS diluted in a buffer containing MgEGTA to abolish any CP or LP activity. Under these conditions, no C3 deposition was observed on WT HAP1 cells, or any of the single (CD46, CD55, CD59) or double (CD46/CD55, CD46/CD59 and CD55/CD59) KO cell lines (Fig. 4A+B). This shows that HAP1 cells are remarkably resistant to spontaneous AP complement activation. As HAP1 cells do not express other surface regulators that can control spontaneous AP complement activation, this leaves fluid phase complement regulator Factor H (FH) as the only candidate for controlling the AP. Indeed, inhibition of FH with a blocking mAb, resulted in strong C3 deposition on the cell surface of double CD46/CD55 KO cells. However, as long as one C3
Figure 3: The effect of knocking out CD46, CD55 and/or CD59 on complement deposition of all three complement pathways. (A+B) WT HAP1 cells were incubated with NHS and a buffer supplement with calcium and magnesium. (A) C3 and C4 deposition in presence or absence of CP inhibitor αC1q. Grey: HI-NHS, solid: NHS, dotted: NHS + αC1q. (B) IgG and IgM binding. Grey: unstained, solid: NHS.
convertase regulator (CD46 or CD55) was present on the cell surface, spontaneous AP activation was completely controlled (Fig. 4C). Surprisingly, upon deletion of CD59 on a background of CD46/CD55 deficient cells (triple KO CD46/CD55/CD59), a subset of cells became positive for C3 deposition upon incubation with NHS in MgEGTA (Fig. 4D), while no C4 deposition was observed (data not shown).

Figure 4: During AP activation only complement deposition was observed on human cells deficient for CD46, CD55 and CD59. Human cells deficient for CD46, CD55 and/or CD59 were incubated with NHS in presence of absence αFH and a buffer supplement with magnesium. C3 deposition was assessed on (A) single KO cell lines, (B) double KO cell lines, (C) double KO cell lines in presence of αFH and (D) triple KO cell line. Grey: HI-NHS, solid: WT NHS, dashed: KO NHS, dotted: KO NHS + αFH. All data are representative FACS histograms, n=3.

← Figure 3, continued

(C+D+E) Human cells deficient for CD46, CD55 and/or CD59 were incubated with NHS and a buffer supplement with calcium and magnesium. C3 and C4 deposition was assessed on (C) single KO cell lines, (D) double KO cell lines and (E) triple KO cell line. Grey: HI-NHS, solid: WT NHS, dashed: KO NHS. All data are representative FACS histograms, n=3.
No off-target effects were shown by the CRISPR/Cas9 approach on the phenotype of the human cells deficient for surface complement regulators. To confirm that the functional effects observed in the previous experiments were mediated by knocking out the specific surface complement regulators and not by off-target effects of the gRNAs used, the individual regulator genes were retrovirally transferred to the human cells triple deficient for CD46/CD55/CD59. Fig. 5A shows that after transduction a subset of cells re-expressed CD46 (positive) and activation of the AP only resulted in C3 deposition on the CD46 negative cells (triple KO CD46/CD55/CD59), and not on the CD46 positive cells (comparable to double KO CD55/CD59). In addition, transduction of CD55 or CD59 resulted in a subset of cells re-expressing CD55 or CD59 (comparable to double KO CD46/CD59 and CD46/CD55 respectively), which also showed no C3 deposition (Fig. 5B+C). These reintroduction experiments rule out any off-target effects of our CRISPR/Cas9 approach.

Figure 5: Retroviral gene transfer restores phenotype for complement regulation. Human cells deficient for CD46/CD55/CD59 were virally transduced with DNA from (A) CD46, (B) CD55 and (C) CD59 and thereafter incubated with NHS and a buffer supplement with magnesium and assessed for marker expression (solid left panel) and C3 deposition (solid right panel). Grey left panel: unstained, Grey right panel: HI-NHS. All data are representative FACS histograms, n=2.
DISCUSSION

In this study we describe the successful generation of genome-edited human complement regulator deficient cell lines. To our knowledge, we are the first that generated 100% genetic single (CD46, CD55, CD59), double (CD46/CD55, CD46/CD59, CD55/CD59) and triple (CD46/CD55/CD59) KOs in a human cell line, providing us with a unique toolset for further complement research. The unlimited availability of complement regulator deficient cell lines may be helpful to test in vitro the role of new therapeutic complement inhibitors and possible novel roles of complement regulatory proteins can be investigated. As an example, we here describe preliminary findings on a potential novel role of CD59 in regulating the AP C3 convertase, next to its role in inhibiting the MAC, which deserves further investigation. As all incubations were performed in the presence of anti-C5, this observed C3 deposition cannot be the result of MAC-mediated cell death. It has been shown before that inhibiting CD59 on healthy RBCs with blocking antibodies resulted in increased C3 deposition\textsuperscript{15-17}. We confirmed that the observed effect of lacking CD59 on C3 deposition is not an off-target effect of the CRISPR/Cas9 approach as by using retroviral transduction of CD59, the phenotype of the human cells deficient for CD46/CD55/CD59 was restored. It still needs to be elucidated by which mechanism CD59 affects C3 regulation and whether this is physiologically relevant.

The observed complement deposition on WT HAP1 was most probably caused by CP activation via antibodies. It has been described that natural antibodies against altered carbohydrate structures on malignant cells can be found in the blood of healthy persons\textsuperscript{18}. As HAP1 cells are a derivative of a chronic myeloid leukemia cell line, this might be the origin of the antibodies found in our study. Human leukocyte antigen antibodies were ruled out as only male healthy volunteers were used to create our nhs pool.

Our data showed that upon CP activation both CD46 and CD55 are important for the regulation of C3 deposition, while only CD46 is important for the regulation of C4 deposition. Barilla-LaBarca et al.,\textsuperscript{19} investigated CHO cells transfected with CD46 or CD55, these cells normally do not express human surface complement regulators, and demonstrated that surface complement regulator CD46 is important for the regulation of C4 deposition and CD55 for the regulation of C3 deposition during CP activation, which is in line with our results\textsuperscript{20}. In contrast though, they have shown that CD46 on their CHO cells did not regulate C3 deposition during CP activation\textsuperscript{19,20}. In line with our study, downregulation of either CD46 or CD55 on breast cancer cells using siRNA resulted in CP-mediated C3 deposition\textsuperscript{21}. We observed that the effect on C3 deposition was much stronger on the CD46 KO cells than on the CD55 KO cells. This is most probably because CD46 directly affects C3 deposition, but also indirectly because there is less C4 activation and thus less formation of the CP C3
convertase. Deleting CD59 had no effect on C3 or C4 deposition upon CP activation as was expected since CD59 is known as inhibitor of late stage complement activation by preventing the formation of the MAC on the cell membrane. Remarkably, the human cells deficient for CD55/CD59 resulted in no increased C3 and C4 deposition compared to WT HAP1, while we at least expected a phenotype of increased C3 deposition from deleting only CD55. The CD55/CD59 KO was generated by targeting CD55 on a background of CD59 confirmed KO cells. Although Sanger sequencing and flow cytometry showed that CD55 was lacking in both CD55 KO and CD55/CD59 KO, the genetic CD55 deficiency was different in both cell lines as they originated from another clone, which might explain why we observed this difference. Another explanation might be that the absence of CD59 negatively modulates C3 deposition on the cell surface. To further investigate the discrepancy between the CD55 single and CD55/CD59 KO, a new double KO should be generated by targeting CD59 on a background of the confirmed CD55 deficient cell line.

By using either CHO cells or rabbit RBCs transfected with human CD46 or CD55, it has been shown that both CD46 and CD55 play a role in the regulation of C3 during AP complement activation. Moreover, when rabbit RBCs were transfected with low concentrations of both CD46 and CD55, these surface complement regulators even had a synergistic function on the regulation of C3 deposition. We however found no AP-mediated C3 deposition upon deletion of CD46 and/or CD55. This result surprised us, as it has been described that C3 deposition occurs on PNH red blood cells, that are deficient for CD55/CD59 and lack CD46 as well, as CD46 is not expressed by RBCs. RBCs do express CD35 which has a similar complement regulatory function as CD46. In the KO HAP1 cells only CD55 and CD59 were deleted, while in PNH cells due to the mutation in the PIG-A gene all proteins that are GPI anchored, including CD55 and CD59 are affected. It is plausible that other GPI-linked proteins have a role in the regulation of C3 deposition, but this needs further investigation. CRISPR/Cas9 may be used to target the PIG-A gene in HAP1 cells and compare the effects on complement activation with CD55/CD59 targeted cells. Most AP-mediated C3 deposition was observed when both CD46 and CD55 were deleted and fluid phase complement regulator FH in the serum was inhibited, confirming that FH is important for the C3 regulation of the AP.

In conclusion, the generation of unlimited available complement regulator deficient cell lines provides a relevant tool for future in vitro complement research to elucidate in depth and under controlled conditions the delicate balance of complement activation and regulation on human cells. Furthermore, these cell lines assist the development of novel therapeutic complement inhibitors for the treatment of complement-mediated diseases and may shed light on possible novel roles of complement regulatory proteins as we here described preliminary findings for a potential novel role of CD59.
Acknowledgement
A.J.F.T. and I.M.B performed experiments and analyzed the results; M.L.J and R.M.S. assisted with the CRISPR/Cas9 approach and viral transduction; A.J.F.T and M.L.J. made the figures; A.J.F.T, S.Z., R.M.S. and D.W. designed the research and wrote the manuscript.

Dr. Robbert Spaapen was supported by a NWO-VENI personal grant (016.131.047) and the rest of this work was supported by an internal grant of Sanquin (PPOC-12-038-PRG).
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