Complement biology in health and disease

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CHAPTER 3

COMPLEMENT C3 CLEAVAGE BY CATHEPSIN-L DOES NOT GENERATE C3A BUT C3A-DESARG AND IS NOT ESSENTIAL FOR SURVIVAL IN A HAP-1 MODEL CELL LINE

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ABSTRACT

Introduction: A novel intracellular role has been described for complement protein 3 expressed in CD4+ T-cells, by indicating that intracellular C3 can be cleaved by Cathepsin-L (CTSL), resulting in signalling by C3a via the intracellular C3aR. This intracellular C3 pathway was hypothesized to be pivotal for the survival of cells. Here, we further explored the CTSL cleavage site in C3 and survival of C3 deficient cells.

Material and methods: Western blots were performed to detect C3 in lysates. C3 protein was incubated together with activated CTSL and the fragments generated were analysed on SDS-page gel and by mass spectrometry (MS). As a model, a C3 knock out cell line was generated from homozygous HAP-1 cells using CRISPR/Cas9. The wild-type cells, mock cells, and C3-deficient cells were analysed regarding morphology, proliferation, and metabolism.

Results: Lysates of cells cultured in the absence of human serum did not reveal the presence of intracellular C3, while lysates of cells incubated in the extracellular presence of normal human serum did reveal C3 bands. *In vitro* exposure of purified C3 to CTSL resulted in the cleavage of C3. Interestingly, this did not result in C3a formation, but in the generation of C3a-desArg, since the arginine was still attached to the N-terminus of the C3b molecule. To study if C3 is essential for cell survival, we generated multiple cells lines from HAP-1 with CRISPR/Cas9. No differences were observed regarding proliferation, morphology and metabolism between the wildtype and C3 deficient HAP-1 cell lines.

Discussion: We demonstrate that processing of C3 by CTSL predominantly generates C3a-desArg and not C3a, and further that C3 or its derived products are not essential for cell survival in a HAP-1 cell line model.

INTRODUCTION

The complement system is an important humoral part of the innate immune system and comprises multiple soluble and membrane bound proteins. Activation of the complement system can occur via three different pathways, each consisting of sequential enzymatic reactions. The main functions described for the complement system are: opsonization, chemotaxis and lysis [1]. Currently, the complement system is no longer viewed as an isolated segment of the innate immune system, as several connections to the coagulation and the adaptive immune system have been identified [2, 3]. This includes the recent description that some complement proteins not only function extracellulary, but also intracellulary independent of the complement system [4, 5].

Studies on intracellular complement have focussed on C3 and C5, referring the new attributed functions as the ''complosome'' [4, 6, 7]. The first description of intracellular complement is provided by Liszewski et al. [5]. Intracellular C3 was reported to be intracellularly cleaved by Cathepsin-L (CTSL) in CD4+ T-cells, resulting in the formation of C3a and C3b. Subsequently, the intracellular C3a bound to the intracellular C3aR where it was involved in the phosphorylation of mTOR. Instead, C3b was reported to shuttle to the cell membrane. Interestingly, CD4+ T-cells from a C3-deficient patient, who is deficient for C3 in the circulation, were reported to be positive for intracellular C3a as analysed by confocal microscopy [5]. In addition, it was found that complement was involved in the metabolism of both CD4+ and CD8+ T-cells via CD46 [8, 9]. The main conclusion of these studies indicate that C3 serves an important role in cell homeostasis research. Subsequently, it was reported that intracellular C3 was only detected after incubation of cells with normal human serum (NHS). Allowing take-up of C3 as hydrolysed C3 ($C3H_2O$) which could then interact with CTSL [10]. Additionally, it was demonstrated that apoptotic cells internalized Factor H (FH), where it could regulate endogenous C3. Interestingly, it was proposed that FH has the potential to bind CTSL and thereby, functions as a cofactor, bringing these two proteins in close proximity to each other [11]. Most recently, intracellular C3 has been connected to the prevention of autophagy in pancreatic islets [12].

The central component of the complement system, C3, is conventionally cleaved by a C3 convertase in to two functional fragments, the larger fragment (170kDa) C3b acts as an opsonin whereas the smaller fragment (10kDa) C3a is a chemoattractant. C3a is, after its generation, quickly modified by carboxypeptidases, removing the C-terminal arginine and thereby generating C3a-desArg [13, 14]. C3a-desArg, also known as Acylation Stimulating Protein (ASP) is no longer able to bind to the C3aR [15]. Conflicting data are reported on the ability of ASP/C3a-to bind the receptor C5L2 [16]. ASP has been linked to metabolism, in which ASP is able to enhance triglyceride synthesis and glucose uptake in adipocytes and cultured skin fibroblasts [17, 18].

Thus, together, a connection between complement and basal mechanisms of the cell homeostasis have been reported. However, different interpretations of reported data have been proposed and expression of intracellular complement components have not been confirmed unambiguously at the protein level. For example, in the study studying the role of C5 in the activation of the inflammasome in CD4+ T-cells, no presence of intracellular C5 has been demonstrated at the protein level [19]. Besides, regarding the presence of intracellular C3, so far this has not been replicated on protein level, also because the antibody originally used for the studies described above is no longer available [5]. Here, we aimed to develop a method to measure C3 in various lysates at the protein level. In addition, we analysed the cleavage product of C3 and CTSL and developed a C3-deficient cell line with the CRISPR/Cas9 system to establish the proposed fundamental requirement of C3 for cell survival / physiology.

MATERIALS AND METHODS

Western blot

Cultured and freshly isolated cells were lysed using NP-40 lysis buffer (Invitrogen, cat#FNN0021) in combination with a protease inhibitor cocktail (Sigma cat#P2714) according to manufacturer's protocol, next these lysates were applied in reduced or non-reduced conditions. For the NHS loaded cells, cells were incubated prior to lysis with NHS at 37°C, after incubation they were washed six times in PBS and the last supernatant was also collected to control for carry over, as previously described [10]. Proteins were separated by SDS/PAGE using Tris-glycine gels (Biorad cat#456-1033). Next proteins were transferred on a Trans-Blot Turbo Transfer pack: mini, 0.2 µM PVDF (Bio-Rad). The membrane was blocked one hour in PBS containing 0.05% Tween and 3% skimmed milk on room temperature. Next, the blot was washed and incubated with the primary antibody overnight on 4°C. After washing with PBS/0.1% Tween the membrane was incubated with the proper secondary antibody labelled with HRP (Dako) for one hour at room temperature. Finally, the blot was washed visualized using ECL Western Blotting Analysis system (GE Healthcare).

Cleavage assay

Protein C3 was cleaved by recombinant CTSL as previously described [5]. Recombinant CTSL and CTSG were activated according to manufacturer's protocol, in short, CTSL (40 ng/µl) was incubated 15 min on ice in the activation buffer (1M MES/5mM DTT/0.0035% Brij35 or 1M MES/5mM /0.2% Rapigest, pH 6.15) without shaking to activate the enzyme. The activated cathepsin was then incubated with commercial purified C3 for different time points at 37°C. If an inhibitor was used, than the inhibitor was incubated with C3 one hour before the activated cathepsin was added. Reactions were stopped by adding 4x Laemmli sample buffer or 10% formic acid, and frozen till further analysis. Reaction mixtures were analysed for C3 fragments using Western blot or Mass spectrometry analysis.

Mass spectrometry

Gel slices containing C3 derived proteins were subjected to reduction with dithiothreitol and alkylation with iodoacetamide using Proteineer DP digestion robot (Bruker). Next, N-terminal amines were acetylated using acetic acid anhydride in order to discriminate between N-termini generated by CTSL (they are acetylated) and peptides generated by trypsin (they are not acetylated) during mass spectrometry work-up. Slices were incubated for 1 hour at room temperature in 20 %(v/v) acetic anhydride, 60 %(v/v) methanol and 10 mM NH4HCO3 pH 8.4. Next, they were washed three times sequentially with acetonitrile and 10 mM NH4HCO3 pH 8.4 prior to protein digestion with trypsin. Peptides were lyophilized, dissolved in 95/3/0.1 v/v/v water/acetonitrile/formic acid and subsequently analyzed by on-line C18 nanoHPLC MS/MS with a system consisting of an Easy nLC 1200 gradient HPLC system (Thermo, Bremen, Germany), and a LUMOS mass spectrometer (Thermo). Fractions were injected onto a homemade precolumn (100 µm × 15 mm; Reprosil-Pur C18-AQ 3 µm, Dr. Maisch, Ammerbuch, Germany) and eluted via a homemade analytical nano-HPLC column (30 cm × 50 µm; Reprosil-Pur C18-AQ 3 um). The gradient was run from 10% to 40% solvent B (20/80/0.1 water/acetonitrile/formic acid (FA) v/v) in 30 min. The nano-HPLC column was drawn to a tip of ~5 µm and acted as the electrospray needle of the MS source. The LUMOS mass spectrometer was operated in data-dependent MS/MS mode for a cycle time of 3 seconds, with a HCD collision energy at 32V and recording of the MS2 spectrum in the orbitrap. In the master scan (MS1) the resolution was 120,000, the scan range 400-1500, at an AGC target of 400,000 @maximum fill time of 50 ms. Dynamic exclusion after n=1 with exclusion duration of 10 s. Charge states 2-5 were included. For MS2 precursors were isolated with the quadrupole with an isolation width of 1.2 Da. First mass was set to 110 Da. The MS2 scan resolution was 30,000 with an AGC target of 50,000 @ maximum fill time of 60 ms.

In a post-analysis process, raw data were first converted to peak lists using Proteome Discoverer version 2.2.0.388 (Thermo Electron), and then submitted to the Uniprot database (452772 entries) using Mascot v.2.2.04 (Matrix Science) for protein and peptide identification. Mascot searches were with 10 ppm and 0.02 Da deviation for precursor and fragment mass, respectively, and trypsin as enzyme with up to two missed cleavages allowed. Methionine oxidation and acetylation were set as variable modification. Carbamidomethyl on cysteïne was set as a fixed modification. False discovery rate was set to 1% and the Mascot ion threshold score to 35. Acetylated spectra were also inspected manually. Data were also processed and visualized using Scaffold 4.10.0 software.

CRISPR/Cas9

Lentiviral vectors for expression of the guide RNAs (**Table 1**) were constructed by cloning synthetic oligonucleotides in the vector pLKO1-Puro-U6-BfuA-stuffer. The pLKO.1-puro U6 sgRNA BfuAI stuffer plasmid was a gift from Rene Maehr & Scot Wolfe

(Addgene plasmid # 50920). This vector allows stable selection for transduced clones using puromycin selection. Lentivirus vectors were produced on 293T cells, titrated, stored, and used as described previously [20]. After several passages with puromycin selection, the Cas9 protein was expressed either via lentiviral (pLV-Cas9-Blast; Sigma-Aldrich Chemie NV, Zwijndrecht, The Netherlands) or adenoviral vectors [21]. Cells were passaged 3 times before, gDNA was collected and clonal cell lines were cultured via limiting dilution.

Table 1. Sequence guides CRISPR

Guide CRISPR	Sequence
C3a Guide 1	TTGGGGTACTTGCCGACTGC GGG
C3a Guide 5	GCAGCTCACGGAGAAGCGAA TGG

T7 Endonuclease I assay

T7 Endonuclease I (T7EI) assay was performed to analyse the CRISPR/Cas9 procedure [22]. Briefly, genomic DNA was extracted from cells using the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's handbook. primers were developed to ensure that the guides were not in the middle of the PCR product. PCR was performed and the PCR product was split in two, one part remained untreated. The other part was denatured and reannealed in NEBuffer 2 (New England Biolabs) using a thermocycler with the following protocol (to re-anneal the PCR products at suboptimal temperature to create mismatched base pairs): 95^{o}C 10min; ↓Ramp to 85^{o}C at 2.0^{o}/s + 85^{o}C 1min; ↓Ramp to 75^{o}C at 0.3^{o}/s + 75^{o}C 1min; ↓Ramp to 65^{o}C at 0.3^{o}/s + 65^{o}C 1min; ↓Ramp to 55^{o}C at 0.3^{o}/s + 55^{o}C 1min; ↓Ramp to 45^{o}C at 0.3^{o}/s + 45^{o}C 1min; ↓Ramp to 35^{o}C at 0.3^{o}/s + 35^{o}C 1min; ↓Ramp to 25^{o}C at 0.3^{o}/s + 25^{o}C 1min; 16^{o}C ∞. The mismatched strands were treated with T7EI for 17 minutes at 37°C (induces double strand breaks at mismatch sites). The initial PCR product and the T7EI product were run on 2% agarose gel to analyse the fragmentation pattern.

Table 2. Sequence primers gDNA C3

Target	5' Forward	3' Reverse
gDNA C3	CCG CCT CCA CCA CCA CCT AGT AAA	TGGCACGAGAACCTCATGGG

Sanger sequencing

C3 from the HAP-1 clonal cell lines was amplified by PCR with primers (**Table 2**). PCR products were visualized on a 1% agarose gel and subsequently sequenced with Sanger sequencing [23]. Sequences were compared to the reference C3 sequence obtained from the UCSS genome browser (NM_000064).

Tritium
The clones were cultured in a flat-bottom 96-wells plate in a concentration of 10.000 cells/well for six hours before incubation with tritium. To a volume of 100uL cell culture, 50uL of 10uCi ^{3}H was added to establish a concentration of 0.5uCi ^{3}H per well. After incubation for 18, 42 or 66hrs, cells were resuspended and transferred to a round-bottom 96-wells plate for harvesting. Cells were harvested with the TOMTEC Harvester 96 according to manufacturer's protocol. 3H-positive cells were counted with the Microbeta Trilux 1450 scintillation counter.

Phase microscopy
Cells were seeded in T75 culture flasks in DMEM, pictures were taken after 24 and 72 hours of culture with Olympus SC30 microscope 20x enlargement.

Flow cytometry
Cells were stained for surface and/or intracellular markers. For intracellular staining the cells were fixed and permeabilized by using the Cytofix/Cytoperm™ Fixation/ Permeabilization Solution Kit (BD). For staining isotype controls were used. After incubation with the fluorescent antibodies, the cells were washed and measured on LSRII. Analyses were performed using FlowJo version 10.

Direct Cellular Metabolism Measurement
Seahorse XF Cell Mito Stress Test (Kit 103015-100, Agilent Technologies, US) was performed according to manufacturer's protocol. In short, cells were seeded in the Seahorse XF Cell Culture Microplate overnight in DMEM. The sensor cartridge was hydrated with Seahorse XF Calibrant at 37°C in a non-CO_2 chamber overnight. Next, the assay medium was prepared (Seahorse XF base medium supplemented 1mM pyruvate/2 mM glutamine/10 mM glucose) and the compounds were loaded in the sensor cartridge (final concentrations: Oligomycin 1µM; FCCP 3 µM; Rotenone & antimycin A both 0,5µM). Standard assay was performed and measured by using the Wave software. Finally, the data was normalized on BCA content, the BCA content was measured using Pierce BCA protein assay kit (cat#23227 Thermo Scientific).

RESULTS

Detection of intracellular C3 in cell lysates
To study the role of intracellular C3, we first set out to replicate the original findings that C3 is present in cell lysates and cleaved by CTSL. Unfortunately, the antibody originally used by Liszweski et al. was not available anymore by the supplier. Therefore, several antibodies were tested for their capacity to detect (intracellular) C3 in lysates. First, we analysed the alternative antibody suggested by Abcam (**Figure 1A**). Unfortunately, this mouse anti-C3d only detectsC3 in the positive control NHS sample, but not in the lysates, despite the use of different lysis procedures. We continued testing various

other antibodies, including the DAKO rabbit anti-C3d seemed promising as it was able to detect C3 fragments on WB and showed positive detection in multiple lysates, both from cell culture and isolated cells (**Figure 1B**). Under reducing conditions, a clear band at the height of 110kDa was visible, corresponding, to the α-chain of C3. Next, we analysed this antibody under non-reducing conditions. As a positive control, lysates from human liver were used, showing a band at 180kDa, corresponding with intact C3. As an additional control for the procedure, C3 was added to Jurkat cells prior to lysis, also giving rise to a clear band at 180 kDa. Unexpectedly, in the lysates from the T-cell lines and $CD4^+$ cells from three different donors, there was a band observed at 110kDa. This band is migrating at the same height as the band observed under reducing conditions (F**igure 1C**). Therefore, as C3 is 180KDa, the protein detected with an apparent molecular weight does not correspond to intact C3. Nonetheless, intact C3 can be detected using this DAKO rabbit anti-C3d in the human liver lysate, the NHS, purified C3 samples as such and spiked in lysates. Thus, together, these data indicate that this rabbit serum recognizes besides intact C3, also another protein with an apparent molecular weight of 110 kDa and hence was not suitable for further use in our studies.

During the course of our experiments it was reported [10] that cells only test positive for C3 upon exposure to NHS, because of ingestion of C3 from the NHS, prior to lysis. Therefore, we continued reproducing these findings using the goat anti-C3 serum (CompTech cat#A213). The cells incubated with NHS were washed repeatedly with PBS and the last supernatant prior to lysis was also run an WB to control for possible carry over of the protein, these ''wash supernatants'' were negative. The lysates of cells, incubated with NHS prior to lysis, show an increase in C3 signal (**Figure 1D**). Additionally, we verified that the mouse anti-C3(a) antibody only recognizes C3, and not the processed form C3b (**Figure 1E**). This mouse anti-C3(a) antibody was used for follow-up experiments, where C3 was incubated in the presence of CTSL *in vitro*.

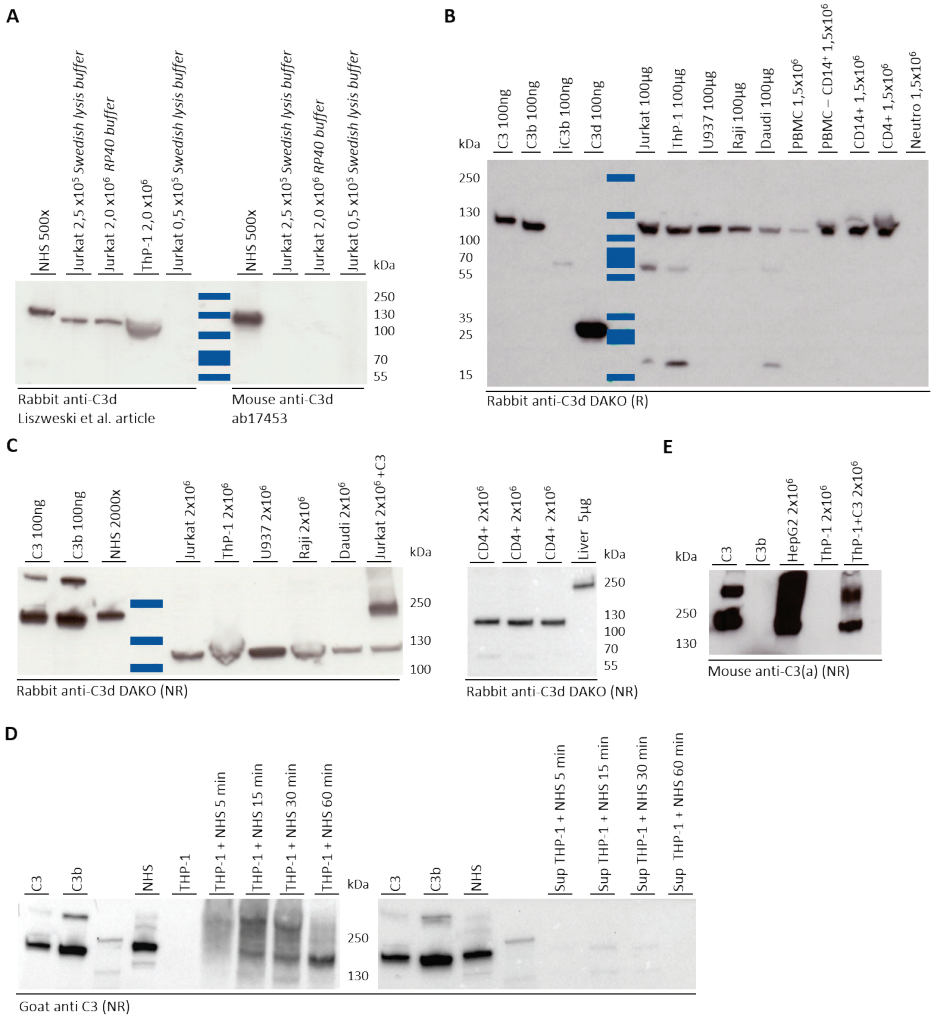

Figure 1. C3 detection in lysates on Western Blot

Western Blot (WB) results from lysates and C3 detection by different antibodies. (**A**) WB of lysates detected by two different antibodies, left is the rabbit anti-C3d from the Liszweski article which is no longer produced and the right part is the alternative (ab17453) offered by the company. (**B**) WB of lysates from various cell lines and freshly isolated cells, detected with rabbit anti-C3d antibody from DAKO under reducing (R) conditions, Peripheral blood mononuclear cells (PBMCs). (**C**) WB of lysates from various cell lines (one Jurkat sample had C3 added during the lysis process to control for the procedure) detected with rabbit anti-C3d antibody from DAKO under non-reducing conditions (NR). (**D**) WB of lysates with or without prior incubation in normal human serum (NHS), the wash supernatants (sup) are on the right. (**E**) WB of proteins and lysates detected with mouse anti C3(a) under NR conditions.

Cathepsin-L cleavage of C3 does not generate C3a

Whether C3 is intracellularly retained after synthesis or taken up from the circulation may both indicate a role for C3-processing in intracellular processes, which is believed to occur after the cleavage of C3 in a convertase-like manner by CTSL. Therefore, we next incubated commercial, purified C3 with activated CTSL and analysed the fragments generated by CTSL-mediated cleavage. Fragments were analysed both by WB and MS. By using the C3(a) antibody we confirm that C3 is cleaved by CTSL and that the reaction can be inhibited with a specific CTSL inhibitor (CTSLi), but not with a non-specific Cathepsin inhibitor (CTSi) (**Figure 2A**). In addition, the disappearance of intact C3 is specific for CTSL and does not occur when C3 is incubated with Cathepsin-G (CTSG) (**Figure 2B**). The products formed by CTSL-cleavage were run on a high percentage SDS-page gel (20%) to visualize the C3a(desArg) using the C3(a) antibody. A relatively faint signal at the height of (commercial) C3a(desArg) was observed after C3-processing by CTSL (**Figure 2C**). To further confirm the conversion of C3 into C3a(desArg), protein N-termini were acetylated using acetic anhydride in order to discriminate between N-termini generated by CTSL and generated during protein digestion. The latter peptides will not contain an acetylated N-terminus. In doing so, we found RSNLDEDIIAEENIVSR which was formed CTSL-dose dependently (**Figure 2D**). Interestingly, CTSL cleaves C3 one amino acid upstream to that of convertase, leaving an N-terminal R on the "C3b α "-chain (**Figure 2E**). These data suggest that C3 is specifically cleaved by CTSL resulting in the generation of C3a-desArg . Although MS analysis could demonstrate that CTSL leaves the arginine (essential for C3a function) on C3b, we have been unsuccessful to detect the C3a-desArg cleavage product despite several attempts on orbitrap and MALDI instruments. In contrast, convertase-generated control C3a could be detected with high yield and full coverage .

CRISPR/Cas9 mediated knockdown of C3 in HAP-1 cells

To analyse the potential role of intracellular C3 we set out to develop C3-/- model cell line(s), by using CRISPR/Cas9. For Cas9 delivery two different viral transvectors were used, either adenovirus or lentivirus. To control for successful use of CRISPR/Cas9, the T7EI assay was performed in the bulk of the cells after Cas9 transfection. Since it has been published that C3 can be taken up from circulation and it is unknown, given the speculated role of C3 in cell survival [5], whether this C3 is essential for survival, both adenoviral and lentiviral generated Cas9 cells were cultured, in limited dilution, in the presence of either FCS or complement active NHS. This resulted in four different culture conditions: adenovirus with FCS (AF); adenovirus with NHS (AN); lentivirus with FCS (LF); lentivirus with NHS (LN). Since all conditions show additional bands after T7EI treatment, CRISPR/Cas9 was successful in all culture conditions (**Supplementary Figure 1**). After limiting dilution, 90 viable clones were collected and gDNA was isolated. Of these 90 clones, 46 were further analysed by Sanger sequencing (**Supplementary Figure 2**), excluding those with double bands or absent bands after PCR. The sequences obtained are summarized in **Table 1**. After CRISPR/Cas9 in 18 out of 46 analysed sequences a

perfect match was found with the reference sequence. In 15 out of 46 sequences we found various mutations in the gDNA: three remained a perfect match on cDNA level; one had a deletion but did not lead to a frameshift mutation; 11 sequences had deletions which resulted in a frameshift mutation (including two sequences with early stopcodons). Interestingly, some mutations were found in more than one clone. For example, clones LNE8.1, LFE5, LFG11, AFH8, ANG11 and AFG8 displayed the same 203bp deletion in the gDNA, resulting in a 11bp deletion on cDNA level and a frameshift which prohibits intact C3 translation. Since this particular mutation is found in all four culture conditions, there seems to be no selection in Cas9 viral delivery and the use of either FCS or NHS supplemented medium. Additionally, clones AND8 and LNA4 had a matching mutation, where there was an even larger deletion of 214bp in gDNA and 22bp in cDNA, prohibiting C3 translation.

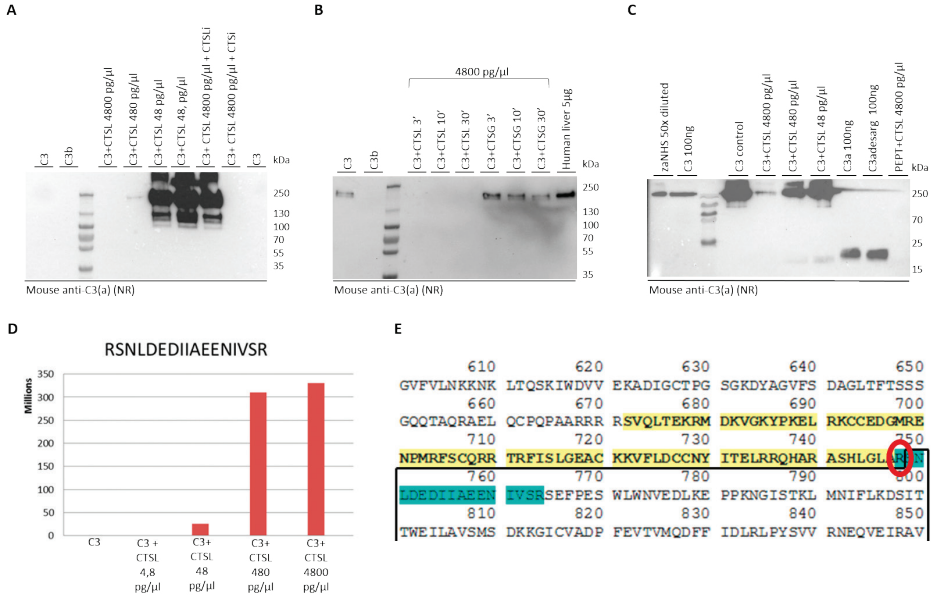

Figure 2. C3 cleavage by Cathepsin-L

(A) Western blot (WB) intact C3 after incubation with Cathepsin-L in various conditions. With decreasing concentration of CTSL, in the presences of a CTSL inhibitor (CTSLi) or non-specific CTS inhibitor (CTSi). (**B**) WB intact C3 after incubation with CTSL or Cathepsin-G (CTSG) for different incubation times. (**C**) WB of C3 with decreasing concentration of CTSL incubated samples analysed on 20% gel, detection of C3 and C3a/C3adesArg. (**D**) Peptide abundance measured by mass spectrometry as a function of increasing concentrations of CTSL. RSNLDEDIIAEENIVSR was the only peptide that showed a dose dependent response to the concentration of CTSL. These results are representative for two independent experiments (**E**) Schematic presentation of the peptide found with MS analysis, the C3a and C3b sequences are separated with the black solid line, the C3a sequence is highlighted in yellow and the N-terminal peptide found for C3b in blue. The arginine (R) that should be on the C3a, but is found attached to the C3b is circled in red.

Table 1. Sequence results of CRISPR-Cas9 of C3a in HAP-1 cell line

Cas9	Culture	Name	Deletion DNA	Deletion cDNA	Insertion nt	Mutation	Frameshift	Comment sequence analysis
Adeno	*FCS*	**AF_A10**	*x*	*x*	*x*	-	*x*	low quality sequence
Adeno	FCS	**AF_A3**	-	-	1 "C"	-	YES	early stopcodon
Adeno	FCS	**AF_A7**	-	-	-	-	NO	perfect match
Adeno	*FCS*	**AF_B5**	-	-	1? "T"	-	YES?	sequence pattern inconclusive
Adeno	FCS	**AF_B6**	-	-	-	-	NO	perfect match
Adeno	FCS	**AF_D10**	-	-	-	-	NO	perfect match
Adeno	FCS	**AF_E10**	-	-	-	-	NO	perfect match
Adeno	FCS	**AF_F12**	-	-	-	-	NO	perfect match
Adeno	FCS	**AF_G10**	-	-	-	-	NO	perfect match
Adeno	FCS	**AF_G11**	-	-	-	-	NO	perfect match
Adeno	*FCS*	**AF_G4**	-	-	-	-	*NO*	sequence pattern inconclusive
Adeno	*FCS*	**AF_G6**	-	-	*3?*	-	*YES?*	sequence pattern inconclusive
Adeno	FCS	**AF_G8**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8
Adeno	FCS	**AF_H8**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8
Adeno	NHS	**AN_C1**	-	-	-	-	NO	perfect match
Adeno	*NHS*	**AN_C11**	-	-	-	*G->C?*	*NO*	sequence pattern inconclusive
Adeno	NHS	**AN_C4**	-	-	-	-	NO	perfect match
Adeno	*NHS*	**AN_D1**	-	-	*1 "T"*	-	*YES?*	sequence pattern inconclusive
Adeno	NHS	**AN_D5**	13	0	-	-	NO	perfect match on cDNA level

Table 1. Continued.

Cas9	Culture	Name	Deletion DNA	Deletion cDNA	Insertion nt	Mutation	Frameshift	Comment sequence analysis
Adeno	NHS	**AN_D8**	214	22	-	-	YES	AN_D8, LN_A4
Adeno	NHS	**AN_F4**	-	-	-	-	NO	perfect match
Adeno	*NHS*	**AN_F9**	-	-	*1? "C"*	-	*YES?*	sequence pattern inconclusive
Adeno	NHS	**AN_G11**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8
Adeno	NHS	**AN_G2**	-	-	1? "G" in intron	-	NO	perfect match on cDNA level (extra G just before start exon)
Adeno	NHS	**AN_H10**	-	-	1? "G" in intron	-	NO	perfect match on cDNA level (extra G just before start exon)
Lenti	FCS	**LF_A10**	-	-	1 "C"	-	YES	early stopcodon
Lenti	FCS	**LF_A12**	-	-	-	-	NO	perfect match
Lenti	FCS	**LF_A3**	-	-	-	-	NO	perfect match
Lenti	FCS	**LF_B4**	-	-	-	-	NO	perfect match
Lenti	*FCS*	**LF_B6**	*222*	*30*	-	-	*NO*	sequence pattern inconclusive
Lenti	*FCS*	**LF_C5**	-	-	-	-	*NO*	sequence pattern inconclusive
Lenti	FCS	**LF_E2**	-	-	-	-	NO	perfect match
Lenti	FCS	**LF_E5**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8
Lenti	FCS	**LF_F3**	73	63	-	-	NO	AA deletion, not leading to frameshift
Lenti	FCS	**LF_G1**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8

Table 1. Continued.

Cas9	Culture	Name	Deletion DNA	Deletion cDNA	Insertion nt	Mutation	Frameshift	Comment sequence analysis
Lenti	NHS	**LN_A4**	214	22	-	-	YES	AN_D8, LN_A4
Lenti	*NHS*	**LN_B4**	-	-	-	-	NO	sequence pattern inconclusive
Lenti	NHS	**LN_C1**	-	-	-	-	NO	perfect match
Lenti	*NHS*	**LN_C7**	-	-	-	-	*NO*	sequence pattern inconclusive
Lenti	*NHS*	**LN_E3**	-	-	-	-	*NO*	sequence pattern inconclusive
Lenti	NHS	**LN_E8-1**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8
Lenti	NHS	**LN_G4**	-	-	-	-	NO	perfect match
Lenti	NHS	**LN_G9**	-	-	-	-	NO	perfect match
Lenti	NHS	**LN_H1**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8
Lenti	NHS	**LN_H10**	-	-	-	-	NO	perfect match
Lenti	*NHS*	**LN_H8**	-	-	*1? "T/C"*	-	*YES?*	sequence pattern inconclusive
n.a.	n.a.	**WT**	-	-	-	-	NO	perfect match

Sequence results from CRISPR/Cas9 clones summarized. Clones were generated by using either adenovirus or lentivirus for Cas9 delivery, subsequently, the clones were culture in either normal human serum (NHS) or fetal calf serum (FCS). The gDNA sequence results were translated to cDNA for further translation of the mutations and analysed whether the mutation resulted in a frameshift. Further comments per sequence are annotated in the last column.

CRISPR/Cas9 mediated knockdown of C3 does not affect survival, morphology and metabolism of model cell line HAP-1

For further analysis of the effects of C3 deficiency on basal cellular processes, two clones (AFE10 (E10) and AND8 (D8)) were selected alongside the WT cell line HAP-1 for further analyses. E10 was selected as a control and D8 as the C3- clone (22bp deletion on cDNA (**Supplementary Figure 2**)). We first explored whether receptors for C3 and C5 anaphylatoxins are expressed intracellularly to determine whether HAP-1 is able to respond to intra-cellular C3a/C5a .In all cell lines tested we observed a clear signal for intracellular C3aR whereas surface staining is negative (**Figure 3**). Likewise, C5aR1/CD88 can be detected intracellularly as an enhanced MFI signal is detected for the intracellular detection. In contrast, surface expression was absent as no specific signal was observed after staining with an anti- C5aR1/CD88-antibody. Similar data were obtained for C5L2. Overall, no differences in the expression patterns of WT, E10 and D8 cell line for C3aR and C5aR-receptors was observed, indicating that all cell lines could, potentially, react in response to C3a/C5a.

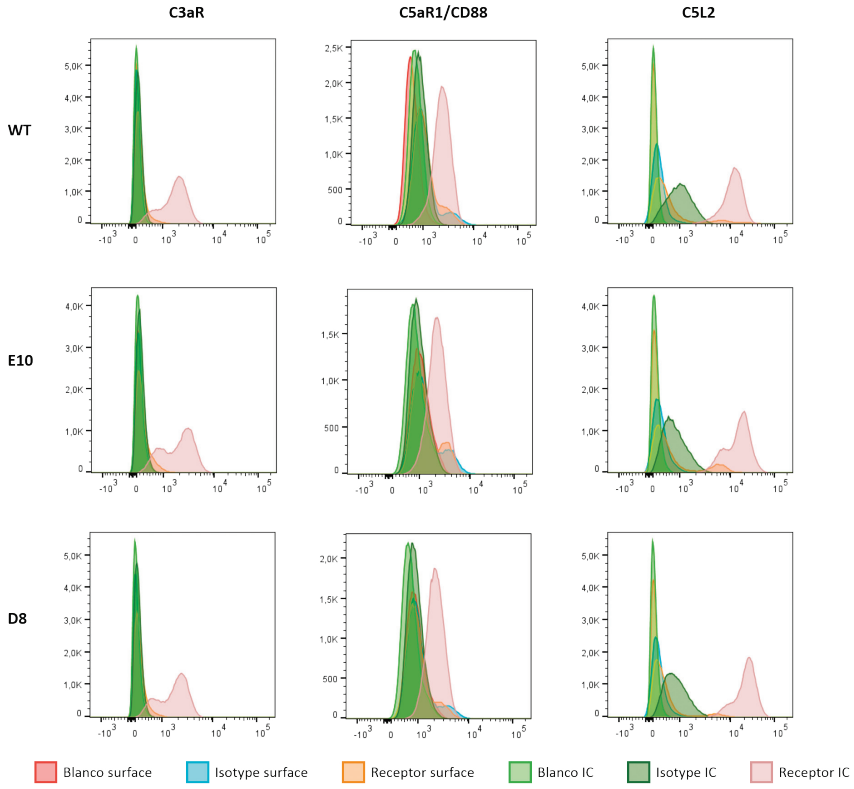

Figure 3. HAP-1 clones intracellular expression C3aR, C5aR1 and C5L2

Histograms of various receptor expression are presented both surface detection and intracellular (IC) after gating for single cells as measured by LSR II an analysed by FlowJo. Wildtype (WT) is the HAP-1 wildtype cell line, E10 and D8 are the clonal cell lines analysed. On the y-axes the counts are depicted and the x-axes show the mean fluorescence intensity.

Next, proliferation of the different cell lines was analysed over a course of 72 hour culture in T75 culture flasks. No differences in proliferative capacity were found between the cell lines in three independent experiments (**Figure 4A**). Next, the basal metabolism of the cells was analysed using the Mito Stress test on a Seahorse XFe96 Analyzer. First, three measurements were performed to obtain a basal measurement per cell line. Next, oligomycin was injected, which inhibits ATP synthase resulting in a reduction in mitochondrial respiration or OCR. The decrease observed in the WT, E10 and D8 cell lines was quite minor. Then FCCP is injected, resulting in the disruption of mitochondrial membrane potential, and uninhibited electron transport chain (ETC) allowing maximum oxygen consumption of cells. The final drugs injected is a combination of Rotenone and Antimycin A, which shuts down the ETC resulting in the non-mitochondrial respiration [24]. Afterwards the BCA content was measured to normalize the results, no differences in mitochondrial respiration or glycolysis was observed between the three cell lines, indicating that C3 does not play a prominent in these basal cellular processes. (**Figure 4B**). Also in this setting no differences were found between the cell lines, it does seem that there is a tendency for D8 to have a higher OCR, but upon the injection of the different drugs, the relative changes in OCR are the same for all three cell lines tested. Likewise, morphology of the cells did not change analysed by phase microscopy after 24 hours of culture and on the forward and side ward scatter with flow cytometry (**Figure 4C, D**). Together, these results indicate that knock out of C3 has no effect on cell survival, morphology, and metabolism in a HAP-1 cell line.

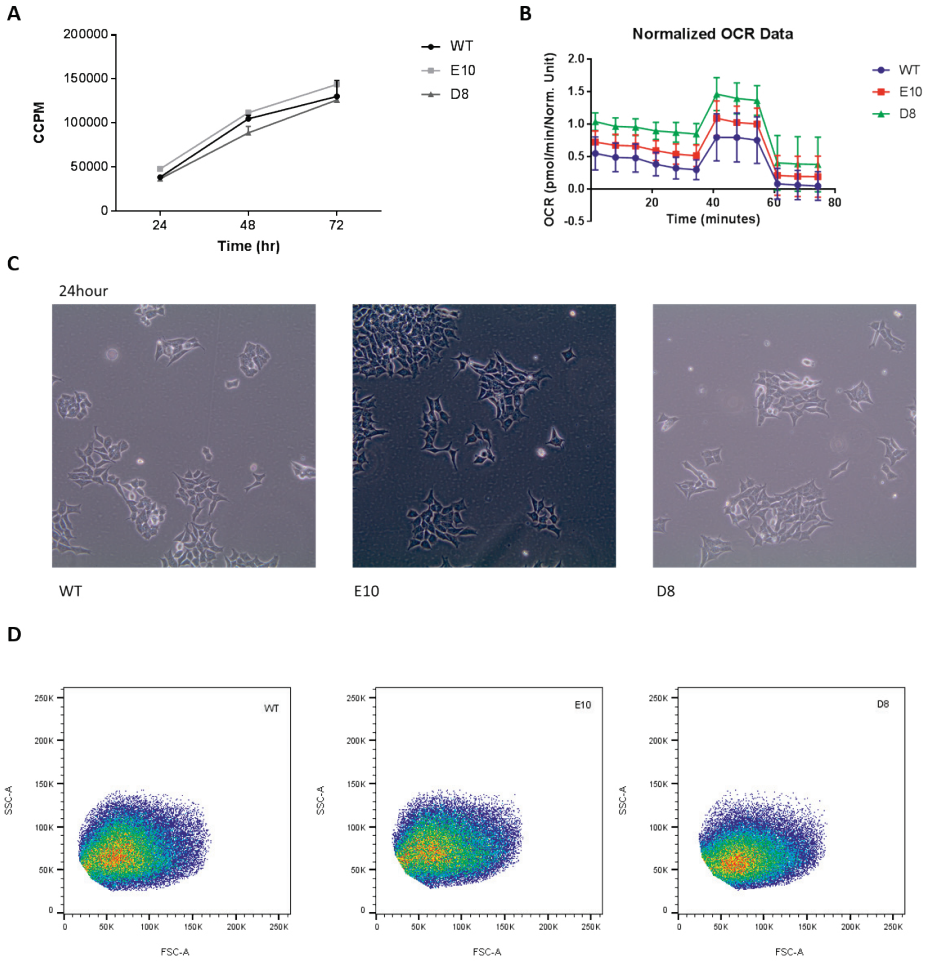

Figure 4. HAP-1 clones gDNA, proliferation, mitochondrial respiration and morphology
(A) Cell proliferative capacity as measured of incorporation of labelled tritium by the different HAP-1 cell lines over the course of 72 hour, taken at 24hour intervals. Shown is a representative of three experiments and all measurements were done in triplicate. (**B**) Oxygen Consumption Rate (OCR) data as measured by the Mitostress test, normalized on BCA content. Olig = Oligomycin, FCCP = Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone, R / A = Rotenone + Antimycin A. (**C**) Images from the different clones taken with the Olympus SC30 phase microscopy after a 24 hour culture period with a seeding concentration of $1x10^6$ cells in 75 cm^2. (**D**) Forward and sideward scatter (FSC and SSC respectively) from the different clones after gating for single cells as measured by LSR II an analysed by FlowJo.

DISCUSSION

Intracellular complement, or the ''complosome'' emerged recently as an interesting new perspective in the field of complement immunology. However, some precaution may be warranted, since replication of published articles remains challenging and necessary. Here, we set out to further explore intracellular C3 with the emphasis on studying the intriguing role that has been attributed to intracellular C3 processing and signalling.

The replication of the findings that C3 is present intracellularly on the protein level using western blot was challenging given the discontinuation of the detection antibody originally used in the studies showing, the presence of intracellular stores containing C3 by confocal microscopy [5]. Therefore, various antibodies were tested for specific reactivity to C3, but, unfortunately, no convincing results by western blot could be obtained. Interestingly, one polyclonal antibody (Dako C3d), which detected the alfa chain at the correct expected molecular weight in almost all lysates, also detected commercially available C3 fragments at the predicted molecular weight. Unfortunately, the polyclonal antibody does not appear to be specific for C3 as it detected a protein with a molecular weight of 110 kDa, (same as the alfa-chain of C3) in lysates of various cellular origins in reducing but also in non-reducing conditions, which is not compatible with C3 [25]. Therefore, we consider it most likely that this antiserum did not visualize C3 in these circumstances. These data are relevant as they point to the absence of C3-expression in these cell lines. Likewise also other antibodies failed to detect a signal in the lysates analysed. Thus, together, we were not able to convincingly show the presence of intracellular C3 on the protein level. In contrast, when the cells were incubated with NHS prior to lysis, we were able to detect C3 in the lysate, indicating that C3 can be detected intracellularly after incubation with NHS as published before [10], which also indicates that the intracellular C3 is either absent or too low to detect prior to this incubation. Thus, together, these data indicate that, under the experimental conditions used, C3-expression cannot be detected in freshly isolated CD4+ T-cells and PBMCs using the various antibodies tested.

Next, we wished to analyse the cleavage of C3 by CTSL and subsequent generation of C3a. We show that CTSL specifically and reproducibly cleaves C3 *in vitro*. However, as shown by MS, the terminal arginine remained attached to the C3b part of the protein, indicating that the fragment generated by CTSL is likely not exactly the same sequence as C3a since. The loss of the terminal arginine on C3a indicates the formation of “ASP”/ C3a-desArg. In contrast to the complete C3a, this variant is not able to bind to the C3aR. Interestingly, ASP has been linked to metabolism [17, 18] and so has (intracellular) complement [8, 9] which could show that metabolism and complement are more intrinsically linked. The observations that CTSL cleavage of C3 produces C3a-desArg instead of C3a is in disagreement with the results and interpretations presented by Liszweski et al. [5], concluding that intracellular C3 processing induced the activation

of intracellular C3aR. The authors argued that intracellular C3 was essential for survival of cells and demonstrated that C3 deficient patients would also express intracellular compartments containing C3 [5]. However, the notion that C3 was essential for cell survival was mainly derived from experiments involving CTSL inhibition and inhibition of C3aR with siRNA. In these experiments, reduced phosphorylation of mTOR was observed, which is essential for cell viability [26]. To study C3 deficiency, we knocked-out of C3 in a model cell line by CRISPR/Cas9. For this purpose, the HAP-1 cell line was used, which is a haploid cell line. By analysing growth, morphology and the metabolic capacity of the cells, no differences between the C3 sufficient and deficient cells was observed, despite the expression of the required receptors intracellular. Nonetheless, although widely used for several purposes, HAP-1 cells are different from other cells due to its haploid nature. Ideally, the use of freshly isolated CD4+ T-cells should be pursued for future CRISPR/Cas9 C3 knock out research. Nonetheless, the ability of cells to survive and grow is also supported by recent studies in which C3 was knock-out, like our study by CRISPR/Cas9, in the lung epithelial cell line A549 cells and an insulin secreting cell line (INS-1). They demonstrated that C3 expression was required for normal autophagy regulation in INS-1 cells [12]. They also state that autophagy can be regulated via mTOR activation, though they found no differences in the phosphorylation of mTOR comparing C3 sufficient and deficient INS-1 cells.

In conclusion, our results indicate the expression of intracellular C3 only after cells are incubated with NHS prior to lysis. We demonstrate that processing of C3 by CTSL is likely generating C3a-desArg. Finally, we showed in a HAP-1 cell line model that C3a is not essential for survival.

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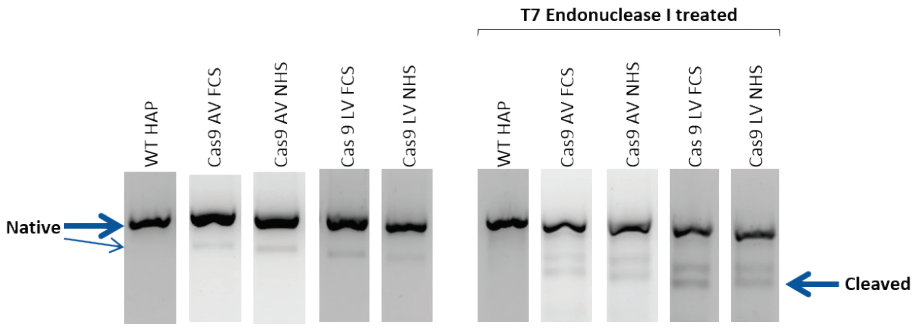
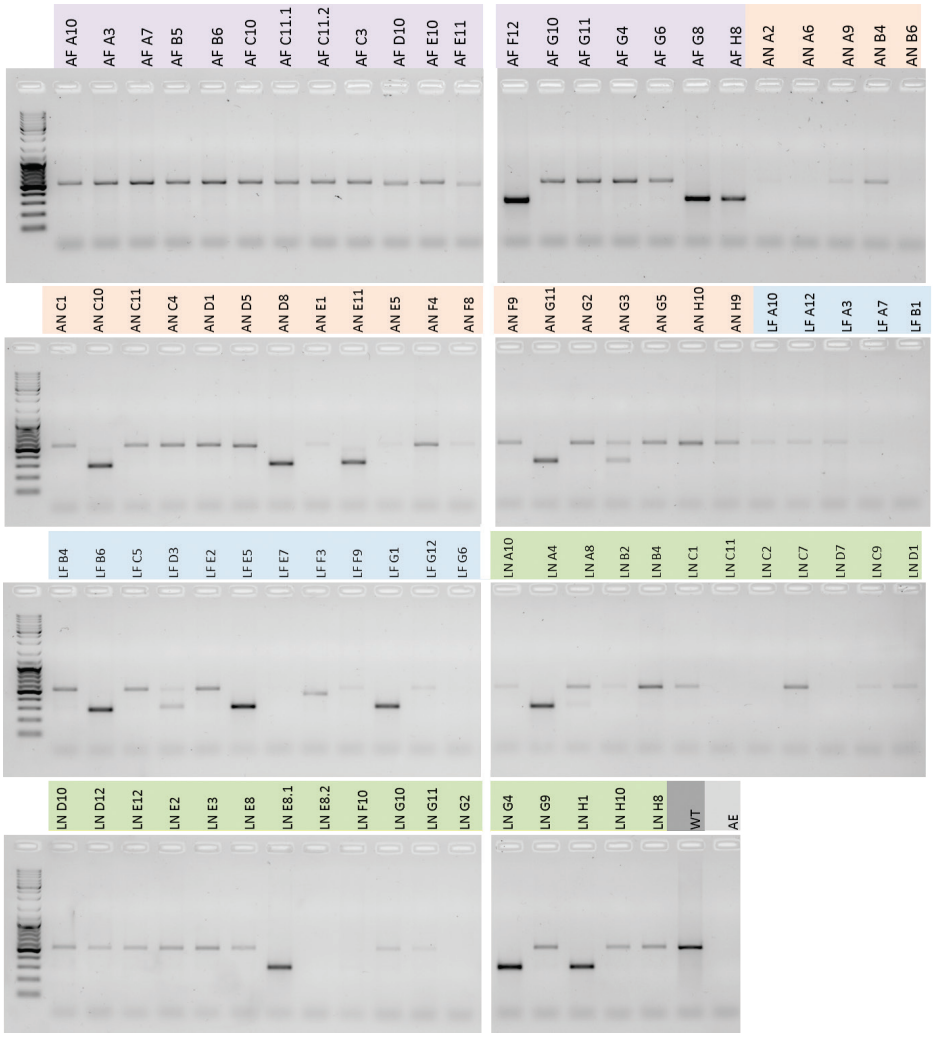
SUPPLEMENTARY FIGURES



Supplementary Figure 1. T7 Endonuclease I assay bulk CRISPR/Cas9 gDNA
The PCR products of the C3a containing the CRISPR sites is visualized on agarose gel for the different culture conditions after CRISPR/Cas9. Bulk genomic DNA was isolated from HAP-1 wildtype (WT); adenovirus with FCS (AF); adenovirus with NHS (AN); lentivirus with FCS (LF); lentivirus with NHS (LN). The PCR product without and with T7 Endonuclease I treatment were run on agarose gel to analyse fragmentation pattern, indicative for successful CRISPR/Cas9 procedure.



Supplementary Figure 2. Agarose PCR product CRISPR/Cas9 clones C3a

The PCR products of the C3a containing the CRISPR sites is visualized on agarose gel for the different clones generated after CRISPR/Cas9. This method resulted in four different culture conditions: adenovirus with FCS (AF); adenovirus with NHS (AN); lentivirus with FCS (LF); lentivirus with NHS (LN). WT is genomic DNA from HAP-1 wildtype and AE is the elution buffer used for genomic DNA isolation.

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