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Anaïs Jiménez-Reinoso, PhD, Ana V. Marin, MSc, Marta Subias, PhD, Alberto López-Lera, PhD, Elena Román-Ortiz, MD, Kathryn Payne, BSc Hons, Cindy S. Ma, PhD, Giuseppina Arbore, PhD, Martin Kolev, PhD, Simon J. Freeley, PhD, Claudia Kemper, PhD, Stuart G. Tangye, PhD, Edgar Fernández-Malavé, PhD, Santiago Rodríguez de Córdoba, PhD, Margarita López-Trascasa, PhD, José R. Regueiro, PhD

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Human plasma C3 is essential for the development of memory B, but not T, lymphocytes

Anaïs Jiménez-Reinoso, PhD\(^a\); Ana V. Marin, MSc\(^a\); Marta Subias, PhD\(^b, c\); Alberto López-Lera, PhD\(^d, e\); Elena Román-Ortiz, MD\(^f\); Kathryn Payne, BSc Hons\(^g\); Cindy S. Ma, PhD\(^h, i\); Giuseppina Arbore, PhD\(^j\); Martin Kolev, PhD\(^k\); Simon J. Freeley, PhD\(^l\); Claudia Kemper, PhD\(^m\); Stuart G. Tangye, PhD\(^n, o\); Edgar Fernández-Malavé, PhD\(^p\); Santiago Rodríguez de Córdoba, PhD\(^q, r\); Margarita López-Trascasa, PhD\(^c, d\); and José R. Regueiro, PhD\(^s\).

From the \(^a\)Department of Immunology, Complutense University School of Medicine and 12 de Octubre Health Research Institute (imas12), Madrid, Spain; the \(^b\)Centro de Investigaciones Biológicas (CSIC), Madrid, Spain; the \(^c\)Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain; the \(^d\)Immunology Unit, Hospital Universitario La Paz, IDiPAZ, Madrid, Spain; the \(^e\)Servicio de nefrología pediátrica, Hospital La Fe, Valencia, Spain; the \(^f\)Immunology Research Program, Garvan Institute of Medical Research, Darlinghurst, NSW, Australia; the \(^g\)St. Vincent’s Clinical School, University of New South Wales, Australia; and the \(^h\)MRC Centre for Transplantation, Division of Transplant Immunology and Mucosal Biology, King’s, College London, London, SE1 9RT, United Kingdom.

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CORRESPONDING AUTHOR:
José R. Regueiro
Department of Immunology
Complutense University School of Medicine
28040 Madrid, Spain.
Telephone no.: +34913941631 Fax no.: +34916941641
E-mail: regueiro@med.ucm.es
CAPSULE SUMMARY:
Primary or secondary plasma C3 deficiency due to mutations in C3 or in complement Factor I impairs memory B, but not T, cell differentiation, but does not preclude intracellular C3 fragment expression in lymphocytes.

KEYWORDS: C3, factor I, immunodeficiency, memory, B cells, T cells
Primary C3 deficiency is an extremely rare autosomal-recessive disease, with less than 50 families described worldwide. Plasma and intracellular C3 are considered BCR and TCR costimulators, respectively, but their contribution to lymphocyte biology remains obscure, particularly in humans. Reduced plasma C3 can be caused by primary C3 deficiency, due to loss-of-function (LOF) C3 mutations, but also by secondary C3 deficiency or C3 consumption, due to gain-of-function (GOF) C3 mutations or to mutations in C3 regulators such as complement Factor I (CFI). We reasoned that comparing B and T cell differentiation and function in primary and secondary plasma C3 deficiency might help to understand the role of plasma and intracellular C3 in adaptive immunity. We report the immunological features of lymphocytes from nine individuals with low plasma C3 belonging to six families with mutations causing primary or secondary C3 deficiency and, in some cases, chronic kidney disease stages 1-3 (see Figure E1, A and Tables E1 and E3 in the Online Repository).

To study whether reduced plasma C3 levels could affect lymphocyte differentiation, patients, healthy carriers (HC) and healthy donors (HD) were immunophenotyped and compared for several T and B cell subsets, including those representing successive differentiation and effector subsets. Absolute lymphocyte numbers were always within normal ranges (not shown). T lymphocyte differentiation was essentially normal, and no significant differences, except for increased Tfh cell frequency, were detected when individuals above (HC+HD) or below (patients) 40 mg/dL plasma C3 (Fig E1, B in the Online Repository) were compared (see Fig E2, in the Online Repository). In contrast, impaired naïve to memory B cell differentiation was
observed in both primary and secondary plasma C3-deficient patients (Fig 1, A), with increased naïve (CD19^+IgD^+CD27^-) B cells, and reduced unswitched (CD19^+IgD^+CD27^+) and switched (CD19^+IgD^-CD27^+) memory B cells. Such changes were statistically significant when plasma C3 levels were below the 40 mg/dL threshold (Fig 1, B).

B cell regulation by complement involves antigen opsonization by C3d and recognition through CD21/CR2, a component of the B cell co-receptor^2, which lowers the threshold for B cell activation^3. To determine a possible role of circulating C3-opsonizing fragments in the generation of memory B cells, we analyzed the correlation between plasma C3 levels and B or T cell subpopulations in our cohort. The results showed a statistically significant positive correlation of plasma C3 levels with the frequency of unswitched memory B cells, and negative correlation with that of naïve B cells, but not with total B cells (Fig 1, C). A non-significant positive correlation was found also with switched memory B cell levels. In contrast, none of the T cell subpopulations analyzed correlated with plasma C3 levels (see Fig E2, C in the Online Repository). Although impaired memory B cell differentiation has been reported in a GOF plasma C3-deficient patient^4, here we demonstrate in a larger cohort that reduced plasma C3 levels, whether primary or secondary, results in a selective defect in the in vivo differentiation of memory B, but not T, lymphocytes.

To explore whether the defect in memory B cell development caused by C3 deficiency was associated to altered T or B cell function, PBMC were studied. Ex vivo T and B cell function, measured as proliferation in response to mitogens (see Fig E3 in the Online Repository), or in vivo B cell function, measured as immunoglobulin or vaccine-
specific antibody levels (see Table E2 in the Online Repository), were mostly normal in our cohort, despite their low plasma C3 levels. Although C3 deficiency has been associated with recurrent bacterial infections\(^1\) and decreased proliferation against specific recall antigens, neither primary nor secondary plasma C3-deficient patients in our cohort (or other's\(^5\)) showed clinical features suggestive of severe T or B cell defects. However, our findings may be clinically relevant to improve patient management in countries where health conditions or vaccinations are below Western standards.

Both B and T lymphocytes contain intracellular C3 fragments\(^6\). Indeed, it was shown that cathepsin-L-cleaved intracellular C3 was required for T cell homeostasis and, upon TCR engagement, increased and participated in Th1 responses. Hence, we generated pure T and B cell lines from our cohort and analyzed their intracellular C3 content by flow cytometry using specific mAb against two C3-associated neo-epitopes (see Fig E4 in the Online Repository). The results showed that all tested cell lines derived from primary or secondary plasma C3-deficient patients, above or below 40 mg/dL plasma C3, expressed intracellular C3 fragments at HD levels (see Fig 2, A, left and Fig E5, A in the Online Repository). In addition, intracellular C3a was increased after TCR activation of C3-deficient T cell lines (see Fig E5, B). These observations are in line with previously unexpected published data\(^6\) and prompted a closer analysis of plasma C3 levels by Western blot and ELISA (Fig 2, A, right). The results showed that even primary, and expectedly secondary, C3-deficient plasmas have very low levels of C3 proteins, which may thus accumulate in cellular compartments and allow intracellular detection. Of note, the presence of iC3b/C3dg fragments in cells from factor I (fI)-deficient patients suggested fI-independent cleavage in lymphocytes.
Intracellular localization studies revealed that C3a and iC3b/C3dg fragments showed different subcellular locations, with iC3b/C3dg, but not C3a (which has previously shown to be located in the lysosomes in T cells\(^6\)), found predominantly associated to the cis-Golgi (Fig 2, B and C). These results suggest a differential intracellular location of the cleaving enzymes generating these complement fragments\(^7\).

Interestingly, T cell lines from primary plasma C3 deficiency showed accumulation of all C3 tested fragments in several subcellular compartments, particularly in lysosomes, which may impair exocytosis of C3 (Fig 2, C, right).

Taken together, these results indicate that although intracellular C3 expression in lymphocytes occurs despite severe plasma C3 deficiency, it cannot replace plasma C3 for memory B cell differentiation. The present data support our previous suggestion that human C3 deficiency is always leaky to some extent and that complete combined intracellular and extracellular C3 (or C5) deficiency may not be viable in humans\(^8\). A mechanism bridging innate and adaptive immunity thus emerges connecting B cell co-receptor recognition of C3 fragments, in a concentration-dependent manner, and memory B cell differentiation. We hypothesize that reduced plasma C3 impairs the generation of C3 activating/opsonizing fragments such as C3dg/C3d. In turn, BCR co-activation through CD21/CR2 would be impaired, hampering sufficient signaling for the generation of memory B cells. Note that this would not affect the generation of antigen-specific antibodies later on (Fig E6). See a Supplemental Discussion in the Online Repository.

In summary, despite the small size of the cohort, we report a side-by-side study of B and T cell features in the very rare conditions of C3 and fI deficiencies. Our results show that plasma C3 is essential for the development of memory B, but not T,
lymphocytes in a plasma C3 level-dependent fashion, and demonstrate that very low plasma C3 levels do not preclude the expression of intracellular C3 fragments in lymphocytes.

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Anaïs Jiménez-Reinoso, PhD\textsuperscript{a}  
Ana V. Marin, MSc\textsuperscript{a}  
Marta Subias, PhD\textsuperscript{b,c}  
Alberto López-Lera, PhD\textsuperscript{d}  
Elena Román-Ortíz, MD\textsuperscript{f}  
Kathryn Payne, BSc Hons\textsuperscript{f}  
Cindy S. Ma, PhD\textsuperscript{f,g}  
Giuseppina Arbore, PhD\textsuperscript{h}  
Martin Kolev, PhD\textsuperscript{h}  
Simon J. Freeley, PhD\textsuperscript{h}  
Claudia Kemper, PhD\textsuperscript{h}  
Stuart G. Tangye, PhD\textsuperscript{f,g}  
Edgar Fernández-Malavé, PhD\textsuperscript{a}  
Santiago Rodríguez de Córdoba, PhD\textsuperscript{b,c}  
Margarita López-Trascasa, PhD\textsuperscript{d}  
José R. Regueiro, PhD\textsuperscript{a}  

From the \textsuperscript{a}Department of Immunology, Complutense University School of Medicine and 12 de Octubre Health Research Institute (imas12), Madrid, Spain; the \textsuperscript{b}Centro de Investigaciones Biológicas (CSIC), Madrid, Spain; the \textsuperscript{c}Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain; the \textsuperscript{d}Immunology Unit, Hospital Universitario La Paz, IdiPAZ, Madrid, Spain; the \textsuperscript{e}Servicio de Nefrología Pediátrica, Hospital La Fe, Valencia, Spain; the \textsuperscript{f}Immunology Research Program, Garvan Institute of Medical Research, Darlinghurst, NSW, Australia; the \textsuperscript{g}St. Vincent's Clinical School, University of New South Wales, Australia; and the \textsuperscript{h}MRC Centre for Transplantation, Division of Transplant Immunology and Mucosal Biology, King's, College London, London, SE1 9RT, United Kingdom. E-mail: regueiro@med.ucm.es  

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REFERENCES


FIG 1. B lymphocyte immunophenotype. A, B cell subset frequencies compared with normal percentiles (n=10 HC + patients vs 12 HD). Symbols as in Fig E1. B, B cell subsets (mean+SD) of individuals below (patients) or above (HD + HC) the 40 mg/dL plasma C3 threshold (gray area in C). ns: non-significant; **p <0.01. C, Correlation analysis (Pearson’s r) of B cell subsets and plasma C3 levels. Solid lines are best-fit linear regressions, dashed lines are 95% confidence intervals.

FIG 2. C3 detection in T cell lines and plasma from C3-deficient patients. A, Left, Intracellular C3 fragments in single cell lines from donors above or below the 40 mg/dL plasma C3 threshold (flow cytometry). Data are mean±SD of GEO-MFI relative to isotype controls (dashed lines). ns: non-significant; Right, Extracellular C3 detection by Western blot (α chain) vs ELISA and nephelometry (mg/dL means or range) in plasma from primary (MS, missense and FS, frameshift) and secondary (MS) C3-deficient patients. B, Subcellular T cell colocalization of C3 fragments with cis-Golgi (confocal microscopy x60 using one C3a- and two iC3b/C3dg-specific mAb, scale bars 5 µm). C, Statistical analysis of C3 fragment colocalization with several subcellular compartments (mean+SD; only r > 0.5 (dashed horizontal lines) were considered; p<0.05, **0.01, ***0.001).
A

B

C

CD19

Naive

Unswitched

Switched

CD19

CD19

Naive

Unswitched

Switched

Plasma C3 levels

r = 0.1548
p = 0.5817

r = 0.6322
*p = 0.0114

r = 0.7236
**p = 0.0023

r = 0.4766
p = 0.0725
ONLINE REPOSITORY MATERIAL

METHODS

Donors, complement profile, genetic diagnosis and lymphocyte isolation

Ten different individuals (nine with low plasma C3 levels, seven below 40 mg/dL and medium/long-term clinically diagnosed disease, see Table E3) belonging to six different families with primary (due to loss-of-function, LOF, C3 mutations) or secondary (due to gain-of-function, GOF, C3 mutations, or due to LOF complement Factor I or CFI mutations) plasma C3 deficiency were studied (Fig E1). In all cases, age- and sex-matched healthy donors (HD) were included (n ≤ 18). Plasma C3 levels and genetic data are summarized in Table E1. Healthy carriers (HC) and patients were classified according to their primary or secondary C3 deficiency, their LOF/GOF mutated or wild type (WT) alleles for C3 or CFI (WT/LOF; LOF/LOF; WT/GOF), but also by an arbitrary 40 mg/dL threshold for very low plasma C3 levels observed in all patients with clinical features (Fig E1, B). All HC and HD had plasma C3 ≥ 40 mg/dL.

In figure legends, full red and red/white symbols denote homozygous or heterozygous individuals, respectively, with plasma C3 < 40 mg/dL and clinical features. Black/white symbols identify HC. Triangles and rhombuses identify individuals with mutations in C3 or CFI, respectively.

C3 and CFI mutations screening and genotyping were performed as described E1, E2. Plasma C3 levels were measured by nephelometry or ELISA. Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood (EDTA) by density-gradient centrifugation (Ficoll Paque Plus, GE Healthcare).

All samples were obtained after written informed consent from the donors or their guardians, and all studies were performed according to the principles expressed in the
Declaration of Helsinki and approved by the Institutional Research Ethics Committees of the hospitals involved.

**Case reports (see Table E3 for further details)**

**C3.1.II:1** is a 18-year-old membranoproliferative glomerulonephritis (MPGN) type I primary plasma C3-deficient patient of Pakistani origin diagnosed at age 9, born to consanguineous parents. From age 12 to 15 he suffered from IgA-negative cutaneous episodes of vasculitis and knee arthralgia resembling Henoch-Schönlein Purpura (HSP). He also suffered one severe episode of pneumonia with pleural effusion that required draining. Despite signs of microhematuria and microalbuminuria, renal function was preserved. Renal biopsies showed C3-negative linear glomerular deposits with intense immunofluorescence staining (4/4) for IgM, C4 and C1q and intermediate (3/4) for IgA, IgG, kappa and lambda chains, as well as focal mesangial deposits weakly staining for IgA, IgG, C4 and C1q. **C3.1.I:1** is his HC father with low plasma C3 levels.

**C3.2.II:1** is a 13 year-old Spanish MPGN immunofluorescence-negative primary plasma C3-deficient patient diagnosed at age 1, who presented macroscopic hematuria and proteinuria in the nephritic range but no signs of renal dysfunction or infectious disease. As observed in C3.1.II:1, he presented recurrent HSP-like IgA-negative vasculitis in the lower limbs. He received a complete immunization with two doses of Prevenar® before 2 years of age and showed high pneumococcus-specific IgG titers after a dose of Pneumococcal polysaccharide vaccine (PPV-23, Pneumovax®23) at 8 years of age. He also displayed non-immune hemolytic anemia associated to splenomegaly. Enzymatic disorder and paroxysmal nocturnal hemoglobinuria was ruled
out. Immunofluorescence staining of renal biopsies was repeatedly negative. C3.2.I:2 is his HC mother with low plasma C3 levels.

C3.3.II:2 is a 40 year-old Spanish MPGN type I secondary plasma C3-deficient patient diagnosed at age 23. When he was 15 years old, he suffered a sepsis episode with generalized HSP and renal insufficiency, presently persistent reduced C3 levels in serum, proteinuria and mild hematuria. A renal cortex biopsy showed a high proportion of sclerotized glomeruli. Non-sclerotized glomeruli showed a lobulated morphology and mesangial proliferation, endocapillary hypercellularity and capillary wall thickening. Direct immunofluorescence revealed intense granular C3 staining in the mesangium and peripheral regions and weakly intense staining of IgM, C1q, kappa chains and IgA in peripheral subendothelial regions.

C3.4.I:1 is a 49 year-old healthy secondary plasma C3-deficient Spanish man with mild proteinuria and microhematuria diagnosed at age 45.

CFI.1.II:1 is 39 year-old woman with secondary plasma C3 deficiency due to a homozygous mutation in CFI reported previously E2, E3. She was diagnosed at 23, but she debuted at 16 years of age with a meningococcal septicemia coincident with menstruation. From age 22, she developed up to 20 monthly recurrent episodes of acute meningitis around the perimenstrual period. Her younger sister aged 30 (CFI.1.II:4) has the same genotype and secondary plasma C3 deficiency.

CFI.2.II:2 is a 53 year-old woman with secondary plasma C3 deficiency due to a homozygous mutation in CFI E2 diagnosed at age 19. She suffered several episodes of
otitis during childhood and was diagnosed of juvenile chronic polyarthritis and HSP at 11. She suffered meningococcal meningitis at 17 and meningococcal pneumonia at 19 years of age. Two of her siblings have a clinical history of infectious diseases (otitis, septic arthritis and lymphocytic meningitis). She is presently asymptomatic.

**Extracellular flow cytometry**

The following mAbs were used: CD3 (A07746), CD4 (A07751, A07752), CD8 (A07756, A07758), CD19 (A07771), and CD45RA (IM0584U) from Beckman Coulter; CD27 (555440), CD31 (555446), IgD (555779), and TCRγδ (333141) from BD Biosciences; TCRαβ (306717) from BioLegend. FACSCalibur instrument (BD Biosciences); and CellQuestPro (BD Biosciences) and FlowJo (Tree Star) software were used for the analysis.

Gating strategy for B immunophenotype: CD19⁺ lymphocytes were analyzed for IgD⁺CD27⁻ (naïve), IgD⁺CD27⁺ (unswitched) and IgD⁻CD27⁺ (switched) cell distributions.

Gating strategy for T immunophenotype: lymphocytes were analyzed for CD3⁺, αβ⁺ or γδ⁺ cell distribution; CD3⁺ lymphocytes were analyzed for CD4⁺ and CD8⁺.

Gating strategy for CD4⁺ T cell subsets: CD4⁺CD45RA⁺CD31⁺ (recent thymic emigrants or RTE), CD4⁺ CD45RA⁺CD31⁻ (central naïve or CN), CD4⁺CD25⁻CD127⁺CXCR5⁻CD45RA⁺ (naïve), CD4⁺CD25⁺CD127⁺CXCR5⁺ CD45RA⁻ (memory), CD4⁺CCR7⁺CD45RA⁻ (central memory), CD4⁺CCR7⁻CD45RA⁻ (effector memory), CD4⁺CD25⁺CD127⁺ (Treg) and CD4⁺CD25⁺CD127⁺CXCR5⁺ CD45RA⁻ (follicular helper T or Tfh)E4. Th1 (CXCR3⁺CCR6⁻), Th2 (CXCR3⁻CCR6⁻), Th17 (CXCR3⁺CCR6⁺) and Th1/Th17 (CXCR3⁺CCR6⁺) subsets were analyzed within...
the subpopulation of memory CD4+ T cells. Analysis and gating strategy for CD8+ T cell subsets was performed as previously described E4, E5.

**Proliferation in response to mitogens and antibody titers**

PBMC were stained with carboxyfluorescein succinimidyl ester (CFSE, from Molecular Probes) and cultured in RPMI-1640 supplemented with 10% FBS for 5 days after stimulation with 1 µg/mL plate-coated anti-CD3 (UCHT-1, OKT3; eBioscience), 5 µg/mL *Phaseolus vulgaris* leucoagglutinin (PHA), 10 ng/mL phorbol myristate acetate (PMA) and 1 µM ionomycin, 8 µg/mL *Phytolacca americana* pokeweed (PWM) (Sigma-Aldrich) or 10 ng/mL superantigens (*Staphylococcal* Enterotoxin B or E, SEB, SEE; Toxin Technology, Inc.). CFSE dilution was analyzed by flow cytometry as reported E6. Vaccine-specific serum antibody titers were determined with ImmunoCAP® Rc208 kit (for anti-Tetanus toxoid IgG levels) (Phadia, Uppsala, Sweden); Human anti-Diphteria Toxoid IgG EIA kit (Binding Site, Birmingham, UK), Rubella ELISA IgG kit (Vircell, Granada, Spain), Varicella ELISA IgG kit from Vircell, Enzygnost® anti-Measles virus IgG kit (Siemens, Marburg, Germany). Anti-pneumococcal IgG titers were measured with an in-house ELISA using Pneumo23® polysaccharide pneumococcal vaccine as antigen and HRP-labelled goat anti-human IgG as detection antibody.

**Generation of transformed T and B cell lines**

Human T cell lymphotropic virus type 1 (HTLV-1)-transformed T cell lines, which have been reported to synthesize higher intracellular C3 than resting or activated primary T cells E7, were generated as reported E8, and maintained in RMPI-1640 supplemented with 10% FBS, 1% L-glutamine, 1% Antibiotic-Antimycotic (Gibco),
and 100 IU/mL recombinant human IL-2 (provided by Craig W. Reynolds, Frederick Cancer Research and Development Center, NCI, NIH, Frederick, Maryland, USA). B cells were transformed as described and maintained in RMPI-1640 supplemented with 20% FBS, 1% L-glutamine, 1% Antibiotic-Antimycotic (Gibco).

**Intracellular flow cytometry**

Cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% saponin, or using commercial kits (Cytofix/Cytoperm, 554722 from BD Biosciences; Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent, 00-5521-00 from eBioscience). Primary and secondary antibody dilutions and washings were performed in saponin-containing buffer. Intracellular C3 detection was performed by flow cytometry using several mouse anti-human C3-specific monoclonal antibodies (mAbs) (Fig E4): SIM320.12.2.1 (iC3b/C3dg) and SIM320.12.3.1 (iC3b/C3dg) were produced in house and detect a fl-cleavage dependent neo-epitope of the C-terminal domain of both iC3b and C3dg fragments (IgG1 isotype); clone 2991 (ab11873 from Abcam, IgG1 isotype) detects a C3 convertase-dependent C3a neo-epitope. CD46 (555948 from BD Biosciences) or CD19 (A07771, PE-Cy5 from Beckman Coulter) were used as positive controls for T or B cell lines, respectively. For purified mAb several PE-conjugated goat anti-mouse IgG secondary antibodies were used: goat F(ab’2) anti-mouse IgG (H+L) (731856 from Beckman Coulter or 12-4010-87 from eBioscience) and goat anti-mouse IgG (550589, BD Biosciences).

**ELISA and Western blot**

96-well plates were coated with a rabbit anti-human C3 antibody (produced in house) in PBS at 4°C. After blockage with Tris-Tween 1% BSA for 1 hour at room
temperature (RT), serial dilutions in Tris-Tween 1% BSA of plasma samples were added and incubated for 1 hour at RT. C3 was detected using a mouse anti-human C3 monoclonal antibody (produced in house, clone SIM27) for 1 hour at RT followed by a peroxidase-labeled goat anti-mouse incubation for 30 minutes at RT. For detection we used OPD substrate. The reaction was stopped with 10% sulfuric acid. Absorbance was measured at 492 nm. Purified human C3 was used for the calibration curve. All samples were tested in duplicate.

Human C3 was detected by Western blot after SDS-PAGE of plasma samples under reducing conditions using a polyclonal rabbit anti-human C3 antibody (produced in house).

**Confocal microscopy**

For subcellular localizations analysis of C3a and iC3b/C3dg fragments, cells were fixed and permeabilized using Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent (00-5521-00 from eBioscience), and blocked with 1X Permeabilization Buffer (00-8333-56 from eBioscience) with 1% hIgG + 10% FBS (Gibco). Primary and secondary antibody dilutions and staining washes were also performed in 1X Permeabilization Buffer. C3a and iC3b/C3dg fragments were detected using the same mAb as for intracellular flow cytometry (Fig 2, B). Subcellular compartments were detected using rabbit anti-human polyclonal antibodies (from Thermo-Fisher Scientific): GM130 (Cis-Golgi, PA1-077), Trans-Golgi Network 46 (Trans-Golgi, PA1-1069), EEA1 (Early endosome, PA1-063A) and Lamp1 (Lysosome, PA1-654A). Alexa Fluor® 488-conjugated goat anti-Mouse IgG (H+L) Superclonal™ and Alexa Fluor® 555-conjugated goat anti-Rabbit IgG (H+L) Superclonal™ were used as secondary antibodies (A28175 and A27039, from Invitrogen). Mouse IgG1
(eBioscience) and rabbit IgG (Abcam) were used as isotype controls. Cells were mounted using ProLong® Gold with DAPI (P36935 from Molecular Probes) and images (represented as maximum intensity projections from Z-stacks) were obtained in the Center for Cytometry and Fluorescence Microscopy of Complutense University with an Olympus FV1200 Confocal Microscope and analyzed with Fiji/ImageJ (NIH, USA). Pearson’s correlation coefficients $R_{coloc}$ from Fiji/ImageJ were used for statistical analysis, and colocalization was accepted when $r > 0.5$ (dashed lines in Fig 2, C).

For intracellular C3a expression analysis after TCR activation, cells were analyzed before or after activation in 48-well plates coated with 2.0 µg/mL mAb against CD3 (OKT3 clone, purified from a hybridoma in Kemper’s Lab), for 36 hours. Cells were fixed and permeabilized using Cytofix/Cytoperm (554722 from BD Biosciences), and blocked with 1X Perm/Wash (554723 from BD Biosciences) with 10% FBS (Gibco). Primary and secondary antibody dilutions and staining washes were also performed in 1X Perm/Wash buffer. C3a and iC3b/C3dg fragments were detected using the same mAb as for intracellular flow cytometry, above and Fig E5); Alexa Fluor® 488-conjugated rabbit anti-mouse IgG (H+L) from Invitrogen (A11059) was used as secondary antibody. Cells were mounted with VECTASHIELD HardSet Antifade Mounting Medium with DAPI (H-1500, from Vector Laboratories, Inc.) and images (represented as maximum intensity projections from Z-stacks) were obtained in the King’s College London Nikon Imaging Centre by confocal microscopy with an A1R Si Confocal Microscope (x100 objective) and analyzed with NIS Elements software (Nikon™, Surrey, UK).

**Statistical analysis**
Results are shown either as scatter dot plots compared with the normal 5th, 50th and 95th percentiles in boxes, or as mean ± or ± standard deviation (SD). Two-tailed Student’s t test, one-way ANOVA with Tukey’s multiple comparison post-hoc test, and Pearson’s correlation coefficient method with two-tailed p-value, were performed in the latter using GraphPad Prism (La Jolla, CA), and significance was considered only when p-values were < 0.05 (*p < 0.05; **p < 0.01; ***p < 0.001).

ONLINE REPOSITORY DISCUSSION

Clinical relevance

The memory B cell development impairment shown in both primary and secondary C3-deficient patients (Fig 1) did not cause an equivalent functional impairment in vitro (Fig E3) or in vivo (Table E2), and it did not correlate with the severity of the kidney disease (Table E3). Correspondingly, despite reports of recurrent bacterial infections in some primary plasma C3-deficient patients1, 4, E10, our cohort, as well as other’s 5, did not show clinical features suggestive of severe B cell defects, such as bacterial, sinopulmonary infections, or gastrointestinal infections by Giardia lamblia or rotavirus. Other examples of impaired B cell transit from naïve to memory stages with normal levels of specific antibodies have been reported, for instance in human CD21/CR2 (the C3 fragment receptor) deficiencyE11. In contrast, human deficiencies of other components of the B cell co-receptor, such as CD19 or CD81, are associated with both impaired memory B cell development and defective antibody responsesE12-14, suggesting discrete roles for each co-receptor chain in B cell differentiation (see graphical abstract in Fig E6). European vaccination guidelines were used in our cohort,
which was carefully followed-up by top-notch specialists. This may explain the absence of overt clinical correlates of B-cell dysfunction, which may be clinically more relevant for the management of complement deficiency patients in countries where health conditions or vaccinations are below Western standards.

It could be argued that some compensatory mechanism could partly replace the lack of plasma C3 in patients to provide signals that support normal T and B lymphocyte responses. However, we find such compensation unlikely because C3 deficiency affects all complement activation pathways (classical, lectin and alternative) as C3 activation is the central step where all converge. Indeed, the alternative complement activation pathway supports up to 80% of all complement activation\cite{15}, and immunoglobulin deposits, as found in most patients in our cohort (Henoch–Schönlein purpura, membranoproliferative glomerulonephritis, Table E3), indicate a defect in immune complex clearance, which depends on the classical complement pathway.

**Impact of C3 mutations**

We report immunological features of patients carrying LOF C3 mutations causing primary C3 deficiency (p.T56TfsX16 and p.G561D), and GOF C3 mutations and LOF CFI mutations causing secondary C3 deficiency (p.W552C and p.R1042G\cite{16} for C3, and p.C247GE2 and p.A258T\cite{2} for CFI, Fig E4, B and Table E1), which were leaky in all cases (Fig 2, A, right), perhaps explaining intracellular C3 detection. LOF C3 mutations reduce plasma C3 levels by generating misfolded proteins, likely degraded. GOF C3 dominant mutations\cite{4, 16} reduce plasma C3 levels by generating abnormal alternative pathway C3 convertases (C3bBb), which cannot be inactivated and thus consume the normal C3 encoded by the normal allele. LOF CFI mutations reduce C3
levels by preventing C3b degradation, allowing C3bBb to consume normal C3. A graphical abstract summarizes these mechanisms (Fig E7).

The LOF C3 mutations resulted in distinct biochemical features (Fig 2, A, right): the frameshift p.T56TfsX16 mutation is very severe but allowed for minute amounts of a slightly larger α C3 protein (normal size is 110 kDa), whereas the missense p.G561D mutation seemed less severe and its normal-sized product was cleaved into C3b (101 kDa α’ chain). However, in all cases, intracellular C3 fragments were detected (Fig 2 and Fig E5), although slightly less in primary C3-deficient T, but not B, cells (Fig 2, A).

In addition, C3 fragments accumulated in degradation compartments, which could impair C3 exocytosis (Fig 2, C, right and graphical abstract in Fig E8). These data strongly support our previous contention that complete (combined intra- and extracellular) C3 (or C5) deficiency may not exist in humans8.

Taken together, our data indicate an essential role for plasma C3 in the development of memory B, but not T, lymphocytes, and support disparate regulation of plasma C3 and intracellular C3 content in lymphocytes.

ONLINE REPOSITORY REFERENCES


FIG E1. Cohort of studied individuals. A, Pedigrees from the six families studied; mutations in C3 or complement Factor I (CFI) are shown below each family tree. Arrows indicate studied individuals (red for patients, black for healthy carriers, HC) in each family. B, All patients showed plasma C3 levels below 40 mg/dL (right), which was considered a threshold for further analyses. HD, healthy donors.

FIG E2. T lymphocyte immunophenotype analysis. A, T cell subset distribution compared with normal 5th, 50th and 95th percentiles (boxes; n=17 HD). Each symbol represents an individual from a single experiment. B, Statistical analysis comparing T cell subsets (mean+SD) of individuals below (patients) or above (HD + HC) the 40 mg/dL plasma C3 threshold; ns: non-significant; *p <0.05. C, Correlation between T cell subsets and plasma C3. r: Pearson’s correlation coefficient. Solid lines are the best fit after linear regression; dashed lines are their 95% confidence intervals. Gray areas mark the < 40 mg/dL plasma C3 range. Symbols as in Fig E1, B.

FIG E3. Lymphocyte proliferation in response to different stimuli. A, Scatter dot plots of patients and HC T (CD3+ and B (CD19+) proliferation in response to the indicated stimuli measured as % CFSE- cells after 5 days compared with normal 5th, 50th and 95th percentiles (boxes; n=18 HD). NA: non-activated. B, Statistical analysis comparing lymphocyte responses (mean+SD) of individuals below (patients) or above (HD + HC) the 40 mg/dL plasma C3 threshold: ns: non-significant.

FIG E4. A, Diagram of native C3 (top) and its fragments and plasma or vesicular enzymes involved, including the binding sites in C3a, iC3b and C3dg of the indicated mAb (clones between brackets). B, The mutations in plasma C3-deficient patients from Table E1 are shown in native C3.

FIG E5. Intracellular C3 analysis in T cell lines from plasma C3-deficient patients. A, Representative intracellular C3a, iC3b/C3dg and CD46 or CD19 expression in T (left) and B (right) lymphoblastoid cell lines derived from the indicated primary or secondary plasma C3-deficient patients compared to isotype controls (gray histograms). B, Intracellular C3a expression using mAb 2991 in T cell lines from primary plasma C3-deficient patients (C3.1.II:1 and C3.2.II:1) and a HD, after TCR activation for 36 hours, measured by confocal microscopy (magnification x100).

FIG E6. Differential effects of primary deficiencies of B cell co-receptor components or ligands in B cell differentiation and antibody synthesis.

FIG E7. Impact of primary and secondary plasma C3 deficiencies due to LOF/GOF C3 and LOF CFI mutations in extracellular C3 activation.

FIG E8. Subcellular accumulation of C3 fragments in vesicular traffic degradation compartments of primary C3-deficient T cells (right) compared to HD T cells (left), combining our results and previous data. Red arrows identify biosynthetic-secretory pathways; blue arrows denote retrieval/recycling pathways; green arrows identify endocytic pathways. C3aR, C3a receptor; CTSL, cathepsin-L.
TABLE E1. Genetic data, plasma C3 and serum fI levels.

<table>
<thead>
<tr>
<th>Family</th>
<th>Mutation</th>
<th>Plasma C3 (mg/dL)§</th>
<th>fI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≥ 40</td>
<td>&lt; 40¶</td>
</tr>
<tr>
<td>C3.1</td>
<td>I:1</td>
<td>HC</td>
<td>C3 65</td>
</tr>
<tr>
<td></td>
<td>II:1</td>
<td>Primary</td>
<td>C3&lt;6</td>
</tr>
<tr>
<td>C3.2</td>
<td>I:2</td>
<td>HC</td>
<td>C3 40</td>
</tr>
<tr>
<td></td>
<td>II:1</td>
<td>Primary</td>
<td>C3&lt;9</td>
</tr>
<tr>
<td>C3.3</td>
<td>II:2</td>
<td>Secondary</td>
<td>C3 17</td>
</tr>
<tr>
<td>C3.4*</td>
<td>I:1</td>
<td>Secondary</td>
<td>C3&lt;6</td>
</tr>
<tr>
<td>CFI.1*</td>
<td>I:2</td>
<td>HC</td>
<td>CFI 97</td>
</tr>
<tr>
<td></td>
<td>II:1</td>
<td>Secondary</td>
<td>CFI 19</td>
</tr>
<tr>
<td></td>
<td>II:2</td>
<td>Secondary</td>
<td>CFI 17</td>
</tr>
<tr>
<td>CFI.2*</td>
<td>II:2</td>
<td>Secondary</td>
<td>CFI 33</td>
</tr>
</tbody>
</table>

Normal local ranges for plasma C3 or serum fI are 75-135 mg/dL or 77-115 % of normal human serum, respectively; values in bold are below normal range. Subject identification (ID) as in Fig E1, patients in bold. *Reported previously. †HC: healthy carrier; ND, not determined. §: a) By effect on structure: FS, frameshift; MS, missense; Sp, splicing; b) By effect on protein function: WT, wild type; LOF, loss-of-function; GOF, gain-of-function. ¶Mean values of 2-6 determinations (nephelometry). All with clinical symptoms.
**TABLE E2.** Serum immunoglobulins and vaccine-specific antibodies.

<table>
<thead>
<tr>
<th>Family</th>
<th>Code</th>
<th>ID</th>
<th>Age*</th>
<th>Immunoglobulins</th>
<th>Bacteria</th>
<th>Virus</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IgG</td>
<td>IgA</td>
<td>IgM</td>
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<tr>
<td>C3.1</td>
<td>I:1</td>
<td>45</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II:1</td>
<td>13</td>
<td>845</td>
<td>203</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>C3.2</td>
<td>I:2</td>
<td>40</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II:1</td>
<td>8</td>
<td>1630</td>
<td>315</td>
<td>116</td>
<td></td>
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<tr>
<td>C3.3</td>
<td>II:2</td>
<td>35</td>
<td>746</td>
<td>230</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>CFI.1</td>
<td>I:2</td>
<td>55</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II:1</td>
<td>36</td>
<td>1130</td>
<td>121</td>
<td>233</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II:4</td>
<td>26</td>
<td>852</td>
<td>226</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>CFI.2</td>
<td>II:2</td>
<td>50</td>
<td>1050</td>
<td>333</td>
<td>110</td>
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</table>

Normal range

<table>
<thead>
<tr>
<th></th>
<th>725-1900 mg/dL</th>
<th>50-340 mg/dL</th>
<th>45-280 mg/dL</th>
<th>&gt; 0.1 µg/mL</th>
<th>&gt; 2.4 µg/mL</th>
<th>&gt; 0.01 IU/mL</th>
</tr>
</thead>
</table>

Identification (ID) as in Fig E1 (patients in bold). *At date of analysis. +: positive. −: negative. ND, not determined.
**TABLE E3.** Clinical and immunological features of plasma C3-deficient patients.

<table>
<thead>
<tr>
<th>Code</th>
<th>Plasma C3 deficiency</th>
<th>Age (years)</th>
<th>Main renal pathology</th>
<th>Renal dysfunction stage‡</th>
<th>Other pathologies</th>
<th>Infections§</th>
<th>Current condition</th>
<th>% Memory B cells¶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AD* PA†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C3.1.II:1</td>
<td>Primary</td>
<td>9 18</td>
<td>MPGN I</td>
<td>1</td>
<td>HSP Arthralgia</td>
<td>Pneumonia (x2)</td>
<td>Normal renal function</td>
<td>Normal renal function</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mild proteinuria</td>
<td>Mild proteinuria</td>
</tr>
<tr>
<td>C3.2.II:1</td>
<td>Primary</td>
<td>1 13</td>
<td>MPGN I</td>
<td>1</td>
<td>HSP Hemolytic anemia, Chronic hemolysis, Splenomegaly</td>
<td>Streptococcal pharyngitis, Viral meningitis, B19 parvovirus</td>
<td>Normal renal function, Chronic hemolysis, Splenomegaly</td>
<td>Normal renal function</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mild proteinuria</td>
<td>Mild proteinuria</td>
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<tr>
<td>C3.3.II:2</td>
<td>Secondary</td>
<td>23 40</td>
<td>C3G</td>
<td>3</td>
<td>HSP Hypertension, Hypercholesterolemia</td>
<td>Septicaemia, Pneumonia</td>
<td>Normal renal function, Chronic hemolysis, Splenomegaly</td>
<td>Normal renal function</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mild proteinuria</td>
<td>Mild proteinuria</td>
</tr>
<tr>
<td>C3.4.I:1</td>
<td>Secondary</td>
<td>45 49</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Asymptomatic</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>CFI.1.II:1</td>
<td>Secondary</td>
<td>23 39</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Meningococcal septicaemia, Meningococcal meningitis (x20)</td>
<td>Asymptomatic</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>CFI.1.II:4</td>
<td>Secondary</td>
<td>14 30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Asymptomatic</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>CFI.2.II:2</td>
<td>Secondary</td>
<td>19 53</td>
<td>-</td>
<td>-</td>
<td>HSP Chronic juvenile arthritis</td>
<td>Meningococcal meningitis, Pneumococcal pneumonia</td>
<td>Asymptomatic</td>
<td>Asymptomatic</td>
</tr>
</tbody>
</table>

MPGN I: membranoproliferative glomerulonephritis. C3G: C3 glomerulopathy. HSP: Henoch-Schönlein purpura. *At diagnosis; †present age; CKD (Chronic Kidney Disease) stage: min 1 - max 5. No correlation was found with memory B cell levels, not shown; §bacterial, viral, other; number of infections are marked between brackets; ¶Normal ranges (5th-95th percentiles) for memory B cells: 8.6-28.4 % unswitched and 11.4-32.6 % switched. Bold means out of range.
EXTRACELLULAR C3 ACTIVATION

Amplification loop

C3bBb, C4bC2b

C3b, C3a

Primary C3 deficiency C3 LOF

Secondary C3 deficiency C3 GOF

Factor I

Secondary C3 deficiency Factor I LOF

iC3b, C3dg

MEMORY B CELL DEVELOPMENT