SUPPLEMENTARY MATERIALS

Nanoparticle physicochemical properties determine the activation of intracellular complement

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The authors declare no conflict of interest

Acknowledgment

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Authors contributions

A.N.I. planned and conducted experiments, as well as analyzed data. A.S. conducted follow up flow cytometry and western blotting experiments. K.C.C. carried out SCX fractionation, J.A.K. performed proteomic sample preparation and LC-MS analysis. J.B. designed proteomics experiments and carried out all relevant data analysis. A.E.E. synthesized triazine dendrimers and performed their characterization. E.E.S. conceived the synthesis and managed all work related to the dendrimer characterization. M.A.D conceived the study, planned experiments, analyzed data and wrote the manuscript. All authors contributed to figure preparation and manuscript writing.

Supplementary Materials and Methods

Reagents

CD3/CD28 beads were from Dynabeads from ThermoFisher Scientific (Waltham, MA). Protease inhibitors, their catalog number, and manufacturers, were as follows: cathepsin L inhibitor sc-364671 and Caspase8/Granzyme B inhibitor sc-3084 – Santa Cruz Biotechnology (Dallas, TX); cathepsin L inhibitor ALX-260-133-M001 – Enzo Life Sciences (Farmingdale, NY); cathepsin L inhibitor SCP0110, and E64 (E3132) – Sigma (St.Louis, MO); Pefablock 11873601001 – Roche (Basel, Switzerland); Futhan 552035 - BD Pharmigen (San Jose, CA); Z-VAD-FMK, ZVD3401 – Invivogen (San Diego, CA); Furin inhibitor I, 344930 – EMD Millipore (Burlington, MA); Bortezomib, S1013 – Selleckchem (Huston, TX); Compstatin 2585, - Tocris Bioscience (Bristol, UK). Antibodies, their catalog numbers, and manufacturers were as follows: anti-CD3 OKT, cat#86883, lots GR52307-4 and GR1917169-1 – Abcam (Cambridge, UK); anti-C3a/C3adesArg, HM2074, clone 2991 – Hycult (Plymouth Meeting, PA); rat anti-mouse IgG1 PE, 12-4015-82 and rat anti-mouse IgG2-APC 17-4210-82 – eBioscience (Santa Clara, CA); mouse anti-human CD3 APC and human intracellular cytokine staining starter kit, 559302 – BD Pharmingen (San Jose, CA); monoclonal antibody to human C3 (C3c) - Quidel (San Diego, CA); anti- C3adesArg antibody clone K13/16 - BioLegend (San Diego, CA); β-Actin (D6A8) rabbit monoclonal antibody, HRP-linked anti-mouse IgG and HRP-linked anti-rabbit IgG - Cell Signaling Technology (Danvers, MA). Recombinant purified human C3c from *E.coli* and human source were purchased from MyBiosource (San Diego, CA) and Abcam (Cambridge, MA), respectively.

Synthesis and characterization of triazine dendrimers.

Synthesis of the dendrimer described has been reported [1, 2]. Briefly, an iterative, divergent approach was employed using a macromonomer, a molecule that provides two generations of growth for every synthetic cycle. The macromonomer comprises three triazines interconnected by a PEG-like diamine, 1,4,7,11-trioxotridecanediamine, such that overall, the macromonomer has a monochlorotriazine at the focus and four protected terminal amines on the periphery. Synthesis is monitored using 1H and 13C NMR spectroscopy, mass spectrometry, fluorescence spectroscopy, high-performance liquid chromatography as well as DLS [3].

Flow cytometry.

To detect intracellular complement activation by flow cytometry, we followed the protocol described by Liszewski M. et al.,[4]. To detect surface expression of complement, cells were incubated with antibodies specific to CD3adesArg, clone 2991. The amount of the antibody was 1µg per sample for 30 min in DPBS with 0.1% BSA at 4ºC. After that the cells were washed twice with PBS and incubated for 30 min at 4 ºC with secondary anti-mouse, PE-labeled IgG1 and APC-conjugated anti-CD3 antibodies. The cells in the positive control samples treated with anti-CD3 OKT antibodies were stained using APC-conjugated anti-mouse IgG2α. Cells were washed twice and resuspended in DPBS. Samples were analyzed using FACS Calibur flow cytometer and Cell Quest software from BD Biosciences (San Jose, CA). Human PBMC were first separated based on their forward and side scatter profiles into lymphocytes and monocytes populations. Cells from the lymphocyte gate were next analyzed for the presence of the T-cell marker CD3. Finally, CD3-positive and negative cells were gated to detect positive cells stained with C3adesArg antibody, clone 2991 and fluorescently -tagged secondary antibody.

Induction and detection of the intracellular cytokines.

Isolated PBMC were pre-treated with anti-human CD3/CD28 beads for 4 hours in full culture media with or without G5-NH2 PAMAM dendrimers at 37ºC, 5%CO2. Brefeldin A was added to culture media to prevent cytokines release from cells. At the end of the incubation, cells were washed and stained using human intracellular cytokine staining starter kit according to the manufacturer's protocol. Briefly, PBMC were stained with CD4-APC antibodies for 30 min at 4ºC. Cells were washed with PBS twice. PBMC were fixed and permeabilized with BD Cytofix/Cytoperm™ Buffer. To maintain cell permeability cells were washed with BD Perm/Wash™ Buffer. Anti-IL2PE and anti-IFNγPE antibodies were added to corresponding samples to detect intracellular cytokines.

Western blotting

Western blotting to determine the intracellular complement levels was performed using whole cell lysates from Jurkat cells treated with 20 µg/mL of G5-NH2 PAMAM dendrimers for 1 hour. Upon termination of the treatment, cells were centrifuged and washed twice with PBS. The cell pellets were lysed with radioimmunoprecipitation assay (RIPA) buffer (Boston BioProducts,

Ashland, MA, USA) supplemented with HaltTM Protease and Phosphatase Inhibitor Single-Use Cocktail (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 10 min at 4ºC followed by sonication for 10 seconds at 1 amplitude to ensure complete lysis of the cellular organelles. Finally, the lysates were centrifuged to remove debris. Protein concentrations in the lysates were measured using BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 50 µg protein along with 1 µg of recombinant human C3c synthesized in *E.Coli* cultures and human source were loaded on Novex 4-20% Tris-Glycine Gels. The protein samples were electrophoresed and transferred on PVDF membranes. The membranes were then blocked in 5% non-fat milk in PBST (0.075% Tween 20 in PBS) overnight at 4°C to reduce nonspecific antibody binding. The blots were probed for appropriate targets using the optimized concentrations of primary and secondary antibodies, and protein bands were visualized using Pierce ECL Plus reagent. The blot images were acquired using G:Box gel documentation system and Genesys software (Syngene USA, Frederick, MD). All the images were adjusted evenly for brightness and contrast throughout the blots.

Isolation of plasma membrane proteins for analysis by Mass Spectrometry

Jurkat cells ($2x10^8$ cells per sample) were treated with G5-NH2 PAMAM (20µg/mL) for 1 hour at 37ºC, 5%CO2. The cells were washed, and plasma membrane protein extraction was performed using plasma membrane protein extraction kit according to a manufacturer's protocol. Briefly, the cells were resuspended in ice-cold Homogenize Buffer Mix and then homogenized on ice using Dounce homogenizer. The total cellular membrane proteins were separated from cytosol fraction by centrifugation at 10,000 x g for 30 min at +4°C. Extraction of the plasma membrane protein fraction from total cellular membrane proteins was performed using Lower and Upper Phase solutions. The isolated pellets of plasma membrane proteins were stored at -80°C.

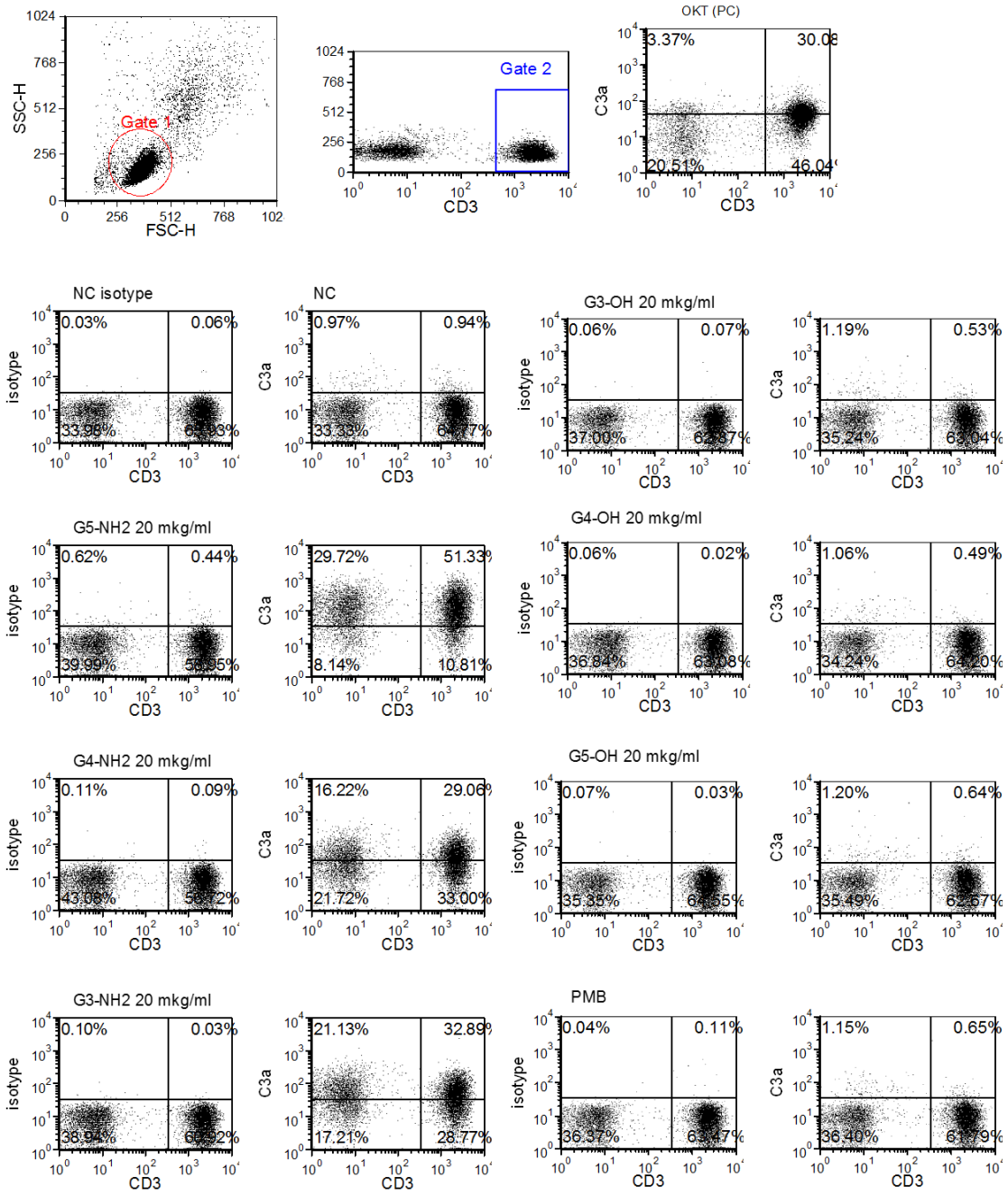
LC-MS proteomics analysis

Membrane proteins from the experimental and control sample were solubilized and digested using trypsin (Promega, Madison, WI) as previously described[5]. Next, peptide digests were enzymatically deglycosylated and dephosphorylated using PNGaseF (New England BioLabs, Ipswich, MA) and thermosensitive alkaline phosphatase (Thermo Fisher Scientific Inc. Waltham, MA) in accordance with manufacturers 'protocols. Finally, peptide digests were fractionated prior

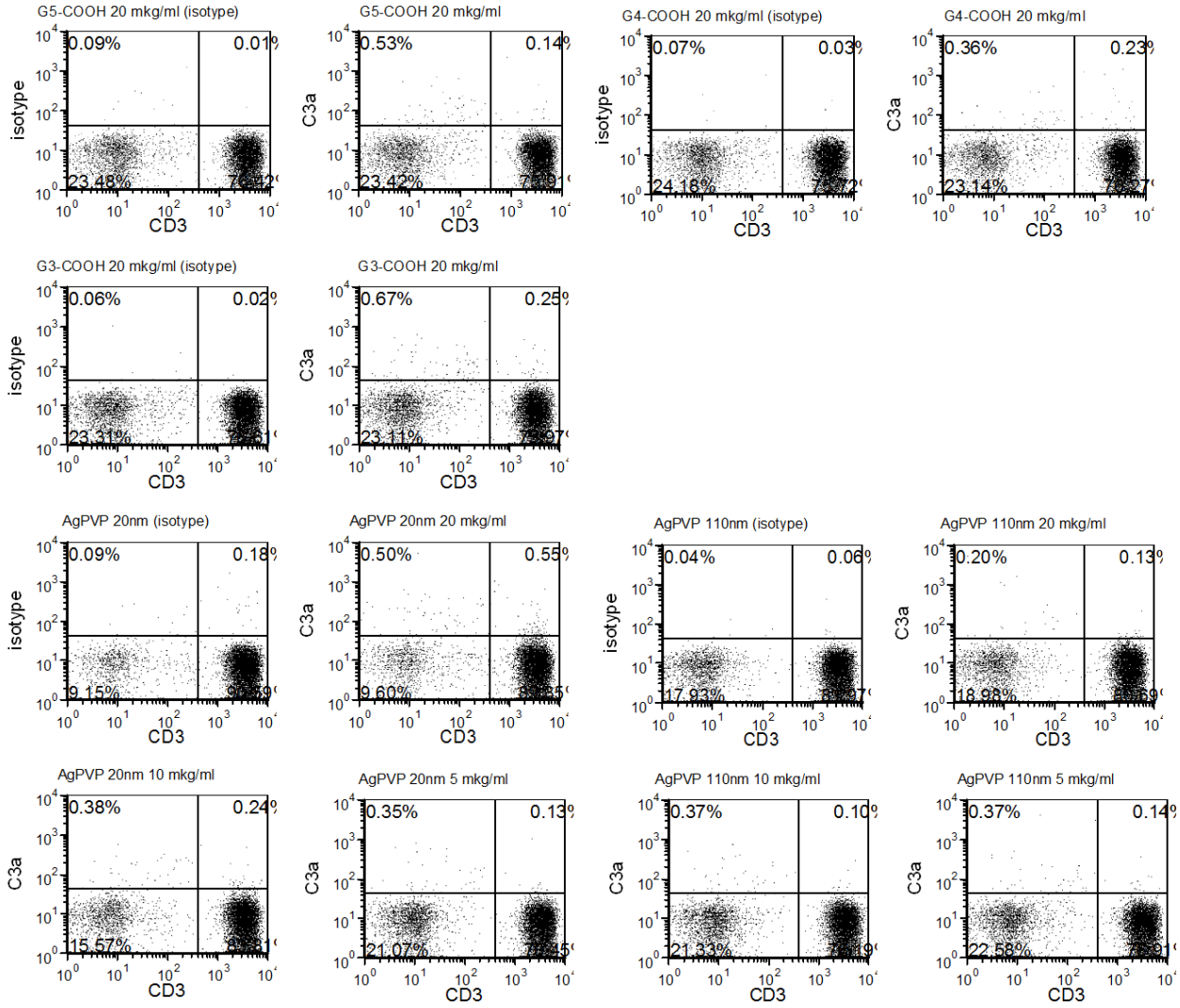
to LC-MS analysis employing off-line strong cation exchange (SCX) chromatography (LC), as previously described[6]. Based on the SCX-LC profile, peptides were pooled into 10 fractions and analyzed in duplicates using an Orbitrap Elite mass spectrometer (ThermoElectron, San Jose, CA) coupled to a nano-flow reversed phase (RP) LC (Agilent 1100, Sanat Clara, CA) as previously described [6]. Proteins were identified using the SEQUEST algorithm incorporated in the Proteome Discoverer 1.4 (Thermo). To search the human proteome database (SwissProt release v57.15). The search thresholds were: for the MS^1 spectra, mass tolerance was set at 5 ppm, and for MS^2 spectra, mass tolerance was set at 0.6 Da. Dynamic amino acid modifications were added for the detection of the following: +0.984 Da for asparagine deamidation, +57.021 Da for cysteine carboxyamidomethylation (i.e., alkylation), and +15.994 Da methionine oxidation. The search allowed for fully tryptic peptides with up to two missed cleavage sites. Proteins identified by a single peptide-spectrum match (PSM) were not included in the final dataset. Strict FDR was set to ≤ 0.01. Protein grouping was employed to increase the quality and reliability of protein identifications and enforce economy in the number of identified proteins. Previously described subtractive proteomic analysis[7] was used to reveal a non-redundant list of proteins identified solely in dendrimer-treated cells.

To expand the coverage of the membrane proteome, we employed subtractive shot-gun membrane proteomics [8]. This strategy has been proven effective in proteomic profiling of complex membrane protein mixtures [9]. A total of ten (10) pooled SCX peptide fractions from dendrimer-treated cells and control (i.e., negative control Jurkat cells) membrane preparation were collected and injected twice (i.e., two technical replicates) to execute LC-MS analysis.

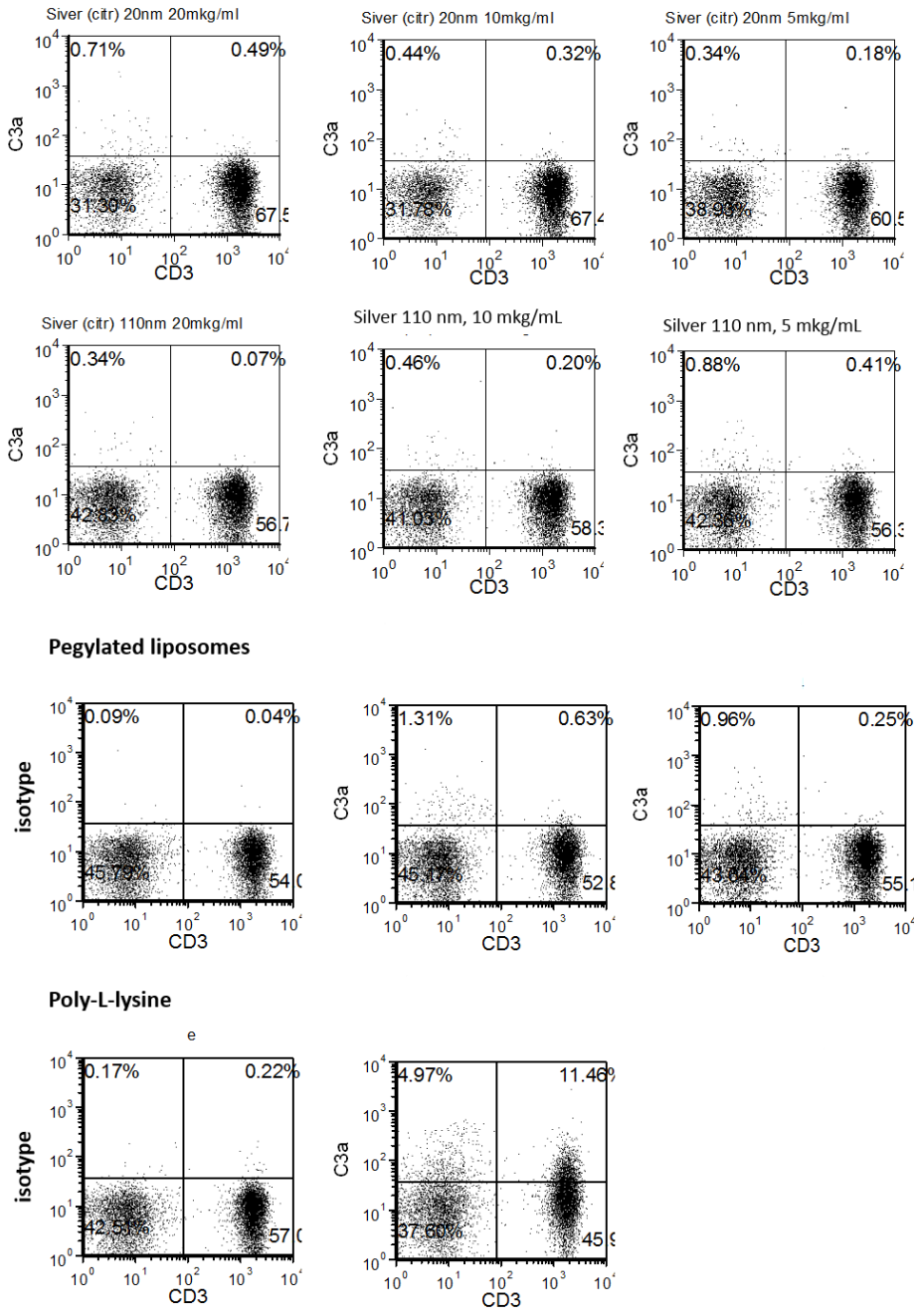
Supplementary Figures and Tables



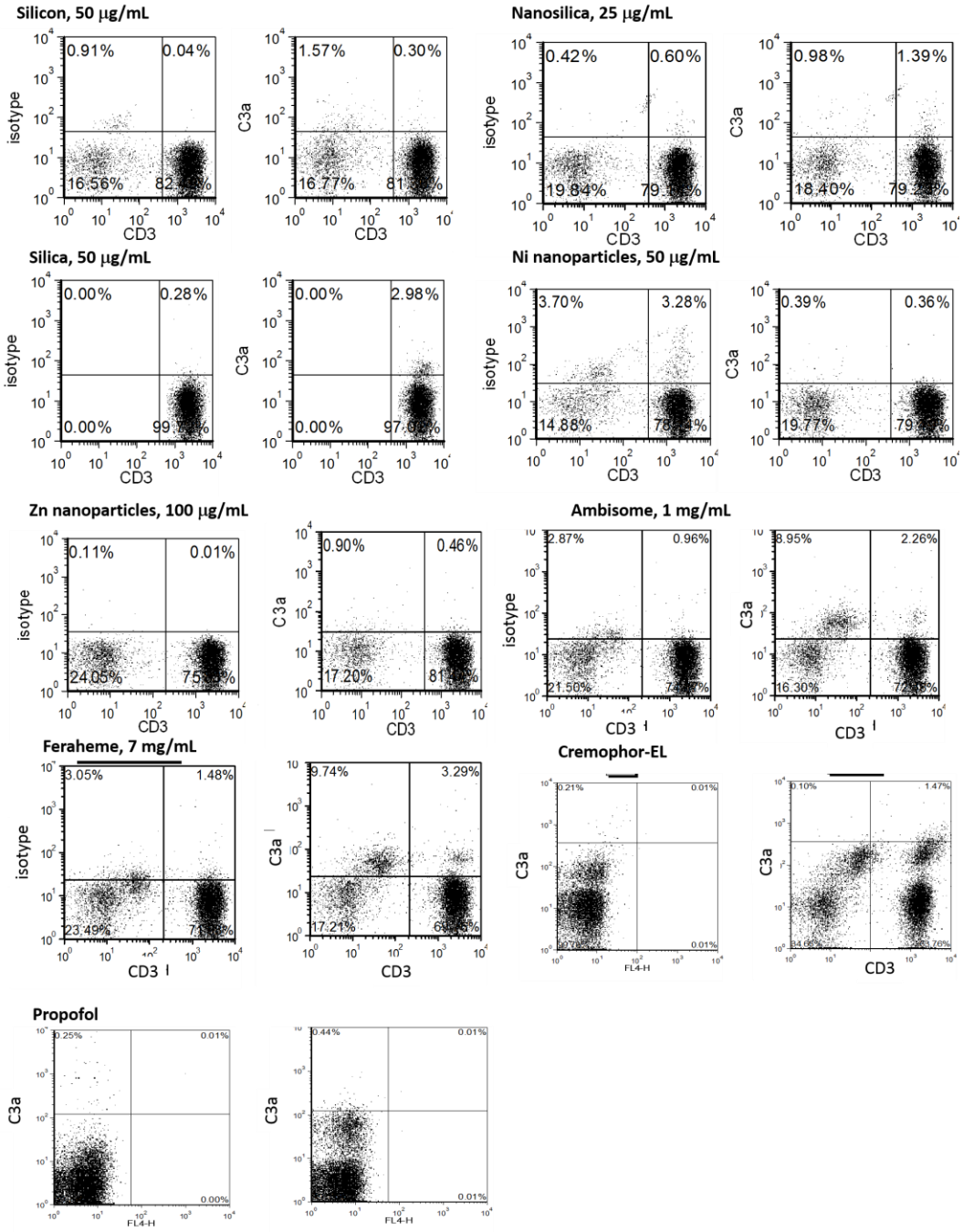
Supplementary Figure 1. Detection of intracellular complement by flow cytometry on the surface of PBMC exposed to different nanoparticles and controls. The experiments were conducted using primary human PBMC as described in materials and methods. Representative flow cytometry plots of both isotype control and C3adesArg-antibody (clone 2991) stained cells are shown. PMB – polymyxin B; G3-NH2, G4-NH2, and G5-NH2 – generation 3, 4 and 5, respectively, amine-terminated PAMAM-dendrimers. G3-OH, G4-OH, and G5-OH- hydroxy-terminated PAMAM dendrimers. OKT3-antiCD3 antibodies (positive control). mkg/mL is concentration in μg/mL.



Supplementary Figure 1 (continues). Detection of intracellular complement by flow cytometry on the surface of PBMC exposed to different nanoparticles and controls. The experiments were conducted using primary human PBMC as described in materials and methods. Representative flow cytometry plots of both isotype control and C3adesArg-antibody (clone 2991) stained cells are shown. Ag-PVP – poly(vinyl)pyrrolidone stabilized colloidal silver nanoparticles. G3-COOH, G4-COOH, and G5-COOH – generation 3, 4 and 5, respectively, carboxy-terminated PAMAM dendrimers. mkg/mL is concentration in μg/mL.



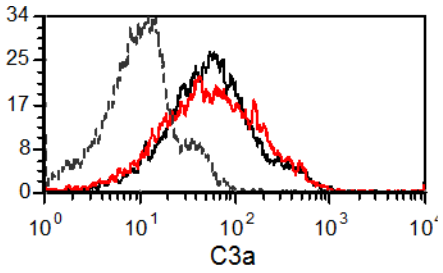
Supplementary Figure 1 (continues). Detection of intracellular complement by flow cytometry on the surface of PBMC exposed to different nanoparticles and controls. The experiments were conducted using primary human PBMC as described in materials and methods. Representative flow cytometry plots of both isotype control and C3adesArg-antibody (clone 2991) stained cells are shown. Silver (citr) – citrate stabilized colloidal silver nanoparticles. mkg/mL is concentration in μg/mL. PEGylated liposomes: the plot in the middle represents Doxil carrier at the concentration 0.7 μg/mL of the DXR equivalent, the plot on the right shows Doxil carrier at the concentration of 0.4 μg/mL of DXR equivalent.



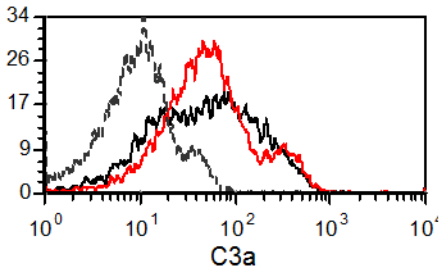
Supplementary Figure 1 (continues). Detection of intracellular complement by flow cytometry on the surface of PBMC exposed to different nanoparticles and controls. The experiments were conducted using primary human PBMC as described in materials and methods. Representative flow cytometry plots of both isotype control and C3adesArg-antibody (clone 2991) stained cells are shown. Each treatment is labeled at the top of the pair of graphs and includes two plots: the plot on the left is the isotype control and the plot on the right is the C3adesArg data for the same set of cells as that shown in the isotype for the given treatment. In isotype control plot of Cremophor-EL, and both plots showing Propofol treatment, CD3 staining was not included and the cells were analyzed from the lymphocyte gate.

Inhibitor	Target	Tested Concentrations
Cathepsin L Inhibitor I (Z-FF-FMK) (Santa Cruz sc-364671)	Cathepsin L	5, 25 μM
Cathepsin L inhibitor ((N-1Naphthalenylsulfonyl)-IW-CHO) (Enzo ALX-260-133-M001)	Cathepsin L	40, 80 nM
Cathepsin L inhibitor (Z-FF-FMK) (Calbiochem 219421)	Cathepsin L	0.5, 15, 25 μM
Cathepsin L inhibitor (RKLLW-NH2) (Sigma SCP0110)	Cathepsin L	15, 25, 50 μM
E64 (trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (Sigma E3132)	Cysteine proteases	10, 50 μM
Pefablock (AEBSF) (Roche 11873601001)	Serine proteases	0.5, 2 mM
Futhan (Fu-175) (BD Pharmigen 552035)	Broad specificity protease inhibitor (NOT cell permeable)	50, 200 μg/ml
Caspase8/Granzyme B inhibitor (Santa Cruz sc-3084)	Caspase8/GranzymeB	50, 100 μM
Z-VAD-FMK (InVivoGen ZVD3401)	Pan-caspase inhibitor	200, 400 μM
Furin inhibitor I (Calbiochem 344930)	Inhibits processing from pre-C3 to C3	
Bortezomib (Selleckchem S1013)	Proteasome inhibitor	2.5, 5, 25 nM
Compstatin (Tocris #2585)	Complement inhibitor (prevents C3 cleavage)	28, 50 μM

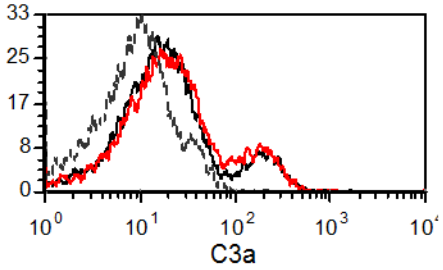
Supplementary Table 1. Protease inhibitors analyzed in our study. The table summarizes protease inhibitors, their suppliers and ordering information, targets and tested concentrations.



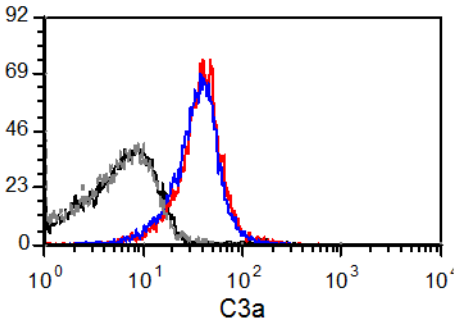
G5-NH 10 mkg/ml - black
G5-NH+inhibitor - red



G4-NH 10 mkg/ml - black
G4-NH+inhibitor - red

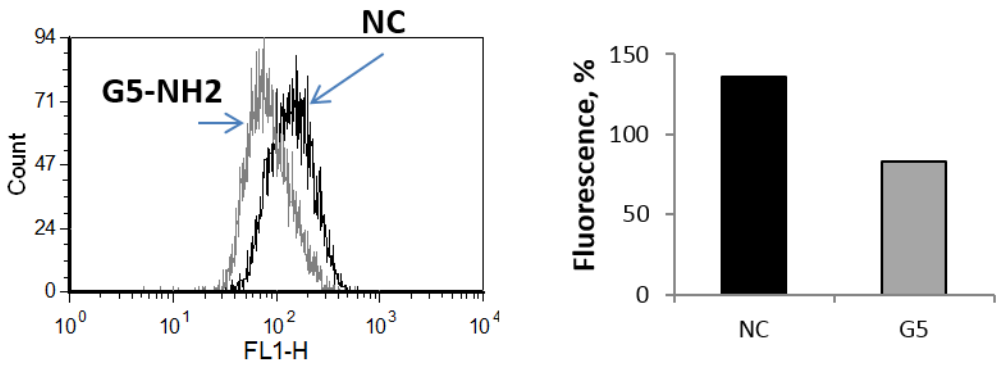


G3-NH 5 mkg/ml - black
G3-NH+inhibitor - red



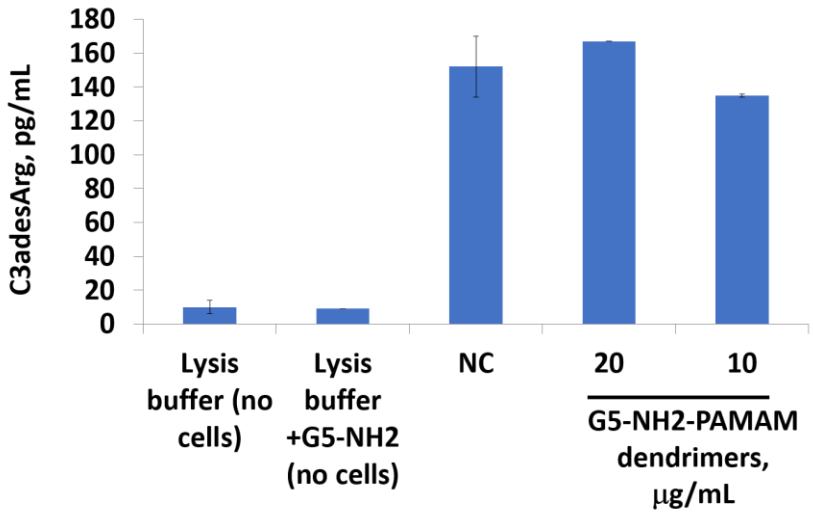
NC - black
isotype - grey (dotted)
red - OKT
blue - OKT+CSTL80nM(30min)

Supplementary Figure 2. Effects of Cathepsin-L inhibitors on OKT and dendrimer-stimulated intracellular complement expression. Cells from healthy donors were either untreated or treated with OKT antibody or dendrimers. Each treatment was also repeated in the presence of cathepsin L inhibitor (+inhibitor or +CTSL). The inhibitor at the final concentration of 80nM was added to the cultures 30 minutes before stimulation with OKT3 or dendrimers. Shown is representative data from cells of one donor. mkg/mL is the concentration in μg/mL

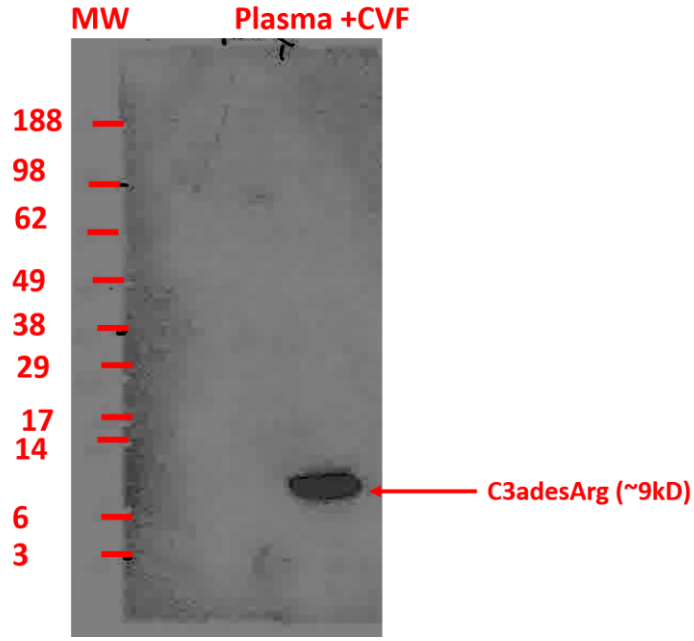


Supplementary Figure 3. Dendrimer treatment results in fluorescein leakage from cells. PBMC from healthy donors were loaded with fluorescein diacetate (FDA). The excess dye was washed away, and cells were treated with PBS as a negative control (NC) or G5-NH2 PAMAM dendrimers (G5 or G5-NH2) for 1h at 37C. The cells were next analyzed by flow cytometry. FDA is cleaved inside the cell by cellular esterase into fluorescein, which cannot pass the cellular membrane unless the membrane is perforated. The decrease in the cellular fluorescence of FDA-loaded cells occurs when fluorescein leaves the cells through the holes in the cellular membrane. Shown are representative data from cells of one donor.

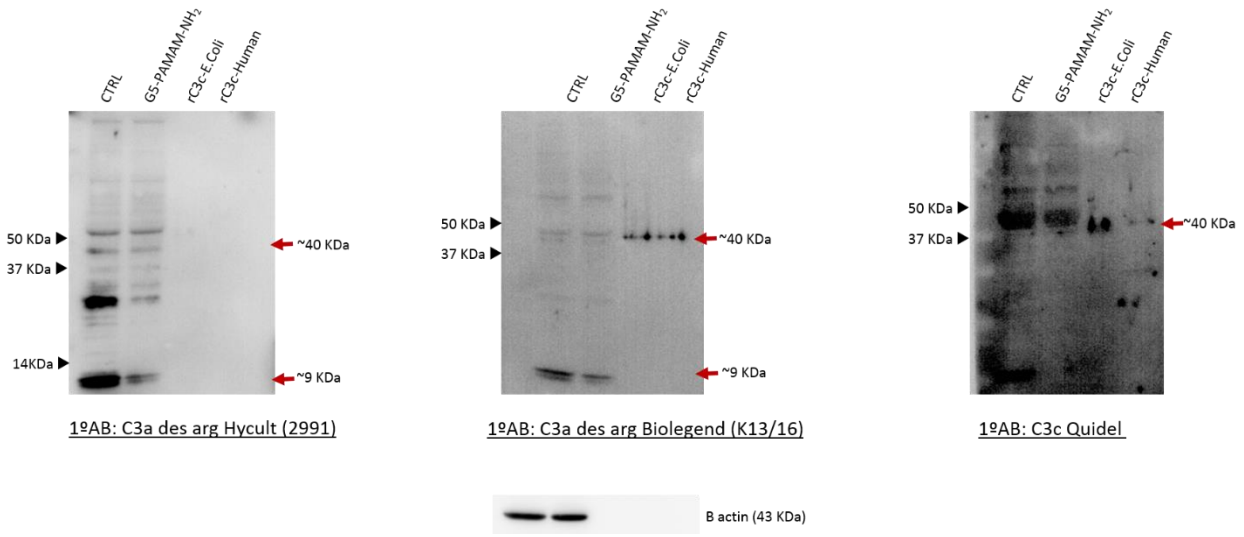
(A)



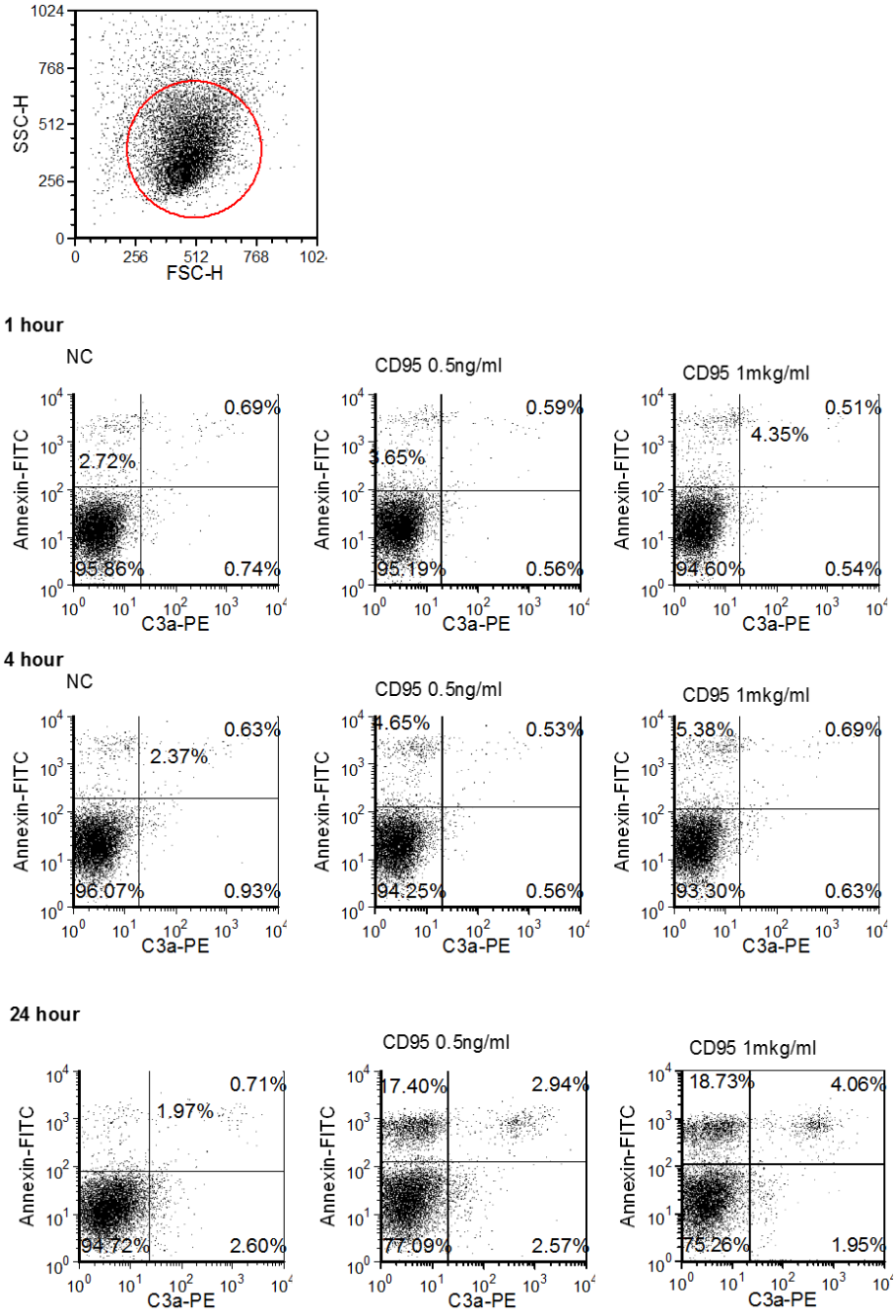
(B)



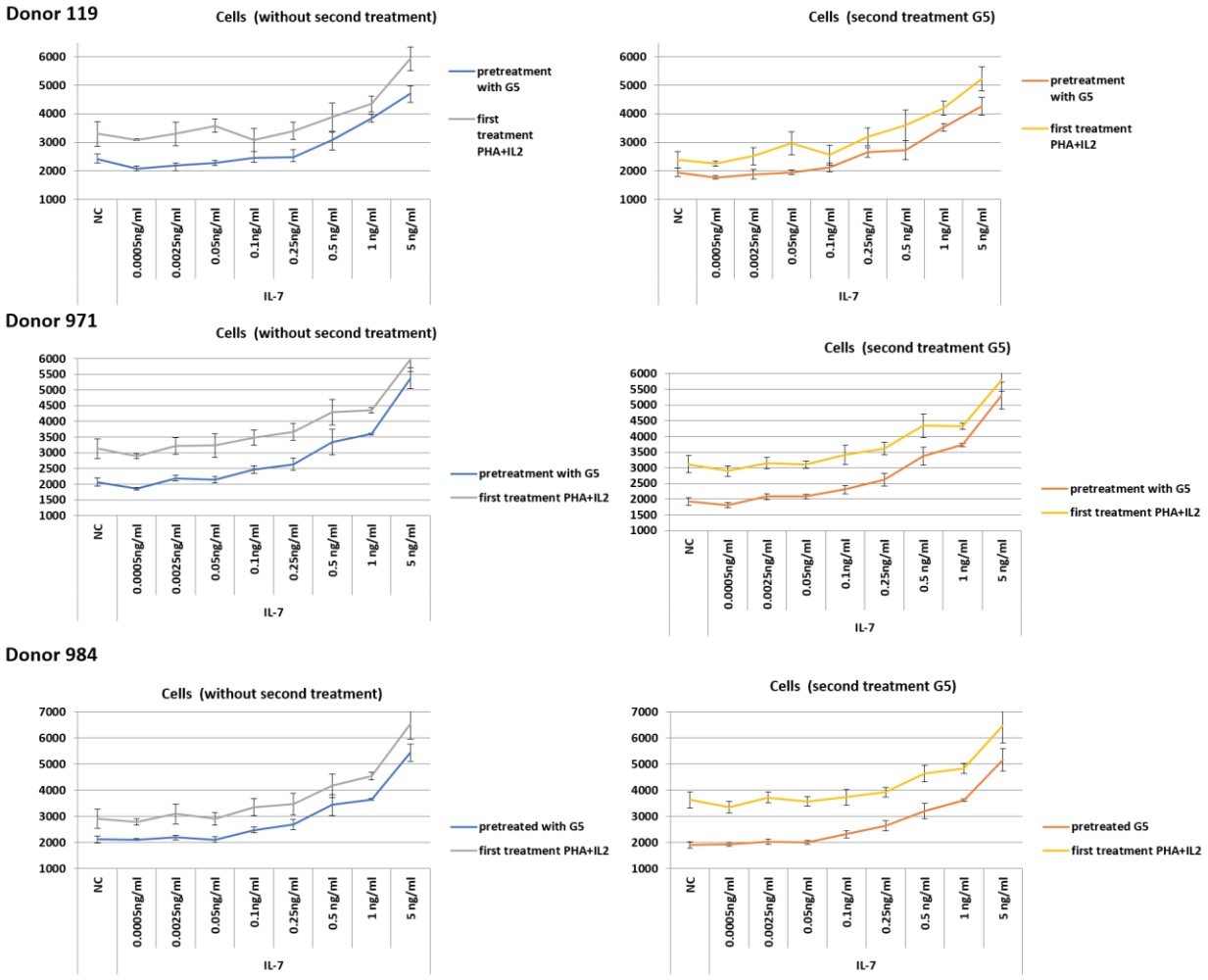
Supplementary Figure 4. Detection of complement by ELISA and Western blotting. (A) PBMC were treated with either negative control, PBS (NC) or amine-terminated PAMAM dendrimers (G5-NH2-PAMAM dendrimers). Cell lysates were prepared as described in materials and methods and tested by commercial ELISA intended for the detection of C3adesArg in plasma and serum samples. Lysis buffer unspiked or spiked with G5-NH2 PAMAM dendrimers were analyzed side-by-side as controls intended to rule out false-positive results. (B) Western blot analysis of plasma sample treated with cobra venom factor using C3adesArg antibody clone 2991 identifies a short peptide, which is consistent with the expectation that this clone detects C3a split product as opposed to the C3a portion of the full C3 protein or its alpha-chain.



Supplementary Figure 5. Western blot analysis of cellular lysates and recombinant C3c proteins using various monoclonal antibodies. The cellular lysates of untreated or cationic G5 PAMAM dendrimer treated Jurkat cells were separated by polyacrylamide gel electrophoresis as described in materials and method. Three gels were run for the same set of samples to increase the throughput. Primary antibodies (1ºAB) with different specificity (C3a desArg vs. C3c) or different clones (C3adesArg clone 2991 vs. K13/16) were used for the analysis. Bands with approximate molecular weight of 40 and 9 kD, highlighted with red arrows, correspond by size to C3c and C3a, respectively. After the analysis using complement specific antibodies, the blots were stripped and re-probed using an antibody specific to beta-actin to verify the protein loading. Shown in the middle is the representative beta-actin image obtained using the same membrane as that probed earlier with C3adesArg antibody clone K13/16. No beta-actin is seen in the lanes containing recombinant C3c proteins and is expected.



Supplementary Figure 6. Induction of apoptosis using CD95 antibody does not induce intracellular complement expression on the cell surface. Lymphocytes were treated with CD95 agonist antibody for various periods. The induction of apoptosis and presence of the intracellular complement on the cell surface were monitored by Annexin V and C3adesArg staining, respectively. Shown are representative data from cells of one donor. mkg/mL is the concentration in μg/mL



Supplementary Figure 7. Analysis of dendrimer effects on IL-7 induced T-cell proliferation. PBMC from healthy donors were studied. The analysis included two treatment scenarios. The first scenario is shown in the left column and marked as Cells (without second treatment). In this scenario, the cells were incubated for 5 days in the presence of phytohemagglutinin L and IL-2 to allow the expansion of the T-cells. One group was incubated only in the presence of PHA and IL-2 (labeled on the graph as first treatment PHA+IL-2) , wheres G5-NH2 PAMAM dendrimers at concentration 10 μg/mL were included into the media in addition to the PHA and IL-2 (labeled on the graph as pretreated with G5). At the end of 5th day, the cells were washed and stimulated with various concentrations of IL-7. In the second scenario, shown on the right, all cells were first expanded in the presence of PHA-L and IL-2, then washed and then restimulated with various concentrations of IL-7. The cells incubated with IL-7 only are labeled on the graph as first treatment PHA+IL-2. The cells, which were incubated with IL-7 in the presence of 10 μg/mL of G5-NH2 PAMAM dendrimers, are shown on the graph as pretreated G5. R&D systems protocol for the detection of T-cell proliferation using resosurin assay was followed in both scenarios.

UniProt BLAST Analysis, CO3 HUMAN vs. CO3 BOVIN: Selected alignment(s). E-value: 0.0, Score: 6745, Ident.: 77.1%, Positives : 87.7%, Query Length: 1663, Match Length: 1661

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P01024 CO3_HUMAN 1140  DMALTAFVLISLQEAKDICEEQVNSLPGSITRAGDFLEANYMNLQRSYTVAIAGYALAQM  1199
                       D++LTAFVLI+L EAKDICE QVNSL  SI KAGDFLE +Y  L+R YTVAIA YALA +
Q2UVX4 CO3_BOVIN 1139  DVSLTAFVLIALHEAKDICEAQVNSLGRSIAKAGDFLENHYRELRRPYTVAIAAYALALL  1198
P01024 CO3_HUMAN 1200  GRLKGPLLNKFLTTAKDKNRWEDPGKQLYNVEATSYALLALLQLKDFDFVPPVVRWLNEQ  1259
                       G+L+G  L KFL TAK+KNRWE+P ++LYNVEATSYALLALL  KD+D  PPVVRWLNEQ
Q2UVX4 CO3_BOVIN 1199  GKLEGDRLTKFLNTAKEKNRWEEPNQKLYNVEATSYALLALLARKDYDTTPPVVRWLNEQ  1258
P01024 CO3_HUMAN 1260  RYYGGGYGSTQATFMVFQALAQYQKDAPDHQELNLDVSLQLPSRSSKITHRIHWESASLL  1319
                       RYYGGGYGSTQATFMVFQALAQYQKD PDH+ELNLDVS+QLPSR+S + HRI WESASLL
Q2UVX4 CO3_BOVIN 1259  RYYGGGYGSTQATFMVFQALAQYQKDVPDHKELNLDVSIQLPSRNSAVRHRILWESASLL  1318
P01024 CO3_HUMAN 1320  RSEETKENEGFTVTAEGKGQGTLSVVTMYHAKAKDQLTCNKFDLKVTIKPAPETEKRPQD  1379
                       RSEETKENE FTV AEGKGQGTLSVVT+YHAK K +++C KFDL+V+I+PAPET K+PQD
Q2UVX4 CO3_BOVIN 1319  RSEETKENERFTVKAEGKGQGTLSVVTVYHAKLKGKVSCKKFDLRVSIRPAPETVKKPQD  1378
P01024 CO3_HUMAN 1380  AKNTMILEICTRYRGDQDATMSILDISMMTGFAPDTDDLKQLANGVDRYISKYELDKAFS  1439
                       AK +MIL+ICT+Y GDQDATMSILDISMMTGF+PD +DLK L+ GVDRYISKYE+++  S
Q2UVX4 CO3_BOVIN 1379  AKGSMILDICTKYLGDQDATMSILDISMMTGFSPDVEDLKTLSTGVDRYISKYEMNRD-S  1437
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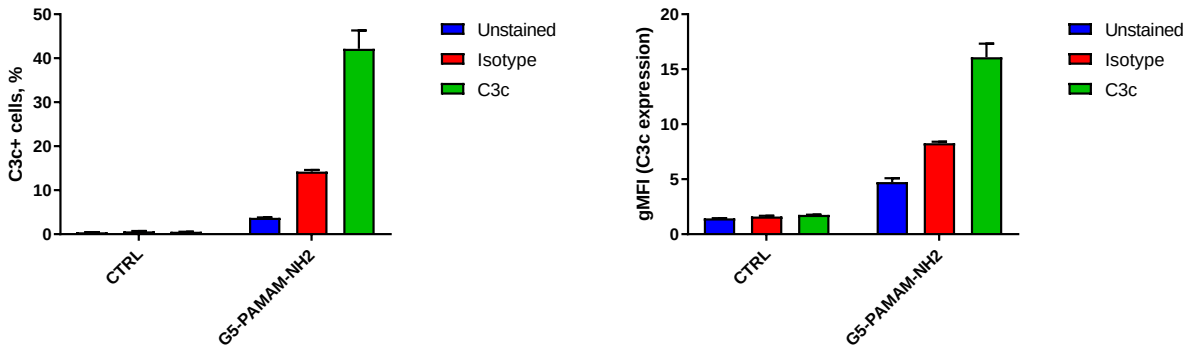
Supplementary Figure 8. UniProt BLAST analysis of peptides identified by mass spectrometry. The analysis was performed as described in materials and methods. Green color highlights the position of complement split products C3c and C3d.

PTM / Processing

Molecule processing

Feature key	Position(s)	Description
Signal peptide	1 – 22	1 Publication
Chain (PRO_0000005907)	23 – 1663	Complement C3
Chain (PRO_0000005908)	23 – 667	Complement C3 beta chain
Chain (PRO_0000430430)	569 – 667	C3-beta-c By similarity
Chain (PRO_0000005909)	672 – 1663	Complement C3 alpha chain
Chain (PRO_0000005910)	672 – 748	C3a anaphylatoxin
Chain (PRO_0000419935)	672 – 747	Acylation stimulating protein
Chain (PRO_0000005911)	749 – 1663	Complement C3b alpha' chain
Chain (PRO_0000005912)	749 – 954	Complement C3c alpha' chain fragment 1
Chain (PRO_0000005913)	955 – 1303	Complement C3dg fragment
Chain (PRO_0000005914)	955 – 1001	Complement C3g fragment
Chain (PRO_0000005915)	1002 – 1303	Complement C3d fragment
Peptide (PRO_0000005916)	1304 – 1320	Complement C3f fragment 1 Publication
Chain (PRO_0000273948)	1321 – 1663	Complement C3c alpha' chain fragment 2

Supplementary Figure 9. Analysis of complement component C3 fragmentation and the position of corresponding peptides. The analysis was performed as described in materials and methods.



Supplementary Figure 10. Analysis of control and cationic G5 PAMAM dendrimer treated Jurkat cells by flow cytometry using C3c-specific monoclonal antibody. The cells were either treated with PBS (CTRL) or 10 mg/mL of amine-terminated G5 PAMAM dendrimers (G5-PAMAM-NH2) for one hour. The cells were then analyzed by flow cytometry using a monoclonal antibody specific to C3c component of the complement. gMFI was used to measure the C3c expression on individual cells, while percent positive cells (C3c+ cells, %) was assessed to estimate the induction of intracellular complement expression in the total population of treated cells. Isotype control antibodies were used to exclude non-specific staining.

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