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Complement activation by carbon nanotubes and its influence on the phagocytosis and cytokine response by macrophages

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Abstract

Carbon nanotubes (CNTs) have promised a range of applications in biomedicine. Although influenced by the dispersants used, CNTs are recognized by the innate immune system, predominantly by the classical pathway of the complement system. Here, we confirm that complement activation by the CNT used continues up to C3 and C5, indicating that the entire complement system is activated including the formation of membrane-attack complexes. Using recombinant forms of the globular regions of human C1q (gC1q) as inhibitors of CNT-mediated classical pathway activation, we show that C1q, the first recognition subcomponent of the classical pathway, binds CNTs via the gC1q domain. Complement opsonisation of CNTs significantly enhances their uptake by U937 cells, with concomitant downregulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory cytokines in both U937 cells and human monocytes. We propose that CNT-mediated complement activation may cause recruitment of cellular infiltration, followed by phagocytosis without inducing a pro-inflammatory immune response.

From the Clinical Editor: This study highlights the importance of the complement system in response to carbon nanontube administration, suggesting that the ensuing complement activation may cause recruitment of cellular infiltration, followed by phagocytosis without inducing a pro-inflammatory immune response.

The unique physical and chemical properties of carbon nanotubes (CNTs) make them very desirable materials in a range of biomedical applications.[1,2] CNT-mediated drug delivery has generated special interest.[1,3–11] By functionalising the outer walls of the nanotubes via attaching target-specific molecules (e.g. antibodies), drugs can be delivered to specific targets. Iron filled CNTs (Fe-MWNTs), filled with a ferromagnetic material, promise magnetic drug delivery and hyperthermia therapy.[3,12,13]

Due to their hydrophobicity and length, making stable dispersions of CNTs in physiological buffers, essential for drug delivery, is a common obstacle. Thus, non-covalent and covalent modifications of the CNT surfaces that include pre-coating with proteins, surfactants, nucleic acids, or introducing new functional groups on the external walls, are used. The biocompatibility of CNTs can be significantly improved when their surface is functionalized.[1,14–16]

Understanding the interactions between nanoparticles and immune system would facilitate their strategic and specific *in vivo* delivery.[17] The innate immune system plays a key role in protection against microorganisms and synthetic particles including CNTs.[18–21] Activation of the complement system, a major component of the innate immunity,[22] can influence therapeutic
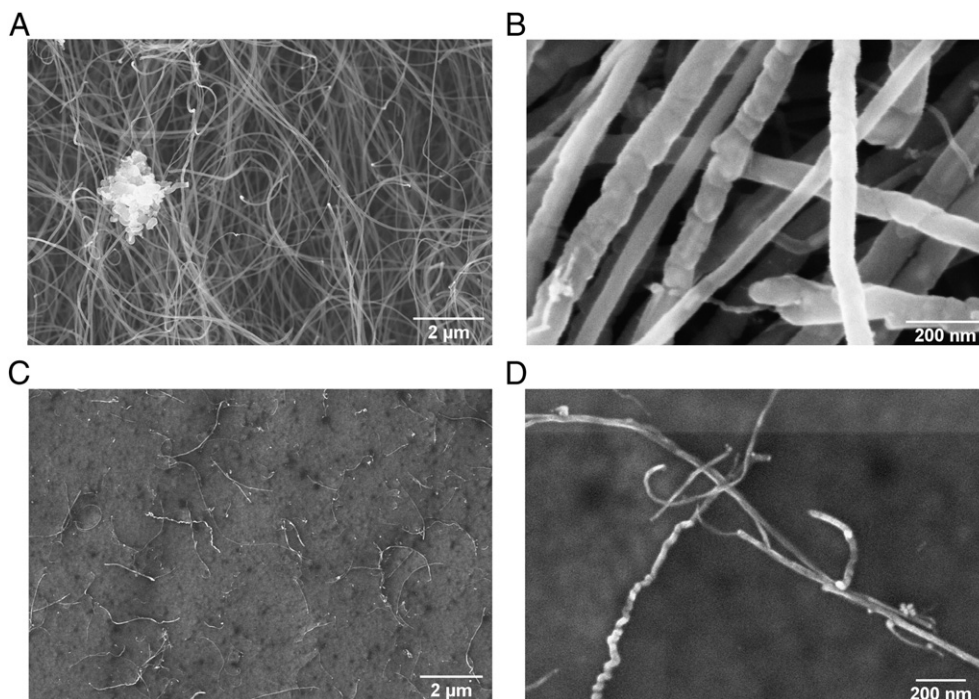
Figure 1. Scanning electron microscopy images of pristine (**A** and **B**) and CMC-coated (**C** and **D**) Fe-MWNTs showing high purity of the pristine samples and high dispersion of the functionalized samples. The dispersions are very stable (non-sedimenting).

activity and effectiveness of CNTs, as it can, in principle, cause nanoparticles to adhere to immune cells. In addition, activation of the complement system releases the bioactive peptides, C3a, C4a, C5a, which contribute to inflammation.[19–22]

The complement system is composed of a group of >40 plasma and cell surface proteins,[22] which recognises and clears non-self (microorganisms) and altered self (apoptotic and necrotic cells, aggregated proteins, etc.). It can be activated via three pathways: classical, alternative or lectin, all of which converge on the formation of a C3 convertase, a protease which activates C3 that gets cleaved into C3b.[23] The target-bound C3b and a degradation product, iC3b are powerful opsonins, i.e. they mediate binding of the target to phagocytic cells. In the classical pathway, C1q binds to charged or hydrophobic clusters on targets,[23] via its globular (gC1q) domain, followed by activation of two protease proenzymes, C1r and C1s, which, with C1q, form the C1 complex. This is followed by cleavage of C4 and C2, to form C4b2a, the C3 convertase. In the lectin pathway, Mannose-Binding Lectin (MBL) and Ficolins recognise neutral sugar and other uncharged features,[24] and form C4b2a via the MBL-associated serine proteases (MASPs). The alternative pathway involves a constant but slow hydrolysis of C3 in solution, which forms $C3(H_2O)$. It forms a complex with Factor B, activated by Factor D (FD) to form $C3(H2O)Bb$ (a C3 convertase).[25] C3b, which binds randomly and covalently to any nearby surface or particle, is stabilised by properdin, an upregulator of the alternative pathway. This stage is followed by generation of C5 convertase to cleave C5 to form C5a and C5b. C5b binds to C6, C7, C8 and C9 to form the C5b-9 complex, or membrane attack complex (MAC), which can insert into the lipid bilayer of the target and cause cell lysis.[25] The small fragments, C3a, C4a and C5a, promote inflammation

as anaphylactic or chemotactic factors. Once C3b is deposited on a complement-activating particle, it is processed to form the products, iC3b and C3dg/C3d. These complement fragments are recognised by different complement receptors on various cell types, and the receptor–ligand interaction can cause the particle to adhere to the cell.[22] The receptors involved include Complement Receptor 1 (CR1) which binds C3b and C4b; CR2, which binds C3dg/C3d, CR3; and CR4 which bind iC3b and CRIg (C3b and iC3b).[26]

Non-functionalized SWNTs (single-walled) and DWNTs (double-walled), when placed in contact with human serum, activate complement via the classical and (to a lesser extent) the alternative pathway.[19] SWNTs, stabilised with several poly (ethylene glycol) derivatives, show no alternative pathway activation but C4 cleavage occurs, suggesting complement activation via the lectin pathway.[27,28] Functionalization (altering the surface properties of the CNTs) can increase or decrease the extent of complement activation,[15,18,27,28] while differing surface modifications can switch complement activation from one pathway to another. The mode of binding of the recognition subcomponents of the three pathways to CNTs, and whether they bind directly or via other deposited (serum) adaptor proteins, remains unclear. According to Ling et al,[29] C1q "crystallizes" on CNTs, but is not bound in a way that allows activation of the next step of the complement cascade. Thus, other serum proteins may form a stable layer on the CNTs, triggering indirect C1 binding and complement activation. Salvador-Morales et al,[18,19] however, observed direct binding of C1q to CNTs and subsequent complement activation.

In this study, we show several types of pristine and non-covalently functionalized CNTs activate the complement system predominantly via the classical pathway, which continues up to the activation of C3 and C5, confirming the findings of

Andersen et al[28] that the entire complement system is activated, leading to the formation of MAC. The interaction of C1q with these CNTs appears to occur via the gC1q domain since the recombinant forms of the gC1q regions of A, B and C chains bind CNTs stably. The binding of globular heads takes place in an orientation that inhibits the binding of serum C1q, thus suppressing C1-mediated classical pathway activation. We also report that complement activation and deposition on the surface of these CNTs enhance their uptake by macrophages (U937 cell line) in a time-dependent manner. Complement opsonisation of CNTs also leads to modulation of pro-inflammatory and anti-inflammatory cytokine expression by U937 cells and human monocytes.

Methods

Carbon nanotube dispersions

Purified catalytic vapour deposition DWNTs and Fe-MWNTs were prepared as described earlier.[30,31] To remove the iron filling (MWNTs), the samples were annealed under Argon to 2500 °C.[32] SWNTs were purchased from Nanocyl (Sambreville, Belgium) and dispersed using non-covalent functionalization via 2 min ultrasonication in PBS containing various dispersants with 5 mM EDTA, pH 7.4. Proteins used for dispersion were 4% w/v human fibrinogen (FBG) (Calbiochem), 4% w/v bovine serum albumin (BSA) (Sigma), and 4% (w/v) human serum albumin (HSA) (Sigma). Other dispersants used were 1% v/v Tween 20 (Sigma), 2% w/v Carboxymethyl cellulose (CMC) (Sigma), 2% w/v phospholipid polyethylene glycol (PL-PEG-NH2) (Avanti Polar Lipids) and 1% w/v baker's yeast RNA (Sigma). After sonication, the samples were centrifuged at 8000 g for 5 min to remove aggregates and the supernatants were washed by vacuum filtering using a 0.2 μm polycarbonate filter (Whatman) with PBS–5 mM EDTA in order to remove an excess of surfactants. Functionalized CNTs were re-suspended in PBS–EDTA and analysed using a Zeiss HR-LEO 1550 FEG Scanning Electron Microscope (SEM) (Figure 1). Length and diameter distributions of the MWNTs were determined from TEM images (data not shown); length 2.3±1.9 μm (n=562) diameter 23 ±11 nm (n=275).

Concentrations and extinction coefficients at 730 nm of all CNTs were determined in triplicates using a gravimetric method.[33] Briefly, CNTs were functionalized using RNA as described above, and after washing, absorption at 730 and 808 nm was measured for a series of dilutions. The solution was incubated with RNAse (1 mg/mL) (Sigma) at 37 °C for 3 h. After extensive washing, CNTs were dried on a Whatman 0.2 μm filter. The extinction coefficients found were used to determine the concentrations of the other functionalized CNT samples.

Opsonization of CNTs with C1q, ghA, ghB and ghC proteins

C1q was purified from human plasma using affinity chromatography on IgG-Sepharose.[34] The C-terminal globular regions of human C1q A, B and C chains, designated ghA, ghB and ghC, were expressed as maltose-binding-protein fusion proteins in E. coli.[35] CMC-MWNTs were incubated with C1q, ghA, ghB or ghC, separately or mixed together, at 1:2 w/w ratios
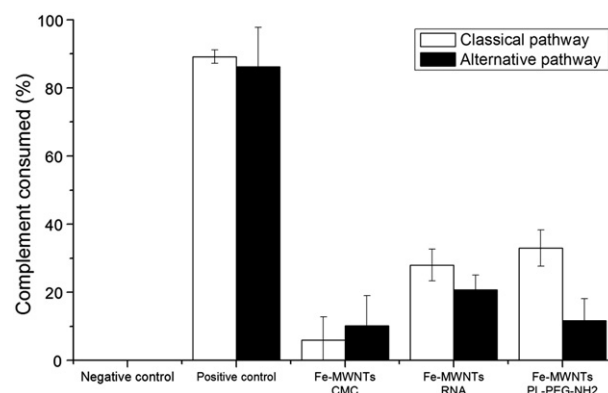

Figure 2. Complement activation via both the classical and alternative pathway for coated Fe-MWNTs. Samples were incubated with human serum for 1 h. Zymosan was used as the positive control. Zero complement consumption was based on a negative control: serum incubated without CNTs. Percentage complement consumption was calculated as $(C − Ci)/C × 100\%$, where C represents the total complement activity in CH50 units of the negative control, Ci is the amount of activity remaining in the supernatant of the sample tested. All experiments were done in triplicate; error bars represent ± standard deviation.

in 50 mM Tris, 100 mM NaCl, 5 mM CaCl₂, pH 7.5 overnight at 4 °C. Excess proteins were removed by washing three times in the same buffer. Protein binding was analysed by SDS-PAGE.

Complement activation and consumption assays for the classical pathway

To investigate whether CNTs activated (consumed) complement in human serum[19], CNTs were incubated with normal human serum (SeraLab) for 1h at 37 °C, removed by centrifugation, and the capacity of the incubated serum to lyse antibody-sensitised sheep erythrocytes (EA) was tested. EA were prepared[19] using sheep erythrocytes from TCS Biosciences and stored in dextrose gelatin veronal buffer (DGVB⁺⁺: 2.5 mM sodium barbital, 71 mM NaCl, 0.15 mM CaCl2, 0.5 mM MgCl2, 2.5% w/v glucose, 0.1% w/v gelatin, pH 7.4) at 10^9 cells/ml concentration.

CNT suspensions (100 μl of 0.1 mg/ml) in PBS were added to 100 μl of 1:1 diluted human serum in DGVB⁺⁺. Zymosan (0.2 mg in 100 μl PBS; Sigma) served as a positive control. Samples were incubated with occasional shaking at 37 °C for 1 h followed by centrifugation (13,000 g, 10 min). The supernatants of each sample were serially double-diluted (1/10 to 1/5120 in DGVB⁺⁺) and placed in microtitre wells. 100 μl of each dilution was incubated with 100 μl of EA (10^8 cells/ml) in DGVB⁺⁺ in U-shaped wells (Fisher Scientific) for 1 h at 37 °C. Next, cells were spun down (700 g, 10 min, RT), and released haemoglobin in the supernatant was read at 405 nm. Total haemolysis (100%) was measured by lysing EA with water. Background spontaneous haemolysis (0%) was determined by incubating EA with buffer only. CH50 values, which correspond to the dilution factor of the serum that results in 50% cell lysis, were calculated and compared.

To investigate whether coating CNTs with C1q or ghA, ghB and ghC modified the activation of complement in human serum induced by CNTs, complement consumption assay was
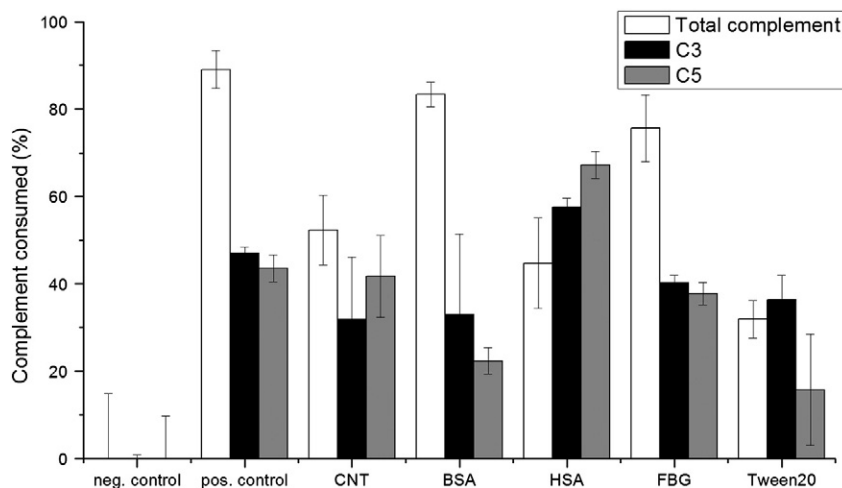
Figure 3. Complement consumption for total complement (classical pathway assay) and C3 and C5 consumption for DWNTs- pristine as well as coated with various plasma proteins (BSA, HSA, FBG) or Tween 20. DWNTs were pre-coated as described in Methods and incubated with human serum for 1 h. Zymosan samples were used as the positive control. Zero complement consumption was based on a negative control: serum incubated without CNTs. Percentage of complement consumption was calculated as $(C - C_i)/C \times 100\%$, where C represents the total complement activity in CH50 units of the negative control, C_i is the amount of activity remaining in the supernatant of the sample tested. All experiments were done in triplicate; error bars represent ± standard deviation.

performed similarly.[19] Samples of pre-coated and uncoated CMC-MWNTs at 100 μg/ml in 50 mM Tris, 150 mM NaCl, and 5 mM CaCl$_2$ pH 7.5 buffer were incubated at 37 °C for 1 h with equal volumes of 1:1 diluted serum in DGVB^{++} to give a total volume of 50 μl followed by centrifugation (13,000 *g*, 10 min), supernatants were collected and assayed.

Complement activation and consumption assay for the alternative pathway

EA$_{IgG}$ cells were made by treating SRBCs with the IgG fraction of rabbit anti-SRBC antibodies (GE Healthcare). EA$_{IgG}$ cells were diluted to 10^9 cells/ml in DGVB-Mg-EGTA (2.5 mM sodium barbital, 71 mM NaCl, 7 mM MgCl$_2$, 10 mM EGTA, 2.5% w/v glucose, 0.1% w/v gelatin, pH 7.4).

Nanotube suspensions (100 μl of 0.1 mg/ml in PBS) were added to 100 μl undiluted human serum and incubated for 1 h at 37 °C. Zymosan (0.2 mg) served as a positive control. The supernatants of each sample were serially diluted (1/5 to 1/320 in DGVB-Mg-EGTA) and placed in microtitre wells. 100 μl of each dilution was incubated with 100 μl of EA$_{IgG}$ (10^9 cells/ml in DGVB-Mg-EGTA) for 1 h at 37 °C. Further steps were performed as above.

C3 and C5 consumption assay

To analyse whether the complement activation by CNTs continued up to the activation of C3 and C5, an adapted haemolytic assay was performed. 20 μl of serum diluted 1/20 in DGVB^{++} was incubated for 1 h at 37 °C with or without CNTs (20 μL 0.1 mg/mL in PBS) and the nanotubes were removed by centrifugation. Then, 20 μl of the supernatant added to 20 μl 1/20 diluted C3-depleted serum (Sigma) or C5 depleted serum (Quidel) and 100 μl EA cells at 10^8 cells/ml in DGVB^{++} and incubated for 1 h at 37 °C. Further procedures and the

calculation of the activity (titre) of C3 or C5 were done as described above.

Phagocytosis assay

To biotinylated carbon nanotubes, MWNTs functionalized with CMC were washed thoroughly to remove excess CMC. CMC-CNTs were dialysed against 0.1 M MES buffer (2-(*N*-morpholino)ethanesulfonic acid, pH 5) and made to 0.2 mg/ml. Pentylamine biotin (Pierce) (10 mg) was added to 10 ml of the CMC-CNTs at 0.2 mg/ml and 100 μl of a 20 mg/ml solution of EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) in 0.1 M MES buffer pH 5 was added. The reaction (Supplementary information S1) was allowed to take place for 2 h at RT with stirring, and was stopped by adding 100 μl of 0.1 M ethanolamine, pH 8.2. The resulting biotin-CMC-CNTs were dialysed into PBS (pH 7.4) to remove remaining reactants and MES.

U937 cells (a monocytic cell line derived from histiocytic lymphoma) were cultured in complete RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate, and passaged before use. In each well of a 24 well plate, 5×10^5 cells were incubated in complete RPMI 1640 or AIM-V AlbuMAX serum free medium (GIBCO) supplemented with glutamine, pyruvate penicillin and streptomycin as above. 20 μg of biotin-CMC-CNTs in 20 μl PBS was added to each well and incubated for 15, 30, 45, 60, 120 or 360 min. Cells were harvested and washed five times in PBS using centrifugation at 300 *g* and stored at −80 °C until further use. A test with the same washing and Sepharose beads (instead of cells) with biotin-CMC-CNT dispersion showed that suspended CNTs remained in dispersion and were washed away by this method. The supernatants were removed and 25 μl lysis buffer (10 mM HEPES, 20 mM NaCl, 0.5 mM EDTA, 1% w/v Triton X 100) was added to the cells.

After lysing the cells, 25 µl of 0.1 mg/ml horse IgG in PBS was added to the dispersion to use in the quantification assay. The IgG was added as a blocking agent to minimise non-specific binding reactions.

An ELISA type assay was developed to quantify the amount of CNTs taken up by cells (see Supplementary material S2). Microtitre wells (NUNC, polysorb) were coated with 100 µl Avidin (Pierce) at 50 µg/ml in 0.1 M Na_2CO_3, pH 9 for 1 h at RT, followed by blocking with 1 mg/ml horse IgG in PBS for 1 h at RT. Next, 50 µl of a solution or cell lysate containing biotin-CMC-CNTs and 50 µl of 0.1 mg/ml horse IgG was incubated for

1 h in each well. The plate was washed 7 times with 0.1 mg/ml IgG in PBS to remove excess CNTs and then incubated with 1:2000 dilution of Streptavidin-HRP (Sigma) for 1 h at RT. Following washing again, O-Phenylenediamine dihydrochloride (OPD) (Sigma) was used as a substrate for the HRP and the yellow 2, 3-Diaminophenazine product was read at 450 nm.

Measurement of cytokine mRNA expression using quantitative PCR analysis

In a 24 well cell culture plate, 20 µg of CMC-MWNTs or RNA-MWNTs in 20 µl PBS was added to each well containing 5×10^5 U937 cells and incubated for 15, 30, 45, 60, 120 or 360 min; control samples were incubated with PBS only for 30 min. Cells were harvested, spun down (3000 g, 5 min), and stored at −80 °C. Total RNA was extracted from frozen cell pellets using the GenElute Mammalian Total RNA Purification Kit (Sigma-Aldrich). Samples were treated with DNase I to remove any contaminating DNA. To inactivate both DNase I and RNase, samples were heated at 70 °C for 10 min, and subsequently chilled on ice. The amount of total RNA was determined at 260 nm using the NanoDrop 2000/2000c (Thermo-Fisher Scientific), and the purity was assessed using the ratio of absorbance at 260 nm and 280 nm. cDNA was synthesized using High Capacity RNA to cDNA Kit (Applied Biosystems) using 2 µg of total RNA extract.

Primer sequences were designed and analysed for specificity using the nucleotide BLAST and Primer-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The following primers were used: 18S forward (5'-ATGGCCGTTCTTAGTTGGTG-3'), 18S reverse (5'-CGCTGAGCCAGTCAGTGTAG-3'), IL-1β forward (5'-GGACAAGCTGAGGAAGATGC-3'), IL-1β reverse (5'-TCGTTATCCCATGTGTCGAA-3'), IL-6 forward (5'-GAAA-GCAGCAAAGAGGCACT-3'), IL-6 reverse (5'-TTTCACCAGG-CAAGTCTCCT-3'), IL-10 forward (5'-TTACCTGGAGGAGG-TGATGC-3'), IL-10 reverse (5'-GGCCTTGCTCTTGTTTTCAC-3'), IL-12 forward (5'-AACTTGCAGCTGAAGCCATT-3'), IL-12 reverse (5'-GACCTGAACGCAGAATGTCA-3'), TGF-β forward
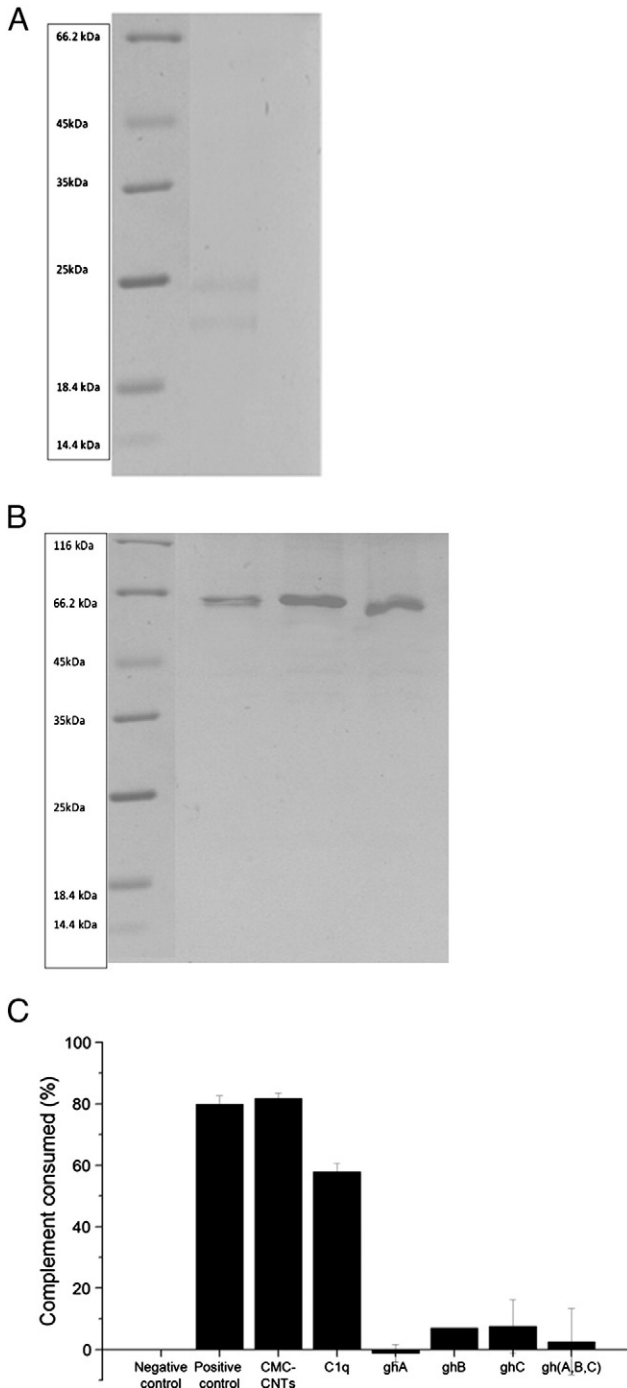

Figure 4. **(A)** Binding of C1q onto CMC-CNTs. Samples of CMC-MWNTs incubated with purified C1q and washed extensively were analysed by SDS-PAGE (8%) in reduced conditions. Lane 1: Molecular weight marker; lane 2: C1q coated CMC-MWNTs; lane 3: CMC-MWNTs. The bands visible are C1q A and B chains (~25 kDa) running above the C chain (~22 kDa). **(B)** Binding of ghA, ghB and ghC onto CMC-CNTs. Samples of CMC-MWNTs incubated with recombinant ghA, ghB or ghC and washed extensively were analysed by SDS-PAGE (12%) under reduced conditions. Lane 1: Molecular weight marker; lane 2: ghA coated CMC-MWNTs; lane 3: ghB coated CMC-MWNTs; lane 4: ghC coated CMC-MWNTs. The bands seen at about 65 kDa are fusion proteins formed from C1q globular heads a, b, or c, and maltose binding protein. **(C)** Complement inhibition assay to show that pre-coating of CNTs with ghA, ghB, ghC or an equimolar mixture of three recombinant proteins inhibits complement activation. CNTs were coated with the proteins in a ratio of 1:2 w/w, by incubating the CNTs with proteins overnight and washed. Samples were incubated with human serum for 1 h. Zero complement consumption was based on a negative control: Serum incubated without CNTs. Percentage of complement consumption was calculated as $(C − Ci)/C \times 100\%$, where C represents the total complement activity in CH50 units of the negative control, Ci is the amount of activity remaining in the supernatant of the sample tested. Zymosan was used as positive control. All experiments were done in duplicate; error bars represent ± standard deviation.
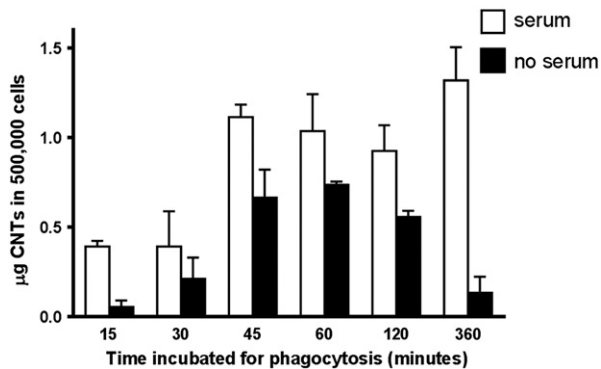
Figure 5. Uptake of CNTs by U937 cells in the presence and absence of serum. U937 cells were incubated with biotinylated CMC-MWNTs for 15, 30, 45, 60, 120 and 360 min. Excess CNTs were washed away and cells were lysed. The amount of CNTs in the lysate was quantified using a newly developed assay (supporting information S2). A standard curve with known concentrations of biotin-CNTs was performed in the same assay to calculate the mass of the CNTs in the cell samples. All experiments were done in triplicate; error bars represent ± standard deviation.

(5'-GTACCTGAACCCGTGTTGCT-3'), TGF-β reverse (5'-GTATCGCCAGGAATTGTTGC-3'), TNF-α forward (5'-AGCC-CATGTTGTAGCAAACC-3') and TNF-α reverse (5'-TGAGG-TACAGGCCCTCTGAT-3').

The qPCR reaction consisted of 5 µl Power SYBR Green MasterMix (Applied Biosystems), 75 nM of forward and reverse primers and 500 ng template cDNA in a 10 µl final reaction volume. PCR was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The initiation steps involved 2 min at 50 °C followed by 10 min at 95 °C. The template was then amplified for 40 cycles, each cycle comprised of 15 s at 95 °C and 1 min at 60 °C. Samples were normalized using the expression of human 18S rRNA. Data were analyzed using the RQ Manager Version 1.2.1 (Applied Biosystems). Ct (cycle threshold) values for each cytokine target gene were calculated and the relative expression of each cytokine target gene was calculated using the Relative Quantification (RQ) value, using the equation: $RQ = 2^{-\Delta\Delta Ct}$ for each cytokine target gene, and comparing relative expression with that of the 18S rRNA constitutive gene product. Assays were conducted in triplicate.

Complement dependent interactions of CNTs with human monocytes and measurement of cytokine mRNA expression

Human monocytes were isolated from fresh blood from a healthy consenting individual using a Ficol-Paque plus (GE

Healthcare) gradient according to the manufacturer's instructions. Lymphocytes and monocytes (PBMCs) obtained from the interface of plasma and Ficol-Paque were washed twice in RPMI 1640 and suspended in RPMI 1640. 2.5×10^6 PBMCs were plated out in each well of a 12 well plate (SPL Life Sciences), pre-coated with human serum overnight. After 2 h of incubation at 37 °C in 5% CO_2, the non-adherent PBMCs were gently removed away from adherent monocytes.

Medium was replaced by complete RPMI 1640 (containing 10% human serum), or complete RPMI 1640 containing 10% heat inactivated human serum (30 min 56 °C) or AIM-V AlbuMAX serum free medium (GIBCO) with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate. 10 µg CMC-MWNTs in PBS was added to each well and incubated for 15, 30, 45 or 60 min, control samples were incubated with PBS only for 30 min. Cells were harvested and spun down (3000 g, 5 min) and stored at −80 °C. Further treatment and preparations were similar to as described above.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software). For the experiments with U937 cells, an unpaired 2-side *t* test was used to compare the means of the cytokine targets with and without serum for any significant difference in expression. *P* values were computed, graphs compiled and analysed.

A 2-way ANOVA test was performed on the data obtained from the monocyte experiments and significant differences in expression at a given time point between serum and no serum-treated samples; and serum and heat-inactivated serum were determined.

Results

Complement (classical and alternative) is activated by various forms of CNTs

The activation of the classical and alternative pathway by Fe-MWNTs coated with CMC, RNA and PL-PEG-NH$_2$ is shown in Figure 2. All the CNT samples tested activated complement through both pathways, but predominantly through the classical pathway. C3 and C5 consumption analysis showed that complement activation continued up to C5, indicating formation of MAC (Figure 3). Differences in complement activation might

Figure 6. In vitro mRNA expression of cytokines by **(A)** U937cells incubated with CMC-CNTs. **(B)** U937 cells incubated with RNA-MWNTs. **(C)** Adherent monocytes incubated with CMC-CNTs. U937 cells were incubated with CMC-MWNTs or RNA-MWNTs in the presence or absence of serum for 15, 30, 45, 60, 120 and 360 min. Monocytes were incubated with CMC-MWNTs for 15, 30, 45 and 60 min in the presence of serum, heat inactivated serum, or without serum. The mRNA expression of cytokines was measured using real time qPCR and the data normalised to 18S rRNA gene expression as a control. Relative expression (RQ) was calculated by using the comparative Ct method with cells incubated with PBS for 30 min as the calibrator (data not shown). The RQ value was calculated using the formula: $RQ = 2^{-\Delta\Delta Ct}$. Assays were conducted in triplicate. Error bars represent ± standard error of the mean. For U937 cells, a two-side *t* test was performed on the data and significant differences in expression at a given time point between serum and no serum-treated samples are shown as follows: All time points showed significant differences in expression, $P \leq 0.01$, except where shown significant, *: $0.01 < P < 0.05$, and ns: not significant. For monocytes, a 2-way ANOVA was performed on the data and significant differences in expression at a given time point between serum and no serum-treated samples and serum and heat-inactivated serum are shown as follows: All time points showed non-significant differences in expression, $P \geq 0.01$, except where shown significant, *: $0.01 < P < 0.05$, and **: $P \leq 0.01$.
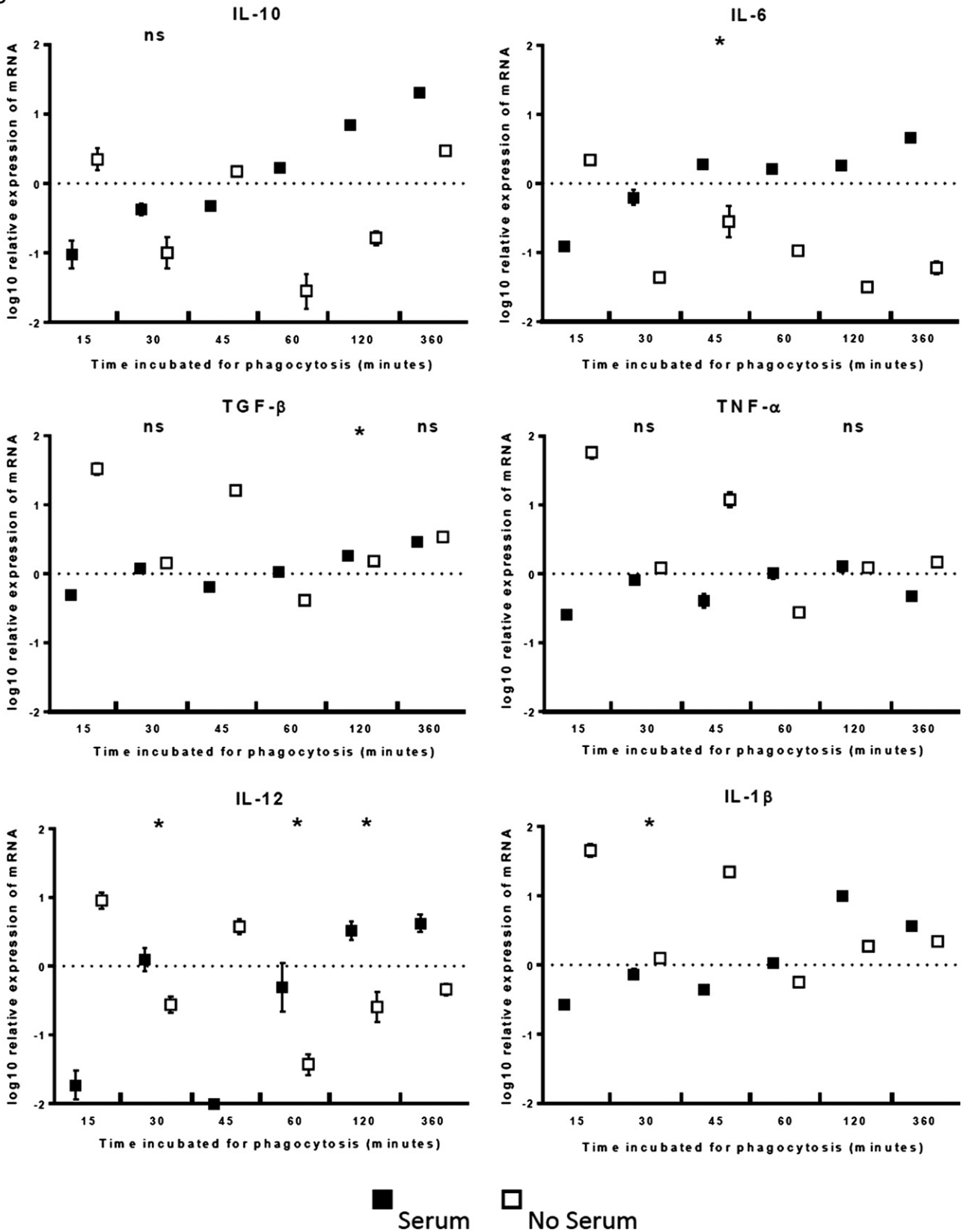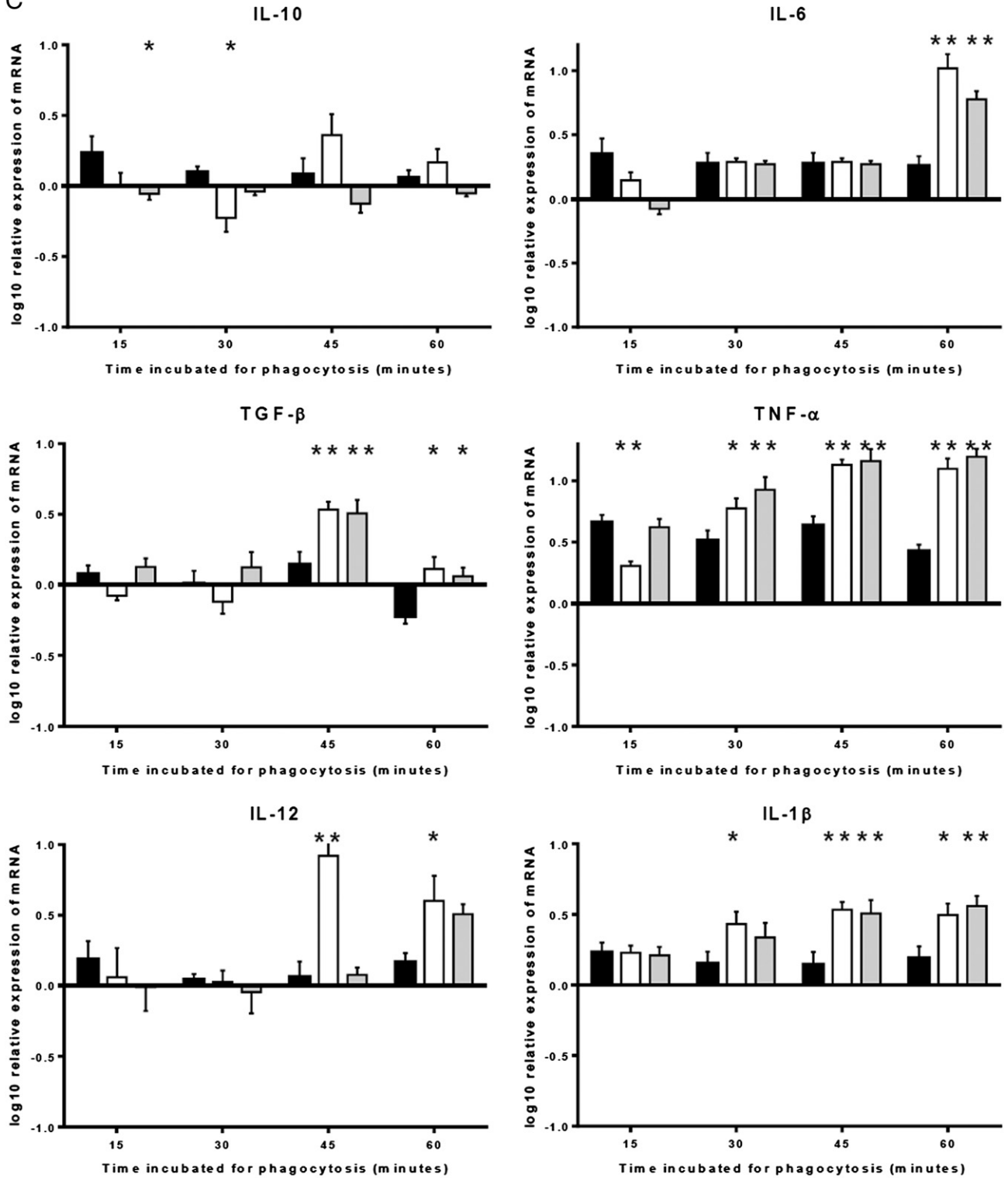
A

B

Fig. 6 (*continued*)

C
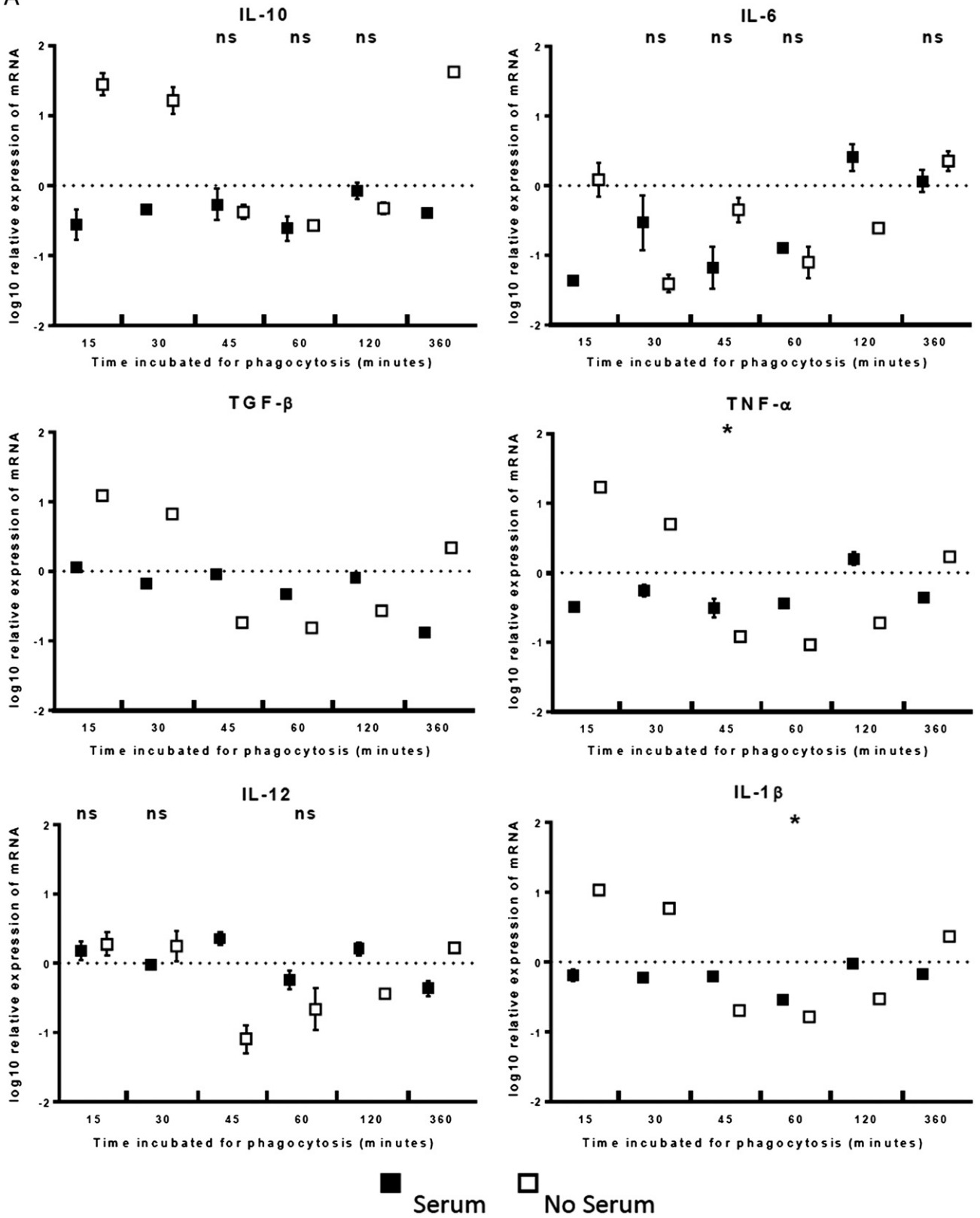
Fig. 6 (continued)

be caused by degree of coating of the CNTs, as well as by the chemical nature of the coating substance.

C1q binds to CNTs via its gC1q domain

The binding of C1q was analysed by incubating CMC-MWNTs with individual C1q globular heads. All globular heads were shown to bind to the CNTs (Figure 4, *A*, *B*), indicating the orientation of C1q on the CNTs with the head regions onto the CNTs. This binding is most likely through charge pattern recognition. In this orientation, binding of C1r and C1rs is possible, and therefore, complement activation via the classical pathway is initiated. Coating MWNTs with globular heads showed very effective inhibition of complement activation (Figure 4, *C*). This opens up a way to reduce the recognition of the particles by the innate immune system.

Complement dependent interactions of CNTs with U937 cells and monocytes, phagocytosis and cytokine expression

CNTs were incubated with U937 cells in the presence and absence of serum, either allowing complement activation to occur or not, and monitored for phagocytosis through a newly developed assay for up to 6 h. The efficiency of phagocytosis of CNTs by U937 cells is enhanced by the presence of serum, strongly suggesting that complement proteins may be involved in this process (Figure 5). In order to examine the pro- and anti-inflammatory responses during this interaction, 6 key cytokines were analyzed for their expression during the course of phagocytosis (Figure 6). The data show that all cytokine responses studied are dampened in the presence of serum. The immune responses also showed similar expression patterns when compared between two types of CNTs. For the RNA-CNT without serum, cytokine responses have a biphasic pattern with a sharp upregulation in the first 15 min. of phagocytosis, which diminishes and then peaks again at 45 min. (Figure 6, *B*). IL-10 is also downregulated in the early stages of phagocytosis, but is slowly upregulated between 60 min. and 360 min. A similar pattern is also observed in IL-12, IL-6 and IL-1β, although the effect seems more subtle in nature. For the CMC-CNT, similar patterns of cytokine expression to those seen with RNA-CNT were observed, although it does not appear to be biphasic in nature (Figure 6, *A*). The initial upregulation appears to be longer in duration (during the first 30 min.), but then seems to diminish and plateau and be slightly downregulated.

In all six cytokines studied for the RNA-CNTs, there is on average a log two-fold difference in expression between serum-treated versus untreated during the first 15 min. of phagocytosis. In the presence of serum, cytokine expression is always downregulated, and this is particularly notable in IL-12 where a log two-fold down regulation is observed. The differences in cytokine expression observed for CMC-CNTs are similar, although the average fold difference between expression in serum treated versus no serum during the first 15 min of phagocytosis was less, at about log one-fold difference. IL-10 is downregulated in the presence of serum quite markedly during the early stages of phagocytosis with a log two-fold difference between serum and no serum.

To further validate the results obtained using CNTs and U937 cells, freshly isolated monocytes were incubated with CMC-CNTs with and without normal human serum, as well as heat-inactivated serum. Heat inactivated serum yielded results comparable with serum free conditions, suggesting that down-regulation of pro-inflammatory cytokines by serum results from complement deposition on the CNTs. This effect was most evident for TNF-α and IL-1β, and also for TGF-β, IL-12 and IL-6 in the late phase.

Discussion

The precise inflammatory properties of CNTs are still being examined but various studies suffer from inconsistent findings. The type of functionalization on the surface of CNTs appears to be one of the key factors in modulating these inflammatory properties.[36] Host serum proteins can also enhance and/or mask the physiochemical characteristics of these CNTs. CNT interactions with complement proteins clearly play a key role in inflammation.

The rationale for the predominant classical pathway activation by CNTs remains an area of investigation. It is established that C1q binds to CNTs [29] but a recent study has reported that CNT-bound C1q fails to activate complement due to the lack of C1 assembly.[28] C1q binds generally by ionic interactions with rearrangement to stronger hydrophobic interaction.[37] C1q is a charge pattern recognition molecule.[38,39] It is likely that derivatisation of CNTs creates diverse C1q-recognisable molecular patterns. Thus, C1q binds these modified CNTs via its gC1q domain as indicated by binding of the recombinant ghA, ghB and ghC, which are able to inhibit classical pathway activation. Thus, in the case of CMC-CNTs, C1q binds via the gC1q domain in an orientation that supports complement activation. It is possible that under certain circumstances, C1q adaptor molecules such as IgG, IgM, CRP, SAP and PTX3 can first bind to CNTs, thus, allowing C1q to dock and then trigger classical pathway activation. However, the classical pathway complement inhibition caused by direct coating of CNTs with recombinant ghA, ghB and ghC appears to suggest a direct interaction between C1q and the CNTs.

C1q binding alone does not guarantee that the whole complement system is activated, or that the nanotube becomes coated with C3b. In order to activate the whole complement system, pristine or surface-modified CNTs need surface features which allow binding of C4 and C3. If, for example, a surface binds and activates C1, then C4 is activated, but if there is no site to which C4b can bind, C4b2a will not form efficiently and there will be very limited turnover of C3.[40] If C4b2a forms on the surface, either by hydrophobic absorption or by covalent attachment of C4b to OH, NH2 or SH groups, C3 will be activated (consumed). The C5 convertase C4b2a3b will be formed, if C4b2a is accessible on the surface to bind C3b. If C3b can bind appropriately, C5 will be activated (consumed). Each complement pathway ends in the formation of the C5-9 complex or MAC. A rise in MAC levels after interaction between PEGylated SWNTs and human serum has been reported, indicating that complement activation proceeds through the entire complement cascade,[27] and further work by Andersen

et al[28] shows clearly that for a variety of derivatised CNT, the complete complement cascade is activated. This is important in understanding the capacity of nanomaterials to generate anaphylatoxins such as complement fragments C4a, C3a and C5a. In our study, we confirm that complement is activated until the consumption of C3 and C5 for both pristine and derivatised CNTs. Consumption of C5 is a clear indicator of formation of MAC. Since C3 and C5 consumption is substantial, C3 and C5 convertases must form on the CNT surface. This, in turn, means that there are binding sites for C4b and C3b. Formation of complement fragments C3a, C4a and C5a promote inflammation, and depending on dose and route of administration, may cause problems when CNTs are used as drug delivery agents.[41] As noted above, C3b and C4b can bind covalently to surface nucleophilic groups, such as OH, NH2. These will be of very low abundance on the pristine CNT surface, but possibly adsorbed dispersant, protein or HDL[19] provides such sites. SDS-PAGE and Western blotting analysis of C3 fragments bound to CNTs (data not included) suggested that C3b does not form covalent adducts with higher molecular weight species (proteins) when bound to the CNT. It may bind covalently to molecules such as unesterified cholesterol (in adsorbed HDL),[42] or it may bind via hydrophobic interactions possibly directly to the CNT surface.

An important role of protein adsorbed on the CNT surface in uptake by phagocytic cells has been noted.[43] CNTs have been shown to be engulfed by phagocytic cells via a range of phagocytic and endocytic mechanisms.[14] The phagocytosis data here are consistent with the known role complement plays in facilitating microbial uptake by macrophages. Thus, serum opsonisation or complement deposition enhanced uptake of labelled CNTs almost by two-fold at the time points tested. Curiously, the quantity of intracellular serum-treated CNTs continued to increase post 60 min. whereas the level of non-opsonised CNTs started to diminish. It will be interesting to track the intracellular route of opsonised and non-opsonised CNTs in order to understand their fate following uptake. Complement or serum protein deposition likely renders CNTs resistant to phagolysosomal degradation of their coating, with possible loss of biotin label. Alternatively, untreated CNTs could potentially be transcytosed by the cells, being recognised as biologically irrelevant debris. Studies to delineate various aspects of cytoplasmic trafficking of CNTs are under way. Reports suggest that CNTs stay in the cytoplasm either free or in endosomes,[8,44] while others state that the CNTs also enter the cell nucleus.[45–47] Exocytosis has not been reported often and the time course for the process varies between simultaneous with endocytosis[48] until after 5 h of incubation.[44]

MWCNTs have been shown to stimulate secretion of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-8 and IL-6.[49,50] CNTs induce an acute and robust inflammatory response in mice[51] as well as by human monocytes in vitro.[52] Here, we report that CMC-CNTs alone induced upregulation of IL-10, TNF-α and IL-1β within 30 min. of the exposure to U937 cells; most of these responses were dampened in the presence of serum/complement deposition. In the case of RNA-CNTs, IL-10 was weakly upregulated by the opsonisation, which may raise the antigen threshold for an adaptive immune response. The biphasic TNF-α and IL-1β response induced by RNA-CNTs was seriously dampened by serum treatment. Serum also appears to have dampened IL-12 production by U937 cells. It appears that complement deposition may sequester CNT-mediated pro-inflammatory response towards an anti-inflammatory environment. Further studies are required to understand roles of specific complement proteins in modulating phagocytic and immune properties of macrophages. It also indicates that complement may facilitate the dampening of inflammation during this phagocytic process, resulting in more efficient cell recruitment. The importance of complement activation and deposition was further analysed by heat inactivation of the serum in the experiments with monocytes. Under these conditions cytokine profiles were similar for heat inactivated and serum free conditions.

These observations have particular importance in targeted tissues and cells, where the use of CNTs in therapy may lead to tissue damage as a result of inflammation. The presence of complement proteins as opsonins for CNTs or the coating of tissues by these proteins, will facilitate a reduction in any possible tissue damage as a result of an exaggerated inflammatory response to the CNTs. It is therefore important to further elucidate the precise immunological signature of these functionalized CNTs, not only to predict their use as a therapeutic vehicle, but also to gain insights into any unwanted pathologies or toxicity, particularly on targeted tissues and cells. The data and assays presented here, may serve as important screens for new functionalized CNTs before they are tested for *in vivo* studies.

CNTs are not biodegradable and are unlikely to be used for multiple-dose administration, but they could be used in prostheses, or in single dose treatments and with the increase of use of CNTs the risk of accidental exposure increases. Studies on complement activation by carbon nanotubes are useful as models for other (carbon-based) nanomaterials as well as for toxicology.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2014.02.010.

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