

Published in final edited form as:

J Immunol. 2009 November 15; 183(10): 6724–6732. doi:10.4049/jimmunol.0902374.

Complement Activation by CpG in a Human Whole Blood Loop System: Mechanisms and Immunomodulatory Effects¹

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Abstract

Phosphorothioate oligodeoxynucleotides can activate complement, and experimental murine studies have revealed differential effects upon simultaneous TLR stimulation and complement activation compared with either event alone. We set out to investigate the immune stimulatory effects of CpG 2006 in fresh non-anticoagulated human blood with or without presence of active complement. We also sought to elucidate the mechanism behind complement activation upon stimulation with phosphorothioate CpG 2006. In a human blood loop system, both backbone and sequence-specific effects by CpG were counteracted by selective inhibition of C3. Furthermore, DNA backbone-mediated CD40 and CD83 expression on monocytes and sequence-specific IL-6 and TNF production were reduced by complement inhibition. CpG-induced complement activation occurred via either the classical or the alternative pathway and deposits of both IgM and properdin, two activators of complement, were detected on CpG after incubation with EDTA plasma. Quartz crystal microbalance with dissipation monitoring demonstrated alternative pathway convertase build-up onto CpG as a likely pathway to initiate and sustain complement activation. Specific inhibition of C3 suppressed CpG 2006 uptake into monocytes indicating that C3 fragments are involved in CpG internalization. The interplay between complement and TLR9 signaling demonstrated herein warrants further investigation.

Single-stranded DNA sequences possess the capacity to stimulate TLR9, and for therapeutic purposes many TLR9 ligands as well as antisense oligos carry a modified backbone for increased stability. Being reliant on the oligonucleotide (ODN)⁴ backbone and the base context flanking the CpG motif, the CpG ODNs gain specific patterns of activity, affecting different cell types and inducing distinct cytokine profiles. Three classes of synthetic ODNs (classes A, B, and C) are described where CpG ODN class B is the only ODN type assessed in clinical trials (1). CpG 2006, also named PF-3512676 or CpG 7909 (when applied as a vaccine), is currently enrolled in multiple clinical trials as single or combination therapeutic

¹This work was supported by the Swedish Cancer Fund and was partially supported by National Institutes of Health Grants GM-62134 and AI-068730 (to J.D.L.).

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Disclosures The authors have no financial conflicts of interest.

⁴Abbreviations used in this paper: ODN, oligonucleotide; AP, alternative pathway; CBA, cytometric bead array; DAF, decay-accelerating factor; DC, dendritic cell; QCM-D, quartz crystal microbalance with dissipation monitoring.

for cancer (2-7). Type B CpGs have also been used in experimental murine tumor models with great success (8-10).

Toxicity tests in primates demonstrate that high levels of phosphorothioate (P-S)-modified oligos result in severe complement-mediated toxicity (11-13). In rodents, however, the most prominent effects are splenomegaly, liver enlargement, and monocytic organ infiltration. Henry et al. (12) suggested a mechanism by which P-S-modified oligos activate complement through the alternative pathway (AP). These investigators argued that reduced levels of factor H, an inhibitor of AP convertase, facilitates complement activation in the circulation. They further reported that complement activation is observed with a number of P-S oligonucleotides of various sequences and length, indicating that the mechanism is dependent of chemical properties rather than nucleotide sequence (11,12).

Complement activation and the resulting split fragments such as anaphylatoxins can either promote or inhibit tumor growth. The importance of C5a in promoting a Th1 milieu has been shown by several investigators (14-16). Additionally, C3aR knockout mice show exaggerated Th2 responses in an OVA sensitization model, and C3aR-deficient OVA-presenting dendritic cells (DCs) induce T cells to secrete more IL-4 and IL-5 compared with wild-type DCs (17). Also, tumors frequently overexpress complement inhibitors that prevent deposition of C3 products on tumor cells as a means to avoid immune activation (18-20). The importance of factor H and complement for tumor evasion is demonstrated by reduced tumor growth in vivo after knockdown of factor H in tumor cells (21).

Contrary, complement may also facilitate tumor growth by inhibiting Th1 responses. For example, C5aR-deficient mice have a more Th1-polarized immunity protecting them from *Leishmania major* infection, and LPS-induced IL-12p70 production by macrophages is inhibited by C5a (29). C5a in the microenvironment attracts myeloid-derived suppressor cells that inhibit CD8⁺ T cell-mediated tumor regression (22). Taken together, an acute inflammation may cause tumor regression and favor Th1 immunity while chronic inflammation facilitates tumor progression (22,23), and the thin line between acute and chronic inflammation may account for the differences reported above.

Of specific interest is the interplay between TLR and complement signaling. In a decay-accelerating factor (DAF)-deficient mouse model, Zhang et al. demonstrated that LPS in combination with an uncontrolled complement system, caused by the DAF deficiency, synergistically elevate IL-6 and TNF levels with decreased IL-12p40 and p70 levels. The same group also evaluated CpG 1826, a murine type B TLR9 agonist, in DAF-deficient mice and found a reduction in IL-12p40 upon TLR9 stimulation and complement activation (24).

Since experiments in murine model systems have demonstrated an interplay between TLR9 and complement, we set out to investigate how the TLR9 agonist CpG 2006 affects complement activation in a human setting. By using a human whole blood loop system we found that complement had a role in the TLR9 induced up-regulation of activation/maturation markers as well as on cytokine secretion. Complement activation was initiated via either the classical or the alternative pathway, and both IgM as well as properdin were shown to bind to the oligo. Furthermore, we demonstrated AP convertase build-up onto CpG 2006 as a means to sustain complement activation. Lastly, selective C3 inhibition could reduce the oligo uptake into monocytes. We conclude that complement plays an important part in human TLR9 biology and must be investigated further.

Materials and Methods

Reagents

CpG 2006 (5'-TCG TCG TTT TGT CGT TTT GTC GTT-3'), GpC (control oligo) (5' TGC TGC TTT TGT GCT TTT GTG CTT 3'), CpG 2006 Cy5, and biotinylated CpG 2006 with complete phosphorothioate backbones were purchased from Cybergene. Additionally, CpG 2006 P-O consisting of a complete phosphodiester backbone and CpG 2216 (GGg gga cga tcg tcG GGG GG), a mixed (P-O and P-S) backbone, with lowercase letters representing phosphodiester bonds, were also purchased from Cybergene. To block complement function, cyclic compstatin Ac-I(CV(1MeW)QDWGAHRC)T (25) and C5aRA AcF-(OPdChaWR) (26) were used at concentrations of 9.2 and 8.4 μM , respectively.

Whole blood and plasma experiments

Loops made of polyvinylchloride tubing (inner diameter 4 mm, length 600 mm) with a Corline heparin inner surface (Corline Systems) were applied. Heparinized polyvinylchloride tubes were preincubated with 0.9% NaCl before experimental initiation. Blood was collected from healthy volunteers into a surface-heparinized Falcon tube with low dose heparin (Leo Pharma), with a final concentration 0.5 U/ml. This heparin concentration has negligible effects on complement activation. Blood (5 ml) filled the loop, and subsequently reagents of choice were added to the blood. Surface-heparinized polyvinylchloride tubes formed loops when connected to each other through an assay-specific surface-heparinized connector. A round wheel with motor and elastic cords immobilizing the loops was installed in a 37°C chamber and the wheel was set to rotate at 18 rpm. At indicated time points (1 or 6 h) loops were taken out of the chamber, samples were collected, and loops were closed and set to rotate again. Blood samples were immediately transferred into tubes containing EDTA (10 mM final concentration) to inhibit further activation of the complement and coagulation systems. Platelets and leukocytes were counted with a Coulter AcT diff analyzer (Beckman Coulter). Samples were spun at 4°C and plasma was stored at -80°C for later analysis.

For the blocking experiment performed in surface-heparinized 96-well plates, CpG 2006 Cy5 was used in whole blood containing 0.5 U heparin/ml (blood collected with the same procedure as described above). Blood was preincubated with 10 $\mu\text{g/ml}$ azide-free anti-CD11b (clone 44; Bio-Legend), azide-free mouse IgG (Jackson ImmunoResearch Laboratories), or compstatin. After 10 min, CpG coupled to Cy5 was added and incubated for 1 h while shaking. Blood was collected into EDTA containing tubes, put on ice, and further analyzed by cell counter and FACS analysis.

To assess the pathway of initial complement activation by CpG, hirudin plasma was stimulated with CpG 2006 in the presence or absence of EGTA, which inhibits the classical pathway of complement, leaving only the alternative pathway operative. Plasma was obtained by collecting blood directly into a surface-heparinized Falcon tube in the presence of the specific thrombin inhibitor hirudin (Refludan; Aventis Pharma) to a final concentration of 50 $\mu\text{g/ml}$. Platelet-free plasma was prepared by centrifugation and transferred into new surface-heparinized tubes. EGTA was added to a final concentration of 10 mM when indicated. The plasma was stimulated 30 min with 8 μM oligo in surface-heparinized 96-well plates at 37°C on a rocking device. Complement activation was terminated with EDTA immediately after experimental ending. The use of human blood was approved by the local ethical committee at Uppsala University, and all experiments followed the regulatory guidelines. Fresh human blood was obtained from healthy volunteers who had received no medication for at least 10 days.

Flow cytometry

For FACS analysis, whole blood was stained using anti-human CD14, anti-human CD40, and anti-human CD83 (all from BioLegend) at room temperature, and erythrocytes were lysed. Samples were washed twice and analyzed using FACSCalibur or FACS LSR II (BD Biosciences). Monocytes were first gated according to size and granula and subsequently gated as CD14⁺ cells. In the CpG Cy5 blocking experiment, cells were immediately stained with anti-CD14 after the end of the experiment and erythrocytes were lysed. Cells were kept on ice to minimize continuing internalization after the end of the experiment. After extensive washing of the cells to remove all surface-bound oligo, the samples were analyzed using the FACSCalibur or FACS LSR II (BD Biosciences). Data were analyzed with the FlowJo software (Tree Star).

Cytometric bead array (CBA)

To detect and quantify IL-12p70, IFN- γ , IL-10, IL-6, IL-8, and TNF- α in plasma, a CBA human inflammation kit (BD Biosciences) was used according to the manufacturer's protocol. For complement activation studies (measuring C3a, C4a, and C5a) the CBA anaphylatoxin kit (BD Biosciences) was used.

Quartz crystal microbalance with dissipation monitoring (QCM-D)

A gold sensor crystal (Q-sense) was placed in the chamber of the Q-sense D600 system and a baseline was monitored in PBS supplemented with 1 mM NiCl₂. The sensor was coated with human fibrinogen (200 μ g/ml; IMCO) for 50 min, in some cases followed by an additional injection of fibrinogen to ensure proper coverage of the sensor surface. The fibrinogen was on-site biotinylated 60 min with 1 mg/ml freshly dissolved sulfo-NHS-LC-LC-biotin (Pierce) with succeeding addition of NeutrAvidin (200 μ g/ml; Pierce) for 50 min, and biotin-CpG 2006 (460 μ g/ml) for 30 min. Biotin-CpG was excluded in control experiments. For rinsing and dilutions throughout the experiment, 1 mM NiCl₂ in PBS was used for stabilization of the AP convertase (27). Complement factor solutions were thawed at 37°C, put on ice, and dissolved to appropriate concentration in PBS supplemented with 1 mM Ni²⁺. Rinsing with PBS-Ni was performed before each new injection if not otherwise stated. AP convertase build-up onto the CpG surface was accomplished according to Andersson et al. (28) with modifications. In short, C3b (200 μ g/ml) was supplemented for 50 min, surface was rinsed, and a sequential build-up of C3 convertase was accomplished by addition of factor B (38 μ g/ml), factor D (10 μ g/ml), and factor C3 (133 μ g/ml) with 20-min intervals without rinsing. C3 was incubated 50 min with the convertase to allow C3 cleavage to C3b and C3a with a following rinsing with Ni-PBS to remove C3a and excess complement factors. The experiments were performed at 25°C. Reagents were injected primarily to the temperature loop to let the temperature stabilize at 25°C; total volume of 1 ml of reagent was then injected to the sensor chamber to allow for complete exchange of reagent in the sensor chamber. Purified complement factors B, D, C3, and C3b were isolated in-house and their purification/preparation is summarized elsewhere (28).

Confocal microscopy

Buffy coats were provided by the Blood Center, Uppsala University Hospital, and PBMCs were prepared by Ficoll separation. PBMCs were plated onto microscope slides with removable chambers. After 2 h of incubation and subsequent adhesion of monocytes, nonadherent cells including media were removed and new media was added. After 24 h, autologous hirudin plasma was added to the cells, and 60 μ g/ml CpG-Cy5 was supplied to the cells. The chambers were not surface-heparinized in this system. To ensure that there was a difference in complement activation between the wells receiving only CpG and the wells receiving CpG plus compstatin, plasma was collected into EDTA-containing tubes at

the end of the experiment and analyzed for C3a and C5a. After 30 min of incubation, cells were stained with anti-CD14 FITC (BioLegend) at 4°C for 30 min and subsequently washed. Cells were immediately fixed with paraformaldehyde and mounted with a 4,6'-diamidino-2-phenylindole (DAPI)-containing mounting media. Slides were analyzed using the Zeiss 510 META confocal microscope using a $\times 63/1.4$ oil lens and Zeiss LSM 510 software.

Oligo-bead assay

Streptavidin-coated polystyrene beads (6 – 8 μm) (SpheroTec) were incubated with biotinylated CpG 2006 and subsequently washed with blocking buffer (0.05% Tween 20 plus 1% BSA in PBS). CpG-coated beads were used in a flow-based detection assay to evaluate IgM and properdin binding to CpG 2006. EDTA plasma was obtained as described above. Plasma was diluted to the indicated concentrations in blocking buffer and subsequently incubated with the CpG 2006 precoated beads for 30 min at room temperature. After incubation, beads were washed with blocking buffer to remove unbound plasma proteins, and the CpG was evaluated for deposits of IgM and properdin, respectively. IgM was detected by anti-human IgM FITC (Dako) and properdin was detected by goat anti-human properdin (BindingSiteSera) with subsequent staining with anti-sheep (cross-reacts with goat) IgG Alexa Fluor Ab (Invitrogen) after washing. Pure properdin was also assessed for binding to CpG 2006. In this case, properdin (Quidel), of indicated concentrations, was incubated with CpG and detected as described. Beads were washed twice after staining with the appropriate Ab and analyzed on a FACS LSR II (BD Biosciences). Data were analyzed with the FlowJo software (Tree Star). All samples had internal controls where the beads were not precoated with CpG, and the value of the internal control was subtracted from the sample value.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 4.03 software (GraphPad Software).

Results

Dose-dependent activation of complement by phosphorothioate ODNs in human whole blood

We sought to determine the effects of CpG 2006 and its corresponding control oligo on complement activity in whole blood using surface-heparinized tubings that allows for the study of complement activity. The concentration of oligo required for complement activation was assessed. Blood was collected at indicated time points and complement activation was determined by measuring levels of C3a and C5a. At an oligo concentration of 20 or 60 $\mu\text{g}/\text{ml}$, complement activity was detected after 15 min while 2 or 6 $\mu\text{g}/\text{ml}$ oligo did not result in detectable levels of anaphylatoxins (Fig. 1A). After 1 h of incubation, 60 $\mu\text{g}/\text{ml}$ induced high levels of C3a and C5a, while 6 $\mu\text{g}/\text{ml}$ oligo did not induce complement activation (not shown). To block C3 or the receptor of C5a (C5aR), compstatin or a C5aR antagonist (C5aRA) was added to the loops simultaneously with oligo. Compstatin effectively blocked the complement activation by P-S oligos while the C5a receptor antagonist did not affect complement cleavage as expected (Fig. 1, B and C). Levels of C4a were not elevated after 1 h of incubation (not shown).

Monocyte count drop during incubation is counteracted by a C3 inhibitor

Cell counter analysis revealed a drop in monocyte counts after 6 h of incubation with CpG or GpC oligo (Fig. 2A). In the presence of compstatin this was not seen. The change in

monocyte count was not dependent on C5a since C5aR blockade also led to a monocyte drop after stimulation with oligo (Fig. 2A). To rule out that complement activation was due to reduced platelet levels and coagulation, the platelet levels were investigated and found to be constant over time (not shown).

Backbone/complement-mediated induction of CD40 and CD83 expression on monocytes

We decided to investigate the immediate effects that complement activation in combination with TLR stimulation may have on monocyte activation. Blood was collected into heparinized loops as described previously. Loops were set to rotate in the 37°C chamber and sampling was performed at indicated time points. After 1 h of incubation, monocytes (CD14⁺) displayed increased CD40 and CD83 expression in loops containing CpG or GpC, while oligo stimulated loops with addition of either compstatin or C5aRA demonstrated no change compared with the initial sample (Fig. 2, B and D). After 6 h, CD40 and CD83 expression was vastly increased upon oligo stimulation (independent of oligo sequence), while compstatin partly inhibited CD40 and CD83 up-regulation on monocytes (Fig. 2, C and E). C5aRA did not affect CD40 or CD83 surface expression (6 h, Fig. 2, C and E). Similar results were obtained for the CD14⁻ monocyte population but cells were few and data not as reliant as for the CD14⁺ population (not shown).

ODN sequence-specific IL-6 and TNF production is complement-dependent

Cytokine release was investigated using CBA. Loops were prepared as previously described. Sampling was performed after 1 and 6 h and levels of IL-12p70, IL-10, IFN- γ , TNF, IL-6, and IL-8 in plasma were measured. After 1 h of incubation, low or no levels of cytokines were present. IL-12p70, IL-10, or IFN- γ was not elevated even after 6 h of incubation (not shown). In contrast, TNF, IL-6, and IL-8 levels were increased after 6 h of incubation with CpG. TNF and IL-6 levels were CG motif-dependent since the control oligo (inverted CG motif) did not initiate cytokine secretion (Fig. 3, A and B). IL-8, however, was produced at similar levels regardless of the CG or GC motif (Fig. 3C), and IL-8 secretion was C5a-dependent since C5aR blockade resulted in a nearly complete block of IL-8 production ($p < 0.05$, unpaired t test comparing CpG and GpC to CpG plus C5aRA or GpC plus C5aRA, respectively (data not shown)). C3 inhibition blocked the CG motif-induced IL-6 and TNF secretion as well as the complement-induced IL-8 secretion (Fig. 3).

Phosphodiester CpG activates complement via the classical pathway while phosphorothioate CpG utilizes either the classical or alternative pathway depending on blood donor

To study if complement can be activated via the classical or the alternative pathway using a modified backbone (P-S in this case), we utilized EGTA to selectively block classical activation. We assessed CpG 2006 (P-S) but also the same oligo with a complete phosphodiester (P-O) backbone as well as type A CpG 2216 (a mixed P-O, P-S backbone). Hirudin plasma was obtained by collecting blood into a surface-heparinized Falcon tube and subsequent centrifugation. Plasma was plated on 96-well surface-heparinized wells. The plate was incubated for 30 min at 37°C with the different oligos in the presence or absence of EGTA. P-S-modified CpG 2006 affected classical activation in donor X but alternative activation in donor Y (Fig. 4A). However, P-O CpG 2006 (Fig. 4B) stimulated complement activation via the classical pathway in both donors, and the same pattern was seen for the CpG 2216 mixed backbone (only donor Y) (Fig. 4C).

Both IgM and properdin interact with CpG 2006

Since CpG 2006 does not activate complement exclusively via the classical pathway, we investigated the binding of IgM and properdin to the oligo. Plasma was diluted in buffer and

added to beads coupled to CpG 2006. Using an anti-IgM FITC Ab, CpG-bound IgM could be detected when incubating CpG with plasma from different donors (Fig. 4D). Donor X that responded with the classical pathway in Fig. 4A showed higher levels of IgM bound to CpG 2006. To assess if properdin interacts with CpG to activate the alternative pathway, pure properdin or EDTA plasma was incubated with beads coated with CpG. An anti-human properdin Ab was used to detect bound properdin. Pure properdin was found to bind CpG-coated beads in a dose-dependent fashion (Fig. 4E). Moreover, plasma-derived properdin was detected on the oligo after incubation with EDTA plasma (Fig. 4F).

Phosphorothioated CpG can mediate AP convertase build-up

Since there is an apparent role for the AP in P-S CpG 2006 complement activation, we decided to evaluate if CpG 2006 allows for AP convertase build-up and subsequent complement activation. To test this hypothesis the technology of QCM-D was used. QCM-D is a system in which protein interactions, multipolymer formation, and biosensor templates can be studied with a straightforward instrument during real-time monitoring of mass deposit and dissipation shifts. This technology was used for monitoring AP convertase build-up.

Fig. 5A demonstrates the layer-on-layer build-up that was used to allow for CpG immobilization as well as to minimize background binding of C3b binding to other materials than CpG. Fibrinogen coating was chosen over albumin to avoid C3b binding since albumin is known to bind C3b (28). Initial adherence of C3b to the CpG-coated sensor demonstrated a 50 Hz decrease in frequency, whereas the corresponding control experiment demonstrated a decrease of only 3 Hz. After rinsing, the values were 40 Hz for C3b addition to CpG vs 1 Hz for the negative control experiment with CpG excluded (Fig. 5B). Consequently, the contribution of nonspecific binding of C3b to the biotinylated fibrinogen or to NeutrAvidin was negligible. AP convertase assembly and the consequent C3b deposition upon C3 addition revealed a further frequency shift of 100 Hz (Fig. 5B).

Complement activation facilitates CpG 2006 internalization

Since antisense oligos as well as CpG sequences based on a P-S backbone strongly activate complement *in vivo*, this reduces their therapeutic window. Compstatin could in theory, by reducing this complement activation, open the door for administration of elevated dosages, thereby enhancing the efficacy with less toxicity. However, there is a possibility that compstatin affects the therapeutic capacity of CpG by modulating the innate immune response by blocking release of cytokines and the activation/maturation of monocytes (Figs. 2 and 3).

Since compstatin inhibits the rapid cytokine release induced by CpG stimuli, we set out to investigate if complement inhibition results in decreased cellular uptake of oligo. Compstatin as well as anti-CD11b blocked the attachment and internalization of CpG 2006 (Fig. 6A, **, $p < 0.01$ compared with mouse IgG, ***, $p < 0.001$ compared with CpG Cy5 alone). A similar experiment but with addition of compstatin or C5aR antagonist together with CpG Cy5 demonstrated similar inhibition of attachment/internalization by compstatin as did the former experiment, while the C5aR antagonist did not affect CpG Cy5 uptake by monocytes (not shown). Confocal microscopy validated the FACS data confirming the uptake of CpG into vesicle-like structures (red) and demonstrated an apparent decrease of uptake upon compstatin addition (Fig. 6B). Three-dimensional confocal images illustrated that vesicle-like structures were almost exclusively in the cytoplasmic region (not shown). Plasma was collected after cell stimulation, and C3a as well as C5a was measured to confirm that compstatin indeed had blocked complement activation in this experiment (data not shown).

Discussion

When considering antisense and CpG ODN therapy, it is important to understand how phosphorothioated oligos interact with complement. CpG ODNs are often injected locally, and systemic toxicity is thereby limited. However, local tissue concentrations may well exceed the threshold for complement activation that can influence therapeutic outcome since C3a and C5a release may affect the local immune response (14-17,22,23,29). Several groups have reported that C5a may have a negative impact on Th1 responses, mostly using LPS-induced TLR4 stimulation and simultaneous complement activation (22,24,29).

P-S oligos prolong coagulation and activate complement when studied in the context of antisense DNA and human blood components (30-32). Both C3a and C5a are known chemoattractants and their receptors, expressed on monocytes/macrophages (33,34), granulocytes (33), and DCs (35,36), as well as lymphocytes (37,38), facilitate cell migration to the site of inflammation. CpG ODNs target pDCs specifically due to their strong TLR9 expression, and CpG 2006 can elicit high levels of C3a and C5a. This favors a model whereby CpG acts not only on TLR9 but also activates complement on site to attract immature APCs. The immature APCs will then respond to TLR9 stimulation as well as complement split products and subsequently mature to functional APCs. Herein, we demonstrate that CpG 2006, at a concentration of 20 $\mu\text{g/ml}$, activates the complement cascade, resulting in elevated C3a levels. At a concentration of 60 $\mu\text{g/ml}$, CpG 2006 strongly initiates activation of the complement cascade with resulting high levels of C3a and C5a. This is an unrealistic i.v. dose, but we argue that this model system mimics the complement activity taking place in the extracellular fluids where in situ injections of CpG result in much higher local concentrations.

To our knowledge, this is the first study investigating CpG 2006 and complement activation in human blood. Previous studies on complement activation by P-S oligos in humans or primates have used antisense oligos with no or weak immune-activating properties, thereby limiting other signaling cascades that could interfere or potentially synergize with the complement split product signaling. The initial observation that complement affects CpG 2006-induced immune responses was established using compstatin, a specific C3 inhibitor. We noted that CpG as well as GpC rapidly mediated a decrease in monocyte counts that was abolished upon C3 inhibition. The change in monocyte morphology/levels was not C5a-dependent since C5aR blockade did not counteract this change. We further demonstrated that CpG as well as GpC caused an up-regulation of the activation marker CD40 as well as the maturation marker CD83 on monocytes after 6 h of incubation in the whole blood loop system. The expression was complement-dependent rather than oligo sequence-specific as demonstrated by a similar up-regulation with GpC. The C5aRA did not inhibit the expression of CD40 and CD83. This warranted further investigation since the expression was complement-dependent and since monocytes are not known to express significant amounts of TLR9. However, we found detectable levels of TLR9 in monocytes upon intracellular staining (supplemental Fig. S1).⁵ This was supported by Saikh et al. (39), who demonstrated low TLR9 expression in human monocytes. Monocyte activation/maturation may, however, be C3a-dependent, since C5aR blockade did not affect CD40/CD83 expression. It would be of interest to study the surface marker expression of pDCs and B cells (known to express higher levels of TLR9, supplemental Fig. S1) to see if there is a complement and TLR-induced synergy in the costimulatory surface marker expression.

Cytokine secretion was also investigated after CpG or GpC stimulation of whole blood and was found to be enhanced in a sequence-specific manner in the case of TNF- α and IL-6. As

⁵The online version of this article contains supplemental material.

expected, C5aRA abolished IL-8 production (not shown). Of interest is the impact of C3 inhibition on cytokine production. The sequence-specific cytokine production of IL-6 and TNF was completely abolished by compstatin, indicating that complement activation fragments are essential for the rapid CpG-induced TNF and IL-6 secretion. Caution must be taken since we are investigating acute immune responses and not prolonged effects of combined complement and TLR9 stimulation. The long-term effects on APC maturation by CpG 2006 plus complement are currently under investigation.

We further observed that complement activation was not exclusively via the classical pathway upon P-S CpG stimulation. Activation may occur via the classical pathway and likely through naturally occurring IgM Abs or C1q (40) binding to the CpG oligo. Activation could also occur via the lectin pathway if the subcomponents (mannose-binding lectin or ficolin) bind to the oligo (not investigated herein), or via the alternative pathway if properdin binds directly to the oligo. The backbone modifications may initiate different pathways of complement activation depending on donor-specific amounts of complement initiators binding to the oligo. In the case of a complete P-O or mixed (P-O and P-S) backbone we demonstrated a clear classical complement activation, suggesting that C1q or IgM might drive complement activation. For P-S CpG 2006 we found that IgM binds to the oligo, with plasma from donor X depositing more IgM onto the oligo than for donors Y, Z, and Q. The differences in IgM deposition could explain why donor X responded by the classical pathway and donor Y by the AP. Additionally, we found that purified properdin as well as properdin from plasma bound in a dose-dependent fashion to P-S CpG. Recent advances in complement biology indicate that properdin not only stabilizes the AP convertase but also binds directly to targets such as necrotic cells, pathogens, and DNA (41,42). The proposed model postulates that properdin binds noncovalently to target surfaces, thereby recruiting C3b and allowing for AP convertase build-up (42,43). Thus, we suggest that donors might react differently to CpG 2006 depending on the precise plasma protein profile of the donor and the corresponding amounts that are deposited onto the oligo. An array of different proteins and their quantity may determine the outcome.

Henry et al. found that phosphorothioated oligos may activate complement via reduced levels of factor H in the circulation, but they did not explain the mechanism behind this phenomenon (12). However, factor H levels cannot decrease in our closed human whole blood loop system, and thus we propose that properdin binds to P-S CpG and allows for C3b adhesion to the oligo. This will set the scene for further AP convertase build-up. By employing QCM-D we demonstrated AP convertase build-up and subsequent complement activation driven by CpG. Upon AP convertase build-up and the sequential cleave-off of C3a and C3b, there were further mass deposits onto the oligo, indicating that C3b may bind directly to CpG.

Complement split products may potentiate the uptake of both CpG and GpC into endosomal vesicles, and CG is questioned as the sole motif for TLR9 interaction. The localization of the DNA might be just as important for TLR9 interaction (44,45). Complement split products may assist in correct endosomal localization of DNA. Thus, C3b adhesion to CpG prompted us to investigate whether complement activation affected the CpG uptake into monocytes. Whole blood incubated with CpG-Cy5 demonstrated a decreased oligo uptake in monocytes upon C3 inhibition. A Mac-1 blocking Ab also reduced the uptake of CpG-Cy5. However, Mac-1 can bind to P-S oligos directly (46) and an indirect pathway remains thus to be elucidated.

DAF deficiency alters the response to TLR4 signaling as illustrated by elevated levels of IL-6, IL-1 β , and TNF- α (24). This notion supports our observation that complement activation can enhance TLR9-induced cytokine production via AP convertase build-up. The

capacity of P-S oligos to sequester factor H (12) also allows for enhanced complement activation and elevated TLR9 responses. Although a rapid i.v. infusion of P-S oligos into monkeys resulted in hemodynamic changes and in some cases death, this can be avoided by slow infusion of the oligos (11) or local administration to avoid extensive systemic complement activation.

In conclusion, our results demonstrate that CpG 2006 can activate complement at high doses and that the initial mechanism for activation is not exclusively the classical pathway as described for unmodified DNA. Complement activation can also involve the alternative pathway as illustrated by the fact that CpG binds to properdin and acts as an AP convertase enhancer. A putative model for these interactions is presented in Fig. 7. Additionally, this complement on-switch adds to the cytokine production initiated by TLR9 stimulation. Hence, upon in situ administration of CpG 2006 in humans, high local concentrations leading to complement activation may be important for DC migration, Th1 polarization, and subsequent antitumor responses. In contrast, prolonged chronic complement activation may have the opposite effect by counteracting the Th1 response, and complement inhibition together with CpG 2006 administration may be required. Thus, the effects of prolonged complement activation after CpG administration need to be assessed both in experimental animal models but also in human tumor biology since complement and TLRs differ between humans and mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Lillemor Funke for valuable help with providing isolated complement components. The authors also thank Ida Mangsbo for graphically designing the last figure.

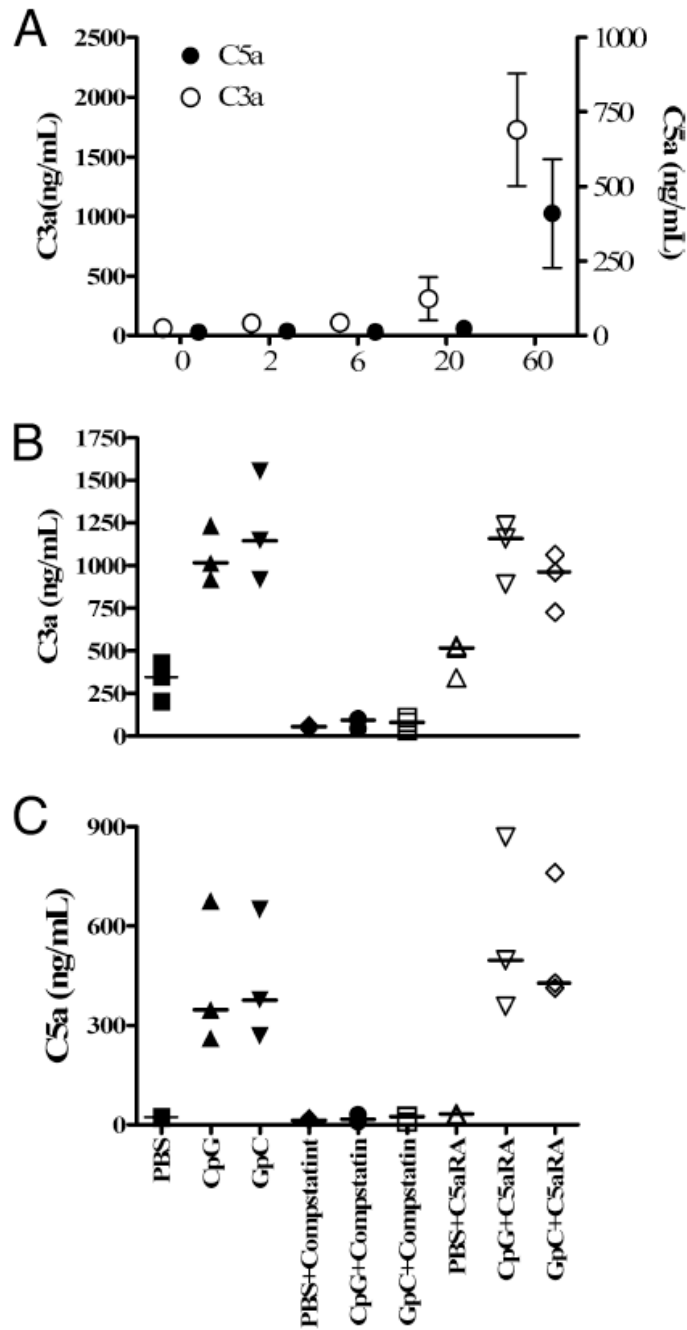
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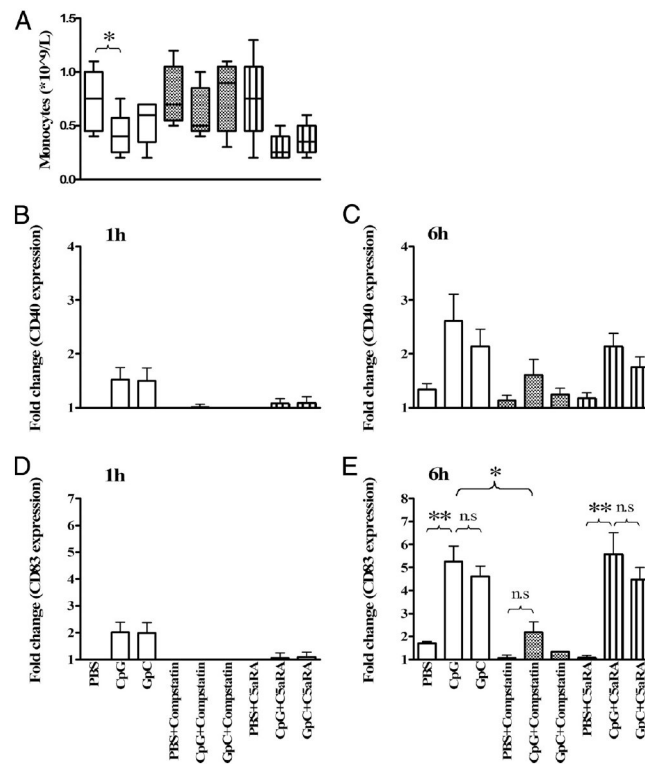
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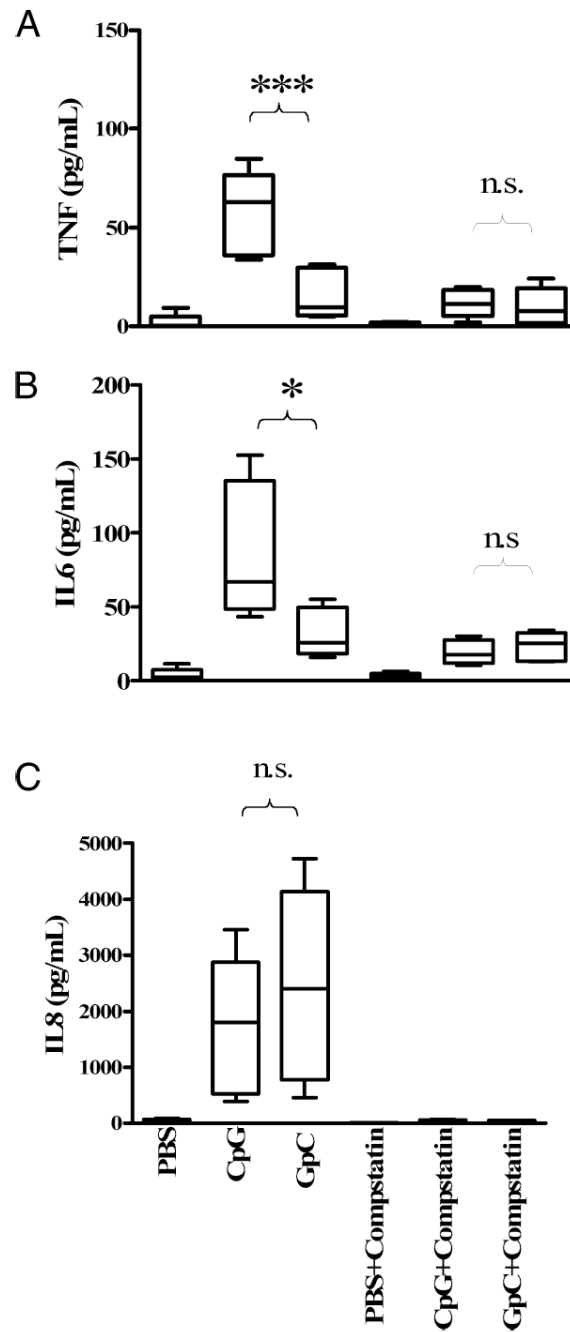
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**FIGURE 1.**

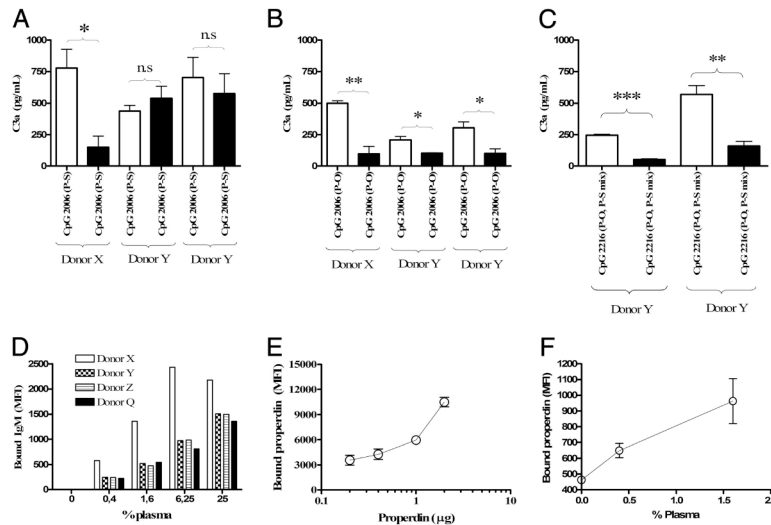
CpG- and GpC-induced complement activation results in increased levels of C3a and C5a. *A*, Increasing amounts of CpG or GpC were incubated in a circulating whole blood loop system. Each concentration represents the mean \pm SD of $n = 4$ (CpG, $n = 2$ and GpC, $n = 2$). Samples were collected after 15 min into EDTA containing tubes and later analyzed by CBA for C3a and C5a levels. *B* and *C*, At the highest concentration ($60 \mu\text{g/ml}$) each individual oligo was investigated separately for complement activation in the whole blood loop system. Compstatin and C5aRA were added to the loops to block C3 and C5aR, respectively. Blood was collected after 1 h and analyzed by CBA. The mean values are shown and data are from three individual experiments.

**FIGURE 2.**

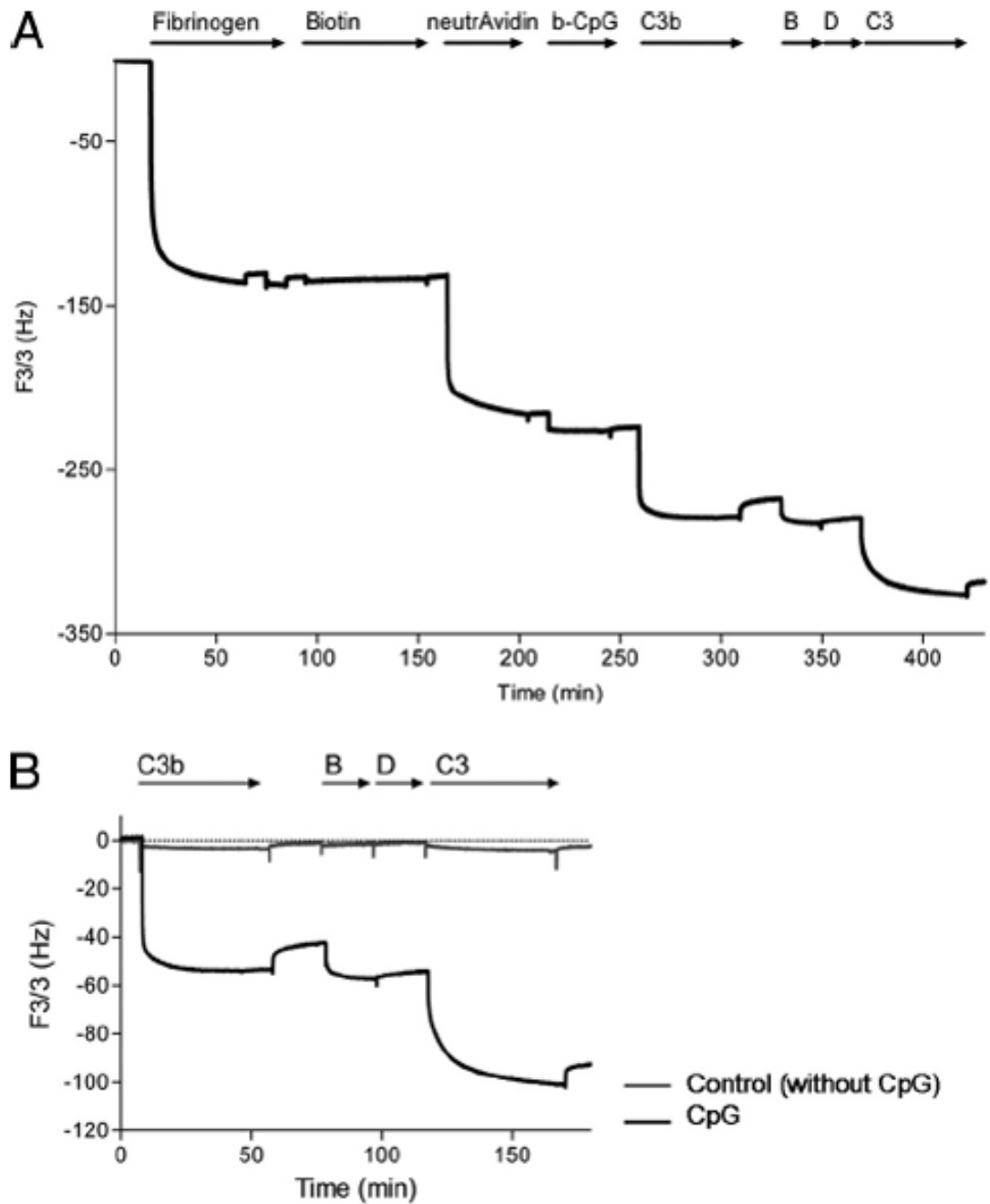
Addition of CpG or GpC to blood loops results in complement induced CD83 and CD40 expression on monocytes. In the surface-heparinized whole blood loop system the effects of CpG or GpC on monocyte counts and their expression of CD40 or CD83 were monitored by FACS analysis. Compstatin was added to block complement activation and C5aRA to block C5aR. After 1 and 6 h blood was collected into tubes containing EDTA and cells were immediately analyzed with a cell counter and subsequently stained for CD40, CD83, and CD14. *A*, Monocyte levels after 6 h of incubation. *B* and *C*, Levels of CD40 on monocytes (CD14⁺) after 1 h (*B*) and 6 h (*C*). *D* and *E*, Levels of CD83 on monocytes (CD14⁺) after 1 h (*D*) and 6 h (*E*). *, $p < 0.05$ and **, $p < 0.01$, unpaired t test (n.s., not significant). Data are from three independent experiments with three different donors ($n = 3/\text{bar}$). Data are presented as fold change compared with a control sample acquired before experiment initiation (mean \pm SEM).

**FIGURE 3.**

Complement activation is essential for a rapid CpG-induced cytokine production in human whole blood loops. Plasma from whole blood loops was analyzed by CBA for IL-6, TNF, IL-8, IL-1 β , IL-12p70, and IL-10 after 1 and 6 h of incubation. Only IL-6, TNF, and IL-8 levels at 6 h are shown in the figure since the other cytokines as well as 1-h samples had low or no cytokine levels. *A*, Levels of TNF (pg/ml) after stimulation. *B*, Levels of IL-6 (pg/ml) after stimulation. *C*, Levels of IL-8 (pg/ml) after stimulation; $n = 5-6/\text{group}$. ***, $p < 0.001$ and *, $p < 0.05$ (n.s., not significant) by unpaired t test.

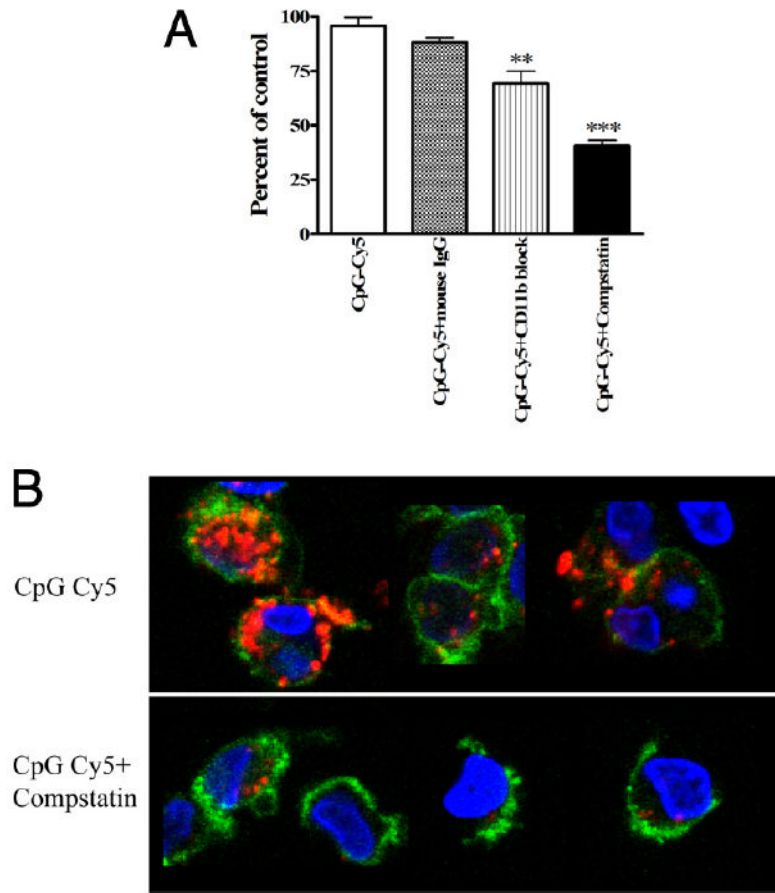
**FIGURE 4.**

P-S CpG 2006 can activate complement both via the alternative and classical pathway in contrast to P-O CpG 2006 and a mixed backbone CpG 2216 that activate complement exclusively via the classical pathway. Hirudin plasma was obtained and plated on a surface-heparinized 96-well plate with oligos added at a final concentration of 8 μ M. The plate was rocked at 37°C for 30 min and complement activation was analyzed by measuring levels of C3a and C5a. C5a measurements demonstrated similar results as the C3a data (data not shown). C3a levels in hirudin plasma after incubation with CpG 2006 P-S backbone (A), with CpG 2006 P-O backbone (B), and with CpG 2216 mixed P-S, P-O backbone (C) are shown. Every bar represents triplicates, and significance was tested using an unpaired *t* test (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; n.s., not significant). Data are shown as means \pm SEM. Histograms represent plasma with (filled bars) and without (open bars) EGTA. D, Streptavidin beads coated with biotinylated CpG 2006 and incubated with EDTA plasma were analyzed with FACS for IgM molecules attached to the oligo. For every dilution of plasma, the mean fluorescence intensity (MFI) levels of the control sample (beads without CpG treated with the same amount of plasma) were subtracted from the sample value. Four different donors and four dilutions were assessed. E, Isolated properdin was analyzed for its capacity to bind to CpG 2006. Streptavidin beads coated with biotinylated CpG 2006 were incubated with a dilution series of properdin and analyzed with FACS for properdin CpG 2006 interaction. Values are shown as mean \pm SEM. One out of two representative experiments is shown. F, Streptavidin beads coated with biotinylated CpG 2006 and incubated with EDTA plasma were analyzed with FACS for properdin attached to the oligo. For every dilution the MFI levels of the control sample (beads without CpG treated with the same amount of plasma) were subtracted from the sample value. Four donors were assessed. Values are shown as means \pm SEM. The experiment was repeated twice and representative data are shown.

**FIGURE 5.**

CpG 2006 facilitates AP convertase build-up. QCM-D was used to study the interaction of CpG 2006 with C3b. *A*, A gold sensor was covered with fibrinogen that was biotinylated in the QCM chamber. Subsequently, NeutrAvidin and biotinylated CpG were added. After that, C3b was injected and incubated. Then the AP convertase was built up using factors B, D, and C3. Each step was followed by washing (except after factors B, D, and C3). Frequency as well as dissipation (not shown) were monitored over time. *B*, C3b binding reflected by a drop in frequency that corresponds to mass increase on the sensor (black line). Control experiment (gray line) where biotinylated CpG 2006 was omitted in the surface build-up is

shown. Normalized frequency was extracted from the third overtone. One representative experiment out of two is shown.

**FIGURE 6.**

Complement inhibition blocks cellular adhesion and uptake of CpG 2006. **A**, Surface-heparinized 96-well plates were applied in this experiment. CpG 2006-Cy5 was added to 200 μ l of whole blood and the plate was placed in 37°C chamber on a rocker and was incubated for 1 h. Compstatin or Abs (10 μ g/ml) were added at $t = -10$ min. At $t = 0$ min CpG was added. After incubation, blood was collected into EDTA tubes, immediately stained for CD14, and analyzed by FACS. ***, $p < 0.001$ compared with CpG-Cy5 alone, and **, $p < 0.01$ compared with CpG-Cy5 plus mouse IgG control ($n = 3$); unpaired t test, mean \pm SD. One representative experiment out of two is shown. **B**, Confocal images of CpG uptake with or without compstatin. Monocytes were prepared by adhesion of PBMCs to slides with removable chambers. Hirudin plasma was added, and subsequently compstatin and CpG Cy5 were added to the chambers. The cells were incubated for 30 min at 37°C with 5% CO₂. The experiment was terminated by the addition of EDTA. Plasma was saved for later analysis and the chambers were removed. After washing, cells were stained with an anti-CD14 Ab and finally cells were fixated in PFA. Cells were analyzed using confocal microscopy. 4,6'-Diamidino-2-phenylindole (DAPI) (blue) was used to visualize nuclei; anti-CD14 (green) represents monocytes and CpG-Cy5 is visualized in red. A Zeiss 510 Meta confocal microscope was used for imaging, and for acquisition the Zeiss LSM 510 software was applied. The data were acquired using a $\times 40$ oil lens.

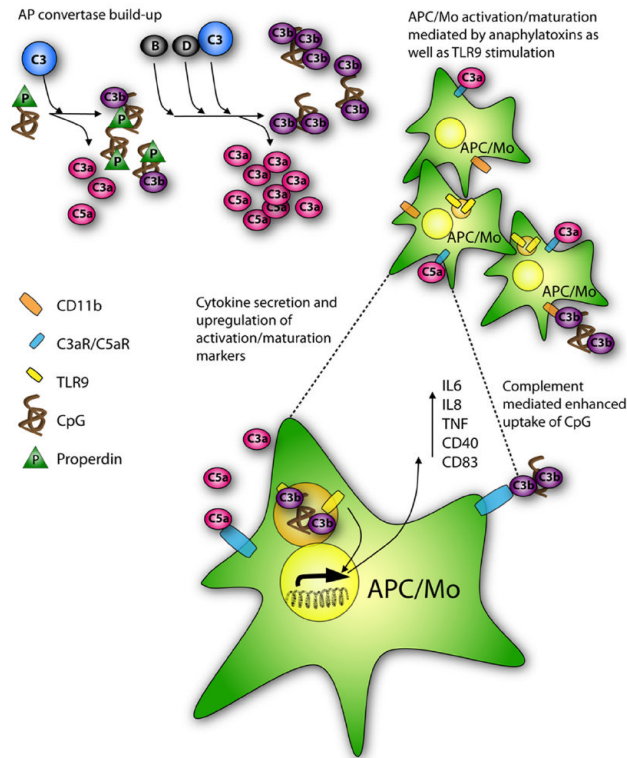


FIGURE 7. Schematic illustration of the different components involved in CpG-induced complement activation and TLR9 activation and the resulting cytokine secretion and APC activation/maturation that shape the immune response. PS oligos, in this case represented by CpG, activate complement leading to release of anaphylatoxins (C3a and C5a) as well as complement split products that are deposited onto the oligo. Additionally, other molecules such as natural Abs, C1q, as well as properdin bind to the oligo, acting as modulators of complement activation. The activation of complement together with TLR9 stimulation enhances immune activation at the site of inflammation. Both migration and maturation of different cell types are affected. Mo indicates monocytes.