Journal of Immunology 2022; 209 (9): 1760-1767

Synthetic oligodeoxynucleotide CpG motifs activate human complement through their backbone structure and induce complement-dependent cytokine release

Eline de Boer*†, Marina Sokolova*†, Huy Q. Quach*†‡, Karin E. McAdam*†, Maximilian P. Götz§, Viktoriia Chaban*†, Jarle Vaage*¶||, Beatrice Fageräng*†, Trent M. Woodruff#, Peter Garred§, Per H. Nilsson†‡**, Tom E. Mollnes*†**††‡‡§§, Søren E. Pischke*†||¶¶

* Institute of Clinical Medicine, University of Oslo, Oslo, Norway

† Department of Immunology, Oslo University Hospital, Oslo, Norway

‡ Linnaeus Centre for Biomaterials Chemistry, Linnaeus University, Kalmar, Sweden

§ Laboratory of Molecular Medicine, Department of Clinical Immunology, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

¶ Division of Physiology, Department of Molecular Medicine, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway

|| Department of Research and Development, Division of Emergencies and Critical Care, Oslo University Hospital, Oslo, Norway

School of Biomedical Sciences, Faculty of Medicine, The University of Queensland, Brisbane, Australia

** K.G. Jebsen Inflammatory Research Center, University of Oslo, Oslo, Norway

†† Research Laboratory, Nordland Hospital Bodø, Bodø, Norway

‡‡ Faculty of Health Sciences, K.G. Jebsen TREC, University of Tromsø, Tromsø, Norway

§§ Center of Molecular Inflammation Research, Norwegian University of Science and Technology, Trondheim, Norway

¶¶ Department of Anaesthesiology and Intensive Care, Division of Emergencies and Critical Care, Oslo University Hospital, Oslo, Norway

Running title: Synthetic CpG motifs activate human complement

ABSTRACT

Bacterial and mitochondrial DNA, sharing an evolutionary origin, act as danger-associated molecular patterns in infectious and sterile inflammation. They both contain immunomodulatory CpG motifs. Interactions between CpG motifs and the complement system are sparsely described and mechanisms of complement activation by CpG remain unclear. Lepirudin-anticoagulated human whole blood and plasma was incubated with increasing concentrations of three classes of synthetic CpGs: CpG-A, -B and -C oligodeoxynucleotides and their GpC sequence controls. Complement activation products were analyzed by immunoassays. Cytokine levels were determined via 27-plex beads-based immunoassay and CpG-interactions with individual complement proteins were evaluated using magnetic beads coated with CpG-B. In whole blood and plasma, CpG-B and CpG-C ($p<0.05$ for both), but not CpG-A ($p>0.8$ for all) led to time- and dose-dependent increase of soluble C5b-9 (sC5b-9), the alternative complement convertase C3bBbP, and the C3 cleavage product C3bc. GpC-A, -B and -C changed sC5b-9, C3bBbP and C3bc to the same extent as CpG-A, -B and -C, indicating a DNA-backbone-dependent effect. Dose-dependent CpG-B binding was found to C1q ($r=0.83$; $p=0.006$) and factor H ($r=0.93$; $p<0.001$). The stimulatory complement effect was partly preserved in C2-deficient plasma and completely preserved in MASP-2-deficient serum. CpG-B increased levels of IL-1β, IL-2, IL-6, IL-8, MCP-1 and TNF in whole blood, which were completely abolished by inhibition of C5 and C5aR1 ($p<0.05$ for all). In conclusion, synthetic analogues of bacterial and mitochondrial DNA activate the complement system via the DNA-backbone. We suggest that CpG-B interacts directly with classical and alternative pathway components, resulting in complement-C5aR1-dependent cytokine release.

KEY POINTS

- CpG ODNs activate the complement system dependent on their backbone structure.
- CpG ODNs bind to C1q and factor H, indicating initial CP and direct AP activation.
- CpG ODNs induced C5-C5aR1 dependent proinflammatory cytokines and chemokines.

INTRODUCTION

Mitochondrial DNA (mtDNA) is released after ischemia/reperfusion injury, surgery, severe trauma, organ failure and sepsis (1-5). Mitochondria evolved from α-proteobacteria via symbiosis (6, 7). Due to its structural similarity to bacterial DNA, mtDNA triggers inflammation (8). The pro-inflammatory effect of bacterial and mtDNA is attributed to their particular CpG sequence, wherein cytosine is unmethylated (9). Synthetic oligodeoxynucleotides (ODN) containing CpG motifs have been used to mimic immunostimulatory effects of bacterial and mtDNA (10). There are three classes of CpG motifs: CpG-A, CpG-B, and CpG-C. They are categorized basing on their lengths, position and spacing between CpG motifs as well as their backbone modifications (11).

CpG ODNs have been shown to activate the innate immune system via pattern recognition receptor (PRR) TLR9, which is primarily expressed in the endoplasmic reticulum of B cells and plasmacytoid dendritic cells (12-14). CpG-TLR9 interaction activates MyD-88 and NFκB signaling pathways, leading to maturation, differentiation, and proliferation of both innate and adaptive immune cells and release of pro-inflammatory cytokines (11, 12, 15).

Many studies have focused on characterization and modulation of the CpG-TLR9 response while little is known about CpG ODNs effect on other PRRs of innate immune responses. The complement system represents a crucial arm within innate immunity and consists of more than 50 soluble and membrane-bound proteins, with pattern recognition, immune clearance and inflammatory actions (16, 17). The complement system is activated through three distinct pathways: the classical (CP), the lectin (LP), and the alternative (AP) pathway. All three pathways converge at the formation of the C3 convertase (i.e., C4bC2a from CP and LP and C3bBb from AP), which cleaves C3 into C3a and C3b. C3b fragments can attach to

pathogen surfaces or bind to C3 convertases to form the C5 convertases, which cleave C5, leading to the release of C5a and the formation of the terminal complement complex C5b-9.

Mangsbo and colleagues have described that in human whole blood CpG-B ODN induced the generation of both C3a and C5a at significant levels and reduced the responsiveness of TLR9 signaling when C3 function was blocked (18). Yet, the exact mechanism of complement activation by CpG remains unclear. Therefore, the aim of this study was to investigate the effect of different classes of CpG ODNs on activation of the complement system in human whole blood and plasma and to identify the mechanism of complement activation by CpGs.

MATERIAL AND METHODS

Activators and inhibitors

Synthetic pyrogen-free ODNs were obtained from InvivoGen (San Diego, CA) except for ODN 4191 (Integrated DNA Technologies, Coralville, IA), which counteracts CpG induced TLR9 activation (Fig. 1, Table I) (19). Murine mAbs against C2 (clone 175-62) and factor D (clone 166-32) were from Genentech (South San Francisco, CA). Anti-MBL (3F8) was kindly provided by Gregory Stahl (20, 21). The C5 inhibitor eculizumab (IgG2/4κ mAb) was obtained from Alexion Pharmaceuticals (Zurich, Switzerland), C5aR1 antagonist PMX-53 (cyclic hexapeptide) was synthesized as previously described (22). Inhibitory CD14 antibodies (clone r18D11) were developed in our lab as described previously (23). Zymosan A 100 μg/ml (Sigma, St. Louis, MO) was used as internal positive control. All dilutions were carried out in Dulbecco´s PBS, pH 7.4 (Merck, Darmstadt, Germany).

Whole blood and plasma experiments

Human whole blood was collected from healthy adult volunteers into NUNC cryotubes (Nalgene NUNC, Roskilde, Denmark) containing 50 μg/ml lepirudin (Refludan, Pharmion, Copenhagen, Denmark). Plasma was prepared by centrifugation at 3000*g* for 15 min at 4°C. Aliquots of whole blood and plasma were incubated with increasing doses of the three classes of CpG ODNs (between 10 and 100 μg/ml) for up to 4 h at 37°C. GpC ODNs and CpG ODNs were used at equimolar concentration (10 μM). To assess the complement pathway involvement, mAbs blocking function of C2, MBL, and factor D were added to plasma and incubated for 5 min at room temperature prior to activation by CpG-B ODN (50 μg/ml) for up to 10 min. Cytokine release was determined in whole blood pre-incubated with TLR9 inhibitor ODN 4191, C5 inhibitor eculizumab, C5aR1 antagonist PMX-53 or anti-CD14 prior

to CpG-B ODN incubation. In all experiments, complement activation was stopped by adding 10 mM EDTA, and aliquots were stored at -70°C until further analyses.

C2 deficient plasma and MASP-2-deficient serum

Plasma from a type I C2-deficient patient (24) was stimulated with CpG-B ODN (50 μg/ml) for 30 min at 37°C. C2 activity was reconstituted with 30 μg/ml purified C2 (Complement Technology Inc., TX). Diluted serum (50%) from a MASP-2-deficient patient (25) was incubated with CpG-B ODN (100 μg/ml) for 60 min at 37°C. MASP-2 activity was reconstituted with 0.5 μg/ml recombinant MASP-2, which was developed as previously described (26, 27).

Enzyme immunoassays

C3bc, C3bBbP and fluid phase C5b-9 (sC5b-9) concentrations in plasma were measured by in-house ELISAs as previously described in detail (28-31). Briefly, the capture mAbs clone bH6, anti-factor P (clone #2, Quidel, San Diego, CA) and clone aE11 were used, they react with neoepitopes C3bc (bH6) and C9 (aE11) respectively, which are exposed after complement activation and not present in the native components. After background OD values were subtracted, standard curves were fitted by a sigmoidal four parameter (logistic) equation using GraphPad Prism version 9 (GraphPad Software) (31). Inter- and intra-assay coefficient of variability below 20% were accepted. When original ODs were inaccurate (>20% differences or above maximum range) and could be responsible for a change in trend, remeasurements were conducted. Cytokines were measured in EDTA plasma samples with a 27-plex kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer´s instructions. Only the cytokines in which the concentrations were significantly statistically increased ($p < 0.05$) compared to untreated samples were selected for further analyses.

Magnetic bead assays

Biotinylated ODN-2006 (3.73×10^{11} molecules) were mixed with streptavidin coated magnetic beads (1.0×10^4 particles, 6.1 µm, Polyscience, Warrington, FL) in RNAse free microcentrifuge tubes (Ambion, Austin, TX) at room temperature on a plate orbital shaker for 15 min. Subsequently, the suspension was washed twice in PBS 0.01% Tween-20 using centrifugation for 5 min at 10,000*g* at 4°C. The resulting pellet was resuspended in 50 µl of non-stimulated pooled EDTA human plasma (1:25 diluted in PBS, 6 donors) and incubated at 37°C for 30 min on a shaking device. After removing unbound proteins by centrifugation, beads were diluted in PBS 1% albumin (pH 7.4) and incubated for 25 min at 4°C with primary antibodies directed against factor H (clone C18/3, Hycult Biotech, Uden, Netherlands), properdin (clone #1, Quidel), C1q (A201, Quidel), C2 (175-62), MBL (3F8) and MASP-2 (produced in the laboratory of Professor Peter Garred, University of Copenhagen). Suspensions were washed twice in PBS 0.01% Tween-20, followed by incubation with corresponding Alexa Fluor 488-conjugated secondary antibodies diluted in PBS 1% albumin (pH 7.4) for 25 min at 4°C. Following two final washes, samples were analyzed using Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA) with the threshold set at forward scatter of 4.0 to exclude unbound substances. Single magnetic bead populations were selected by means of FSC area against FSC height plots. Data was analyzed using FlowJo software (Becton, Dickinson and Company, Ashland, OH). Fluorescence was expressed as median fluorescence intensity. To correct for non-specific binding, negative controls were implemented at each step in which PBS was used as substitute for biotinylated ODN-2006, plasma, capture antibody or detecting antibody. Each experiment was performed in triplicate and repeated three times at different days.

Ethical statement

This study was designed and performed according to the ethical guidelines from the declaration of Helsinki. Informed written consent was obtained from the blood donors. This study was approved by the Regional Ethic Committee (REK S-04114).

Statistical analysis

Complement levels in whole blood or plasma over time were compared with their control group using generalized linear mixed model analyses. Time, group and time-by-group interaction was handled as fixed intercept and donor as random intercept to calculate complement trends within each donor over time. All treatment groups were compared with non-inhibited CpG ODN groups using paired t-test. As this is an exploratory in vitro study with small sample sizes, no post hoc testing was performed. No statistical analysis was performed with the gathered data from the C2 and MASP-2 deficient experiments since the plasma and serum was collected from only one individual. Pearson coefficients were calculated for assessing the correlation between CpG ODN/bead ratio and fluorescence emitted upon complement protein binding. All statistical analyses were conducted using GraphPad Prism 9 (GraphPad Software, San Diego, CA) and IBM SPSS Statistics for Macintosh (IBM Cooperation, Armonk, NY). P values less than 0.05 were considered statistically significant.

RESULTS

Complement activation by different classes of CpG ODNs in whole blood and plasma.

CpG-B and CpG-C ODN led to a significant dose-dependent increase in C3bc, C3bBbP and sC5b-9 levels in whole blood ($p < 0.01$ for all, Fig. 2) and plasma (Fig. S1), whereas CpG-A ODN had no effect on complement activation ($p > 0.05$) in neither whole blood nor plasma.

Backbone mediated effect accountable for complement activation by CpG ODNs.

Similar to CpG-A, equimolar concentrations of sequence-modified GpC-A did not result in complement activation (Fig. 3). GpC-B ODN resulted in a similar extent of complement activation as measured by C3bc, C3bBbP and sC5b-9 and were not different from CpG-B ODN. A similar trend in C3bc, C3bBbP and sCb-9 levels was observed for sequence-modified GpC-C ODN (Fig. S2). In further experiments, only CpG-B ODNs were used as the backbone is identical to CpG-C ODNs.

Classical and alternative complement pathways are activated by CpG-B ODN.

Contribution from the C2-dependent CP and LP in complement activation was assessed in C2 deficient plasma. CpG-B ODN led to increased levels of C3bc and sC5b-9 over time (Fig. 4A). Reconstitution of C2 led to a 2- to 3-fold increase in complement activation, indicative for contribution of CP and/or LP. The contribution of the lectin pathway was examined in MASP-2 deficient serum. A time-dependent increase of C3bc and sC5b-9 levels was observed, yet reconsitution with MASP-2 did not increase complement activation (Fig. 4B). These data suggest that the lectin pathway is not required for CpG ODN induced complement activation. The degree of contribution of each complement pathway was further investigated by pre-incubation of plasma from healthy individuals with specific inhibitory antibodies against C2 (inhibition of both CP and LP), MBL (LP inhibition), or factor D (AP inhibition).

Upon CpG-B ODN stimulation, C2 inhibition led to markedly reduced levels of C3bc ($p = 0.021$) and sC5b-9 ($p = 0.027$) (Fig. 5). MBL inhibition showed no statistical effect on complement activation. Factor D inhibition led to an overall inhibition of C3bc, C3bBbP and sC5b-9 formation ($p = 0.010$, $p = 0.046$, $p = 0.039$, respectively).

Direct binding of C1q and factor H to CpG-B ODN.

Binding of CpG-B ODN to various complement components in EDTA-plasma was investigated by flow cytometry. C1q and factor H showed a significant positive correlation between the concentration of CpG-B and either C1q or factor H ($p = 0.006$, $p < 0.001$, respectively) (Fig. 6). No binding of C2, properdin, MBL or MASP-2 to CpG-B ODN could be detected (Fig. S3).

Complement dependent cytokine release upon CpG-B ODN incubation in whole blood.

Complement- and TLR mediated cytokine responses were investigated in human whole blood using a TLR9 inhibitor (ODN 4191), an antibody neutralizing the important TLR co-receptor CD14, and a C5 cleavage inhibitor. CpG-B ODN significantly increased pro-inflammatory cytokines (IL-1β, IL-2, IL-6, TNF) and chemokines (IL-8 and MCP-1) (Fig. 7). Only C5 inhibition reduced CpG ODN induced cytokine release ($p < 0.05$, for all). Importantly, the selective C5aR1 inhibitor PMX-53 led to cytokine reductions comparable to C5 inhibition, indicating that the effect was mediated primarily through the C5a-C5aR1 signaling axis (Fig. 8).

DISCUSSION

In this study, we showed that CpG ODN class-B and -C induced dose- and time-dependent complement activation both in human whole blood and plasma of healthy donors. These complement effects were dependent on the backbone of CpG ODNs, but independent of the CpG sequence. CpG ODNs were capable of binding to C1q and factor H, indicative of initial CP and direct AP driven complement activation. CpG ODN induced proinflammatory cytokines and chemokines were significantly suppressed by complement inhibition at the level of C5-C5aR1, demonstrating that the biological effect of CpG was mediated through this complement axis.

CpG-B and CpG-C ODN led to significant dose- and time-dependent increase in the activation products C3bc, C3bBbP and sC5b-9 in whole blood and plasma, whereas CpG-A ODN had no effect on complement activation. To our knowledge, this is the first study that demonstrates a similar dose-dependent increase of complement in whole blood and plasma upon CpG stimulation, and in particular the plasma results indicate that CpG ODN induced complement activation did not depend on CpG deposition on activating cell surfaces. While other studies have focused on the CpG sequence and its association to complement and synergistic TLR9 activation, the present study highlights that the CpG ODN structure itself, i.e. the backbone, is responsible for the complement activation which might include multiple levels of the complement system cascade (18, 32, 33). Unlike CpG-A, both CpG-B and CpG-C share a complete phosphorothioate (P-S) backbone, explaining the differential effects on complement activation. These findings are also consistent with previous studies assessing P-S ODNs in primates and showing ODN backbone-dependent complement activation (34-36). A different magnitude of complement activation was observed for CpG-B and CpG-C, and this might be explained by the palindrome sequence comprised in CpG-C ODN. In the presence

of a palindrome sequence, secondary hairpin structures with a stem and loop could form which may contribute to additional nuclease stabilization (37). This may have positively affected complement protein binding affinity. In contrast to our results, one prior study described that CpG-A ODN incubation led to increased C3a and C5a levels, although notably this was only conducted in the plasma from one individual (18). The lack of immunostimulatory potential in our model may be related to the phosphodiester (P-O)/P-S chimeric backbone of CpG-A ODNs. Compared to P-O ODNs, P-S ODNs are more likely to bind to DNA-binding proteins, i.e. complement products (38). Additionally, P-S ODN-protein complexes dissociate at a much lower rate (38). We argue that these functional differences between the backbone structures may explain that the apparent threshold needed for CpG-A ODN induced complement activation was not reached in our model.

C3bc and sC5b-9 activation substantially increased upon C2-reconstitution in C2-deficient plasma but not in MASP-2 deficient serum upon MASP-2 reconstitution. A CP-mediated complement activation by CpG ODNs is thus plausible. CpG ODNs were only found to form direct complexes with C1q, a recognition molecule of the CP, suggesting that this initial activation was through CP. In a study that supports our findings, Jiang *et al*., observed that both single and double stranded DNA fragments were capable of binding to C1q, resulting in the activation of the complement system via CP (39). DNA motifs that support C1q interaction have not been characterized yet, but our results imply that the activation is dependent on physical properties of DNA rather than its sequence (40). Contrary to our data, Tang *et al.* documented that CpG-B ODNs were able to bind to MBL, although excess mannan interfered with this binding (41). Unfortunately, the use of purified components precludes interactions within physiological conditions, by contrast, our model comprises close to physiologic conditions (28).

Further, CpG ODNs were found to bind directly to factor H, a negative regulator of the AP. This pathway is constitutively activated in the fluid phase by low-rate spontaneous hydrolysis of C3 to C3(H2O). To regulate this mechanism, C3(H2O) is rapidly inactivated to iC3(H2O) by factor H in conjunction with factor I (17). We speculate that when factor H binds to CpG ODN, its interaction with C3b could be impaired, leaving the C3(H2O)Bb to split more C3 leading to spontaneous self-amplification and extensive sC5b-9 formation. This presumption would explain the observed complement activation in C2 deficient plasma and absence of complement products when AP function was counteracted by the inhibition of factor D. However, further research is needed to confirm this hypothesis. In support of this, studies investigating toxicity of P-S ODNs in primates observed complement activation together with decreased plasma levels of factor H. Moreover, the addition of purified factor H prevented ODN induced complement activation (35). Overall, our results show that CpG ODNs induce initial activation of the CP as well as direct activation of the AP.

We found complement-dependent cytokine release upon CpG ODN incubation, which was independent of TLR9 and CD14. The lepirudin based whole blood model used here is physiologically relevant to assess cross-talk between complement and TLR signaling pathways since lepirudin does not interfere with the complement cascade (28, 42, 43). Inhibition of C5 reduced release of all cytokines induced by CpG ODN, indicating that these cytokine responses were complement-dependent. This complement-dependent effect appeared to be mediated entirely through C5aR1 since specific C5aR1 antagonism led to reduction of cytokine levels comparable to C5 inhibition. Similar interactions between complement and TLR signaling have been reported; decay-accelerating factor deficient mice showed higher levels of IL-12 than wild-type mice in response to CpG ODNs (44). Of

interest, the lack of C5aR1 on dendritic cells led to significant lower levels of IL-12 in the presence of CpG ODNs (45). In contrast, others have found evidence for C3/C3a dependent CpG ODN signaling as the cellular uptake of CpG ODNs in monocytes was markedly reduced upon C3 inhibition as well as the CpG ODN induced cytokine responses, while C5aR1 blockage had only a minor effect (18, 44). As discrepancies to aforementioned studies exists, future studies should account for TLR-9 expression levels on monocytes, anticoagulants used and the role of the involved complement products upon CpG ODN induced TLR-9 signaling.

Given that synthetic CpG-B and -C ODN share common structural features with bacterial DNA such as backbone P-S modification, we propose that the mechanism by which bacterial DNA or mtDNA activate the complement system could be similar (46). Future studies should assess the effect of isolated mtDNA on the complement system. A recent study concluded that the complement system mediated undesired inflammatory responses elicited by cell-free mtDNA in COVID-19 patients (47). These future findings can be of great relevance, as they might form the basis for a novel therapeutic strategy, i.e., complement inhibition in a broad spectrum of diseases in which mtDNA release contributes to pathology (1-4, 8). In addition, our findings contribute to the current understanding on the mechanism of immunogenicity induced by CpG ODNs, when used as adjuvants in vaccines against life-threatening diseases such as: cancer, malaria, hepatitis and COVID-19 (48, 49).

A limitation of this study is that we did not measure complement activation products specific for the CP and LP. Unfortunately, there is scarcity of reliable assays that reflect CP and in particular LP activation. However, by using pathway-specific antibodies e.g., C1q, MBL, MASP-2 and deficient-C2 plasma and -MASP-2 serum we could distinguish between initial

activation of CP and LP. Purified C2 itself has been reported to induce low-grade spontaneous complement activation (50). Therefore, the levels of complement activation in reconstituted C2 deficient plasma might be partially attributed to the purified protein.

In conclusion, we found that CpG ODN class-B and -C activated the complement system in a dose- and time-dependent manner. The effect appears to be CpG ODN class-dependent and triggered by physical properties of the ODN backbone. Complement activation was classical and alternative pathway mediated through interactions with C1q and factor H, respectively. CpG ODN induced cytokines release appeared to be secondary to complement activation, mediated through C5aR1 and independent of TLR9 stimulation. Further elucidation on underlying immunological mechanisms behind sterile inflammation by CpG ODNs may offer unexplored opportunities to modulate the innate immunity in pathogen induced and sterile inflammatory processes.

REFERENCES

1. Hu, Q., Q. Zhou, J. Wu, X. Wu, and J. Ren. 2019. The Role of Mitochondrial DNA in the Development of Ischemia Reperfusion Injury. *Shock* 51: 52-59.
2. Bliksoen, M., L. H. Mariero, I. K. Ohm, F. Haugen, A. Yndestad, S. Solheim, I. Seljeflot, T. Ranheim, G. O. Andersen, P. Aukrust, G. Valen, and L. E. Vinge. 2012. Increased circulating mitochondrial DNA after myocardial infarction. *Int J Cardiol* 158: 132-134.
3. Hu, Q., H. Ren, J. Ren, Q. Liu, J. Wu, X. Wu, G. Li, G. Wang, G. Gu, K. Guo, Z. Hong, S. Liu, and J. Li. 2018. Released Mitochondrial DNA Following Intestinal Ischemia Reperfusion Induces the Inflammatory Response and Gut Barrier Dysfunction. *Sci Rep* 8: 7350.
4. Zhao, M., Y. Wang, L. Li, S. Liu, C. Wang, Y. Yuan, G. Yang, Y. Chen, J. Cheng, Y. Lu, and J. Liu. 2021. Mitochondrial ROS promote mitochondrial dysfunction and inflammation in ischemic acute kidney injury by disrupting TFAM-mediated mtDNA maintenance. *Theranostics* 11: 1845-1863.
5. Baysa, A., A. Fedorov, K. Kondratov, A. Ruusalepp, S. Minasian, M. Galagudza, M. Popov, D. Kurapeev, A. Yakovlev, G. Valen, A. Kostareva, J. Vaage, and K. O. Stenslokken. 2019. Release of Mitochondrial and Nuclear DNA During On-Pump Heart Surgery: Kinetics and Relation to Extracellular Vesicles. *J Cardiovasc Transl Res* 12: 184-192.
6. Lang, B. F., E. Seif, M. W. Gray, C. J. O'Kelly, and G. Burger. 1999. A comparative genomics approach to the evolution of eukaryotes and their mitochondria. *J Eukaryot Microbiol* 46: 320-326.
7. Boguszewska, K., M. Szewczuk, J. Kazmierczak-Baranska, and B. T. Karwowski. 2020. The Similarities between Human Mitochondria and Bacteria in the Context of Structure, Genome, and Base Excision Repair System. *Molecules* 25.
8. Zhang, Q., M. Raoof, Y. Chen, Y. Sumi, T. Sursal, W. Junger, K. Brohi, K. Itagaki, and C. J. Hauser. 2010. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* 464: 104-107.
9. Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374: 546-549.
10. Shirota, H., and D. M. Klinman. 2014. Recent progress concerning CpG DNA and its use as a vaccine adjuvant. *Expert Rev Vaccines* 13: 299-312.
11. Klinman, D. M. 2004. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat Rev Immunol* 4: 249-258.
12. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408: 740-745.
13. Latz, E., A. Schoenemeyer, A. Visintin, K. A. Fitzgerald, B. G. Monks, C. F. Knetter, E. Lien, N. J. Nilsen, T. Espevik, and D. T. Golenbock. 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol* 5: 190-198.
14. Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 5: 987-995.
15. Huang, X., and Y. Yang. 2010. Targeting the TLR9-MyD88 pathway in the regulation of adaptive immune responses. *Expert Opin Ther Targets* 14: 787-796.
16. Ricklin, D., G. Hajishengallis, K. Yang, and J. D. Lambris. 2010. Complement: a key system for immune surveillance and homeostasis. *Nat. Immunol.* 11: 785-797.

17. Merle, N. S., S. E. Church, V. Fremeaux-Bacchi, and L. T. Roumenina. 2015. Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Front Immunol* 6: 262.
18. Mangsbo, S. M., J. Sanchez, K. Anger, J. D. Lambris, K. N. Ekdahl, A. S. Loskog, B. Nilsson, and T. H. Totterman. 2009. Complement activation by CpG in a human whole blood loop system: mechanisms and immunomodulatory effects. *J. Immunol.* 183: 6724-6732.
19. Ashman, R. F., J. A. Goeken, E. Latz, and P. Lenert. 2011. Optimal oligonucleotide sequences for TLR9 inhibitory activity in human cells: lack of correlation with TLR9 binding. *Int Immunol* 23: 203-214.
20. Zhao, H., N. Wakamiya, Y. Suzuki, M. T. Hamonko, and G. L. Stahl. 2002. Identification of human mannose binding lectin (MBL) recognition sites for novel inhibitory antibodies. *Hybrid Hybridomics* 21: 25-36.
21. Fung, M., P. G. Loubser, A. Undar, M. Mueller, C. Sun, W. N. Sun, W. K. Vaughn, and C. D. Fraser, Jr. 2001. Inhibition of complement, neutrophil, and platelet activation by an anti-factor D monoclonal antibody in simulated cardiopulmonary bypass circuits. *J Thorac Cardiovasc Surg* 122: 113-122.
22. Li, X. X., J. D. Lee, N. L. Massey, C. Guan, A. A. B. Robertson, R. J. Clark, and T. M. Woodruff. 2020. Pharmacological characterisation of small molecule C5aR1 inhibitors in human cells reveals biased activities for signalling and function. *Biochem Pharmacol* 180: 114156.
23. Lau, C., K. S. Gunnarsen, L. S. Hoydahl, J. T. Andersen, G. Berntzen, A. Pharo, J. K. Lindstad, J. K. Ludviksen, O. L. Brekke, A. Barratt-Due, E. W. Nielsen, C. R. Stokes, T. Espevik, I. Sandlie, and T. E. Mollnes. 2013. Chimeric anti-CD14 IGG2/4 Hybrid antibodies for therapeutic intervention in pig and human models of inflammation. *J Immunol* 191: 4769-4777.
24. Harboe, M., P. Garred, E. Karlstrom, J. K. Lindstad, G. L. Stahl, and T. E. Mollnes. 2009. The down-stream effects of mannan-induced lectin complement pathway activation depend quantitatively on alternative pathway amplification. *Mol Immunol* 47: 373-380.
25. Csuka, D., L. Munthe-Fog, M. O. Skjoedt, E. Hein, J. T. Bay, L. Varga, G. Fust, and P. Garred. 2013. A novel assay to quantitate MASP-2/ficolin-3 complexes in serum. *J Immunol Methods* 387: 237-244.
26. Ma, Y. J., M. O. Skjoedt, and P. Garred. 2013. Collectin-11/MASP complex formation triggers activation of the lectin complement pathway--the fifth lectin pathway initiation complex. *J Innate Immun* 5: 242-250.
27. Götz, M. P., M. O. Skjoedt, R. Bayarri-Olmos, C. B. Hansen, L. Pérez-Alós, I. Jarlhelt, T. Benfield, A. Rosbjerg, and P. Garret. 2022. Lectin pathway enzyme MASP-2 and downstream complement activation in COVID-19. *J. Innate Immunity* In press.
28. Mollnes, T. E., O. L. Brekke, M. Fung, H. Fure, D. Christiansen, G. Bergseth, V. Videm, K. T. Lappegard, J. Kohl, and J. D. Lambris. 2002. Essential role of the C5a receptor in E coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood* 100: 1869-1877.
29. Garred, P., T. E. Mollnes, T. Lea, and E. Fischer. 1988. Characterization of a monoclonal antibody MoAb bH6 reacting with a neoepitope of human C3 expressed on C3b, iC3b, and C3c. *Scand J Immunol* 27: 319-327.
30. Mollnes, T. E., T. Lea, S. S. Froland, and M. Harboe. 1985. Quantification of the terminal complement complex in human plasma by an enzyme-linked immunosorbent

assay based on monoclonal antibodies against a neoantigen of the complex. *Scand J Immunol* 22: 197-202.
31. Bergseth, G., J. K. Ludviksen, M. Kirschfink, P. C. Giclas, B. Nilsson, and T. E. Mollnes. 2013. An international serum standard for application in assays to detect human complement activation products. *Mol. Immunol.* 56: 232-239.
32. Ruan, B. H., X. Li, A. R. Winkler, K. M. Cunningham, J. Kuai, R. M. Greco, K. H. Nocka, L. J. Fitz, J. F. Wright, D. D. Pittman, X. Y. Tan, J. E. Paulsen, L. L. Lin, and D. G. Winkler. 2010. Complement C3a, CpG oligos, and DNA/C3a complex stimulate IFN-alpha production in a receptor for advanced glycation end product-dependent manner. *J. Immunol.* 185: 4213-4222.
33. Kim, T. H., J. Park, D. Kim, A. Gautam, M. Akauliya, J. Kim, H. Lee, S. Park, Y. Lee, and H. J. Kwon. 2019. Anti-Bacterial Effect of CpG-DNA Involves Enhancement of the Complement Systems. *Int J Mol Sci* 20.
34. Galbraith, W. M., W. C. Hobson, P. C. Giclas, P. J. Schechter, and S. Agrawal. 1994. Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey. *Antisense Res Dev* 4: 201-206.
35. Henry, S. P., M. A. Jagels, T. E. Hugli, S. Manalili, R. S. Geary, P. C. Giclas, and A. A. Levin. 2014. Mechanism of alternative complement pathway dysregulation by a phosphorothioate oligonucleotide in monkey and human serum. *Nucleic Acid Ther* 24: 326-335.
36. Henry, S. P., P. C. Giclas, J. Leeds, M. Pangburn, C. Auletta, A. A. Levin, and D. J. Kornbrust. 1997. Activation of the alternative pathway of complement by a phosphorothioate oligonucleotide: potential mechanism of action. *J Pharmacol Exp Ther* 281: 810-816.
37. Kandimalla, E. R., L. Bhagat, Y. P. Cong, R. K. Pandey, D. Yu, Q. Zhao, and S. Agrawal. 2003. Secondary structures in CpG oligonucleotides affect immunostimulatory activity. *Biochem Biophys Res Commun* 306: 948-953.
38. Stein, C. A. 1996. Phosphorothioate antisense oligodeoxynucleotides: questions of specificity. *Trends Biotechnol* 14: 147-149.
39. Jiang, H., B. Cooper, F. A. Robey, and H. Gewurz. 1992. DNA binds and activates complement via residues 14-26 of the human C1q A chain. *J Biol Chem* 267: 25597-25601.
40. Paidassi, H., P. Tacnet-Delorme, T. Lunardi, G. J. Arlaud, N. M. Thielens, and P. Frachet. 2008. The lectin-like activity of human C1q and its implication in DNA and apoptotic cell recognition. *FEBS Lett* 582: 3111-3116.
41. Tang, Y., D. Ma, S. Ming, L. Zhang, J. Zhou, G. Shan, Z. Chen, X. Lu, and D. Zuo. 2015. Mannan-binding lectin reduces CpG DNA-induced inflammatory cytokine production by human monocytes. *Microbiol Immunol* 59: 231-237.
42. Edens, R. E., R. J. Linhardt, and J. M. Weiler. 1993. Heparin is not just an anticoagulant anymore: six and one-half decades of studies on the ability of heparin to regulate complement activity. *Complement Profiles* 1: 96-120.
43. Engstad, C. S., T. J. Gutteberg, and B. Osterud. 1997. Modulation of blood cell activation by four commonly used anticoagulants. *Thromb Haemost* 77: 690-696.
44. Zhang, X., Y. Kimura, C. Fang, L. Zhou, G. Sfyroera, J. D. Lambris, R. A. Wetsel, T. Miwa, and W. C. Song. 2007. Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo. *Blood* 110: 228-236.
45. Briukhovetska, D., B. Ohm, F. T. Mey, J. Aliberti, M. Kleingarn, M. Huber-Lang, C. M. Karsten, and J. Kohl. 2020. C5aR1 Activation Drives Early IFN-gamma

Production to Control Experimental Toxoplasma gondii Infection. *Front Immunol* 11: 1397.

46. Yang, Y., G. Xu, J. Liang, Y. He, L. Xiong, H. Li, D. Bartlett, Z. Deng, Z. Wang, and X. Xiao. 2017. DNA Backbone Sulfur-Modification Expands Microbial Growth Range under Multiple Stresses by its anti-oxidation function. *Sci Rep* 7: 3516.

47. Scozzi, D., M. Cano, L. Ma, D. Zhou, J. H. Zhu, J. A. O'Halloran, C. Goss, A. M. Rauseo, Z. Liu, V. Peritore, M. Rocco, A. Ricci, R. Amodeo, L. Aimati, M. Ibrahim, R. Hachem, D. Kreisel, P. A. Mudd, H. S. Kulkarni, and A. E. Gelman. 2021. Circulating Mitochondrial DNA is an Early Indicator of Severe Illness and Mortality from COVID-19. *JCI Insight* 22;6.

48. Kuo, T. Y., M. Y. Lin, R. L. Coffman, J. D. Campbell, P. Traquina, Y. J. Lin, L. T. Liu, J. Cheng, Y. C. Wu, C. C. Wu, W. H. Tang, C. G. Huang, K. C. Tsao, and C. Chen. 2020. Development of CpG-adjuvanted stable prefusion SARS-CoV-2 spike antigen as a subunit vaccine against COVID-19. *Sci Rep* 10: 20085.

49. Scheiermann, J., and D. M. Klinman. 2014. Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer. *Vaccine* 32: 6377-6389.

50. Lappegard, K. T., D. Christiansen, A. Pharo, E. B. Thorgersen, B. C. Hellerud, J. Lindstad, E. W. Nielsen, G. Bergseth, D. Fadnes, T. G. Abrahamsen, E. A. Hoiby, L. Schejbel, P. Garred, J. D. Lambris, M. Harboe, and T. E. Mollnes. 2009. Human genetic deficiencies reveal the roles of complement in the inflammatory network: lessons from nature. *Proc Natl Acad Sci U S A* 106: 15861-15866.

FOOTNOTES

1. This work was supported by the Research Council of Norway (274352).

2. Corresponding author: Søren E. Pischke, Department of Immunology and Institute of Clinical Medicine, Oslo University Hospital, P. B. 4950 Nydalen, 0424 Oslo, Norway. E-mail: s.e.pischke@medisin.uio.no; phone number: +47 454 86 579.

3. Abbreviations used in this paper: mtDNA, mitochondrial DNA; ODN, oligodeoxynucleotide; PRR, pattern recognition receptor; CP, classical pathway; LP, lectin pathway; AP, alternative pathway; sC5b-9, fluid phase C5b-9; P-S, phosphorothioate;

FIGURE LEGENDS

Figure 1. Backbone and sequence characteristics of CpG-A, -B and -C ODNs.

CpG class A ODNs contain a mixed backbone of P-O and P-S combined with two CpG motifs. CpG class B and CpG class B ODNs contain a complete PO backbone, along with a variable number of CpG motifs. This figure was created with BioRender.com

Figure 2. CpG-B and CpG-C ODNs induced complement activation in whole blood.

Human whole blood was incubated with increasing concentrations of CpG-A, -B and -C ODNs. C3bc (upper panel), C3bBbP (middle panel) and sC5b-9 (lower panel) levels significantly increased dose- and time-dependently upon incubation with CpG-B and -C ODN and zymosan, but remained at the level of negative controls upon CpG-A ODN treatment. Data present the mean of individual complement responses (mean +/- SEM, n=6). One data point was excluded (sC5b-9 CpG-A 10 μg/ml > +/- 3 times SD). General mixed model analyses. ** = $p < 0.01$, *** = $p < 0.001$. Zymosan as positive control.

Figure 3. Immunostimulatory effect of CpG ODNs on the complement system is dependent on ODN structure rather than sequence.

Lepirudin-anticoagulated plasma from 6 healthy donors was incubated with 10 μM/ml CpG-A and CpG-B or GpC-A and GpC-B ODN for 1 h. C3bc, C3bBbP and sC5b-9 were not significantly increased over time upon stimulation with CpG-A and GpC-A and did not differ from unstimulated lepirudin-anticoagulated plasma (A). C3bc, C3bBbP and sC5b-9 concentrations were significantly increased time-dependently upon stimulation with CpG-B and GpC-B, no differences were found in the level of complement formation between the ODNs (B). Values are shown as mean +/- SEM. Statistical significance was measured using a general mixed model analysis. *** = $p < 0.001$.

Figure 4. CpG-B ODN induced complement activation in C2-deficient plasma and MASP-2 deficient serum.

Plasma and serum from one C2-deficient and MASP-2-deficient individual was used to assess the route of complement activation by CpG-ODN. C2-deficient plasma was stimulated with CpG-B ODN (50 µg/ml) alone or together with purified C2 (A). A time-dependent increase of C3bc and sC5b-9 levels was observed, although these levels were more markedly increased upon the reconstitution with C2. MASP-2-deficient serum was stimulated with CpG-B ODN (100 µg/ml) alone or together with purified MASP-2 (B). CpG-B ODN led to increased levels of C3bc and sC5b-9 over time, no differences were found in the level of complement formation upon the reconstitution with MASP-2. As these experiments include only one donor, statistical analyses were not conducted.

Figure 5. Interplay between classical and alternative complement pathways upon CpG ODN induced complement activation.

Plasma from healthy donors was incubated with CpG-B ODNs (50 µg/ml) in the absence or presence of anti-C2 (50 µg/ml), anti-MBL (10 µg/ml) or anti-factor D (25 µg/ml). C2 inhibition resulted in a signficant reduction of C3bc and sC5b-9 levels. Factor D inhibition resulted in overall significant decrease in complement formation, while no inhibitory effect was evident for MBL. The horizontal lines represent the mean, one data point was excluded (C3bc + MBL > +/- 3 times SD), and one data point was #N/A (sC5b-9 + factor D). Left panel n=6, middle panel n=6, right panel n=5. Paired t-tests comparing complement levels of CpG-B ODNs with complement inhibitors. * = $p < 0.05$, ** = $p < 0.01$.

Figure 6. CpG-B ODN binds complement proteins factor H and C1q.

Biotinylated CpG-B ODNs coupled to magnetics beads were pre-incubated in normal human plasma. The upper panel depicts median fluorescence representative curves of the highest CpG-B ODN dose and the negative control in the presence of factor H and C1q antibodies. The lower panel shows that the increase of median fluorescence levels for factor H and C1q is directly correlated to the dose of CpG-B ODN. Experiments were repeated three times and performed in triplicates. The Pearson's correlation coefficient was calculated to test for significance.

Figure 7. CpG-B ODN leads to complement dependent cytokine release.

Whole blood from 5 healthy donors was incubated with CpG-B ODN in the absence or presence of TLR-9 inhibitor ODN 4191 (30 μg/ml), anti-CD14 (15 μg/ml) or anti-C5 eculizumab (100 μg/ml) for 4 h, only cytokines revealing significant effects are shown. C5 inhibition led to significantly reduced levels of IL-1β, IL-2, IL-6, IL-8, TNF and MCP-1. Data are presented as box plots (25-75th percentiles), whiskers (10-90th percentiles), horizontal line (50th percentile) and plus-sign (mean). Paired t-test was used to compare the cytokine values of all groups. * = $p < 0.05$.

Figure 8. CpG induced cytokine responses were mediated through the C5a-C5aR1 signaling pathway.

Lepirudin-anticoagulated whole blood from 6 healthy donors was incubated with CpG-B ODN in the absence or presence of anti-C5aR1 (10 μg/ml) or anti-C5 (eculizumab, 100 μg/ml) for 4 h. CpG-B ODN induced a significant release of IL-1β, IL-2, IL-6, IL-8, MCP-1 and TNF inhibition of C5 or C5aR1 counteracted this effect. Data are presented as box plots with whiskers depicting the 10th-90th percentile, the horizontal line shows the 50th percentile and the

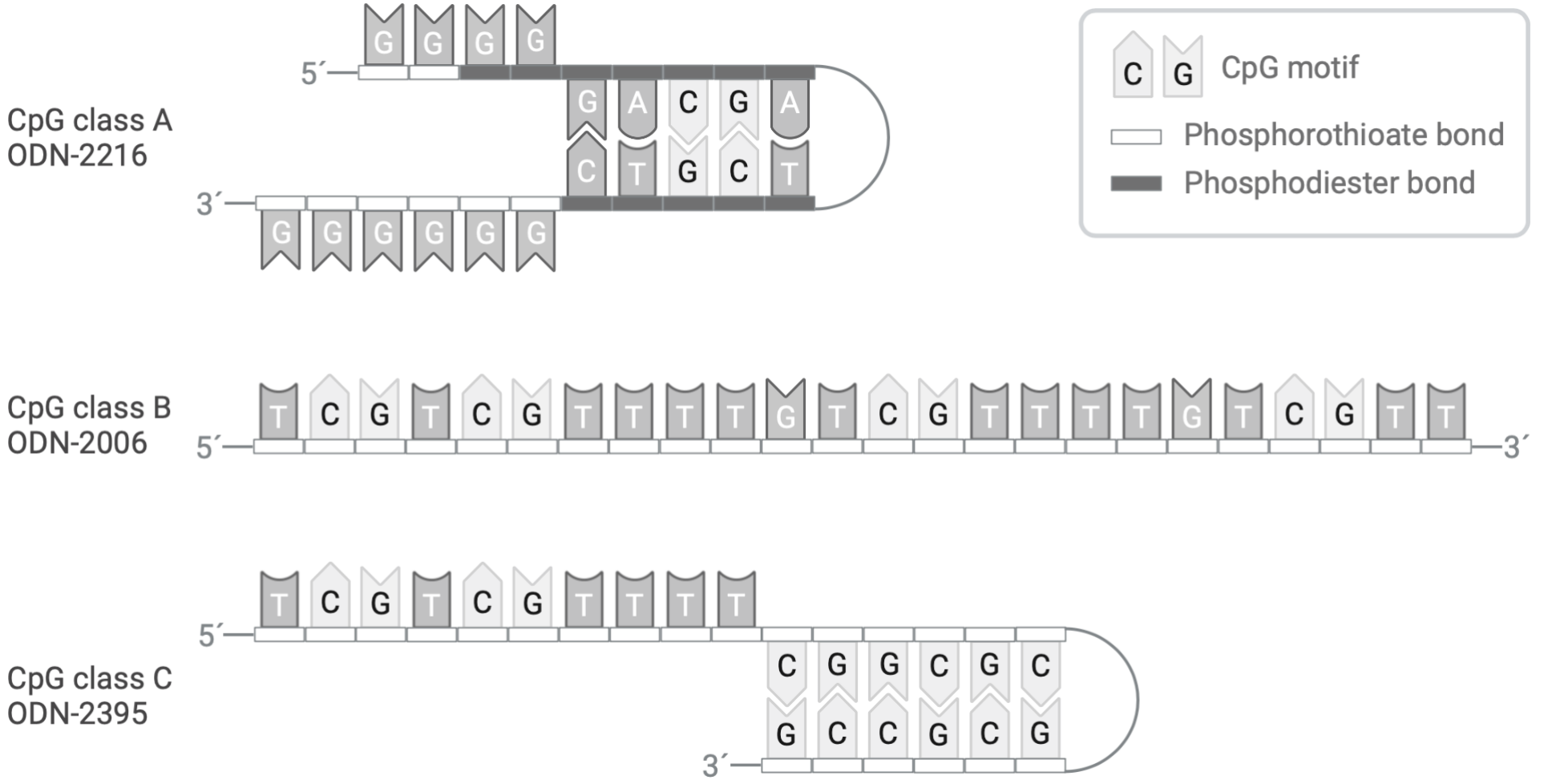
580 plus-sign represents the mean. Paired t-test comparing the cytokine values between groups. *

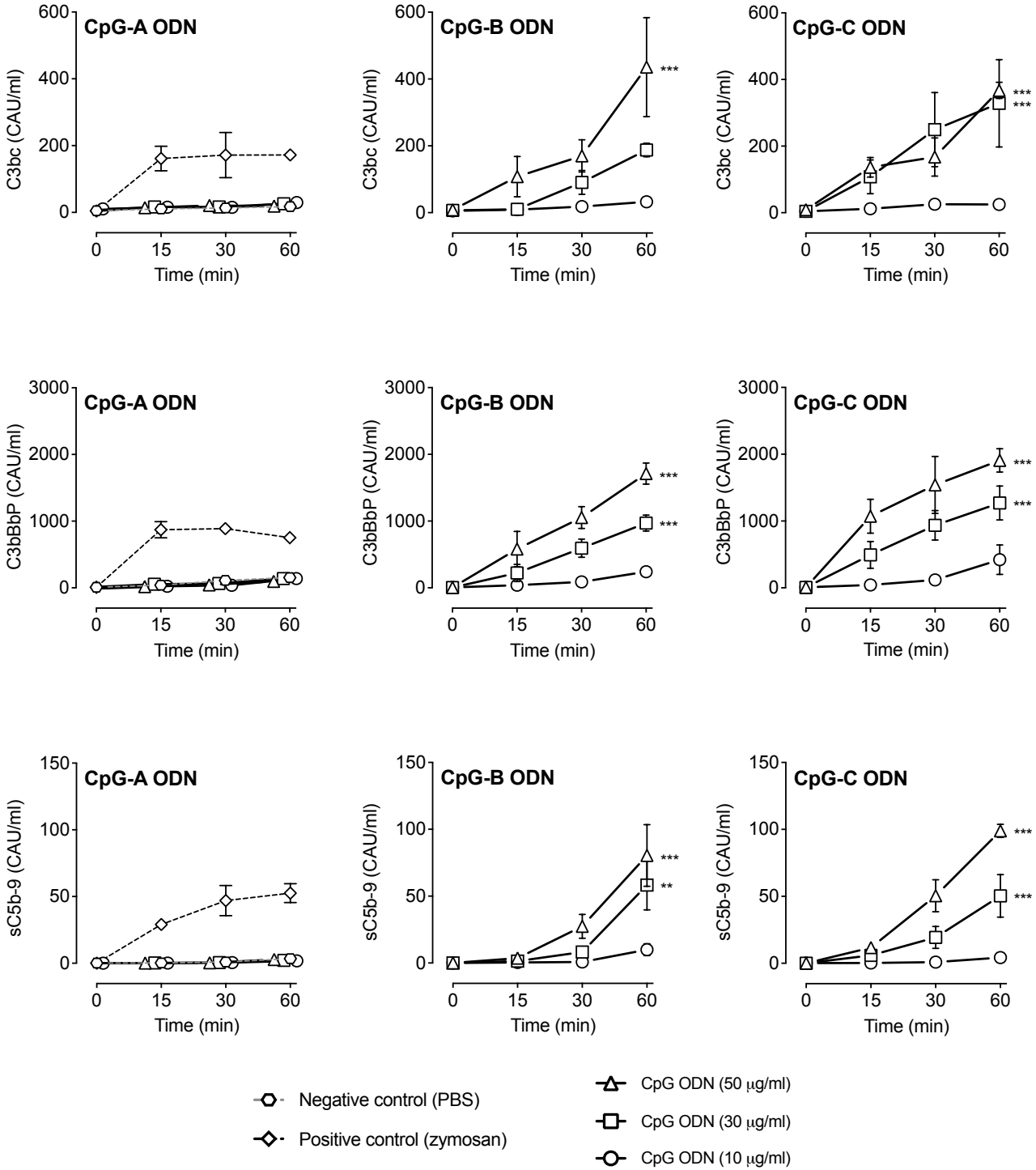
581 = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

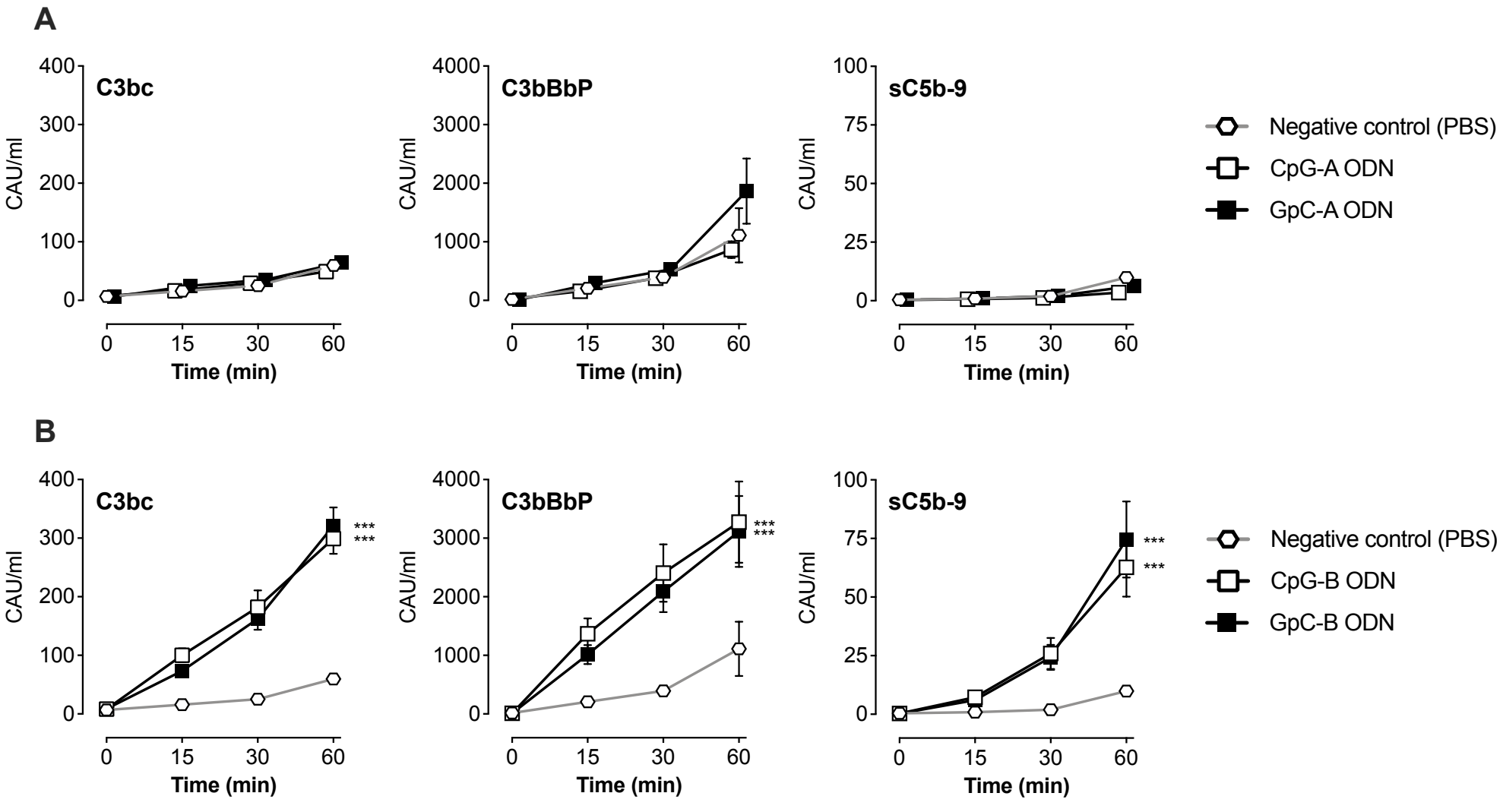
Table I. Sequence list of oligonucleotides

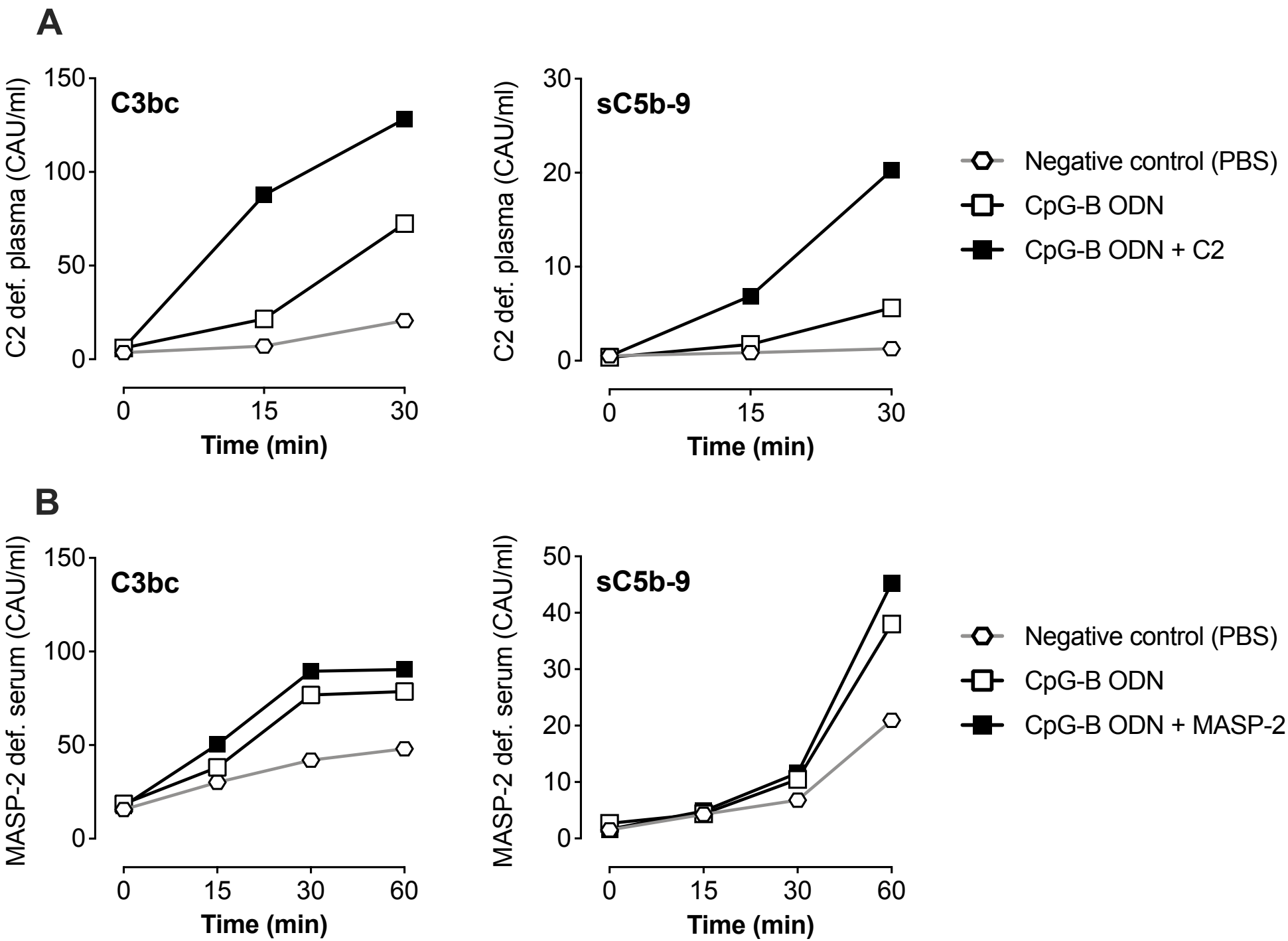
No.	ODN	Sequence[1]
1	Class-A 2216	5’-ggGGGACGA:TCGTCgggggg-3’
2	Class-B 2006	5’-tcgtcgttttgtcgttttgtcgtt-3’
3	Class-C 2395	5’-tcgtcgttttcggcgc:gcgccg-3’
4	Class-A 2243	5’-ggGGGAGCATGCTGggggggg-3’
5	Class-B 2137	5’-tgctgcttttgtgcttttgtgctt-3’
6	Class-C 2395c	5’-tgctgcttttgggggggcccccc-3’
7	Inhibitor 4191	5’-TCCTATCCTGGAGGGGAAG-3’

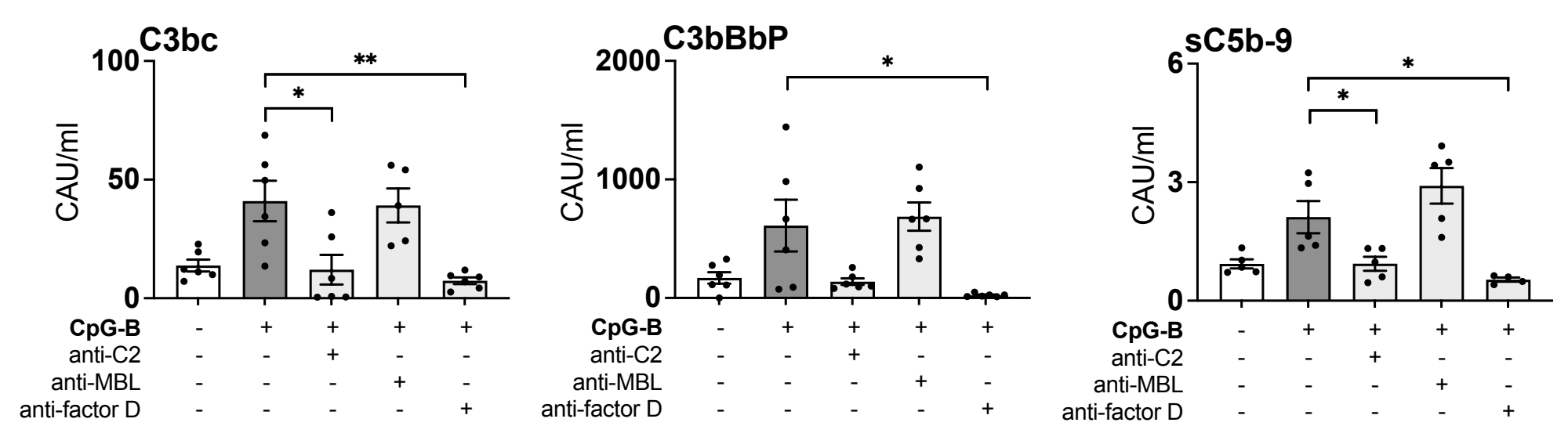
[1] Capital letters indicate phosphodiester and those in lower case phosphorothioate bonds. Underlining stands for palindromic sequence.

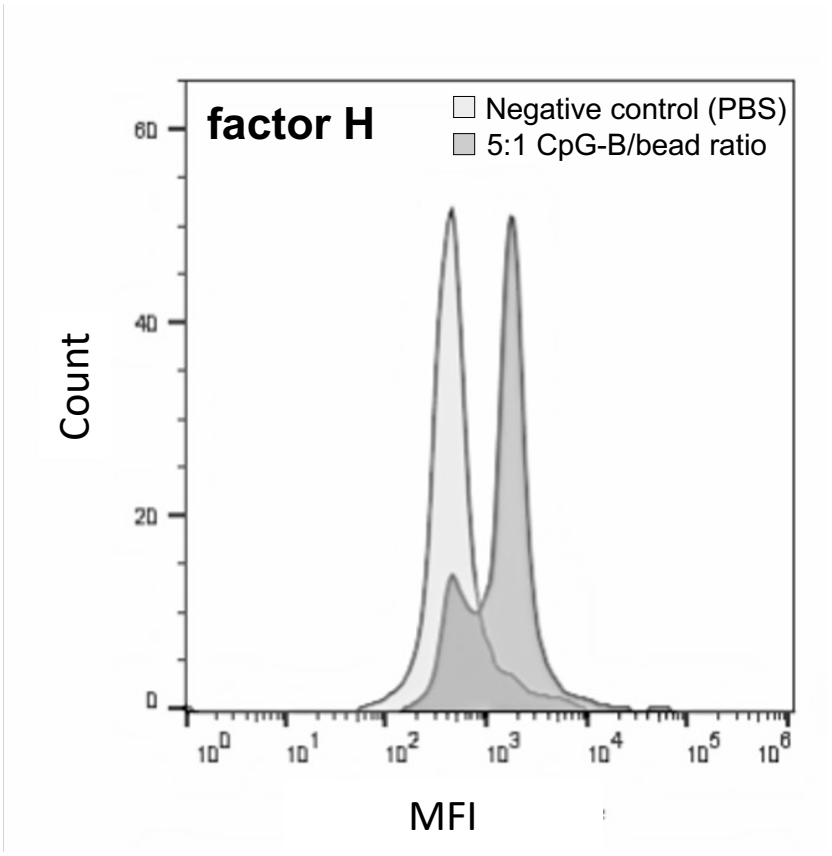

CpG class A
ODN-2216
5´
3´
G G G G
G A C G A
C T G C T
G G G G G G
CpG class B
ODN-2006
5´
3´
T C G T C G T T T T G T C G T T T T G T C G T T
CpG class C
ODN-2395
5´
3´
T C G T C G T T T T
C G G C G C
G C C G C G
C G CpG motif
Phosphorothioate bond
Phosphodiester bond

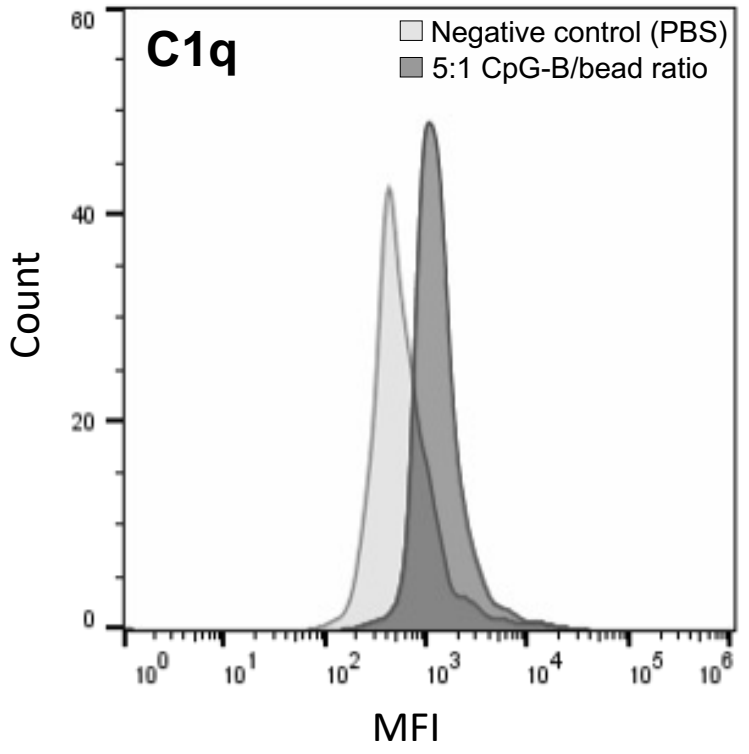

CpG-A ODN
CpG-B ODN
CpG-C ODN
C3bc (CAU/ml)
C3bBbP (CAU/ml)
sC5b-9 (CAU/ml)
Time (min)
0
15
30
60
***
**
Negative control (PBS)
Positive control (zymosan)
CpG ODN (50 μg/ml)
CpG ODN (30 μg/ml)
CpG ODN (10 μg/ml)

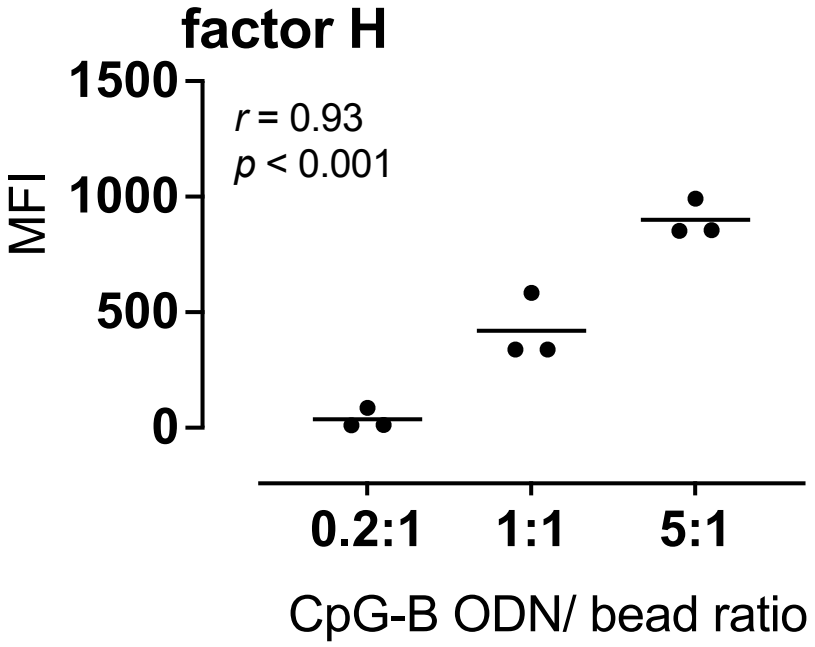

A
C3bc
C3bBbP
sC5b-9
CAU/ml
Time (min)
Negative control (PBS)
CpG-A ODN
GpC-A ODN
B
C3bc
C3bBbP
sC5b-9
***
***
Negative control (PBS)
CpG-B ODN
GpC-B ODN

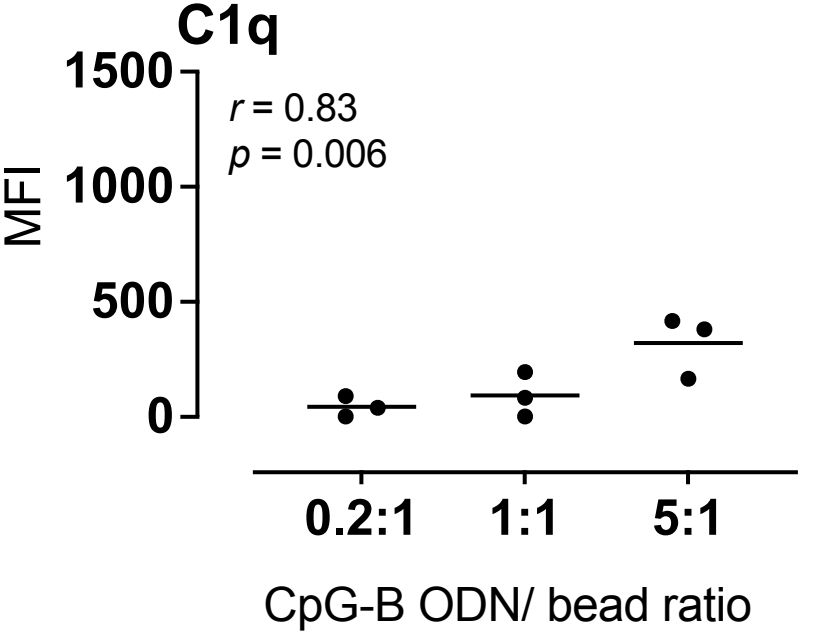

A
C3bc
C2 def. plasma (CAU/ml)
150
100
50
0
0
15
30
Time (min)
sC5b-9
C2 def. plasma (CAU/ml)
30
20
10
0
0
15
30
Time (min)
Negative control (PBS)
CpG-B ODN
CpG-B ODN + C2
B
C3bc
MASP-2 def. serum (CAU/ml)
150
100
50
0
0
15
30
60
Time (min)
sC5b-9
MASP-2 def. serum (CAU/ml)
50
40
30
20
10
0
0
15
30
60
Time (min)
Negative control (PBS)
CpG-B ODN
CpG-B ODN + MASP-2

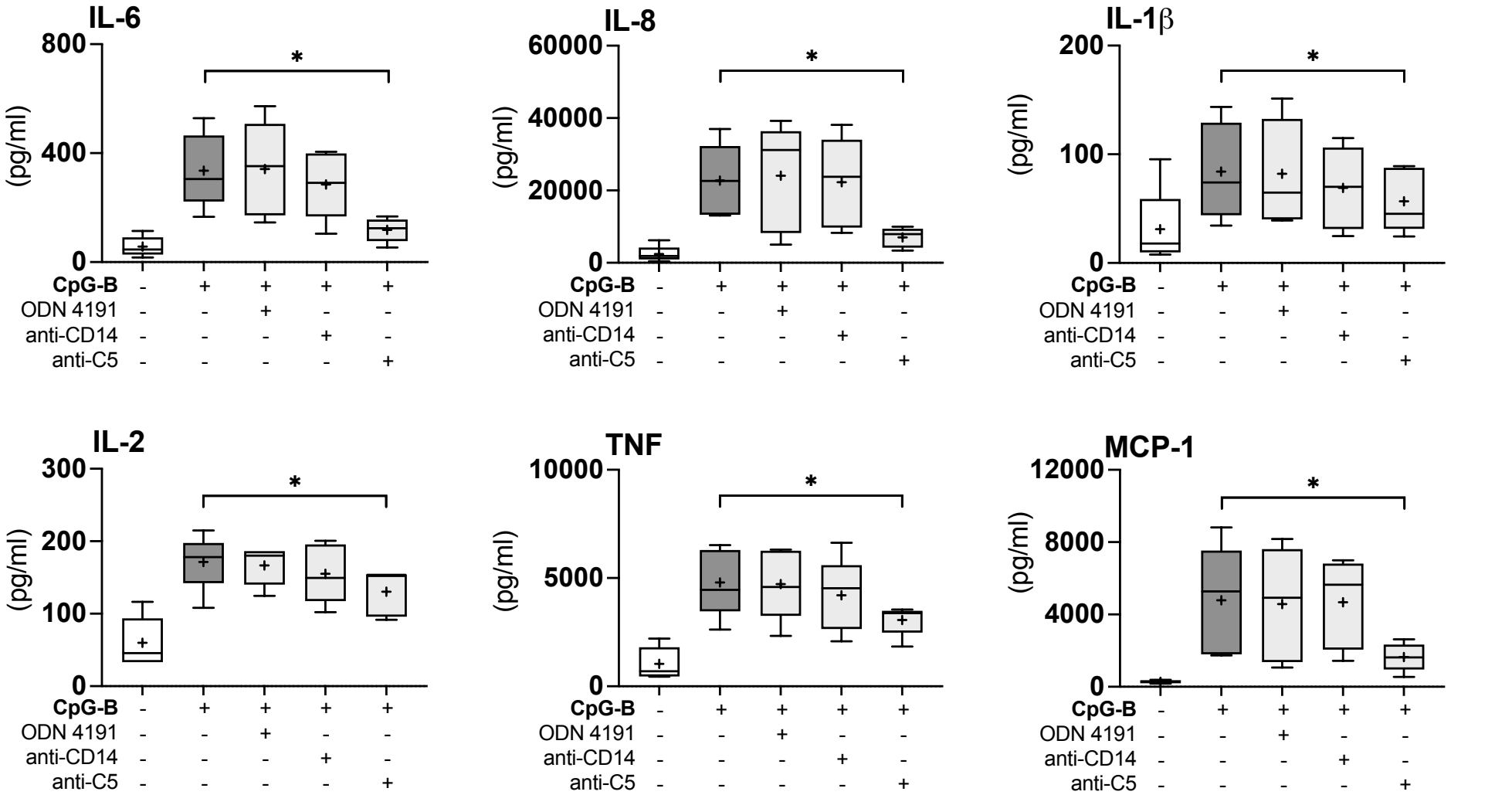

C3bc
CAU/ml
100
50
0
**
*
CpG-B - + + + +
anti-C2 - - + - -
anti-MBL - - - + -
anti-factor D - - - - +
C3bBbP
CAU/ml
2000
1000
0
*
CpG-B - + + + +
anti-C2 - - + - -
anti-MBL - - - + -
anti-factor D - - - - +
sC5b-9
CAU/ml
6
3
0
*
*
CpG-B - + + + +
anti-C2 - - + - -
anti-MBL - - - + -
anti-factor D - - - - +

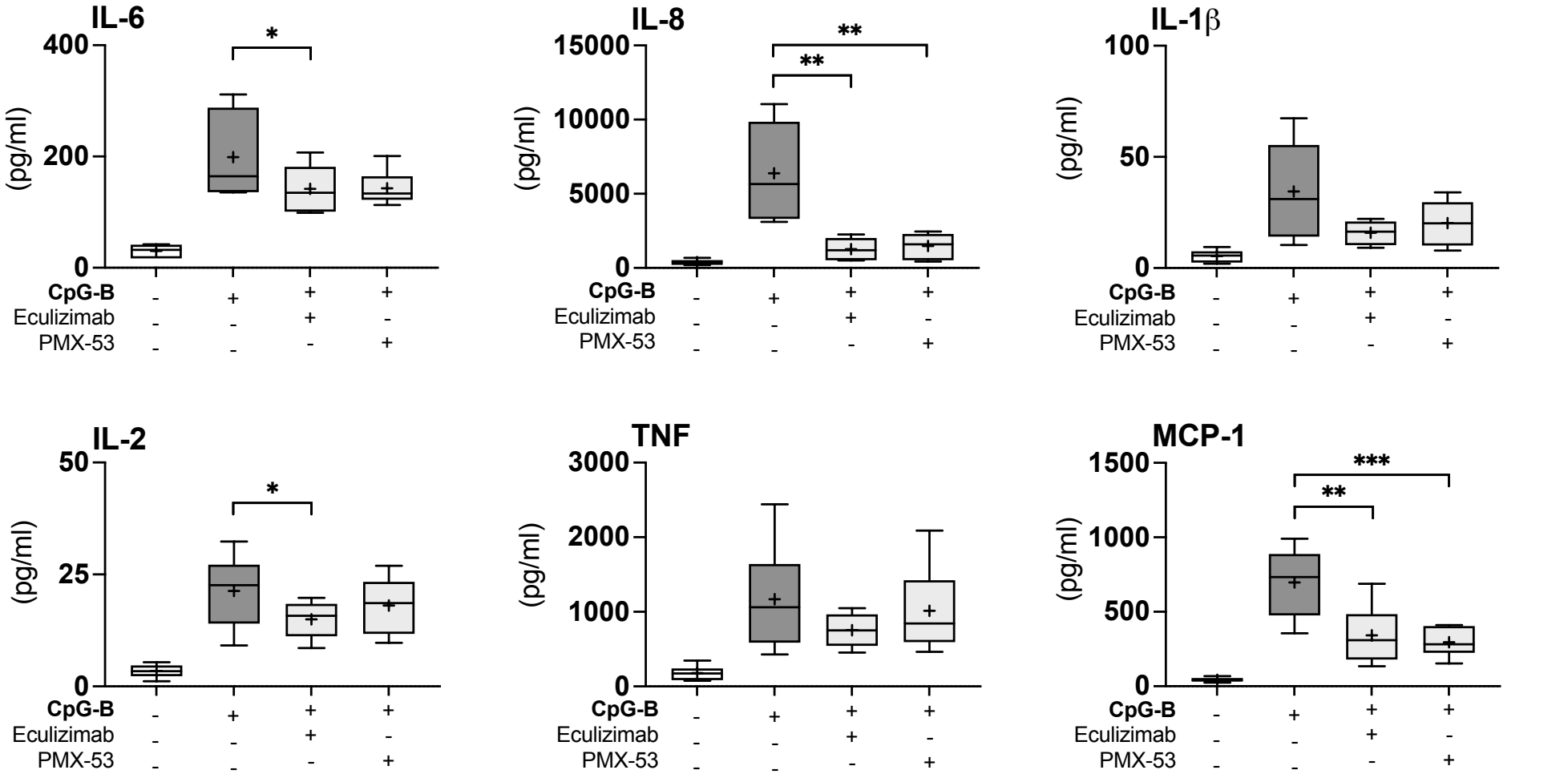

factor H
Negative control (PBS)
5:1 CpG-B/bead ratio
Count
MFI


C1q
Negative control (PBS)
5:1 CpG-B/bead ratio
Count
MFI

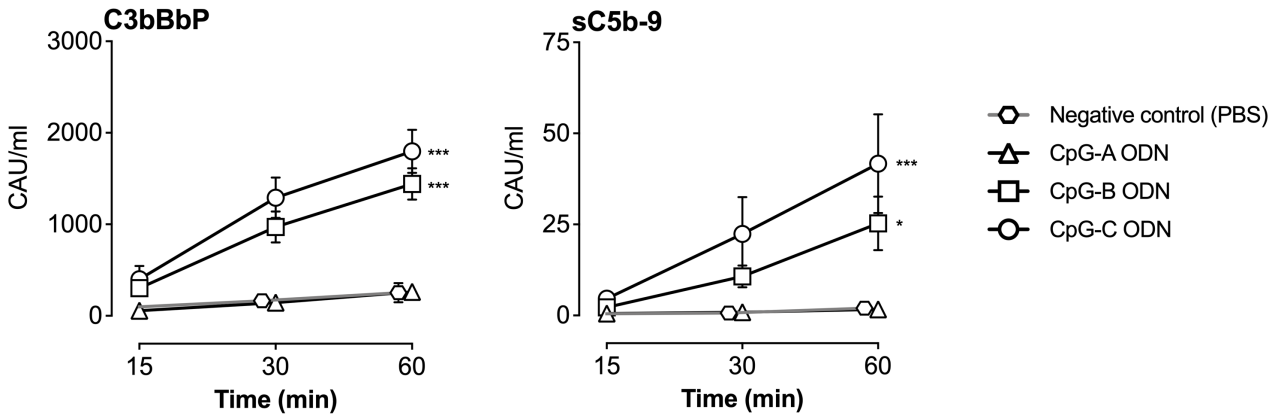

factor H
MFI
r = 0.93
p < 0.001
0.2:1
1:1
5:1
CpG-B ODN/ bead ratio


C1q
MFI
r = 0.83
p = 0.006
0.2:1
1:1
5:1
CpG-B ODN/ bead ratio


IL-6
800
400
0
(pg/ml)
*
CpG-B - + + + +
ODN 4191 - - + - -
anti-CD14 - - - + -
anti-C5 - - - - +
IL-8
60000
40000
20000
0
(pg/ml)
*
CpG-B - + + + +
ODN 4191 - - + - -
anti-CD14 - - - + -
anti-C5 - - - - +
IL-1β
200
100
0
(pg/ml)
*
CpG-B - + + + +
ODN 4191 - - + - -
anti-CD14 - - - + -
anti-C5 - - - - +
IL-2
300
200
100
0
(pg/ml)
*
CpG-B - + + + +
ODN 4191 - - + - -
anti-CD14 - - - + -
anti-C5 - - - - +
TNF
10000
5000
0
(pg/ml)
*
CpG-B - + + + +
ODN 4191 - - + - -
anti-CD14 - - - + -
anti-C5 - - - - +
MCP-1
12000
8000
4000
0
(pg/ml)
*
CpG-B - + + + +
ODN 4191 - - + - -
anti-CD14 - - - + -
anti-C5 - - - - +


IL-6
400
200
0
(pg/ml)
*
IL-8
15000
10000
5000
0
(pg/ml)
**
**
IL-1β
100
50
0
(pg/ml)
IL-2
50
25
0
(pg/ml)
*
TNF
3000
2000
1000
0
(pg/ml)
MCP-1
1500
1000
500
0
(pg/ml)
***
**
CpG-B - + + +
Eculizimab - - + -
PMX-53 - - - +

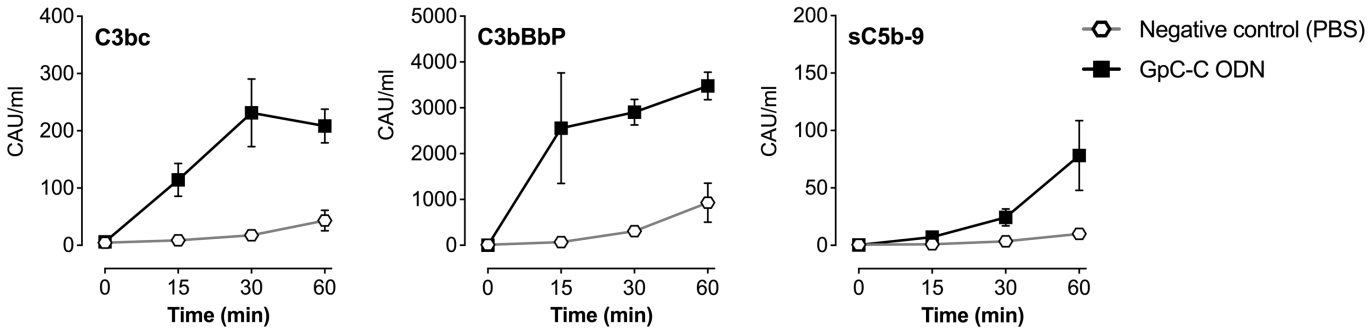
Supplementary Figure 1.



CpG-B and CpG-C ODN induced complement activation in plasma.

Plasma from 6 healthy donors was incubated with 50 µg/ml of CpG-A, CpG-B and CpG-C ODN. C3bBbP and sC5b-9 levels were significantly increased over time in the presence of CpG-B and CpG-C, whereas CpG-A had no effect upon complement formation. Data are presented as mean +/- SEM (n=6). General mixed model analysis. * = $p < 0.05$, *** = $p < 0.001$.

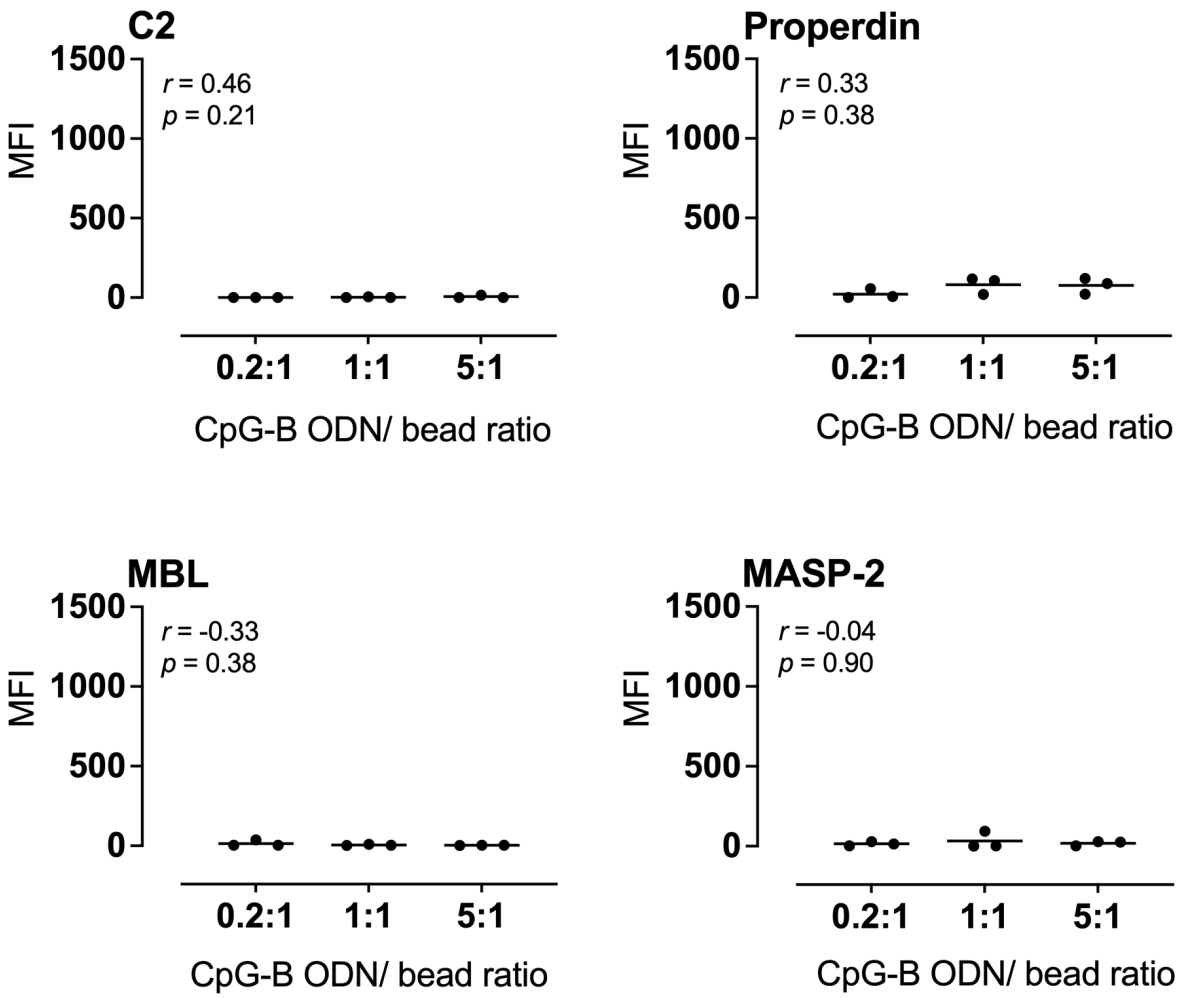
Supplementary Figure 2.



Class-C ODN induced complement activation in whole blood is dependent on ODN structure.

Lepirudin-anticoagulated plasma from 3 healthy donors was incubated with 10 µM/ml GpC-C ODN for 1 h. C3bc, C3bBbP and sC5b-9 increased over time and seem to differ from unstimulated lepirudin-anticoagulated plasma. Data are presented as mean +/- SEM. As this experiment included only three donors, no statistical analyses were conducted.

Supplementary Figure 3.



CpG-B ODN does not bind to complement proteins C2, properdin, MBL or MASP-2.

Biotinylated CpG-B ODNs coupled to magnetics beads were pre-incubated in normal human plasma in the presence of specific complement antibodies. No significant correlations in median fluorescence levels were observed between CpG-B ODNs and C2, properdin, MBL or MASP-2. Experiments were repeated three times and performed in triplicates. The Pearson's correlation coefficient was calculated to test for significance.