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The induction of cytokines by polycation containing microspheres by a complement dependent mechanism

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ABSTRACT

The cytokine-inducing potential of various microspheres were evaluated in a short-time screening assay of lepirudin-anticoagulated human whole blood utilizing the Bio-Plex Human cytokine 27-plex system. The inflammatory cytokines IL-1β, TNF and IL-6; the anti-inflammatory mediators IL-1ra and IL-10; the chemokines IL-8, MIP-1α and MCP-1; and the growth factor VEGF were induced by polycation (poly-L-lysine or poly(methylene-co-guanidine)) containing microspheres. Alginate microspheres without polycations did not induce the corresponding cytokine panel, nor did soluble alginate. By inhibiting complement C3 using compstatin analog CP20, a total inhibition of complement activation as well as the inflammatory mediators was achieved, indicating that complement activation alone was responsible for the induced cytokines. A strong deposition of C3c on the poly-L-lysine containing surface, while not on the microspheres lacking polycations, also points to the formation of C3 convertase as involved in the biomaterial-induced cytokine induction. These results show that complement is responsible for the induction of cytokines by polycation containing microspheres. We point to complement as an important initiator of inflammatory responses to biomaterials and the lepirudin anticoagulated whole blood assay as an important tool to identify the most tolerable and safe materials for implantation to humans.

1. Introduction

A major challenge of biomedical materials is related to the immunological responses directed to the material that can be detrimental for its function. Although the host responses are following recognizable patterns with cellular events of a normal inflammatory and wound healing process [1], the mechanisms initiating the inflammation as well as leading to a chronic condition is less well understood. In order to design biomaterials with the most optimal function for its performance, i.e. biomaterials provoking a low host response, or biomaterials directed to synergize with the host tissue (i.e. vascularization or tissue interaction), it is most critical to get an understanding of the mechanisms behind the host reactions.

The interplay between inflammatory and wound healing cells is guided by large networks of cytokines in continuous and dynamic processes. The biomaterial-induced cytokines are anticipated to be important for the performance of a biomaterial *in vivo*. How the cytokine profiles are affected by the biomaterial surfaces are investigated by use of animal models [2–5], *in vitro* monocyte/macrophage cultures [6–8] or monocyte/fibroblast co-culture systems [9]. A particularly efficient tool is the whole blood model, containing the immediate protein cascades including complement, coagulation, fibrinolysis and contact activation systems, suggested to be important for the initial inflammation [10]. Upon exposure to plasma [11] or peritoneal fluid [12], proteins will bind and potentially initiate the reactive protein cascades [11] or serve as cellular anchor points [13]. A whole blood model based on anticoagulation by the hirudin analog lepirudin [14], inhibiting thrombin and

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rendering the other proteins and the immune cells able to mutually interact, is particularly suitable to study biomaterial surface interaction with the host immune system. This method has been used to reveal mechanisms behind the polyvinyl chloride (PVC) tube activation [15–17] and recently also for implantable sensors [18,19]. In addition to being an efficient tool for revealing mechanisms behind the host response, the human whole blood assay is beneficial for overcoming species differences [20], as well as for screening a set of different biomaterials under equal conditions.

The aim of the present study was twofold. Firstly, we explored the tolerability of microspheres with potential in cell encapsulation therapy [21,22] by screening for inflammatory and anti-inflammatory mediators, i.e. pro-inflammatory cytokines, anti-inflammatory cytokines, chemokines and growth factors in human fresh blood anticoagulated specifically by inhibiting thrombin. Secondly, we searched for a causal connection between the activation of the complement cascade and the secretion of these mediators, since we previously found microspheres containing polycations (poly-L-lysine; PLL or poly(methylene-co-guanidine); PMCG) to activate complement [23].

2. Materials and methods

2.1. Reagents

Ultrapure sodium alginates; Pronova UP-MVG (67% guluronic acid, intrinsic viscosity 1105 ml/g, endotoxin <43 EU/g), Pronova UP-LVG (≥60% guluronic acid, intrinsic viscosity 830 ml/g, endotoxin <100 EU/g) or Pronova UP100M (Macrocystis pyrifera, 56% mannuronic acid, endotoxin < 26 EU/g) were obtained from FMC BioPolymer AS (Novamatrix, Norway). The protein contents were less than 0.3% for all alginates as specified by the manufacturer. Cellulose sulfate (CS) sodium salt was from Acros Organics (Geel, Belgium), and PMCG supplied as 35% aqueous solution was from Scientific Polymer Products Inc. (Ontario, NY, USA). Sodium chloride, calcium chloride, barium chloride and sodium citrate of analytical grades were from Merck, Darmstadt, Germany. PLL (P2658, lot nr. 96H5502), Tween 20, Zymosan A (Z-4250), LPS was from *E. coli* 0111-B4 (Invivogen), PBS with calcium and magnesium, EDTA, paraformaldehyde and LDS-751 were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Other reagents: mannitol (HPLC grade, BDH Anala R.) was obtained from VWR International Ltd. (Pool, England), non-pyrogenic sterile saline (0.9% NaCl) was from B. Braun (Melsungen, Germany), and lepirudine was from Celgene Europe (Boudry, Switzerland). The C3 inhibitor compstatin analog CP20, Ac-Ile-[Cys-Val-Trp(Me)-Gln-Asp-Trp-Sar-Ala-His-Arg-Cys]-mlle-NH2 [24] and a corresponding control peptide Sar-Sar-Trp(Me)-Ala-Ala-Asp-Ile-His-Val-Gln-Arg-mlle-Trp-Ala-NH2 was synthesized in the laboratory of Prof. John D. Lambris. Anti-CD14 F(ab′)$_2$ (clone 18D11) and its isotype control, in addition to the anti-human C5b-9 clone aE11 were purchased from Diatech (Oslo, Norway). The FITC-conjugated rabbit anti-human C3c (F0201) and FITC conjugated poly-rabbit anti-mouse (F0261) were from Dako (Glostrup, Denmark). Biotinylated 9C4 was an "in-house" antibody previously described in [25]. Equipment for blood sampling: Polypropylene vials (NUNC, Roskilde, Denmark) and BD vacutainer top (Belliver Industrial Estate, Plymouth, UK).

2.2. Microsphere preparations

Alginate microbeads (Ca/Ba Beads and Ba Beads) and microcapsules (Alginate-Poly-L-lysine-Alginate; APA and Alginate-Poly-L-lysine; AP) were made as previously described [23,26] using 5 ml of 1.8% UP-MVG alginate/0.3 M mannitol and an electrostatic bead generator (7 kV) with 4 × 0.4 mm needles and flow 10 ml/h. Gelling solutions varied according to microsphere type; Ca/Ba Beads: 1 mM $BaCl_2$/50 mM $CaCl_2$/0.15 M mannitol; Ba Beads: 20 mM $BaCl_2$/0.15 M mannitol; APA and AP: 50 mM $CaCl_2$/0.15 M mannitol. AP microcapsules were made by incubating the alginate microbeads in 25 ml 0.05% PLL for 10 min. APA microcapsules were made by incubating the AP microcapsules in 10 ml 0.1% Pronova UP100 M/0.15 M mannitol for 10 min. The PMCG microcapsules were made as previously described [27] using a multi-loop reactor with 40 s complexation time, after same protocol as described in [23] with following specifications; polyanions 0.90% UP-LVG alginate/0.90% CS/0.9% NaCl; polycation 1.2% PMCG/1% $CaCl_2$/0.9% NaCl/0.025% Tween 20. The membrane composition was equilibrated by incubation in 50 m M sodium citrate/0.9% NaCl solution for 10 min, with subsequent coating with 0.1% CS/0.9% NaCl 10 min. Alginate microspheres were washed, aliquoted and stored in 0.9% NaCl. The resulting diameter of the microspheres (mean ± SD of $N = 30$) were as follows; Ca/Ba Beads: 500 ± 30 μm; Ba Beads: 490 ± 20 μm; APA: 500 ± 30 μm; AP: 510 ± 30 μm; PMCG microcapsules 820 ± 40 μm.

2.3. LAL assay

The microspheres storing solutions were endotoxin tested by the Endpoint chromogenic assay (LAL assay, Lonza) following the manufacturer's protocol. The endotoxin levels were between 11 and 24 pg/ml. In comparison, the endotoxin level in sterile medical water was measured to16 pg/ml.

2.4. C3 deposition

Microspheres were incubated in pooled lepirudin anti-coagulated plasma ($N = 3$) for 21 h at 37 °C under continuously rotation. Plasma was removed, and microspheres washed twice in saline and added 50 μg/ml FITC-conjugated poly-rabbit anti-human C3c or its respective control (FITC-poly-rabbit anti-mouse). C3 deposition was visualized by a CLSM, Zeiss LSM 510 confocal laser scanning microscope from Carl Zeiss MicroImaging GmbH (Göttingen, Germany) with a 488-nm laser source (BP 505–530). Identical settings were used for the different microspheres and controls.

2.5. Whole blood model

The whole blood model, first described in [14], is utilizing the anti-coagulant lepirudin which specifically inhibits thrombin. Lepirudin is a preferred anti-coagulant for studying effects of complement activation on cellular responses. In contrast to heparin, lepirudin has no effect on the complement activation. The whole blood model was recently adapted for study of alginate microspheres [23]. Shortly described, blood was drawn from healthy volunteers using lepirudin as anti-coagulant (50 μg/ml). Immediately after blood withdrawal, blood (500 μl) was incubated with 100 μl PBS and 100 μl saline solution containing microspheres (50 μl), alginate (900 μg), zymosan 10 μg or only saline as described previously [23]. Samples were incubated for 30, 120 and 360 min. For inhibition studies, blood was pre-incubated for 5 min with either the compstatin analog CP20 (25 μ M), a 13-amino acid cyclic peptide that binds to C3 and inhibits its cleavage [24], or the Anti-CD14 F(ab')$_2$ (10 μg/ml) binding to the TLR4 co-receptor CD14, or their respective controls, control peptide (25 μ M) or control F(ab′)$_2$ (10 μg/ml). Blood (500 μl) was then added AP or PMCG microcapsules or the controls (Zymosan 10 μg, LPS 100 ng/ml or saline). Samples were incubated in 240 min at 37 °C. Aliquots of plasma was stored at −80 °C before analysis. In all studies, five healthy donors were used, and the data presents the mean ± SEM of 5 donors ($N = 5$).

2.6. Inflammatory mediators

Plasma samples were analyzed using ELISA kit for IL-8 or TNF (both R&D), or a multiplex cytokine assay (Bio-Plex Human cytokine 27-Plex Panel; Bio-Rad Laboratories, Hercules, CA) containing the following analytics: IL-1 beta (IL-1β), IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin (CCL11), basic fibroblast growth factor (bFGF), granulocyte colony stimulating factor (GCSF), granulocyte-macrophage colony stimulating factor (GMCSF), interferon gamma (IFN-γ), chemokin (C-X-C motif) ligand 10 (IP-10 or CXCL10), monocyte chemoattractant protein 1 (MCP-1, or CCL2), macrophage inflammatory protein-1-alfa (MIP-1α, or CCL3), macrophage inflammatory protein-1-beta (MIP-1β, or CCL4), platelet derived growth factor-BB (PDGF-BB), regulated upon activation T-cell expressed and secreted (RANTES, or CCL5), tumor necrosis factor alpha (TNF), and vascular endothelial growth factor (VEGF). The multiplex analyzes were performed as recommended by the producer using half amounts of beads. In order to detect low activation species, the standard was diluted four times more for the inhibition experiments.

2.7. Terminal sC5b-9 complex (sTCC) EIA

The sTCC was quantified in an EIA using mAb aEll specific for C9, and biotinylated 9C4 specific for C6, in the sC5b-9 complex. The assay has been described in detail previously [28] and performed according to a later modification [25]. The results are given in arbitrary units (AU/ml), which is related to a standard of maximally activated serum defined to contain 1000 AU/ml.

2.8. Statistical methods

Results were analyzed using GraphPad Prizm version 5.0 from GraphPad Software (San Diego, CA). Data were analyzed using one-way, repeated measurements ANOVA followed by Dunnett's multiple comparison test using saline as control. The effect of the complement inhibitor compstatin was analyzed using a paired *t*-test against the results in the absence of inhibitor. The data were transformed logarithmically if the normality test failed. $P < 0.05$ was considered statistically significant.

2.9. Ethics

The use of human whole blood for basal experiments was approved by the regional ethics committee in Norway Regional Health Authority. The experiments were performed in accordance with their guidelines and following the Helsinki Declaration.

3. Results

3.1. Cytokine responses

We have previously demonstrated that microcapsules containing polycations are potent activators of the complement system [23]. In the present study, we examined the microcapsule-induced cytokines in a fresh human whole blood model and investigated the role of complement activation in the cytokine response. Addition of PLL containing APA and AP microcapsules to whole blood for 360 min led to significantly enhanced amount ($P < 0.05$) of the inflammatory cytokines TNF, IL-1β and IL-6 (Fig. 1). In contrast, alginate microbeads (Ca/Ba Beads or Ba Beads) did not enhance these cytokines compared to the saline control (Fig. 1). Furthermore, comparable amounts of soluble alginate (UP-LVG) also did not enhance the amount of inflammatory cytokines (Fig. 1).

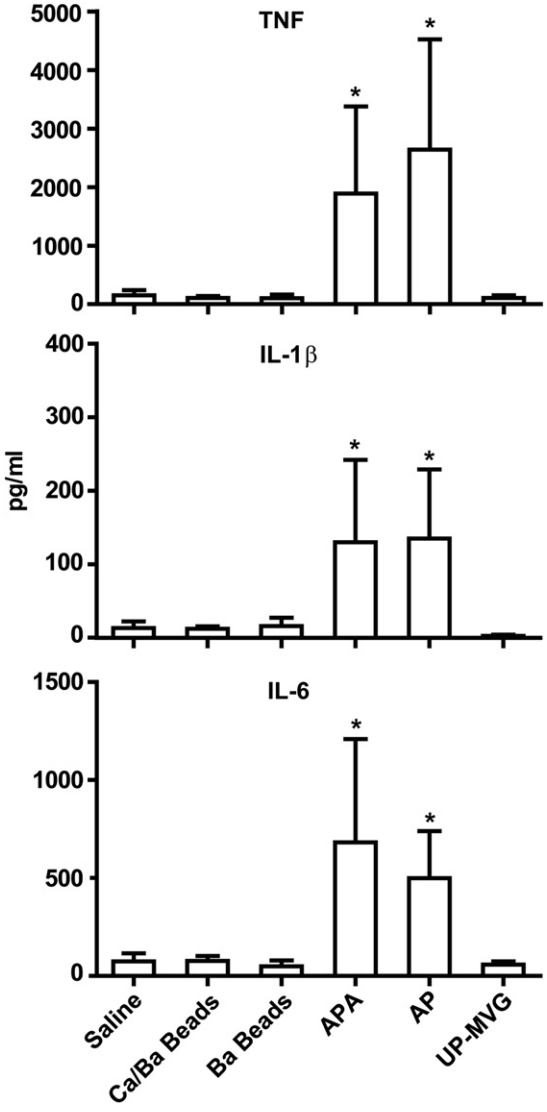

Fig. 1. Effect of various microspheres (Ca/Ba Beads, Ba Beads, APA; Alginate-Poly-L-lysine-Alginate, AP; Alginate-Poly-L-lysine) and alginate (UP-MVG) on inflammatory cytokines (TNFα, IL-1β and IL-6) in plasma after 360 min in lepirudin anti-coagulated fresh human blood. The plasma baseline and positive control values (pg/ml); TNFα 16.8 ± 8.1 (baseline) and 28893 ± 6549 (zymosan), IL-1β 1.6 ± 1.4 (baseline) and 2675 ± 337 (zymosan), IL-6 6.5 ± 4.2 (baseline) and 21737 ± 3033 (zymosan). Data are expressed as the mean ± SEM ($N = 5$) of separate experiments with different donors. Significant values are given as $P < 0.05$ (*).

IL-1ra and IL-10 are well recognized as anti-inflammatory mediators. APA and AP microcapsules significantly enhanced the IL-1ra and IL-10 levels, while the Ca/Ba Beads, Ba Beads as well as the UP-LVG alginate had no effect (Fig. 2).

Furthermore, APA and AP microcapsules enhanced IL-8, MIP-1α, MCP-1 and VEGF levels, while Ca/Ba Beads, Ba Beads or the soluble alginate had no effect (Fig. 3). APA and AP microcapsules significantly increased IL-8 and MIP-1α levels, whereas MCP-1 was significantly increased by APA and VEGF by AP (Fig. 3).

The IP-10 secretion pattern showed a lower induction upon addition of microspheres compared to saline or soluble alginate (Fig. S1). RANTES was significantly elevated after incubation with Ca/Ba Beads and Ba Beads, APA and AP microcapsules for 120 min (Fig. S1), while after 360 min incubation no significant differences were found (data not shown). A slight non-significant elevation of IFN-γ, MIP-1β and PDGF-BB was found after addition of APA and AP microcapsules (Fig. S1). A slight non-significant elevation was detected for Ca/Ba Beads and UP-LVG alginate for eotaxin (Fig. S1).

The following cytokines were either not detected or boarder detected with no increase over time measured after 30, 120 and 360 min incubation; IL-2, IL-4, IL-5, IL-7, IL-9, IL-13, IL-15, bFGF, GM-CSF and G-CSF.

3.2. C3 deposition

Substantial amount of C3c was detected on the surface of the PLL containing microspheres after 21 h incubation in plasma (Fig. 4A and B), while C3c was not detected on the alginate microbead

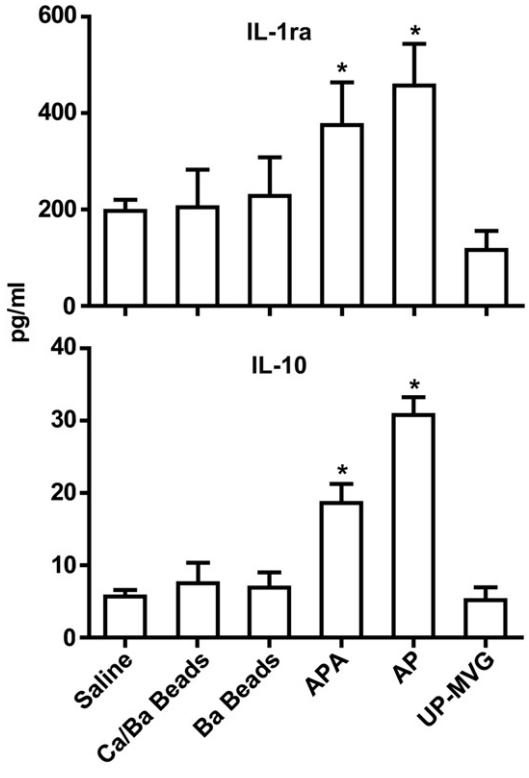

Fig. 2. Effect of various microspheres (Ca/Ba Beads, Ba Beads, APA; Alginate-Poly-L-lysine-Alginate, AP; Alginate-Poly-L-lysine) and alginate (UP-MVG) on the anti-inflammatory interleukin-1 receptor antagonist (IL-1ra) and the anti-inflammatory cytokine IL-10 secretion in plasma after 120 min incubation in lepirudin anti-coagulated fresh human blood. The plasma baseline values and positive control values (pg/ml); IL-1ra 63.8 ± 21.9 (baseline) and 552 ± 69.8 (zymosan) and IL-10 1.3 ± 0.7 (baseline) and 21.2 ± 2.9 (zymosan). Data are expressed as the mean ± SEM ($N = 5$) of separate experiments with different donors. Significant values are given as $P < 0.05$ (*).

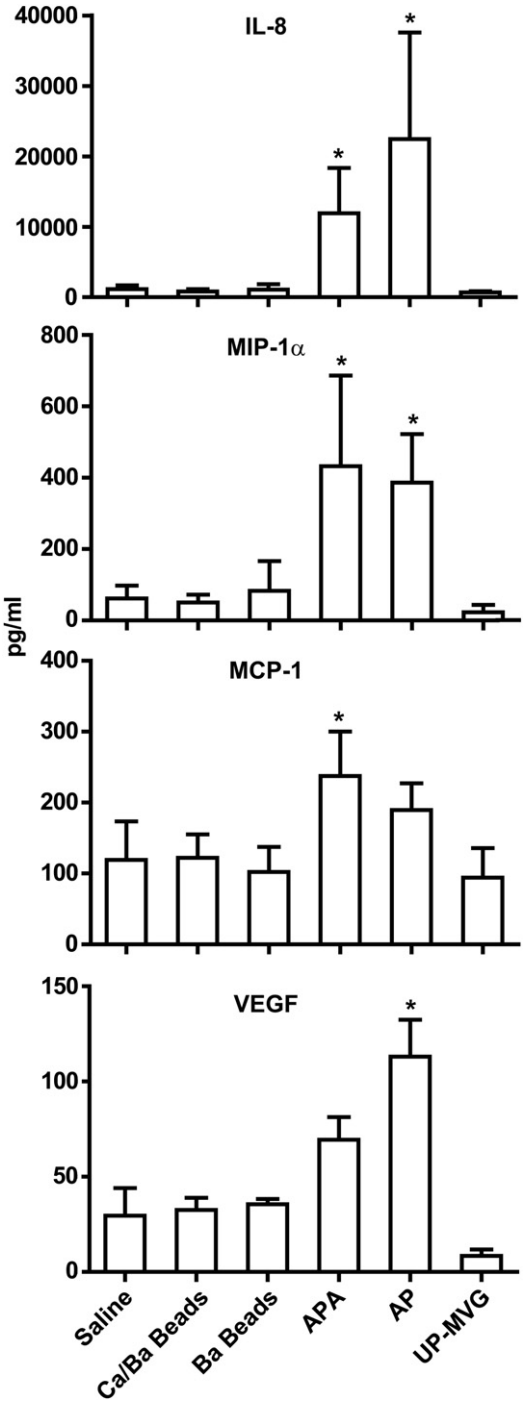

Fig. 3. Effect of various microspheres (Ca/Ba Beads, Ba Beads, APA; Alginate-Poly-L-lysine-Alginate, AP; Alginate-Poly-L-lysine) and alginate (UP-MVG) on chemokines (IL-8, MIP-1α and MCP-1) and the growth factor VEGF secretion in plasma after incubation for 360 min in lepirudin anti-coagulated fresh human blood. The plasma baseline and positive control values (pg/ml); IL-8 40.6 ± 14.4 (baseline) and 16624 ± 1762 (zymosan), MIP-1α 0 ± 0 (baseline) and 1987 ± 238 (zymosan), MCP-1 28 ± 12.5 (baseline) and 908 ± 483 (zymosan) and VEGF 1.4 ± 0.9 (baseline) and 51.8 ± 8.3 (zymosan). Data are expressed as the mean ± SEM ($N = 5$) of separate experiments with different donors. Significant values are given as $P < 0.05$ (*).

surface (Fig. 4C and D). On the PLL containing microspheres at shorter incubation times, gradually increasing amount of C3c was detected, indicating that the C3c accumulated by time (results not shown). The corresponding control of the AP microcapsule incubated for 21 h and stained with the isotype control confirms that the C3c staining was specific (Fig. 4E). At higher magnification of the PLL containing surface taken at 4 h incubation in plasma, the C3c was found to deposit in spots in various amounts (Fig. 4F). This is probably reflecting the establishment of the C3 convertases at various times during incubation. While the C3c is present in both native C3 and the activated C3b, our data points to a formation of an active C3 convertase on the PLL containing surface since; 1) The PLL containing microcapsules induce C3a and C5a [23] which is consistent with the formation of active C3b and C5b convertases; 2) The PLL containing microcapsules induce the activated complement (sTCC) as shown here and in [23]; 3) The spotted deposition of C3c found at short incubation time (Fig. 4F). Overall, the results indicate that active C3 convertases are built up on the PLL membrane, while not on the alginate surface.

3.3. Connection between complement and cytokines

Complement activation has been shown to be involved in the induction of chemokines and growth factors [15,16,29]. Since polycation containing microcapsules are potent complement activators [23], we examined whether their cytokine responses were dependent on the ability to induce complement activation. The role of CD14 was also examined, since inhibition of CD14 has been shown to reduce cytokine expression, particularly in combination with complement inhibition [29,30]. In the next set of experiments we selected AP and PMCG microcapsules and incubated them in whole blood for 240 min in the absence and presence of the C3 inhibitor compstatin analog CP20, anti-CD14 $F(ab')_2$, or in combination. The PMCG microcapsules contain the polycation PMCG and the polyanions cellulose sulfate and alginate [27]. Both the PMCG and AP microcapsules significantly enhanced complement activation measured as sTCC (Fig. 5). Concomitantly, significant elevation of the inflammatory cytokines TNF, IL-1β and IL-6 was observed (Fig. 5). Addition of compstatin analog CP20 completely abolished TCC, TNF, IL-1β and IL-6 after incubation with AP or PMCG microcapsules (Fig. 5). In addition, significant inhibition by compstatin analog CP20 was detected for the saline control, consistent with the modest complement activation induced by the polypropylene vials. Anti-CD14 $F(ab')_2$ did not inhibit microspheres-induced cytokines, whereas LPS-induced cytokine secretions (positive control) was efficiently inhibited as expected (data not shown). These results indicate that CD14 was not involved. Further on the data indicates that complement activation was completely responsible for the microsphere-induced cytokine secretion.

The PMCG microcapsules significantly enhanced IL-1ra, while only slightly increased IL-10 levels (Fig. 6). Again, complement inhibition significantly reduced the AP microcapsule-induced IL-1ra and IL-10, and PMCG microcapsule-induced IL-1ra and IL-10 levels. The concentrations of IL-10 after 4 h incubation were lower in the inhibition study than in the first experiments after 2 h incubation, indicating the inter-assays variations. However, since the effect was consistent between donors and upon complement inhibition, we conclude that this was a specific response generated by the AP microcapsule. Inhibition with anti-CD14 $F(ab')_2$ fragment had no effect on microspheres-induced IL-1ra or IL-10 secretion, while the LPS-induced secretion was inhibited by the anti-CD14 $F(ab')_2$ fragment (not shown). Our results indicate therefore that CD14 was not involved in the microsphere-induced secretion of IL-1ra or IL-10, whereas the effect could be explained solely by complement activation.

Incubation with AP and PMCG microcapsules significantly increased IL-8, MIP-1α and MCP-1 whereas only addition of the AP microcapsules significantly increased the VEGF (Fig. 7). Inhibition with compstatin analog CP20 totally abolished the secretion of IL-8, MIP-1α, and MCP-1, as well as most of the VEGF secretion (Fig. 7).

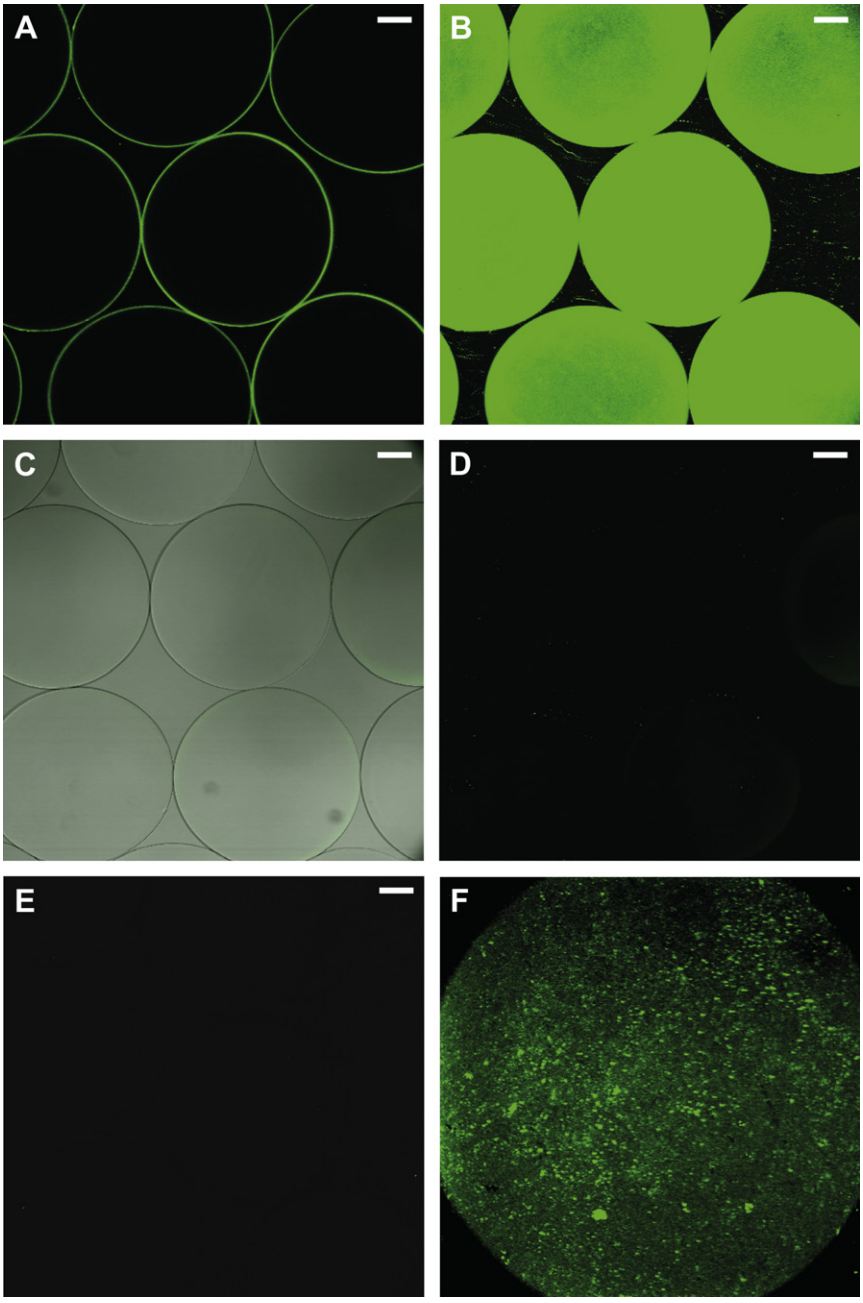

Fig. 4. Deposition of C3c on AP (Alginate-poly-L-lysine) microcapsules and Ca/Ba microbeads after incubation in lepirudin anti-coagulated plasma. (A) Equatorial section of AP microcapsules after 21 h incubation in plasma followed by FITC anti-C3c staining. (B) Corresponding 3D projection made by section through the entire AP microcapsules. (C) Ca/Ba beads stained with FITC anti-C3c after 21 h incubation in plasma and given as equatorial section overlaid transmitted light. (D) Corresponding 3D projection of Ca/Ba beads. (E) Negative control; 3D projections of AP microcapsules stained with FITC anti-mouse. (F) Closer image of the AP microcapsule surface taken after 4 h incubation and shown as 3D projection, revealing spots of C3c deposition. Bar is equal to 100 μm.

The anti-CD14 F(ab')$_2$ fragment did not inhibit chemokine or growth factor secretion from the microspheres while the LPS-induced secretion (positive control) was inhibited (not shown), indicating that CD14 was not involved in the microsphere-induced cytokine secretion.

Several chemokines and growth factors were modestly induced by the microspheres, as shown in supplementary (Fig. S1). Complement inhibition was consistently effective, showing the clearest inhibition for IFN-γ and IL-17 with significant differences for AP microcapsules (Fig. S2). IL-17 was detected in the inhibition studies, whereas its levels were partly under detection limit in the first set of analyses. The elongation of standard dilutions might have contributed to higher detection efficiency in the inhibition experiments. Moreover, complement inhibition significantly increased IP-10 after incubation with AP microcapsules, although this was not found for PMCG microcapsules (Fig. S2).

4. Discussion

As shown by the present data, the inflammatory potential of different microspheres can be effectively revealed by use of fresh human whole blood anticoagulated with lepirudin. By specifically inhibiting proteins and receptors, whole blood represents a potent tool for unmasking the underlying mechanisms leading to secretion

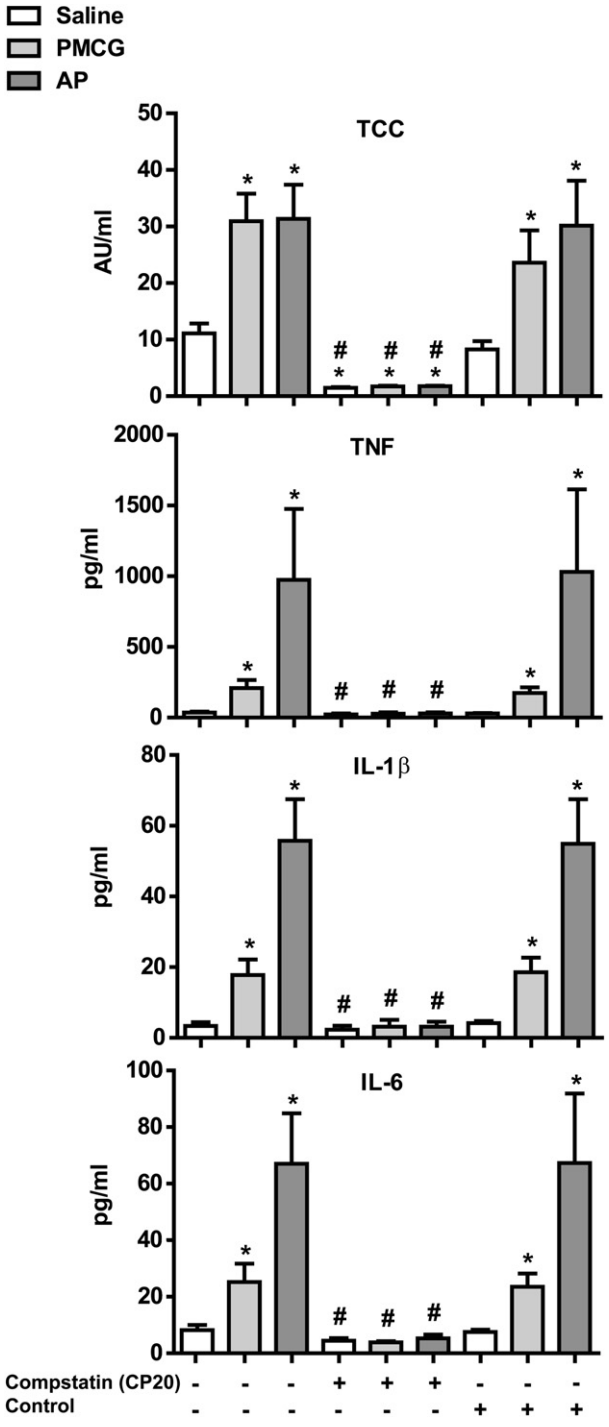

Fig. 5. Effect of the C3 inhibitor compstatin analog CP20 on complement (sTCC) and inflammatory cytokines (TNFα, IL-1β and IL-6) after 240 min incubation of AP (Alginate-Poly-L-lysine) or PMCG microcapsules in lepirudin anti-coagulated fresh human blood. A corresponding control peptide to compstatin analog CP20 was used as control. The plasma baseline and positive control values given as AU/ml for sTCC and pg/ml for the cytokines were respectively: sTCC; 1.3 ± 0.6 (baseline), 75.4 ± 11.2 (zymosan), 9.9 ± 2.3 (LPS), TNFα; 5 ± 2.3 (baseline), 19213 ± 1961 (zymosan), 19407 ± 3641 (LPS), for IL-1β; 0.02 ± 0.02 (baseline), 559 ± 132 (zymosan), for 1226 ± 370 (LPS), and for IL-6 1.2 ± 0.4 (baseline), 11392 ± 1660 (zymosan), 20570 ± 3622 (LPS). Data are expressed as mean ± SEM ($N = 5$, for baseline values $N = 3$) of separate experiments with different donors. Significant values are given as at $P < 0.05$, *compared to saline control #effect of compstatin compared to the same condition without compstatin.

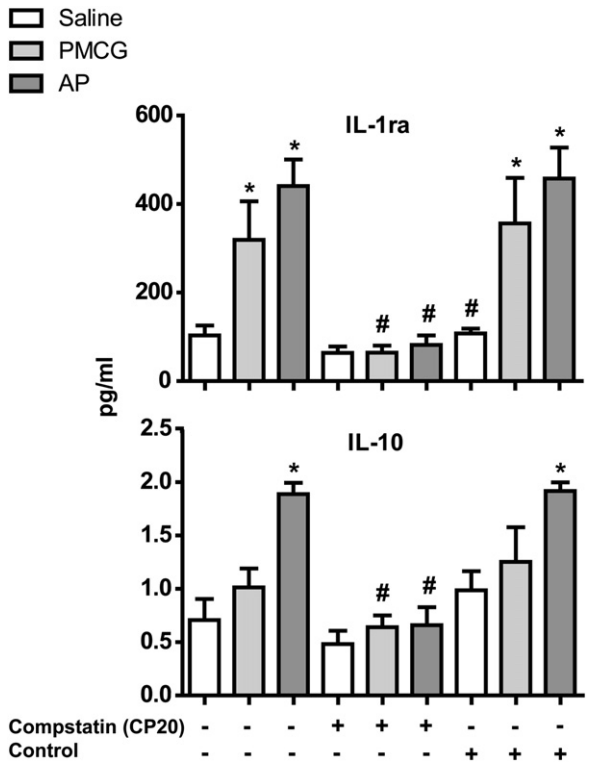

Fig. 6. Effect of C3 inhibition (Compstatin analog CP20) on IL-1ra and IL-10 after after 240 min incubation with AP and PMCG microcapsules in lepirudin anti-coagulated fresh human blood. A corresponding control peptide to compstatin analog CP20 was used as control. The plasma baseline and positive control values (pg/ml): IL-1ra; 32 ± 7 (baseline), 1245 ± 23 (zymosan), 2161 ± 341 (LPS), IL-10; 0.2 ± 0.1 (baseline), 6.5 ± 1.2 (zymosan), for 5.22 ± 0.93 (LPS). Data are expressed as the mean ± SEM ($N = 5$, for baseline value $N = 3$) of separate experiments with different donors. Significant values are given as $P < 0.05$ *compared to saline control #effect of compstatin compared to the same condition without compstatin.

of inflammatory mediators like inflammatory- and anti-inflammatory cytokines, chemokines and growth factors. Collectively, the data indicate that complement activation is responsible for the broad inflammatory response induced by the polycation containing microspheres.

A clear trend was detected by the different microspheres. Alginate microbeads without polycations did not induce significant increase in inflammatory mediators compared to the saline control while a significant elevation was found after incubation with polycation containing microcapsules. The inflammatory cytokines (TNF, IL-1β and IL-6), the anti-inflammatory cytokines (IL-1ra and IL-10), the chemokines (IL-8, MIP-1α, MIP-1β, MCP-1, RANTES) and the growth factors (VEGF and PDGF) were significantly elevated after addition of APA and AP microcapsules containing the polycation PLL in its outer membrane. The polycation containing PMCG microcapsules also significantly increased TNF, IL-1β, IL-6, IL-8, MIP-1α, MCP-1 and IL-1ra. The polycation containing microcapsules have previously been shown to activate complement while alginate microbeads do not activate the complement cascade [23]. In the present study we show C3c to be present in abundant amounts on the PLL containing surface, while not on a surface containing solely alginate. This finding is likely a result of activated C3 convertase on the PLL containing surface, since both C3a and C5a are produced [23], indicating formation of C3 and C5 convertases. Overall, the increased cytokine levels corresponded well with the complement activation profiles. To explore whether a direct link could be found between complement activity and cytokines, we introduced the specific C3 inhibitor compstatin analog CP20 [24]

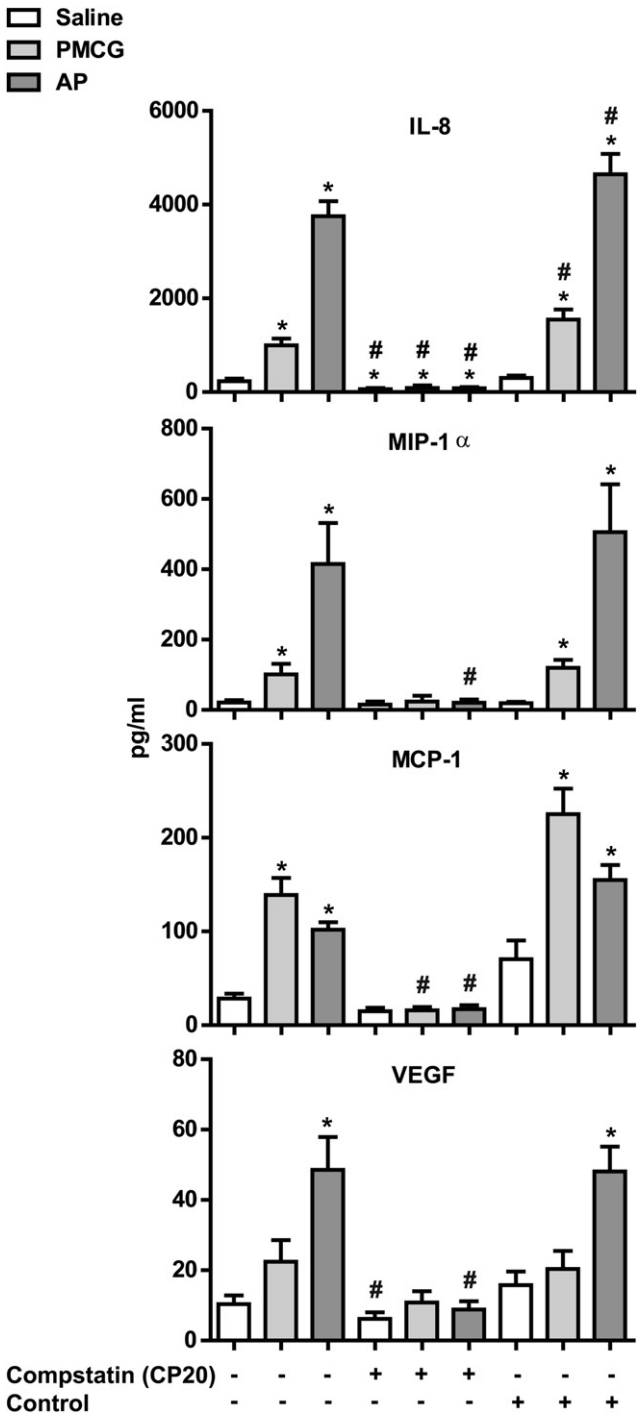

Fig. 7. Effect of C3 inhibition (Compstatin analog CP20) on chemokines (IL-8, MIP-1α, MCP-1) and the growth factor VEGF after 240 min incubation of AP or PMCG microcapsules in lepirudin anti-coagulated fresh human blood. A corresponding control peptide to compstatin analog CP20 was used as control. The plasma baseline and positive control values (pg/ml): IL-8; 3.5 ± 0.8 (baseline), 5607 ± 407 (zymosan), 2084 ± 338 (LPS), MIP-1α; 0.2 ± 0.2 (baseline), 3308 ± 559 (zymosan), for 3922 ± 584 (LPS), MCP-1 21.7 ± 3.4 (baseline), 235 ± 77 (zymosan), 473 ± 75 (LPS), and VEGF 1.2 ± 0.3 (baseline), 19.8 ± 3.7 (zymosan), 21.2 ± 3.4 (LPS). Data are expressed as the mean ± SEM ($N = 5$, for baseline values $N = 3$) of separate experiments with different donors. Significant values are given as $P < 0.05$ *compared to saline control #effect of compstatin compared to the same condition without compstatin.

and incubated it with the polycation containing AP and PMCG microcapsules. A complete inhibition of complement activation and cytokine induction was achieved (Figs. 5–7). Our data thus strongly points to complement activation alone as the activation mechanism for the pro-inflammatory cytokines (TNF, IL-1β, and IL-6), the anti-inflammatory cytokines (IL-1ra, IL-10), the chemokines (IL-8, MIP-1α, MCP-1) and the growth factor (VEGF). A strong complement dependency related to the secretion of chemokines IL-8, MIP-1α and MCP-1 induced by other biomaterials has previously been demonstrated [15,17], and recently also a partially complement dependence has been shown for the inflammatory cytokines [19]. To the best of our knowledge, however, this work show for the first time that the secretion of inflammatory cytokines from polycation containing alginate microspheres can be solely dependent on complement.

Secretion of pro-inflammatory cytokines could potentially be caused by endotoxin contamination from Gram-negative bacteria activating the Toll-like receptor 4 (TLR4) on leukocytes. We can, however, exclude contamination by LPS as a cause of the pro-inflammatory cytokine by the polycation containing microcapsules since; 1) the endotoxin level was low for all microspheres (≤24 pg/ml); 2) inhibition of CD14 (LPS co-receptor for TLR4) did not change microcapsules-induced cytokines but inhibited LPS-induced cytokines and finally; 3) addition of the complement inhibitor compstatin analog CP20 completely inhibited the cytokine secretions. Our results thus demonstrate that complement activation alone could trigger inflammatory cytokine secretion. This total complement dependency in cytokine responses was also recently shown by single-stranded DNA sequence CpG 2006 giving a complete inhibition of the inflammatory cytokines by use of the C3 inhibitor compstatin [31].

Products from complement activation have been shown to amplify TLR-mediated cytokine responses as TNF, IL-1β, IL-6 and IL-12 [32]. There is emerging evidence that cross-talk between complement and TLRs is commonly involved in innate and adaptive immune responses [33,34]. Cross-talk between complement and the TLR co-receptor CD14 has also been demonstrated [29,30], which lead us to include anti-CD14 inhibition in the present study. In addition, we had previously found a link to CD14 by soluble PLL stimulating monocyte cultures to secrete TNF [35], thus a cross-talk between complement and CD14 could be expected. However, we did not find any effect of anti-CD14 alone or in combination with compstatin analog CP20, illustrating that the cytokine responses were independent of CD14 for the polycation containing microcapsules. The cytokine secretion was only dependent on the complement. This inconsistency with the previous findings could be due to the state of the PLL, which on the present study was complexed in the outer membrane with alginate, while in the previous study was used in its soluble form.

By inhibiting the complement C3 with compstatin, formation of split products from C3 and the C5 are inhibited. The split products anaphylatoxins C3a and C5a are pro-inflammatory polypeptides with diverse effects on innate immunity functions [34,36]. Their effects are induced by binding to the corresponding G-protein coupled C3a receptor and C5a receptor, while both can bind the C5L2 receptor [37]. We have previously demonstrated that the PLL containing microcapsule-induced C3a and C5a [23], and thus they are potential candidates responsible for the microcapsule-induced cytokine formation. The anaphylatoxins themselves may stimulate cytokine secretion as indicated by the ability of C3a and C5a per se to induce NFκB activation in human peripheral blood monocytes [38]. Further on, C3a and C5a is shown to induce an up-regulation of IL-8, IL-1β and RANTES mRNA in human umbilical vein endothelial cells, with dose and synergistic effect of C3a and C5a upon the IL-8 induction [39]. In mice, C5a is shown to promote inflammation by antagonizing the effects of IL-6 and IFN-γ [40], or of TLR ligands [32,41] leading to elevated secretion of various cytokines. The different anaphylatoxin receptors seem also to be involved in the regulation of the cytokine secretion profiles [41]. At last, the

effect of C5a might depend on the cell type, since C5a increased the stimulation in monocytes whereas inhibited the stimulation in neutrophils upon LPS treatment [42]. Altogether, this illustrates that the anaphylatoxins are hot candidates for the complement mediated cytokine secretion in the present study. Synergistic effects of C3a and C5a could possibly explain our results, but also a possibility of different cell types responding opposite upon stimulation with the complement fragments exists.

The variation in cytokine levels between the AP and PMCG microcapsules despite a similar degree of complement activation (sTCC) might be explained by differences in the availability of the anaphylatoxins. Previously we have found the anaphylatoxin levels to be low for the PMCG microcapsules while increased for the AP microcapsules [23]. Since the split products are formed prior to the formation of sTCC, the anaphylatoxins must have been formed also by addition of the PMCG microcapsules. One explanation for the low level of anaphylatoxins could be their highly positive charges attaching to the negatively charged cellulose sulfate coat on the PMCG microcapsules. Reduced amounts of the anaphylatoxins are thus probably available for the leukocyte C3a, C5a, or C5L2 receptors, leading to reduced amount of cytokines. Since the MCP-1 secretion was opposite with most increase by addition of the PMCG microcapsules, we speculate in additional mechanisms being involved. Soluble complexes have been found to leak from the PMCG microcapsules (unpublished observations) and could be one candidate for the observed MCP-1 stimulation. For the PMCG microcapsules, the complement activation might be caused partly by the leaking complexes, since the C3 deposition pattern on the PMCG microcapsules are not accumulating with time [23]. This is in contrast to the APA and AP microcapsules showing C3 accumulation on the surface of APA and AP microcapsules, indicating that the C3 convertase is building up with time.

Other complement activation products might also contribute to cytokine stimulation. For example iC3b and C4b were shown to be involved in IP-10 induced secretion, by inversely inhibiting the IP-10 [43]. An inversely regulation of IP-10 by complement was also observed with Gram-negative bacteria [30]. In the present study, the various microspheres-induced lower IP-10 than saline or soluble alginate (Fig. S2). The lowest IP-10 secretion was induced by the AP microcapsules, an effect which was significantly counteracted by inhibiting the C3 by compstatin analog CP20. This indicates that complement was involved in the AP-induced IP-10 response. Since the alternative pathway has been identified as responsible for the AP-induced complement activation [23], iC3b is the most likely candidate for the AP-induced effect on IP-10.

The low cytokine secretion from alginate microbeads corresponded well with a low complement response [23]. However, since the Ca/Ba and Ba microbeads induced a lower level of complement activation compared to saline control in the previous study, we cannot fully exclude the possibility of a small stimulation from the alginate microbeads through mechanisms independent of complement. RANTES, a chemokine involved in T-lymphocyte activation and proliferation, was the only cytokine with a significant elevation after incubation with Ca/Ba Beads and the Ba Beads, although not by soluble alginate (Fig. S1). Since alginate films have been shown to activate RANTES secretion by dendritic cells [44], it is possible that the increased secretion in our study reflects an alginate effect on this mediator. The activation in our study was however transient, therefore more studies should be done in order to confirm the RANTES response. An activation potential by the Ca/Ba beads and Ba beads might also be indicated by the IP-10 response. As already discussed, an inverse response seems to indicate activation of IP-10, thus the lower levels of IP-10 found after incubation with Ca/Ba beads and Ba beads do indicate some activation potential. Overall, however, our data on alginate microbeads show that their activation on most mediators is low or absent.

Transferred to an *in vivo* situation, it is likely that activation of complement and subsequent cytokine responses by PLL containing microcapsules, but not alginate microbeads, significantly contributes to their lower tolerability, as manifested by cell overgrowth/fibrosis [35,45–48]. Complement has been shown to affect fibroblast activation, extracellular protein matrix deposition and macrophages influx in a renal fibrosis model using C5 deficient mice [49]. In addition, the expression of growth factor TGF-β_1 and growth factor subunit PDGF-B mRNA was also affected by the C5 deficiency. These findings show the importance of complement in fibrosis. Since complement also is responsible for the induction of cytokines normally involved in an inflammatory response as shown in our study, it is also likely that these mediators play a role.

The interplay and dynamic expressions of inflammatory- and anti-inflammatory cytokines, chemokines and growth factors might be of importance for the cellular response toward the biomaterial, although the impact of the various mediators in this dynamic situation is mostly unknown. Monocytes have a large repertoire of cytokines and we would point to the monocytes as probably the most important cytokine producer in the present study.

The biomaterials surface properties seem to impact the cytokine profiles since variability's in whole blood studies are found between the PVC tubing's [15–17], cellulose ester or polyamide membranes [19] and presently the polycation containing alginate microspheres. The growth factors G-CSF, GM-CSF and bFGF have been shown only to be induced by the PVC tubing's [17], whereas the pro-inflammatory cytokines (TNF, IL-1β, IL-6) have not. Instead the pro-inflammatory cytokines are induced by the cellulose ester and the polyamide membranes [19], as well as by the polycation containing microspheres. The mediators most commonly secreted seem to be IL-8, MCP-1, MIP-1α and RANTES, as well as the growth factors VEGF and PDGF-BB. Among these, the strongest complement dependencies are found for IL-8, MIP-1α and VEGF. The most consistent and strong response is found for the chemokine IL-8 [16,17,19]. We would therefore point to IL-8 as an important mediator for predicting the biomaterial tolerability. IL-8 is produced by cell types as monocytes and endothelial cells, whereas in whole blood the endothelial cells are lacking thus the monocytes probably are the main producers. IL-8 is a strong chemoattractant for neutrophils, the most abundant cell in circulation and early responder upon inflammation. T-lymphocytes, basophiles and endothelial cells also have receptors for IL-8 with influence on chemotaxis [50] and angiogensis [51]. In a lung disease model, the chemokine receptor for IL-8, CXCR2, has been shown to have a bimodal function recruiting neutrophils in early events and inducing fibrotic and tissue granulation [52]. It is therefore likely that IL-8 is an important contributor both in early inflammatory events, as well as being involved in later events in the host response to biomaterials. Our data suggest that IL-8 is an efficient mediator for predicting biomaterial tolerability, however, we would also stress that the different cytokines in combinations might have other effects than each cytokine alone, and thus the interplay between the various mediators should not be underscored. Using the "holistic" whole blood model, we have an efficient tool for measuring the outcome of the interplay between the various cells and mediators.

Our results points to a general effect of polycation containing biomaterials and their potential ability to initiate an inflammatory response through complement activation. In the field of microencapsulation, one should be aware of this possibility. Various encapsulation protocols including polycations exist, and some might be good in reducing the complement activation of the

polycation by strong interaction with polyanions. Therefore, we cannot infer that every capsule protocol containing polycations will be stimulating. However, we would recommend that the potential activation potential is unveiled using the lepirudin based whole blood assay. The whole blood assay is an efficient tool for optimization of the microspheres to reduce their inflammatory potential that is of importance for the use of biomaterials and microcapsules in a clinical setting.

5. Conclusions

This study show that the polycation containing microspheres are capable of inducing the inflammatory cytokines (TNF, IL-1β, IL-6),the anti-inflammatory mediators (IL-1RA, IL-10), the chemokines (IL-8, MIP-1α, MCP-1) and the growth factor VEGF in a totally complement dependent manner. In contrast, the corresponding cytokines are not induced by the complement inert alginate microbeads. These results can explain why alginate microbeads generally are more tolerated than polycation containing microcapsules *in vivo*. Further on, the results emphasize complement as a valuable tool for revealing inflammatory potentials and the tolerability of the biomaterial. The fresh human whole blood model is efficient for screening microspheres, biomaterials or their components, and represents a powerful tool for identifying effectors for design of tolerable biomaterials.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2012.10.012.

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